

Green Biosynthesis of Nanoparticles

Mechanisms and Applications

Edited by
Mahendra Rai and Clemens Posten



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Preface

There are physical and chemical methods for the synthesis of nanomaterials. But, due to the damage these methods cause to the environment, there is a pressing need for a green nanotechnology that is clean and eco-friendly for the development of nanomaterials. More precisely, green nanotechnology can be developed to minimize the potential environmental and human health risks associated with the fabrication and use of nano-based materials and products.

Recently, biological synthesis has attracted the focus of scientists. The importance of biological synthesis is being emphasized globally, because chemical methods are capital-intensive, use toxic chemicals and have low productivity. Thus, the need for clean, eco-friendly, cost-effective and biocompatible synthesis of metal nanoparticles has encouraged researchers to exploit biological sources as nanofactories. Biological synthesis of nanoparticles is quite novel, leading to a truly green approach that provides advancement over chemical and physical methods, as it is cheaper, environment friendly and easily scaled up for large-scale synthesis. In these methods there is no need to use high pressure, energy, temperature and toxic chemicals.

Different biological systems are exploited for the rapid synthesis of nanoparticles, including bacteria, fungi and plant extracts. Microbes are the 'nanofactories' for the synthesis of nanoparticles.

This book includes the green synthesis of nanoparticles by algae, diatoms, bacteria and plants. Moreover, the mechanisms behind the synthesis of nanoparticles have been discussed.

The book should be immensely useful for students, researchers and teachers of biology, chemistry, chemical technology, nanotechnology, microbial technology and those who are interested in green nanotechnology.

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1 Green Technology for Nanoparticles in Biomedical Applications

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Introduction

A growing number of scientists and engineers are exploring and tweaking material properties at the atomic scale to create designer materials that ultimately might increase the efficiency of current energy sources or make new energy sources practical on a commercial scale. At the nanoscale, fundamental mechanical, electrical, optical and other properties can differ significantly from their bulk material counterparts, and materials can self-assemble spontaneously into ordered structures. Nanostructured materials also have enormous surface areas per unit weight or volume, so that vastly more surface area is available for interactions with the other materials around them. That is useful, because many important chemical and electrical reactions occur only at surfaces and are sensitive to the shape and texture of a surface as well as its chemical composition (Van Hove, 2006; Ashby *et al.*, 2009).

Green technologies have been around since the first public health projects were set up in cities to provide people with clean drinking water. Since then, many other green techniques such as scrubbers for smokestacks, catalytic converters for cars, recycling

plants, solar panels and energy-efficient appliances have been introduced. To date, a new generation of green technologies is imminent, as pressures on resources grow and investors see a healthy profit in a wide range of innovative products.

With the development of science and technology, a growing number of researchers are merging green chemistry and green engineering with nanotechnology, and these researchers see a bright future for a new field known as 'green nano'. Some want to help green up industries that use emerging nanotechnologies. Others who are working on green technologies such as solar cells, remediation techniques and water filters are turning to nanotechnology in order to achieve their goals of creating better devices to help the environment. These researchers assert that a strong marriage between nanotechnology and green chemistry/engineering holds the key to building an environmentally sustainable society in the 21st century.

Nanoparticles

Unlike bulk materials, nanoparticles have characteristic physical, chemical, electronic,

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electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties (Chuang and Chen, 2011). Decreasing the dimension of nanoparticles has a pronounced effect on the physical properties, which differ significantly from the bulk materials. These physical properties are caused by their large surface atom, large surface energy, spatial confinement and reduced imperfections. Nanoparticles have advantages over bulk materials due to their surface plasmon resonance (SPR), enhanced Rayleigh scattering and surface-enhanced Raman scattering (SERS) in metal nanoparticles and their quantum-size effect in semiconductors and supermagnetism in magnetic materials. Therefore, nanoparticles are considered as building blocks of the next generation of optoelectronics, electronics and various chemical and biochemical sensors, etc. (Hahn *et al.*, 2011; Kameya and Hanamura, 2011).

By now, an astonishing multitude of materials ranging from inorganic to polymeric nanoparticles, biological building blocks and nanostructured thin films with many different electronic, magnetic, optical and bio properties have been synthesized and characterized in great detail. The pivotal point is the directed assembly or self-assembly of these systems into hierarchically ordered and/or arbitrarily defined structures. The production and use of nanostructured and nanoscaled materials has become a key technology in many more fields, for example pharmacy (Kathiravan *et al.*, 2011; Tran *et al.*, 2011), regenerative medicine (Harrison and Sirivisoot, 2011), diagnostics (Zhang and Kataoka, 2009), cosmetics (Kokura *et al.*, 2010) and food technology (Dudo *et al.*, 2011).

Progress in nanotechnology is aiming not only at miniaturization but also at systems with increased complexity. This is not only just a matter of geometrical structuration but also a matter of specific functionalities that are positioned at discrete locations and in defined distances. Nature and its highly precise mechanisms of life, mainly based on two classes of biomacromolecules, proteins or polypeptides and polynucleic acids, set the benchmark for functional structures down to atomic scales. Thus, the use of biomolecules is considered as an obvious step in the synthesis and construction of next-generation nanomaterials and devices.

A whole new branch termed 'bionanotechnology' seeks for scientific as well as economic breakthroughs in the development of bioinorganic nanomaterials with novel properties for computation and nanotechnology, new methods in diagnosis and analytics or new drugs and drug delivery systems (Mahmoud *et al.*, 2011).

In medicine, nanoparticles can be used in bioanalysis and as biosensors. Bioanalysis can have a variety of applications. For example, nanoparticles can be used to induce signal transduction, as quantitation identifiers, in bioassays, and finally they can be used for specific functions in biological systems (Penn *et al.*, 2003). Maxwell *et al.* (2002) showed that colloidal gold could be used to create biosensors to identify specific DNA sequences and base mutations. It has also been shown that copper-gold bimetallic nanoparticles can be used as oligonucleotide labels for the electrochemical stripping detection of DNA hybridization (Cai *et al.*, 2003).

Related to health and environmental issues, it has been shown that nanoparticles can be used in the remediation of organic pollutants in the environment (Obare and Meyer, 2004). Iron nanoparticles have been proven to be effective in the dechlorination of polychlorinated biphenyls. It has also been shown in the literature that bimetallic nanoparticles can be used for groundwater treatment (Elliott and Zhang, 2001). Results show the destruction of trichlorethylene (TCE) and other chlorinated hydrocarbons using bimetallic nanoparticles. The results of this study showed that 96% of the TCE was eliminated within 4 weeks of injecting the nanoparticles.

Over the past few decades, nanoscale particles have elicited much interest due to their distinct chemical, physical and biological properties. A variety of nanoparticles (NPs) with various shapes such as spheres, nanotubes, nanohorns and nanocages, made of different materials, from organic dendrimers, liposomes, gold, carbon, semiconductors, silicon to iron oxide, have already been fabricated and explored in many scientific fields, including chemistry, materials sciences, physics, medicine and electronics.

The novel properties of NPs, attributed primarily to the quantum size effect, are

confronted by their conventional ecologically hazardous synthesis protocols (Jackson and Halas, 2001). Endeavours are under way to develop greener avenues in the domain of nanotechnology. It is pertinent to mention that carbohydrate-templated silver NPs (Babu *et al.*, 2010) have carved a unique niche in the domain of nanobiotechnology with an immense spectrum of applications, particularly as antimicrobial biopolymer nanocomposite. Macromolecules such as starch, when used for encapsulation or entrapment of inorganic particles, can impart novel properties to the latter (Ziolo *et al.*, 1992). Enhanced compatibility, reduced leaching and protection of surfaces from damage, with concomitant improvement in dispersibility and stability of the NPs, are a few of the desired facets of polymer-templated nanomaterial over uncoated counterparts (Bourgeat-Lami and Lang, 1998).

Green Nanoparticles

Silver nanoparticles

Nanotechnology is emerging as a cutting-edge technology interdisciplinary with biology, chemistry and material science. Silver nanoparticles (Ag-NPs) are important materials that have been studied extensively. Such nanoscale materials possess unique electrical, optical, as well as biological properties and are thus applied in catalysis, biosensing, imaging, drug delivery, nanodevice fabrication and medicine (Smith *et al.*, 2010). Due to strong antimicrobial activity, Ag-NPs are also used in clothing, the food industry, sunscreens and cosmetics (Kokura *et al.*, 2010). Additionally, Ag-NPs have been shown to undergo size-dependent interactions with HIV-1 and inhibit binding to the host cell *in vitro* (du Toit *et al.*, 2010; Shegokar *et al.*, 2011).

Although different techniques such as ultraviolet irradiation, aerosol technologies, lithography, laser ablation, ultrasonic fields and photochemical reduction have been used successfully to produce metal NPs, they remain expensive and sometimes involve the

use of hazardous chemicals (Butkus *et al.*, 2003; Ashby *et al.*, 2009). Consequently, green synthesis of NPs has received increasing attention due to the growing need to develop an environmentally benign technology in nanoparticle synthesis. Several biological systems including bacteria, fungi, yeast and plants have been used in this regard (Nabikhan *et al.*, 2010; Shivaji *et al.*, 2011; Zaki *et al.*, 2011). Although the green synthesis of Ag-NPs by various plants has been reported, the potential of plants as biological materials for the synthesis of NPs is yet to be explored fully. In addition, information on the biological response of human cells to green synthesized Ag-NPs is also very limited.

Furthermore, it should be noted that lack of access to potable water is a leading cause of death worldwide. Dehydration, diarrhoeal diseases, contaminated water sources, waterborne pathogens, water needed for food production (starvation) and water for sanitation are just some of the factors that impact health. The water–health nexus is crucial for the survival of humanity. Meanwhile, people all over the world face profound threats to the availability of sufficient safe and clean water, affecting their health and economic well-being. The problems with providing clean water economically are growing so quickly that incremental improvements in the current methods of water purification could leave much of the world with inadequate supplies of clean water in mere decades. Recent advances strongly suggest that many of the current problems involving water quality can be addressed and potentially resolved using nanosorbents, nanocatalysts, bioactive nanoparticles, nanostructured catalytic membranes and nanoparticle-enhanced filtration, among other products and processes resulting from the development of nanotechnology (Zäch *et al.*, 2006; Ashby *et al.*, 2009; Van Hove, 2009). Moreover, nanotechnology solutions are essential because the abiotic and biotic impurities most difficult to separate in water are in the nanoscale range. At the same time, nanotechnology has enabled the development of a new class of atomic-scale materials capable of fighting waterborne disease-causing microbes. The explosive growth in nanotechnology research has opened the doors to new strategies

using nanometallic particles for oligodynamic disinfection (Anshup, 2009; Diallo *et al.*, 2009). The excellent microbicidal properties of the oligodynamic NPs qualify their use as viable alternatives for water disinfection. Oligodynamic metallic NPs such as silver, copper, zinc, titanium, nickel and cobalt are among the most promising nanomaterials with bactericidal and viricidal properties owing to their charge capacity, high surface-to-volume ratios, crystallographic structure and adaptability to various substrates for increased contact efficiency. This new class of nanometallic particles produces antimicrobial action referred to as oligodynamic disinfection for their ability to inactivate microorganisms at low concentrations. When oligodynamic metals with microbicidal, bactericidal and viricidal properties are reduced to nanosize scale, they show tremendous advantages in disinfection capacity due to the greater surface area, contact efficiency and often better elution properties. These qualities enable materials such as silver (Ag), copper (Cu), zinc (Zn), titanium (Ti) and cobalt (Co) to be considered as viable alternative disinfectants. New combinatorial oligodynamic materials consisting of these nanometallic particles have been deployed among a number of substrates for their use in water disinfection (Kim *et al.*, 2006). Materials such as Ag deposited on titanium oxide and Ag-coated iron oxide have displayed faster kinetics and greater efficiency in eliminating bacteria.

To date, Ag is the most widely studied oligodynamic material due to its wide range in microbicidal effectiveness, low toxicity and ease of incorporation on various substrates in a host of dynamic disinfection applications. Furthermore, the systems supported with nanometallic Ag particles are effective in reducing the presence of target microorganisms in a wide variety of water disinfection applications. The main known negative health effect from Ag is argyria, which is an irreversible darkening of the skin and mucous membrane resulting from overexposure to ionic silver (Ag(I), Ag⁺) (Butkus *et al.*, 2005).

Typically, Ag-NPs are derived from silver salts (silver nitrate (AgNO₃), silver chloride (AgCl), silver bromide (AgBr) and silver

iodide (AgI)), and a variety of the substrates that Ag is deployed on, such as activated carbon, activated carbon fibres (ACFs), polyurethane, zeolites and ceramics in point of entry (POE) and point of use (POU) applications, display the effective inactivation of pathogens in water (Byeon and Kim, 2010).

Wang *et al.* (1998) prepared viscose-based activated carbon fibre supporting silver (ACF(Ag)) by pretreatment, carbonization, activation, vacuum impregnation and decomposition processes. The ACFs were then subjected successively to a vacuum impregnation treatment in unsaturated silver nitrate (analytical grade) aqueous (AgNO₃) solutions (NH₄H₂PO₄ 3.3 g l⁻¹, (NH₄)₂SO₄ 6.7 g l⁻¹) with varying concentrations for different times and were finally heated to different temperatures for decomposition, thus producing ACF(Ag). Moreover, ACF(Ag) containing as little as 0.065 wt% of Ag exhibits strong antibacterial property against *Escherichia coli* and *Staphylococcus aureus*.

Garlic (*Allium sativum*) has long been considered a herbal remedy to prevent and treat various metabolic diseases such as thrombosis, hypertension, diabetes, dementia and atherosclerosis. Garlic is a very good source of vitamin C and vitamin B₆, along with beta-carotene, thiamine, riboflavin, niacin and folate, which function as antioxidants. Recently, Ahamed *et al.* (2011) studied a simple, cost-effective and environmentally benign synthesis of Ag-NPs at ambient conditions using garlic clove extract as a reducing and stabilizing agent in order to apply the biological response of Ag-NPs in human lung epithelial (A549) cells.

Furthermore, Guidelli *et al.* (2011) investigated a totally green synthesis of colloidal Ag-NPs using the natural rubber latex (NRL) extracted from *Hevea brasiliensis*. The synthesis was fast and occurred at a relatively low temperature (water boiling temperature). Moreover, it was very simple, inexpensive and environmentally benign, devoid of photochemical, electrochemical or irradiation processes. The colloidal particles could be used and stored in their liquid form, or even as a film obtained by drying the starting solution. Combining the angiogenic properties of the NRL and Ag-NPs, the nanostructured material

obtained could be used in a wide range of applications such as a hybrid biopolymer, and was aimed at the fabrication of a wound dressing with potential healing action. Also, the Ag-NPs could protect the wound against microorganism contamination.

The results showed that the formation of Ag-NPs was increased on raising the NRL and AgNO₃ content. The particle size seemed to be related to the AgNO₃ concentration, in such a way that larger Ag particles were produced when more AgNO₃ was added to the reaction medium. The dynamic light scattering technique and the transmission electron microscope (TEM) micrographs suggested the formation of aggregates of Ag-NPs by increasing the AgNO₃ concentration.

Gold nanoparticles

Today, a cadre of research scientists and engineers is working to develop cutting-edge methods for the green manufacturing of nanoelectronics and other nanoproducts that are more people/planet friendly, such as gold (Au) NPs, which are promising materials for use in new kinds of electronics and medical imaging. It is well known that Au-NPs have been considered an important area of research due to their unique and tunable surface plasmon resonance (SPR) and their applications in biomedical science, including drug delivery, tissue/tumour imaging, photothermal therapy and immunochromatographic identification of pathogens in clinical specimens (Chen *et al.*, 2010; Kim *et al.*, 2010; Cao *et al.*, 2011). The use of Au compounds and Au-NPs, with respect to their potential therapeutic applications such as anti-angiogenesis, as an antimalarial agent, an anti-arthritis agent, and as an agent in biohydrogen production, has driven various breakthroughs in the field of nanotechnology. The standard method to synthesize Au-NPs uses large amounts of toxic solvents that can be flammable and explosive.

An innovative synthesis method investigated by J.E. Hutchison uses non-toxic solvents, a new catalyst and purification by nanoporous filtration. This technique not only has proved to be greener, safer and faster

than the old method but also is much cheaper, showing that the green synthesis of nanomaterials can boost efficiency and save money (Hutchison, 2008).

As one of the world's healthiest foods, the major constituents of honey are fructose and glucose, and it also contains amino acids that help build up calcium (Ca) in the body. All over the world, honey has been subjected to extensive study of its ingredients, physico-chemical properties, vitamins, mineral content and quality control. It is reported to benefit human longevity due to its high energy and presence of chemical elements, vitamins and enzymes. Honey is rich in vitamin C and the important minerals present are potassium (K) and magnesium (Mg). Also, it contains ingredients that can function as antioxidants, which play a vital role in the prevention of cancer. Philip (2009) investigated a greener synthesis method for the preparation of Au-NPs in water using natural honey, which acted as both a reducing and a protecting agent. In addition, the synthesis was carried out at room temperature.

The typical TEM images obtained for colloids (g₁) and (g₄) are shown in Fig. 1.1. The decrease in anisotropy and particle size with the increase in the quantity of honey is evident from the images. Colloid (g₁) consists of a larger propensity of triangular NPs (Fig. 1.1a) when compared with those in the TEM image of colloid (g₄) shown in Fig. 1.1b. Colloid (g₄) consists of almost spherical NPs with an average size ~15 nm. Sintering of Au-NPs and their adherence to the nanotriangle in colloid (g₁) is evident from Fig. 1.2. The blunt-angled nanotriangles in Fig. 1.1a are a result of the shrinking process arising from the minimization of surface energy.

Recently, Narayanan and Sakthivel (2011) took the facile environmentally friendly greener synthesis of anisotropic nanostructures and isotropic spherical Au-NPs using the cell-free filtrate of the fungus *Sclerotium rolfsii*. The results show that modulation of size and shape can be achieved by varying the ratio of Au salt and the cell-free filtrate of the fungus (*S. rolfsii*). The production of anisotropic and isotropic Au-NPs is quite stable in aqueous solution for 2 months. This simple, efficient, eco-friendly process is very rapid

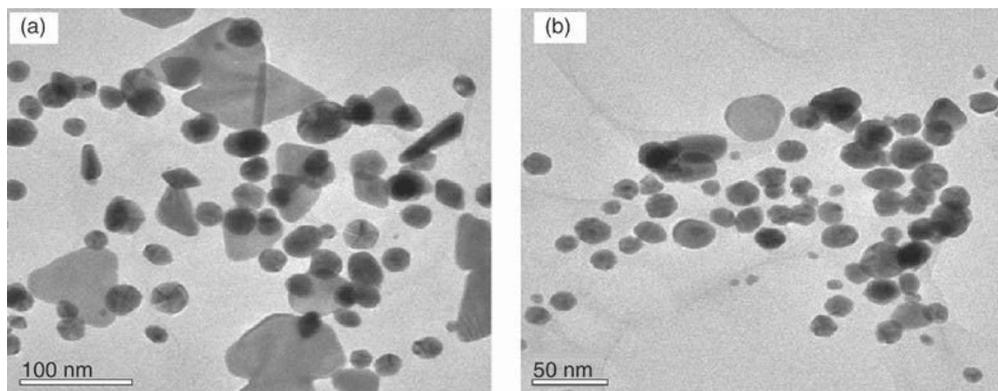


Fig. 1.1. TEM images of colloids: (a) g_1 ; (b) g_4 (Philip, 2009).

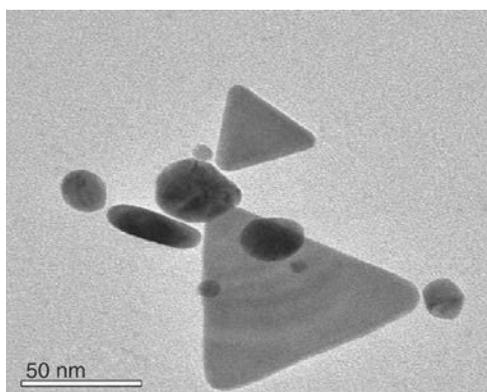


Fig. 1.2. Gold nanotriangle in colloid g , showing adherence of smaller particles (Philip, 2009).

and completes in 10–15 min. In addition, Kalishwaralall *et al.* (2010) employed the response surface methodology (RSM) and central composite rotary design (CCRD) to optimize a fermentation medium for the production of α -amylase by *Bacillus licheniformis* at pH 8 to evaluate the relationship between a set of controllable experimental factors and observed that a limited number of experiments resulted in the rapid and enhanced synthesis of Au-NPs.

Greener Nanoparticles

Researchers at Monash University (Australia) have produced metallic nanostructures at

room temperature by the spontaneous reduction of Ag^+ and Au^{3+} in a 'green' ionic liquid (Bhatt *et al.*, 2007). Room-temperature ionic liquids (RTILs) are an increasingly important area for chemistry research as possible replacements for conventional solvents as they are more environmentally friendly and can be used at lower temperatures. Unfortunately, the advantageous properties of RTILs become disadvantages when removal of solvents from synthesized products is required.

However, a special class of 'distillable' ionic liquids, dialcarbs, exists. Unlike conventional RTILs, these can be easily purified, recovered and separated to their constituent parts by low-temperature distillation. Using Ag^+ in the dialcarb, DIMCARB, the Monash researchers observed spontaneous chemical reduction of Ag^+ to Ag nanostructures. This alternative synthesis route using an ionic liquid negates the need for the high temperatures and large quantities of toxic and volatile organic solvents normally associated with nanostructure formation. In addition, the distillable nature of the solvent allows facile removal of the solvent at relatively low temperatures. Although the work has been expanded to include Au nanostructure synthesis from Au^{3+} , a number of areas still require research, including looking at other metals of interest and the use of solvents other than DIMCARB. The eventual aim is to find optimal conditions for nanoparticle and/or nanowire synthesis.

Greener Nanostructures

The fabrication of relatively large quantities of organic nanostructures is now possible using a new method that combines tools from microelectronics manufacturing and organic chemistry invented by Joseph DeSimone (Gratton *et al.*, 2007; DeSimone *et al.*, 2011). His general-purpose 'moulding' technique, called particle replication in non-wetting templates (PRINT), as a low-waste, green method that can be used to manufacture a broad range of organic NPs, enables the rapid, error-free reproduction of NPs of any shape. Moreover, functional groups can be added to tailor nanomaterials for biomedical applications. His team has successfully fabricated nano- and microparticles containing bioactive compounds.

Whereas monofunctional NPs provide a single function – a quantum dot can exhibit high fluorescence but it cannot be removed from a matrix using a magnetic field – multifunctional nanoparticles (MF-NPs) are able to achieve a mixed effect using one system. In these systems, variable strategies are used to attain a combination of targeting specificity, optimized optical, electrical and/or magnetic properties and analysis capability.

MF-NPs are not a new nanotechnological innovation. Perusal of the literature demonstrates that the unique properties of MF-NPs, along with the size effect of NPs, have already opened exciting avenues for developing new and advanced nanoparticle probes for biomedical imaging and drug delivery, which have great potential for therapy in areas such as cancer, diagnostics and neuropathologies. Great effort is also devoted to the characterization, understanding and improvement of the structural properties of such multifunctional nanostructures. However, the unique features of these MF-NPs remain practically unexplored in analytical chemistry and applications to the development of new analytical methodologies and/or devices with the aim of determining species in solution are really scarce (Han *et al.*, 2007; de Diosa and Díaz-García, 2010).

The number of different types of NPs is increasing rapidly. However, from the analytical standpoint, they can mostly be classified into two major types: particles that

contain inorganic elements, usually metals and metal oxides, as a core (Fe_3O_4 , semiconductors, Ag, Au, TiO_2 , SiO_2) and those that are based on organic molecules (carbon nanotubes, dendrimers, liposomes) as major building materials.

The Drawbacks of Nanoparticles

Several particle types and structures have been discovered. Noteworthy structures include polymeric micelles, dendrimers, quantum dots (QDs) and solid NPs. Although these structures may promise endless opportunities, their safety should not be ignored. The reactivity of these tiny particles may be due to their large surface area in comparison to their overall mass. Semiconductor metals such as colloidal gold and iron oxide crystals are commonly used and have demonstrated toxicity. Additionally, these tiny particles permeate the skin and blood–brain barrier easily, leading to several potential toxicities. Research should be carried out to investigate fully any toxicity issues associated with these structures.

Quantum dots

Quantum dots (QDs), approximately 2–100 nm in diameter, are luminescent semiconductor nanocrystals. QDs have advanced optical properties compared with traditional organic fluorophores: (i) a high brightness due to the extinction coefficient and quantum yield; (ii) broad absorption characteristics and a narrow line width in emission spectra; (iii) continuous and tunable emission maxima due to quantum size effects; and (iv) a longer fluorescence lifetime ranging from 10 to 40 ns. Their controllable tiny size (in nanoscale) gives QDs good biocompatibility; some QDs can pass biological barriers such as cell membranes easily (Jaiswal *et al.*, 2003), for which it is impossible for bulk particles to pass. The surface coating is a key factor in the biological utilization of QDs, as the modifications form a functionalized surface for the particle, which is important for delivering QDs to

target tissues (Gao *et al.*, 2004). Based on the many novel properties of QDs, their applications in biological fields have become a hot issue for recent research. QDs are being developed as tumour diagnostic agents (Gao *et al.*, 2004), specific bioindicators (Cai *et al.*, 2006), drug delivery systems (Voura *et al.*, 2004) and therapeutic medicines (Sershen *et al.*, 2000).

QDs are expected to be developed for imaging and therapeutic applications. However, most studies of the biological applications of QDs were performed by *in vitro* cell culture experiments. Even for *in vivo* studies using animal models, only the nanoproperties of QDs before uptake by experimental animals were considered, but the states and biobehaviours *in vivo* were generally ignored. Due to the complex biological environment, entirely different effects for the same NPs may be obtained in *in vitro* and *in vivo* studies. Because the biological behaviours of NPs (e.g. QDs) are difficult to detect and control after they enter a body, there are still many unknowns, such as whether QDs keep their original characteristics *in vivo* or become other states and how QDs interact with organism tissues. These questions have not yet been answered clearly.

Shih *et al.* (2010) adopted a process applied to fabricate QDs from water-soluble precursors. In the manufacturing process of the invention, any salt of a metal suitable for use in a QD, that is soluble in water, may be employed as a starting material. Exemplary water-soluble metal salts that may be employed in the invention are metals that can form sulfides, such as $\text{Cd}(\text{NO}_3)_2$, $\text{Cd}(\text{ClO}_4)_2$, CdCl_2 , CdSO_4 , cadmium acetate, $\text{Zn}(\text{NO}_3)_2$, $\text{Zn}(\text{ClO}_4)_2$, ZnSO_4 , ZnCl_2 , zinc acetate, $\text{Mn}(\text{NO}_3)_2$, $\text{Mn}(\text{ClO}_4)_2$, MnSO_4 , MnCl_2 , manganese acetate, $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{ClO}_4)_2$, PbSO_4 , PbCl_2 and lead acetate. Any suitable water-soluble sulfide may be used as a reactant in the invention process. Exemplary water-soluble sulfides that may be employed in the invention are those such as CdS, NaS, ZnS and PbS. Also, sulfide gases, such as H_2S , may be bubbled through the aqueous solution in the invention process. The addition of sulfide is preferably done gradually, such as by titration, with stirring, and may take, for example,

about 2 h. Generally, it is desirable to use about a stoichiometric amount of the sulfide. However, varying the amount of sulfide from a stoichiometric amount can, in some cases, produce desirable variations in the sizes of the particles in the QDs, and thus it may be useful to use anywhere from 0.1 to 10 times the stoichiometric amount of sulfide, more preferably 0.5–5 times but most preferably about 0.8–1.2 times. The stoichiometric amount is based on the reaction of the sulfide with the metal to form the metal sulfide. Also, any thiol-functionalized molecule with a charged group, preferably on the opposite end, may be used as a reactant in the process of the invention, as long as the thiol-functionalized molecule is water soluble.

Although QDs hold promise in medical imaging, solar cells and sensing and electronic devices, the most useful kinds, such as cadmium selenide, are highly toxic. In order to seek a cheaper and greener route for the synthesis of QDs, Wang *et al.* (2008) chose *N,N*-dimethyl-oleoyl amide (DMOA) as the solvent, which was cheaper, more environmentally friendly and more stable in the atmosphere than solvents such as trioctylphosphine oxide (TOPO) or octadecene (ODE). Oleic acid, instead of hexylphosphonic acid (HPA) or tetradecylphosphonic acid (TDPA), was selected to dissolve the reddish CdO powder and form homogeneous cadmium oleate solution, which also acted as the capping ligand in the formation of the CdSe QDs. The obtained CdSe QDs have a zinc blende structure rather than the wurtzite structure obtained in ODE/TOPO/TOP/TDPA systems with TOP-Se and CdO. Compared with the trioctylphosphine (TOP) and tributylphosphine (TBP) method, the proposed method can be carried out under Ar or open to air, due to the relative air stability of oleic acid and DMOA in the atmosphere. The new route is a more low-cost synthesis route, which is of great value for both laboratory research and industrial application in terms of green chemical principles.

Moreover, Liu *et al.* (2011) developed the synthesis of a low-cost, green and reproducible, non-injection, one-pot synthesis of high-quality CdS QDs based on a conventional non-injection, one-pot method without

nucleation initiators simply by mixing cadmium stearate and S powder into a new solvent *N*-oleoylmorpholine. The *N*-oleoylmorpholine was synthesized through a conventional amidization of OA with an excess of morpholine. The as-prepared crude product was purified by rotary evaporator at a temperature as high as 220°C to remove adequately the unreacted morpholine as well as the water produced in the reaction. The results show that the reported synthetic route, without the injection of precursors and the selection of any expensive reagents, is easy for small-scale laboratory synthesis. More importantly, it is suitable for the industrial production of high-quality CdS QDs.

In order to overcome the disadvantages of the CdSe QD produced by the traditional organometallic method, such as high temperature set-ups (300–400°C), highly toxic and expensive precursors, Saran and Bellare (2010) presented a novel green stabilization and recovery technique to prepare highly fluorescent, water-soluble and biotaggable CdSe and CdSe–CdS QDs at room temperature, from dioctyl sulfosuccinate sodium salt (AOT)/water/*n*-heptane microemulsion.

In addition, Yuan *et al.* (2010) described a new approach of combining QD technology with anticancer drug therapy. Monodispersed ZnO QDs of size 2–4 nm were synthesized successfully by a chemical hydrolysis method that exhibited a strong blue emission at ~440 nm. The water-dispersed ZnO-QD–chitosan–folate carrier loaded with anticancer drug (DOX) was fabricated successfully. The experimentally observed drug-loading efficiency was ~75%. Chitosan enhances the stability of the QDs because of its hydrophilicity and cationic charge characteristics. The drug release response of DOX-loaded ZnO-QD–chitosan–folate carrier was characterized by an initial rapid drug release followed by a controlled release.

Current and Future Developments

The need to diagnose diseases and medical conditions at an early stage is becoming more and more crucial, which is well in accordance with the saying: prevention is better than cure. Hence, the earlier a condition is

diagnosed the better a chance is gained to prevent a serious condition. New bionanotechnologies are needed to speed up the diagnostic processes and help scientists and clinicians in the initiation of targeted treatments and in the follow-up of treatment responses.

Current developments

Improved diagnosis is required in the developed and developing world. Quick and accurate diagnosis benefits individual patients by improving their treatment, in addition to ensuring the efficient use of resources and limiting the spread of infectious diseases. Screening and early detection of breast, cervical, colorectal and possibly oral cancers can reduce mortality. Better systems are required that can be used in resource-limited settings to detect diseases as early as possible and to monitor the effectiveness of treatments. Technology breakthroughs in detection include identifying relevant biomarkers and developing sensitive analytical tools for early diagnostics, which require smaller samples and will deliver more complete and accurate data from a single, non-invasive measurement. Further advances could lead ultimately to information-rich point-of-care diagnostics, resulting in a reduction in the need for diagnosis and subsequent treatment in hospital, with the associated costs. Improved biomonitoring techniques could also allow the identification of disease risks and predisposition, along with other individual genetic traits. The combination of basing treatment on a targeted genotype, rather than a mass phenotype, and an increased focus on chemical genetics could result in personalized treatment and medication tailored to the specific needs of the individual.

Future prospects

One of the most far-reaching developments is a growing understanding of how we can extract health-related information from the nano-sized offspring of genomic activity – nucleic acids and proteins. Moreover, the idea

is to build a communications bridge with cells or cell tissues that is indistinguishable from the biological system itself. This could open the door to monitoring cell activities and responses to medications in real time. A sensor package might, for instance, monitor the blood continuously for markers of anything from flu to cancer. And depending on the results, a device would adjust the flow of a therapeutic substance automatically to optimize treatment, which could do for heart diseases or cancers by giving an at risk individual a skin patch that would have a read-out and connection with a drug delivery system. This goes well beyond today's state of the art, which is based on the use of huge hundreds of microns probes that cause scarring and degrade quickly.

Conclusion

Through a confluence of experimental knowledge from biology, chemistry, physics and computer science, the processes of life are understood in sufficient detail to harness biomolecules for people's use. Ultimately, green nanotechnology for bionanotreatments and diagnostics must bypass or control the host

immune response to fulfil their function over a desired timeline. Though many of the treatments currently under development have yet to reach their envisioned performance at a research level, the potential clinical application of such interventions provides sufficient promise to ensure that green nanotechnology will become a dominant focus area for technological innovation worldwide and will impact all of our lives on a daily basis. Moreover, as society moves towards becoming more eco-friendly, scientists and engineers will be required not only to develop sustainable solutions but also to find more efficient ways of producing, refining and using these during the transition. At the same time, ways of increasing traditional efficiency and recycling should be sought. Renewable resources should be utilized more and more efficiently by green nanotechniques to diminish the side effect of traditional processing techniques. Moreover, finding new green nanotechnology will require new breakthrough technologies. In the foreseeable future, research on green nanotechnologies to prevent contamination and remedy the already existing environmental problems will be realized to make this new, clean ecoscience and technology practical on a commercial scale in our daily life.

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2 Multiple Strategic Approaches for Green Synthesis and Application of Silver and Gold Nanoparticles

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Introduction

The era of nanotechnology began late in the 20th century with the discovery of scanning tunnel microscopy and atomic force microscopy (Meyyappan, 2006). Nanotechnology, coined by Norino Taniguchi (Taniguchi, 1974), refers to all technological developments performed at nanoscale (10^{-9} m). The development of nanotechnology has been a boon to mankind as its significance paved the way for several applications in therapeutics (Liu *et al.*, 2010), catalysis (Shin *et al.*, 2009), microelectronics, biosensing devices (Zhou *et al.*, 2011), air and water purifiers, paints (Kumar *et al.*, 2008), etc. As an antimicrobial agent, silver nanoparticles are powerful nanoweapons in the destruction of multiple drug-resistant bacteria (Rai *et al.*, 2012). Due to their smaller size and larger surface area, nanoparticles have improved catalytic activity; as in the case of gold, its activity is high only in the order of nanometres (Haruta, 2003). Nanoparticles can be synthesized by physical, chemical or biological methods. The physical methods initially used gave low yield (Mallick *et al.*, 2004). Chemical methods use various chemical agents to reduce metallic ions to nanoparticles.

This comprises certain drawbacks as there will be use of toxic chemicals and generation of hazardous by-products (Mallick *et al.*, 2004). From the medical aspect, applications of nanoparticles increased tremendously only when the biological approach for nanoparticle synthesis came into focus. The various resources available naturally for the green synthesis of nanoparticles are plants, plant products, bacteria, fungi, algae, yeast and viruses (Thakkar *et al.*, 2010). Though there is a large platform for a greener synthesis of nanoparticles, the most commonly preferred way is bacterial synthesis, as bacteria are easy to handle and genetic manipulation is also possible (Parikh *et al.*, 2008; Pugazhenthiran *et al.*, 2009).

Historical Aspects

The history of nanoparticles dates back to Roman times, where they were used mainly for decorative staining of glassware (Giljohann *et al.*, 2010). However, the concept of nanoparticles was unknown during those days. A revolution in nanotechnology took place only after the work done on colloidal gold by Faraday in 1857, who ignited the idea of

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metallic nanoparticles (Faraday, 1857). Later works by Feynman on quantum electrodynamics (Drexler, 1992) and electron microscopic studies gave a deeper insight into nanoparticles. The idea of microbial synthesis of nanoparticles came through experiments on the biosorption of metals like silver, cadmium and copper by competent Gram-positive and Gram-negative microbes, which were used mainly for remediation of heavy metal salts from the environment (Beveridge and Murray, 1976; Doyle *et al.*, 1980; Beveridge and Fyfe, 1985; Mullen *et al.*, 1989). The adsorbed metal ions were found mostly on the cell surface and, in rare cases, in the cytoplasm as colloidal aggregates. Comparative studies on the adsorption of various metals on the cell wall of competent microbes revealed that Gram-positive microbes such as *Bacillus subtilis* had a higher binding capacity to Gram-negative bacteria such as *Escherichia coli* (Beveridge and Fyfe, 1985). These adsorbed heavy metals were not recognized as nanoparticles during that time (Mullen *et al.*, 1989).

Silver and gold nanoparticles were the most largely used metallic nanoparticles, due to their wide range of applications. The antimicrobial property of silver has been known since ancient times, and silver nitrate solution was used as a disinfectant in World War II (Margraff and Covey, 1977; Deitch *et al.*, 1987; Chu *et al.*, 1988; Silver, 2003; Atiyeh *et al.*, 2007; Law *et al.*, 2008). The nanoparticles of metals were found to possess unique properties when compared to bulk metals, which gave the idea of metal nanoparticle synthesis. The first instance of silver nanoparticle synthesis was established in 1984 when Haefeli and his colleagues synthesized silver nanoparticles from the bacteria *Pseudomonas stutzeri* AG259 strain isolated from silver mines (Haefeli *et al.*, 1984; Nair and Pradeep, 2002; Zhang *et al.*, 2005).

Advantages of the Biogenesis of Nanoparticles

As stated earlier, there are three methods to synthesize nanoparticles. The physical methods include spark discharging, pyrolysis, etc.

The chemical methods are electrochemical reduction, solution irradiation, cryochemical synthesis, etc. The major process involved in chemical synthesis is the reduction of metal ions to nanoparticles and preventing the aggregation of metallic nanoparticles. The former is done with the help of various reducing agents like sodium borohydride (Kim *et al.*, 2007), methoxypolyethylene glycol (Mallick *et al.*, 2004), potassium bitartrate (Tan *et al.*, 2003) or hydrazine (Li *et al.*, 1999), and the latter is proceeded with the use of stabilizers like sodium dodecyl benzyl sulfate (Tan *et al.*, 2003) or polyvinyl pyrrolidone (Li *et al.*, 1999).

The major disadvantage in the physical method is the low yield, and in the chemical method is the use of toxic solvents like thio-glycerol, 2-mercaptoethanol and also the generation of hazardous by-products (Mallick *et al.*, 2004). Moreover, synthesized nanoparticles were found to possess chemicals on their surfaces (Singh *et al.*, 2010). All these factors limit the medical applications of synthesized nanoparticles.

Biogenesis refers to the biological synthesis of nanoparticles. The major implication of this biological approach is its relative simplicity in the synthesis of nanoparticles, and it is less time-consuming. In addition to this, the high yield, low toxicity, low cost and its biocompatibility adds to its value (Kalimuthu *et al.*, 2010). Another advantage is that the size of the nanoparticles synthesized can also be controlled easily by various parameters like pH and temperature (Gurunathan *et al.*, 2009a,b). The use of stabilizers to prevent aggregation is not required as the proteins in the system act as stabilizers (Kalishwaralal *et al.*, 2010). Nanoparticles with smaller radius of curvature have higher catalytic activity; hence, angular shapes are preferable due to their smaller radii of curvature compared to spherical particles of the same volume.

Biogenerators of Nanoparticles

Various prokaryotic and eukaryotic systems like plants, plant products, algae, fungi, yeast, bacteria and even viruses (Thakkar *et al.*, 2010) are regularly used as biogenerators of nanoparticles (Table 2.1).

Table 2.1. A list of organisms synthesizing nanoparticles.

2.1.a Bacteria			
Species	Nanoparticle	Size in nm	Reference
<i>Pseudomonas stutzeri</i> AG259	Ag	200	Tanja <i>et al.</i> (1999)
<i>Bacillus megaterium</i>	Ag	46.9	Fu <i>et al.</i> (1999)
<i>Lactobacillus</i> strains	Ag	500	Nair and Pradeep (2002)
<i>Corynebacterium</i> sp.	Ag	10–15	Zhang <i>et al.</i> (2005)
<i>Pseudomonas aeruginosa</i>	Au	15–30	Husseiny <i>et al.</i> (2007)
<i>Rhodopseudomonas capsulata</i>	Au	10–20	Shiyong <i>et al.</i> (2007)
<i>Klebsiella pneumonia</i>	Ag	50	Ahmad <i>et al.</i> (2007)
<i>Bacillus licheniformis</i>	Ag	50	Kalimuthu <i>et al.</i> (2008)
<i>Geobacter sulfurreducens</i>	Ag	200	Law <i>et al.</i> (2008)
<i>Morganella</i> sp.	Ag	15–25	Parikh <i>et al.</i> (2008)
<i>Rhodopseudomonas capsulata</i>	Au nanowires	10–20	He <i>et al.</i> (2008)
<i>Escherichia coli</i>	Ag	1–100	Gurunathan <i>et al.</i> (2009b)
<i>Bacillus licheniformis</i>	Au	10–100	Kalimuthu <i>et al.</i> (2009)
<i>Bacillus subtilis</i>	Ag	5–60	Saifuddin <i>et al.</i> (2009)
<i>Proteus mirabilis</i>	Ag	10–20	Samadi <i>et al.</i> (2009)
<i>Bacillus</i> sp.	Ag	5–15	Pugazhenthiran <i>et al.</i> (2009)
<i>Bacillus cereus</i>	Ag	4 and 5	Ganesh Babu and Gunasekaran (2009)
<i>Staphylococcus aureus</i>	Ag	1–100	Nanda and Saravanan (2009)
<i>Lactobacillus fermentum</i>	Ag	11.2	Sintubin <i>et al.</i> (2009)
<i>Brevibacterium casei</i>	Ag	50	Kalishwaralal <i>et al.</i> (2010)
<i>Klebsiella pneumoniae</i>	Se	100–550	Fesharaki <i>et al.</i> (2010)
<i>Bacillus cereus</i>	Se	150–200	Soniya and Swaranjit (2010)
2.1.b Fungi			
Species	Nanoparticle	Size in nm	Reference
<i>Verticillium</i> sp.	Ag	25 ± 12	Mukherjee <i>et al.</i> (2001)
<i>Fusarium oxysporum</i>	Ag	5–50	Ahmad <i>et al.</i> (2003)
<i>Aspergillus fumigatus</i>	Ag	5–25	Bhainsa and D'Souza (2006)
<i>Phanerochaete chrysosporium</i>	Ag	100	Vigneshwaran <i>et al.</i> (2006)
<i>Aspergillus flavus</i>	Ag	8.92 ± 1.61	Vigneshwaran <i>et al.</i> (2007)
<i>Fusarium acuminatum</i>	Ag	5–40	Ingle <i>et al.</i> (2008)
<i>Aspergillus niger</i>	Ag	20	Gade <i>et al.</i> (2008)
<i>Fusarium semitectum</i>	Ag	10–60	Basavaraja <i>et al.</i> (2008)
<i>Trichoderma asperellum</i>	Ag	13–18	Mukherjee <i>et al.</i> (2008)
<i>Cladosporium cladosporioides</i>	Ag	10–100	Balaji <i>et al.</i> (2009)
<i>Penicillium fellutanum</i>	Ag	1–100	Kathiresan <i>et al.</i> (2009)
<i>Penicillium brevicompactum</i> WA 2315	Ag	23–105	Shaligram <i>et al.</i> (2009)
<i>Fusarium solani</i>	Ag	5–35	Gade <i>et al.</i> (2009)
<i>Phoma glomerata</i>	Ag	60–80	Birla <i>et al.</i> (2009)
<i>Aspergillus niger</i> NCIM 616	Au	12.79 ± 5.61	Bhambure <i>et al.</i> (2009)
<i>Trichoderma viride</i>	Ag	5–40	Fayaz <i>et al.</i> (2010)
<i>Aspergillus clavatus</i>	Ag	10–25	Verma <i>et al.</i> (2010)
<i>Penicillium brevicompactum</i> WA 2315	Au	30–50	Liangwei <i>et al.</i> (2011)
<i>Fusarium culmorum</i>	Ag	5–25	Bawaskar <i>et al.</i> (2010)
<i>Pestalotia</i> sp.	Ag	10–40	Raheman <i>et al.</i> (2011)

Continued

Table 2.1. Continued.

2.1.c Plants			
Species	Nanoparticle	Size in nm	Reference
<i>Azadirachta indica</i>	Ag	50	Shankar <i>et al.</i> (2004)
<i>Embllica officinalis</i> fruit extract	Ag	10–20	Ankamwar <i>et al.</i> (2005)
	Au	15–25	Ankamwar <i>et al.</i> (2005)
<i>Cinnamomum camphora</i> leaf	Ag	55–80	Huang <i>et al.</i> (2007)
Coriander leaf	Au	6.75–57.91	Narayanan and Sakthivel (2008)
<i>Glycine max</i> (soybean) leaf extract	Ag	25–100	Vivekanandhan <i>et al.</i> (2009)
<i>Jatropha curcas</i>	Ag	10–20	Bar <i>et al.</i> (2009)
<i>Phyllanthus amarus</i>	Ag	18–38	Kasthuri <i>et al.</i> (2009)
<i>Carica papaya</i>	Ag	60–80	Mude <i>et al.</i> (2009)
<i>Gliricidia sepium</i>	Ag	10–50	Raut <i>et al.</i> (2009)
<i>Datura metel</i>	Ag	16–40	Kesharwani <i>et al.</i> (2009)
<i>Coriandrum sativum</i> leaf extract	Ag	26	Sathyavathi <i>et al.</i> (2010)
<i>Ocimum sanctum</i> leaf	Au	<30	Daizy and Unni (2010)
<i>Ocimum sanctum</i> leaf	Ag	10–20	Daizy and Unni (2010)
<i>Zingiber officinale</i>	Au	10	Singh <i>et al.</i> (2010)
<i>Murraya koenigii</i>	Ag	40–80	Bonde <i>et al.</i> (2012)
<i>Ocimum tenuiflorum</i>	Ag	25–40	Patil <i>et al.</i> (2012)

Bacteria

As soon as the role of bacteria on nanoparticle biosynthesis came into the limelight, a lot of metal ions like gold, silver and cadmium were synthesized in the nanoscale. Bacteria are the nanofactories primarily used, as genetic manipulation is possible and handling is relatively easy (Parikh *et al.*, 2008; Pugazhenthiran *et al.*, 2009). Nanoparticles are synthesized by two ways: the top-down approach, i.e the bulk material is broken down to nano-sized materials; and the bottom-up approach, i.e molecules are grouped together to form nanoparticles. The bottom-up approach is applied mainly for the biogenesis of nanoparticles. This is carried out by both extra- and intracellular methods. Extracellular biosynthesis of silver nanoparticles was revealed in *E. coli*, where the culture supernatant was separated by centrifugation and a low concentration of silver nitrate added to obtain silver nanoparticles (Gurunathan *et al.*, 2009a). Later, the synthesized nanoparticles were characterized using transmission electron microscope (TEM) and Fourier transform infrared spectroscopy (FTIR). Finally, the size distribution of the

nanoparticles was found using a Malvern Zeta sizer ZS compact scattering spectrometer and the size of silver nanoparticles was found to be 50 nm (Gurunathan *et al.*, 2009a). *Bacillus licheniformis* is known to produce intracellularly silver nanoparticles of 50 nm (Kalimuthu *et al.*, 2008). The comparative studies of extra- and intracellular synthesis revealed that extracellular synthesis was simpler, as the isolation and characterization were easy and the steps required for downstream processing were comparatively less. Furthermore, intracellular synthesis of nanoparticles has inadequate applications in the medical field, due to the presence of endotoxins (Vallhov *et al.*, 2006). This enhances the possibilities of using extracellular-mediated synthesis of nanoparticles.

Actinomycetes

Actinomycetes are a group of Gram-positive bacteria that show important characteristics of both bacteria and fungi. Extracellular synthesis of gold nanoparticles is seen in the actinomycetes species *Thermomonospora*. Under alkaline conditions (Sastry *et al.*, 2003),

they have also produced monodispersed gold nanoparticles (Ahmad *et al.*, 2003). The intracellular synthesis of monodispersed gold nanoparticles has been observed in the species *Rhodococcus* sp. (Ahmad *et al.*, 2003). Spherical nanoparticles of silver and gold have been synthesized from *Brevibacterium casei*, whose size is in the range of 10–50 nm for both particles (Kalishwaralal *et al.*, 2010).

Plants

Plants have been shown to produce nanoparticles of gold, silver, copper and gold-silver-copper alloy (Anderson *et al.*, 1998; Gardea-Torresdey *et al.*, 2002; Haverkamp *et al.*, 2007; Sharma *et al.*, 2007; Manceau *et al.*, 2008; Haverkamp and Marshall, 2009). A few plants, like *Brassica juncea* (Indian mustard), *Medicago sativa* (lucerne) and *Helianthus annuus* (sunflower), were seen to produce silver, nickel, cobalt, zinc and copper nanoparticles. Plant products like leaf extract of the plant species *Ocimum tenuiflorum* have been shown to synthesize silver nanoparticles in the range of 25–40 nm (Patil *et al.*, 2012). Extracts of *Terminalia chebula* have been found to synthesize silver nanoparticles that show good antimicrobial activity against both Gram-positive and Gram-negative bacteria (Mohan Kumar *et al.*, 2012). The plant, *B. juncea*, is a hyperaccumulator (Bali and Harris, 2008). Silver nanoparticles produced from the plant species *Acalypha indica*, in the range of 20–30 nm, show antimicrobial property against various waterborne pathogens (Krishnaraj *et al.*, 2010). *Jatropha curcas* has been shown to produce silver nanoparticles in the range of 15–50 nm (Bar *et al.*, 2009). The reduction of metal nanoparticle in plants is due to the presence of phytochemicals like flavones, ketones, aldehydes, amides and carboxylic acids. The effects of these phytochemicals on silver nanoparticle synthesis have been studied in a few plant species, like *Bryophyllum* sp., *Cyprus* sp. and *Hydrilla* sp. In *Bryophyllum* sp., the presence of an anthraquinone, emodin, led to the production of silver nanoparticles. In *Cyprus* sp., studies revealed that benzoquinones like cyperoquinone,

diethoxyquinone and remirin were present. *Hydrilla* species have catechol and protocatechaldehyde. Nanoparticles in the range of 2–5 nm were synthesized in all these species (Jha *et al.*, 2009b). Water-soluble phytochemicals like flavones, organic acids and quinones require less time for reduction of metal ions when compared to enzymes of bacteria and fungi. However, the drawback in this approach is that no reliable processes have been reported to recover nanoparticles from higher plants (Lamb *et al.*, 2001; Marshall *et al.*, 2007). Though procedures have been reported to extract nanoparticles from plant sources, it has been proven that releasing nanoparticles from cells is difficult. Besides, the plant materials could be dried and burnt to isolate the nanoparticles, but this could cause fusion and angular changes in them.

Fungi

Many fungi, like *Fusarium oxysporum* (Ahmad *et al.*, 2003), *Aspergillus fumigatus* (Bhainsa and D'Souza, 2006), etc., have been shown to form gold and silver nanoparticles (Mohanpuria *et al.*, 2008; Reith *et al.*, 2009). Intracellular synthesis of gold nanoparticles has been shown in *Verticillium luteoalbum* (Gericke and Pinches, 2006). A few marine fungi, like *Penicillium fellutanum*, have also been shown to produce silver nanoparticles intracellularly (Kathiresan *et al.*, 2009). A larger production of nanoparticles is achievable in fungi compared to bacteria, as the enzymes responsible for nanoparticle production are secreted in larger proportion (Mohanpuria *et al.*, 2008). Balaji and his fellow workers speculated in 2009 that proteins, polysaccharides and organic acids released by fungus regulated the growth and shapes of nanocrystals (Balaji *et al.*, 2009). Highly medicinal mushrooms like *Ganoderma lucidum* have been shown to synthesize extracellularly nanoparticles of the size range 10–70 nm, with an average size of 45 nm; in addition, amide linkages and protein capping enhances the stability of the produced nanoparticles (Karwa *et al.*, 2011). Extracellular synthesis of silver nanoparticles has been shown in the fungal species, *Aspergillus*

terreus, where spherical nanoparticles in the range 1–20 nm are produced (Li *et al.*, 2012).

Yeasts

Extracellular-mediated synthesis of silver nanoparticles is seen in the silver-tolerant yeast strain MKY3 (Kowshik *et al.*, 2003), and gold nanoparticles in marine yeast, *Yarrowia lipolytica* NCIM 3589 (Agnihotri *et al.*, 2009). *Schizosaccharomyces pombe* were able to synthesize CdS nanocrystals in their mid-log phase (Kowshik *et al.*, 2003). *Saccharomyces cerevisiae* has been known to synthesize Sb₂O₃ nanoparticles of 2–10 nm in size (Jha *et al.*, 2009a). Gold nanoparticles, which can be stabilized easily using small cysteine molecules, are synthesized using the fungal species *Hansenula anomala* (Sathish-Kumar *et al.*, 2011).

Algae

Certain algae like *Sargassum wightii* have been shown to produce gold nanoparticles extracellularly (Singaravelu *et al.*, 2007). Microalgae are primitive microscopic plants that can be used to produce nanoparticles. Some of the lucrative properties of microalgae that make them easier for nanoparticle synthesis are their faster doubling time (Chisti, 2007), well developed, large-scale culture (Chisti, 2007, 2008), easy harvestable nature (Molina *et al.*, 2003) and the cells can be readily disrupted (Chisti and Moo-Young, 1986). The microalgae *Chlorella vulgaris* has been shown to synthesize nanoparticles of gold in the size range of 40–60 nm in diameter (Luangpipat *et al.*, 2011).

Enzymatic synthesis of nanoparticles

Nanoparticles can be also synthesized using enzymes. By the use of the enzyme α -NADPH-dependent nitrate reductase and a chelator phytochelatin in silver nitrate solution, silver nanoparticles in the range of 10–25 nm in diameter were synthesized (Anil Kumar *et al.*, 2007). An enzyme urokinase (URAK) synthesized from *Bacillus cereus* NK1

was found to produce both gold and silver nanoparticles. Incubation of this enzyme with silver nitrate created silver nanoparticles of 60 nm in size, and with gold chloride (HAuCl₄) produced gold nanoparticles of 20 nm in size. Alkaline condition is found to exhibit an increased rate of nanoparticle production in URAK-mediated synthesis (Deepak *et al.*, 2011). The enzyme α -amylase produced in *B. licheniformis* is found to be a key compound responsible for the synthesis of gold nanoparticles (Rangnekar *et al.*, 2007). Besides this, the protease enzyme from *Actinobacter* sp. is also found to synthesize gold nanoparticles (Bharde *et al.*, 2007). This proves that enzymes are the major factors responsible for the synthesis of nanoparticles in biological systems that are capable of reducing the metal ions to nanoparticles.

Biotemplate for Nanoparticle Synthesis

Proteins

Certain proteins can be used as biotemplates for the synthesis of nanoparticles. The synthesis is mediated by using the cavity in a few proteins like ferritin, ferritin-like-protein (FLP) and chaperonin, which act as templates (Granier *et al.*, 1997; Hempstead *et al.*, 1997; Ilari *et al.*, 2000). Among these, the frequently used proteins are ferritin and apoferritin, which have been used to synthesize nanoparticles of iron sulfide, manganese oxide and uranyl hydroxide (Meldrum *et al.*, 1991). Nanoparticles of iron vanadate, phosphate, molybdate and arsenate have been synthesized using ferritin as a template (Wong and Mann, 1996; Polanams *et al.*, 2005). Apoferritin templates have been used to synthesize semiconductor nanoparticles (Iwahori *et al.*, 2005) and hollow nanoparticles (Kim *et al.*, 2005). Cobalt oxide/hydroxide and iron oxide nanoparticles are synthesized using ferritin-like proteins as templates (Allen *et al.*, 2002, 2003). A study by Padalkar and co-workers (2009) reported the use of fibrillar proteins as biotemplates for the synthesis of Zn nanoparticle chains in the size range of 30–165 nm.

The mechanism of nanoparticle synthesis using templates has been studied using ferritin, the outer wall of which is positively charged while its core is negatively charged. The outer and inner surfaces are connected by six positively charged 4F-channels and eight negatively charged 3F-channels. The cations enter the cavity through the 3F-channel and bind to the cavity's inner surface. Crystallization takes place in the cavity, after which nanocrystals of the size of the cavity, i.e. 8 nm, are formed (Allen *et al.*, 2002; Kim *et al.*, 2005). In the protein, ferritin, silver nanoparticles are difficult to produce, as silver cations would not be able to bind to the inner surface of protein. In those cases, modifications in the proteins, like introducing a peptide, assist binding and direct the synthesis (Kramer *et al.*, 2004).

Viruses

The shell cavities of some viruses can be used as biotemplates for the synthesis of nanoparticles. Cowpea chlorotic mottle virus (CCMV) has been used successfully as a biotemplate (Slocik *et al.*, 2005). It has an outer protein shell and an inner cavity, which can be used to develop nanocrystals after the removal of RNA. Both the inner and outer surfaces are connected with channels, which would open at pH greater than 6.5 and close at lower pH levels (Speir *et al.*, 1995). The positively charged inner surface enables the oxoanions to enter the cavity, where they accumulate to form nanocrystals. Douglas and fellow workers have synthesized polytungstate nanoparticles using CCMV as biotemplates (Douglas and Young, 1998). The positively charged inner surface might hamper the synthesis of iron nanoparticles, which can be overcome through the mutagenesis of the inner protein surface (Douglas *et al.*, 2002). By the use of viral capsids as templates, Prussian blue nanoparticles in the size range of 16.3–19.7 nm were produced (de la Escosura *et al.*, 2008).

Biogenesis of Nanopolymers

Polymers in the size of nanometres are referred to as nanopolymers. Polyhydroxybutyrate

(PHB), a polymer of polyester class, can be synthesized *in vitro* by the use of the microbe, *B. casei* SRKP2. Further transmission electron microscope (TEM) analysis revealed them to be in the size range of 100–125 nm (Deepak *et al.*, 2009). An additional advantage is that it is completely biodegradable, biocompatible with medical instruments (Steinbüchel, 1995; Gouda *et al.*, 2001) and is also produced from a renewable carbon source (Kim, 2000). Further, PHB nanoparticles can be used to immobilize various enzymes. The immobilized enzymes show higher caseinolytic activity (Deepak *et al.*, 2009). In general, enzyme activity decreases when immobilized, but when enzymes are immobilized on PHB nanopolymers, its activity increases. For example, the immobilization of URAK on PHB nanopolymers enhanced the activity of the enzyme by 1.5-fold, and immobilization of nattokinase on PHB nanoparticles increased its activity by 20% (Hsieh *et al.*, 2009). The immobilized enzymes also maintained stability at high pH ranges (Gianfreda and Scarfi, 1991). When immobilized, the enzyme showed thermal stability up to 70°C when compared to free enzyme, which is thermally stable up to 50°C (Deepak *et al.*, 2009).

Principle Behind Biogenesis of Nanoparticles

The main underlying principle behind the biogenesis of nanoparticles is the reduction of metal ions by various biomolecules like enzymes/proteins, amino acids, polysaccharides and vitamins present in the organisms. Therefore, the reduction potential and capacity of the system determines the effectiveness of the biogenerators. However, this would be possible in only those organisms that have silver resistance machinery against the respective metal ions, provided that the metal concentration does not exceed the threshold limit. As stated, the primary source for reduction is the enzymes secreted by the organisms. The effect of the enzymes on nanoparticle synthesis has been studied and it was found that the enzymes alone could be responsible for the reduction of metal ions to their nanoparticles (Anil Kumar *et al.*, 2007). Many enzymes have been reported to catalyse

nanoparticle synthesis like nitrate reductase, protease, urokinase, α -amylase, etc.

Mechanism of Nanoparticle Synthesis

Biogenesis of silver nanoparticles

Enzymatic synthesis

Many mechanisms involving many biomolecules have been proposed for the biosynthesis of nanoparticles, but the most accepted mechanism involves nitrate reductase-dependent reduction of silver ions (Anil Kumar *et al.*, 2007; Kalimuthu *et al.*, 2008). Nitrate reductase is an enzyme that is co-factored by nicotinamide adenine dinucleotide (NADH) (Anil Kumar *et al.*, 2007) and is capable of reducing silver ions to silver nanoparticles. Basically, it is an enzyme that is responsible for the conversion of nitrate in the nitrate cycle (Durán *et al.*, 2005). This mechanism has been clearly studied in *B. licheniformis* (Kalimuthu *et al.*, 2008), as it secretes many NADH-dependent enzymes in which the effect of α -NADH-dependent nitrate reductase on silver ions also has been clearly studied. The enzyme nitrate reductase converts nitrate to nitrite and will transfer an electron to silver ions (Ag^+) to form free silver (Ag^0). However, the synthesis would take only at lower concentrations of silver nitrate: at higher concentration, it would lead to cell destruction (Kalimuthu *et al.*, 2008; Pandian *et al.*, 2010).

The synthesis of nanoparticles could be considered rather as a defence mechanism by organisms against silver ions. Silver ions, due to their antimicrobial activity, could be harmful to these organisms. Hence, these organisms develop some mechanisms to protect them by neutralizing the ions. Silver ions can exhibit their antimicrobial activity in many ways. They bind to the negatively charged DNA molecules, altering their structure, which inhibits DNA replication. Free silver ions bind to the thiol containing protein groups and inhibit their function and, most importantly, they produce reactive oxygen species, thus forming highly reactive radicals like hydrogen peroxide, which can destroy the cell. These radicals are produced in the

respiratory system when the NADH dehydrogenase is inhibited by silver ions (Gautam and Sharma, 2002; Matsumura *et al.*, 2003). These highly reactive oxygen species are found to have degenerative effects on the cells. This could be neutralized by catalase, which would catabolize those reactive oxygen species. In *B. licheniformis*, the increase in silver ion concentration is accompanied by an increase in catalase synthesis, and when the silver ion concentration increases beyond the threshold level, the catalases cannot help the cell to survive. Thus, apoptosis could be induced at higher levels of silver ions. In order to prevent this, silver ions are converted to their inactive silver by their defence mechanisms.

Studies on the fungus *F. oxysporum* confirmed that the reduction of metal ions was carried out by a nitrate-dependent reductase enzyme and an extracellular shuttle quinone (Durán *et al.*, 2005). The synthesized silver nanoparticles were further stabilized by fungal proteins (Durán *et al.*, 2007). TEM analysis showed N and S atoms around silver nanoparticles, which indicated the linkage of these atoms at the surface. The action of nitrate reductase was confirmed by its presence in the fungal filtrate with commercial nitrate reductase discs (Ingle *et al.*, 2008).

Peptide-mediated synthesis

The use of peptides for the biogenesis of nanoparticles was first shown by Naik and co-workers. The peptides with reducing amino acid moieties like argenine, cysteine, lysine and methionine can readily reduce metal ions (Naik *et al.*, 2002). These peptides bind over the silver clusters and facilitate easy reduction of ions over the surfaces to build up silver nanoparticles. The amino acid tyrosine acts as a reducing agent at high pH and assists the reduction of silver ions by ionizing the phenolic groups in tyrosine (Selvakannan *et al.*, 2004).

Biogenesis of gold nanoparticles

Enzymatic synthesis

The synthesis of gold nanoparticles is generally followed by the reduction of gold salts in

a suitable solvent, followed by the addition of stabilizers to prevent aggregation. The most commonly used stabilizing agents are thiol-modified ligands, as they form gold–sulfur bonds on the surface of gold nanoparticles (Templeton *et al.*, 2000). The size of nanoparticles synthesized can be controlled by varying the gold/thiol and the size of the side chain. The higher the number of thiol groups, the smaller the particle size (Brust *et al.*, 1994), and the bulkier the side chain (double-chain stabilizers), the smaller the particle size (Hostetler *et al.*, 1998).

A model for enzymatic gold nanoparticle synthesis and stabilization in *Stenotrophomonas maltophilia* was proposed by Nangia *et al.* in 2009. The enzyme involved is NADPH-dependent reductase, which reduces Au^{3+} to Au^0 . The peak value of zeta potential for gold nanoparticles is -16.7 mV. Hence, it has to be capped by negatively charged groups, and the phosphate ions of NADP serve this purpose (Nangia *et al.*, 2009).

Extracellular biosynthesis and characterization of gold nanoparticles was made in *Rhizopus oryzae* (Das *et al.*, 2009). After the addition of AuCl_4^- in fungal culture, amide I, II and III groups appeared, along with loss of carboxyl groups in the mycelium, thus showing the importance of polypeptides/proteins in the formation of gold nanoparticles. Also, in spectral analysis, shifts in infrared peaks indicated the involvement of phosphate bonds in the reduction. Thus, this shows that surface-bound protein molecules are important factors in gold nanoparticle synthesis (Das *et al.*, 2009).

Biotemplate-mediated synthesis

As mentioned earlier, viral capsid can be used as a template for gold nanoparticle synthesis. The active tyrosine residues on the potentiated viral capsid surface reduce AuCl_4^- and form gold nanoparticles on the surface. This reduction is enhanced by incorporating tyrosine sites within the capsid cavity or surfaces. Furthermore, the ratio of tyrosine to histidine residues can be varied to enhance gold nanoparticle production (Slocik *et al.*, 2005). It was also revealed that tyrosine-containing oligopeptides could only synthesize nanoparticles,

thus showing the importance of reducing amino acids for nanoparticle synthesis (Ray *et al.*, 2006).

Size-controlling Parameters in the Biogenesis of Nanoparticles

Several parameters are involved in controlling the size and rate of synthesis of nanoparticles, such as the quantity of the enzyme present, the concentration of silver ions and the alkalinity of the environment. The rate of synthesis of the nanoparticles is linked directly to the increase in silver ion concentration, but as the concentration exceeds the threshold limit, the rate decreases due to the induction of apoptosis in the cells by various mechanisms. For example, in *B. licheniformis*, the optimum concentration of silver nitrate is found to be 5 mM, above which production decreases. Further, it has been found that alkaline conditions enhance nanoparticle synthesis, and maximum synthesis is observed at pH 10 (Gurunathan *et al.*, 2009 a,b), beyond which synthesis decreases. Moreover, agitation is not required, as the alkaline environment is sufficient to increase the rate and production of nanoparticles. Both the chemical and biological synthesis methods can control the size of the nanoparticles. With variation in the temperature and the pH of the environment, the size of the synthesized nanoparticles can be varied.

Studies on silver nanoparticles indicate that the size of silver nanoparticles synthesized at 60°C is 15 nm when compared to 50 nm at room temperature. At acidic pH, the size of the nanoparticle synthesized is 45 nm, whereas at pH 10 it is just 15 nm. This phenomenon can be explained as being due to the formation of nucleation centres. The nucleation centre increases at higher temperature and pH. As the nucleation centre increases, the reduction of silver ion to silver nanoparticle also increases (Gurunathan *et al.*, 2009a,b).

Applications of Nanoparticles

The unique properties of nanoparticles enable them to encompass innumerable applications.

In the medical scenario, biologically synthesized nanoparticles are found to be more effective. The antimicrobial activity of silver nanoparticles can be exploited and they could be used for wound dressings, bandages, ointments, food containers, water treatment, implant coatings and many other uses. A few also have FDA (Food and Drug Administration) approval. Due to their anti-angiogenic properties, they have been applied in visual impairment cases like diabetic retinopathy (Gurunathan *et al.*, 2009a). The anticancerous properties of silver nanoparticles stand out above all other properties, as they have innumerable medical applications for cancer therapy (Gusseme *et al.*, 2010). Silver nanoparticles are also found to inhibit certain viruses like hepatitis B (Lu *et al.*, 2008), respiratory syncytial virus (Sun *et al.*, 2008), herpes simplex virus type 1 (Baram-Pinto *et al.*, 2009) and monkey pox virus (Rogers *et al.*, 2008). Silver nanoparticles may also be used as coatings for solar energy absorption, in batteries as intercalating materials, as an optical receptor, a chemical reaction catalyst and also in biolabelling (Klaus *et al.*, 1999).

Applications of gold nanoparticles can be extended for their anti-angiogenic, antimalarial and anti-arthritic properties (Navarro *et al.*, 1997; Mukherjee *et al.*, 2005; Tsai *et al.*, 2007). They can be used as an agent for biohydrogen production (Zhang *et al.*, 2007), as sensors, as a photochemical material (Kuwahara *et al.*, 2001), as a photo catalyst (Kamat, 2002), as chromopores (Anil Kumar *et al.*, 2007), and in gene delivery (Ghosh *et al.*, 2008) and biolabelling. Cancer detection and treatment are the major medical applications of gold nanoparticles.

Nanoparticles can be used as effective catalysts. Chemiluminescence emission from the luminal-H₂O₂ system was enhanced by the catalytic activity of silver nanoparticles (Guo *et al.*, 2008). Gold nanoparticles of less than 5 nm in size, stuck over base metal oxide or carbon, form active catalysts. Gold nanoparticles are used in gas sensors for their extreme property to detect a number of gases like CO, NO_x (Thompson, 2007). Silver nanoparticles coated with Pd are used as a catalyst for the remediation of trichloroethane (TCE) and organic pollutants in groundwater (Nutt *et al.*, 2005).

Certain enzymes can be immobilized on nanoparticles, which increases their application tremendously as they increase their stability. They provide sustained release of enzymes and also protect immobilized enzymes from degradation, which extends their application to drug delivery (Tamber *et al.*, 2005). In certain filters, nanoparticles are impregnated to increase the antimicrobial property of the filter and prevent the formation of biofilm. Gold nanoparticles, due to their interesting electrical properties, can also be used as novel biosensors (Guo and Wang, 2007).

Future Prospects

Nanotechnology is a booming field as a large amount of money is being invested by both the public and private sectors. Additionally, applications of nanotechnology in health care and life science have flourished greatly in recent years and have a great potential to revolutionize the health sector. Nanoparticles comprise properties different from those of bulk materials, thus increasing their application on a large scale. The 21st century has already seen the advent of nanoparticles in our daily lives, as they are used in many cosmetics (Perugini *et al.*, 2002), therapeutics (Czupryna and Tsourkas, 2006), drug delivery to specific sites in the body and gene delivery systems (Jin and Ye, 2007), biocompatible replacements for body parts and fluids, material for bone and tissue regeneration, and biosensors (Prow *et al.*, 2006). Nanoparticles are efficient drug-delivering vehicles, as they have the potential to infiltrate the blood-brain barrier (BBB) and deliver drugs to the brain without any loss in the characteristics of the drug molecule (Lockman *et al.*, 2004). This phenomenon enables nanoparticles to treat brain diseases effectively. Nanotechnology has great impact in the agricultural field, as pesticides formulated at nanoscale have greater and improved efficiency. The antimicrobial, antifungal, anti-inflammatory and antiviral properties extend their application for treating many lethal diseases. Even cancer treatment can be made easy with the advent of nanoparticles. Further, the applications of nanoparticles

keep increasing day by day, especially in environmental and medical scenarios. Thus, in the near future, nanobiotechnology will dominate due to its immense, wide-scale applications.

Conclusion

The field of nanobiotechnology has broadened after the emergence of nanoparticles in biogenesis. This eco-friendly approach possesses several advantages, such as low toxicity, high yield and simplicity in synthesis. Also, various bioresources are available for the synthesis of nanoparticles, like bacteria, fungi, plants, algae and even viruses. Studies have revealed that bacterial synthesis of nanoparticles has greater significance to other

biological resources. *In vitro* synthesis has been found to be comparatively more efficient than *in vivo* synthesis. Also, the size of the nanoparticles can be controlled easily in biogenesis. Biologically synthesized nanoparticles can be used readily in medical applications due to their minimal toxicity. This environment-benign technology can also be applied industrially. Thus, the development of a greener route to nanoparticle synthesis has been a boon to mankind and has a strong impetus for the future.

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3 Role of Natural Products in Green Synthesis of Nanoparticles

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Introduction

Nanotechnology is one of the most active fields of research in materials science. Nanoparticles have completely new and improved properties based on their size, distribution and morphology. Nanotechnology is developing day by day and is making a prominent impact on our lives. Applications of nanoparticles, particularly nanomaterials, are being enhanced rapidly (Nalwa, 2000; Jain *et al.*, 2009). Metal nanoparticles have been found to have incredible sensitivity in the field of biomolecular detection, diagnostics, therapeutic antimicrobials, catalysis and microelectronics (Jain *et al.*, 2009; Sundrarajan and Gowri, 2011).

The synthesis of metal nanoparticles has attracted the attention of scientists in various fields, particularly in the chemical, physical, materials, biological and medical, optical, engineering and mechanical sciences (Jain *et al.*, 2009; Sundrarajan and Gowri, 2011). The properties of nanoparticles depend mainly on their size, shape, morphology, composition and crystalline phase, as well as surface area. The high surface area and fractions of atoms/molecules of metal nanoparticles are responsible for their unusual properties such as electronic,

catalytic and magnetic activities, as well as having wide applications in water and air purification (Jain *et al.*, 2009; Sundrarajan and Gowri, 2011). Metal oxide nanoparticles like titania nanoparticles possess interesting chemical stability and have optical, dielectric, catalytic and biological properties, as well as being used as pigment, fillers, catalyst supports and photocatalysts (Sundrarajan and Gowri, 2011).

Metal and metal oxide nanoparticles are usually synthesized by various physiochemical methods. Some of the synthetic methods commonly used are the sol-gel, non-sputtering, reduction and electrochemical techniques (Sundrarajan and Gowri, 2011). Besides these, a number of other methods are also available for the synthesis of metal nanoparticles; for example, reduction in solutions, chemical and photochemical reactions, electrochemical and thermal decomposition, sonochemical and radiation- and microwave-assisted processes. However, these methods have limitations as they are costly, toxic and hazardous, and require high energy and pressure.

Nevertheless, the routinely physicochemical techniques for nanoparticle production, such as photochemical reduction, laser ablation, electrochemistry, lithography

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and high-energy irradiation, either remain expensive or employ hazardous substances such as organic solvents and toxic reducing agents like sodium borohydride and *N,N*-dimethylformamide. In addition, due to the high surface energy of nanoparticles, these tend to form aggregates, so surface passivation and capping reagents are frequently added to the reaction systems to avoid coalescence. Nanotechnology also requires the synthesis of nanomaterials of different chemical compositions, sizes and morphology, with an excellent control over these characteristics. The development of reliable, eco-friendly processes for the synthesis of nanomaterials is an important aspect of nanotechnology (Bhattacharya and Gupta, 2005).

Due to the growing need to minimize or eliminate the use of environmental risk substances, as green chemistry principles describe, the synthesis of nanoparticles using biological entities has received increasing attention in the last decade (Mohanpuria *et al.*, 2008). The biosynthetic procedures involve either living organisms, such as bacteria, fungi and plants, or biomass, like plant extracts. Biological syntheses of nanoparticles have emerged as a simple alternative to more complex physico-chemical approaches to obtain nanomaterial. The interactions between metals and microorganisms have been well studied, and the ability of microorganisms to accumulate metals is already employed in biotechnological processes such as bioremediation and bioleaching (Gericke and Pinches, 2006). The environment-friendly approach has much more importance due to its economic advantage and its biocompatibility and viability in the long run, as well as its lack of negative effects during application, particularly in the medical field. Green synthesis of nanoparticles is an unmatched approach where redox is the main reaction (Sundrarajan and Gowri, 2011). Phytochemicals with anti-oxidant or reducing properties and microbial enzymes are usually responsible for the preparation of metal nanoparticles (Sundrarajan and Gowri, 2011). The underlying eco-friendly principles for the green synthesis of nanoparticles are the choice of the solvent, reducing agent and non-toxic material. Green synthesis has been achieved with bacteria, fungi and

actinomycetes (Sundrarajan and Gowri, 2011). The use of plant extracts for nanoparticle synthesis obtained from neem, *Camellia sinensis*, *Coriandrum sativum*, *Nelumbo nucifera*, *Ocimum sanctum* and several other species has been studied. Plant extracts have advantages such as ease of availability and safe handling, and they possess a broad viability of natural products. The phytochemicals responsible for the synthesis of nanoparticles are mainly flavonoids, terpenoids, ketones, aldehydes and amides (Sundrarajan and Gowri, 2011).

Green synthesis has paved the way for the sustainable safe production of nanoparticles and has been proven the better method due to its slower kinetics, ease of manipulation and control over crystal growth for nanoparticle stabilization. The use of environment-friendly materials, particularly plant extract, fungi, bacteria and enzymes, for the synthesis of metal nanoparticles has numerous benefits in pharmaceutical and other biomedical applications. Green synthesis is superior to chemical and physical methods as it is environment friendly, cost-effective, easily scaled up for large-scale synthesis and there is no need to use high pressure, energy, temperature or hazardous chemicals (Singh *et al.*, 2010).

Biosynthesis of Nanoparticles

Nanoparticles can be synthesized using pure chemical compounds, but this method has many limitations, such as being costly and hazardous to health. An alternative method for the green synthesis of nanoparticles using a biological source has been discovered recently. The biological production of metal nanoparticles is becoming a very important field in chemistry, biology and materials science. Metal nanoparticles have been produced physically and chemically for a long time. However, their biological production has been investigated only very recently. The biological reduction of metals by plant extracts has been known since the early 1900s. However, the reduction products have not yet been studied. The synthesis of metal nanoparticles using natural products has been shown to produce similar

results as those produced by chemical or physical methods. The utilization of plant extracts for the biosynthesis of metal nanoparticles has gained considerable importance due to the enhancement of the chemical, physical and biological properties of particles synthesized by this method. Metal nanoparticles used as conductors and sensors have great importance for their unusual size and shape-dependent properties. They are also found to have remarkable applications in biological and chemical sensing, even of a single molecule, controlled drug delivery catalysis and drug testing and diagnostics. Metal nanoparticles are attractive due to their ease of synthesis, modifications and size as well as shape distribution (Bar *et al.*, 2009; Kumar *et al.*, 2011).

The biosynthesis of nanoparticles has received considerable attention due to the dire need for environmentally proven technologies in material synthesis. Therefore, to develop environment-friendly processes for nanoparticle synthesis without using toxic chemicals is the growing need of the day (Bar *et al.*, 2009; Kumar *et al.*, 2011).

The environmentally sustainable synthesis of metal nanoparticles has led to a few novel approaches. One of the fundamental steps in the biosynthesis of nanoparticles is bioreduction. Green nanoparticle synthesis, using microorganisms and plant extracts or natural compounds, has been conducted for many reasons, including eco-friendly ease of handling and cost-effectiveness. Further, it is also superior by eliminating the processes of maintaining microbial cultures and can also be suitably set up for large-scale nanoparticle synthesis (Ingle *et al.*, 2009).

There have been recent reports on the biosynthesis of metal nanoparticles using plant extracts. Nanoparticles have been synthesized using various natural products like extracts from the leaves of coriander (*Coriandrum sativum*), *Aloe vera*, lemongrass (*Cymbopogon citratus*), leguminous shrub (*Sesbania drummondii*), *Cinnamomum camphora*, *Emblica officianalis*, *Mangifera indica*, *Hibiscus rosa-sinensis*, *Murraya koenigii* and *Ocimum sanctum*, as well as purified compound extracted from henna leaves (*Lawsonia*

inermis), green tea (*Camellia sinensis*), natural rubber, neem leaf broth and starch (Bar *et al.*, 2009; Kumar *et al.*, 2011).

Potential of Plant Extracts in Nanoparticle Synthesis

A wide variety of physical and chemical processes have been developed for the synthesis of metal nanoparticles, but these methods are expensive and require the use of toxic and aggressive chemicals as reducing agents. Therefore, green chemistry should be integrated into nanotechnologies, especially when nanoparticles are to be used in medical applications such as imaging, tissue repair, drug delivery, disinfection and metal sensing. The manufacturing of nanoparticles under totally 'green' principles can be achieved via the selection of an environmentally acceptable solvent system with eco-friendly reducing and stabilizing agents. Therefore, biological approaches to nanoparticle synthesis have been suggested as valuable alternatives to physical and chemical methods. Most previous studies employed biomolecules (proteins, amino acids, carbohydrates and sugars), different types of whole cells of various microorganisms or dissimilar plant resources (roots, leaves, flowers, bark powders, seeds and fruit) for the synthesis of metal nanoparticles. In particular, naturally grown plant species, which are a vital source of phytochemicals, may serve as environmentally benign reservoirs for the production of metallic nanoparticles. Further, it minimizes hectic processes and does not need elaborate processes, multiple purification steps and maintenance of microbial cell cultures (Ghodake and Lee, 2011).

An important branch of the biosynthesis of nanoparticles is the potential of plant extracts in the biosynthesis reaction; for example, the synthesis of nanoparticles using purified apiin compound, extracted from plants such as henna leaf at ambient conditions. Using green tea, *Camellia sinensis*, extract as a reducing and stabilizing agent produced gold nanoparticles and silver nanostructures in aqueous solution at

ambient conditions. Plant extracts from live lucerne, the broths of lemongrass, geranium leaves and others have served as green reactants for nanoparticle synthesis. The reaction of aqueous AgNO₃ with an aqueous extract of leaves of a common ornamental geranium plant, *Pelargonium graveolens*, formed Ag nanoparticles. A vegetable,

Capsicum annum L., was also used to synthesize Ag nanoparticles. The synthesis of silver and gold nanoparticles by an environmentally friendly process has also been studied (Sharma *et al.*, 2009; Logeswari *et al.*, 2012). The potential plant extracts used in nanoparticle synthesis are summarized in Table 3.1.

Table 3.1. Potential plant extracts used in nanoparticle synthesis.

S. No	Plant species	Parts/extracts	Nanoparticle synthesized	Reference
1.	<i>Cinnamomum camphora</i>	Leaf extracts	Au-NPs	Kumar <i>et al.</i> , 2011
2.	<i>Emblica officianalis</i>	Whole-plant extracts	Au-NPs	Kumar <i>et al.</i> , 2011
3.	<i>Mangifera indica</i>	Leaf extracts	Au-NPs	Kumar <i>et al.</i> , 2011
4.	<i>Hibiscus rosa-sinensis</i>	Whole-plant extracts	Au-NPs	Kumar <i>et al.</i> , 2011
5.	<i>Murraya koenigii</i>	Leaf extracts	Au-NPs	Kumar <i>et al.</i> , 2011
6.	<i>Ocimum sanctum</i>	Whole-plant extracts	Au-NPs	Kumar <i>et al.</i> , 2011
7.	<i>Zingiber officinale</i>	Whole-plant extracts	Au-NPs	Kumar <i>et al.</i> , 2011
8.	<i>Camellia sinensis</i>	Whole-plant extracts	Ag-NPs	Logeswari <i>et al.</i> , 2012
9.	<i>Azadirachta indica</i>	Leaf extracts	Ag-NPs	Logeswari <i>et al.</i> , 2012
10.	<i>Azadirachta indica</i>	Whole-plant extracts	Ag-NPs	Logeswari <i>et al.</i> , 2012
11.	<i>Hevea brasiliensis</i> (natural rubber)	–	Ag-NPs	Logeswari <i>et al.</i> , 2012
12.	Starch	–	Ag-NPs	Logeswari <i>et al.</i> , 2012
13.	<i>Jatropha curcas</i>	Whole-plant extracts	Ag-NPs	Logeswari <i>et al.</i> , 2012
14.	<i>Quercus virginiana</i>	Whole-plant extracts	Au-NPs	Logeswari <i>et al.</i> , 2012
15.	<i>Magnolia grandiflora</i>	Whole-plant extracts	Au-NPs	Logeswari <i>et al.</i> , 2012
16.	<i>Pueraria lobata</i>	Whole-plant extracts	Au-NPs	Logeswari <i>et al.</i> , 2012
17.	<i>Pinus taeda</i>	Whole-plant extracts	Au-NPs	Logeswari <i>et al.</i> , 2012
18.	<i>Pelargonium graveolens</i>	Whole-plant extracts	Ag-NPs	Safaepour <i>et al.</i> , 2009
19.	<i>Trachyspermum ammi</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
20.	<i>Papaver somniferum</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
21.	<i>Crossandra infundibuliformis</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
22.	<i>Acalypha indica</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
23.	<i>Rhizophora mucronata</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
24.	<i>Mentha piperita</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
25.	<i>Stevia rebaudiana</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
26.	<i>Chenopodium album</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
27.	<i>Cassia fistula</i>	Whole-plant extracts	Ag-NPs	Vijayaraghavan <i>et al.</i> , 2012
28.	<i>Terminalia chebula</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
29.	<i>Macrotyloma uniflorum</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
30.	<i>Anacardium occidentale</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
31.	<i>Cinnamomum zeylanicum</i>	Bark extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
32.	<i>Allium sativum</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
33.	<i>Murraya koenigii</i>	Leaf extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
34.	<i>Mangifera indica</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
35.	Mushroom	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
36.	<i>Coleus amboinicus</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
37.	<i>Medicago sativa</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
38.	<i>Citrus sinensis</i>	Whole-plant extracts	Ag-NPs	Mohan <i>et al.</i> , 2012
39.	<i>Coccinia grandis</i>	Leaf extracts	Ag-NPs	Arunachalam <i>et al.</i> , 2012
40.	<i>Citrullus colocynthis</i>	Whole-plant extracts	Ag-NPs	Satyavani <i>et al.</i> , 2011

Antioxidants (Reductants) and Nanoparticle Synthesis

Natural antioxidants such as ascorbic acid, α -tocopherol, carotenoids, flavonoids and other phenolic constituents are effective in their mode of action. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have begun to be restricted. Vitamin E is an effective natural antioxidant, but it has limited usage. Therefore, there is considerable interest in the food and pharmaceutical industries for the development of antioxidants from natural sources (Haraguchi *et al.*, 1997).

Many plant species contain different structure types of antioxidative compounds (reductant) like flavonoids, coumarins, xanthenes, anthraquinones and terpenoids. Flavonoids are naturally occurring phenolics, which are widely distributed in a variety of plants at high levels and are commonly present in vegetables, fruit, and beverages such as tea and wine. Antioxidative plant phenolics, different types of flavonoids, for example flavone, flavanone, flavonol, flavanonol, and other classes having 5,7,3',4'-hydroxy substituents, have shown a strong reducing and antioxidant capacity. Among them, quercetin (1), isorhamnetin (2), kaempferol (3) and morin (4) have shown potent antioxidant power (Tringali, 2001c). Isoflavones are also major members of naturally occurring flavonoids. Isoflavan derivatives glabridin (5), hispaglabridin A (6), hispaglabridin B (7), 4'-O-methylglabridin (8) and 3'-hydroxy-4'-O-methylglabridin (9) have shown potent antioxidative activity (Tringali, 2001c) and can be fruitful in nanoparticle synthesis (Fig. 3.1).

Another class of natural product, coumarins, which are also called benzopyrones, are found mostly in the plant kingdom. Coumarins with various hydroxyls and other substituents have strong antioxidative property. Among the plant-derived coumarins, O-dihydroxy substituents, esculetin (10), daphnetin (11) and fraxetin (12), were found to be the most active (Tringali, 2001b). Similarly, the xanthenes class of natural product consists of pyrone-like flavonoids and exhibits a wide range-reducing property. Among them, 1,2,5-trihydroxyxanthone (13), 1,2-dihydroxy-5,6-dimethoxyxanthone (14)

and 1,8-dihydroxy-6-dimethoxyxanthone (15) were effective in preventing oxidation (Tringali, 2001b) (Fig. 3.2). This shows that the coumarins and xanthenes have excellent potential for the preparation of metal nanomaterials such as silver or gold nanoparticles.

Phenylpropanoids are distributed widely and unevenly in edible plants and fruit-bearing plants. A variety of phenolic compounds of this class have shown antioxidative capability, particularly phenylpropanoid glycosides. They demonstrated excellent reducing activity and it was noted that their antioxidative activity was potentiated by an increase in the number of phenolic hydroxyl groups in the molecule. It is therefore concluded that the phenylpropanoid compounds can show the best results in the green synthesis of nanoparticles.

Terpenoids are also widely distributed in a variety of plants. In comparison to hydrophilic flavonoids, lipophilic terpenoids have been revealed to possess potent antioxidative activities and protective effects against oxidative stresses. Sesquiterpenoids, 7-hydroxy-3,4-dihydrocadalin (16), 7-hydroxycadalin (17) and 2,7-dihydroxy- ρ -calacoren (18), also showed potent antioxidative activities against linoleic acid autoxidation (Tringali, 2001d). Abietane-type diterpenes, carnosol (19), rosmannol (20), isorosmannol (21), rosmariquinone (22), carnosic acid (23) and epirosmannol (24), were found to be effective antioxidants (Tringali, 2001d). Similarly, totarane-type diterpenes, totarol (25), totaradiol (26), 19-hydroxytotarol (27), totaral (28) and 4- β -carboxy-19-nortotarol (29) (Fig. 3.3), showed potent antiperoxidative activities and were proved to be powerful reductants (Tringali, 2001a,d). The powerful reducing property of the above-mentioned compounds belonging to different classes of natural products justified their use as a good natural source for nanoparticle synthesis.

Natural Products as a Reductant

It can be concluded from the above discussion that many of the plant species contain different structure types of antioxidative compounds (reductant) like flavonoids,

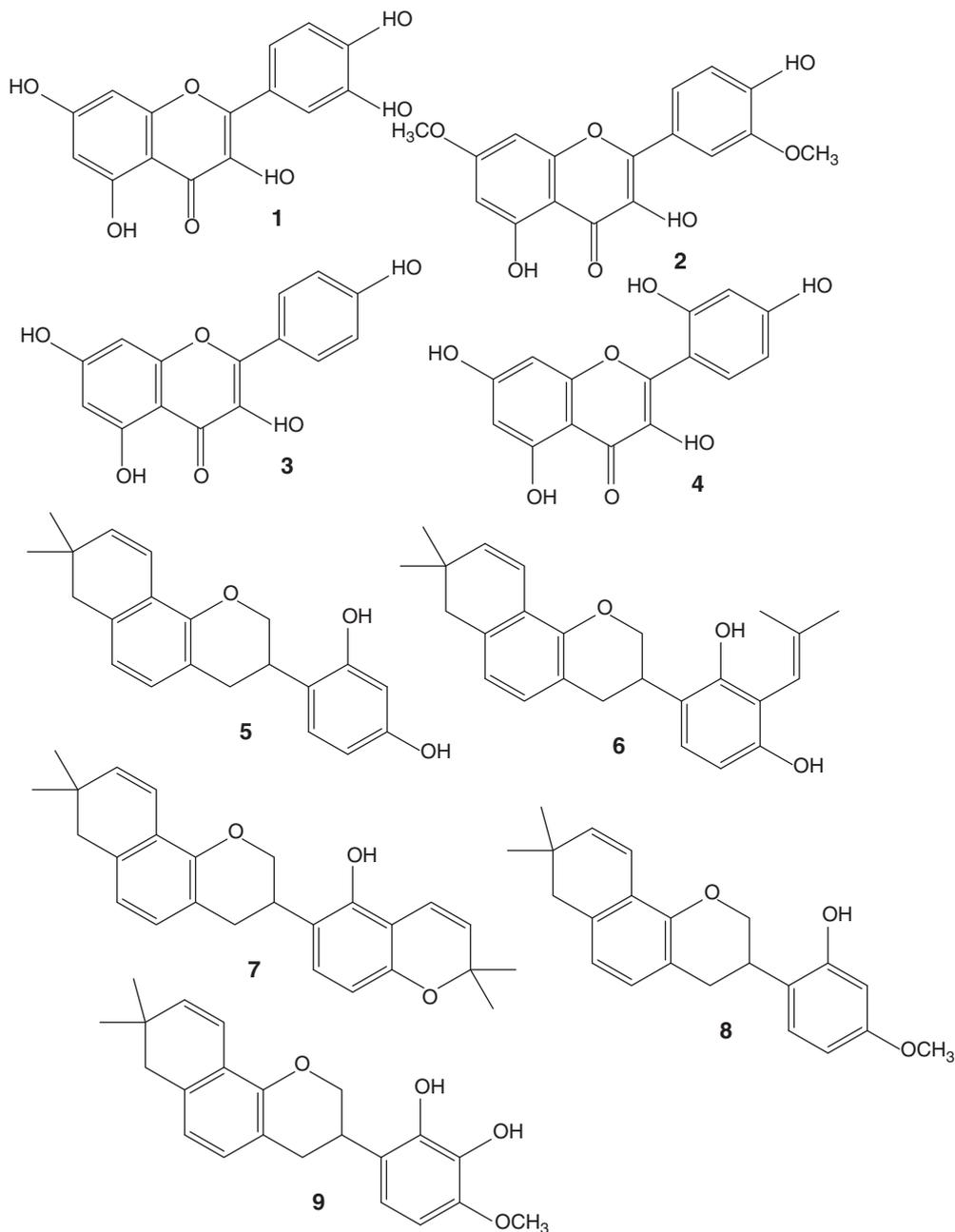


Fig. 3.1. Antioxidant flavonoids from natural sources.

coumarins, xanthenes, anthraquinones and terpenoids. Flavonoids actively scavenge oxygen radicals, terpenoids strongly prevent lipid peroxidation and both have different modes of reduction. The combined effects of

the different nature in hydrophobic terpenoids and hydrophilic flavonoids contribute to the total antioxidative action of these plants. Antioxidative constituents in plants have other various biological activities,

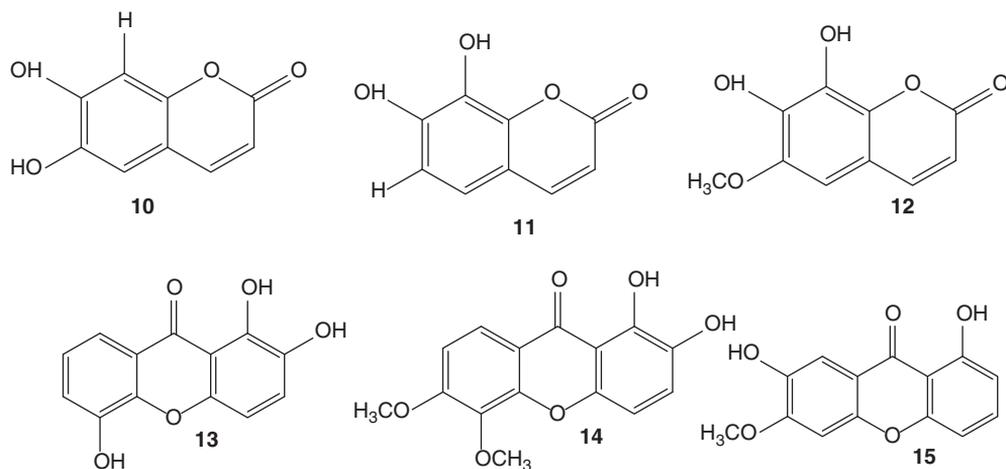


Fig. 3.2. Antioxidant coumarins and xanthenes isolated from natural sources.

especially in the case of flavonoids. These additional activities of antioxidative phytochemicals contribute to their nanoparticle synthesis. A brief discussion about natural products is provided below.

Natural products

Natural products are the complex organic compounds produced by living organisms. They are divided mainly into two categories. First, those compounds that are present in all living organisms and play a key role in cell metabolism and reproduction, called primary metabolites, e.g. nucleic acids, amino acids, carbohydrates, etc. Second, those compounds that are the characteristics of a limited range of species, called secondary metabolites. Primary metabolites have a biological effect within the cell or organism and are mainly responsible for their production. On the other hand, secondary metabolites exert their biological effect on other organisms.

Biologically active compounds have been studied from plants of commercial and medicinal importance. Most of them were secondary metabolites isolated from living sources. A number of bioactive principles have been obtained and structurally identified with the help of various chemical and physical methods. These principles are now the

active molecules in modern medicines. In the past two decades, several compounds such as cyclosporin A, clavulanic acid, mevinnolin, taxol and ivermectin have been discovered by natural product screening approaches.

Chemical Structure and Diversity of Natural Products

The classes of natural products

Secondary metabolites seem to be very diverse, but actually most of them belong to one or another class based on the basic skeleton of their structure. Natural products are classified into different groups on the basis of their biosynthetic origin. The four groups are alkaloids, phenylpropanoids, polyketides and terpenoids. Since more than 200,000 structures of natural products from plants are known, only selected groups and compounds are presented here (Hanson, 2003d).

Alkaloids

Alkaloids are a class of natural product widely found in the plant kingdom. Millions of people around the globe use alkaloids every day by drinking a cup of coffee or tea. Alkaloids are the secondary metabolites biosynthetically derived from amino acids. They

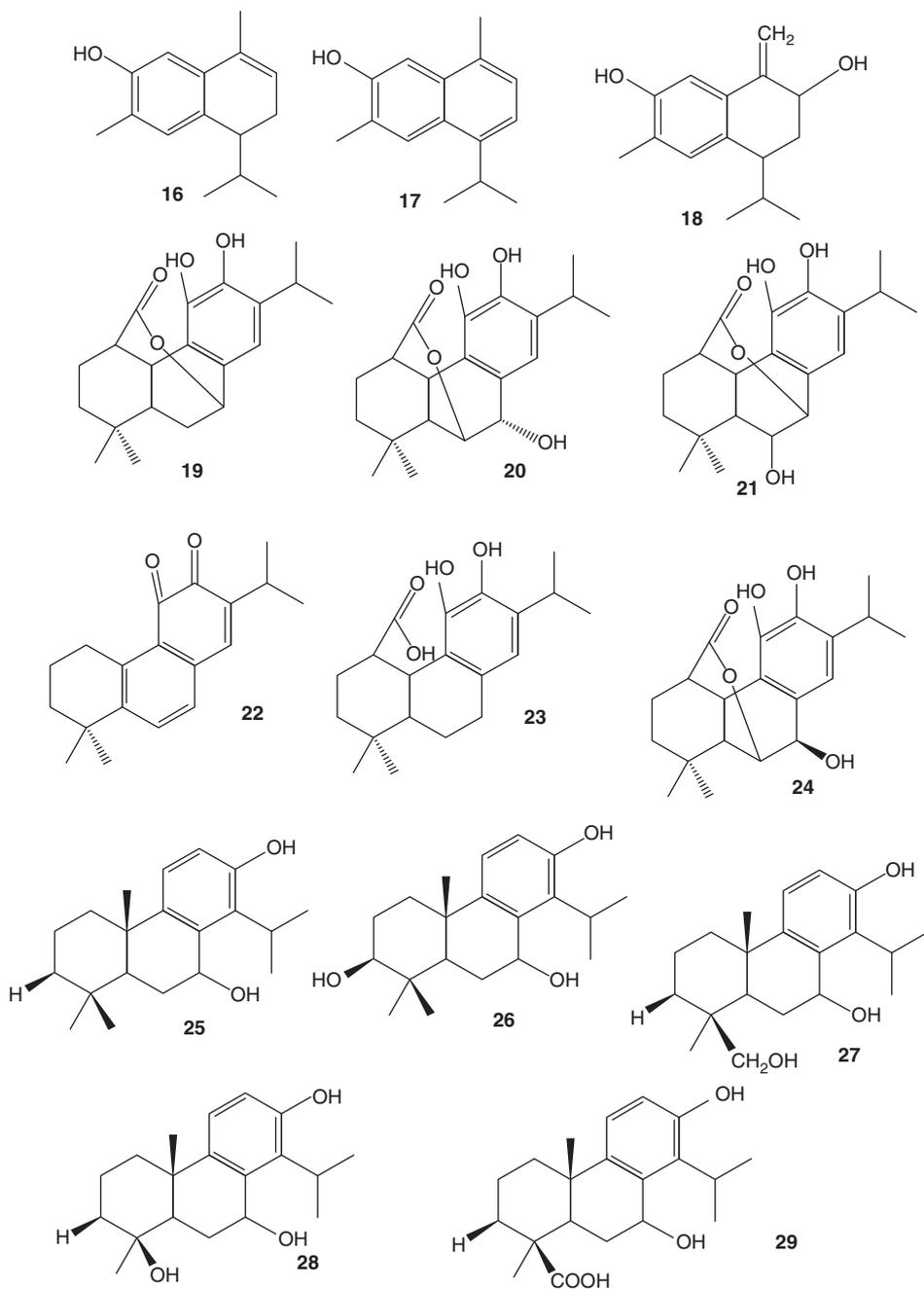


Fig. 3.3. Antioxidant terpenoids from natural sources.

are classified according to the biosynthetic pathways where they are derived from amino acids, which provide their nitrogen atom and part of their skeleton.

The terpenoid, steroid and purine alkaloids are also important. Alkaloids are grouped based either on the plants of origin, like *Aconitum*, *Senecio*, *Cinchona*, *Carare* and

ergot, or on the ring structure; for example, pyrrolidine (30) piperidine (31), tropane (32), pyrrolizidine (33), indol (34), isoquinoline (35) and quinoline (36) alkaloids. Over 5000 alkaloids have been identified in numerous plant families, mostly in the angiosperms particularly found in *Papaver*, *Aconitum*, *Senecio*, *Cinchona*, *Cararea*, *Mandragora*, *Atropa*, *Cephaelis*, *Nicotiana*, *Berberis* and coffee species (Hanson, 2003d) (Fig. 3.4).

Phenylpropanoids

Compounds containing an aromatic ring and at least three carbon side chains are grouped as phenylpropanoids. The simplest compounds

belonging to this class are cinnamic acid (37) and its derivatives, present in almost the entire plant kingdom. The cyclization of side-chain alkenes may lead to the biosynthesis of coumarins such as umbelliferone (38), present in plants of Umbelliferae, and the next type of subclass compound within this class. Oxidative coupling of two phenylpropanoid units may lead to the production of lignan, exemplified by pinoresinol (39) (Fig. 3.5). Lignans are reported mostly from higher plants of the family Pinaceae and Taxaceae.

Flavonoids belong to phenylpropanoids produced biosynthetically by the condensation of simple phenylpropanoid units. Flavonoids are polyphenolic compounds

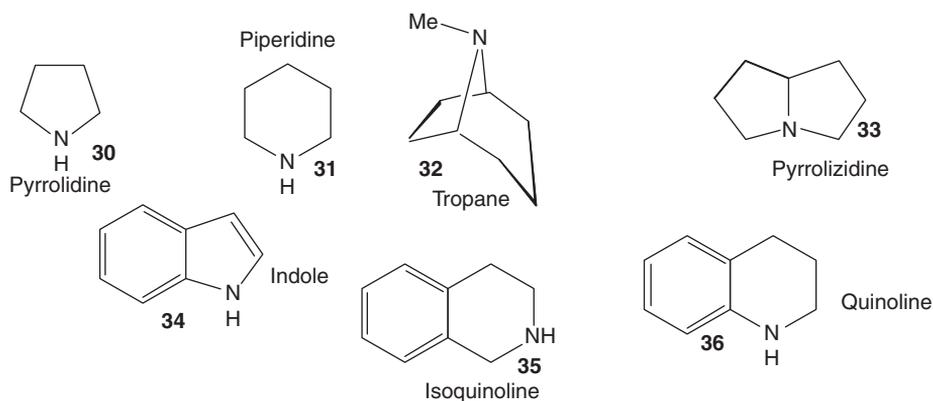


Fig. 3.4. Structures of some common alkaloids.

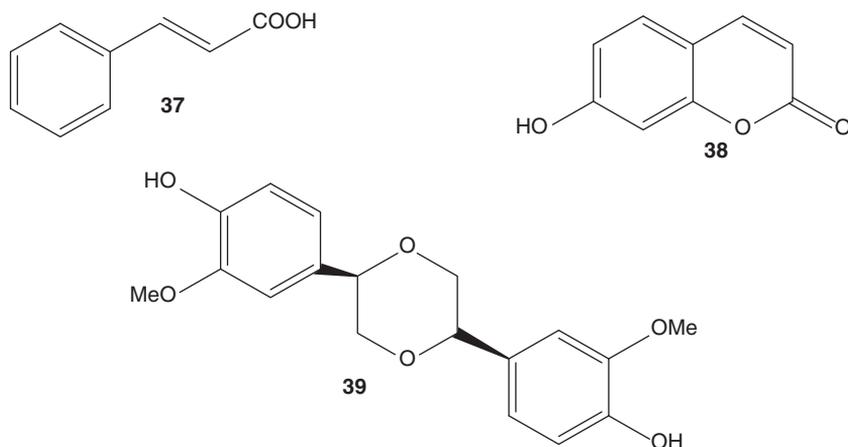


Fig. 3.5. Structures of some common phenylpropanoids.

possessing 15 carbon atoms; two benzene rings joined by a linear three-carbon chain (40). The subclasses of flavonoids differ in the pattern of substitution of ring (C), as well as the level of oxidation, while compounds of the same class or within a class differ in the pattern of substitution of the other two rings (A and B). On the basis of structural arrangement, flavonoids are classified chalcones (41), flavones (42), flavonols (43), flavanones (44), isoflavones, flavanonols, flavan-3-ols and anthocyanidins (45) (Fig. 3.6). Flavonoid is one of the most abundant classes of compound present in higher plants. Most of them are recognized easily as flower pigments in flowering plants. However, their occurrence is not restricted to flowers and could be present in all parts of the plant (Hanson, 2003c).

Polyketides

Polyketoids are natural compounds formed by the stepwise condensation of ethanoate units. The acetate origin of these compounds leads to the formation of an even number of carbon chains. Many plant-based oils contain long-chain carboxylic acids, called fatty acids. Fatty acids are the derivatives of carboxylic acid with aliphatic chains. They are present either in saturated fatty acids, for example butyric (46), lauric (47), myristic (48), palmitic (49), stearic (50) and arachidic (51), or in unsaturated fatty acids such as alpha-linolenic acid (52), linoleic acid (53), arachidonic acid (54), oleic acid (55) and erucic acid (56) (Fig. 3.7). The chain of the skeleton may be short, as butyric acid (four carbons), or long, having as many as 100 carbons. Natural fatty acids have an even number of carbon atoms

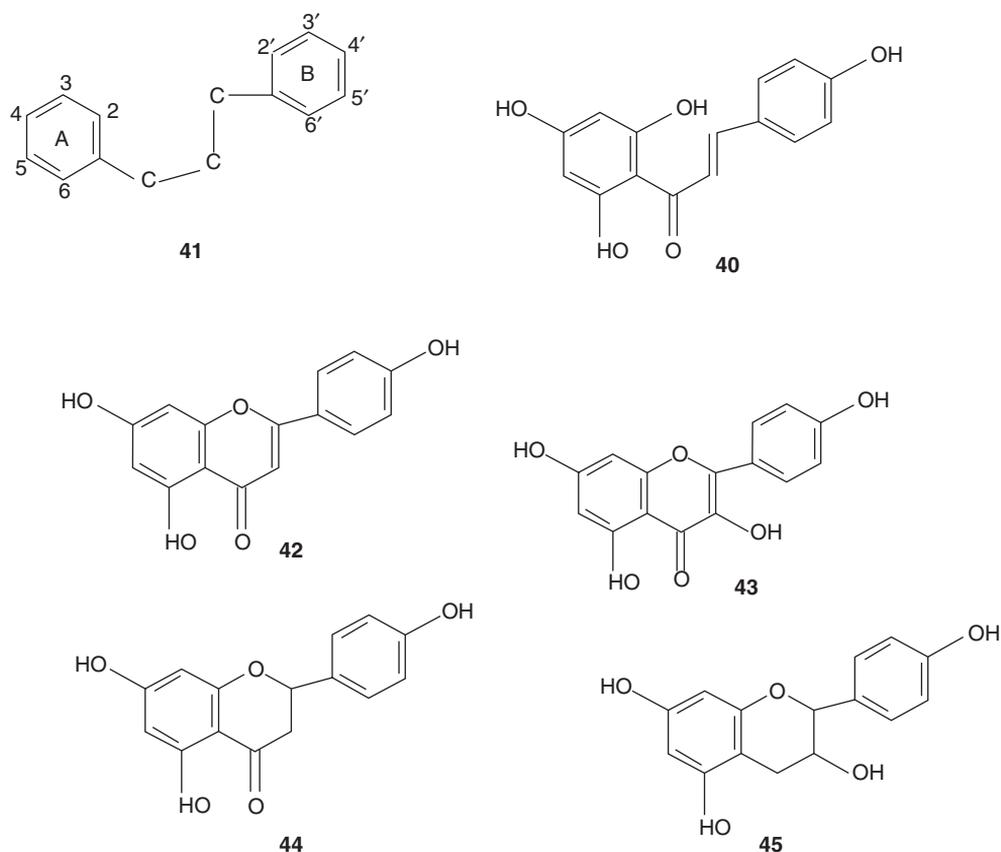


Fig. 3.6. Structures of different classes of flavonoids.

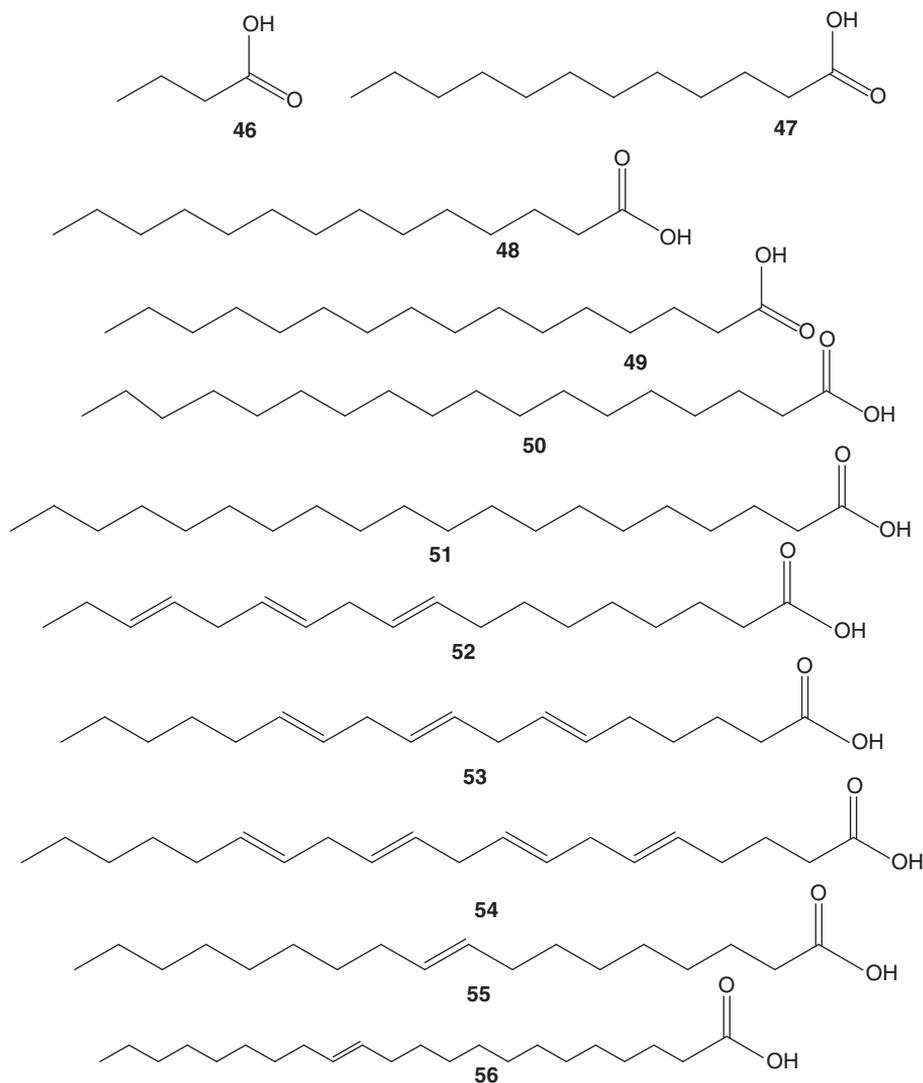


Fig. 3.7. Structures of some common fatty acids.

as their biosynthesis involves acetate, which has only two carbon atoms. Natural fatty acids are also assumed to have at least eight carbon atoms (Hanson, 2003b).

Terpenes and terpenoids

Terpenes cover most of the range of natural products and are one of the largest classes of hydrocarbons, widely distributed in plants and also in some insects, such as

swallowtail butterflies. They are present mainly in turpentine produced from plant resin. Terpenoids are obtained from the oxidation of terpenoids or rearrangement of the carbon skeleton.

Terpenes are widely distributed in the essential oils of a variety of plants, particularly flowers. Essential oils have many applications and are used widely as traditional medicines, food additives, fragrances and in aromatherapy.

Terpenes are made of a small unit called isoprene (C_5H_8). Biosynthetically, they are derived from this small unit. The basic molecular formula of terpene is the multiples of isoprene (C_5H_8) n , where n is the number of linked units. This rule is called the isoprene rule. The terpene is further classified according to the number of carbons, as given below.

Classification starts from a simple unit, as hemiterpenes consist of a single isoprene unit. Isoprene itself is considered the only hemiterpene; other examples of hemiterpene are hemiterpenoids, the derivatives of isoprene such as prenol and isovaleric acid.

Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. Geraniol is the simplest monoterpene alcohol. The geranyl prefix indicates two isoprene units. Monoterpenes are the major components of aroma plants, known as essential oils. Thus, geraniol (57) is the major component isolated from the oil of geranium (*Pelargonium graveolens*), and its isomer, linalool (58), was obtained from a garden herb. Citrol (59) was isolated from lemon oil extracted from lemongrass (*Cymbopogon flexuosus*), whereas

menthol (60) was obtained from mint (*Mentha arvensis*) (Hanson, 2003a) (Fig. 3.8).

Sesquiterpenes consist of three isoprene units and have the molecular formula $C_{15}H_{24}$. Farnesol is an example of sesquiterpene alcohol. This farnesyl prefix indicates three isoprene units. Sesquiterpenes are present in the higher boiling portions of essential oils of aroma plants, particularly the genus *Artemisia*. Examples are exemplified by caryophyllene (61) from clove oil, humulene (62) from hop oil, cederen (63) from cedar wood oil (*Cedrus deodara*) and longifoline (64) from turpentine oil (*Pinus ponderosa*) (Hanson, 2003a) (Fig. 3.9).

Diterpenes are a subclass of terpene and are composed of four isoprene units with the general formula $(C_5H_8)_4$. They are present in wood resins and are derived from geranyl pyrophosphate. Diterpenes like abiaetic acid (65) and pimaric acid (66) are obtained from the resin of *Pinus* and *Abies* species. Other diterpenoids are also reported from *Ajuga*, *Salvia*, *Scutellaria* and *Teucrium*. Diterpene is one of the important sub-classes of terpene because it is composed of biologically active compounds such as retinol (67), retinal (68) and phytol (69) (Hanson, 2003a) (Fig. 3.10).

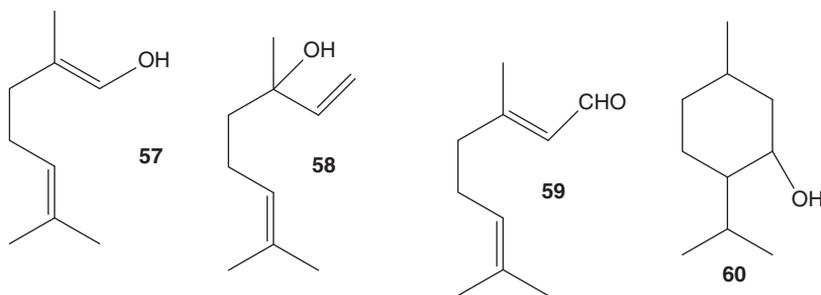


Fig. 3.8. Structures of compounds classified as monoterpenes.

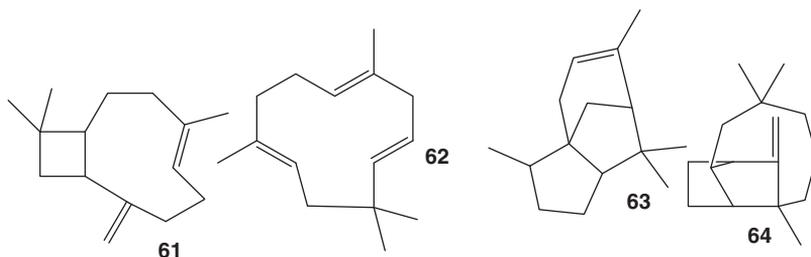


Fig. 3.9. Structures of compounds classified as diterpenes.

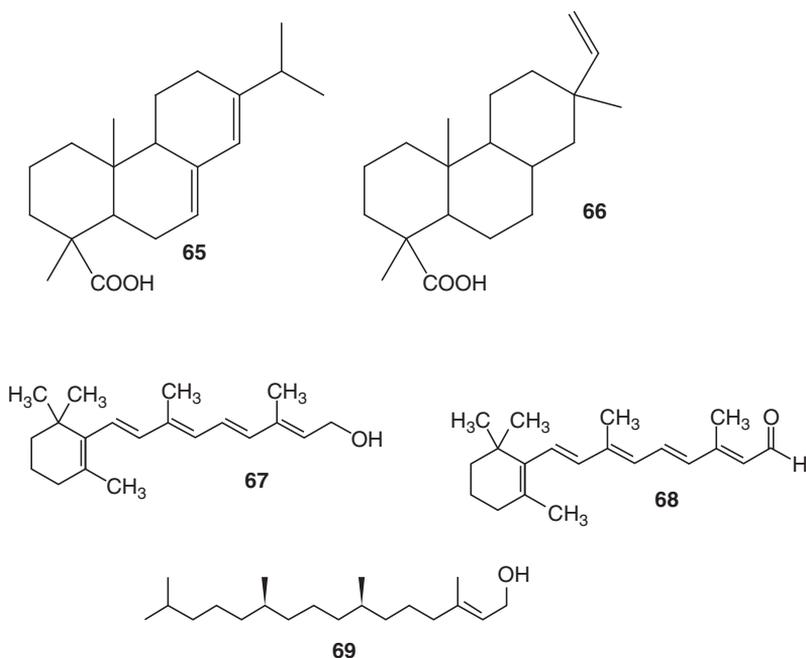


Fig. 3.10. Structures of compounds classified as sesquiterpenes.

Terpenes having 25 carbons and five isoprene units are called sesquiterpenes. They are rare compared to the other sizes.

Triterpenes are made of six isoprene units and have the molecular formula $C_{30}H_{48}$. They are present in the fats of animals and the resins of plants. The linear triterpene squalene (70) is derived from the coupling of two molecules of farnesyl pyrophosphate and is present mainly in shark liver oil. Lanosterol (71) is obtained biosynthetically from squalene, which is the structural precursor of steroids. Amyrins (72) are reported from wood resin and the bark of many trees. Triterpenes are also reported from the Meliaceae and Rutaceae families, as well as fungi (Hanson, 2003a) (Fig. 3.11).

Tetraterpenes are composed of eight isoprene units and have the molecular formula $C_{40}H_{56}$. Examples of tetraterpenes are acyclic lycopene, the monocyclic gamma-carotene and the bicyclic alpha- and beta-carotenes.

Terpenes having many isoprene units are called polyterpene, for example natural rubber and gutta-percha.

Bioassay-guided isolation

Plant secondary metabolites are currently the subject of much research interest, but their extraction as part of a phytochemical or biological investigation presents specific challenges that must be addressed throughout the solvent extraction process. Successful extraction begins with careful selection and preparation of plant samples and a thorough review of the appropriate literature for indications of which protocols are suitable for a particular class of compound or plant species. During the extraction of plant material, it is important to minimize interference from compounds that may interact with the target compounds and to avoid contamination of the extract, as well as to prevent decomposition of important metabolites or artefact formation as a result of extraction conditions or solvent impurities.

Selection, collection and identification of plant materials

A variety of methods are applied in the selection, collection and identification of

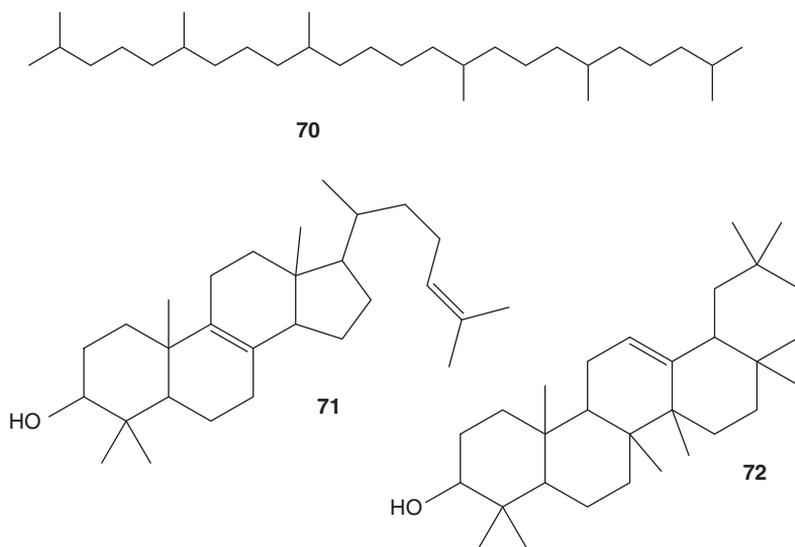


Fig. 3.11. Structures of some triterpenes.

plant species. The reproducibility of phytochemical research is greatly affected by the choice of method. Any flaw in this connection can lead to erroneous results, which can reduce the scientific importance of the study. A specific part of the plant contains specific secondary metabolites. It is necessary to know which part of the plant contains the highest level of metabolite to ensure that the extracts obtained from the plant are representative of the range of phytochemicals; collection of multiple plant parts or the whole plant seems prudent. Quantitative and qualitative variation of the specific class of phytochemical exists among closely related plant species. Therefore, great care is recommended in making broad inference whether a specific class of natural product is present or absent in plant species under investigation. A number of approaches are employed in the selection of plant species for drug discovery, for instance:

1. The investigation of plants traditionally used for food, medicine or poison based on a literature review or interview of local traditional people.
2. The systematic or random collection of plant samples from a comparatively uncharted ecological region.

3. The selection of a plant species that is in a phylogenetic relationship to a species known to have produced a specific compound.

4. Selection based on studies and reports of biological activity of a species given in the literature (Sarker *et al.*, 2006a).

Drying and grinding

Scientific protocols should be used for the drying and grinding of plant materials. The following principles must be followed:

1. The plant material should be dried at temperatures below 30°C to avoid the decomposition of thermolabile compounds.
2. It should be protected from sunlight because of the potential for photochemical transformations.
3. Air circulation around the plant material is essential to avoid heat and moisture accumulation. It is also necessary to use a fan or other means to provide air flow around or through the drying sample.
4. Fresh plant material could also be extracted, but it is advisable to extract it as soon as possible using organic solvents such as methanol (MeOH) or ethanol (EtOH), which will deactivate the enzymes present in the plant. The resulting extract will contain a

significant portion of water and it can be partitioned with an organic solvent that is immiscible with hydroalcoholic mixtures.

5. Small quantities of plant material can be milled using an electric coffee or spice mill, or in a mortar and pestle, in which case the addition of a small amount of sand may aid the process.

6. Large quantities of plant material are usually best carried out using an industrial-scale grinder.

7. Grinding into powder is necessary because it improves the efficiency of extraction by increasing the surface area of the plant material. It also decreases the amount of solvent needed for extraction by allowing the material to pack more densely (Sarker *et al.*, 2006b).

Extraction

Various techniques having a different cost and level of complexity are used to extract plant material. Simple techniques such as percolation and maceration are very effective and economical. However, in some cases more sophisticated and costly extraction techniques are required using specialized equipment such as large-scale steam distillation and supercritical fluid extraction (SFE). Extraction is a continuous process, and once an equilibrium is reached between the concentration of solute inside the plant material and the concentration in the solvent, the extraction stops at that point and will need the replacement of fresh solvent for further extraction. The resulting solution should be filtered to remove any remaining particulate matter. This step is mandatory, irrespective of the extraction technique used. Plant extracts should not be stored in the solvent for long periods, because of the accompanying increased risk of artefact formation and decomposition or isomerization of extract constituents. Extracts can be concentrated at reduced pressure on a rotary evaporator or dried under a stream of nitrogen. If a rotary evaporator is used, it is advisable to keep the water bath temperature below 50°C to prevent the decomposition of thermolabile components. Especially when extracting large amounts of a single sample, the solvent

collected from the rotary evaporator condenser during the concentration of one extraction batch may be recycled for further extraction of that same sample, but use of recovered solvent in the extraction of other samples is not advised, because it may lead to cross-contamination of later extracts. The extract obtained is subjected to evaluation of biological activity. The active fraction/extract is then subjected to chromatography to isolate the active constituents (Sarker *et al.*, 2006c).

Choice of solvent

The choice of solvent for extraction is one of the most important steps. The principle of 'like dissolves like' is the key rule to be followed. The following factors should be considered for the solvent selection of plant materials:

1. Solubility of the target constituents.
2. Safety and ease of working with the solvent.
3. The potential for artefact formation.
4. The grade and purity of the solvent.
5. A solvent is chosen that maximizes the yield of the compounds of interest while minimizing the extraction of unwanted compounds.
6. Literature related to the phytochemical investigation of a particular species, genus or family can provide clues to the choice of solvent. A careful review of the literature related to the species and compound classes under investigation may save time and energy.
7. Solvents with low boiling points are generally easier to use from the standpoint that they are more easily concentrated. Acetone, chloroform (CHCl₃), dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-hexane/petroleum ether evaporate relatively quickly, whereas water and butanol are more difficult to remove (Sarker *et al.*, 2006d).

Fractionation and isolation

Generally, the powdered plant materials need exhaustive extraction with methanol approximately three to four times. The methanolic fraction is suspended in water partitioned with hexane, chloroform or dichloromethane, ethyl acetate and *n*-butanol to obtain fractions (Fig. 3.12). The extracts are usually concentrated

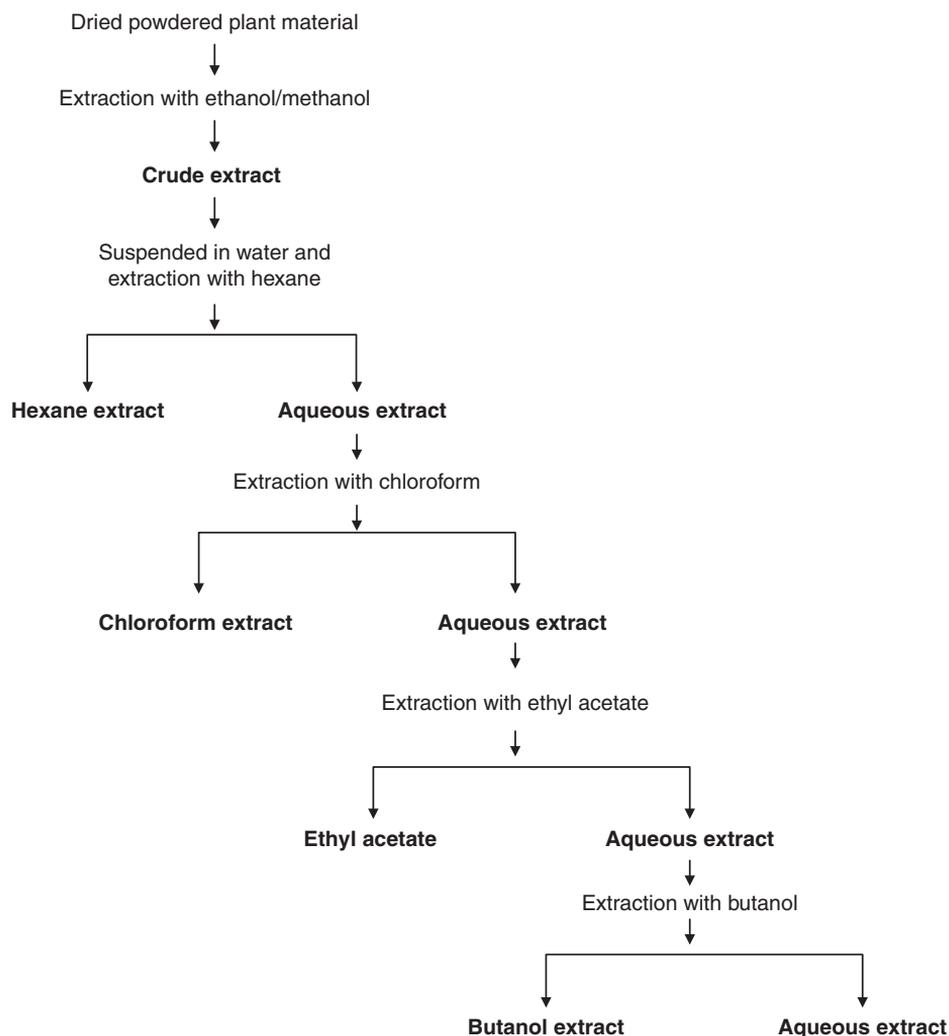


Fig. 3.12. Extraction and fractionation of plant materials.

under vacuum to yield the residue of fractions. The conventional method of chromatography is used for separation. The fractions are loaded on column chromatography over silica gel eluting with solvent in increasing order of polarity of a solvent system already optimized by thin-layer chromatography (TLC). Chromatographic separations are carried out by column chromatography using column silica gel and flash silica gel. Re-distilled commercial solvents and reagents of analytical grade can be used. Fractions

collected from column chromatography are then compiled using TLC. The subfractions are then re-chromatographed until a single spot is obtained on TLC. TLC is usually performed on silica gel GF254 plates. The purity of the samples is checked on the same pre-coated plates. TLC plates can be viewed under ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Dragendorff's solution, ceric sulfate, stannum chloride solution and other spraying reagents are generally used

to detect the spots on the TLC plates. Other methods for separation and purification are gas chromatography (GC), GC mass, high-performance liquid chromatography (HPLC) and LC-mass, high-speed counter-current chromatography (Ali *et al.*, 2011).

Structure Determination and Instrumentation

Isolation and purification are not the final goals for chemists. It is the structure of synthesized and isolated compounds from a natural source that needs to be identified. In fact, this final step is the most difficult. Among these structures, elucidation is the most difficult step. Until the latter half of the 20th century, chemists had no sufficient resources to deal with this problem.

A number of structures were reported to be incorrect, even after long-term trials. Nowadays, the situation is quite changed due to the development of a variety of spectroscopic techniques. Nuclear magnetic resonance (NMR) especially is one of the best methods for structure elucidation. X-ray crystallography has been found to be most useful for solid examples. Prior to spectroscopic methods, structure determination by comparison with compounds of a known structure based on identity in physical and chemical properties was a common practice. This approach failed in the case of structure determination of a wholly new compound. These traditional methods being tedious, complex and time-consuming have been replaced nowadays by more sophisticated assay and safe spectroscopic and diffraction methods, mainly ultraviolet-visible (UV-Vis), infrared (IR), NMR and mass spectrometry (MS).

UV-Vis spectroscopy deals with the excitation of the electrons in molecules. UV determines the unsaturation and conjugation of organic compounds. This method is very sensitive and hence very suitable for analytical purposes.

Infrared spectroscopy also does not provide absolute structure elucidation but gives information about the presence of functional groups. The IR spectrum may be divided into two parts. The region from 4000 to 1600 cm^{-1} is

known as the functional group region, while the region from 1600 to 625 cm^{-1} is called the fingerprint region. The functional group region for various functionalities is summarized in Table 3.2. The functional group region gives information about the class of organic compounds, whereas the fingerprint region is used to confirm them. The fingerprint region provides a set of absorption bands that is uniquely characteristic of each compound. NMR spectroscopy is the only spectroscopy that gives information about the exact structure elucidation. NMR depends mainly on active nuclei. On the bases of active nuclei, NMR spectroscopy is termed after the active nucleus used, i.e. ^1H , ^{13}C , ^{11}B , ^{14}N , ^{15}N , ^{17}O , ^{19}F , ^{23}Na , ^{29}Si , ^{31}P , ^{35}Cl , ^{113}Cd , ^{129}Xe , ^{195}Pt NMR. ^1H and ^{13}C NMR are the most common and useful used in organic chemistry.

The ^1H NMR spectrum provides three types of information about the compounds: (i) the kinds of proton; (ii) the number of protons; and (iii) the number of surrounding protons. The type of proton can be identified by the chemical shift (Table 3.3). The number of protons causing the particular absorption peak can be determined by the area under the peak. The area under the absorption peak is directly proportional to the number of protons giving rise to that peak. The number of neighbouring protons can be determined from the spin-spin splitting patterns. The coupling constant plays a key role in identifying the types of surrounding proton. Similarly, the ^{13}C NMR gives information about the nature of carbon, whether it is saturated, unsaturated or even quaternary. Two-dimensional NMR spectroscopy gives information of the connectivity of protons and carbons. Combining all this information and these data, one can establish the tentative structure of organic compounds. The final structure can be confirmed using a set of four spectroscopic techniques. Mass spectrometry tells us about the molecular mass and molecular formula; IR tells us about the functional groups, whereas UV-Vis spectra give information about unsaturation and conjugation. Using this information as a support to NMR spectroscopy, the structure of organic compounds can be established easily.

Table 3.2. Expected region of frequencies of various functional groups in IR spectroscopy.

Infrared correlation chart			
Stretching frequencies of functional groups			
(A) SINGLE BOND TO HYDROGEN			
Base value		RANGE	SINGLE BOND TO HYDROGEN
O-H	3600	3750–3000	{ O-H { Alcohols, phenols Free H-Bonded Carboxylic acids 3650–3600 (m-s) 3400–3200 (m) 3300–2500 (m)
N-H	3500		
			{ N-H { Pri-, Sec-, Amines Amines 3500 (m) 3000–3500 (w-m)
S-H		2550	
C-H	3000	3300–2700	{ C \equiv C — H Alkyne 3300 (m-s) { C = C — H Alkene 3100–3080 (w) { Ar-H Aromatic 3030–3000 (w) { CH ₃ , CH ₂ Alkane 3000–2850 (s) { — C(=O) — H Aldehydic 2700–2800 (w)
(B) TRIPLE BONDS			
C \equiv C	2250	2400–1900 (2500–1950)	TRIPLE BONDS C \equiv C Alkyne 2250–2100 (m-w) C \equiv N Nitrile 2260–2210 (w)
C \equiv N	2150		
X = Y = Z			
(C) DOUBLE BONDS			
C = O	1715	1900–1600	DOUBLE BONDS (a) Carbonyl group: Aldehyde 1740–1720 (s) Ketone 1725–1705 (s) Carboxylic acid 1750–1735 (s) Ester 1700–1735 (s) Amides 1700–1640 (s) Anhydrides 1810, 1760 (two)(s) Acid halides 1890–1815 (s)
C=C	1615	1675–1580	(b) Other double bonds C=C Alkene 1680–1600 (w-m) Aromatic 1600–1400 (w-m) C=N Schiff's base 1640 (w-m) N=N Azo group 1600–1500 (s) N=O Nitro group 1400–1300 (s)
C=N	1615		
N=O			

Notes: Key to intensities: (s) strong, (m) medium, (w) weak, (v) variable.

Potential of Pure Plant-based Natural Products in Metal Nanoparticle Synthesis

The synthesis of metal nanoparticles by an environmentally friendly procedure involving the reduction of metal ion by plants like *Azadirachta indica*, *Glycine max*, *Cinnamomum zeylanicum*, *Camellia sinensis*,

Ocimum tenuiflorum, *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica* and *Citrus sinensis* has been studied (Logeswari *et al.*, 2012). This green synthesis of nanoparticles has shown many advancements over the chemical and physical methods, as it is easy to handle, less toxic, less expensive and, further, there is no need for pressure, energy, temperature or toxic chemicals (Jayaseelan *et al.*, 2011).

Table 3.3. Expected chemical shift of different types of protons.

Characteristic proton chemical shifts		
Type of proton	Structure	Chemical shift (ppm)
Cyclopropane	C ₃ H ₆	0.2
Primary	R-CH ₃	0.9
Secondary	R ₂ -CH ₂	1.3
Tertiary	R ₃ -C-H	1.5
Vinylic	C=C-H	4.6–5.9
Acetylenic	Triple bond, CC-H	2–3
Aromatic	Ar-H	6–8.5
Benzylic	Ar-C-H	2.2–3
Allylic	C=C-CH ₃	1.7
Fluorides	H-C-F	4–4.5
Chlorides	H-C-Cl	3–4
Bromides	H-C-Br	2.5–4
Iodides	H-C-I	2–4
Alcohols	H-C-OH	3.4–4
Ethers	H-C-OR	3.3–4
Esters	RCOO-C-H	3.7–4.1
Esters	H-C-COOR	2–2.2
Acids	H-C-COOH	2–2.6
Carbonyl compounds	H-C-C=O	2–2.7
Aldehydic	R-(H-)C=O	9–10
Hydroxylic	R-C-OH	1–5.5
Phenolic	Ar-OH	4–12
Enolic	C=C-OH	15–17
Carboxylic	RCOOH	10.5–12
Amino	RNH ₂	1–5

Green synthesis also does not need complicated processes like intracellular synthesis and multiple purification steps to maintain microbial cell cultures (Ghodake and Lee, 2011).

Many reports have been published about the biogenesis of silver nanoparticles using several plant extracts such as discussed earlier. However, there is still a need for an economic, commercially viable as well as an environmentally clean route to synthesize metal nanoparticles and to find the capacity of their pure natural reducing constituents to form silver nanoparticles, and this has not yet been studied (Safaepour *et al.*, 2009). The limited study of the pure natural product for the synthesis of metal nanoparticles has been discussed herein. The purpose of the accumulation of data is to encourage our readers to isolate and utilize pure natural product for the green synthesis of metal nanoparticles.

The plant system is the best example of a nanofactory. Recently, much work has been done with regard to the plant-assisted reduction of metallic nanoparticles and responsible candidate phytochemicals (Jha *et al.*, 2009). The participation of phenolics, proteins and other reducing agents in the synthesis of metal nanoparticles has been speculated. In the biosynthesis of metal nanoparticles, in particular silver and gold, oil of tansy, which contains a variety of terpenes, can be used as an agent in the reduction of metal ion to metal nanoparticles. Reduction may involve the conversion of C O group of the terpenes to -C(O) O group and may be responsible for the reduction of Ag⁺ and Au⁺ to Ag⁰ and Au⁰, respectively (Dubey *et al.*, 2010). It is also possible that terpenoids may play a role in the reduction of metal ions by oxidation of the aldehydic groups in molecules to carboxylic acids (Shankar *et al.*, 2004).

Terpenoids are believed to be the surface-active molecules that stabilize nanoparticles, and reducing these and the sugars present in neem leaf broth possibly enables the reaction of the metal ions (Song *et al.*, 2009). The biological synthesis of anisotropic gold and quasi-spherical silver nanoparticles by using apiin as the reducing and stabilizing agent has been also reported (Kasthuri *et al.*, 2009). Similarly, flavonoids such as quercetin, daizeol and puerarin can act as reductants for the enlargement of gold nanoparticles (Wang *et al.*, 2007).

Silver nanoparticles were synthesized successfully from silver nitrate solution through a simple green route using the latex of *Jatropha curcas* as a reducing as well as a capping agent. Small silver nanoparticles are stabilized mostly within the cavity of the cyclic peptides, whereas larger ones are stabilized by the enzyme, curcain (Bar *et al.*, 2009).

The role of enzymes like reductase and reducing equivalent living organisms involved in nanoparticle synthesis has been discovered (Kumar and Yadav, 2009); for example, the reduction of silver nitrate to silver ions by using nitrate reductase enzyme isolated from a fungus (*Fusarium oxysporum*) has been documented (Ingle *et al.*, 2008). Besides this, natural products like naphthoquinones and anthraquinones with excellent redox properties have also been reported (Krishnaraj *et al.*, 2010). The involvement of natural products like proteins, polyphenols and carbohydrates in the synthesis of metal nanoparticles has been properly documented (Patil *et al.*, 2012). Flavonoids like quercetin, polysaccharides and caffeine (alkaloid) have been used for silver nanoparticle synthesis (Kumar and Yadav, 2009). The capability of several flavonoids, including quercetin, to chelate various metal ions is well documented, and further studies have shown that chelate complexes are formed in the reduction reaction of flavonoids (Egorova and Revina, 2000). Further abundant sugars and proteins in the extract of rose petals, which were measured qualitatively, were likely to be the reducing agents responsible for metal ion reduction (Noruzi *et al.*, 2011).

Tannic acid is an ideal reducing and stabilizing agent under basic conditions at room temperature. Several reactions have been reputed as being due to the antioxidant and chelating properties of tannic acid. On the one hand, gallic acid under basic conditions reduces silver nitrate into silver nanoparticles, whereas on the other hand, glucose is a weak reducing agent at room temperature but a good stabilizing agent in alkaline medium (Sivaraman *et al.*, 2009). Recently, the synthesis of silver nanoparticles using pure compound geraniol has been studied (Safaepour *et al.*, 2009). The synthesis of nanoparticles using plant extracts and fractions has been studied thoroughly. However, there are limited reports available on the synthesis of nanoparticles using pure products.

Conclusion and Future Prospects

Recently, there has been increasing interest in using metal nanoparticles for different medical and industrial applications. In particular, many reports have been published about the biogenesis of silver and gold nanoparticles using several plant extracts, such as those discussed earlier. However, there is still a need for an economic, commercially viable and environmentally clean route to synthesize metal nanoparticles and to find the capacity of their pure natural reducing constituents to form silver nanoparticles, which has not yet been studied. The purification of active compounds from a natural matrix needs a deep understanding of the chemistry of different compounds and knowledge of phytochemistry. The limited study of the pure natural product in the synthesis of metal nanoparticles has been discussed herein. Significant variations have been detected in the chemical compounds of plants collected from different parts of the world. Plant extract prepared from one species in various laboratories may lead to different results. This is the main drawback of using crude natural products for the biosynthesis of nanoparticles, and it needs to be resolved.

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4 Biological Synthesis of Nanoparticles Using Algae

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Introduction

Nanotechnology is an important field of modern research dealing with the design, synthesis and manipulation of particle structure ranging from approximately 1 to 100 nm. Nanoparticles have a wide range of applications in areas such as health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, single electron transistors, light emitters, non-linear optical devices and photoelectrochemical systems (Hamilton and Baetzold, 1979; Wang and Herron, 1991; Hoffman *et al.*, 1992; Schmid, 1992; Colvin *et al.*, 1994; Mansur *et al.*, 1995).

Nanoparticles are synthesized and stabilized through chemical, physical and biological synthetic methods. These methods have been developed to obtain nanoparticles of various shapes and sizes, including laser ablation, gamma irradiation, electron irradiation, chemical reduction, photochemical methods, microwave processing and biological synthetic methods. Most of these methods are still in the development stage, and the problems experienced are with the stability

and aggregation of nanoparticles and the control of crystal growth, morphology, size and size distribution. Furthermore, the extraction and purification of nanoparticles produced for further applications are still important issues. Selection of the solvent medium and of eco-friendly, non-toxic reducing and stabilizing agents are the most important issues that must be considered in the green synthesis of nanoparticles.

An important area of research in nanotechnology deals with the synthesis of nanoparticles of different chemical compositions, sizes and monodispersity (Sastry *et al.*, 2003; Korbekandi *et al.*, 2009; Iravani, 2011). There is a growing need to develop eco-friendly processes that do not use toxic chemicals in the synthesis protocols. Synthesis of nanoparticles through biological methods is an environment friendly and economically viable alternative method without the use of harsh, toxic and expensive chemicals. Increasing pressure to develop green chemistry and eco-friendly methods for nanomaterials synthesis has encouraged researchers to shift to biological systems. Biotechnological applications such as the bioremediation of toxic metals have been employed for a long time (Beveridge and Murray, 1980; Aiking *et al.*, 1982;

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Mehra and Winge, 1991; Southam and Beveridge, 1994; Niemeyer, 2005; Shankar *et al.*, 2005). However, the possibility of using such microorganisms in the deliberate synthesis of nanoparticles is a relatively new procedure. The potential of organisms in nanoparticle production ranges from simple prokaryotic bacterial cells to complex eukaryotes (Mohanpuria *et al.*, 2008; Korbekandi *et al.*, 2009; Iravani, 2011). Some examples of nanoparticle production include using: bacteria for gold, silver, cadmium, zinc, magnetite and iron; yeasts for lead and cadmium; fungi for gold, silver and cadmium; and algae for gold (Debaditya and Rajinder, 2005). This chapter reviews the green synthesis of nanoparticles using algae.

Important Aspects in the Biosynthesis of Nanoparticles

The ability of organisms in nanoparticle synthesis has opened a new, exciting approach towards the development of these natural nanofactories. It seems that the optimization of reaction conditions can control the morphologies and other properties of nanoparticles. Therefore, researchers have focused their attention on finding optimal reaction conditions and cellular mechanisms involved in the reduction of metal ions and the formation of nanoparticles (Riddin *et al.*, 2006; Govender *et al.*, 2009). Important aspects that might be considered in the process of producing well-characterized nanoparticles are as follows:

1. Selection of the best organisms: in order to choose the best candidates, researchers have focused on some of the organisms' important intrinsic properties, including growth rate, enzyme activities and biochemical pathways (Mohanpuria *et al.*, 2008).
2. Selection of the biocatalyst state: it seems that the organisms' enzymes (the biocatalysts) are the major agents in nanoparticle biosynthesis. Biocatalysts can be used as either whole cells, crude enzymes or purified enzymes. It seemed that using culture supernatant or cell extract of the cell could increase the rate of reaction; however, these nanoparticles did not show long-term stability. Moreover, the release of nanoparticles from the cells was an important aspect that might be considered in

the case of intracellularly produced nanoparticles. Most of the reactions responsible for nanoparticle production seem to be bioreductions. In bioreductions, we need coenzymes (such as NADH, NADPH, FAD, etc.) to be supplied in stoichiometric amounts. As they are expensive, the use of whole cells is preferred, because the coenzymes will be recycled during the pathways in live whole cells.

3. Optimal conditions for cell growth and enzyme activity: we need to produce greater amounts of enzymes, which can be accomplished by the production of more biomass. Thus, the optimization of growth conditions is crucial. Nutrients, inoculum size, pH, light, temperature, buffer strength and mixing speed should be optimized. The induction of responsible enzymes seems to be crucial as well. The presence of substrates or related compounds in subtoxic levels from the beginning of growth increases activity. Harvesting time is important in the case of using whole cells and crude enzymes. Therefore, it might be necessary to monitor enzyme activity during the course of growth.

4. Optimal reaction conditions: it is better to harvest the cells (the biocatalysts) to remove unwanted residual nutrients and metabolites in order to avoid adverse reactions and to provide a cleaner medium for better and easier analysis. In order to use organisms for the production of nanoparticles on an industrial scale, yield and production rate are important issues to be considered. Therefore, we need to optimize bioreduction conditions in the reaction mixture. Substrate concentration (to be at subtoxic level for the biocatalyst), biocatalyst concentration, electron donor (and its concentration), exposure time, pH, temperature, buffer strength, mixing speed and light all need to be optimized. Researchers have used some complementary factors such as visible light or microwave irradiation and boiling, which could affect morphology, size and rate of reaction.

Biosynthesis of Nanoparticles Using Algae

The suspension of dried cells of *Chlorella vulgaris* in HAuCl₄ solution accumulated elemental gold in the cells (Hosea *et al.*, 1986).

The accumulation of Au(0) by lyophilized preparations of *C. vulgaris* has been investigated. Gold was bound to the algae by suspending dried algal cells in solutions containing hydrogen tetrachloroaurate (III). Relative amounts of ionic and atomic algal-bound gold were determined by thiourea extraction. It was found that the amount of algal-bound atomic gold produced from ionic gold increased over time. The effect of algal-bound gold concentration on the rate and extent of gold reduction was observed. The effect of Au(0) accumulation on the binding ability of gold-bound algae was also investigated and an apparent enhancement of gold-binding ability was reported (Hosea *et al.*, 1986). Morphological control over the shape of Au nanoparticles has been well established using *Plectonema boryanum* UTEX485, blue-green algae, while treated with aqueous Au (S₂O₃)₂³⁻ and AuCl₄ solutions (Lengke *et al.*, 2006a). The mechanism involves interaction with aqueous Au(III) chloride solution, which promotes the precipitation of nanoparticles of amorphous Au(I) sulfide at the cell walls and finally deposits metallic Au in the form of octahedral platelets near cell surfaces and in solutions (Lengke *et al.*, 2006b).

Diatoms are single celled photosynthesizing microorganisms belonging to the group of brown algae (division Chromophyta, class Bacillariophyceae), which produce an intricately structured cell wall made of nanopatterned silica. Phytochelatin (PC)-coated CdS nanocrystallites were formed in a marine phytoplanktonic alga, *Phaeodactylum tricorutum*, in response to Cd (Hosea *et al.*, 1986). *P. tricorutum* exposed to Cd formed Cd-PC complexes in which sulfide ions (S²⁻) could be incorporated to stabilize PC-coated CdS nanocrystallites. Native CdS particles exhibited differences in their particle size, sulfide content and optical spectroscopic properties. Characterization of these complexes showed that CdS nanoparticles were coated mainly with γ -glutamyl peptides. The PC₂ oligopeptide was not found to be a coating peptide. CdS particles were stable in the pH range from 6 to 8 and showed half-dissociation at pH 4.9. *In vitro* reaction with S²⁻ easily converted native, sulfide-free Cd-PC complexes to PC-coated CdS nanocrystallites but was less effective in restricting

particle accretion (Scarano and Morelli, 2003). Native PC-coated CdS crystallites from *P. tricorutum* exhibited heterogeneity in particle size. By using the reported relationship between λ_{\max} and the radius of nanocrystallites (Efros and Rodina, 1989; Bae and Mehra 1998), formation of PC-coated CdS nanocrystallites was reported for the freshwater microalga *Chlamydomonas reinhardtii* (Hu *et al.*, 2001) and for the marine microalga *P. tricorutum* (Morelli *et al.*, 2002). PC-coated CdS crystallites from *P. tricorutum* were similar to CdS nanocrystallites isolated from several plants and yeasts (Reese and Winge, 1988; Reese *et al.*, 1988; Dameron *et al.*, 1989; Dameron and Winge, 1990; Winge *et al.*, 1992; Rauser, 2000). The formation of PC-coated CdS crystallites in *P. tricorutum* could explain the low sensitivity of the algae to Cd, as compared with other marine diatoms (Torres *et al.*, 1997). The role of these particles in Cd tolerance was supported by the enhanced production of PC-coated CdS crystallites in Cd-resistant mutants of yeast, *Candida glabrata*, and in the metal-tolerant ecotypes of *Silene vulgaris* (Verkleij *et al.*, 1990; Mehra *et al.*, 1994), and by the inability to synthesize CdS complexes of Cd-sensitive mutants of *Schizosaccharomyces pombe* (Ortiz *et al.*, 1992; Speiser *et al.*, 1992).

Rapid formation of gold nanoparticles through extracellular biosynthesis has been made feasible in a marine alga, *Sargassum wightii* Greville (Singaravelu *et al.*, 2007). *S. wightii* was able to form high-density and extremely stable gold nanoparticles (8–12 nm) extracellularly, in a short timespan (Singaravelu *et al.*, 2007). The reduction of AuCl₄⁻ ions during exposure to *S. wightii* powder might be followed easily by ultraviolet-visible (UV-Vis) spectroscopy. The UV-Vis spectrum of the aqueous medium containing gold ion showed a peak at 527 nm, corresponding to the plasmon absorbance of gold nanoparticles. It has been well established that surface plasmon resonance of metallic gold nanoparticles exhibits a ruby red colour and gives rise to an absorption band at 510–540 nm (Richard, 1978). The fact that the gold nanoparticles peak remained close to 527 nm even after 15 h of incubation demonstrated that the nanoparticles were well dispersed in the solution, with no significant aggregation.

S. wightii is a marine water alga rich in reductases. Nitrate reductases are the most influential enzymes that are considered to be scaffold or nucleating agents, allowing an NADH-dependent reduction of Au⁺³ to Au⁰. Oza *et al.* (2012) reported that nitrate reductase was involved in reducing and stabilizing gold ions to gold nanoparticles.

When *Spirulina platensis* biomass was exposed to 10⁻³ M aqueous AgNO₃ and HAuCl₄, extracellular formation of spherical gold (6–10 nm), silver (7–16 nm) and bimetallic Au–Ag nanoparticles (17–25 nm) resulted after 120 h at 37°C at pH 5.6 (Govindaraju *et al.*, 2008). Proteins might be responsible for the reduction and stabilization of nanoparticles. Studies on the biosorption and bioreduction of Au(III) ions are ongoing, and *Fucus vesiculosus*, a brown alga, is important in this respect (Mata *et al.*, 2009). Bioreduction with *F. vesiculosus* could be used as an alternative eco-friendly process for recovering Au from dilute hydrometallurgical solutions and leachates of electronic scraps. In another study, the extracellular biosynthesis of self-assembled spherical gold nanoparticles (15–20 nm) was accomplished using the aqueous extract of the brown alga *Laminaria japonica*. The results of the FTIR analysis suggested that the peptides and/or proteins carried out the dual function of effective Au(III) reduction and successful capping of the gold nanoparticles (Gajanan and Sung, 2012).

El-Rafie *et al.* (2012) reported the synthesis of silver nanoparticles using polysaccharides extracted from four marine macroalgae, namely, *Pterocladia capillacea*, *Jania rubens*, *Ulva fasciata* and *Colpomenia sinuosa*, as reducing agents for silver ions, as well as stabilizing agents for synthesized silver nanoparticles. Hot water extracts of the algae *P. capillacea*, *J. rubens*, *U. fasciata* and *C. sinuosa*, were studied for their polysaccharide content and were found to be 6.46, 5.63, 8.84 and 4.33%, respectively. GLC analysis of these extracted polysaccharides revealed that rhamnose (46.88% in *P. capillacea*), galactose (30.2% in *U. fasciata*; 22.23% in *J. rubens*) and fucose (10% in *C. sinuosa*) constituted the major sugars, comprising part of a structural polysaccharide. In another study, the synthesis of silver nanoparticles (13 ± 3 nm) through a green route

using sulfated polysaccharide isolated from marine red algae (*Porphyra vietnamensis*) was reported (Venkatpurwar and Pokharkar, 2011). FTIR spectra revealed the involvement of the sulfate moiety of polysaccharide for the reduction of silver nitrate. The capping of anionic polysaccharide on the surface of nanoparticles was confirmed by zeta potential measurement (–35.05 mV) and was responsible for electrostatic stability. The silver nanoparticles were highly stable at a wide range of pH (2–10) and electrolyte concentration (up to 10⁻² M of NaCl). The dose-dependent effect of the silver nanoparticles produced revealed strong antibacterial activity against Gram-negative bacteria as compared to Gram-positive bacteria.

Gelidiella acerosa (Forsskal) is an abundant seaweed growing in the coastal areas of south India. *G. acerosa* has been used as a gelling agent to make jellies, a calorie-free cookery ingredient, a valuable antioxidant for treating reactive oxygen species (ROS)-mediated diseases and a useful post-coital contraceptive. It has been reported that *G. acerosa* could be used for the biological synthesis of spherical silver nanoparticles (approximately 22 nm). The nanoparticles produced had antifungal activities against *Humicola insolens*, *Fusarium dimerum*, *Mucor indicus* and *Trichoderma reesei* (Vivek *et al.*, 2011). In another study, gold nanoparticles (approximately 79 nm) were synthesized in a short period using cell extract of the marine microalga *Tetraselmis suecica* as a reducing agent of HAuCl₄ (chloroauric acid) solution. Transmission electron microscopy and particle-size distribution patterns determined by the laser-light-scattering method confirmed the formation of well-dispersed gold nanoparticles (Shakibaie *et al.*, 2010). Senapati *et al.* (2012) have reported the intracellular synthesis of gold nanoparticles (5–35 nm) using *Tetraselmis kochinensis*. The nanoparticles produced are more concentrated on the cell wall than on the cytoplasmic membrane, possibly due to the reduction of metal ions by the enzymes present in the cell wall and the cytoplasmic membrane.

Extracellular biosynthesis of gold nanoparticles using *Padina gymnospora* has been attempted and rapid formation of gold nanoparticles was achieved in a short time frame

(Singh *et al.*, 2013). AFM analysis showed the results of particle sizes (53–67 nm) and average height of particle roughness (60 nm). Participation of the polysaccharides of the algal cell wall in the reduction process was confirmed by FTIR analysis of the biomass after gold recovery. Algal pigments, such as fucoxanthins, a type of carotenoid rich in hydroxyl groups, could also have participated in the gold reduction. These pigments have reductive properties and are released to solution by diffusion. These soluble elements could have acted as capping agents, preventing the aggregation of nanoparticles in solution and playing a relevant role in their extracellular synthesis and shaping. For instance, the pigment C-phycoerythrin (C-PE), extracted from the marine cyanobacterium *Phormidium tenue* NTDM05, was used to synthesize CdS nanoparticles (about 5 nm). Essentially, it was found that the pigment stabilized the CdS nanoparticles (Mubarak Ali *et al.*, 2012).

A few reports are available regarding gold accumulation using algal genera including cyanobacteria as the biological agent. Cyanobacteria and eukaryotic alga genera such as *Lyngbya majuscula*, *Spirulina subsalsa*, *Rhizoclonium heiroglyphicum*, *C. vulgaris*, *Cladophora prolifera*, *Padina pavonica*, *S. platenis* and *Sargassum fluitans* can be used as cost-effective means in the biorecovery of gold from aqueous solutions, as well as in the formation of gold nanoparticles (Niu and Volesky, 2000; Lengke *et al.*, 2006a,b; Chakraborty *et al.*, 2009). In the case of *L. majuscula*, TEM images indicated the formation of spherical gold nanoparticles in a size range of <20 nm. In previous studies, the bioaccumulation and bioreduction of gold by *Rhizoclonium riparium*, *Navicula minima* and *Nitzschia obtusa* have been reported (Chakraborty *et al.*, 2006). It was demonstrated that accumulation of gold on *R. riparium* was almost pH independent, and was slightly higher at basic pH. Accumulation of gold was studied with ^{198}Au radiotracer, at 0.1, 1 and 5 ppm concentrations of gold (Nayak *et al.*, 2006). In another study, *N. obtusa* showed better accumulation of gold in acidic pH in comparison to neutral and basic pH. Maximum accumulation was observed with 1 mg kg⁻¹ or less gold concentration.

Furthermore, *N. minima* was found to be a better accumulator of gold in wide ranges of pH and substrate concentration of the media. It was reported that the gold accumulation by diatoms was due mainly to adsorption by biosilica (siliceous frustules of dead diatom cells) (Chakraborty *et al.*, 2006). In one study, the cyanobacteria *Phormidium valderianum*, *P. tenue* and *Microcoleus chthonoplastes*, and the green algae *Rhizoclonium fontinale*, *Ulva intestinalis*, *Chara zeylanica* and *Pithophora oedogoniana*, were exposed to hydrogen tetrachloroaurate solution and were screened for their suitability for producing gold nanoparticles (Parial *et al.*, 2012). All three cyanobacteria genera and two of the green algae (*R. fontinale* and *U. intestinalis*) produced gold nanoparticles intracellularly. *P. valderianum* synthesized mostly spherical nanoparticles, along with hexagonal and triangular nanoparticles, at basic and neutral pHs (pH 9 and pH 7, respectively). Medicinally important gold nanorods were synthesized (together with gold nanospheres) only by *P. valderianum* at acidic pH (pH 5). This was determined initially by two surface plasmon bands in UV-Vis spectroscopy and later confirmed by TEM. Spherical to somewhat irregular particles were produced by *P. tenue* and *U. intestinalis*. *C. zeylanica* and *P. oedogoniana* were found unable to produce nanoparticles. Synthesis of nanoparticles by algae is more environmentally friendly than purely chemical synthesis. However, the choice of algae is very important. In Table 4.1, important examples of nanoparticle biosynthesis using algae are listed.

Extraction of Biosynthesized Nanoparticles

The extraction of nanoparticles from organisms for further applications has not been well investigated, but further studies may solve these problems and find the best ways of extracting and purifying the nanoparticles. In order to extract the produced nanoparticles from the cells, some researchers used physico-chemical methods, including freeze–thawing, heating processes and osmotic shock. It seems that these processes may interfere with the

Table 4.1. Some important examples of nanoparticle biosynthesis using algae.

Algae	Nanoparticle	Size (nm)	References
<i>Chlamydomonas reinhardtii</i>	Cadmium sulfide	–	Hu <i>et al.</i> , 2001
<i>Chlorella vulgaris</i>	Gold	–	Mandal <i>et al.</i> , 2006
<i>Gelidiella acerosa</i>	Silver	22	Vivek <i>et al.</i> , 2011
<i>Phaeodactylum tricornutum</i>	Cadmium sulfide	–	Mandal <i>et al.</i> , 2006
<i>Sargassum wightii</i>	Gold	8–12	Singaravelu <i>et al.</i> , 2007
<i>Spirulina platensis</i>	Silver	7–16	Govindaraju <i>et al.</i> , 2008
<i>Spirulina platensis</i>	Gold	6–10	Govindaraju <i>et al.</i> , 2008
<i>Spirulina platensis</i>	Gold–silver alloys	17–25	Govindaraju <i>et al.</i> , 2008
<i>Tetraselmis kochinensis</i>	Gold	5–35	Senapati <i>et al.</i> , 2012
<i>Tetraselmis suecica</i>	Gold	79	Shakibaie <i>et al.</i> , 2010

structure of the nanoparticles, and some events such as aggregation, precipitation and sedimentation could happen. These may change the shape and size of the nanoparticles and interfere with their properties. It appears that surfactants and organic solvents might be good candidates for both the extraction and stabilization of nanoparticles. It should be noted that in the case of the extracellular production of nanoparticles, centrifuge could be used for extraction and purification, but this causes a problem with aggregation.

Conclusion

Biosynthetic methods of producing nanoparticles provide a new possibility of conveniently synthesizing nanoparticles using

natural reducing and stabilizing agents. As possible environmentally friendly alternatives to chemical and physical approaches, the biosynthesis of metal and semiconductor nanoparticles using algae has been suggested. Monodispersity and particle size and shape are very important parameters in the evaluation of nanoparticle synthesis. Therefore, efficient control of the morphology and monodispersity of nanoparticles must be explored, and reaction conditions should be optimized. By using screened algae with a high production capability and by controlling reaction conditions, well-characterized nanoparticles can be obtained by synthesis rates faster than or comparable to those of chemical approaches. This eco-friendly method potentially can be used in various areas, including pharmaceuticals, cosmetics, foods and medical applications.

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5 Synthesis of Metallic Nanoparticles by Diatoms and Chrysophytes – Prospects and Applications

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Introduction

The ability of many different organisms to form inorganic materials from different precursors either intra- or extracellularly has been well known for more than three decades and is well documented in the available literature (Wilbur and Simkiss, 1979). Many biotechnological applications and waste processing technologies, such as the remediation and reclamation of toxic metals from soils or polluted waters, employ bacteria and other microorganisms (Pérez-de-Mora *et al.*, 2006; Vijayaraghavan and Yun, 2008) or plants (Sheoran *et al.*, 2009).

Microbe–metal interactions are widely utilized by researchers in a broad range of

scientific fields such as biotechnology, nanotechnology and materials and environmental engineering. The recently discovered technique of nanoparticle biosynthesis and the thoroughly investigated biosorption shows the apparent connection between metal salts reduction and nanoparticle formation.

By viewing the processes inside viruses, microbes, plants and other living organisms or their parts and extracts, scientists were inspired to develop new biosynthesis methods for the fabrication of nano- and micro-scale inorganic materials (Mandal *et al.*, 2006; Vigneshwaran *et al.*, 2007; Mohanpuria *et al.*, 2008; Nadagouda and Varma, 2008; Vijayaraghavan and Yun, 2008; Narayanan and Sakthivel, 2010; Thakkar

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et al., 2010; Amarnath *et al.*, 2011; Valodkar *et al.*, 2011; Venu *et al.*, 2011).

Regarding the nature of biosynthesis reactions, we can consider the biosynthesis of nanoparticles among chemical, 'bottom-up' methods of nanomaterial preparation. However, biosynthesis is very specific, due to the fact that it takes place either inside a living organism or in a complex system of cell metabolites. The complex system inside any living cell contains thousands of different molecules with varied functional groups such as amine, hydroxyl, thiol, carboxyl, etc. These functional groups can be involved in redox processes of metal reduction. Furthermore, the active participation of reducing enzymes, such as nitrogenase reductase complex, should also be taken into account (Mohanpuria *et al.*, 2008).

A large number of factors complicate the localization and prediction of the particular process. The product – biosynthesized nanoparticles – is therefore mixed with the complex system of cell debris containing metabolic products and other biomolecules. This mixture of proteins, polysaccharides, polyphenols and other molecules is unique for any organism and can affect strongly the properties of the biosynthesized nanoparticles (NPs) (size, charge, toxicity, biocompatibility, etc.). Furthermore, the surrounding chemicals (capping agents) can influence NP stability and protect the NPs against aggregation (Das and Marsili, 2010; Durán *et al.*, 2011). Therefore, further biosynthesis studies employing different organisms can lead to nanomaterials with very interesting potential applications.

Regarding the aforementioned facts, it is generally difficult to separate these tiny metallic particles from the cell debris. However, the isolation of NPs is not necessary for certain applications; for example, whole nanocomposites can be used in catalysis (Sharma *et al.*, 2007; Lloyd *et al.*, 2011; Narayanan and Sakthivel, 2011; Zhang *et al.*, 2011; De Corte *et al.*, 2012; Hosseinkhani *et al.*, 2012). Noble metals have been known as efficient catalysts involved in many reactions and industrial applications. Nanoparticles, with their high surface to volume ratio, have remarkably higher efficiency in catalytic reactions, so a smaller amount of catalyst is needed for the particular application.

The biosynthetic method of metallic NP preparation can represent a relatively cheap,

sustainable and eco-friendly process and can contribute to the further development of modern nanotechnology. In this chapter, we demonstrate a novel synthesis of metallic NPs and nanocomposites by biologically driven processes employing two diatom strains (*Diadesmis gallica* and *Navicula atomus*) and a silica-scaled chrysophyte (*Mallomonas kalinae*).

To provide the context for the results, the silica-based algae will be described briefly, with a particular focus on their morphology, and the current potential of these organisms for nanotechnology will be reviewed.

Diatoms – Characterization, Morphology and Ecology

Diatoms are unicellular or colonial photosynthesizing microorganisms belonging to the group of heterokont algae (kingdom Chromista, class Bacillariophyceae). Their brown colour is due to the presence of an accessory pigment, fucoxanthin, that masks chlorophylls *a* and *c*. The diatom cell is encased by a characteristic siliceous case called a frustule, which can be reminiscent of a box with an overlapping lid. Diatoms are practically ubiquitous and form the most widespread group of algae. They can be found in marine habitats, brackish waters, all varieties of fresh water, on wet rocks and in soils (van den Hoek *et al.*, 1995). Diatoms often represent a dominant component of the phytoplankton, epiphyton (growing on submerged vegetation), as well as benthos, and are probably the most abundant group of aquatic eukaryotic organisms. Marine planktonic diatoms play a crucial role in the world's net primary production. Diatoms fix approximately 1015 g of CO₂ into organic carbon every year, equivalent to roughly 40% of marine primary production (Granum *et al.*, 2005). For their ubiquity and specific ecological demands (pH, conductivity, etc.), diatoms are used as sensitive bioindicators in the monitoring of water quality (Karleskint *et al.*, 2009).

There have been approximately 12,000 species of diatoms described. The fossil record has revealed that diatoms only evolved in the Jurassic and became more common in the mid-Cretaceous (Round *et al.*, 1990). However, estimated diatom diversity exceeds

this number by an order of magnitude (Katharine and David, 2007). The traditional classification recognized two morphologically distinct types, radially symmetrical centric and bilaterally symmetrical pennate diatoms. Molecular approaches have contributed to our understanding of relationships within the diatoms. The centric group was revealed to be paraphyletic, but there is a monophyletic group (sharing a common ancestor) comprising most diatoms traditionally considered to be pennates (Gordon *et al.*, 2009). Classification and species recognition is still based mostly on the structured frustule morphology, studied after the cell content is removed. Light microscopy under high magnification (at least 1000 \times) or, in

modern diatomology, scanning electron microscopy are used (Fig. 5.1).

The frustule of a diatom is composed of two halves, referred to as the epitheca and the hypotheca. Each of these halves is composed of a flat upper portion called the epivalve (hypovalve respectively), and the circular pieces of silica (girdle bands) form the side portion called the epicingulum (hypocingulum respectively) (Fig. 5.2). When classifying diatoms, characteristic surface structures such as extensions, perforations, thickenings or thin areas in the wall, as well as the final pattern, together with a frustule shape characteristic of each species, is observed when the epivalve or hypovalve is uppermost (van den Hoek *et al.*, 1995).

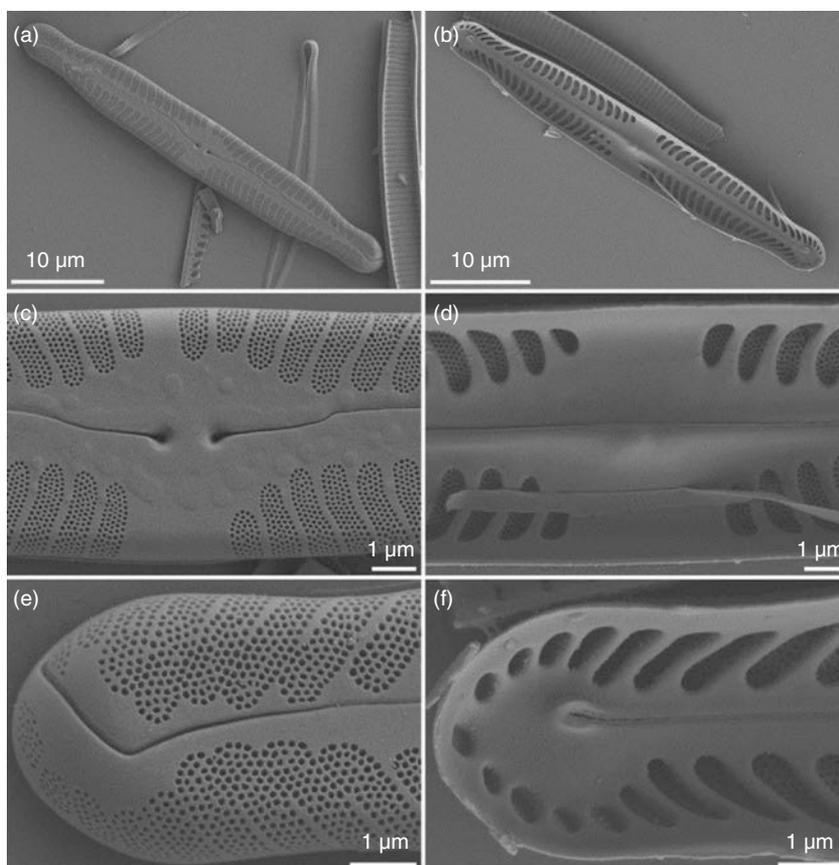


Fig. 5.1. (a–f) Micrographs taken with a JEOL7401 scanning electron microscope, kindly provided by Jana Veselá from the University of South Bohemia. (a) Pennate diatom *Pinnularia subcapitata* from the valval view. (b) The inner side of the valve. (c) Detail of the microstructured central part of the valve from outside and (d) inside. (e) Detail of the microstructured apical part from outside and (f) inside.

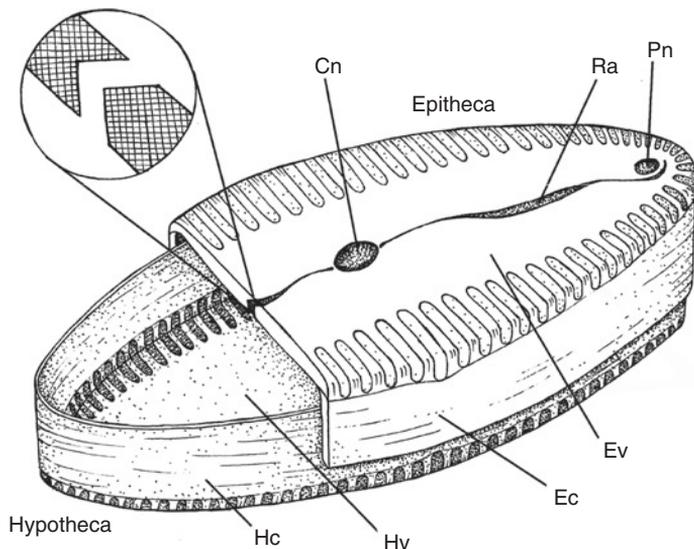


Fig. 5.2. Scheme of a pennate diatom (drawing by Markéta Bohunická): Cn = central nodule; Ec = epicingulum; Ev = epivalve; Hc = hypocingulum; Hv = hypovalve; Ra = raphe; Pn = terminal nodule.

Precise forms are produced through a unique mechanism of silica acquisition and processing, which has been described in detail previously (Volcani, 1981; Hildebrand, 2003; Gordon *et al.*, 2009). Diatoms are likely the only eukaryotic organism fully dependent on the availability of silica present in their environment in an accessible form. Although silica is one of the most abundant minerals on the planet, its soluble forms, mainly occurring as an orthosilicic acid ($\text{Si}[\text{OH}]_4$), are rare in most watery environments. It has not been clarified exactly in which form the silica is taken up by the diatom cells, but it is known that the transport is active through the plasmatic membrane (Karleskint *et al.*, 2009).

The formation of a new diatom frustule starts after cell division and cytokinesis of the mother protoplast in flattened vesicles, called silica deposition vesicles (SDVs), in which colloid silica material is collected, gradually polymerized into silica spheres of 30–50 μm diameter and subsequently deposited on to the forming diatom frustule (Karleskint *et al.*, 2009). It is likely that the precise formation of the valve pattern is enabled by organic macromolecules of the SDV matrix. Exocytosis follows the completion of the siliceous frustule and two daughter cells are separated,

each containing maternal epitheca and newly formed hypotheca. During the process, the inner membrane of the SDV is transformed into new plasmalemma, and primary coatings around the silica are formed by the outer membrane (Foo *et al.*, 2004). Frustulins, the proteins associated with the mature cell wall, form the building blocks and contain a highly conserved domain of repeated amino acid sequences (Kröger *et al.*, 1996; van de Poll *et al.*, 1999). The mechanism of diatom frustule formation has been further investigated as a model for biomimetic synthesis of silica nanostructures (El Rassy *et al.*, 2005; Crawford *et al.*, 2009).

Chrysophytes – Characterization, Morphology and Ecology

Silica-scaled chrysophytes comprise two separate lineages (Paraphysomonadaceae and Synurales) within the class Chrysophyceae (also called golden algae), in which cells are covered with an armour of silica scales attached in a fairly organized manner to the plasma membrane. They represent free-living or colonial flagellates (Fig. 5.3a–d), residing predominantly in fresh waters such as pools,

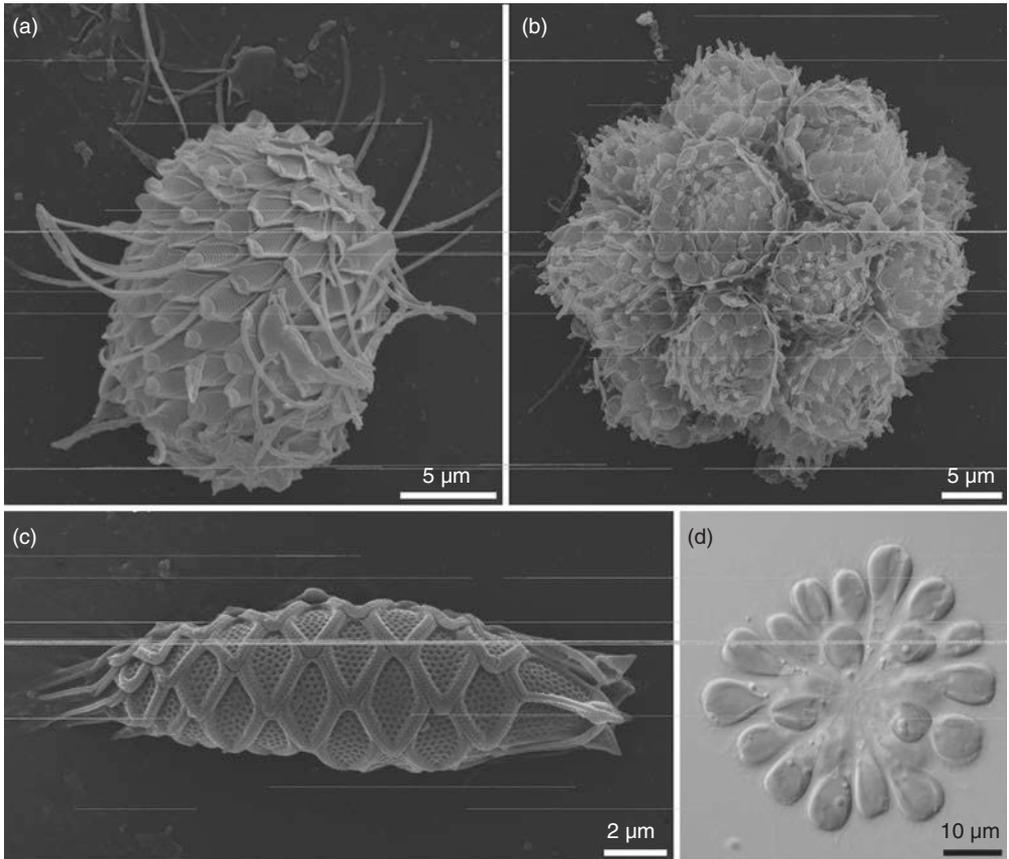


Fig. 5.3. (a) The whole cell of the single living flagellate, *Mallomonas striata*, in a scanning electron microscope (SEM). (b) The colonial flagellate, *Synura echinulata*, in SEM. (c) *Mallomonas clavus* in SEM, note precisely organized silica scales on the surface of the cell. (d) *Synura* sp. observed in a light microscope.

ponds, rivers and lakes. Endogenously formed silicified resting stages, stomatocysts (Fig. 5.4a–c), are produced as a result of unfavourable environmental conditions or sexual reproduction (Sandgren, 1989). Palmella stages (non-flagellated cells enclosed in mucilage) have also been reported. Palmella-stage formation has been induced in laboratory conditions by gradual desiccation. Wee *et al.* (2005) postulated that this life history stage might be utilized by chrysophytes for dispersal by semi-aquatic animals (e.g. waterfowl).

Silica scales are formed inside specialized silicon deposition vesicles (Fig. 5.4d) (Leadbeater, 1986). Silicon in the form of weak orthosilicic acid ($\text{Si}[\text{OH}]_4$) is actively taken up into the cell (Reynolds, 2006). As the scales

mature, they are transported to the plasma membrane and extruded on to the cell surface. Scale formation is similar to frustule formation in the diatoms. In contrast to diatoms that have an absolute requirement for silicon during cell division, silica-scaled chrysophytes grow well and divide in silicate-limited environments. Colonies bearing less silicified scales, or even actively growing naked colonies, were demonstrated in laboratory experiments with *Synura petersenii* (Sandgren *et al.*, 1996). Genera *Mallomonas* and *Synura* differ from other silica-scaled chrysophytes in the construction of the scale case. In an undisturbed scale case, the imbrication of the scales is so precise that it resembles the tiles on a roof. When the cell divides, each of the daughter cells receives

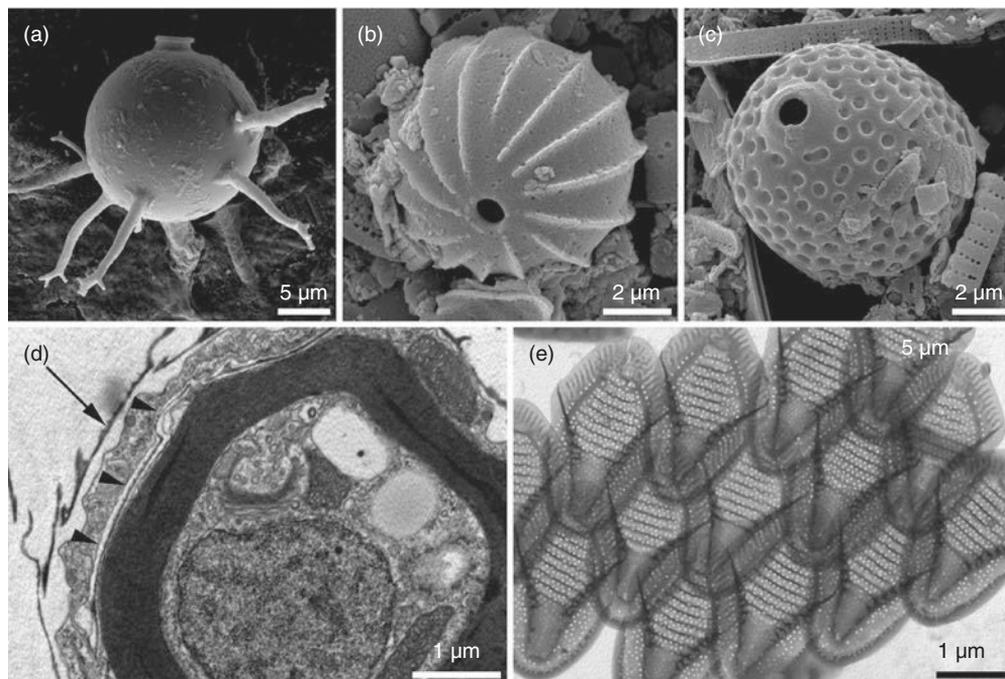


Fig. 5.4. (a–c) Silicified resting stages – stomatocysts. (a) *Ochromonas tuberculata* stomatocyst. (b–c) Unidentified stomatocysts of two different species. (d) A transverse section of the cell viewed in a transmission electron microscope. Production of a silica scale in a silicon deposition vesicle (arrowheads), note the scale that is already extruded to the cell surface (arrow). (e) Silica scales of *Mallomonas actinoloma* ‘glued’ together by an adhesive proteinaceous material.

approximately half of the parental scales and adds the newly produced scales to the existing scale case (Leadbeater and Barker, 1995). This process is still poorly understood. In *Mallomonas*, some or all scales may harbour a bristle (Fig. 5.5f) that is produced in a separate silicon deposition vesicle and is fastened to the scale additionally on the cell surface. On the other hand, the spine in *Synura* scales is an indivisible part of the scale and is produced in the same silicon deposition vesicle (Fig. 5.5e,g). The distal surface of the scale is covered by an adhesive proteinaceous material or ‘glue’ that binds scales to one another and to the plasma membrane (see Fig. 5.4e). The bristle is similarly glued to the scale (Wetherbee *et al.*, 1995). The number of scales per scale case may vary according to the cell size within a single species. Between various *Mallomonas* species, the average number of scales per cell varies from 30 to 160 (Siver, 1991).

The morphology of silica scales is species specific and thus provides a valuable tool for species determination (Fig. 5.5a–e). Silica scales are too small to be identified correctly by a light microscope (from approximately 2.5 µm in *Mallomonas pumilio* to more than 6 µm in *Mallomonas insignis*). Both scanning and transmission electron microscopes are used to study scale morphology. The silica scales resemble flat, bilateral, symmetrical or almost symmetrical objects. The side of the scale adjacent to the plasma membrane is smooth, while the other side is structured (Fig. 5.5f,g). Some species possess scales with miniature pores (c.70 nm in diameter) bisecting the scale transversely (e.g. *Synura echinulata*, Fig. 5.5g,i, and *Mallomonas heterospina*, Fig. 5.5h), while other species have compact scales with no transverse pores (e.g. *Mallomonas striata*, Fig. 5.5f).

The shift in the species composition of silica-scaled chrysophytes along environmental

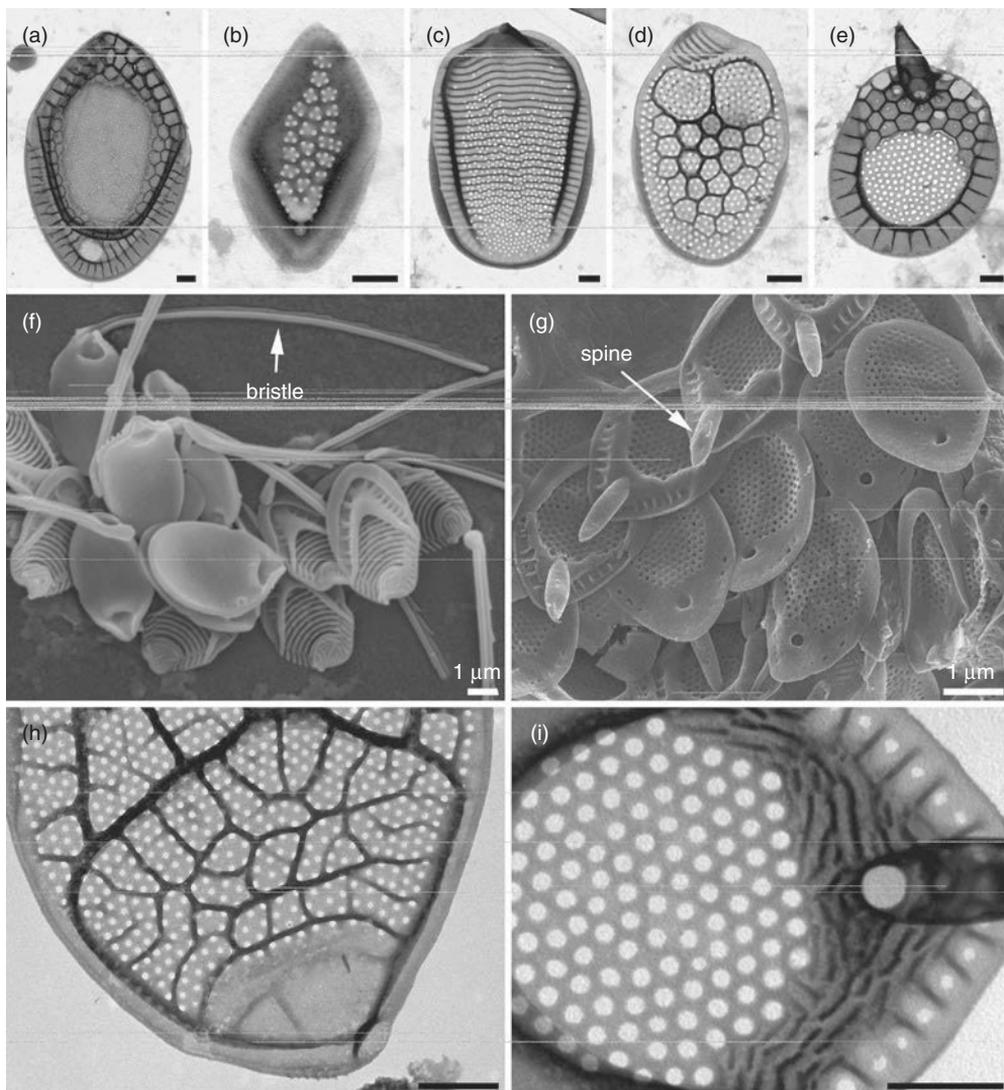


Fig. 5.5. Species-specific silica scales of different morphology (TEM images). (a) *Mallomonas insignis*. (b) *Mallomonas pumilio*. (c) *Mallomonas transsylvanica*. (d) *Mallomonas multiunca*. (e) *Synura uvella*. (f) Scales of *Mallomonas striata* from inner side (smooth) and outer side (structured), note the depression on the inner side where the bristle is attached. (g) Scales of *Synura echinulata* from inner and outer sides, note the miniature pores (c.70 nm in diameter) bisecting the scale transversely. (h–i) Detailed view of the transverse pores: (h) *Mallomonas heterospina*; (i) *Synura echinulata*. Scale bar = 0.5 mm, unless stated otherwise.

gradients is well documented (Siver, 1991, 1995). Conductivity (correlating with the level of nutrients), pH, temperature and irradiance seem to be the primary factors controlling the occurrence and distribution of these organisms in natural localities. Many taxa are distributed within a narrow range of

ecological conditions, rendering them useful as bioindicators of changes in lake water conditions (Kristiansen, 1986). The scales are preserved in sediments for a prolonged period of time and are used in palaeoecology to assess eutrophication, acidification and shifts in climate (Smol, 1995). For example, abundance

and species composition of silica-scaled chrysophytes in Hall Lake, Washington, USA, changed with the historical development of the lake, as was revealed from a core of the recent sediment (Munch, 1980). Silica-scaled chrysophytes responded to disturbance around the lake, such as the establishment of a sawmill or extensive road construction.

Diatom Nanotechnology and Other New Applications with Silica-based Algae

Diatoms have been used to detect various metals from rivers, lakes and other bodies of water where metals are below detection limits, and their capability of adsorption (biosorption) and absorption is well known and verified (Chakraborty *et al.*, 2006; Yuan *et al.*, 2010). Chakraborty *et al.* (2006) employed two diatom strains for gold biorecovery experiments. Based on previous studies and limitations due to the detection and biosorption of other radioactive heavy metals, gold radionuclide ^{198}Au insertion was proposed. The subsequent study of Chakraborty *et al.* (2009) describes the Au-NP formation process and compares biorecovery abilities between prokaryotic and eukaryotic algal genera. Another study describes gold biosorption and bioreduction with brown alga, *Fucus vesiculosus* (Mata *et al.*, 2009), focusing on pH dependence and particular phases of the bioreduction process. All of these results suggest the living, treated or modified algal biomass to be a viable and low-cost substrate for metal biorecovery technologies.

However, due to the significant progress in understanding diatom properties and the silica biomineralization process, there has recently been a strong shift in biotechnology towards nanotechnology methods. Utilization of their unique processes and properties can encourage the development of new diatom-based materials and devices, with significant potential applications.

According to Gordon *et al.* (2009), the cross-disciplinary area of diatom nanotechnology or diatom nanoscience emerged in 2005. This new interdisciplinary field employs

collaborations and connection between biology (diatomologists), biochemistry, biotechnology, physics, chemistry and materials science and engineering. The diatom frustules made of amorphous clear silica glass are more structured than the majority of man-made engineered materials. Furthermore, diatom frustules are made effectively with a minimum consumption of energy. Some species can be cultivated easily under laboratory conditions. However, a less expensive bulk source of silica produced by diatoms is diatomite, or diatomaceous earth, which is formed by diatom fossils (Yuan *et al.*, 2010).

Valuable optical properties of the silica frustules and the diversity of their structures have led to the recent concern of scientists. The high, three-dimensional symmetry of micro- and nanostructured pores can be used in sensing and photonic applications as photonic crystals or optical sensors. Utilization of these highly organized 'devices' is enabled due to their low cost and wide distribution. Certain strains of diatoms are also easy to culture and maintain. All these facts make diatoms to be excellent candidates in practical micro- and nanotechnology. The characteristics discussed above have been utilized to construct photoluminescence diatom gas sensors (De Stefano *et al.*, 2009) and probes for biomolecule detection (De Stefano *et al.*, 2008; Gale *et al.*, 2009).

Diatom frustules also have promising properties for the medical field. Due to their box structures, porosity, chemical stability and biocompatibility, frustules can be used for drug delivery applications. The surface of amorphous silica can be easily functionalized, covered, protected and designed for drug release through the nanopores. Partially unstable and reactive hydroxyl groups on the surface of diatom frustules can be modified chemically through chemical cross-linkers with specific functional groups (De Stefano *et al.*, 2008).

The use of diatom frustules as templates for the deposition or insertion of different chemicals and the modification of diatom silica to obtain more progressive functional materials is also investigated. For the alteration of silica structure materials such as TiO_2 , BaTiO_3 , SrTiO_3 , MgO , NiSO_4 or mixtures

Zn₂SiO₄:Mn, Y₂SiO₅:Eu³⁺ were used and different modification techniques were proposed (Gutu *et al.*, 2009).

To the best of our knowledge, there is not a direct connection between chrysophytes and nanoscience in application to date, except the utilization of *Mallomonas kalinae* in precious metal nanoparticle biosynthesis performed by our research group, despite the fact that chrysophytes are also extremely interesting organisms with silica-based structures with high application potential.

Although the biosynthesis of nanoparticles through phototrophic organisms such as cyanobacteria (Lengke *et al.*, 2006, 2007; Brayner *et al.*, 2007; Parial *et al.*, 2012) and algae (Govindaraju *et al.*, 2010; Merin *et al.*, 2010) has been noted previously, this study describes the very first experiments carried out using chrysophytes. Fabrication of nanoparticles in the presence of siliceous structures provides occasions for novel bionanocomposite formation and their further use.

Biosynthesis of Metallic Nanoparticles Using Diatoms

Formation of nanoparticles and bionanocomposites

We conducted the biosynthesis of gold nanoparticles successfully by means of two diatom strains mixed with aqueous H₂AuCl₄ in laboratory conditions. The interaction of diatoms with aqueous salt promoted the precipitation of metallic nanoparticles. A detailed description of the methods and concentrations, as well as light and electron microscopy characterization, is given in Schröfel *et al.* (2011). Similarly, silver metallic nanoparticles were also synthesized by means of *Diademes gallica* diatom strain (Schröfel, 2012).

The presented method of tetrachloroaurate and silver nitrate reduction by diatoms appears to be an effective and low-cost technique for bionanocomposite preparation. Additionally, performing the described method is very simple since it utilizes organisms commonly living in streams and ponds worldwide and can be performed at room

temperature and in physiologic pH. It is also environmentally friendly compared to other chemical methods that use toxic reagents.

Biosynthesis experiments have led to the development of fully functional bionanocomposites. Reduction, capping and immobilization of metallic nanoparticles are performed in a single, fast step. As far as we know, this is the first record of biomaterial preparation using such a technique. The resulting material can be further stored either in solution or dried in the form of a powder. Nanoparticles are stable for weeks due to support from the silica matrix and biomolecular capping agents.

Chemical composition of golden-based bionanocomposites, their shapes, sizes and interaction with siliceous frustules and extracellular polymeric substances (EPS) of the diatoms described by means of transmission electron microscopy (TEM), scanning electron microscopy (SEM) and X-ray diffraction (XRD) techniques are given in Figs 5.6a,c, 5.7a and 5.8, respectively.

Material modification and utilization

Fabricated bionanocomposite was modified successfully to obtain magnetically responsive material (Figs 5.6 and 5.7). The resulting material is easily recoverable with a magnet (Fig. 5.9). Therefore, it is suitable for applications in which it is necessary to recollect the reagent (catalyst, antimicrobial material). Basic magnetic characterization of magnetically modified bionanocomposite was performed by the vibrating sample magnetometer and is shown in Fig. 5.10 (Schröfel, 2012).

Due to its unique properties, bionanocomposite can be used in a variety of experimental conditions. Diatom frustules are very chemically stable, but their porous morphology offers a large surface for nanoparticles to attach. Unlike the commonly used polymers (such as polyvinyl alcohol), silica frustules are expected to resist high temperatures. This fact can be of concern due to possible catalytic applications. Material can be used in dry or wet conditions, stored as a powder, frozen or in methanol (Schröfel, 2012).

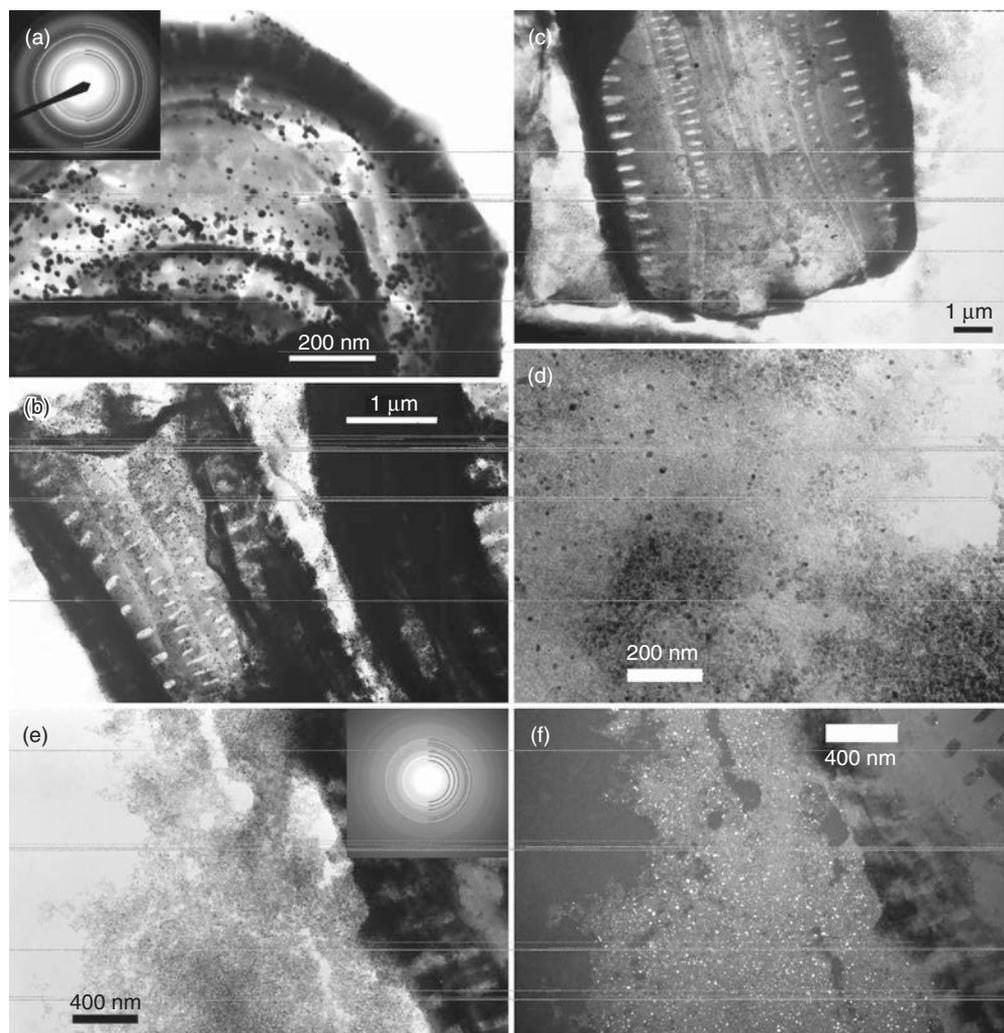


Fig. 5.6. TEM micrographs of samples containing (a–b) silica frustules and gold nanoparticles, (c–d) silica frustules and gold nanoparticles modified with ferrofluid and (e–f) silica frustules modified with ferrofluid. (a) Detail of gold nanoparticles associated with the diatom frustule, selected area electron diffraction (SAED) pattern corresponding with cubic gold. (b) Larger part of diatom frustule with gold nanoparticles. Grey ferrofluid veil together with gold nanoparticles (dark dots) on the frustule surface (c) and in detail (d). Ferrofluid nanoparticles by the diatom surface in (e) bright and (f) dark field. (e) SAED pattern of maghemite. This figure is reproduced from Schröfel (2012) with permission of the author.

Biosynthesized composite material was used successfully as an oxidation nanocatalyst (Schröfel, 2012). Reduction of 4-NP in the presence of sodium borohydrate was conducted successfully using both silver- and gold-based bionanocomposites (Figs 5.10 and 5.11). These preliminary results are promising for additional reactions and applications, for example nitroaromatic removal from contaminated wastewater,

degradation of halogen derivatives, etc. (Corma and Garcia, 2008). Due to its physical properties, bionanocomposite can be used as a magnetically recoverable nanocatalyst, a chromatography column filling or for usage in dry filters.

Furthermore, silver bionanocomposite has been proven to inhibit bacterial growth of four different bacterial strains effectively (Schröfel, 2012). Disk diffusion tests and

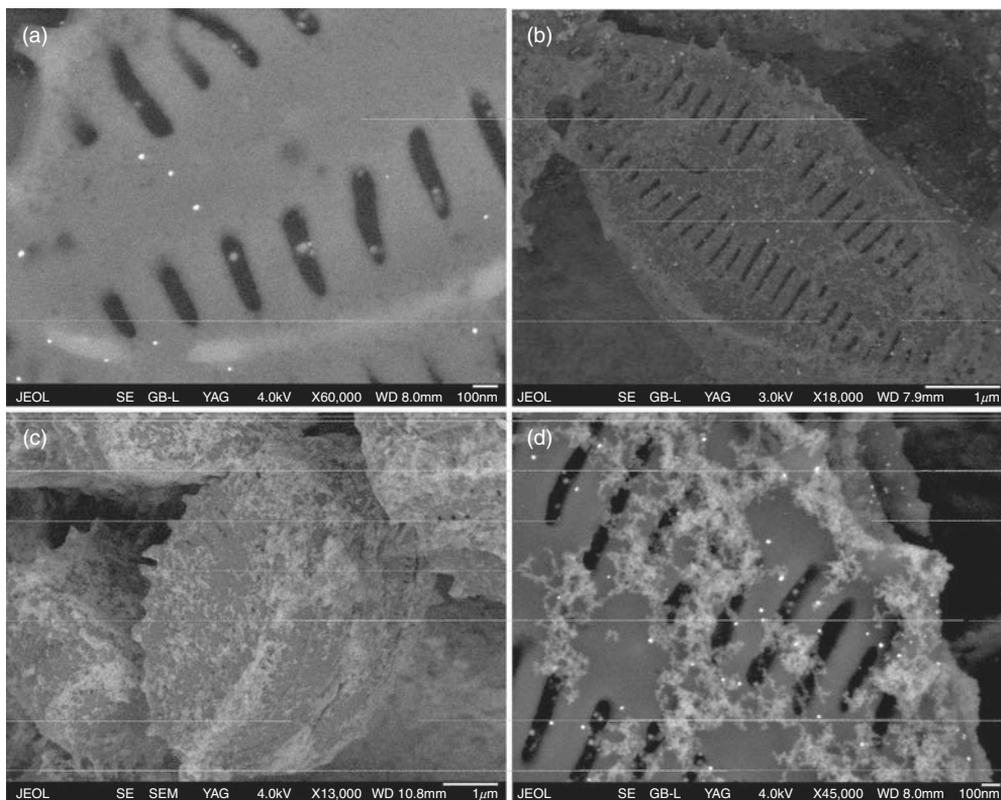


Fig. 5.7. SEM micrographs of samples containing (a–b) silica frustules and gold nanoparticles, (c–d) silica frustules and gold nanoparticles modified with ferrofluid. This figure is reproduced from Schröfel (2012) with permission of the author.

minimal inhibition concentration assessments were performed for more detailed descriptions of biocomposite antibacterial properties and showed promising results for further investigation (Table 5.1). For this reason, bio-nanocomposite material could possibly be used in water purification (disinfection) systems, membranes or wastewater treatment plants (Lohmueller *et al.*, 2011).

Biosynthesis of Metallic Nanoparticles with *Mallomonas kalinae*

Regarding many other interesting organisms with silica-based structures, we focus further on biosynthesis experiments with the chrysophyte *Mallomonas kalinae* (MK) (Rezacova,

2006). The biosynthesis protocol is very similar to the one using diatoms. One of the anticipated advantages is the very fast formation of metal nanoparticles immediately after metal salt addition to the MK biomass, even faster than in the case of tested diatoms. In the course of a few minutes, the colour change caused by Au-NP formation in the reaction suspension can be observed.

These experiments have shown the extensive potential of these brown algae for nanoparticle biosynthesis. Regarding our experiences with magnetic modification of different varieties of biomass, it can be assumed that preparation of magnetically responsible nanocomposites formed by MK cells and precious metal NPs will also be feasible.

Figure 5.12 clearly shows biosynthesized gold nanoparticles documented by means of

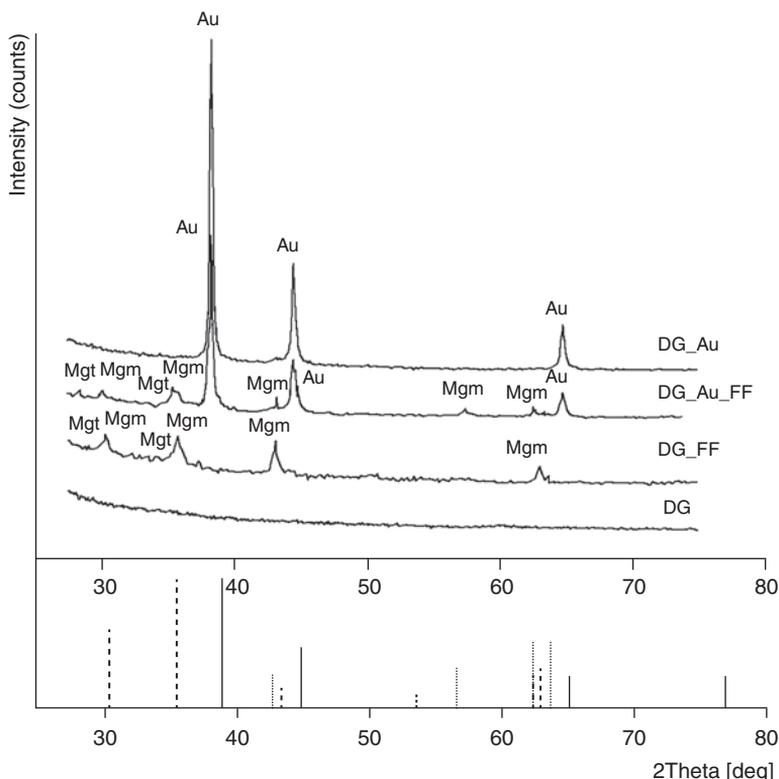


Fig. 5.8. XRD diffractogram of bionanocomposite samples containing gold nanoparticles (Au) and ferrofluid. The lower graph represents theoretical diffraction patterns for cubic gold (Au; solid line), maghemite (Mgm; dotted line) and magnetite (Mgt; dashed line). This figure is modified from Schröfel (2012) with permission of the author.

TEM. Based on the selected TEM micrographs, image analysis was also performed and the NP size distribution was assessed (Fig. 5.12d).

Considering future applications of MK_AuNP composites or suspensions with Au-NP synthesized by the use of MK, preliminary experiments of soman hydrolysis were performed (data not shown). Soman, a nerve agent (GD, *O*-pinacolyl methylphosphonofluoridate), was decomposed effectively using MK_AuNP in the aqueous environment. These experiments illustrate the potential of these golden algae for new functional nanomaterial preparations; therefore, optimization of the biosynthesis protocol and other analyses and tests will be performed in the near future.

Conclusions and Future Prospects

Based on this chapter and other cited literature, we anticipate the further development of biosynthesis methods for metallic nanoparticle preparation. We have shown important considerations in the possible utilization of these nanomaterials in a multitude of applications. In particular, we have reported the fabrication of multi-purpose materials containing noble metal nanoparticles stabilized with cellular matrix on the biogenous silica scaffold. This biosynthesis approach and usage of silica-based algal strains takes advantage of a simple three-in-one method for material preparation, where nanoparticle formation, stabilization and immobilization take place simultaneously. The resulting material is solid and can be



Fig. 5.9. Illustration of ferrofluid modified bionanocomposite behaviour in external magnetic field. This figure is reproduced from Schröfel (2012) with permission of the author.

stored as a powder or suspension. Unlike the colloidal solutions of nanoparticles, the solid state of the bionanocomposite also implies further transformation – we performed magnetic modification, which resulted in the magnetically recoverable material. Examples of catalytic and antimicrobial applications were also illustrated. This method of bionanocomposite preparation can be utilized in many similar (catalysis, medical) applications due to its feasibility, simplicity and effectiveness.

For many reasons, diatoms and chrysophytes are remarkable and interesting creatures. They represent the variety and adaptability of all living organisms. The ubiquity of diatoms in a range of widely varied environments clearly illustrates the success of their survival strategy; building their homes from glass using renewable energy resources is just one example. Diatoms have been utilized more frequently in recent technological applications, demonstrating the increasing significance of nature to scientists in providing inspiration with biogenous materials. Any single diatom or chrysophyte cell can create nanostructured shapes, the likes of which cannot even be replicated by current lithography

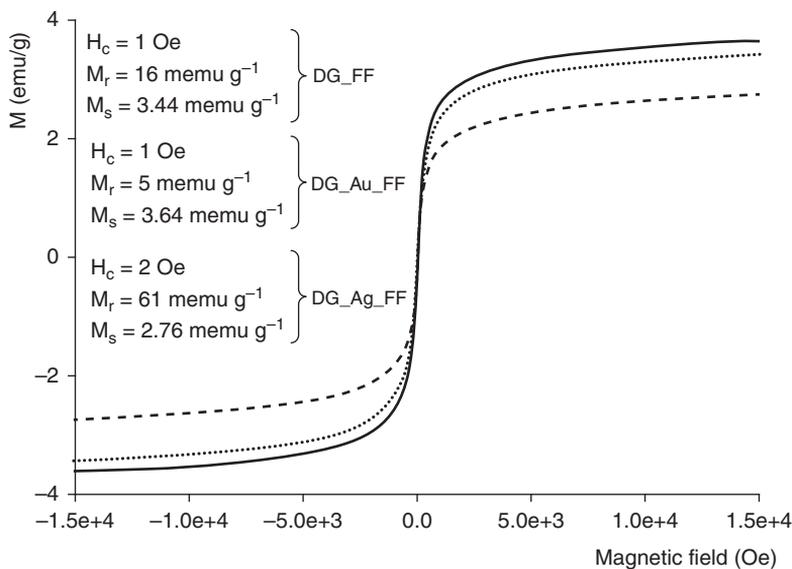


Fig. 5.10. Magnetic saturation curves of magnetically modified bionanocomposites (BNCs) in external magnetic field (15,000 Oe). DG_FF (solid line) = sample containing magnetically modified diatom cells; DF_Au_FF (dotted line) = sample containing magnetically modified diatom cells with gold nanoparticles; DF_Ag_FF (dashed line) = sample containing magnetically modified diatom cells with silver nanoparticles. This figure is modified from Schröfel (2012) with permission of the author.

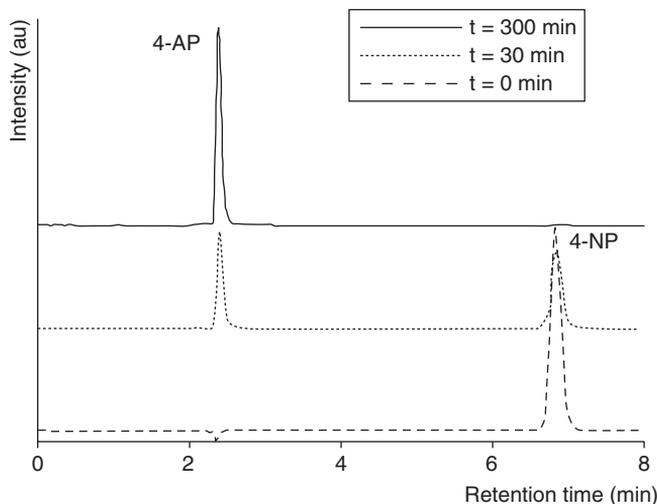


Fig. 5.11. HPLC chromatogram – conversion of 4-NP (0.1 mM) to 4-AP in the presence of NaBH_4 (8 mM) and golden diatom-based bionanocomposite (4.5 g l^{-1}). This figure is reproduced from Schröfel (2012) with permission of the author.

Table 5.1. Minimum inhibitory concentration assessment, silver-based diatom nanocomposite, 37°C .

Bacterial culture	1 day			2 days			3 days		
	10%	3.33%	1.12%	10%	3.33%	1.12%	10%	3.33%	1.12%
<i>Bacillus anthracis</i> ^a									
<i>Staphylococcus aureus</i>									
<i>Streptococcus agalactiae</i>									
<i>Escherichia coli</i>									
<i>Pseudomonas aeruginosa</i>									
Slowing down	Inhibition			No effect					

Notes: ^aBacterial spores.

techniques. This chapter should therefore serve as motivation for deriving more inspiration from the nature surrounding us, which could lead to a better understanding of complex natural processes and the development of novel biotechnology methods.

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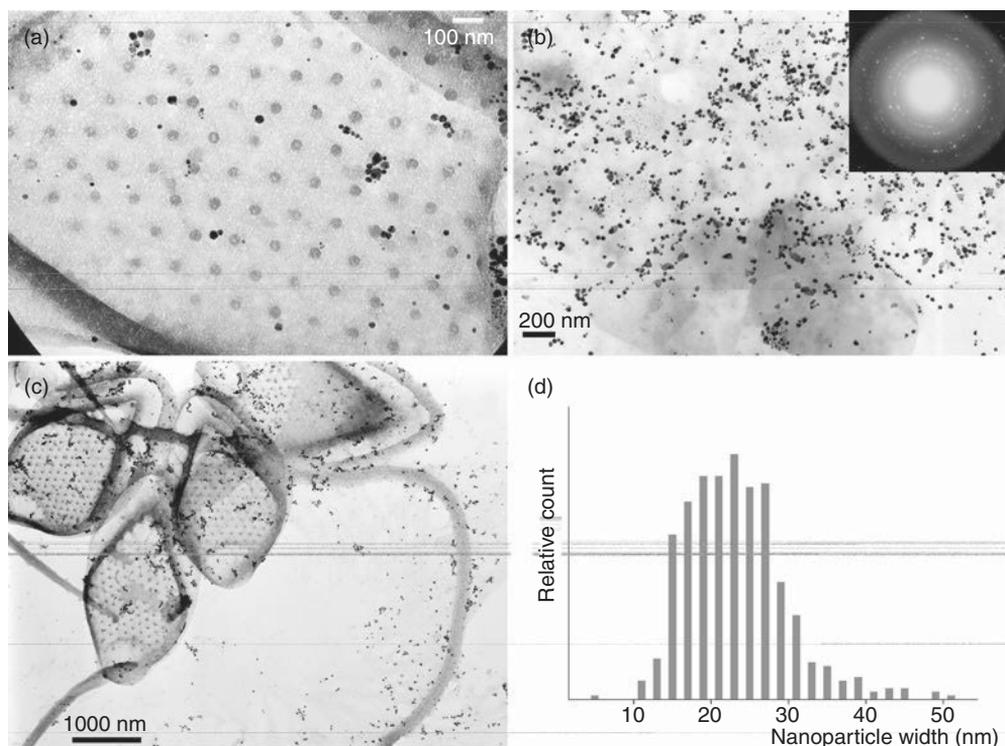


Fig. 5.12. TEM diffractograms of samples containing chrysophyte cells and gold nanoparticles. (a) Detail of the silica scale with pores in the structure and attached gold nanoparticles. (b) Detail of gold nanoparticles synthesized by *Mallomonas kalinae*. SAED (insert) confirms only Au nanocrystalline particles. (c) Assembly of a few silica scales of *Mallomonas kalinae* with biosynthesized gold nanoparticles. Arrangement of some nanoparticles into chains can be observed. (d) Histogram of gold nanoparticle size distribution; the mean size of nanoparticle according to image analysis is approximately 23 nm.

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6 Green Synthesis of Silver and Gold Nanoparticles Using Plant Extracts

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Introduction

The production of metallic silver and gold nanoparticles with different morphologies and sizes has been realized during the last decade using different routes (Mohanpuria *et al.*, 2008; Narayanan and Sakthivel, 2010; Zhang *et al.*, 2011; Gan and Li, 2012). These synthetic methods involve the reduction of an ionic form of silver and gold in an appropriate medium using various reducing agents. The most environmentally benign method uses plant extract as a reducing agent (Thakkar *et al.*, 2010). This biological approach to silver and gold nanoparticle synthesis is ideal in terms of green chemistry (Medina-Ramirez *et al.*, 2009; Rani and Rajasekharreddy, 2011; Abdel-Mohsen *et al.*, 2012; Vijayaraghavan *et al.*, 2012). Research in the past decade has provided evidence that a large number of biological species, including bacteria (Narayanan and Sakthivel, 2011), fungi (Bhainsa and D'Souza, 2006), algae (Singaravelu *et al.*, 2007), yeast (Mohanpuria *et al.*, 2008) and plant extract (Song and Kim, 2009; Narayanan and Sakthivel, 2011) are active in the reduction of both Ag and Au to nanocrystals.

Although various techniques including chemical and physical means have been developed, the biosynthesis of metal nanoparticles

continues to be used by researchers worldwide (Kumar, 2009). The idea of using plant extract for the biosynthesis of metal nanoparticles is based on the observation that plants can uptake and bioreduce metal ions from soils and solutions. For instance, lucerne plants were grown in an AuCl_4^- -rich environment. The research data confirmed the nucleation and growth of Au nanoparticles inside the plant (Gardea-Torresdey *et al.*, 2002). The use of plant extracts for nanoparticle synthesis can be advantageous over other biological processes because it eliminates bacteria, fungi and yeast harvesting.

The biosynthesis of metal nanoparticles involves three main steps: (i) the selection of reducing agents; (ii) the selection of the solvent medium; and (iii) the selection of stabilizing reagents. Generally, plant extract contains both a reducing agent and a stabilizing polymer. For these reasons, plant extracts are responsible for the nanoparticle synthesis. This chapter reviews plant extract preparation methods.

Plant Extract Preparation

Gardea-Torresdey and co-workers (Gardea-Torresdey *et al.*, 2002, 2003) first reported the synthesis of silver and gold nanoparticles

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using live lucerne plants. After being crushed, lucerne seeds were placed in contact with a nutrient solution containing gold or silver salts. The lucerne plants were harvested 9 days after germination. The formation of silver and gold nanoparticles at the sprouts was supported by X-ray absorption spectroscopy (XAS) analysis.

The extracellular synthesis of silver nanoparticles using geranium leaf extract was reported by Shankar and co-workers (Shankar *et al.*, 2003). Fresh and washed *Pelargonium graveolens* leaves (20 g) were added to 100 ml of sterile distilled water and then boiled for 1 min. After cooling, the water extract was ready to use as a reduction reagent. Next, 5 ml of extract was added to 100 ml of 10^{-3} M AgNO_3 solution.

The synthesis of nanoparticles using a plant extract eliminates the need for the elaborate processes of cell culture preparation and microbial cell growth. The protocol for plant extract preparation can vary. Fresh leaves are generally finely cut and extracted by distilled water. For instance, the extract preparation procedure using *Aloe vera* leaves was as follows: a 30 g portion of *Aloe vera* leaves were finely cut and boiled in 100 ml of distilled water. The obtained extract was used for nanoparticle synthesis (Chandran *et al.*, 2006).

Pioneering works on silver and gold nanoparticle synthesis using plant broths have been carried out by Sastry and his group. Shankar and co-workers (Shankar *et al.*, 2004) reported metal nanoparticle synthesis by the reduction of silver and gold ions using neem (*Azadirachta indica*) leaf broth. The neem leaf broth was prepared by placing 20 g of fresh and finely cut *A. indica* leaves in a 500 ml Erlenmeyer flask with 100 ml of sterile distilled water. The mixture was boiled for 2 min before the broth was decanted. The morphologies and aggregation kinetics of the Ag-nanoparticles were controlled by the addition of a conventional surfactant (cetyltrimethylammonium bromide, CTAB). The effects of the pre- and post-micellar concentration of CTAB were investigated (Khan *et al.*, 2012a).

Acalypha indica from the Euphorbiaceae family is a traditional South Indian plant (Krishnaraj *et al.*, 2010). The freshly collected

leaves (10 g) were used to prepare an aqueous extract. The biomass was boiled with 100 ml of distilled water at 60°C for 5 min. The water extract was filtered through a nylon filter (0.45 μm) and used for silver nanoparticle synthesis.

Magnolia (*Magnolia kobus*) and persimmon (*Diospyros kaki*) leaves were used by Song and co-workers (Song *et al.*, 2009) to achieve a quicker synthesis of silver and gold nanoparticles. They reported that silver nanoparticles could be synthesized rapidly using plant extract. The broth solution was prepared by placing 5 g of leaves in a 300 ml Erlenmeyer flask with 100 ml of sterile distilled water. The mixture was boiled for 5 min before finally decanting it to a pure solution. The pure extract was stored at 4°C and used within a week.

The leaves were collected from different places. Shenoy and co-workers have reported a one-step protocol for the preparation of extract (Shenoy *et al.*, 2011). The fresh leaves of *Anacardium occidentale* were collected from a rural area of Thiruvananthapuram, India. *A. occidentale* belongs to the Anacardiaceae family. This plant has great economic and medicinal value. The water extract was obtained using 10 g of the homogenized leaves, which were stirred with 100 ml of deionized water for 5 min.

Silver nanoparticles have been developed from *Artemisia nilagirica* leaves (Vijayakumar *et al.*, 2013). *A. nilagirica* belongs to the Asteraceae family. The fresh leaves were collected and dried for 10 days before being placed in an oven at 60°C for 24–48 h. The dry leaves were cut into fine pieces and placed in 100 ml of doubly distilled water. This mixture was boiled for 5 min and decanted. The pure extract was cooled and filtered through Whatman No. 1 filter paper. The extract was stored in a refrigerator.

The preparation of broth from *Cassia fistula* leaves was described by Lin and co-workers (Lin *et al.*, 2010). These trees were cultivated by the Department of Virescence, Xiamen University, China. The fresh *C. fistula* leaves were exposed to the sun. After 2 weeks, the completely dried biomaterial was crushed into powder and screened by a 20-mesh sieve. The obtained powder was used for silver

nanowire synthesis. First, 1 g of powder was added to 25 ml of deionized water in a 100 ml beaker. The mixture was boiled for 5 min. After boiling, the broth was filtered and the appropriate amount of deionized water was added. The total volume of the broth was 25 ml. The broth pH was approximately 5.7.

Chenopodium album, belonging to the Amaranthaceae family, is a widely distributed weed in Asia, North America and Europe. The leaves of *C. album* were collected during the months of October and November in Lucknow (Uttar Pradesh), India. The fresh leaf extract was prepared from 20 g of clean leaves. The leaf broth was boiled for 30 min and the cold suspension was filtered with Whatman No. 40 filter paper. This preparation of *C. album* broth was described in Dwivedi and Gopal's paper (Dwivedi and Gopal, 2010).

The biosynthesis of silver nanoparticles was carried out using the extract from *Cinnamomum camphora* (Lauraceae family) leaves, which were dried by exposure to the sun. The dry biomass was crushed and sieved (20 mesh). The leaf powder was used for the preparation of silver and gold nanoparticles (Huang *et al.*, 2007).

The leaves of *Coccinia grandis*, from the Cucurbitaceae family, were collected from a garden, washed by deionized water and finely cut for extraction. A 20 g sample of this green biomass was placed in 100 ml of hot water (80°C) for 3 min. The suspension was then filtered and the filtrate was used as a reducing agent for nanoparticle synthesis (Arunachalam *et al.*, 2012).

Cassia auriculata leaves were collected from the forest area of Vellore, Tamil Nadu, India. The dry *C. auriculata* leaves were ground into a powder and used for synthesis experiments. The leaf extract was prepared by adding 3 g of leaf powder to 30 ml of distilled water. Next, 3 ml of methanol was added to the solution for effective separation of the compounds. The mixture was shaken for 1 h, after which the extract was filtered. The *C. auriculata* extract was prepared by Kumar and co-workers (Kumar *et al.*, 2011) and used for nanoparticle synthesis.

Coriander (*Coriandrum sativum*) leaves, belonging to the Apiaceae family, were used

for extract preparation (Narayanan and Sakthivel, 2008). In this procedure, 20 g of fresh leaves were chopped and boiled in 100 ml of sterile, doubly distilled water for 3 min. The extract was filtered and used for the synthesis of gold nanoparticles.

The leaves of *Coscinium fenestratum* (from the Menispermaceae family) were collected from Banacaud Estate, Trivandrum, Kerala, India. This material was shade-dried and ground into powder. Next, 5 g of the powder was boiled for 10 min in 100 ml of sterile distilled water. The final suspension was filtered and the filtrate was used for silver nanoparticle synthesis (Jacob Packia *et al.*, 2012a).

A green silver nanoparticle synthesis using *Cycas* leaves was presented by Jha and Prasad (Jha and Prasad, 2010). *Cycas*, belonging to the family Cycadaceae, is a commercial source of sago. A 5 g sample of freshly collected *Cycas* leaves was used for the extract preparation. These leaves were washed thoroughly and cut into small pieces. The leaf sample was added to 200 ml of 50% ethanol solution and boiled for 15–20 min until the solution turned brown. This solution was filtered through activated charcoal. The pure extract was cooled to room temperature and used in silver nanoparticle synthesis.

Native medicine uses hibiscus effectively against hypertension, pyrexia and liver disorder (Philip, 2010). *Hibiscus rosa-sinensis*, from the Malvaceae family, is widely grown as an ornamental plant in East Asia. First, 20 g of leaf was finely cut and mixed with 200 ml of deionized water at 27°C for 1 min. Then, the mixture was filtered and used for nanoparticle synthesis.

Madhuca longifolia (Sapotaceae family) is commonly known as 'mahwa'. The leaf extract from this tropical tree was used to prepare gold nanoparticles (Fayaz *et al.*, 2011). A 20 g sample of finely cut leaves was dispersed in 1000 ml of ultrapure water. After 15 days, the supernatant was separated from the biomass. The polysaccharides were removed from the extract and the pure extract was concentrated by lyophilization.

The procedure for the synthesis of silver and gold nanoparticles using plants has been well explored. A leaf extract prepared from *Murraya koenigii* (Rutaceae family) was

reported by Philip and co-workers (Philip *et al.*, 2011). A 9 g sample of the leaves was cut and stirred with 200 ml of deionized water at 27°C for 1 min, after which the mixture was filtered and stored. The filtrate was used for silver and gold nanoparticle synthesis.

A new, simple silver and gold nanoparticle synthesis procedure using *Rosa damascena* (Rosaceae family) was proposed by Ghoreishi and co-workers (Ghoreishi *et al.*, 2011). *R. damascena* flowers were collected from Kashan (Iran). The flowers were dried at 30°C. A 10 g sample of the sun-dried flowers was passed through a 30-mesh sieve and mixed with 100 ml of deionized water at 30°C for 5 min. The fresh extract was filtered and stored for nanoparticle synthesis.

Piper betle (Piperaceae family) leaves were purchased from a local market in New Delhi, India (Khan *et al.*, 2012b). A 3 g sample of piper leaves was chopped into fine pieces and mixed with 250 ml of doubly distilled water. The mixture was heated for 20 min at 60°C. A raw extract was filtered and stored in a brown glass bottle. *P. betle* L. leaf extract was used for Ag-protein spherical nanoparticle production (Rani and Rajasekharreddy, 2011).

The green synthesis of Ag and Au nanoparticles using *Rosa rugosa* leaf extract (Rosaceae family) was reported by Dubey *et al.* (2010b). *R. rugosa* occurs in its natural form in East Asia, from Ochotsk and southern Kamchatka to Korea and northern China, as well as northern Japan. Fresh *R. rugosa* leaves were collected from Mikkeli, Finland, to prepare the leaf extract. First, 50 g of the leaves was placed in a 500 ml Erlenmeyer flask and boiled in 250 ml of ultrapure water for 20 min. Then, the extract was filtered and stored at -15°C.

Stevia rebaudiana leaves, from the sunflower Asteraceae family, were used successfully for the green synthesis of gold nanoparticles (Yilmaz *et al.*, 2011). The shade-dried leaves were crushed and powdered. A 0.1 g sample of the stevia leaf powder was added to 50 ml of deionized water and stirred for 1 h at room temperature. The suspension was centrifuged at 10,000 rpm for 30 min. Equal volumes of the stevia leaf water extract and 0.1 mM AgNO₃ solution were mixed together.

Stable monometallic Au and Ag nanoparticles and Au/Ag bimetallic alloy nanoparticles were synthesized using mahogany leaf extract. *Swietenia mahagoni* is a tree belonging to the Meliaceae family that is found in several Asian countries. The mahogany leaves were collected and shade-dried and then the dried leaves were powdered. The extract was prepared using 1 g of powder mixed with 15 ml of doubly distilled water and boiled at 100°C for 5 min. The suspension was filtered and the brown solution was used for the synthesis of Au and Ag nanoparticles and for bimetallic Au/Ag synthesis (Mondal *et al.*, 2011).

Tea leaf broth, produced from an aqueous infusion of tea leaves, contains a variety of organic compounds such as caffeine, flavonoids, chlorophyll and tannic acid. The broth was extracted using ethyl acetate followed by CH₂Cl₂. Both organic extracts contained polyphenols and caffeine. The broth was prepared by boiling 180 g of dried tea leaves in 400 ml of water (Begum *et al.*, 2009).

Extract Preparation from Leaf Powder

The leaves of *Iresine herbstii*, from the Amaranthaceae family, were collected in the villages of the Bankura (West Bengal, India). The collected leaves were washed and cleaned using detergent. The cleaned leaves were dried at room temperature (25–28°C) for 10 days. The dry leaves were pulverized using an electrical blender (Dipankar and Murugan, 2012). A 25 g sample of the powder was continuously extracted using ethanol. The extraction was conducted using a Soxhlet apparatus at the boiling point of the extract for 12–16 h. The ethanol leaf extract was concentrated under reduced pressure with a rotary evaporator. The water extraction was performed parallel to the ethanol extraction. Then, 5 g of powdered leaf was mixed with 100 ml of sterile deionized water. The suspension was boiled for 5 min, cooled and filtered. The extract was used for the preparation of silver nanoparticles.

The morphologies of the silver nanoparticles synthesized using *Bacillus subtilis* and using leaf extract from the *Catharanthus roseus*

plant were compared by Kannan and co-workers (Kannan *et al.*, 2011). The herb *C. roseus* belongs to the Apocynaceae family and is commonly known as the Madagascar periwinkle. The selected leaves were dried in the shade for 1 week to ensure complete dehydration. The dried leaves were pulverized in a mortar. The leaf extract was prepared by mixing 10 g of the powdered leaves with 100 ml of deionized water. The suspension was boiled for 10 min and then filtered.

The preparation of plant extract from the leaves of *Mentha piperita* (Lamiaceae family) was described by MubarakAli and co-workers (MubarakAli *et al.*, 2011). The clean leaves were dried in the shade for 5 days. Next, the dried leaves were powdered using a kitchen blender. The leaf powder was sterilized at 121°C for 15 min. For the extract preparation, 20 g of powder was taken and mixed with 200 ml of deionized water. The suspension was kept at 60°C in a water bath for 10 min. The extract was filtered and stored at 4°C.

Morinda citrifolia, from the Rubiaceae family and known by the name 'noni', has therapeutic value. The healthy leaves were collected from the Bharathidasan University campus, Tiruchirappalli, Tamil Nadu, India (Sathishkumar *et al.*, 2012). The leaves were cleaned with distilled water and shade-dried for 10 days. For the preparation of the water extract, 20 g of the dry leaves was mixed with 200 ml of distilled water and kept in a hot water bath at 60°C for 10 min.

Aqueous *Piper longum* (Piperaceae family) leaf extract was used for the rapid synthesis of silver nanoparticles (Jacob Packia *et al.*, 2012b). The leaves of *P. longum* were shade-dried and powdered. Next, 5 g of the leaf powder was mixed with 100 ml of sterile distilled water and boiled for 10 min. The water extract was filtered twice (25 and 0.6 µm filters) and used for nanoparticle synthesis.

Apiin (apigenin-7-apiosyl-glucoside) was obtained from the air-dried powdered leaves of henna (*Lawsonia inermis*). A 3 kg sample of leaf powder and 15 l of methanol were used for apiin extraction at room temperature. The methanolic solution was concentrated at or below 40°C. Pure apiin was crystallized from ethyl acetate solution (Kasthuri *et al.*, 2009).

Eclipta alba and *Eclipta prostratata*, belonging to the family Asteraceae, are common weeds. These plants have potential medicinal properties as herbs (Jha *et al.*, 2009). The reducing reagent was prepared by washing and drying 5 g of *Eclipta* leaves, then adding the dry biomass to a 250 ml beaker containing 200 ml of a 50% ethanol/water mixture. The beaker was placed in a boiling water bath for 15–20 min. The initial broth solution was treated with activated charcoal to remove chlorophylls. The suspension was filtered through cloth and the pure solution was treated as the reducing reagent.

The synthesis of silver nanoparticles using the bark extract and powder of *Cinnamomum zeylanicum* was described by Sathishkumar and co-workers (Sathishkumar *et al.*, 2009). *C. zeylanicum* bark is widely used as a spice, and essential cinnamon oil has antimicrobial properties. *C. zeylanicum* bark was purchased from a local Indian market. The bark was grained and sieved using a 20-mesh sieve to obtain a fine fraction. To produce the aqueous extract, 2.5 g of powder was mixed with 100 ml of sterile distilled water and boiled for 5 min.

Gold nanotriangles were synthesized using sun-dried *C. camphora* leaves collected at ambient temperature (Huang *et al.*, 2007). The dried *C. camphora* leaf powder was added to 50 ml of 10⁻³ M aqueous AgNO₃ or HAuCl₄ solution at room temperature.

The use of plant extract for the rapid synthesis of gold nanoparticles has been presented by Sneha and co-workers (Sneha *et al.*, 2010). *P. betle*, belonging to the Piperaceae family, has been used for extract preparation. Fresh leaves were purchased from a local market (Jeonju, Korea), washed and sun-dried for a week. Next, the leaves were powdered in a mixer and sieved. A 20-mesh fraction was used for the plant extract preparation. Then, 2.5 g of *P. betle* powder was added to 100 ml of sterile distilled water and the suspension was boiled for 2 min and allowed to cool to room temperature.

The rowan tree (*Sorbus aucuparia*), a member of the Rosaceae family, is ubiquitous in Europe and western Asia. The corresponding extract was prepared from 45 g of washed rowan leaves in a 500 ml Erlenmeyer flask

and boiled in 180 ml of water for 15 min. The rowan extract was filtered with Whatman No. 40 filter paper and stored at -15°C . The metal nanoparticle synthesis was optimized using different extract qualities, metal solution concentrations, temperatures and contact times (Dubey *et al.*, 2010a).

Extract Preparation from Plant Fruit Body

A new approach to the biosynthesis of silver and gold nanoparticles is the use of plant fruit bodies. The dried fruit bodies from the plant *Tribulus terrestris* in the Zygophyllaceae family were used for water extract preparation (Gopinath *et al.*, 2012). The fruits were collected from a local market in Chennai, India, and sun-dried for 1 week. The dried fruits were pulverized to obtain a powder. Then, 5 g of powder sample was mixed into 100 ml of deionized water and the suspension was boiled for 10 min. The extract was cooled and filtrated. The extract was stored at 4°C for further nanoparticle synthesis.

Banana peels (*Musa paradisiaca*) are rich in polymers such as lignin, hemicellulose and pectins. The extract from banana peels has been proposed for use in the synthesis of silver nanoparticles (Bankar *et al.*, 2010). In the initial stage of extract preparation, 100 g of banana peel was boiled in distilled water for 30 min at 90°C . Next, the peels were crushed and added to 100 ml of pure water. After filtration, the water extract was treated with an equal volume of chilled acetone. The resultant precipitate was separated and dried to a powder. This powder was used for nanoparticle synthesis.

The biosynthesis of silver and gold nanoparticles using *Asparagus lettuce* peel was described by Sun and co-workers (Sun *et al.*, 2012). The peel biomass was cut into small pieces and ground into a powder. The extract solution was prepared using 5 and 10 g of the ground powder in 100 ml of distilled water. The suspension was boiled and filtered. The final solution was stored at 4°C .

Annona squamosa (Annonaceae family) is an edible fruit distributed throughout America and Asia. The *A. squamosa* fruits

were collected from Melvisharan, Vellore District, Tamil Nadu, India. A 4 g sample of the powdered peels was collected and mixed with 40 ml of doubly distilled water. The mixture was shaken for 1 h, after which the extract was filtered through Whatman filter paper (Kumar *et al.*, 2012).

Orange (*Citrus sinensis* from the Rutaceae family) peel has also been used for extract preparation (Konwarh *et al.*, 2011). Approximately 0.2 g of orange peel was washed with deionized water and ground using a domestic blender. The peel pulp was added to 50 ml of hot water (50°C) for approximately 20 min; the aqueous extract was filtered through a muslin cloth at ambient temperature. Pure extract was used for silver nanoparticle synthesis.

The aqueous extract of fruits from *Terminalia chebula* (Combretaceae family) trees was used for the green synthesis of silver nanoparticles (Edison and Sethuraman, 2012). A 0.4 g sample of *T. chebula* powdered fruit was extracted using 100 ml of distilled water. The extraction process was carried out at 50°C for 2 min. The extract was filtered before use in silver nanoparticle synthesis.

A rapid, room temperature synthesis was realized by treating AgNO_3 solution with *Citrus limon* (lemon) extract (Prathna *et al.*, 2011). Lemons are a rich source of citric acid and ascorbic acid. Fresh lemons were purchased from a local market in Vellore, India, and squeezed to obtain lemon juice. This juice was filtered and centrifuged at 10,000 rpm for 10 min. The pure supernatant was used for synthesis experiments. The citric acid concentration in the lemon extract was 1.8–2.0%.

Extract Preparation from Rhizomes, Stem Bark and Tubers

The biosynthesis of gold nanoparticles by *Zingiber officinale* (ginger) rhizomes from the Zingiberaceae family was proposed by Kumar and co-workers (Kumar *et al.*, 2011). Fresh *Z. officinale* rhizomes were washed with deionized water. A 20 g sample of the pure rhizomes was cut and boiled in 250 ml of deionized water. The broth was filtered to

obtain a pure extract. *Z. officinale* possesses many therapeutic abilities.

Silver nanoparticles were also prepared using *Curcuma longa* (Zingiberaceae family) tuber powder and extract (Sathishkumar *et al.*, 2010). *C. longa* tubers were purchased from the local market in Jeonju (Republic of Korea). The tubers were sun-dried for a week. The dry tubers were cut into small pieces and powdered in a mechanical mixer. The obtained powder was sieved using a 20-mesh sieve. The final powder fraction was used for biosynthesis.

Sugarbeet (*Beta vulgaris*, Amaranthaceae family) pulp is a residue obtained during sugar production. The sugarbeet pulp was provided by the Azucarera Ebro Agricola plant in Toro (Zamora, Spain). The pulp was collected directly from the final drying line. The pulp was washed and filtered to remove the molasses. It was then dried in a stove at 60°C and pulverized (or ground) in an agate mortar. A 0.25 g sample of sugarbeet pulp was added to 50 ml of AuCl₄ solution to prepare gold nanoparticles (Castro *et al.*, 2010).

The stem bark of *Callicarpa maingayi* was used for the extract preparation. *C. maingayi* belongs to the Verbenaceae family and is found in Malaysia and Singapore. According to Shameli and co-workers (Shameli *et al.*, 2012), the stem bark was ground into a powder. This powder (20 g) was extracted with a methanol/water mixture (ratio 80:10 v/v) at room temperature for 72 h. The suspension was then filtered and concentrated using a rotary vacuum evaporator at 40°C. The concentrated extract was stored at 4°C until use.

Extract Preparation from Seeds

Aqueous extracts of *Trachyspermum ammi* and *Papaver somniferum* were prepared (Vijayaraghavan *et al.*, 2012). These seeds were purchased from a local market (Chennai, Tamil Nadu, India), washed and sun-dried for 5 h to remove the residual moisture. The seeds were powdered and sieved to obtain a fine powder fraction. Next, 25 g of the powder was added to 100 ml of

hot, sterile distilled water. The water extract was stored at 4°C for further nanoparticle synthesis.

Gold nanoparticles of various morphologies were formed during the incubation of wheat (*Triticum aestivum*) biomass with Au(III) ions at pH 2–6 (Narayanan and Sakthivel, 2011).

A green method for the synthesis of silver nanoparticles using aqueous seed extract of *Jatropha curcas*, from the Euphorbiaceae family, was presented by Bar and co-workers (Bar *et al.*, 2009). *J. curcas* is commercially important; biodiesel is produced from *J. curcas* seeds on an industrial scale. A 50 g sample of the seeds was fragmented and boiled with 500 ml triply distilled deionized water for 2 h. The mixture was filtered and stored for further use.

Plant Latex Preparation

Crude latex obtained by cutting the green stems of *J. curcas* plants was used for biosynthesis. The milky white latex was stored at –20°C until use. In a typical reaction procedure 1 ml of crude latex was diluted to 300 ml using triply distilled deionized water to make a 3% latex solution. Next, 20 ml of this latex solution was mixed with 20 ml of 5 × 10^{–3} M aqueous silver nitrate solution. The mixture was heated at 85°C with constant stirring for 4 h in an oil bath (Bar *et al.*, 2009).

Gum kondagogu trees (*Cochlospermum gossypium*) are naturally found in the forests of India. Natural hydrocolloid gum kondagogu was used for silver, gold and platinum nanoparticle synthesis (Vinod *et al.*, 2011). The plant-derived natural product was collected and cleaned; the gum kondagogu samples were collected from the Girijan Co-operative Corporation, Government of Andhra Pradesh Undertaking, Hyderabad, India. The collected material was powdered using a high-speed mechanical blender. The powder was classified and the –250 µm fraction was used for the biosynthesis procedure.

A simple method for producing silver nanoparticles using latex and the light from a

xenon lamp was proposed by Almeida de Matos and co-workers (Almeida de Matos *et al.*, 2011). The latex was obtained from *Euphorbia milii*, which belongs to the Euphorbiaceae family. A 3% plant latex aqueous solution was added to an AgNO_3 solution. The mixture was stirred vigorously for 10 min and subjected to irradiation from a xenon lamp.

Apiin (aspiigenin-7-glucoside) was isolated as follows. A 3 kg sample of air-dried powdered henna leaves was extracted with 15 l of methanol at room temperature. The methanolic extract was concentrated and 1 l of water was added. The apiin was extracted with petroleum ether, chloroform and ethyl acetate. The ethyl acetate fraction contained cosmosiin and apiin. Both products were separated by fractional crystallization. Cosmosiin was separated out first and the subsequent fraction contained apiin (Kasthuri *et al.*, 2009).

Plant leaf extracts of pine (*Pinus densiflora*; Pinaceae family), ginko (*Ginkgo biloba*; Ginkgoaceae family) and platanus (*Platanus orientalis*; Platanaceae family) were prepared by adding 5 g of pure, finely cut leaves to a 300 ml Erlenmeyer flask with 100 ml of

distilled water. The mixture was boiled for 5 min and the extract was stored at 4°C for a week (Song and Kim, 2009).

Silver and Gold Nanoparticle Biosynthesis

The following procedures were used for silver and gold nanoparticle syntheses. Several millilitres of leaf extract were added to AgNO_3 or H_3AuCl_4 aqueous solutions of a concentration of $1\text{--}2.5 \cdot 10^{-3}$ M. The majority of the synthesis was carried out at room temperature. The nanoparticle preparation kinetic was observed by the change in the ultraviolet-visible (UV-Vis) spectra as a function of time. The silver surface plasmon resonance band occurred at 420 nm (Fig. 6.1). In the case of gold nanoparticles, the band occurred at 550 nm. The sharpness of the peak of the UV-Vis spectra depended on the leaf extract concentration. The UV-Vis spectra exhibited a red or blue shift, depending on the particle size. The size and shape of the nanoparticles can be controlled by varying the ratio of metal ions to plant extract.

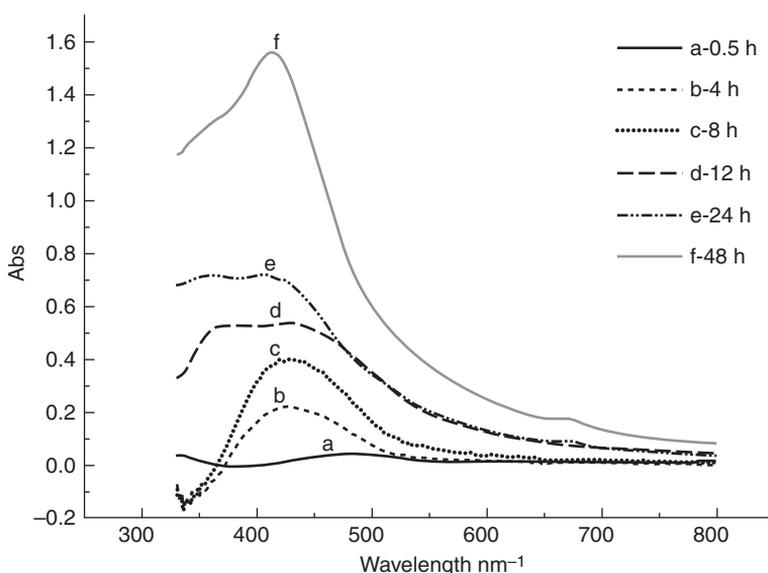


Fig. 6.1. Absorption spectra of silver nanoparticles prepared with *Cassia fistula* leaf extract. (Modified from Lin *et al.*, 2010, with permission of the publisher.)

The second method for preparing metal nanoparticles involved directly adding the leaf powder to silver or gold salt solutions. The formation of gold nanoparticles by *C. camphora* leaf powder depended strongly on the amount of dried biomass used (Huang *et al.*, 2007). The effect of temperature on the synthesis rate and particle size of the gold nanoparticles was also studied (Song *et al.*, 2009), revealing that spherical gold nanoparticles were obtained at 95°C.

The obtained silver and gold nanoparticles were characterized by X-ray diffraction (XRD) analysis. The XRD patterns of silver and gold nanoparticles showed the Bragg reflections corresponding to (111), (200), (220), (311) and (222) (Fig. 6.2).

Zeta potential studies showed that the surface charge of the formed nanoparticles was highly negative and that the isoelectric point was approximately pH 2 (Sadowski *et al.*, 2008).

Fourier transfer infrared spectroscopy (FTIR) analyses were used to identify the biomolecules responsible for the reduction of the Ag^+ and AuCl_4^- ions. The strong absorption bands were assigned to hydroxyl, amine and carbonyl groups. The results suggested that polysaccharides, alkaloids, terpenoids, flavonoids, polyphenols and

amines might be responsible for the bio-reduction (Fig. 6.3).

Conclusions

The biosynthesis of metal nanoparticles using plant extracts is an emerging area of nanotechnology. The synthesis of silver and gold nanoparticles using leaf extracts is an example of a facile green process. These extracts contain both reducer and stabilizer reagents, which are responsible for the production of the silver and gold nanoparticles. The plant extract preparation method has an important effect on nanoparticle morphology. However, the diverse compositions of plant extracts do not explicitly answer what is responsible for the metal ions reduction. This question needs more investigation.

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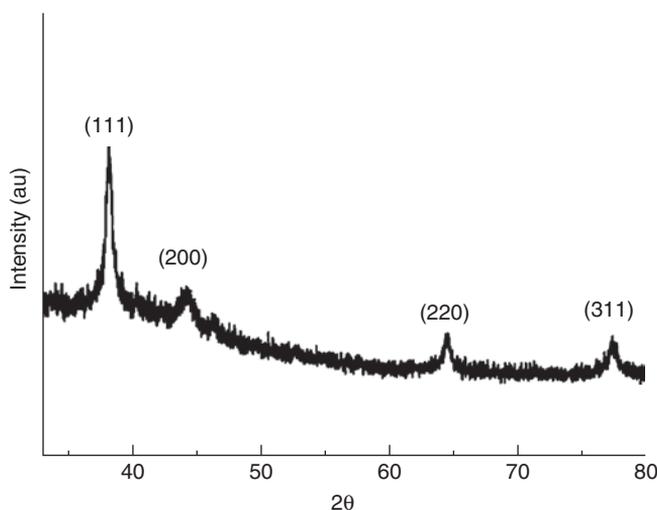


Fig. 6.2. XRD pattern of Ag-NPs prepared with *Cassia fistula* leaf extract. (Reproduced from Lin *et al.*, 2010 with permission of the publisher.)

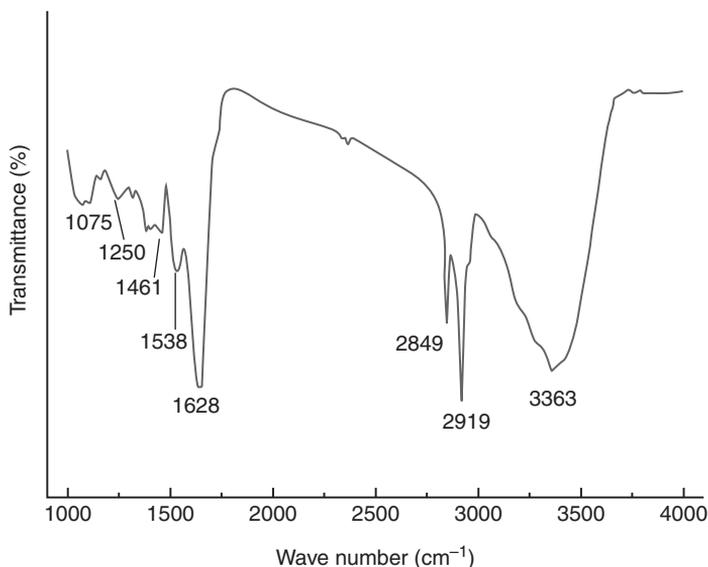


Fig. 6.3. FTIR absorption spectrum of silver nanoparticles prepared with *Cassia fistula* leaf extract. (Reproduced from Lin *et al.*, 2010 with permission of the publisher.)

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7 Rolls and Sandwiches: Cages and Barrels

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Introduction

Nanobiotechnology denotes a multidisciplinary man-made characterization, synthesis and application of nano-sized (10^{-9} m) functional particles for industrial and medical purposes, and there is a whole spectrum of new opportunities in health care, environmental biology, chemical and materials sciences and communications (Sahoo *et al.*, 2007). More specifically, nanobiotechnology, or perhaps it would be cogent to use the term 'nanomedicine', can be exploited in the areas of drug and/or gene delivery (de Kozak *et al.*, 2004; Feng *et al.*, 2004; Garcia-Garcia *et al.*, 2005), the preparation and properties of metal nanoparticles (Prabha and Labhasetwar, 2004; Deng *et al.*, 2009; Fan *et al.*, 2011), imaging (Lin and Datar, 2006; Mulder and Fayad, 2008), dendrimers (Quintana *et al.*, 2002; Samad *et al.*, 2009), molecular diagnostics (Jain, 2003, 2005; Johnson *et al.*, 2008), nano-proteomics (Johnson *et al.*, 2008), cardiac therapy (Wickline *et al.*, 2006; Hoshino *et al.*, 2007), biosensors (Nam *et al.*, 2003), labels for cell and biomolecules (Elechiguerra *et al.*, 2005) and antimicrobial agents (Shrivastava *et al.*, 2007).

A key role to understanding the properties of nanoparticles and the nanoparticle biomacromolecule (proteins, enzymes) interactions has emerged with an understanding of the 'corona' or dynamic layer that is conceived as being the effective unit of interest (Lynch and Dawson, 2008). Several studies of such interactions have appeared in the literature (Cedervall *et al.*, 2007; Lindman *et al.*, 2007; Lynch, 2007; Calzolari *et al.*, 2010), but they have not defined what is actually happening at the biomolecular–nanoparticle interface. The binding of molecules to nanoparticles affects their structure to varying degrees (Fei and Perrett, 2009). As the particle becomes smaller and smaller, not only does its association with the biomacromolecule change but also the organizational composition changes as well. Inevitably, this leads the biomolecules to behave differently and to have totally different biological properties when compared to a native one (Elechiguerra *et al.*, 2005; Blackman, 2009). Very small nanoparticles (4–8 nm) can pass unhindered through cell membranes and access a greater array of biological processes. Materials like carbon or silicon at the nanoscale demonstrate novel properties such as increased strength, electrical conductivity,

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chemical reactivity and other properties that are not observed under normal micro- or macro-level conditions (Aitken *et al.*, 2004).

A large number of versatile and highly stable materials such as nanowires, nanotubes and other nanomaterials have already been produced. These include nanoclusters, defined as semi-crystalline nanostructures with dimensions between 1 and 10 nm; nanopowders that result from the aggregation of non-crystalline nanomaterials with dimensions between 10 and 100 nm; nanocrystals that are single crystalline nanomaterials with dimensions between 100 and 1000 nm; nanorods, nanocups, nanospheres, nanodiamonds, nanostars and quantum dots. Moreover, the coupling of nanoparticles to biomolecules like antibodies, carbohydrates, peptides or proteins and DNA through a number of methods involving covalent and hydrogen bonding has been shown to extend their versatility and stability (Fig. 7.1) (Vinogradov *et al.*, 2002; Ishii *et al.*, 2003). Nanotechnology has recently been extended into biomimicry and certain nanostructures have been shown to mimic enzymes with enhanced activity and improved properties with greater tolerance to extremes in temperature, pH and sensitivity to proteases. Examples include peroxidase ferromagnetic nanoparticles (Gao *et al.*, 2007), oxidase mimetics (He *et al.*,

2011), human drug-metabolizing enzymes (Rentmeister *et al.*, 2011), nanobiosensing (Ju *et al.*, 2011), inorganic nanoparticles (Kotov, 2010), bacterial hydrogenases (Le Goff *et al.*, 2009) and fungal enzymes (Whiteley *et al.*, 2011).

Due to their huge potential and benefits to nanotechnology, nanoparticles have come under intense scrutiny as far as applications across various disciplines are concerned. In biochemistry, for example, they are considered to be better catalysts (Astruc, 2008) and good biological and chemical sensors (Nam *et al.*, 2003; Nie *et al.*, 2007); in information systems, their size and magnetic properties are being explored in the production of data storage devices where the issue of miniaturization is posing an overwhelming challenge (Mayes and Mann, 2004); in medicine, their potential as drug delivery agents has been reported (Nie *et al.*, 2007).

Synthesis of Nanoparticles

Since metal nanoparticles display unusual physical and chemical properties that depend on their size and shape, it has become expedient to synthesize uniform nanoparticles with controlled morphology. The synthesis of nanoparticles can occur either as part of the 'top-down' or 'bottom-up' process. The former

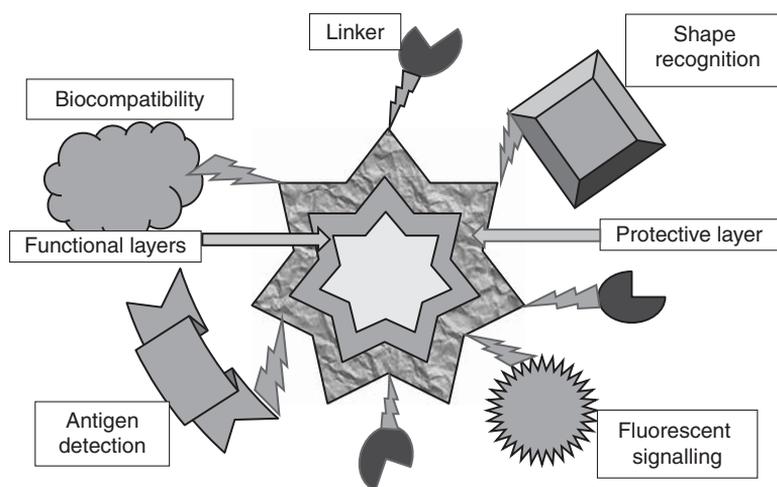


Fig. 7.1. Versatility of nanoparticles conjugated to various biomolecules such as antibodies in antigen detection, fluorescent signalling probes and peptides. (Adapted from Salata, 2004.)

means the production of nanoparticles from bulk material and involves the breakdown of this material into smaller pieces by the use of chemical or mechanical means. The bottom-up approach involves the synthesis of nanomaterials by the chemical reactions between atomic or molecular species. This approach allows for the gradual growth of the precursor particles (nucleation). Both methods can be carried out in liquid, gas or supercritical fluids, solids or even in a vacuum (Luther, 2004).

Noble metal nanoparticles have been synthesized using both chemical and biological approaches, with the former reported to be environmentally hazardous and with limited appeal (Ahmad *et al.*, 2003; Sastry *et al.*, 2003; Riddin *et al.*, 2006; Rashamuse and Whiteley, 2007; Vigneshwaran *et al.*, 2007).

Chemical

The major chemical approaches available for the synthesis of nanoparticles can be grouped into two main areas: the sol-gel method and gas phase synthesis. Various types of nanoparticles of diameters in the range of 1–10 nm, with uniform crystal structure and a high level of monodispersity have been generated by these methods, with about 20% variation in their size; however, for achieving a much better quantum confinement, this value must be reduced to about 5% or less (Murray *et al.*, 1993). The chemical method of synthesis greatly depends on the availability of the right metal/metal-organic precursors (Theodore and Kunz, 2005), with the main disadvantages being the very high temperature and pressure and the use of highly flammable organic solvents. These processes may lack scalability, and control over crystalline dispersion is very limited (Mukherjee *et al.*, 2001).

Sol-gel processing is a wet chemical synthetic method that differs from other chemical methods as nanomaterials can be produced at low temperatures (Luther, 2004; Theodore and Kunz, 2005). Precursors used for sol-gel processing can follow either an inorganic route, using aqueous

metal salt solutions as raw material, or a metal-organic route, using a metal alkoxide in an organic solvent as the starting material. The size distribution of the nanoparticles produced by this method can be controlled by the introduction of a doping agent (Kyprianidou-Leodidou *et al.*, 1994) or by treatment with heat (Wang and Ying, 1999).

Gas phase synthesis of metal nanopowders, first reported in 1930 (Luther, 2004), incorporates a vacuum chamber with a heating element, the precursor metal to be made into nanoparticles or nanopowder and equipment for the collection of the powder. An inert gas is also used at pressures high enough to boost the formation of nanoparticles, while at the same time low enough to enable the generation of spherical nanoparticles. Then, the precursor metal is placed on the already heated element and melted quickly. The melted metal is adjusted rapidly to temperatures far above the melting point of the metal but less than its boiling point, so as to allow enough vapour pressure to be attained easily. At this point, a continued supply of gas is introduced into the chamber, with excess gas removed by pumps in such a way that the gas flow removes the evaporated metal from the heating element. Nanoparticles begin to form as the gas cools the metal vapour, though the nanoparticles are still liquid as a result of the very high initial temperature involved. The particles still in the liquid phase collide and fuse together in a fixed environment, such that the nanoparticles can grow in a specified manner and thus remain spherical with smooth surfaces. As these liquid nanomaterials continue to cool, growth stops and, since they are very reactive, they must be coated to prevent aggregation with each other or with other materials (Horst, 1997; Robert and Wendelin, 2006; Wegner *et al.*, 2006).

Other chemical techniques used in the synthesis of nanoparticles are sonochemical processing (Suslick, 1997), cavitation processing (Sunstrom *et al.*, 1996), microemulsion processing (Kishida *et al.*, 1998) and high-energy ball milling (Leslie-Pelecky and Reike, 1996; Ying and Sun, 1997).

Biological

Microorganisms/plant extracts

Various biosynthetic methods for highly stable gold, silver, platinum, palladium, selenium, titanium and other metal nanoparticles are being exploited through the bioreduction of metal salts by microorganisms, either intracellularly or extracellularly, such as actinomycetes, bacteria, fungi, viruses and yeasts (Ahmad *et al.*, 2003; Sastry *et al.*, 2003; Riddin *et al.*, 2006, 2009, 2010; Govender *et al.*, 2009, 2010; Narayana and Sakthivel, 2010; Rai *et al.*, 2010, 2012a,b; Duran *et al.*, 2011; Gade *et al.*, 2011; Whiteley *et al.*, 2011). Although nanoparticles synthesized by microorganisms are very stable, studies have shown that these particles are not monodispersed and the rates at which they are synthesized are quite slow (Phillips, 2009). One of the ways by which this challenge may be overcome is to optimize some factors involved in synthesis, such as the cultivation method of the microorganism and the technique of harvesting or the use of a combination approach such as the photobiological method (Narayana and Sakthivel, 2010; Vaidyanathan *et al.*, 2010). Understanding the mechanism by which the nanoparticles are synthesized by these microbes at the cellular, biochemical and molecular level may provide information on how to improve the rate of synthesis, quality and intrinsic properties of the nanoparticles produced (Narayana and Sakthivel, 2010). Rapid synthesis of silver, gold and other noble metallic nanoparticles using plant leaf, fruit extracts and plant biomass has also been reported (Parsons *et al.*, 2007; Gardea-Torresdey *et al.*, 2008; Jae and Beom, 2009; Dubey *et al.*, 2010; Narayana and Sakthivel, 2010). These routes, however, do not adequately address the issue of particle monodispersity and uniformity, which is extremely crucial in the applications of these nanoparticles.

Enzymes/proteins

Enzymes, as natural biocatalysts, must be regarded as prime players in support of 'green' nanotechnology, though their use is still in its

infancy and there is always room for significant improvements. Even though the hazardous and toxic NaBH_4 reducing reagent is regarded as being cheap, highly selective and 'historically safe', the use of enzymes in a 'green' synthesis of nanoparticles can only really be supported if the use of this chemical is avoided. It is well documented that enzymes not only may influence the size-controlled formation of nanoparticles but also may serve both as extremely efficient stabilizing agents for the various nanoparticles as well as being a reactive ingredient associated with a specific biomimetic enzyme reaction (Willner *et al.*, 2006) (Fig. 7.2).

The preparation and subsequent study of engineered 'green' nanoparticles offers the abundance of potential and already defined applications in biomedicine, biology, materials science, electronics and biosensors, the pharmaceutical, food and cosmetic industries, and the environmental remediation field. Biocatalytic nanoparticle–enzyme conjugates also act as biorecognition for antigen–antibody and/or DNA–nucleic acid associations.

There are many enzyme classes that have been used as biocatalytic templates in the 'green' synthesis of nanoparticles. In this chapter, it is not possible to offer a complete exhaustive account of all of the examples, but it is worthy to recognize the structural architectures of the enzymes that are responsible. CATH, the protein structure classification

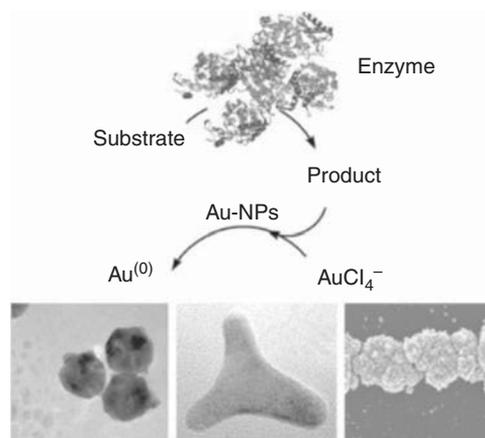


Fig. 7.2. Schematic representation of enzyme-mediated generation of gold nanoparticles (Willner *et al.*, 2006).

(Orengo *et al.*, 1997), defines three secondary structural levels – mainly alpha (α), mainly beta (β) and mainly alpha, beta (α,β ; $\alpha + \beta$), all of which may be further subdivided into architectural domains such as sandwiches, rolls, cages and barrels.

Mainly alpha (α)

LYSOZYME. The amphiphilic enzyme lysozyme acts through enzymatic cleavage of peptidoglycans surrounding the bacterial cell membranes. Due to the fact that lysozyme can adsorb on to hydrophobic surfaces, it is an ideal agent to synthesize extremely stable and biocompatible silver nanoparticles, even without any reducing agent (Fig. 7.3) (Eby *et al.*, 2009a). Lysozyme–silver nanoparticles retained the hydrolase function of the enzyme and were effective in inhibiting the growth of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus anthracis* and *Candida albicans*. Lysozyme–silver nanoparticles are non-toxic to human epidermal keratinocytes at concentrations sufficient to inhibit microbial growth. These results expand the functionality of nanomaterials for biological systems and represent a novel antimicrobial composite for potential aseptic and therapeutic use in the future (Eby *et al.*, 2009b).

CAGES. Biomineralization involving the use of cage-like proteins as a biotemplate/limiting growth field in the synthesis of nanostructured materials may be the key to a potential method for obtaining particles with a homogeneous distribution in size (Yoshimura, 2006), as the protein shell serves

as a guide to control particle growth and to prevent aggregation between the nanoparticles by coating each one as it is produced. Examples of proteins such as ferritin, ferritin-like-protein (FLP), chaperonin and viruses such as cowpea chlorotic mottle virus (CCMV), which have cavities in the centre, have been used to synthesize various types of nanoparticles, most especially that of metals (Ishii *et al.*, 2003; Ueno *et al.*, 2004; Gálvez *et al.*, 2008; de la Escosura *et al.*, 2009). Protein-encapsulated nanoparticles have proven to be an efficient carrier of food and drugs to specific targets in the biological system (Jahanshahi *et al.*, 2008; Rahimnejad *et al.*, 2009). One of the properties that makes them useful in this vein is their biodegradability after delivery. The orientation and size/diameter of the interior cavity of these proteins often determines the morphology of the nanoparticles produced within them. Other properties that make nanoparticles synthesized in protein cages important in delivery includes their non-toxicity, non-antigenicity, high stability (provided by the protein of encapsulation) and a longer shelf life. Encapsulated nanoparticles may have possible biomedical applications as a reactive oxygen scavenger (ROS), in bioimaging and as a vehicle for drug delivery (Galv ez *et al.*, 2008; Liu *et al.*, 2011).

APOFERRITIN/FERRITIN. The most commonly used ‘cage’ is ferritin and apoferritin (HSAF). These two protein templates have exactly the same structure, except that ferritins possess an iron core while apoferritin does not (Fig. 7.4). Apoferritin has a molecular

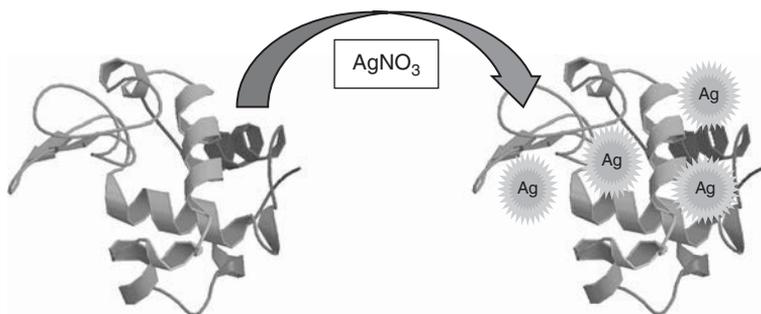


Fig. 7.3. All α -structure for lysozyme (pdb: 2cds) indicating formation of lysozyme–silver nanoparticle conjugate.

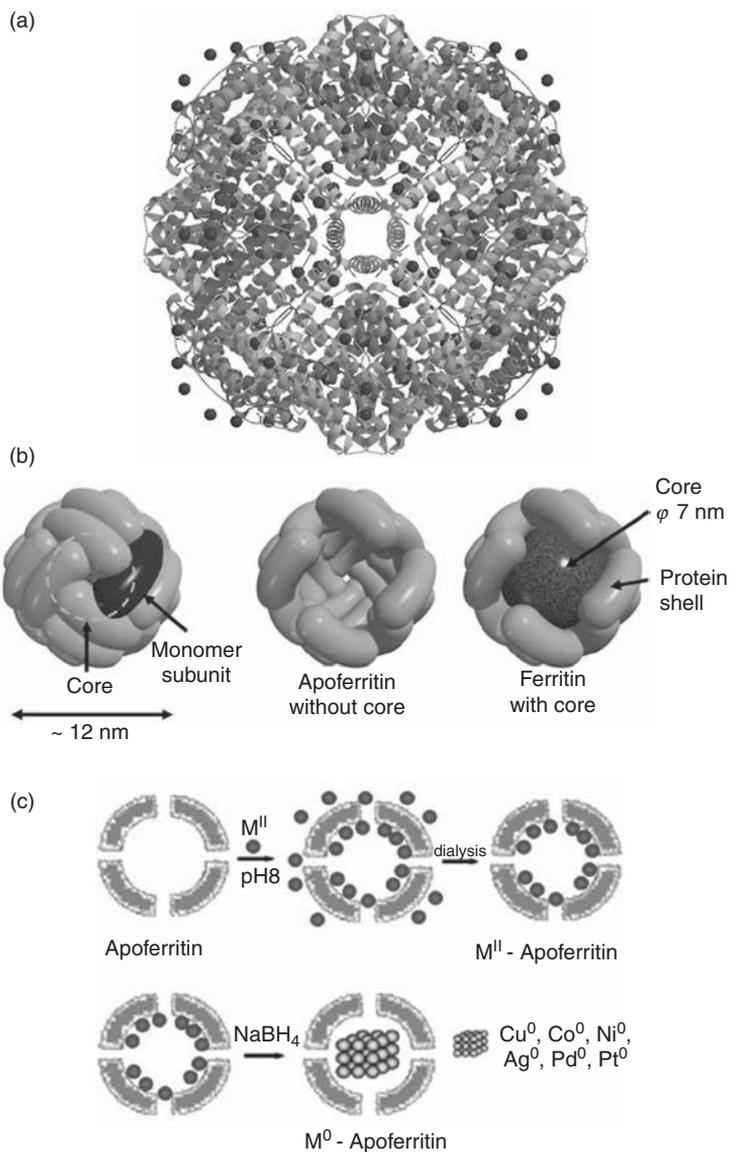


Fig. 7.4. (a) Three-dimensional representation of the all alpha cage-like structure of apoferritin. (b) An illustration of the cage-shaped protein apoferritin without iron core and ferritin with iron core (Iwahori and Yamashita, 2007). (c) Schematic representation of metallic nanoparticle synthesis in apoferritin (Gálvez *et al.*, 2008).

weight of approximately 440 kilodaltons (kDa) and consists of 24 identical subunits each with a molecular weight of $\sim 18,500$ daltons (Bryce and Crichton, 1973; Gálvez *et al.*,

2006). These subunits form a spherical protein shell surrounding an aqueous cavity with an inner diameter of about 8 nm and an outer diameter of 12 nm (Fig. 7.4) (Gálvez

et al., 2006; Iwahori and Yamashita, 2007). Apoferritin has been reported to bind metal ions in its cavity at specific sites, with stoichiometric binding not higher than 60 atoms per apoferritin at pH 7.4 (Pead *et al.*, 1995). When HSAF is treated with Cu(II), Co(II), Ni(II) or Ag(I) and the pH dynamically adjusted to 8, the number of metal ions per apoferritin reaches values of about 300 (Gálvez *et al.*, 2008). Theoretical computations have shown that the potential of the outer surface of apoferritin is net positive, while the inner surface has a negative net charge (Douglas and Ripoll, 1998; Gálvez *et al.*, 2006). The inner and outer surfaces are connected by channels, generated by the multi-subunit construction of the apoferritin shell. Four positively charged and eight negatively charged (also called hydrophilic channels) of about 4 Å provide a pathway for cations and molecules of sufficiently small size into the cavity of apoferritin (Chaspeen, 1998; Gálvez *et al.*, 2006). Encapsulated cations (mostly metal(II)-apoferritin) are susceptible to reaction with an appropriate reducing agent to give rise to the nucleation and growth of a new metal(0)-apoferritin nanoparticle of uniform size since particles are confined to the diameter of the apoferritin core (Fig. 7.4).

Several zero-valent, apoferritin-encapsulated metallic nanoparticles formed by the reduction of charged metal salts within the apoferritin cavity have been reported. These include palladium (Ueno *et al.*, 2004), copper (Gálvez *et al.*, 2005), cobalt and nickel (Gálvez *et al.*, 2006), cadmium (Iwahori and Yamashita, 2007), gold (Zhang *et al.*, 2007), platinum (Deng *et al.*, 2009; Liu *et al.*, 2011; Sennuga *et al.*, 2012a, 2013) and silver (Domínguez-Vera *et al.*, 2007).

Whiteley's group (Sennuga *et al.*, 2013) has synthesized platinum, gold and silver nanoparticles at pH 8 by the incubation of respective metal salts with an aqueous solution of apoferritin, followed by reduction with NaBH₄. The alkaline pH was necessary in order to increase the electronegativity of the interior of apoferritin, thereby improving electrostatic attraction between positive metal cations and the core of the apoferritin shell, leading to an increase in the uptake of metal

atoms. Excess metal salts were removed by dialysis and any aggregated metal particles not stabilized by the apoferritin removed by centrifugation. Different molar concentrations of metal salts were incubated with a fixed concentration of apoferritin in order to vary the size of the nanoparticles and to determine the saturation point in terms of the number of metal atoms apoferritin could encapsulate.

Nanoparticles of metallic origin have been shown to exhibit unusual properties that they normally would not display in their bulk form (Elechiguerra *et al.*, 2005; Blackman, 2009). Indeed, Whiteley's group has indicated a somewhat unexpected change in the activity of several specific enzymes in the presence of nanoparticles that appears to be particle size dependent.

FERROXIDASE. Whiteley's group (Sennuga *et al.*, 2012a, 2013) also showed that gold, silver and platinum nanoparticles, when enclosed within HSAF, were not only stabilized against aggregation but also had an influential effect on its ferroxidase activity. There was an increase in ferroxidase activity with an increase in the molar ratios of Pt:HSAF nanoparticles, with a specific activity of 360 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (ninefold increase over the control) seen at a molar ratio of 1000:1 (Pt:HSAF). At a Pt:HSAF ratio of 500:1, the specific activity was 200 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (fivefold), while at a ratio of 250:1 the specific activity was 80 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ (twofold). Any increase in Pt:HSAF above 1000 led to nanoparticle aggregation, with the ferroxidase activity reverting to that of the control. There was also a ninefold increase of ferroxidase activity with a molar ratio Au:HSAF of 500:1, and a lesser influence noted when this ratio decreased to 250:1 or increased to 1000:1. When the ratio increased (>1000), the activity of the ferroxidase reverted to that of the control. In the case of silver nanoparticles, a dramatic 110-fold increase in ferroxidase activity, facilitated by HSAF, was realized with a molar ratio of Ag:HSAF of 1000:1 and a lesser influence when this ratio decreased to 500:1 (90-fold) or 250:1 (29-fold) or increased to 2000:1 (67-fold); when the ratio increased (>2000), the activity of the ferroxidase reverted to that of the control (Fig. 7.5).

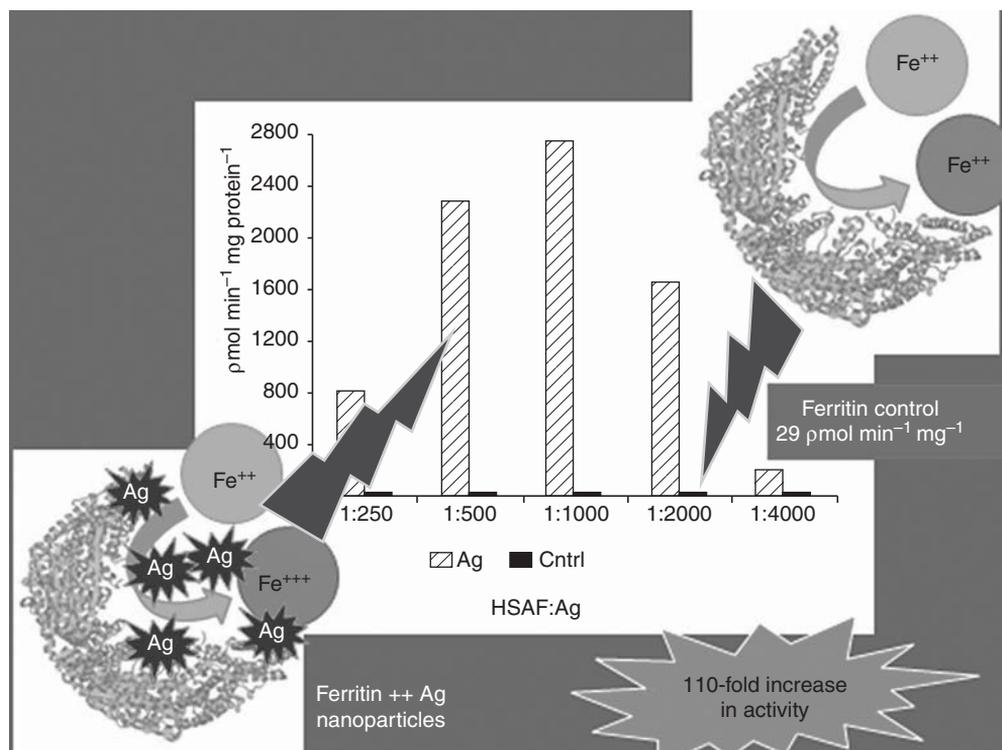


Fig. 7.5. Graphical representation of the influence of Ag nanoparticles on ferroxidase activity with respect to the ferritin.

The release of metal nanoparticles after the uptake of iron further confirmed that these nanoparticles were initially present within the core of the HSAF. It may also suggest the ability of the interior of HSAF to accommodate more than one type of metal atom (Fe and Ag, or Au or Pt) within its cavity, especially at M:HSAF ratio <1000. The functional groups responsible for the coordination of silver/gold/platinum and iron within the core of HSAF may not be involved in the storage and oxidation of iron in HSAF core. A different mechanism of Fe²⁺ uptake and oxidation by apoferritin proposed that the basic amino acid residues within the core of apoferritin were responsible for its ferroxidase activity (Niederer, 1970; Macara *et al.*, 1972). This site was different from that containing acidic amino residues reported to be implicated in the uptake, nucleation and stabilization of nanoparticles.

Platinum is a widely used catalyst that exhibits increased catalysis (Eustis and El-Sayed, 2006; Mohanty *et al.*, 2010; Sau *et al.*, 2010; Fratoddi *et al.*, 2011) with increased surface area, and so it is not surprising that this metal, at the nanoscale level, shows enhanced activity of ferroxidase (Sennuga *et al.*, 2012a, 2013). The size and shape of the platinum nanoparticles confirmed that HSAF was able to control nanoparticle growth within its interior. The encapsulation of the nanoparticles within the cavity of HSAF did not affect its overall protein integrity and structure but did improve an *in vivo* iron uptake, as indicated by an enhanced ferroxidase activity. Furthermore, novelty manifests itself with possible clinical applications in the treatment of diseases associated with poor iron absorption (anaemia) and possible decrease in oxidative stress associated with the toxic levels of iron (haemochromatosis) in biological systems.

Studies on metal nanoparticles stabilized by HSAF have suggested divalent precursor metal salts are preferably accommodated within the core of HSAF. This may be due to the fact that HSAF, in its normal biological function, takes up iron in its Fe^{2+} state that is later converted to Fe^{3+} within its core. Thus, HSAF may have a greater affinity for divalent metal ions within its cavity.

HSAF remained intact after incubation with the metal salts, and all of the nanoparticles (gold, silver and/or platinum) remained bound to HSAF. It was known that acidic amino acids were present within the core of the HSAF, giving the protein a net negative charge at physiological pH, suggesting that the metal nanoparticles interacted with the carbonyl group of the carboxylic side chains of acidic amino acids (Douglas and Ripoll, 1998; Gálvez *et al.*, 2006).

A comparison of these biological processes for synthesis of the metallic nanoparticles of Pt, Au and Ag with apoferritin/ferritin over the conventional chemical method of synthesis concluded that the methods employed were generally mild and eco-friendly in terms of pH and temperature.

DNA-BINDING PROTEINS (DPS). These are supra-molecular size-constrained cages with similar functional roles to ferritin with only 12 subunits (Fig. 7.6). The *Listeria* Dps has dimensions of 9 nm diameter (exterior) by 6 nm diameter (interior) and though there is no sequence homology with the mammalian ferritin, it does contain a ferroxidase reactive centre with similar structural motifs (Ceci *et al.*, 2010; Kasyutich *et al.*, 2010). The formation of nanoparticles of Fe_3O_4 (Allen *et al.*, 2002) and Co_3O_4 (Allen *et al.*, 2003) with about 500 atoms/cage and a size distribution of 4.5–6.0 nm have been reported.

BARRELS. Molecular chaperones promote the proper folding of nascent or denatured proteins into their active native forms. The process of the refolding and release of native proteins requires a co-chaperone, ATP and other co-factors such as K^+ and Mg^{2+} or Mn^{2+} . Chaperones are used in biotechnology for obtaining active recombinant proteins (Hoffmann and Rinas, 2004)

and have been reported to prevent *in vivo* aggregation of proteins at elevated temperatures associated with diseased conditions in the biological system (Melkani *et al.*, 2003; Fei and Perrett, 2009).

GROEL. One of the best characterized chaperonins is GroEL from *E. coli* (a Hsp60 homologue). This is a symmetric tetradecamer made up of two supramolecular rings, with each ring consisting of seven subunits (each subunit has a molecular mass of 60 kDa). These rings stack on each other in a double-decker architectural fashion, forming a three-dimensional barrel structure with a hydrophilic interior of about 4.5 nm in diameter and a wall thickness of 4.6 nm (Fig. 7.7) (Ortac and Severcan, 2007). The structure of GroEL is hybridized by its co-chaperone, GroES (Hsp10), forming a capping assembly on either side of its barrel-like cavity. Misfolded or denatured proteins are encapsulated within the barrel-like cavity of GroEL through hydrophobic or electrostatic interactions and are refolded into their native forms. After refolding, the substrate proteins, still in their inactive forms when bound to GroEL, are released into their active states by the addition of ATP and sometimes GroES (Mendoza *et al.*, 1996; Melkani *et al.*, 2003; Fan and Mark, 2006).

A previous study has mimicked the chaperonin function of GroEL, from *E. coli* and *Thermus thermophilus* HB8, by using its cavity for the encapsulation, folding and ATP-assisted release of CdS semiconductor nanoparticles (Ishii *et al.*, 2003). Another study used a modified cavity of GroEL-like proteins to size-selectively bind and arrange metallic and semiconductor nanoparticles into ordered nanoparticle arrays (McMillan *et al.*, 2002). Both studies have proved beneficial, particularly in the field of materials science, and may be employed in the generation of new bioresponsive, electronic and photonic devices. Furthermore, since the release of many GroEL-bound proteins is dependent on the hydrolysis of ATP, it may be expected that a faster rate of ATP hydrolysis of GroEL might improve its chaperonin function. Two reports on the role of nanoparticles as assistants to help protein refolding (Fei and Perrett, 2009) have appeared in the literature.

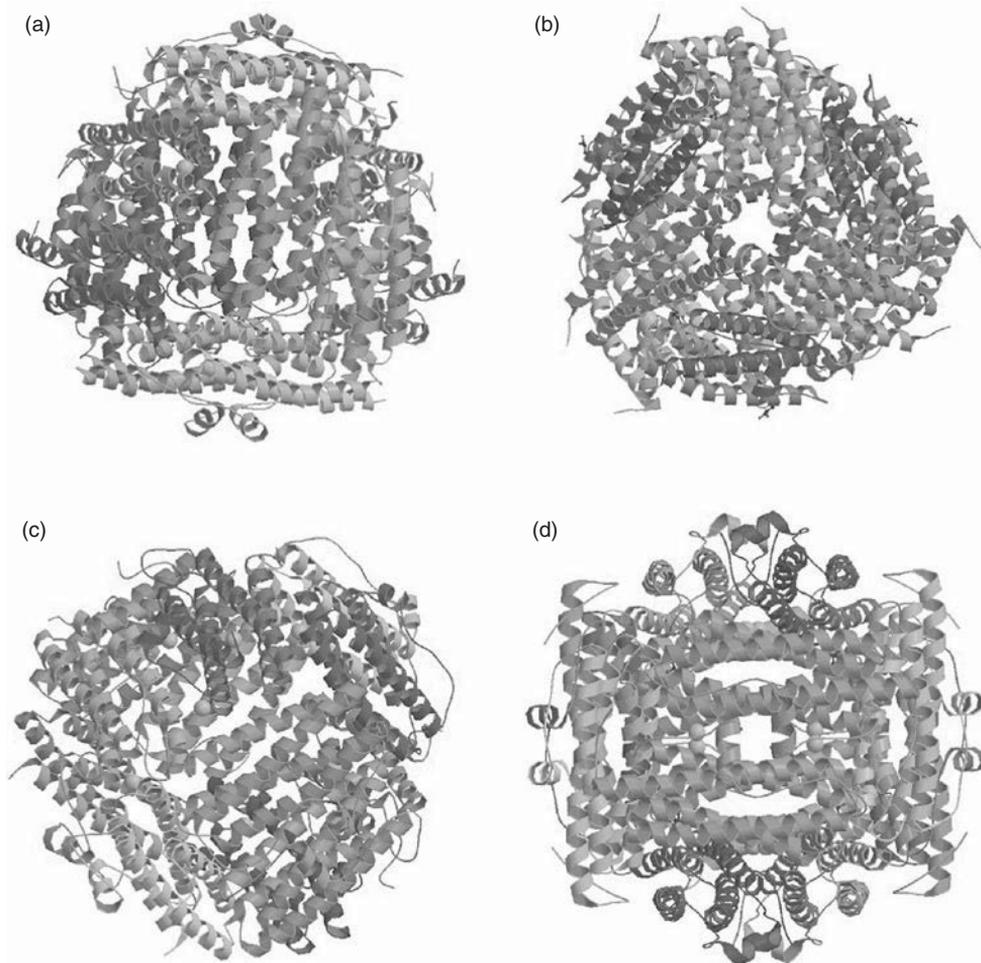


Fig. 7.6. All α -Dps protein: (a) *Mycobacterium arborescens*; (b) *Staphylococcus aureus*; (c) *Listeria*; (d) *Agrobacterium tumefaciens*.

One nanogel formed by the self-aggregation of pullulan bearing a cholesterol group (Akiyoshi *et al.*, 1999; Nomura *et al.*, 2003; Ikeda *et al.*, 2006), and the second a gold nanoparticle functionalized with 2-(10-mercaptodecyl) malonic acid (De and Rotello, 2008).

Whiteley's group incubated a fixed concentration of GroEL with varying concentrations of platinum, silver and/or gold salt (K_2PtCl_4 ; $AgNO_3$; $AuCl_3$) to give GroEL:metal ratios of 1:5–1:2000 (Pt) and 1:14–1:1000 (Ag, Au), followed by reduction with $NaBH_4$ at 4°C to afford a slow change from colourless or pale yellow to a brown solution that occurred concurrent with an increase in

absorbance at 260 nm and with the amount of metal salt (Sennuga *et al.*, 2012b). GroEL-nanoparticles generated with the greater amounts of K_2PtCl_4 (>1:2000) aggregated and precipitated rapidly, indicating that GroEL had stabilized the nanoparticles with a different physical/chemical property and had reached full saturation at this ratio of 1:2000. The colour and absorbance change observed for GroEL:platinum nanoparticle complexes was consistent with that of synthesized platinum nanoparticles reported from Whiteley's group (Deng *et al.*, 2009) and elsewhere (Xie *et al.*, 2005; Fan *et al.*, 2011). Apart from an obvious increase in absorption maxima, there

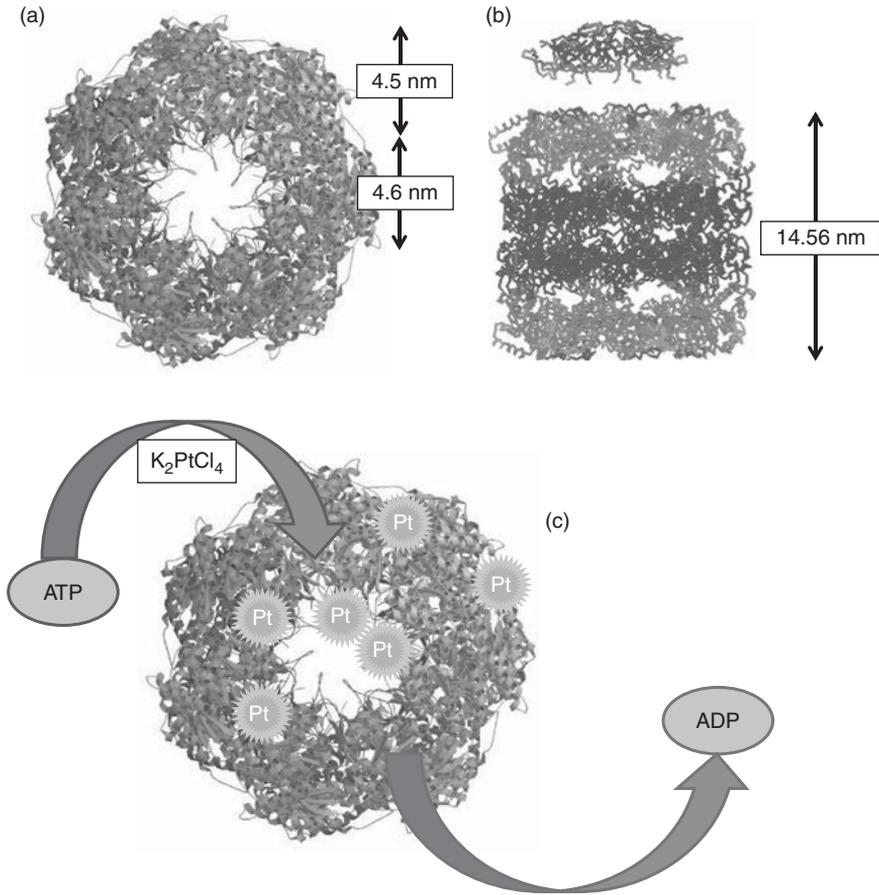


Fig. 7.7. Schematic representation of GroEL. (a) Top view; (b) side view (adapted from Ishii *et al.*, 2003; Ortac and Severcan, 2007); (c) theoretical scheme for ATPase activity of GroEL and Pt nanoparticles.

was a slight shift towards the red region, especially noteworthy for 1:2000 (GroEL: platinum nanoparticles), which provided information on the physical property of the particles such as the size, shape, aggregation state and dielectric constant of the surrounding medium (Alvarez *et al.*, 1997; Safavi and Zeinali, 2010; Prathna *et al.*, 2011).

ATPASE. Whiteley's group have also shown the effect of platinum nanoparticles on the ATPase activity of GroEL, with an 87% activity increase over a control at the molar ratio of GroEL:platinum nanoparticles of 1:25 (Sennuga *et al.*, 2012b). A lesser influence was noted either side of this ratio, with 1:50 ratio offering a 50% increase in activity, both the

1:10 and 1:250 ratio indicating a 37% increase and only about an 8% increase being realized at a ratio of 1:500. The same group went on to show a 47% increase was observed as the highest ATPase activity increase with Au at molar ratios of 1:50 and 1:100 (GroEL:Au); this was followed by only 15% at ratio 1:200. There was no observed activity increase in Ag-nanoparticles-GroEL at all molar concentrations used. Rather, a decrease in activity was observed at higher molar ratios of GroEL:Ag (i.e. >1:50), suggesting an inhibition of the ATPase activity of GroEL at these ratios.

It would be of interest to investigate if nanoparticles, acting either alone or in conjunction with chaperones themselves, would

improve the efficiency of protein folding by increasing the ATPase activity of GroEL (Sennuga *et al.*, 2012b).

Noble metal nanoparticles, especially those of platinum, are increasingly reported in the literature for their outstanding catalytic properties (Wang *et al.*, 2009; San *et al.*, 2011), and so it was not surprising that Pt at the nanoscale level showed enhanced activity of the ATPase activity of GroEL. Platinum nanoparticles acted as an inhibitor of the ATPase activity of GroEL at high molar ratios (>1:1000, GroEL:Pt); in fact, no activity was observed at ratios above the molar ratios of 1:1000. This suggested that the effects of platinum on the ATPase activity of GroEL might be concentration dependent. Furthermore, with an increase in the molar ratio (GroEL:Pt), there may have been an increase in nucleation within the GroEL cavity during synthesis, which interfered with vital amino acid residues important for ATPase activity. In addition, an earlier study had reported an increased ATPase activity of GroEL by divalent cations that stabilized its structure (Melkani *et al.*, 2003). Further, this may explain why a greater enhancement of the ATPase activity of GroEL of almost twofold (85%) was observed with platinum particles compared to that observed with Au, since a divalent metal salt of Pt was used to generate the nanoparticles. A theoretical scheme for formation of platinum nanoparticles and their influence on ATPase activity of GroEL is represented in Fig. 7.7c.

On the other hand, metallic gold in its bulk state does not possess any catalytic properties whatsoever, and consequently the increased ATPase activity of Au-nanoparticles-GroEL further corroborates the increased focus on the mechanism of action of gold nanoparticles as both homogeneous and heterogeneous catalysts (Cortie and van der Lingen, 2002; Hashim, 2005; Thompson, 2007; Campbell *et al.*, 2011). Activity increase was observed to the same degree at molar ratios of 1:50 and 1:100, while a decrease in catalysis, however, was observed at molar ratios >1:200.

ATPase activity of GroEL was inhibited by Ag nanoparticles. The highest inhibition of 64% was at a molar concentration ratio of 1:200 (GroEL:Ag), followed by a 45% inhibition at

ratio 1:500 and 28% at ratio 1:100 (Whiteley *et al.*, private communication). Nanoparticles of silver are well known for their optical and antimicrobial properties (Liz-Marzán, 2004; Jain and Pradeep, 2005) and only recently have their catalytic properties been reported (Zhou *et al.*, 2008; Li *et al.*, 2012). It was quite unexpected that Ag nanoparticles did not increase the ATPase activity of GroEL. Most catalytic properties of silver nanoparticles reported in the literature are usually of particle sizes between 20 and 80 nm. Another study has reported an inhibitory effect of silver nanoparticles of 1–10 nm with proteins/enzymes in viruses and bacteria; this forms the basis for their antimicrobial properties (Elechiguerra *et al.*, 2005). The inhibition was concentration dependent since the highest inhibition was at a molar ratio of 1:200 (GroEL:Ag) and less inhibition was observed at a molar ratio of 1:500. No significant effect was observed on the ATPase activity of GroEL at lower molar concentration ratios <1:100 and at 1:1000. An increased ATPase activity was observed at smaller molar ratios of GroEL:Pt.

Mainly beta (β)

CAGES

Mj heat shock protein (MjHsp). The small heat shock protein cage from *Methanococcus jannaschii* (MjHsp) consisted of 24 subunits, each composed of 147 amino acids, which self-assembled to form a cage with octahedral symmetry (Fig. 7.8). It was found to have an exterior diameter of 12 nm with unusually large 3 nm diameter pores at the threefold axes (Uchida *et al.*, 2011). Analogous to ferritin, MjHsp acted as a size-constrained nano-reactor for the mineralization of iron with a size of about 9 nm. Air oxidation of Fe(II) in the presence of MjHsp led to the formation of ferrihydrite encapsulated within the cage (Flenniken *et al.*, 2003).

β -BARRELS

Aspartic protease. Stable Au nanoparticles have been synthesized using aspartic protease from *Aspergillus satoii*, with the enzyme retaining substantial biocatalytic activity when complexed as the bioconjugate material (Gole *et al.*, 2001).

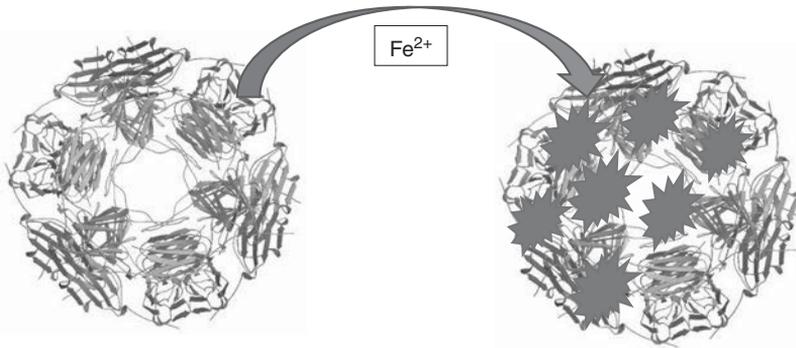


Fig. 7.8. All β -architectural structure of Mj heat shock protein (MjHsp) with suggested complex with iron nanoparticles.

β -SANDWICHES

Superoxide dismutase (SOD). Whiteley's group (private communication) showed the effect of different concentrations of silver nanoparticles on SOD and expressed their results as the rate of reduction of WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) with samples with no SOD, representing 100% reduction. With no nanoparticles (only SOD present), the reduction of WST was 84%, while in the presence of 5.0 μM silver nanoparticles, SOD activity increased by 136%. Any change in the percentage of WST activity implied that the silver nanoparticles interacted and activated the SOD with an activator constant (K_{np}) calculated from Eqn 7.1, indicating the binding affinity of enzyme for nanoparticles:

$$K_{np} = (np \cdot V_m^{app}) / (V_m - V_m^{app}) \quad (7.1)$$

where np = the concentration of silver nanoparticles, V_m and V_m^{app} = the maximum enzyme rate in the absence and presence of the respective concentration of nanoparticles. A negative K_{np} value would represent an activation of the enzyme.

The relative uniform size and shape of silver nanoparticles confirmed that SOD was able to control nanoparticle growth within its interior. The encapsulation of the nanoparticles within the core of SOD did not affect its overall protein integrity and structure, but led to an enhanced human SOD (hSOD) yet decreased *Plasmodium falciparum* (PfSOD) activity (Fig. 7.9). This may prove beneficial

in clinical applications in the treatment or management of diseases associated with malaria and/or oxidative stress.

Mainly α, β

α, β -BARREL

α -Amylase. The synthesis and simultaneous surface functionalization of spherical Au nanoparticles using a pure α -amylase enzyme with the preserved enzymatic activity was reported (Rangnekar *et al.*, 2007). It was found that the hydrolysis was nearly complete in 400 min and the rate of starch digestion by both the functionalized nanoparticle as well as the free enzyme was similar, indicating that the enzyme was stable and biologically active for more than 6 h. These observations suggested that the presence of free and exposed SH groups was necessary in the reduction of the gold salt to Au nanoparticles. The enzymatic functional group of α -amylase was close to the free and exposed SH group, which made it ideal for the production of Au nanoparticles, the binding of the enzyme to these particles via Au-S bond and also retention of the biological activity of the enzyme. We would like to suggest that the five cystine bridges (Fig. 7.10) influence the formation of gold nanoparticles, reducing the -S-S- to sulfhydryls (-SH).

Triosephosphate isomerase (TIM). Though perhaps the pioneer model for the α, β -barrel architectural domain, very little has been reported in the literature regarding the preparation of metal nanoparticles using this

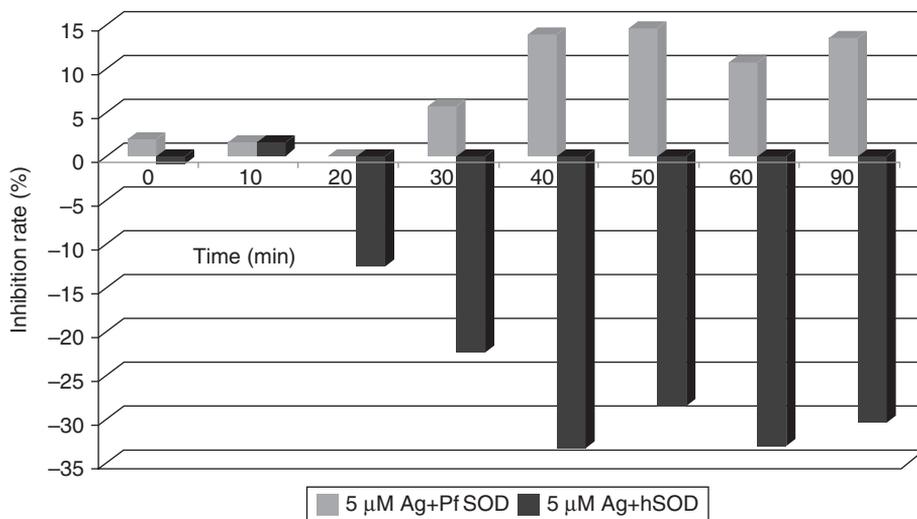


Fig. 7.9. Rate of inhibition (%) for superoxide dismutase (SOD) from *Plasmodium falciparum* (PfSOD) and human (hSOD) in the presence of 5 μM Ag nanoparticles. Data with a negative inhibition rate represent enzyme activation.

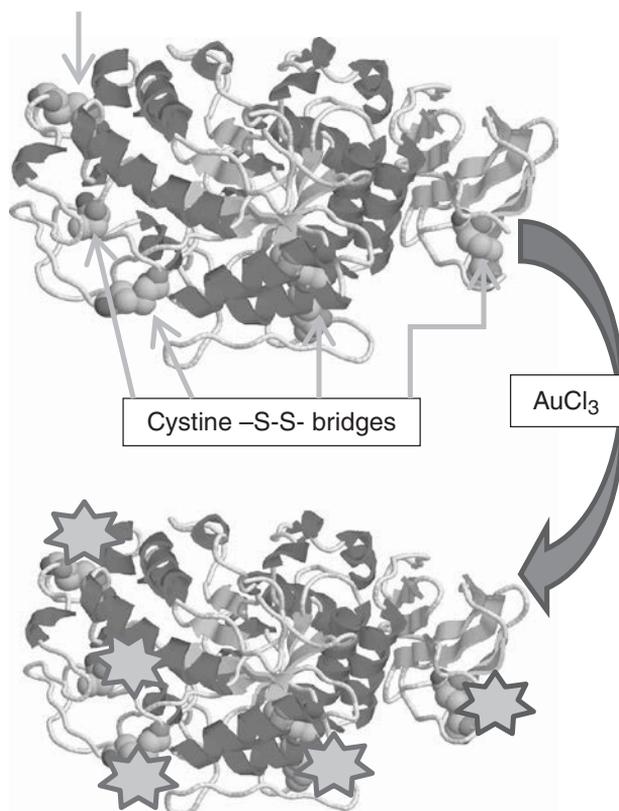


Fig. 7.10. Schematic representation of α,β -barrel structure for α -amylase (pdb: 1HNY) and subsequent interaction with AuCl_3 to form Au nanoparticles associated with the five cystine -S-S- bridges.

enzyme (Fig. 7.11). Since it is regarded as a critical enzyme in the glycolysis cycle, it has been the target of considerable investigation as regards antimalarial drugs. Whiteley's group has studied and exploited the role of the barrel-like structure to synthesize Au and Ag nanoparticles and has illustrated the difference between this enzyme from the malarial parasite, *P. falciparum* (PfTIM), and that from the human (hTIM) (private communication). Once again, strategic positioning of sulfur-containing cysteine amino acids, in particular (Cys¹²⁶) within the barrel core, may appear crucial to the mechanism of reactivity (or lack thereof) (Fig. 7.11) with nanoparticles (Samanta *et al.*, 2011).

α,β -SANDWICH

Lumazine synthase. This enzyme synthesizes lumazine, which is a precursor for riboflavin, with dimensions of 7.8 nm (inner) and 14.7 nm (outer), respectively (Fig. 7.12). Following the same concept as that for apoferritin, this icosahedral 60-subunit bacterial enzyme-based nano-reactor had a hollow porous shell that was

a suitable template for the synthesis of nano-sized iron oxide crystals. The iron ions pass through hydrophilic funnel-shaped channels lined with glutamic acid residues and become encapsulated in the cavity as Fe^{III} oxide during the synthesis of lepidocrocite (γ -FeOOH). The capsid increases in size from 15 to 30 nm in diameter through the formation of a higher order structure as the concentration of Fe^{III} increases (Shenton *et al.*, 2001). If the concept that sulfur and cysteine amino acids may be crucial for the generation and/or stabilization of Au and/or Ag nanoparticles and that these residues play a role in the mechanism for the difference in activity (to the native enzyme), then lumazine synthase is suitably structured. Within the core of the shell and symmetrically placed are 12 sets of five cysteine amino acid residues (six sets per half; 60 cysteines in total; each from a different chain) – with the respective sulfur atoms pointing in towards the cavity and just 5–6 Å apart (Fig. 7.12b) – ideally suited for binding to nanoparticles.

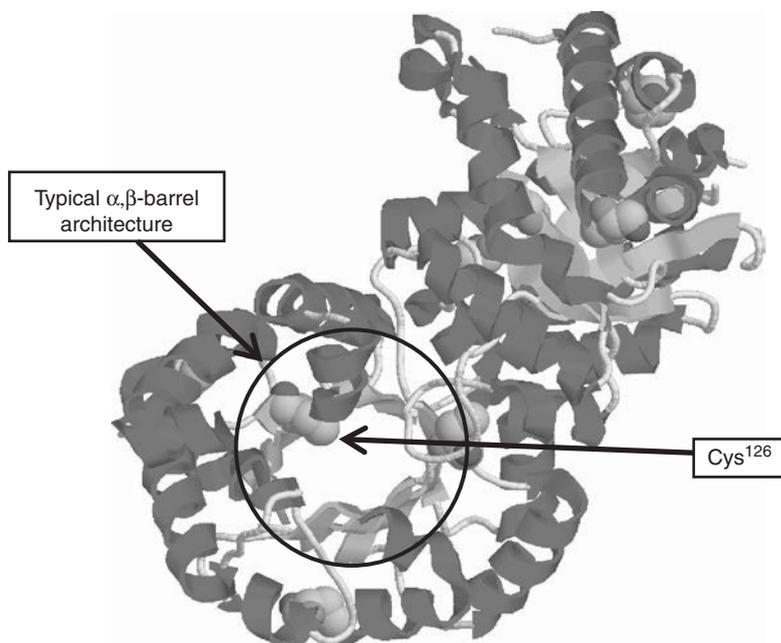


Fig. 7.11. Triosephosphate isomerase (pdb: 8TIM) of *Plasmodium falciparum* showing typical α,β -barrel. Conserved amino acid Cys¹²⁶ is illustrated with the sulfur pointing in towards the barrel.

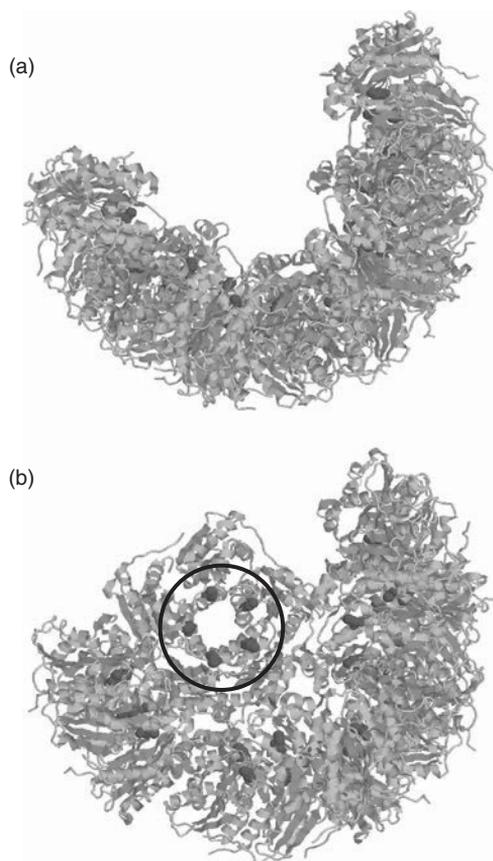


Fig. 7.12. Structure of one-half of lumazine synthase from *Bacillus anthracis* (pdb: 1VSW). (a) Shows the cross-sectional view; (b) illustrates the inside of the shell with six sets each of five symmetrical positioned cysteine amino acids (dark dots) from different chains with sulfur atoms (dark dots) pointing in towards the cavity.

Glutathione reductase. Glutathione reductase (Fig. 7.13a) is an active redox nanoreactor (enzyme) where the metal nanoparticles, synthesized by reduction of the respective metal ions, appear bound at the enzyme's active site (Scott *et al.*, 2008).

Glucose oxidase. A deposition of Au nanoparticles was generated by means of the product H_2O_2 from glucose oxidase (Fig. 7.13b) activity acting as a reducing agent (Willner *et al.*, 2006). Glucose oxidase was used to stabilize Pt nanoparticles (4.0 nm) with hydrogen as the reductant noting

that the enzyme nanoparticle complex retained a similar, if not greater, electrocatalytic activity (Karam *et al.*, 2008). This feature has obvious implications in biosensor technology. A flavin adenine dinucleotide (FAD)-Au-nanoparticle cofactor functionalized with *N*-hydroxysuccinimide, when added with glucose oxidase, acted as a nanocollector and as an electron relay to a macroelectrode (Xiao *et al.*, 2003). Furthermore, with oxygen as an electron acceptor, this reconstituted glucose oxidase nanoparticle enzyme complex had a sevenfold increase in electron transfer rate when compared to the native enzyme. Clearly, a functionalized nanoparticle in the presence of the redox enzyme greatly increased its maximum turnover rate.

Tyrosinase. The enzyme, which is viewed as a specific marker for melanocytes and melanoma cells, hydroxylates tyrosine to L-dopamine (DOPA) using O_2 (Angeletti *et al.*, 2004) as the oxygen source (Fig. 7.14a). It was found that the enzyme activity was far more sensitive when the product (L-DOPA) was in the presence of Au nanoparticles.

Acetylcholinesterase (AChE). Any inhibitors of this enzyme may lead to an interference with the regulation of the neural response system. The enzyme-mediated acetylcholinesterase (AChE) (Fig. 7.14b) production of Au nanowires from AuCl_3 in the presence of glucose has been reported (Willner *et al.*, 2006). The formation and growth (from 2–3 nm to 10–30 nm) of the Au nanoparticles (from 2 to 20 nm in 10 min) was significantly faster in the presence of an electron relay than when H_2O_2 was used as the reducing agent. This formation and growth of the Au nanoparticles was used to follow AChE activity.

The anticholinesterase effect of Au, Ag and Pt nanoparticles made with apoferritin, GroEL and RNase has been investigated by Whiteley's group. All showed some significant reduction in the hydrolysis activity of AChE at certain concentrations, with least inhibition in HSAF and GroEL at 30 and 35%, respectively, compared to that obtained with M-nanoparticles-RNase of 57%. This suggested

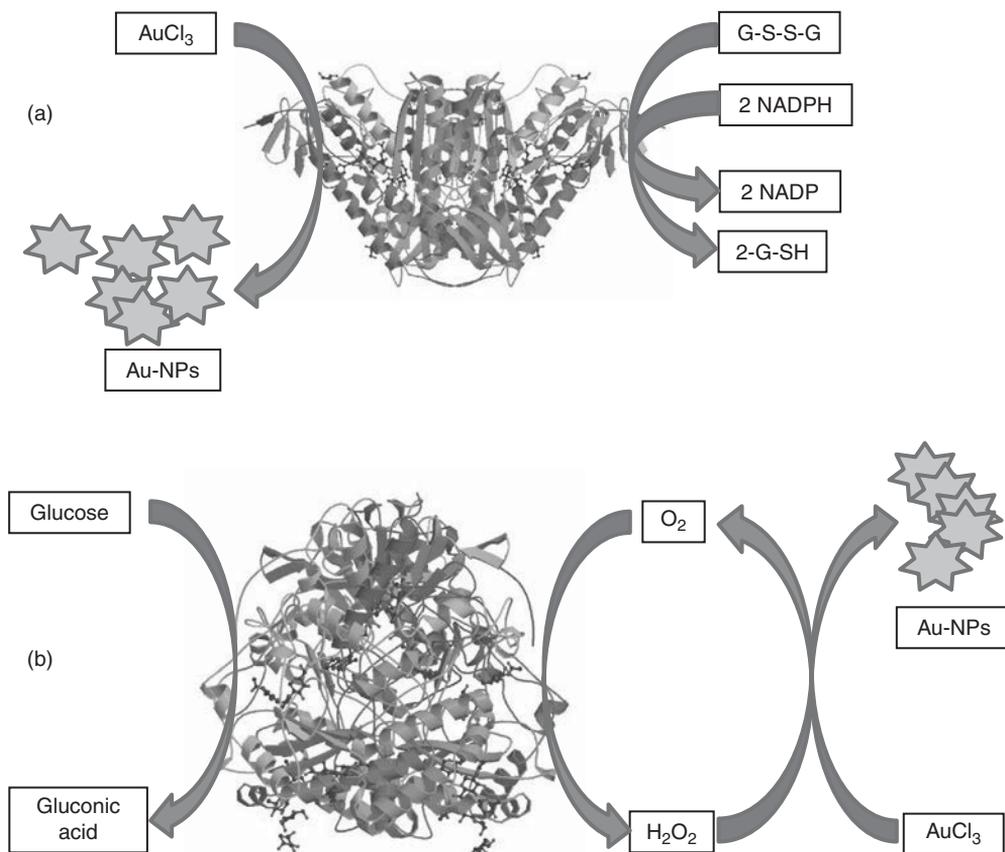


Fig. 7.13. α,β -sandwich structure of (a) glutathione reductase (pdb: 1K4Q) and (b) glucose oxidase (pdb: 1GPE) and their assumed association with the formation of Au nanoparticles.

that the encapsulation of nanoparticles by the cage-like structure of HSAF and the barrel-like structure of GroEL had lowered the inhibitory effect of these metal nanoparticles as opposed to the 'naked' nanoparticles obtained with non-caged RNase. Furthermore, no significant effect on the activity of AChE was noted in the presence of these proteins without nanoparticles. These results were comparable to those that were reported on the cytotoxic properties of protein-encapsulated versus chemically synthesized Pt nanoparticles using polyvinylpyrrolidone (PVP) as a stabilizer (Zhang *et al.*, 2010; Liu *et al.*, 2011).

The degree of inhibition of AChE by biologically synthesized nanoparticles was much lower than that reported (Wang *et al.*,

2009) using chemically synthesized nanoparticles. This supported the fact that a biological approach involving the encapsulation of nanoparticles by proteins or other biomolecules might prove less neurotoxic than chemically produced nanoparticles in various environmental and biomedical applications.

Lipases. Similar retention of enzymatic activity (in fact, comparable to that of the free enzyme) was observed by a lipase from *Thermomyces lanuginosus* (Brennan *et al.*, 2006), with seven fully active lipase molecules attached per nanoparticle. By means of a 1,2,3-triazole cycloaddition copper catalysed reaction, these researchers incubated an acetylene derivatized lipase enzyme with

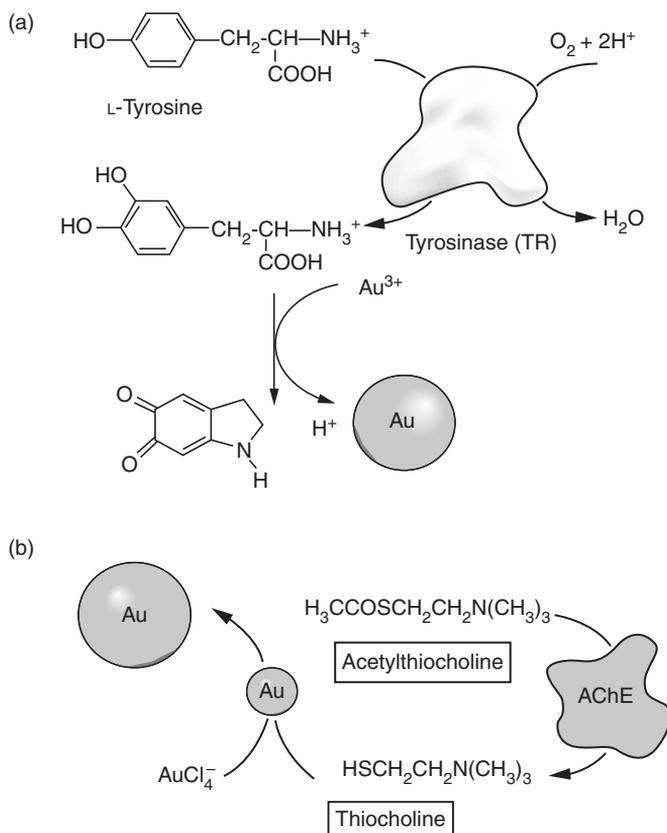


Fig. 7.14. Schematic representation of the role of (a) tyrosinase and (b) acetylcholinesterase in the formation of gold (Au) nanoparticles (Willner *et al.*, 2006).

an azide-functionalized water-soluble gold nanoparticle suspension (Fig. 7.15).

Alkaline phosphatase. Several examples appear in the literature regarding this enzyme and its preparation and role with nanoparticles (Hill and Shear, 2006; Hunter *et al.*, 2011; Zaccheo and Crooks, 2011; Nam *et al.*, 2012). The product from the enzyme alkaline phosphatase reaction on *p*-aminophenol phosphate (1) is capable of catalysing the reduction of silver and/or gold to yield respective nanoparticles (Fig. 7.16) (Basnar *et al.*, 2006).

Lactate and alcohol dehydrogenase. The reduction of metal salts to generate nanoparticles is an electron-transfer process and since

an electron transfer within redox enzymes (dehydrogenases, oxidases, reductases) is usually mediated by means of cofactors (NAD/NADH ; NADP/NADPH ; FMN/FMNH_2 ; FAD/FADH_2), these enzyme systems are prime examples for the synthesis of nanoparticles. The shape of the produced nanoparticles was found to be pH dependent (Xiao *et al.*, 2005). This has found extensive use in biosensor technology and/or increased sensitivity for the determination of substrate-ethanol/lactate/NADH concentration. Gold nanoparticles can transfer electrons between themselves and the active site of a biocatalytic redox enzyme (dehydrogenase) to provide an electrically active biomaterial that would be suitable as a biosensor (Willner *et al.*, 2006) (Fig. 7.17a,b).

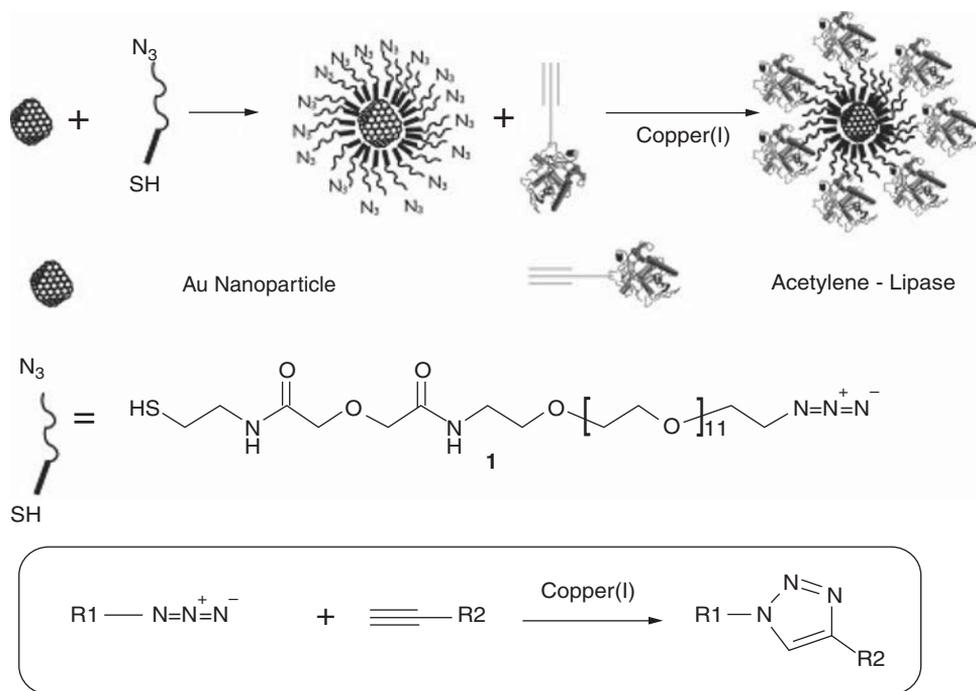


Fig. 7.15. Functionalization of gold nanoparticles with **1**, and linking of lipase to these nanoparticles using click chemistry (Brennan *et al.*, 2006).

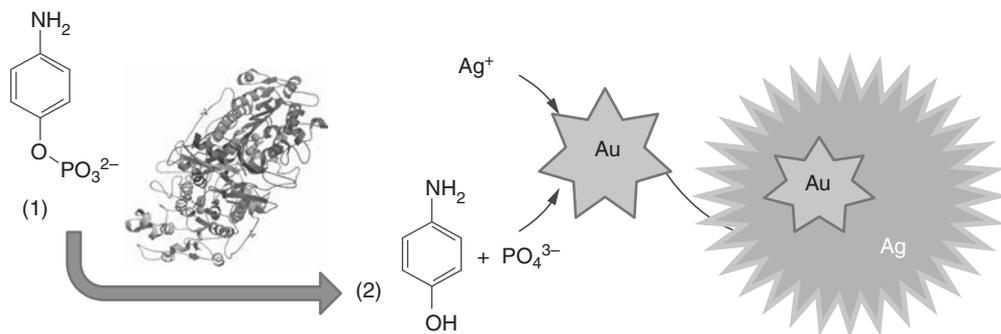


Fig. 7.16. Biocatalytic growth of Ag on gold nanoparticles by alkaline phosphatase with *p*-aminophenol phosphate (1). The *p*-aminophenol product (2) acted as an *in situ* reducing agent (Basnar *et al.*, 2006).

Leucine aminopeptidase. Platinum nanoparticles have been encapsulated by a bacterial aminopeptidase to formulate a hybrid process involving catalysed hydrogenation and peptide hydrolysis for multi-step synthesis (Fig. 7.18). This concept allows for the design and implementation of novel multifunctional materials (San *et al.*, 2011).

α,β -ROLL

RNase. Ribonucleases (RNases) catalyse the hydrolytic cleavage of ribonucleic acid (RNA) molecules and are implicated in many biological functions like angiogenic, antitumour or antibiotic activities (Benito *et al.*, 2005; Cho and Zhang, 2007; Monti *et al.*, 2009). These enzymes are relatively small (molecular weight = 13.7/15.5 kDa; 124 amino acids), kidney-shaped, with their

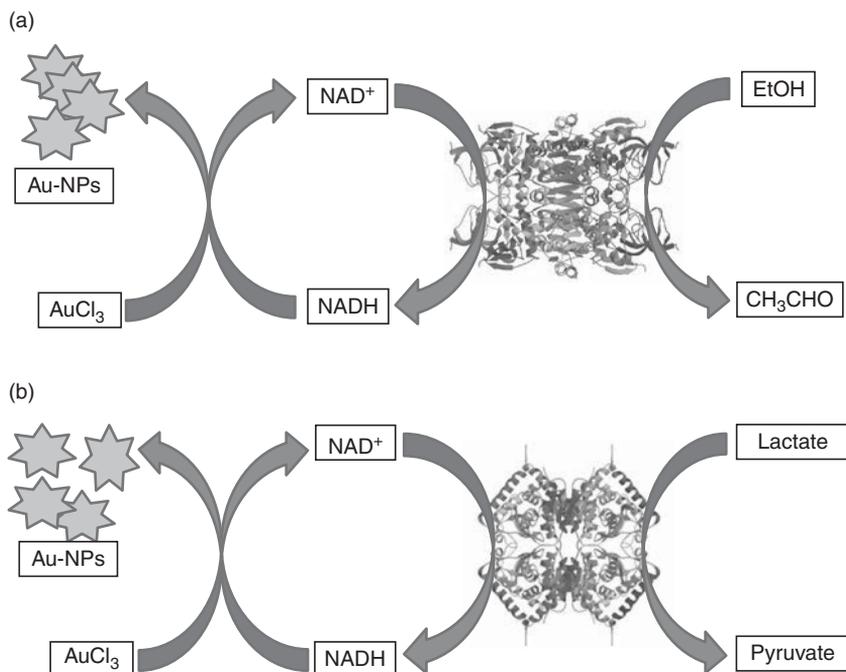


Fig. 7.17. Schematic representation of the NADH/NAD-mediated synthesis of gold nanoparticles with (a) alcohol dehydrogenase (pdb: 1YE3) and (b) lactate dehydrogenase (pdb: 2X8L).

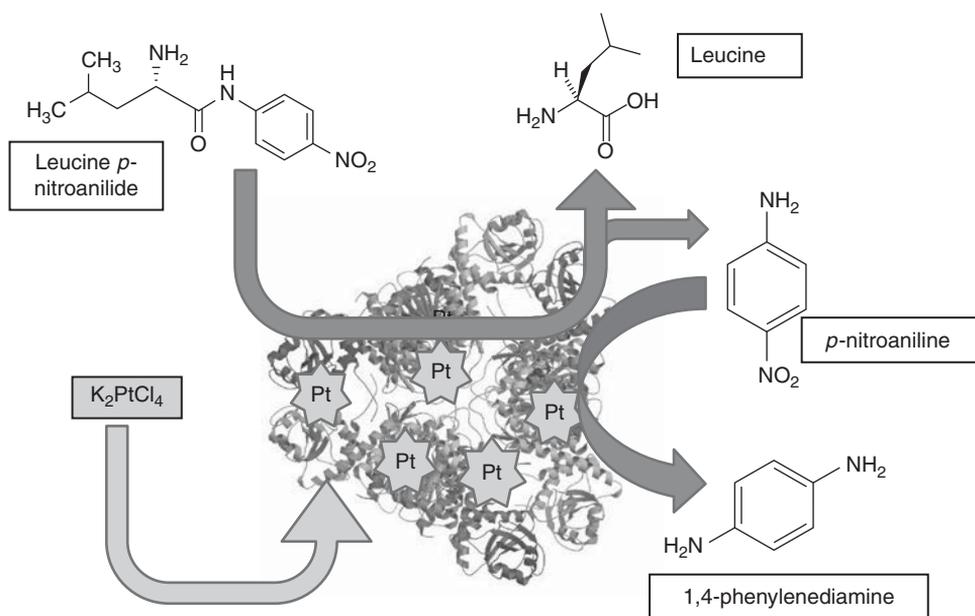


Fig. 7.18. Schematic representation showing encapsulated platinum nanoparticles in leucine aminopeptidase (pdb: 3H8E) and the hybrid multifunctional (hydrogenation/peptidase) bioinorganic catalyst.

active sites in a cleft (Joao and Dwek, 1993; Raines, 1998). Their secondary structure is made up of four-stranded, antiparallel beta-sheets flanked by two short alpha-helices and may be classified as an α,β -roll. The active site is defined by a third N-terminal alpha-helix and one edge of the beta-sheet. The structure of RNase is stabilized by four disulfide bonds, two of which contribute to its thermal stability (Klink *et al.*, 2000).

Whiteley's group incubated RNase with metal salts of Pt, Au and Ag, followed by a subsequent reduction to generate Pt, Au and Ag nanoparticles, respectively. The observed colours of the respective nanoparticle solutions were consistent with what was reported in the literature for Pt (brownish black), Au (reddish brown) and Ag (yellow/orange) (Zhang *et al.*, 2007; Gálvez *et al.*, 2008; Deng *et al.*, 2009; Fan *et al.*, 2011). Colour intensity also increased with an increase in the concentration of precursor metal salts at varying molar concentration ratios of metal salts to RNase, except with Au and Ag at molar ratios >50:1 (M:RNase), where a less intense colour was noticed. Ag nanoparticles were the least stabilized by RNase, followed by Au nanoparticles with Pt being the greatest stabilized.

Whiteley's group (private communication) also showed an effect of nanoparticles on the hydrolytic activity of RNase. Results revealed a general decrease in the RNA hydrolysis activity by RNase in M-nanoparticles-RNase compared to the control. This decrease in RNase activity was also found to be concentration dependent with respect to the molar concentration ratio of metal to RNase. Au nanoparticles had the highest inhibitory effect of 48% to almost 100%, followed by Pt-nanoparticles-RNase with 20–95% inhibition and about 18–90% with Ag nanoparticles. Heavy metal ions are known to be one of the most potent inhibitors of RNases, which might be the reason why the activity of RNase is strongly inhibited, particularly at higher molar concentrations of metals to RNase.

Furthermore, an explanation for the inhibition of RNase activity by metallic nanoparticles might be due to the interaction/stabilization of nanoparticles by vital amino acids responsible for its enzymatic function. It was suggested that basic amino acid residues

(e.g. histidine, lysine and arginine) present at the active region of the enzyme were responsible for nanoparticle stabilization (Raines, 1998), and hence the observed inhibitory effect of metal nanoparticles on RNase activity.

Liposomes

An initial study on liposomes or lipid vesicles originated in the 1960s when there was a need to understand new types of polymeric nanocontainers. Initially, liposomes were used as model systems to study biological membranes but by 1970 had been developed into a medium for the transportation of drugs (Graff *et al.*, 2004). Liposomes, in general, had been reported to be highly useful in biophysics as a good model system in understanding the properties of cell membrane and channels. In chemistry, they served as an excellent illustration of catalysis, energy conversion and photosynthesis, while in biochemistry they improved the understanding of the biological function of proteins and were a good representation of secretion, cell function, trafficking and signalling, gene delivery and function in cell biology (Graff *et al.*, 2004).

Liposomes have various extensive applications in the pharmaceutical industry as directed delivery agent for drugs such as anti-cancer, antifungals and vaccines. They are also useful in cosmetics in the manufacturing of shampoos and other skincare products. They are very important tools in diagnostics as they are able to degrade in the cells once delivery has been carried out (Graff *et al.*, 2004; Salata, 2004). Liposomes were said to be the first synthesized nanoparticles used for drug delivery, but a major limitation was their tendency to fuse together in aqueous environments and release their contents before getting to the target site. This has led to the search for either a replacement or a method of stabilization using newer substitute nanoparticles.

Viral Capsids

A capsid is the protein shell of a virus and consists of several oligomeric structural subunits called *protomers*. The observable

three-dimensional morphological subunits, which may or may not correspond to individual proteins, are called *capsomeres*. The cavity of icosahedral viral capsid architectures is also an ideal size-constrained environment where the interior surface can direct the attachment or nucleation of nanoparticles. Cowpea chlorotic mottle virus (CCMV), a plant virus, is the most widely used icosahedral viral capsid. CCMV virions are 28 nm in diameter and the protein shell defines an inner cavity of approximately 24 nm. This virus is composed of 180 identical coat protein subunits, which encapsulate the RNA. Each coat protein subunit presents at least nine basic N-termini (arginine and lysine) to the interior of the cavity, which in turn creates a positively charged interior cavity surface in order to provide an interface that can be utilized for mineral deposition. The outer surface of the capsid is not highly charged; thus, the inner and outer surfaces of this molecular cage provide electrostatically dissimilar environments that control the mineralization reactions within the protein cage. It is beyond the scope of this review to explore all of the possibilities of the synthesis of nanoparticles using viral capsids, except to list several recent reviews and publications (de la Escosura *et al.*, 2008; Grasso and Santi, 2010; Li *et al.*, 2010; Wang *et al.*, 2011).

Mechanistic Aspects

The enhanced activity shown by several enzymes in this review may be due to the increased conformational rigidity of the enzyme, a reduction of substrate inhibition and/or stabilization of the transition state.

Gold and silver nanoparticles not only have catalytic properties (Campbell *et al.*, 2011; Li *et al.*, 2012) but also have shown extensive popularity in nanomedicine as anticancer/antitumour and antimicrobial agents (Visaria *et al.*, 2006; Cai *et al.*, 2008; Eby *et al.*, 2009b; Buu *et al.*, 2011). From the study by Whiteley's group on apoferritin and ferroxidase (Sennuga *et al.*, 2012a, 2013), on GroEL/ATPase (Sennuga *et al.*, 2012b) and on the relative activity between hSOD and PfSOD, it was not clear why there was a considerable increase in activity

of ferroxidase, ATPase and hSOD in the presence of platinum, gold and silver nanoparticles.

From a structural and mechanistic point of view, the distance between critical amino acid residues and water molecules within the active regions of these enzymes was about 4.0–6.0 Å and well within the range for the nanoparticles to bind (Liu and Thiel, 2004). This would, in turn, enhance the negativity of these amino acids in the core, increasing the binding of Fe²⁺ (ferroxidase) and/or Cu⁺ (hSOD) to these nanoparticles, consequently enhancing the rate of electron removal and oxidation to Fe³⁺ and/or Cu²⁺. The interaction of the nanoparticles would facilitate the addition of a water molecule to enhance the formation of a respective hydrolysis product (FeOOH; H₂O₂) (Baaghil *et al.*, 2003). There is a propensity of attraction of sulfur towards silver, lending support that the nanoparticles interact with any proximal cysteine amino acids in the active region. In hSOD, Cys⁵⁷ and Cys¹⁴⁶ are 2.08 Å apart, and nanoparticle binding changes the conformation of the reactive core and increases the rate of addition of oxidant. These two amino acids are not present in PfSOD. With respect to ferritin/ferroxidase, three Cys¹²⁶ residues from three different chains are juxtaposed within 4–5 Å from the Fe²⁺/Fe³⁺ core, suggesting that the gold/silver nanoparticles interact at these positions and with these exposed –SH groups, leading to an enhanced activity.

Until recently, nothing was known about the effect of nanoparticles within the cavity of apoferritin on its natural biological function to sequester and mineralize Fe(O)OH into its cavity. Earlier studies have also mimicked this function by an *in vitro* demonstration of the oxidation of Fe²⁺ by apoferritin under conditions not complicated by auto oxidation (Bryce and Crichton, 1973; Macara *et al.*, 1973; Bakker and Boyer, 1986; Hanna *et al.*, 1991). These studies were able to establish the mechanism by which the uptake of iron was achieved by apoferritin. Further, these studies pointed to the fact that apoferritin possessed catalytic properties and was regarded as a ferroxidase enzyme with products that remained associated. Thus, an enhanced *in vitro* ferroxidase activity of apoferritin in the presence of some metallic nanoparticles,

as demonstrated by Whiteley's group (Sennuga *et al.*, 2012a, 2013), could translate to an *in vivo* prevention of any toxic build-up of iron in the cytosol and serum that might lead to oxidative stress and eventual death of individual cells of tissues.

During the synthesis of Au nanoparticles using a pure α -amylase enzyme, it was suggested that the presence of free and exposed SH groups, close to the enzyme functional active site, was a necessity in the reduction of gold salt to Au nanoparticles. Furthermore, retention of activity of the enzyme was retained throughout (Rangnekar *et al.*, 2007).

Though there is no apparent reason for the enhanced activity in the presence of nanoparticles, of all of the other enzymes listed in this review it is tempting to suggest possible mechanisms. In all cases, critical positioning of cysteine amino acids and the presence of free -SH groups must play a crucial role. Throughout this review, suggestions and tentative mechanisms have been proposed.

Concluding Comments

An efficient, cheap, uncomplicated, environmentally friendly, biologically 'green' mode of synthesis of metal nanoparticles is still a prime target in nanobiotechnology. The properties of nanoparticles are highly dependent on their morphology and, consequently, this 'green' mode of synthesis must provide

and allow control over their mechanism of formation. Though still in its infancy, with room for significant improvements, enzymes, as natural biocatalysts, must be regarded as prime players in support of 'green' nanotechnology. Enzymes regarded as nanoreactors not only may influence a size-controlled formation of nanoparticles but also may serve both as extremely efficient stabilizing agents for various nanoparticles as well as being a reactive ingredient associated with a specific biomimetic enzyme reaction. There are many enzyme classes that have been used as biocatalytic templates in the 'green' synthesis of nanoparticles. According to CATH protein structure classification, there exist three secondary structural levels – alpha (α), beta (β) and α,β – and architectural domains such as cages, barrels, rolls and sandwiches. This review has focused on the syntheses of metal nanoparticles by means of these different protein structural architectures and has attempted to illustrate the influence that such particle-enzyme/protein bioconjugates have on enzyme activity. The mechanistic aspects and reasons behind these differences in reactivity have also been presented. The preparation and subsequent study of engineered 'green' nanoparticles offers an abundance of potential and already defined applications in pharmaceuticals, biomedicine, biology, materials science, electronics and biosensors, the food and cosmetic industries, and the environmental remediation field.

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8 Understanding the Involved Mechanisms in Plant-mediated Synthesis of Nanoparticles

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Introduction

Phytodiversity is a unique gift that nature has bestowed on humanity. Plants have helped the surviving congeners by all means possible and they have indeed acted as the crux of human's survival on this planet. Not only have different plants been silent participants in the exponential process of evolution but also they have themselves survived the extremities of environmental rigours by prudently treasuring different valued metabolites in order to remain in the drive of evolution. These metabolites (both primary and secondary) have played a significant role for humanity. Plants are widely distributed along ecological boundaries, are easily available and safe to handle, are equipped with a broad weaponry of metabolites and, above all, they are truly green while undertaking any chemical protocol. Their own metabolic status (in order to circumvent environmental rigours) is indeed an ignored treasure which is yet to be utilized to full capacity for the purpose of synthesizing metal/oxide nanoparticles. Making use of plant tissue culture

techniques and downstream processing procedures, it would be possible to synthesize metallic as well as oxide nanoparticles on an industrial scale in the immediate future, once such issues are properly addressed. Recent reports on different plants, plant parts, fruit and food beneficiaries have assisted syntheses of inorganic nanomaterials (Shankar *et al.*, 2003, 2004; Armendariz *et al.*, 2004; Ankamwar *et al.*, 2005a; Chandran *et al.*, 2006; Arangasamy and Munusamy, 2008; Jha *et al.*, 2009a,b; Song and Kim, 2009; Jha and Prasad, 2010, 2011a, 2012) but few have been able to address the accomplished procedure. Therefore, proper knowledge of metabolites and metabolic fluxes would help the emergence of a better insight into synthesis involving raw metabolites in a suspension culture, leading to scaling up by making use of the plant as a whole (in case of algae) or in part (vegetative/reproductive parts), which could be a green and useful option.

This chapter focuses on the plant-mediated synthesis of different inorganic nanoparticles and the understanding of their involved biosynthetic mechanisms.

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Plant Metabolites and Their Role – A Brief Introduction

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites, are often distributed differentially among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. In contrast, primary metabolites such as phytosterols, acyl lipids, nucleotides, amino acids and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident (Croteau *et al.*, 2000).

We have summarized in a tabular form the various metabolites present in different plants (Table 8.1).

Secondary metabolites play a major role in the adaptation of plants towards the changing environment and in overcoming stress constraints (Velikova *et al.*, 2008). This flows from the large complexity of chemical types and interactions underlying various functions: structure stabilization, determined by polymerization and condensation of phenols and quinones, or by electrostatic interactions of polyamines with negatively charged loci in cell components; photo-protection, related to absorbance of visible light and UV radiation due to the presence of conjugated double bonds; antioxidant and anti-radical, governed by the availability of $-OH$, $-NH_2$ and $-SH$ groupings, as well as aromatic nuclei and unsaturated aliphatic chains. Earlier research embracing several plant–abiotic stress stimuli systems has shown the multiplicity of biochemical mechanisms involved in the protective role of secondary metabolites: the condensation of chlorogenoquinone with proteins, yielding brown pigments that limit the spread of stress-induced tissue damage in tobacco; the accumulation of polyamines and the formation of phenylamides in tobacco and bean subjected to water stress and heat shock, respectively, with phenylamides performing ROS-scavenging ability; the accumulation of anthocyanins in the leaves of cotton suffering

an Na/K imbalance and the shift from mono- to orthodihydroxy substitution in the B-ring of anthocyanin aglycone, thus conferring a higher ROS-scavenging capacity; and the relation of drought tolerance in cotton to the level of ROS-scavenging polyphenol compounds. The protective effect of a gaseous secondary metabolite, isoprene, against ozone fumigation and heat shock has been shown, and the ability of isoprene to scavenge singlet oxygen has been demonstrated. Altogether, the data provide evidence that secondary metabolites, through their diversity of functions, can be involved in non-enzymatic plant defence strategies (Edreva *et al.*, 2008). Table 8.1 illustrates, as examples, the candidate chemicals and/or metabolic status present in different plant systems.

Biosynthesis of Metal and Oxide Nanoparticles Using Plants

A known weight of healthy plant/plant part from any suitable category of choice (including lower plants) is taken and washed thoroughly in running water. This is followed by another wash in sterile distilled water. Then, plant samples are placed in a suitable volume of 50% Et-OH on boiling steam bath until the colour of the solvent changes. They are allowed to cool at room temperature, gently pressed and filtered, first through sterile serene cloth and then through Whatman filter paper. These solutions are treated as source broth and used in further biosynthetic procedures.

Source broth from plants is doubled in volume by adding sterile distilled water. To this broth, a known volume of molar salt solution is added and the pH is measured. Now, the temperature and pH are suitably modulated in order to obtain metal or oxide nanoparticles. Accordingly, distinct deposits of nanoparticles will appear in due course, which need to be filtered, dried and taken for further characterization. A suitable capping agent can be taken in to use for capping an oxido-reductively labile species at this stage. A general biosynthetic procedure for the synthesis of metal and/or oxide nanoparticles is illustrated in Fig. 8.1.

Table 8.1. Candidate chemicals present in different plants/plant systems.

Name of source plant	Candidate chemicals	Name of source plant	Candidate chemicals
<i>Acalypha indica</i>	Alkaloids, acalypus, achalyphine, gamma-sitosterol acetate.	<i>Cajanus cajan</i> (Arhar)	Globulins: cajanin, caneajanin. Seed: nitrogenous matter, carbohydrates.
<i>Agave americana</i>	Sugar yielding alcohol, agavose, saponins, steroids.	<i>Datura alba</i>	Alkaloid: hyoscyamine, hyocine, atropine. Alkaloid principle: scopolamine. Seed: fixed oil, allantoin. Leaf: Vitamin C.
<i>Anacardium occidentale</i>	Cardol, anarcadic acid.	<i>Emblica officinalis</i>	Tannin, ascorbic acid.
<i>Aloe vera</i>	Aloin (anthroquinone glycoside), emodin, chrysophanic acid, pentosides-barbolin, isobarbolin, beta barbolin.	<i>Elettaria cardamomum</i> (Elaichi)	Essential oil: terpeniol, terpinine, lemonine, linalool acetate.
<i>Avena sativa</i>	p-Hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, p-coumaric acid, ferulic acid.	<i>Eucalyptus globulus</i>	Volatile oil: cineole, alcohols, aldehydes, ketone, phenols, terpenes, eugenol, sesquiterpene, tannin, resin. Gum: kino-tannic acid, catechin, pyrocatechin.
<i>Anogeissus latifolia</i>	Tannin, gum.	Geranium leaf	Organic acid, emodin.
<i>Azadirachta indica</i>	Margosine, azadirachtin, other compounds.	<i>Hibiscus rosa-sinensis</i>	Quercetin, cyanidin, kaempferol.
<i>Argemone mexicana</i>	Alkaloid: barberine, protopine, argemone oil.	<i>Indigofera tinctoria</i>	Glucoside: indican oxidized from luc-indigo, indigotin.
<i>Acacia arabica</i> (Babool tree)	Gum: arabic acid, malic acid, sugar. Stembark: tannin. Fruit: tannin.	Leaf (pine, persimmon, ginkgo, magnolia and platanus)	Flavones, catecheutic acid, ascorbic acid, etc.
<i>Acacia catechu</i> (Katha)	Catechutannic acid, catechin, quercetin, gum, tannin.	<i>Medicago sativa</i>	Alpha-tocopherol, polypeptides, organic acids.
<i>Aegle marmelos</i> (Bael)	Skimmianine, aegelin, lupeol, cineole, citral, citronellal, cuminaldehyde, eugenol, marmesinin.	<i>Mangifera indica</i>	Vitamin A, B and C. Stembark, tannin seed, tannin, gallic acid, fat, sugar, gum, starch.
<i>Achyranthes aspera</i>	Achyranthine (water-soluble alkaloid). Seed: oleanolic acid, galactose, glucose, saponin.	<i>Moringa oleifera</i> (drumstick/ sahjan)	Alkaloids: morigine, moringinine. Flower: amorphous base, essential oil.
<i>Brassica juncea</i>	Glucoside: myrosin, sinigrin, volatile oil, fixed oil, brassic acid.	<i>Mentha viridis</i>	Menthol, menthone, essential oils.
<i>Boerhavia diffusa</i>	Potassium nitrate. Alkaloid: punarnavine.	<i>Momordica charantia</i>	Bitter glucoside, resin, ash, albuminoids, carbohydrates. Alkaloid: momoridine, saponin, essential oil.
<i>Capsicum annum</i> L.	Capsaicin, capsorubin, capsanthin, cryptocapsin, capsidiol, solanine, volatile oil, ascorbic acid, vitamin K, fatty acid, resin.	<i>Morus alba</i> (Shehtoot)	Essential oil, sugar, pectin, citrates, malates.

Continued

Table 8.1. Continued.

Name of source plant	Candidate chemicals	Name of source plant	Candidate chemicals
<i>Cicer arietinum</i>	Amines, polypeptides, organic acids.	<i>Oryza sativa</i>	Alkaloid: oridine. Base: oryzynine. Glucoside: nukain.
<i>Cinnamomum camphora</i> leaf	Terpenoids	<i>Ocimum basilicum</i>	Essential oil, terpinene, linalool.
<i>Cymbopogon flexuosus</i>	Citral, isocitral, decinal, valeric, citronella caryophyllene, phellandrene, oxobisabolene, methyl heptenone, myrcene, quercetin, kaempferol, apigenine, elemicin, catecol, hydroquinone, etc.	<i>Ocimum sanctum</i>	Essential oil: camphor, eugenol, borneol, citronellic acid, dipentene, terpenolin, mucilage, crithmene, limonene, other terpenes.
<i>Citrus indica</i> (Neembu)	Citric acid, citrene, citrol, cymene, citronellal, etc.	Oat and wheat biomasses	Proteins, polypeptides, soluble sugars, etc.
<i>Cinnamomum camphora</i>	Different terpenoids.	<i>Opuntia dillenii</i>	Malate of Mn, fatty acid, citric acid, wax, resinous matter, sugar. Fruit: carbohydrates, albuminoids, fat, ash.
<i>Coriandrum sativum</i> (Dhaniya)	Coriantrol, oxalic acid, calcium, vitamin C, carotene, essential oil, tannin, malic acid, ash.	<i>Piper nigrum</i> (Kali mirch)	Volatile alkaloids: piperine, piperidine, balsamic essential oil, lignin, gum. Resin: chavisin.
<i>Cuminum cyminum</i> (Jeera)	Thymene, thymol, oil, resin, gum.	<i>Pelargonium graveolens</i>	Organic acid, emodin.
<i>Chilopsis linearis</i>	Terpenoids, quinones.	<i>Phyllanthus fraternus</i>	Phyllanthin, alkaloids, hypophyllanthin, 4-methoxy securinine, 4-methoxy norsecurinine, glycoflavones.
<i>Centella asiatica</i> (Mandookparni)	Bitter substance-hydrocotylin, resin, ascorbic acid. Fresh leaf: glucoside, asiaticoside, essential oil, fatty oil, sitosterol, tannin.	<i>Papaver somniferum</i>	Raw poppy oil. Alkaloids: morphine, narcotine, codiene, codamine, paramorphine, tropine, narceine, cryptopine, gum, pectin, pseudomorphine, paudanine, laudanocine, groscofine, albumin, oil, wax, fat.
<i>Cedrus deodara</i> (Deodar)	Aloe-resin, turpentine, atlantone, sesquiterpenes like himachalol.	<i>Parthenium</i> leaf	Partheinin, organic acids.
<i>Carica papaya</i>	Papain, alkaloid, carpaine, glucoside, carposide, carpesamine.	Quercetin	Compound itself.
<i>Curcuma longa</i> (Haldi)	Colouring matter: curcumin, terpenoids, aromatic turmeric oil, juvabiane, ferulic acid.	<i>Sesbania</i>	Tannins, phenols, phytochelatin.
<i>Cannabis sativa</i> (Bhang)	Cannabinol, cannabidiol, canbigerol, canabidiolic acid, resin.	<i>Triticum aestivum</i>	Proteins, free sugars, polypeptides.
<i>Calotropis gigantea</i> (Aak)	Mudarine, glucosides, calotropin, usenarin, calotoxin.	<i>Tamarindus indica</i>	Tartaric acid, other compounds.

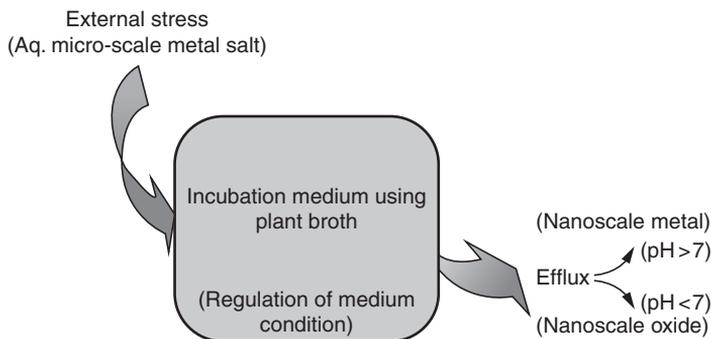


Fig. 8.1. Schematics for the biosynthesis of different nanoparticles using a plant system.

Plants, Their Metabolic Treasures and Involved Mechanisms

Every member of the plant kingdom is ready to negotiate a nanotransformation either in the form of a metal or an oxide, depending on its own metabolic treasure – the sole factor responsible for its survival against the gamut of environmental rigours. Several plants have been used successfully for the efficient and rapid extracellular synthesis of silver and gold nanoparticles. Leaf extracts of geranium (*Pelargonium graveolens*) (Shankar *et al.*, 2003), lemongrass (*Cymbopogon flexuosus*) (Shankar *et al.*, 2005), *Cinnamomum camphora* (Huang *et al.*, 2007), neem (*Azadirachta indica*) (Shankar *et al.*, 2004), *Aloe vera* (Chandran *et al.*, 2006), tamarind (*Tamarindus indica*) (Ankamwar *et al.*, 2005b) and fruit extract of *Emblica officinalis* (Ankamwar *et al.*, 2005a) have shown potential in reducing Au(III) ions to form gold nanoparticles Au(0) and silver nitrate to form silver nanoparticles Ag(0). Biomasses of wheat (*Triticum aestivum*) and oat (*Avena sativa*) (Armendariz *et al.*, 2009), lucerne (*Medicago sativa*) (Gardea-Torresdey *et al.*, 2003), native and chemically modified hop biomass (Lopez *et al.*, 2005) and remnant water collected from soaked Bengal gram bean (*Cicer arietinum*) (Ghule *et al.*, 2006) have also been used for gold nanoparticle synthesis. However, *M. sativa* (Gardea-Torresdey *et al.*, 2002), *Chilopsis linearis* (Rodriguez *et al.*, 2007) and *Sesbania* seedlings (Sharma *et al.*, 2007) have shown synthesis of gold nanoparticles inside living

plant parts. *M. sativa* sprouts (Gardea-Torresdey *et al.*, 2003) and *Brassica juncea* germinating seeds (Sharma *et al.*, 2007) have been used for silver and Ag–Au–Cu alloy nanoparticle synthesis.

An immediate reduction of silver ions as reported in a few recent investigations might have resulted due to water-soluble phytochemicals like flavones, quinones and organic acids (oxalic, malic, tartaric, protocatechuic) present in the plant tissues. Phyllanthin- and hypophyllanthin (lignan)-mediated synthesis of silver and gold nanoparticles have been reported very recently (Kasthuri *et al.*, 2009). Using *Eclipta* leaf extract, silver nanoparticles were synthesized and candidate phytochemicals were accounted to be flavonoids (Jha *et al.*, 2009a). Higher plants belonging to xerophyte, mesophyte and hydrophyte categories were recently assayed for their nanotransformation promises and all were found suitable for biosynthesis, meeting their own metabolic obligations (Jha and Prasad, 2010). An immediate reduction of silver ions in these investigations might have resulted due to water-soluble phytochemicals like flavones, quinones and organic acids (oxalic, malic, tartaric) present in the leaf parenchyma. While the responsible candidate phytochemicals have been broadly ascertained to be terpenoids (citronellol and geraniol), flavones, ketones, aldehydes, amides and carboxylic acid in the light of exhaustive infrared studies, many others that may contribute remain broadly unreported/unexplored.

Recently, Ag and Au nanoparticles have been synthesized from *C. camphora* (Huang *et al.*, 2007) leaf biomass and broth. Coriander leaf extract (Narayanan and Sakthivel, 2008) and *Cinnamomum zeylanicum* bark extract and powder (Sathishkumar *et al.*, 2009), and quite recently *Trianthema decandra* root extract (Narayanan and Sakthivel, 2008), have been used to synthesize silver nanoparticles. Both coriander and cinnamon are rich in terpenoids like coriandrol and cinnamomum, along with cinnamic acid, vitamin C, tannin and oxalic acid, which are quite labile in negotiating a nanotransformation, as found earlier (Jha and Prasad, 2011b), while roots of *Trianthema* might contain a rich treasure of catechin and hydroxyflavones (Geethalakshmi and Sarada, 2010). Earlier, nitrogenase-rich *Gliricidia sepium* (Jacq.) was also employed for preparing silver nanoparticles (Raut *et al.*, 2009). Recently, our group has reported the synthesis of gold nanoparticles employing broth of bael (*Aegle marmelos*) leaf, and the candidate phytochemicals were ascertained to be skimmianine, aegelin, lupeol, cineole, citral, citronellal, cuminaldehyde, eugenol and marmesinin (Jha and Prasad, 2011a). Silver nanoparticles using the aqueous extract of gum olibanum (*Boswellia serrata*), a renewable natural plant biopolymer, has been reported. The plant's resinous exudates have been shown to contain many promising terpenoids having potential analgesic/anti-inflammatory properties (Kora *et al.*, 2012). Similarly, nanocrystalline silver and palladium production using acaricidal, insecticidal and larvicidal efficacy of *Annona squamosa* L. aqueous peel extract as the biomaterial has also been reported (Kumar *et al.*, 2012; Roopan *et al.*, 2012). A rapid biosynthesis route for the preparation of gold nanoparticles by aqueous extract of cypress leaves at room temperature has been carried out (Noruzi *et al.*, 2012). In addition, the biomimetic synthesis of silver nanoparticles by employing aqueous extract of *Citrus limon* (lemon) has been taken up (Prathna *et al.*, 2011). Gold nanoparticles using *C. zeylanicum* (Smitha *et al.*, 2009), *Macrotyloma uniflorum* (Aromal *et al.*, 2012) leaf broth and *Benincasa hispida* seed (Aromal and Philip, 2012) have also been reported.

Plant-mediated synthesis of gold nanoparticles has been demonstrated successfully using not only fruit but also fruit parts (which are generally wasted) such as banana peel extract (Bankar *et al.*, 2010). Barbated skullcup herb extract-mediated biosynthesis of gold nanoparticles and its primary application in electrochemistry has been reported (Wang *et al.*, 2009). Biodirected synthesis of platinum nanoparticles using aqueous honey solutions and their catalytic applications has emerged (Venu *et al.*, 2011). Bioinspired synthesis of highly stabilized silver nanoparticles using *Ocimum tenuiflorum* and *Ocimum sanctum* leaf extract and their antibacterial activity has also been reported (Singhal *et al.*, 2011; Patil *et al.*, 2012; Soundarrajan *et al.*, 2012). The bioprospective of *Sorbus aucuparia* leaf extract in the development of silver and gold nanocolloids has been studied (Dubey *et al.*, 2010). The biosynthesis, characterization and antibacterial effect of plant-mediated silver nanoparticles using *Artemisia nilagirica*, a promising medicinal plant, has been reported (Vijayakumar *et al.*, 2013), and the biosynthesis of anisotropic gold nanoparticles using *Madhuca longifolia* extract and their potential in infrared absorption has also been reported (Fayaz *et al.*, 2011). Biological syntheses of platinum and gold nanoparticles using *Diopyros kaki* and *Magnolia kobus* leaf extract have been presented (Song *et al.*, 2009, 2010). Green synthesis of silver nanoparticles through *Calotropis gigantea* leaf extracts has emerged, which raises the hope further that even common weeds can contribute towards the synthesis of different nanomaterials (Baskaralingam *et al.*, 2012). Not only leaves but also fruit may contain promising phytochemicals, leading to a successful nanotransformation. We have reported this using chilli fruit (*Capsicum annum* L.) broth (Jha and Prasad, 2011b) and orange juice (Jha *et al.*, 2011).

The main idea behind the plant-mediated synthesis of metallic and/or oxide nanoparticles is first to ascertain their candidate chemicals, metabolic status (e.g. Table 8.1) and their gross chemistry (like tautomeric conversion, resonance energy stabilization, oxido-/nitroreductase, etc.), regardless of their phylogenetic or ecological distribution. The synthesis, encapsulation and dispersion

of metallic/oxide nanoparticles are solely dependent on the milieu of the incubation medium and the available chemicals elaborated by the plants in the incubation/transformation mixture. Accordingly, the medium conditions can be regulated suitably to obtain the desired (metal/oxide) nanoparticles (Fig. 8.1). With the aim of understanding the mechanism of plant-mediated biosynthesis of metal and oxide nanoparticles, *Cycas* leaf has been taken as an example. *Cycas* leaves have been found to contain amentoflavone and hinokiflavone as characteristic biflavonyls that ascertain the synthesis of silver nanoparticles (Jha and Prasad, 2010). Reduction is accomplished due to the phytochemicals (flavonoids or other polyphenols)

or phytochelatins/glutathiones/metallothioneins present in the parenchyma of *Cycas* leaves. Reduction of particle size by the leaf broth compared to bacteria or fungi may be perceived as an appreciable advancement. By pursuing suitable modulation of parameters like pH and temperature, oxide nanoparticles can be synthesized (Fig. 8.2).

Conclusion

In the light of the recent literature, it is emphatically clear that plant systems are committed to respond to their immediate chemical environment according to their own metabolic fluxes or metabolic treasures, otherwise

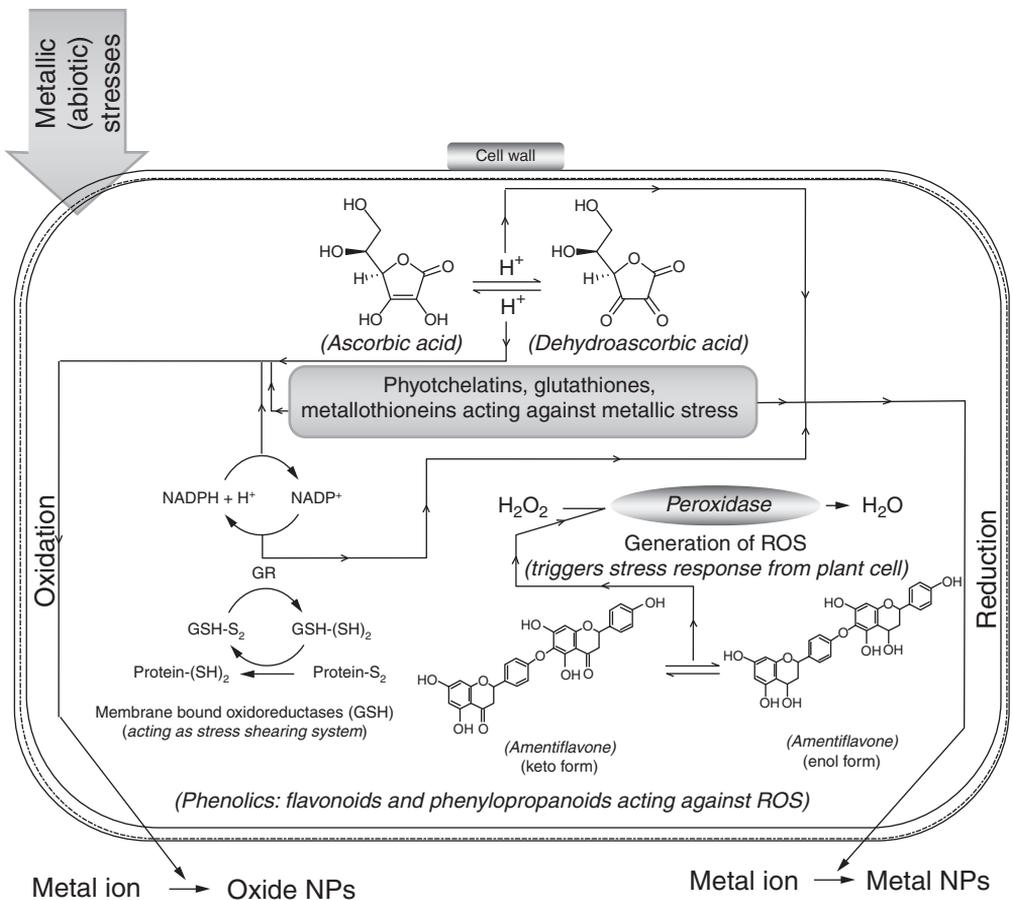


Fig. 8.2. Mechanism for the biosynthesis of metal and/or oxide nanoparticles using *Cycas* leaf broth (modified after Jha and Prasad, 2010).

called adaptability. While bacterial/fungal systems respond by triggering their stress-shearing mechanisms, plants do the same by molecular-level modifications of their metabolic weaponry, resulting in the synthesis of either a metallic or an oxide nanoparticle, or sometimes both in succession, based broadly on the modulation of parameters. All these facts bring us to the indisputable conclusion that it is the specific chemical/net chemical status that makes an organism a

suitable candidate to undertake a nano-transformation. Further, if we employ a suitable tissue culture technique for this purpose, this not only would check any unnecessary wastage of phytodiversity but also would open an avenue for the mass-scale production (scaling up) of nanomaterials using crude/fine phytochemicals. Many more may follow in the immediate future. It is true that all of nature seems ready to precipitate a nanotransformation.

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9 Synthesis of Nanostructured Calcite Particles in Coccolithophores, Unicellular Algae

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Introduction

Coccolithophores are unicellular planktonic algae that belong to the phylum Haptophyta. They can surround themselves with elaborately crafted calcite platelets, so-called coccoliths (Fig. 9.1). Coccoliths feature diameters of only 1–10 µm (Young and Henriksen, 2003). Despite this, they exhibit complex substructures on a nanoscale.

The first reliable records of fossil coccoliths date back some 220 million years (Bown, 2005). An enormous production of coccoliths since the Jurassic circa 150 million years ago (Morse and Mackenzie, 1990) resulted in the formation of thick sediment layers that were responsible for the formation of massive chalk cliffs. Today, coccolithophores are among the most productive calcifiers in the open ocean.

A question that has been discussed extensively in the past, but still remains more or less unanswered, is ‘Why do coccolithophores calcify?’ Several hypotheses concerning this question have been put forth as, for instance, protection-related functions, biochemical functions of coccolith formation, floating-related functions, as well as light regulation (Young, 1994). While many of these hypotheses

have been shown to be unlikely, such as a protection against predators (Hansen *et al.*, 1997) and viruses (Bratbak *et al.*, 1996) or a CO₂ supply to photosynthesis via calcification (Trimborn *et al.*, 2007, and references therein), other hypotheses are more challenging to test and remain unanswered to this date. However, the answer to this question may well explain the evolutionary success of coccolithophores.

The present chapter focuses on the intracellular mechanisms underlying the synthesis of nanostructured coccoliths. While some of the processes and structures involved have been discovered already, much remains unknown.

Excursus into carbonate chemistry

In aqueous solutions, carbon dioxide (CO₂) exists in four different forms: CO₂, carbonic acid (H₂CO₃), bicarbonate ions (HCO₃⁻) and carbonate ions (CO₃²⁻). They convert via the following equilibrium reactions:



Since the concentration of H₂CO₃ is comparably small, it is commonly incorporated into the CO₂ term. The carbonate system acts as

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a buffer system in aqueous solutions. The so-called Bjerrum plot (Fig. 9.2) visualizes the variation of the three main carbon species with the pH value of the solution.

Temperature and salinity influence the pK values. The latter are a measure for the strength of an acid; they give the pH value at which the concentrations of two components in an acid/base system equal each other. Figure 9.2 shows the carbonate system at a salinity of 35 and a temperature of 25°C, as given in Zeebe and Wolf-Gladrow (2001).



Fig. 9.1. Coccolith of *Emiliana huxleyi*.

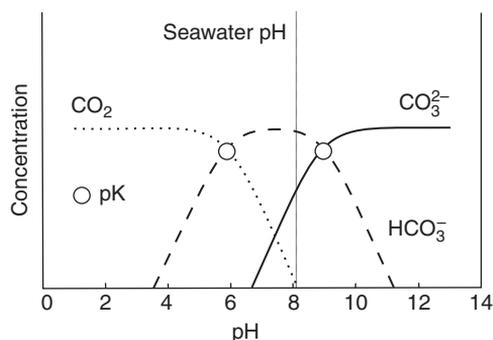


Fig. 9.2. A Bjerrum plot visualizes the composition of the carbonate system in equilibrium and the corresponding pH values. The three main components of the carbonate system are CO₂, HCO₃⁻ and CO₃²⁻. (Figure reproduced from Zeebe and Wolf-Gladrow, 2001.)

The vertical line visualizes the composition of seawater. The pH value of the cytosol is commonly assumed to be seven. However, since the carbonate system of the cytosol is unlikely to be in equilibrium, the Bjerrum plot cannot be used to visualize its composition in terms of inorganic carbon. Anyway, the predominant carbon species inside the cytosol is assumed to be HCO₃⁻. Seawater and cytosol are separated from each other mainly via a biomembrane (see below for an explanation) in the case of unicellular planktonic algae.

An important point is the very slow conversion between CO₂ and HCO₃⁻ (~10 s) compared to the almost instantaneous conversion between HCO₃⁻ and CO₃²⁻ (~10⁻⁷ s) (Zeebe and Wolf-Gladrow, 2001).

Excursus into the cellular constitution of coccolithophores

All cells are surrounded by a plasma membrane separating the inside of the cell, the *cytoplasm*, from its environment. These so-called *biomembranes* consist mainly of amphiphilic lipids. In an aqueous solution, their hydrophilic regions arrange towards the aqueous solution while the hydrophobic regions are shielded away. Thereby, c.10 nm-thick lipid bilayers result, which form spontaneously in aqueous solutions due to the given energetic circumstances.

Biomembranes further contain different proteins that feature versatile functions. Small, non-polar molecules such as O₂ and CO₂ can diffuse passively through biomembranes. The passage of larger and polar molecules, in contrast, is controlled by proteins, which pervade the lipid bilayers, so-called channels or transporters. Biomembranes envelop the cell itself, as well as all cellular organelles. Thereby, chemically optimized milieus can be created for different cellular functions. The main compartments of haptophyte algae are presented in Fig. 9.3.

Chloroplast(s) are typical organelles of plant cells and can be defined as the site of photosynthesis (the process that converts the electromagnetic energy of light into chemical energy). Meanwhile, inorganic carbon in the form of CO₂

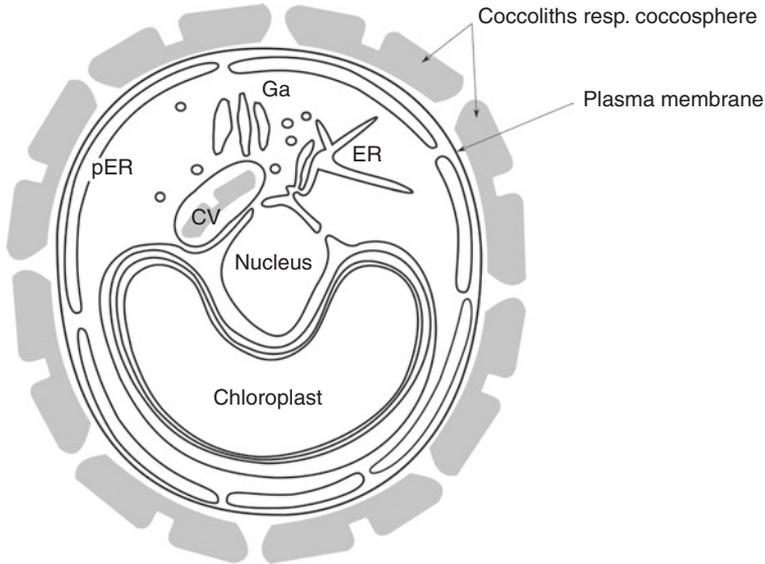


Fig. 9.3. Simplified haptophyte cell. Coccoliths are synthesized in Golgi (Ga)-derived coccolith vesicles (CV) and afterwards expelled to the coccosphere. ER = endoplasmic reticulum; pER = peripheral endoplasmic reticulum.

is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) into low-molecular-weight sugars.

The *endoplasmic reticulum* (ER) serves, besides protein and lipid synthesis, to sequester cytosolic Ca^{2+} , which is facilitated by means of Ca^{2+} buffering molecules inside the ER. Since Ca^{2+} acts as a second messenger between different physiological pathways, the cytosolic $[\text{Ca}^{2+}]$ needs to be very low.

The *peripheral ER* (pER) is a cellular structure often observed in haptophyte algae (van den Hoek *et al.*, 1978). Its function remains more or less unknown. Brownlee and Taylor (2004) proposed an involvement in Ca^{2+} uptake into the endomembranous system.

The ER interacts vividly with the *Golgi apparatus* (Ga) via vesicle transport. Inside the Ga, many proteins and lipids from the ER are modified and sorted. In calcifying haptophytes, it is suspected to play an essential role in the supply of Ca^{2+} for calcification. Furthermore, the *coccolith vesicle* (CV), in which coccolith synthesis takes place, is a descendant of the Ga. In some species, it is

associated with a tubular, membranous system known as the *reticular body* (Rb).

Providing Calcium and Inorganic Carbon for Coccolith Synthesis

During their life cycle, coccolithophores undergo two distinct stages: a diploid stage and a haploid stage. While the diploid stage exhibits two DNA copies, the haploid stage contains only one. However, both stages feature very different coccolith types: the diploid cell characteristically covers itself with heterococcoliths, which consist of different nanosize crystal units arrayed in a radial symmetry. The haploid stage, in turn, has a cell covering made of holococcoliths with a simple crystal shape (Young *et al.*, 1999). Both coccolith types are typically made of the mineral calcite (CaCO_3 ; Young *et al.*, 1999) and organic material (Marsh, 2003).

In the following, we will limit ourselves to the processes underlying heterococcolith synthesis, which occur intracellularly.

Coming to the actual subject of this section, we will discuss first the Ca^{2+} supply for coccolith synthesis in *Pleurochrysis carterae* and *Emiliana huxleyi*, the two model organisms, followed by a general discussion of the inorganic carbon (C_i) supply. Ca^{2+} supply has been discussed extensively in the past (Brownlee and Taylor, 2004), especially regarding the obvious difficulty of transporting Ca^{2+} to the intracellular site of coccolith synthesis through the cytosol, the free $[\text{Ca}^{2+}]$ of which should remain as low as $c.100 \text{ nmol l}^{-1}$ (Brownlee *et al.*, 1995) to prevent toxication. Seawater $[\text{Ca}^{2+}]$, in turn, is rather high with 10 mmol l^{-1} , meaning that the overall supply to the cell should not be limited. The passage of C_i through the cell, taking into account the conversion of individual carbon species into one another, has so far not been discussed in detail.

For *P. carterae* (Fig. 9.4), it seems to be clear that Ca^{2+} transport into the CV is exhibited via vesicles (diameter 400 nm) filled with coccolithosomes (Outka and Williams, 1971; Marsh, 2003). Coccolithosomes are small granules (diameter 20 nm) consisting mainly of two polysaccharides, PS1 and PS2, the latter of which 'sequesters more calcium than any polyanion yet described' (Marsh and Dickinson, 1997). Van der Wal *et al.* (1983a) found the $[\text{Ca}^{2+}]$ of coccolithosomes to be at least 6 mol l^{-1} . Comparing this concentration

to the one of elemental calcium (39 mol l^{-1}) reveals the magnitude of this value. Those 400 nm vesicles have been observed to fuse with the CV and to provide Ca^{2+} in the form of coccolithosomes to the growing coccoliths (Outka and Williams, 1971; Marsh, 1994; Marsh and Dickinson, 1997). *P. carterae* actually synthesizes several coccoliths at once in *medial* to *trans* Golgi cisternae CV (Marsh, 2003).

Nevertheless, prior to the formation of coccolithosomes, Ca^{2+} first has to enter the Ga. The pathway of Ca^{2+} into the cytosol of calcifying coccolithophores probably occurs via Ca^{2+} -permeable channels (Brownlee and Taylor, 2004). One possibility for the further pathway is an active uptake of Ca^{2+} against a concentration gradient into the pER (Brownlee and Taylor, 2004). Inside the pER, which is likely to be connected to the ER, Ca^{2+} may be bound to specialized molecules (not PS1 or PS2) buffering the prevailing $[\text{Ca}^{2+}]$. From here, small transporting vesicles filled with Ca^{2+} could be transported to the Ga. The polysaccharides PS1 and PS2, which are synthesized inside the Ga (Marsh, 1994), then bind the imported Ca^{2+} and form coccolithosomes.

Another possibility is the diffusion/transport of Ca^{2+} (free ions or bound to cytosolic molecules) from the peripheral space (space between the plasma membrane and pER)

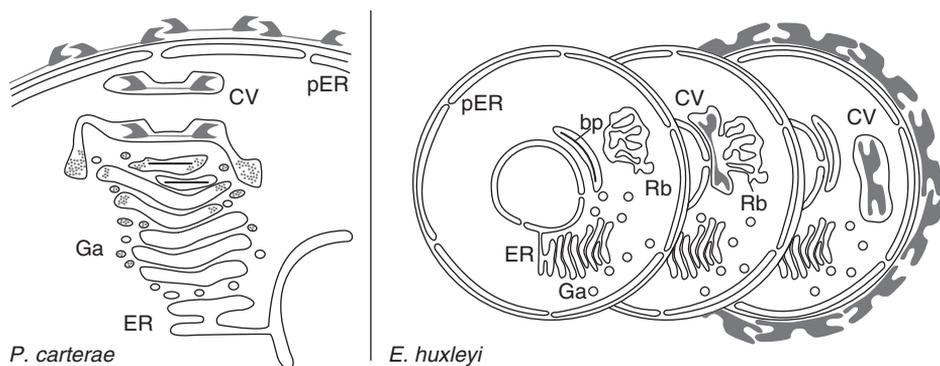


Fig. 9.4. Calcifying haptophyte algae show different types of coccolith vesicles (CV). *Emiliana huxleyi* exhibits a reticular body (Rb), while *Pleurochrysis carterae* shows coccolithosomes that are transported via vesicles from the Golgi apparatus (Ga) to the CV. *E. huxleyi* cells typically show diameters of 4–5 μm , with coccoliths of comparable size (2.5–4.5 μm) (Paasche, 2002). *P. carterae* cells, in turn, exhibit diameters of $c.10 \mu\text{m}$, with coccolith diameters of $c.1 \mu\text{m}$ (Outka and Williams, 1971). bp = base plate; ER = endoplasmic reticulum; pER = peripheral endoplasmic reticulum. (Figure changed after Marsh, 2003.)

to the cytosol, from where it would have to be taken up by the Ga against a concentration gradient.

Currently, both hypotheses cannot be evaluated qualitatively due to a lack of essential data such as the $[Ca^{2+}]$ inside the peripheral space, the pER, detailed knowledge about the location of transporters and their specific kinetic abilities and so on. Nevertheless, Table 9.1 lists theoretical arguments for and against both hypotheses.

The mechanism underlying Ca^{2+} supply in *E. huxleyi* deviates from the one found for *P. carterae*. According to Marsh (2003), *E. huxleyi* 'lacks acidic polysaccharide equivalents of PS1 and PS2' but instead exhibits the membranous system of the Rb, which is closely associated to the CV during coccolith synthesis. Due to its high surface:volume ratio, the Rb would be perfectly suited to import large amounts of Ca^{2+} . The CV-Rb complex of *E. huxleyi*, which is also observed in *Coccolithus braarudii*, is located on top of the shaping nucleus. Both species exhibit sequential coccolith synthesis, in contrast to *P. carterae* (Taylor *et al.*, 2007). Different theories for the supply of Ca^{2+} towards the growing coccolith of *E. huxleyi* are under discussion, one of which is the uptake of cytosolic Ca^{2+} directly across the membranes of the CV and the Rb. The implications of this possibility are

discussed in Table 9.1 for hypothesis (2). The position of the Rb, facing towards the periphery of the cell, could be of advantage here, because the distance from the peripheral space is relatively short. Mackinder *et al.* (2010) proposed a quite inventive mechanism for the import of Ca^{2+} directly across the CV membrane: a high $[H^+]$ is established in an early-stage CV by means of membrane transporters pumping H^+ against a concentration gradient into the CV. Driven by the established H^+ gradient, Ca^{2+} could be imported by means of Ca^{2+}/H^+ exchangers. Both transporter types have been shown to be upregulated in calcifying cells of *E. huxleyi* (Mackinder *et al.*, 2011). However, this theory requires the accumulation of (almost) all Ca^{2+} needed for the synthesis of one complete coccolith in the CV, which Gussone *et al.* (2006) have calculated to be impossible.

Another possibility for the Ca^{2+} transport towards the growing coccolith in *E. huxleyi* actually resembles the vesicle transport between ER/pER and Ga, as discussed for hypothesis (1) in Table 9.1. The only difference is the destination of the vesicles: here, it is the growing CV, while it is the Ga in Table 9.1.

The intracellular pathway of C₁ in coccolithophores is even more speculative than is the one of Ca^{2+} . This can be attributed to a lack of knowledge about the intracellular

Table 9.1. Transport of calcium ions (Ca^{2+}) from the plasma membrane to the Golgi apparatus (Ga) in *P. carterae*.

	Ca^{2+} uptake into ER/pER, then vesicle transport to Ga (1)	Ca^{2+} diffusion through cytosol, then uptake into Ga (2)
Advantages	Ca^{2+} does not encounter the bulk of the cytosol.	Overall process might be less costly, since vesicle transport occurs only once.
Disadvantages	The costly and highly controlled vesicle transport occurs twice. Transport from ER/pER to Ga requires even higher membrane cycling than from Ga to CV, since Ca^{2+} is not yet associated to PS1 or PS2. Moreover, those transport vesicles are probably smaller, implying a higher membrane:volume ratio.	Ca^{2+} diffuses through cytosol. Ca^{2+} uptake into Ga has to be fast, despite low cytosolic $[Ca^{2+}]$; this requires transporters with either a high transport velocity and a high affinity towards Ca^{2+} , or many transporters with high affinities and low transport velocities; such detailed knowledge is missing.

Notes: CV = coccolith vesicles; ER = endoplasmic reticulum; Ga = Golgi apparatus; pER = peripheral endoplasmic reticulum.

distribution of C_i . Sekino and Shiraiwa (1994) have determined the bulk concentration of intracellular dissolved inorganic carbon (DIC, sum of CO_2 , HCO_3^- and CO_3^{2-}) of *E. huxleyi* to be around 13 mmol l^{-1} . However, this value is not sufficient, as it gives no information on the distribution of intracellular DIC.

As shown in Fig. 9.2, surface seawater exhibits a high content in HCO_3^- and CO_3^{2-} . *E. huxleyi* takes up external HCO_3^- and CO_2 (Rokitta and Rost, 2012). While the intracellular process of photosynthesis is a sink of seawater HCO_3^- and CO_2 , coccolith synthesis is thought to rely predominantly on seawater HCO_3^- (Paasche, 2002; Rost *et al.*, 2002). It may be possible that HCO_3^- and CO_2 travel through the cytosol without being converted into each other, because of the slow conversion between both carbon species. Further assuming that chloroplast and CV actively import HCO_3^- , the additional usage of CO_2 could result from CO_2 diffusion towards the C_i fixing enzyme, RubisCO, which is located inside the chloroplast. In terms of the CV, HCO_3^- import would have to be accompanied by an export of H^+ (or import of OH^-) to prevent the CV from acidification (Taylor *et al.*, 2011), the latter being an unfavourable situation for calcite precipitation. However, a transporter with this stoichiometry would lead to an electric charging of the CV, a problem that could be overcome when simultaneously importing Ca^{2+} into the CV. Nevertheless, to the best of our knowledge such a membrane transporter has so far not been found.

Coming back to the C_i import across the plasma membrane, the findings of Herfort *et al.* (2002) hint at an anion exchanger being responsible for HCO_3^- uptake in *E. huxleyi*. Theoretically, CO_2 can diffuse into the cell if the cytosolic $[CO_2]$ is lower than seawater $[CO_2]$. A higher cytosolic $[CO_2]$ implies a leakage of CO_2 from the cytosol. While active CO_2 uptake is assumed for different algae, such as green algae (Sültemeyer *et al.*, 1989), cyanobacteria (Espie *et al.*, 1989) and *E. huxleyi* (Schulz *et al.*, 2007), the underlying mechanisms remain unclear.

The results of Herfort *et al.* (2002) further hint at the activity of extracellular carbonic

anhydrase (eCA) under low external [DIC], with eCA being an enzyme accelerating the otherwise very slow conversion between CO_2 and HCO_3^- inside the diffusive boundary layer of the cell. Other authors, as for instance Rost *et al.* (2002, 2003), did not find evidence of eCA activity in *E. huxleyi*. However, depending on the pH value of the thin water layer surrounding the plasma membrane (cellular boundary layer), eCA may convert CO_2 to HCO_3^- or vice versa. Under high pH values, CO_2 leaking out of the cell could be converted to HCO_3^- , which may then be taken up again by the anion exchanger. Thereby, net CO_2 leakage from the cell may be reduced. When in turn assuming an acidified cellular boundary layer, external HCO_3^- could be converted to CO_2 , which may then enter the cell via diffusion or lower CO_2 leakage from the cell.

Summing up, neither the intracellular passage of Ca^{2+} nor the one of C_i is known in detail, but Ca^{2+} and C_i have to be transported into the CV during coccolith synthesis.

Chemical Control of Calcite Precipitation

Supersaturation

A $CaCO_3$ crystal or precipitate may form when a solute exceeds its solubility in the aqueous solution. The solubility of an inorganic salt is determined by an equilibrium constant, the solubility product (K_{sp}).

For the precipitation reaction,



the solubility product, which is a function of temperature and salinity of the solution, is given by:

$$K_{sp} = [Ca^{2+}]_{eq} \cdot [CO_3^{2-}]_{eq} \quad (9.2)$$

with $[Ca^{2+}]_{eq}$ and $[CO_3^{2-}]_{eq}$ being the effective concentrations (activities) of ions in a solution in equilibrium with the solid phase. A measure for the level of supersaturation of a solution is the difference between the ion activity product of the solution,

$$Q = [\text{Ca}^{2+}]_{\text{sol}} \cdot [\text{CO}_3^{2-}]_{\text{sol}} \quad (9.3)$$

and the equilibrium position of Q as defined by K_{sp} . The saturation state of the solution is generally defined by the saturation ratio,

$$\Omega = \frac{Q}{K_{\text{sp}}} \quad (9.4)$$

The thermodynamic driving force for calcite precipitation to occur is the change of free energy due to the reaction in Eqn 9.1,

$$\Delta G_r = -RT \ln \frac{Q}{K_{\text{sp}}} = -RT \ln \Omega \quad (9.5)$$

Equation 9.1 will proceed in the forward direction, i.e. precipitation reaction will be favoured, if $\Delta G_r < 0$. Therefore, calcite is precipitated from the solution as long as $Q > K_{\text{sp}}$ or $\Omega > 1$. In this respect, Ω is a measure of the extent to which a solution is out of equilibrium and represents the driving force for precipitation.

Inorganic nucleation

A supersaturated solution does not transform spontaneously to a solid crystal. A precondition of crystal growth is the formation of a crystal nucleus of a critical size. Nucleation occurring in a supersaturated solution due to spontaneous nuclei formation is termed homogeneous nucleation. Heterogeneous nucleation, on the other hand, occurs when nucleation is initiated via foreign surfaces. Since biomineralization usually involves foreign substrates, the theoretical discussion

given here considers only heterogeneous nucleation. The principles of this process provide the basis for understanding nucleation at the surface of an organic template, as is assumed for coccolith synthesis.

The free energy change, ΔG_n , associated with nucleation arises from two sources: one depends on ΔG_r , the free energy (per mole) corresponding to precipitation, the other results from the interfacial free energy, ΔG_s , of the surface separating the solid phase from the liquid. The free energy of the reaction, ΔG_r , is negative when the solute exceeds its solubility in the aqueous solution. On the other hand, the free energy of the surface, ΔG_s , is always a positive quantity. The value of ΔG_n is therefore given by

$$\Delta G_n = \frac{V}{v_m} \Delta G_r + \Delta G_s \quad (9.6)$$

where V is the volume of the crystal cluster and v_m is the molar volume.

We consider a cap-shaped crystal cluster on a perfect substrate, as shown in Fig. 9.5. It represents a segment of a sphere with the radius of the curvature r and contact angle θ . The latter is defined by the balance of the tensional forces at the liquid–crystal–substrate triple contact and can be calculated by means of the well-known relation of Young (Young, 1804):

$$\sigma_{sl} = \sigma_{sc} + \sigma \cos \theta \quad (9.7)$$

where σ is the crystal–liquid interfacial tension, σ_{sc} is the substrate–crystal interfacial tension and σ_{sl} is the substrate–liquid interfacial tension. The change of free energy

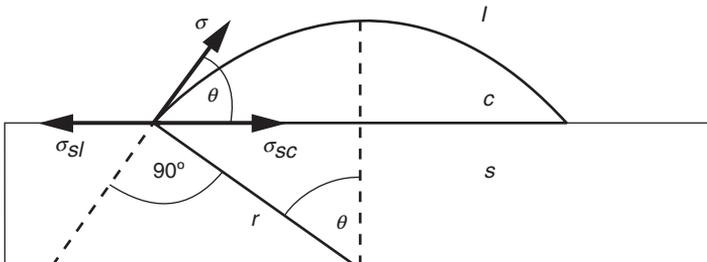


Fig. 9.5. Schematic cap-shaped crystal cluster (c) forming on a flat substrate (s) from a liquid (l).

corresponding to the cap-shaped cluster is made up of the volumetric free energy gain,

$$\frac{V}{v_m} \Delta G_r = \frac{\pi r^3}{3v_m} (1 - \cos\theta)^2 \times (2 + \cos\theta) \Delta G_r \quad (9.8)$$

and the surface free energy

$$\Delta G_s = 2\pi r^2 \sigma (1 - \cos\theta) - \pi r^2 \sigma \cos\theta (1 - \cos^2\theta) \quad (9.9)$$

Substituting the above expressions into Eqn 9.6 gives

$$\Delta G_n = \left(\frac{4\pi r^3}{3v_m} \Delta G_r + 4\pi r^2 \sigma \right) \left(\frac{2 - 3\cos\theta + \cos^3\theta}{4} \right) \quad (9.10)$$

Note that the first term in parenthesis is the free energy change for the spontaneous formation of a spherical cluster during homogeneous nucleation. Hence, ΔG_n for heterogeneous (het) nucleation is equal to the product of ΔG_n for homogeneous (hom) nucleation and a function of the contact angle θ :

$$\Delta G_{n,\text{het}} = \Delta G_{n,\text{hom}} \phi(\theta) \quad (9.11)$$

where

$$\phi(\theta) = \frac{2 - 3\cos\theta + \cos^3\theta}{4} \quad (9.12)$$

Figure 9.6 shows the homogeneous and heterogeneous free energies of nucleation as a function of cluster size, from which it can be seen that ΔG_n exhibits a maximum ΔG_n^* at a critical radius $r = r^*$. A cluster of solid matter with $r < r^*$ is unstable because the cluster will shrink to reduce its free energy. On the other hand, a cluster with $r > r^*$ can grow steadily with a decrease in free energy and is therefore stable. A crystal cluster having $r < r^*$ is called an embryo. When the cluster reaches the critical size $r = r^*$, it becomes a nucleus. The formation of the critical size nucleus requires a statistical fluctuation to transform an embryo into the nucleus. At constant temperature and pressure, the energy of a closed system is constant. However, the energy fluctuates around

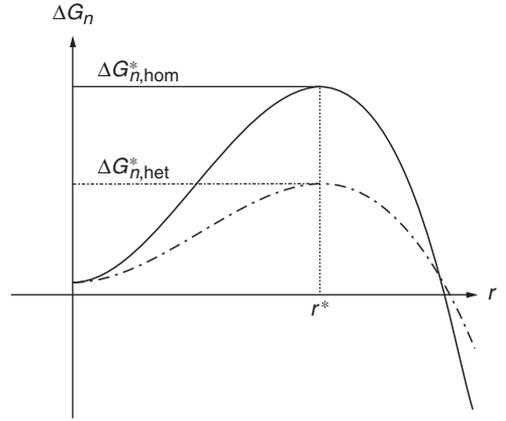


Fig. 9.6. Free energy change (ΔG_n) of homogeneous nucleation (solid line) and heterogeneous nucleation (dashed line) as a function of cluster size (r).

the average value and it is therefore possible that some of the clusters in the fluid acquire sufficient energy to overcome the energy barrier ΔG_n^* . The radius of the critical nucleus is obtained when $d\Delta G_n/dr = 0$, i.e.

$$\frac{d\Delta G_n}{dr} = 0 = \left(\frac{4\pi r^2}{v_m} \Delta G_r + 8\pi r \sigma \right) \phi(\theta) \quad (9.13)$$

Thus, ΔG_n is maximal at the critical cluster size

$$r^* = -\frac{2v_m\sigma}{\Delta G_r} = \frac{2v_m\sigma}{RT \ln \Omega} \quad (9.14)$$

which does not depend on the contact angle and therefore remains unchanged for homogeneous and heterogeneous nucleation. In both cases, the critical cluster size is decreased with reduced σ , assuming a constant level of supersaturation is given. Inserting Eqn 9.14 into Eqn 9.10 gives the critical energy barrier:

$$\begin{aligned} \Delta G_n^* &= \frac{16\pi\sigma^3 v_m^2}{3(RT \ln \Omega)^2} \phi(\theta) \\ &= \frac{16\pi\sigma^3 v^2}{3(kT \ln \Omega)^2} \phi(\theta) \end{aligned} \quad (9.15)$$

where v is the molecular volume and k is the Boltzmann constant. The energetic advantage of heterogeneous nucleation arises from the term $\phi(\theta)$, describing the potential of

the surface to catalyse nucleus formation. As follows from Eqn 9.12, $\phi(\theta)$ varies from 0 to 1 when θ varies from 0 to π . If $\theta = 0$, the crystal phase wets the surface and there is no barrier to nucleation. In the other extreme, if $\theta = \pi$ (complete non-wetting), there is no reduction in energy and we have in practice homogeneous nucleation. Then,

$$\Delta G_{n,\text{het}}^* = \Delta G_{n,\text{hom}}^* = \frac{16\pi\sigma^3 v^2}{3(kT \ln \Omega)^2} \quad (9.16)$$

If the clusters are assumed to be in equilibrium, the Boltzmann distribution can be used to express the rate of nucleation, J_n , in terms of ΔG_n^* :

$$J_n = J_0 \exp\left(-\frac{\Delta G_n^*}{kT}\right) \quad (9.17)$$

where J_0 is a constant. It follows from Eqn 9.15 that ΔG_n^* depends strongly on the interfacial energy (proportional to σ^3) and on the contact angle θ . Relatively small changes in σ can have a significant effect on J_n . Also, an increase in the level of supersaturation decreases ΔG_n^* and therewith increases the rate of nucleation,

$$J_n = J_0 \exp\left[-\frac{16\pi\sigma^3 v^2}{3k^3 T^3 (\ln \Omega)^2} \phi(\theta)\right] \quad (9.18)$$

The above equations for ΔG_n^* and J_n indicate that the interfacial energy of the critical nucleus and the degree of supersaturation are the factors that need to be influenced by the algal cells if calcite precipitation is to be regulated during coccolith formation. These factors can be fine-tuned in biologically controlled calcite precipitation by using organic templates to catalyse nucleus formation and by regulating the transport of ions and their chemical activities in the CV.

Coccolith Morphogenesis

In the following, the term 'coccolith' will refer to 'heterococcolith' only. Holococcoliths are of essentially different morphology and might be produced by different cellular machinery (Rowson *et al.*, 1986). A typical coccolith consists primarily of a radial array

of complexly shaped interlocking calcium carbonate crystals (Fig. 9.7b; Young and Henriksen, 2003). The polymorph of these crystals is calcite (Young *et al.*, 1991), the unit cell of which has a rhombohedral symmetry (Fig. 9.7a). The identification of rhombic crystal faces in coccoliths (Fig. 9.7c; Henriksen *et al.*, 2004) suggests the importance of inorganic crystal growth in coccolith morphogenesis. However, it is also obvious that the overall shape of a coccolith crystal clearly deviates from an inorganically grown calcite rhomb (Fig. 9.7d). By entailment, this means that the cell exerts a rigorous control on coccolith morphogenesis, a fact that is also reflected in the diversity of coccolith morphology among coccolithophores (Young *et al.*, 2003).

When Huxley discovered coccoliths in the 19th century, he assumed they were of inorganic origin (Huxley, 1868). This possibility was rendered unlikely by the discovery of living coccolithophores (Wallich, 1877), and clearly proven wrong by the observation that coccoliths were produced inside the cell and, on completion, extruded (i.e. exocytosed; Dixon, 1900). The advent of transmission electron microscopy half a century later led to the notion that coccoliths were produced in a specialized cellular compartment, the CV (Wilbur and Watabe, 1963). Inside the CV, the morphogenesis of a coccolith is accomplished through two discrete processes, namely crystal nucleation and crystal growth. Both processes are under strict cellular control. Please note that despite diversity among different species in the way in which this control is exerted in particular, there is a general pattern, which will be described in the following.

Nucleation

The nucleation of coccolith crystals is heterogeneous. The surface, which is involved in the formation of calcite nuclei, is the so-called organic base-plate scale. This base plate serves as an organic template and is localized inside the CV (Westbroek *et al.*, 1984). Nucleation occurs on the rim of the base plate and, initially, simple crystals are formed.

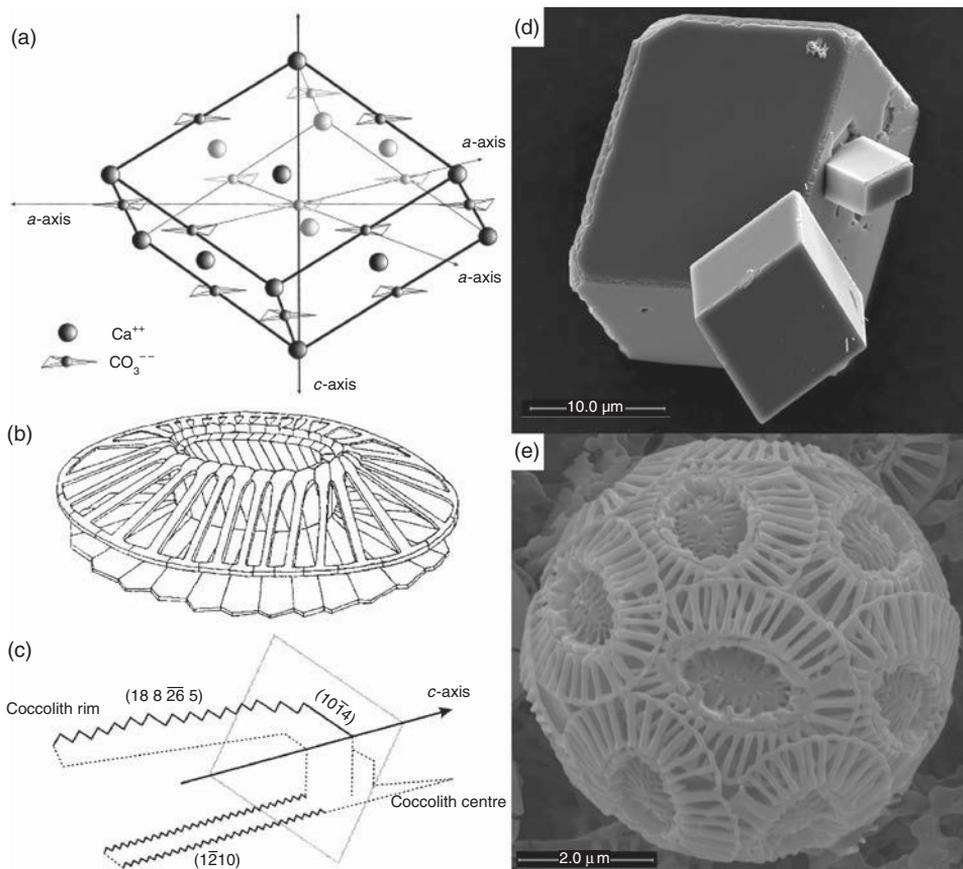


Fig. 9.7. (a) Sketch of a three-dimensional array of a very small portion of CaCO_3 atoms in their spatial arrangement in calcite (reproduced from Young *et al.*, 1999). (b) Drawing of a coccolith of *Emiliana huxleyi* (reproduced from Young *et al.*, 1999). (c) Schematic cross section through an *E. huxleyi* crystal showing its crystallographic relation to the calcite rhomb (reproduced from Henriksen *et al.*, 2004). (d) SEM image of inorganically precipitated calcite (courtesy of Gernot Nehrke). (e) SEM image of *E. huxleyi*.

These crystals are arranged in a structure termed the proto-coccolith ring. The latter consists of a series of crystals with alternating vertical (V-unit) and radial (R-unit) c -axis orientations relative to the base-plate margin (Young and Bown, 1991). This pattern, which came to be known as the V/R model, was first discovered in an extinct *Watznaueria* species in Jurassic clay samples (Young and Bown, 1991) but was subsequently also observed in extant *E. huxleyi* (Young *et al.*, 1992) and *P. carterae* (Marsh, 1999). It was proposed that the V/R model was a fundamental feature of coccolith nucleation and had been

conserved throughout the more than 200 million year history of coccolithophores (Young *et al.*, 1992). Extensive light and electron microscopic studies combined with selected area electron diffraction have rendered the V/R model a well-established feature of coccolith morphology (Young, 1992; Didymus *et al.*, 1994; Davis *et al.*, 1995).

It is worthwhile noting that nucleation controls not only c -axis orientation of the proto-coccolith ring crystals but also a -axis orientation (Mann and Sparks, 1988; Davis *et al.*, 1995; Henriksen *et al.*, 2003). Hence, nucleation exerts total control on crystallographic

orientation of the proto-coccolith ring crystals. This control includes also the determination of handedness (i.e. chirality) in coccolith morphology. No species is known that produces both left- and right-handed coccolith morphotypes (Young *et al.*, 1999). It was originally assumed that handedness was determined by crystal growth (i.e. element growth) as opposed to nucleation (Young *et al.*, 1992). Subsequent in-depth studies of *E. huxleyi* coccoliths, however, revealed that the R-unit *c*-axes were not oriented strictly radially relative to the proto-coccolith rim (as predicted by the original V/R model) but were rotated 20° clockwise (Didymus *et al.*, 1994). Therefore, chirality of the mature coccolith is ultimately a product of certain features of the nucleation site (but can only be expressed through inorganic crystal growth). It is still an essentially open question as to what these specific features of the nucleation site are. It is generally hypothesized that nucleation occurs on an organic substrate, which leads to oriented nucleation on its surface (Klaveness, 1976; Mann and Sparks, 1988). The idea basically is that there is a close geometrical correspondence between Ca²⁺-binding sites of the organic substrate and lattice sites in the crystal faces of the nuclei (Mann and Sparks, 1988). The latter authors also hypothesized that aligned polysaccharides on the template surface could serve that purpose. The so-called template hypothesis was adapted to fit the V/R model in two different versions (Young *et al.*, 1992; Marsh, 1999). However, the actual chemical structure of the organic template remains to be discovered.

Growth

Although controlled nucleation can account for many features of coccolith morphology, the latter is also evidently a product of crystal growth. This growth is essentially inorganic, as inferred from the fact that there is a relationship between morphology and crystallographic directions (Fig. 9.7; Mann and Sparks, 1988; Davis *et al.*, 1995; Henriksen *et al.*, 2003). The expression of handedness, i.e. chirality, in coccoliths, which is determined by chiral nucleation, is only possible if crystal growth

is related to crystallographic orientation. Hence, handedness provides further evidence in support of inorganic crystal growth processes during coccolithogenesis. However, the diversity in coccolith morphology between and even within species (Young *et al.*, 2003, 2009) cannot be explained by controlled nucleation and inorganic crystal growth alone. The discovery of a narrow organic coating, which seems to be a typical feature of coccoliths throughout the group of coccolithophores, led to the notion of a three-dimensional organic matrix that predefined coccolith morphology (Outka and Williams, 1971). This matrix hypothesis seemingly leaves little room for the influence of inorganic growth on coccolith morphology, and what is more, is not entirely consistent with another conspicuous feature of coccolithogenesis, namely the fact that the shape of the CV adopts the shape of the growing coccolith (Outka and Williams, 1971; Klaveness, 1972; Westbroek *et al.*, 1984; Probert *et al.*, 2007). As a consequence, there is only a very small space between the vesicle membrane and the coccolith surface (Langer *et al.*, 2006), rendering a matrix fully grown prior to calcite precipitation rather unlikely. In support of this inference, it was observed that the organic coating was most conspicuous in *P. carterae* (Outka and Williams, 1971) but very thin in many other species (Manton and Leedale, 1969; Klaveness, 1976; Henriksen *et al.*, 2004). For *E. huxleyi*, it was shown that polysaccharides (Fichtinger-Schepman *et al.*, 1981) were located between the vesicle membrane and the growing coccolith (van der Wal *et al.*, 1983b). These polysaccharides (coccolith-associated polysaccharide, CAP) can inhibit crystal growth (Borman *et al.*, 1982) and influence crystal morphology by site-specific attachment to crystallographic steps (Henriksen *et al.*, 2004). In accordance with the above-mentioned observations, the matrix hypothesis was abandoned in favour of the notion that organic polysaccharide coatings functioned as inhibitors of crystal growth (Westbroek *et al.*, 1984; Marsh, 1994). The latter role is a passive one with respect to control of overall coccolith shape, leaving the question open as to how the latter is determined. A first clue pointing towards a

possible answer was provided by the observation that the vesicle membrane was in close contact with the growing coccolith (Outka and Williams, 1971; Klaveness, 1972; Westbroek *et al.*, 1984; Probert *et al.*, 2007). Taken together with the occurrence of a fibrillar structure adjacent to the CV, it was hypothesized that the CV functioned as a dynamic mould for the growing coccolith and that the fibrillar structure exerted the necessary force to shape the CV membrane and therewith the coccolith (Klaveness, 1972, 1976). This hypothesis was subsequently adopted and worked out in greater detail in many studies (e.g. Westbroek *et al.*, 1984; Young *et al.*, 2009). It is now widely believed that the cytoskeleton (Remak, 1843; Freud, 1882; modern textbooks on cell biology) comprises the fibrillar structure described by Klaveness (1972) (e.g. Westbroek *et al.*, 1984; Young *et al.*, 2009). The latter assumption has only recently been tested experimentally (Langer *et al.*, 2010). It was shown that poisoning coccolithophores with microtubule as well as actin inhibitors (microtubules and actin filaments are two major constituents of the cytoskeleton) caused malformations in coccolith morphology (Langer *et al.*, 2010). This observation represents strong evidence in favour of the dynamic mould hypothesis and the role cytoskeleton elements might play.

In summary, coccolith morphogenesis is a sophisticated interplay between template-mediated nucleation, inorganic crystal growth and crystal growth modifications by means of (probably *inter alia*) polysaccharides, CV membrane and cytoskeleton elements.

Outlook

Abiotic CaCO_3 precipitation can be induced easily under laboratory conditions. However, without further control, precipitation proceeds in uncoordinated ways. Nucleation, steady

substrate supply and control of crystal growth are the principal tasks a cell has to accomplish for coccolith formation. Although cell biologists have rough ideas about the nature of the components involved (organic base plates, substrates, ion transporters and macromolecular structures), a detailed understanding of the process is lacking. This section identifies major knowledge gaps that prevent researchers from transferring the principle of coccolith synthesis to biomimetic applications. Also highlighted is how these gaps could be filled using newly available methods.

Clearly, more knowledge is required on the subcellular level, i.e. about the organelle machinery involved in Ca^{2+} supply. For such research, a wide array of (electron-) microscopic techniques is available that not only allow high-resolution morphological studies but also subsequent localization of distinct macromolecules, e.g. by antibody staining or fluorescence hybridization. While these methods require fixation of the cells, more recently published *in situ* methods make use of fluorescent dyes; for example, for staining newly formed CaCO_3 (de Nooijer *et al.*, 2009) or indicating short-term pH changes in the cytosol (Suffrian *et al.*, 2011). However, to influence the material outcome, ultimately one needs a better understanding of the chemical environment and the dynamic processes in the CV. To investigate suborganellar properties, molecular biological innovations, especially the so-called 'functional -omics' (Fig. 9.8; Dettmer *et al.*, 2007; Jammers *et al.*, 2009), have over the last decades been developed to a state at which they can be applied broadly in a cost-effective, high-throughput manner.

Genomics, i.e. gene sequence exploration *latu sensu*, have been applied successfully since the 1980s (e.g. Aiba *et al.*, 1982) and have been extended lately to a vast number of microorganisms. These studies have until now yielded comprehensive insights into the molecular machinery available to cells (Jammers *et al.*, 2009).

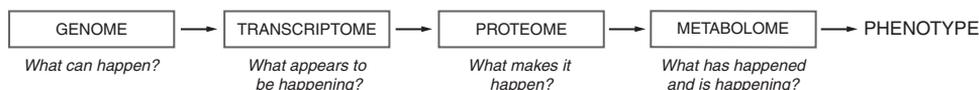


Fig. 9.8. The 'omics' cascade as described by Dettmer *et al.* (2007).

Regarding coccolithophores, the globally dominant species *E. huxleyi* has been the target of intensive sequencing effort: the genomes of 13 strains (DOE Joint Genome Institute, 2012; Read *et al.*, 2013) have been sequenced to identify core genes constituting the *E. huxleyi* 'morphospecies complex'.

Transcriptomics, i.e. studies of gene expression activity, are the first step beyond raw sequence data. These techniques involve the isolation and purification of messenger RNA (mRNA), i.e. complementary transcripts of genetic information that are supposed to be translated into functional proteins. Transcriptomics can be approached with or without knowledge of genetic sequences. If sequences are known, probes can be designed to detect expression levels in single gene assays (e.g. by quantitative polymerase chain reaction, PCR) or whole-transcriptome profilings (e.g. using microarray). If sequences are unknown, so-called 'next-generation sequencing' can be performed on mRNA to yield sequences of active transcripts. The obtained data can then be treated bioinformatically and queried against Internet databases. Already, shortly after the first genetic sequence data on *E. huxleyi* were published (Wahlund *et al.*, 2004), several workers have pioneered transcriptomic exploration of calcification (Dyhrman *et al.*, 2006; Quinn *et al.*, 2006). Calcification-relevant genes were, for instance, shown to follow light–dark fluctuations (Richier *et al.*, 2009). Furthermore, cells utilize a repertoire of HCO_3^- -transporters and carbonic anhydrases (CA) regulated according to external conditions (Mackinder *et al.*, 2011). High-throughput methods, for example microarrays, can yield a holistic, pathway-based understanding of metabolism and its regulation. Until now, such understanding is still lacking, at least with respect to coccolithophores.

Proteomics investigate not only the abundance but also the regulatory modifications of proteins. To this end, protein extracts are usually separated in a two-dimensional gel electrophoresis, based on their overall charge (first dimension) and molecular weight (second dimension). After staining, spots can be analysed using versatile combinations of chromatography and

mass-spectrometry to yield sequences of analysed proteins. These can then be treated bioinformatically and queried against Internet databases. As translation of mRNA into proteins must be highly controlled, cells apply many regulatory instances (e.g. messenger lifetime and translation rate). Therefore, proteomics bridge a crucial gap of knowledge by yielding information on protein–protein interactions and especially post-translational modifications (e.g. phosphorylation or acetylation). Regarding coccolithophores, Jones *et al.* (2011) were the first to apply mass spectrometry-based proteomics in *E. huxleyi*, and they identified a number of abundant key proteins from various biological pathways, including proton pumps putatively involved in calcification.

Metabolomics, the last step of the 'omics' cascade (Dettmer *et al.*, 2007), investigate the metabolite compositions of cells. Analytes range from low molecular weight (<100 Da) compounds like glycerol, to larger components (~1000 Da) like lipids, oligosaccharides and cofactors. The versatile chemical nature of the named compounds already indicates that methods for extraction, separation and detection must be well chosen. If only one known metabolite is to be measured, procedures can be standardized easily with high sensitivity and performed in a high-throughput manner. If, however, many metabolites are to be measured, one has to find a balance between resolution, throughput and analytic complexity: while comparably easy methods can be used to assess classes of compounds (carbohydrates, isoprenoids, polar lipids, etc.) in so-called metabolic fingerprinting (Fiehn, 2002), finer resolution and more analytes require different extraction and separation procedures, or even instrumentation. This is probably one reason why standardized metabolomic techniques are not yet applied broadly in microalgal research (Jamers *et al.*, 2009). However, this developing field promises great advances in cellular physiology: knowledge of the metabolome yields insights not only into pathway activities but also into the signalling cascades that control them. Metabolomics may further help to resolve the constituents of organelles, for example the CV.

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10 Phytotoxic Effects of Metal Nanoparticles in Plants

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Introduction

Nanotechnology can be defined as an intersection of technology involving different fields such as physics, chemistry, electronics, biology and medicine (Basavaraja *et al.*, 2007; Bar *et al.*, 2009a,b; Bankar *et al.*, 2010; Verma *et al.*, 2010; Duran *et al.*, 2011; Badole and Dighe, 2012). It has become a fast-developing industry, posing a substantial impact on economy, society and the environment (Bhattacharya and Mukherjee, 2008; Chen and Schluesener, 2008; Egger *et al.*, 2009; Vasseharan *et al.*, 2010; Yadav and Rai, 2011; Zhang, 2011; Dinesh *et al.*, 2012; Rajesh *et al.*, 2012). Thus, with the advent of newer nanotechnology-based products ranging from health care products to cosmetics and dietary supplements, the exposure of nanoparticles to the environment has become a driving concern (Haverkamp *et al.*, 2006; Harris and Bali, 2007; Haverkamp and Marshall, 2009; Gubbins *et al.*, 2011). Nanomaterials, when exposed to the environment through industrial or domestic waste, tend to disrupt the microflora of soil and water, which in turn may lead to negative effects on the food chain and may also disrupt plant productivity by disrupting nitrogen assimilation and metabolism (Ghosh *et al.*, 2010; Lee *et al.*, 2012).

Nanoparticles are atomic- or molecular-scale particles with at least one dimension between 1 and 100 nm, and they show drastically improved physiochemical properties compared to bulk material (Kasthuri *et al.*, 2008; Esteben-Tejeda *et al.*, 2009; Jha and Prasad, 2010; Satyavathi *et al.*, 2010; Mallikarjuna *et al.*, 2011; Renugadevi and Aswini, 2012). Nanoparticles typically below 100 nm have been harnessed in various fields of science and technology with an increasing number of commercial applications, including tissue engineering, targeted drug delivery, biodetection of pathogens, etc. (Mohanpuria *et al.*, 2008; Gajbhiye *et al.*, 2009; Lara *et al.*, 2010; Prasad and Elumalai, 2011). In recent years, there has been an extensive debate on the risks and benefits of manufactured nanoparticles in the environment (Navarro *et al.*, 2008; Mazumdar *et al.*, 2011a,b). Due to the extensive use of nanoparticles in consumer products, they have made their way into the aquatic, terrestrial and atmospheric environments, where their behaviour and fate are completely unknown. Hence, the organisms that interact with nanoparticles are potentially affected due to their exposure in various environments (Ruffini and Ruberto, 2009; Miralles *et al.*, 2012).

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The aim of this review is to provide a short insight about the toxicity effects of nanoparticles in plants as they interact strongly with the nanoparticles in atmospheric, terrestrial and aquatic environment and are expected to be greatly affected due to this exposition.

Nanotoxicity

Nanotoxicity is an emerging discipline and many researchers are focusing on nanoparticles such as fullerenes, single-walled carbon nanotubes and TiO_2 (titanium dioxide) as test materials to reveal nanotoxicity mechanisms (Sahandi *et al.*, 2011; Slomberg and Schoenfisch, 2012) (Fig. 10.1). However, the mechanism of nanotoxicity still remains unknown, but it depends largely on the chemical composition, chemical structure, particle size and surface area of the nanoparticle (Zhu *et al.*, 2008; Corredor *et al.*, 2009; Lee *et al.*, 2012). Thus, the toxicity of nanoparticles may be related to two different points: (i) chemical toxicity based on the chemical composition and release of nanoparticles; and (ii) stress caused due to the surface, size and/or shape of the nanoparticles. Phytotoxicity is also a constituent of nanotoxicity and involves the use of plants in the study of toxicity (Lin and Xing, 2007; Xingmao *et al.*, 2010; Boonyanitipong *et al.*, 2011).

Phytotoxicity studies in plants generally involve toxicity studies related to seed germination and root elongation (Mazumdar and Ahmed, 2011a). Seed germination is a physiological process beginning with water imbibition by seeds and resulting in the emergence of rootlets. In this process, the seed coat also plays a major role in protecting the embryo from harmful external factors (Lin and Xing, 2007; Stampoulis *et al.*, 2009; Sahandi *et al.*, 2011). Thus, toxic nanoparticles obviously show an inhibitory effect on root growth but germination is not greatly affected. Since roots are the first tissues to confront toxic nanoparticles, the cytotoxic effects appear more in roots rather than shoots (La Rosa *et al.*, 2011; Mazumdar and Ahmed, 2011b; Wang *et al.*, 2012) (Fig. 10.2).

State-of-the-Art

Nanoparticles are released into the environment in different ways, which may be either intentional release or accidental release, but which result in toxic effects to organisms. The toxicity effects of nanoparticles depend mainly on the nature, the size and the concentration of the particles and the exposure time (Miralles *et al.*, 2012).

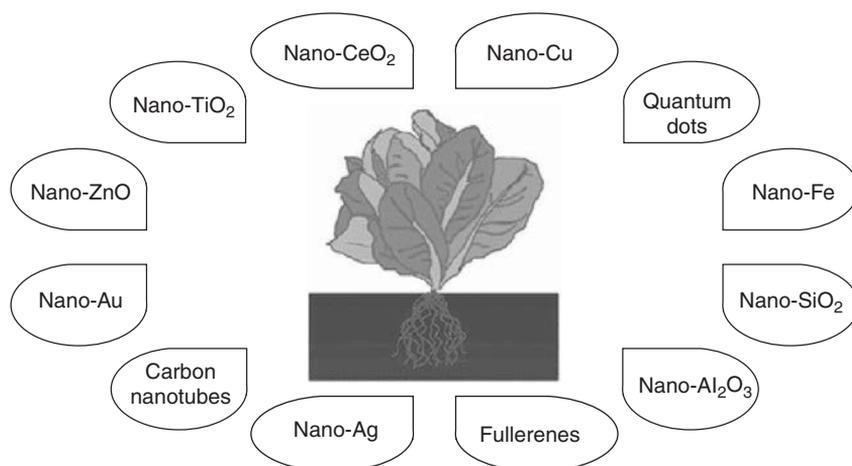


Fig. 10.1. Different types of metal nanoparticles affecting the plant system (Miralles *et al.*, 2012, reprinted with permission from American Chemical Society).

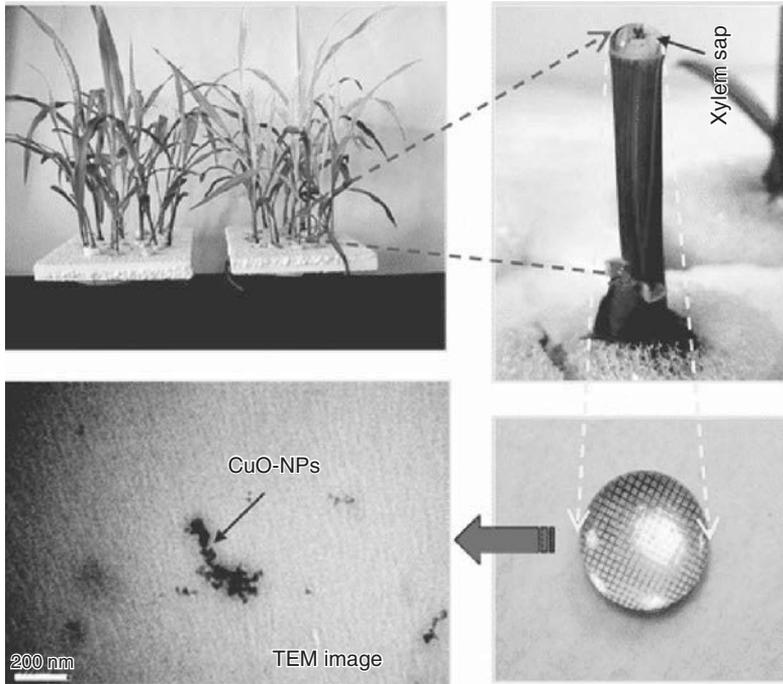


Fig. 10.2. Effect of nanoparticles on plant germination (Wang *et al.*, 2012, reprinted with permission from American Chemical Society).

Zhu *et al.* (2008) reported the uptake of magnetite nanoparticles using pumpkin plants and their subsequent translocation and accumulation in various plant tissues. The authors observed that a significant amount of magnetite nanoparticles were taken up by the pumpkin plants and translocated to various plant tissues. The nanoparticles accumulated mainly near roots as well as leaves. González-Melendi *et al.* (2008) also evaluated the effect of carbon-coated Fe nanoparticles on pumpkin plants. The authors observed the uptake and penetration of nanoparticles in the complete plant and the plant tissues. The nanoparticles were visualized by reflection on a confocal microscope, clusters of nanoparticles were observed in an electron microscope, possibly due to the iron core. The preliminary results of the study depicted the presence of nanoparticles in both the extracellular space and in some cells (Fig. 10.3).

Corredor *et al.* (2009) analysed the penetration and movement of carbon-coated iron nanoparticles in specific parts of plants using application methods like injection and spraying. The penetration of carbon-coated nanoparticles was tracked using correlative microscopy;

after 48 h of injection, isolated nanoparticles in the cytoplasm of individual cells near vascular core were observed. Also, in a spraying application method, isolated nanoparticles were found in the cytoplasm of epidermic cells. While studying the response of cells and tissues to the presence of nanoparticles, the authors observed that the nanoparticles moved away from the application point to the interior stem. Also, dense cytoplasm with starch containing organelles and nanoparticle aggregates were observed, which suggested the movement of nanoparticles from cell to cell.

Ghosh *et al.* (2010) reported the genotoxic effects of TiO₂ nanoparticles on plants. For the study, *Allium cepa* bulbs and *Nicotiana tabacum* seedlings were used as the test plant systems. A root inhibition test on *N. tabacum* depicted significant reduction in root elongation at treatment doses of 6 mM and 8 mM, while for *A. cepa* bulbs, chromosomal aberrations were observed at a concentration of 10 mM. The DNA-damaging effects of TiO₂ nanoparticles were also analysed by DNA laddering. For *A. cepa*, the DNA damage was observed at a concentration of 4 mM, followed by subsequent

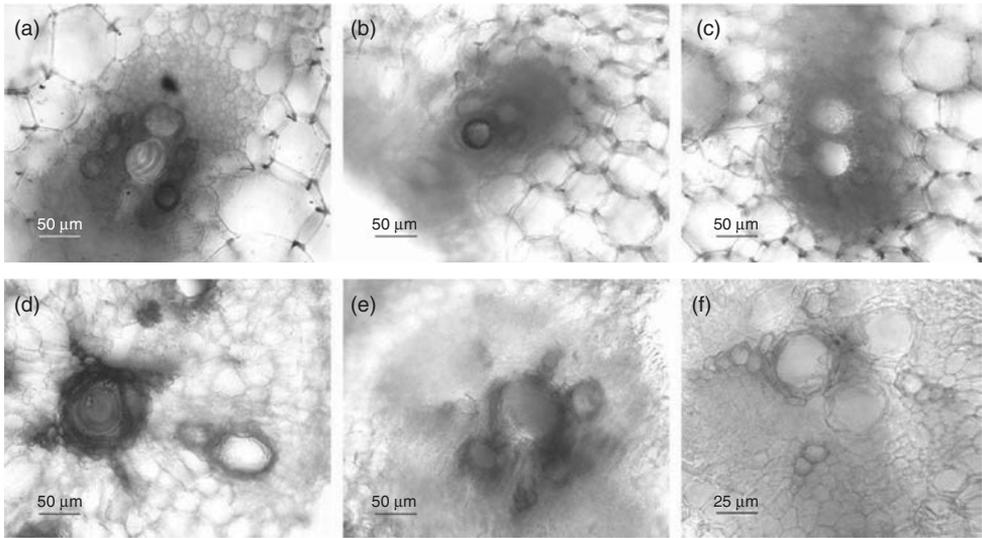


Fig. 10.3. Penetration of carbon-coated Fe nanoparticles in vascular tissues of *Cucurbita pepo* (González-Melendi *et al.*, 2008, reprinted with permission from Oxford University Press). Hand-cut sections of petioles (a–c) and roots (d–f) of pumpkin plants treated with bioferrofluid. (a) Detail of vascular tissues at the application point. Dark coloration indicates accumulation of bioferrofluid. (b) Detail of vascular tissues adjacent to a magnet. Bioferrofluid is concentrated in xylem vessels. (c) Detail of vascular tissues opposite a magnet placement. No bioferrofluid accumulation is observed. (d) Detail of root vascular tissue preceding magnet localization. Bioferrofluid appears distributed through the xylem vessels as a dark staining. (e) As (d) but at the point of the magnet placement. Again strong presence of bioferrofluid is observed. (f) As (d) but after the point of the magnet placement. No bioferrofluid is observed, indicating its flux was mainly stopped at the magnet placement.

reduction in the extent of DNA damage with increasing treatment concentrations. For *N. tabacum*, DNA fragmentation was clearly observed at highest concentration of 10 mM. The atomic force microscopy (AFM) image of *N. tabacum* clearly indicated the presence of 90–100 nm sized spherical nanoparticles and also a few large aggregates. The lipid peroxidation (LPO) assay of *A. cepa* also affirmed the genotoxicity of TiO₂ nanoparticles. The LPO assay of *A. cepa* was measured by determining the amount of malondialdehyde (MDA). In the test, *A. cepa* roots depicted an initial increase in concentration of MDA at a treatment concentration of 4 mM, followed by a decrease in up to the highest concentration of 10 mM.

Boonyanitipong *et al.* (2011) also investigated the toxicity effects of ZnO (zinc oxide) and TiO₂ nanoparticles on germinating seeds of *Oryza sativa*. The authors conducted the phytotoxic study based on three parameters, i.e. seed germination percentage, root length and number of roots. For ZnO nanoparticles toxicity test, nano-ZnO suspensions at various

concentrations (10, 100, 500 and 1000 mg l⁻¹) were tested, and also nano-TiO₂ (100, 500 and 1000 mg l⁻¹) concentrations were tested. For the nano-TiO₂ treatments, 100% germination of seeds was observed. However, with the increase in soaking time, there was a slight decrease in root length. With nano-ZnO, increasing concentrations of nanoparticles greatly affected root length and root growth.

Mazumdar and Ahmad (2011a) studied the phytotoxic effects of silver nanoparticles on *O. sativa*. The authors experimented with the phytotoxic effects of silver nanoparticles on *O. sativa* seedlings by directly exposing them to different concentrations of silver nanoparticle solution. It was observed that a phytotoxicity period of 12 days with 1000 μg ml⁻¹ concentration of silver nanoparticle solution led to adverse effects on the plants. Smaller particles of size 25 nm were also found to be toxic to the plants. The Fourier transform infrared (FTIR) spectra of the treated and untreated plants depicted a shifting of peaks corresponding to the involvement of carboxyl, hydroxyl, amine and carbonyl groups

in binding the silver nanoparticles to the root cell. Transmission electron microscopy (TEM) images also revealed the presence of different sized nanoparticles deposited inside the root cells. It was also observed that the nanoparticles penetrating the root cells damaged the cell wall while entering the small pores of the cell wall.

Sahandi *et al.* (2011) studied the effect of silver nanoparticles and silver nitrate on seed yield and the abscission of borage. Silver nitrate at concentrations of 0, 100, 200 and 300 ppm were used and silver nanoparticles at concentrations of 0, 20, 40 and 60 ppm were sprayed on the seeds of borage plants at growth stage. The results of the above study depicted that increased concentration of silver nitrate from 100 to 300 ppm caused a decrease in seed yield, while an increase in the concentration of silver nanoparticles from 20 to 60 ppm caused an improvement in seed yield. Also, with an increase in the concentration of silver nitrate, the polyphenol contents of the borage seeds increased, but enhanced concentration of silver nanoparticles led to a reduction in the polyphenol content of seeds.

Gubbins *et al.* (2011) reported the phytotoxicity of silver nanoparticles to *Lemna minor*. The authors observed that growth of *L. minor* plants, when exposed to smaller (~20 nm) and larger (~80 nm) sized nanoparticles at low concentrations, was evident, but with an increased exposure to nanoparticles, plant growth became acute. La Rosa *et al.* (2011) also evaluated the toxicity effects of ZnO nanoparticles in desert plants. The authors selected three plants, *Parkinsonia florida* (blue palo verde), *Prosopis juliflora-velutina* (velvet mesquite) and *Salsola tragus* (tumbleweed), to determine the phytotoxicity of ZnO nanoparticles on germination rate and root elongation. X-ray absorption spectroscopy (XAS) was performed for the preliminary determination of nanoparticle biotransformation within plant tissues. The results depicted that the germination of seeds was not affected significantly in all three plant species. While root elongation in blue palo verde was reduced by up to 16% at a ZnO concentration of 4000 mg l⁻¹, root size of tumbleweed was reduced by up to 14% and 16% at ZnO concentrations of 500 and 2000 mg l⁻¹,

and for velvet mesquite, root elongation reduced in all the concentrations.

Lee *et al.* (2012) reported the phytotoxicity effects of silver nanoparticles on *Phaseolus radiatus* and *Sorghum bicolor* in agar and soil media. The growth of the seedlings of both the test species was affected adversely when exposed to silver nanoparticles. In the agar test, both the test plants depicted concentration-dependent growth inhibition. However, in soil studies, the growth of the plants was not affected within the concentrations tested. Slomberg and Schoenfish (2012) studied the phytotoxicity effect of silica nanoparticles on *Arabidopsis thaliana*. The authors evaluated 14, 50 and 200 nm sized silica nanoparticles in concentrations of 250 and 1000 mg l⁻¹. Reduced development and chlorosis was observed in hydroponically grown plants treated with 50 and 200 sized nanoparticles regardless of nanoparticle concentration. However, the nanoparticles were no longer toxic to the plants after the removal of silanol from the surface of silica nanoparticles or by adjusting the pH of the growth medium to 5.8 (Fig. 10.4).

Future Prospects

With the advancement of research on the toxicology of nanomaterials, many researchers are focusing on the toxicity of nanoparticles to plants. However, there are still many unresolved issues and challenges. Future toxicity studies should be directed towards designing the mechanism for phytotoxicity of nanoparticles related to the size distribution of nanoparticles and its effect on phytotoxicity, possible uptake and translocation of nanoparticles in plants. Interactions between nanoparticles and plants also deserve in-depth study. Moreover, the mechanism of uptake of nanoparticles and its interaction with plant tissues and at the cellular level needs to be investigated precisely. Proper attention also needs to be given to designing experiments related to the toxicity of nanoparticles and the interpretation of data to develop improved scientific understanding of the biological effects of nanoparticles.

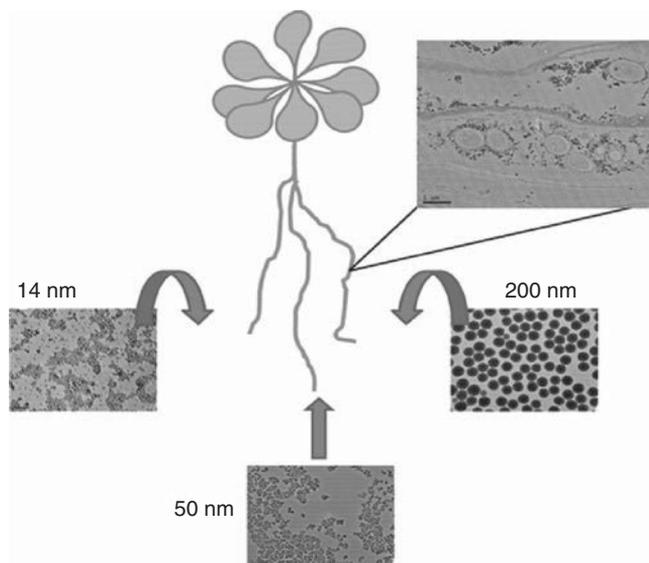


Fig. 10.4. Effect of different sized (14, 50 and 200 nm) silica nanoparticles on growth of *Arabidopsis thaliana* (Slomberg and Schoenfisch, 2012, reprinted with permission from American Chemical Society).

Conclusion

In recent years, remarkable progress has been achieved in the field of nanotechnology. Moreover, the use of nanomaterials in a number of commercial applications has led to the discharge of these materials into the environment. The impact of nanoparticles on plant systems has raised considerable concerns regarding the risk assessment of nanoparticles in the environment. The impact of nanoparticles on plants generally depends on the composition, size and shape of the

nanoparticles and their concentration. Thus, to support the sustainable development of nanotechnology, toxicity focused research considering all the relevant aspects of concern needs to be performed.

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11 Biomineralization, Properties and Applications of Bacterial Magnetosomes

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Introduction

Magnetotactic bacteria are a diverse assemblage of aquatic, Gram-negative, motile prokaryotes that biomineralize intracellular, membrane-bounded crystals of a magnetic iron oxide or iron sulfide mineral. The mineral crystals, together with their associated membrane, are referred to as magnetosomes (Balkwill *et al.*, 1980) and cause the bacteria to orient and swim along the earth's geomagnetic and external magnetic field lines (Frankel, 1984). Salvatore Bellini first described these organisms in 1963 in a publication of the Istituto di Microbiologia of the University of Pavia, Italy (Bellini, 2009a,b). He observed large numbers of bacteria swimming in a consistent, single, northward direction. Bellini referred to these microorganisms as 'batteri magnetosensibili' (magnetosensitive bacteria) and speculated that their unusual behaviour was due to an internal 'magnetic compass'. This internal 'magnetic compass' was later confirmed by Richard P. Blakemore, who independently rediscovered magnetotactic bacteria in 1974 and was the first to observe and describe magnetosomes within the cells of these microorganisms (Blakemore, 1975).

Magnetotactic bacteria are global in distribution and ubiquitous in sediments of freshwater, brackish, marine and hypersaline habitats, as well as in chemically stratified water columns of these environments (Bazylinski and Frankel, 2004). Although not generally considered to be common in extreme environments, moderately thermophilic and alkaliphilic species have recently been found (Lefèvre *et al.*, 2010, 2011a). The presence of magnetotactic bacteria is dependent on the existence of an oxic-anoxic interface (OAI) that represents opposing concentration gradients of oxygen from the surface and reducing compounds (usually reduced sulfur species) in sediments or water columns. The highest numbers of magnetotactic bacteria are usually found at or just below the OAI of sediments or chemically stratified water columns (Moskowitz *et al.*, 2008).

The great diversity of magnetotactic bacteria is demonstrated by their large number of cell morphologies, which include coccoid-to-ovoid cells, rods, vibrios and spirilla of various sizes. The magnetotactic bacteria even include a group of multicellular forms referred to as multicellular magnetotactic prokaryotes (MMPs) (Abreu *et al.*, 2007).

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The phylogenetic diversity of cultured and uncultured magnetotactic bacteria based on their 16S rRNA gene sequences is also extensive. To date, representatives of the magnetotactic bacteria are associated phylogenetically with five major lineages within the Domain Bacteria, with three within the Proteobacteria. Although most known cultured and uncultured magnetotactic bacteria belong to the Alpha-, Gamma- and Deltaproteobacteria classes of the Proteobacteria phylum, several uncultured species are affiliated with the Nitrospirae phylum (Amann *et al.*, 2007; Lefèvre *et al.*, 2012) and one to the candidate division OP3, part of the Planctomycetes-Verrucomicrobia-Chlamydiae (PVC) superphylum (Kolinko *et al.*, 2012). None have yet been found to belong to the Archaea.

The physiology of known magnetotactic bacteria, including that determined experimentally with cultured strains and that inferred from uncultured types, is also quite diverse. Different types of metabolism found in the magnetotactic bacteria include chemoorganoheterotrophy, chemolithoautotrophy and even chemoorganolithoautotrophy (Bazylynski and Williams, 2007). Many species metabolize sulfur compounds in some way, i.e. many are sulfide-oxidizing or sulfate-reducing bacteria (Sakaguchi *et al.*, 2002; Bazylynski and Williams, 2007; Lefèvre *et al.*, 2011a,b, 2012). In general, most are microaerophilic, facultatively anaerobic or obligately anaerobic (Bazylynski and Williams, 2007). Many physiological studies of magnetotactic bacteria demonstrate their great potential in the cycling of key elements including iron, sulfur, nitrogen and carbon in natural habitats (Bazylynski and Williams, 2007).

This chapter briefly reviews many of the properties of magnetotactic bacteria and their magnetosomes that make them useful in so many biotechnological applications, as well as describing many of these applications.

Structure and Composition of Bacterial Magnetosomes

Magnetosomes have an organic membranous and an inorganic mineral component. The mineral component is composed of tens-of-nanometre-sized crystals of an iron oxide and/or

an iron sulfide. Iron oxide-type magnetosome crystals consist solely of magnetite, Fe_3O_4 , while magnetosomes of iron-sulfide producing magnetotactic bacteria contain greigite, Fe_3S_4 , and several non-magnetic iron sulfide precursors to greigite (Pósfai *et al.*, 1998a,b). The mineral composition of the magnetosome crystals in magnetotactic bacteria is species specific, indicating that it is under genetic control; some cultured magnetite-producing species do not synthesize greigite, even in the presence of hydrogen sulfide (Meldrum *et al.*, 1993a,b). Some magnetotactic bacteria, including the MMP and a group of large, slow-swimming rods affiliated phylogenetically with the Deltaproteobacteria (Lefèvre *et al.*, 2011b), produce both mineral crystals in separate magnetosomes, although they are aligned within the same chain or chains in the cell (Bazylynski *et al.*, 1993b, 1995). Environmental conditions, that is, whether the cells are under oxic or anoxic conditions or if oxygen and/or sulfide are present, appear to affect what and how much of each mineral is biomineralized in these organisms (Bazylynski *et al.*, 1991, 1995; Lefèvre *et al.*, 2011b).

Most known freshwater magnetotactic bacteria biomineralize magnetite in their magnetosomes. Others, especially those from marine, estuarine and saltmarsh species, produce iron sulfide-type magnetosomes containing crystals consisting primarily of the magnetic iron sulfide, greigite, Fe_3S_4 (Heywood *et al.*, 1990; Mann *et al.*, 1990b; Pósfai *et al.*, 1998a,b), although these organisms have recently been found in non-marine environments (Lefèvre *et al.*, 2011b). Reports of non-magnetic iron pyrite (FeS_2) (Mann *et al.*, 1990b) and magnetic pyrrhotite (Fe_7S_8) (Farina *et al.*, 1990) probably represent misidentifications of additional iron sulfide species occasionally observed with greigite in the cells (Pósfai *et al.*, 1998a,b) that include mackinawite (tetragonal FeS) and a possible sphalerite-type cubic FeS, both possible mineral precursors to greigite (Pósfai *et al.*, 1998a,b). Based on transmission electron microscopy, electron diffraction and known iron sulfide chemistry (Berner, 1967, 1984), the reaction scheme for greigite formation in the magnetotactic bacteria appears to be: cubic FeS \rightarrow mackinawite (tetragonal FeS) \rightarrow greigite (Fe_3S_4) (Pósfai *et al.*, 1998a,b).

Currently recognized greigite-producing magnetotactic bacteria include the MMP (Rodgers *et al.*, 1990, 1991; DeLong *et al.*, 1993; Abreu *et al.*, 2007) and a variety of large, rod-shaped, anaerobic, sulfate-reducing bacteria (Heywood *et al.*, 1990; Bazylinski *et al.*, 1993a,b; Lefèvre *et al.*, 2011b) (Fig. 11.1d).

The specificity for iron in magnetosome mineral crystals is very high. Despite this, there are several reports of the presence of other transition metal ions in magnetite and greigite magnetosome crystals in both cultured and uncultured magnetotactic bacteria. Trace amounts of titanium were found in magnetite particles of an uncultured freshwater magnetotactic coccus collected from a wastewater treatment pond (Towe and Moench, 1981). The incorporation of small amounts of cobalt in surface layers of magnetosome magnetite crystals was demonstrated in three *Magnetospirillum* species (Staniland *et al.*, 2008). Uncultured magnetotactic bacteria in microcosms were exposed to $MnCl_2$ and up to 2.8% atomic manganese in ultrathin sectioned cells, and magnetosomes were detected via localized energy dispersive X-ray analysis (Keim *et al.*, 2009). Significant amounts of copper

were found in greigite magnetosome crystals of some uncultured MMPs collected from a salt marsh in California (Bazylinski *et al.*, 1993a).

Biologically produced magnetosome mineral particles display a range of well-defined morphologies (Fig. 11.1), which are, however, extraordinarily consistent in cells of a single bacterial species or strain (Bazylinski *et al.*, 1994). Three general morphologies of magnetite or greigite crystals are known in magnetotactic bacteria (Mann *et al.*, 1990a; Bazylinski *et al.*, 1994), including: (i) roughly cuboidal (cuboctahedral) (Balkwill *et al.*, 1980; Mann *et al.*, 1984a,b; Heywood *et al.*, 1990) (Fig. 11.1a); (ii) parallelepipedal or elongated-prismatic (rectangular in the horizontal plane of projection) (Moench and Konetzka, 1978; Towe and Moench, 1981; Bazylinski *et al.*, 1988; Moench, 1988; Heywood *et al.*, 1990) (Fig. 11.1b); and (iii) bullet-shaped (also described as anisotropic) (Mann *et al.*, 1987a,b; Thornhill *et al.*, 1994; Pósfai *et al.*, 1998a,b; Lefèvre *et al.*, 2011c) (Fig. 11.1c). The morphological variation of bullet-shaped magnetite particles (Fig. 11.1c) is more complex than that of the other types. These crystals can be further subdivided into those with one pointed

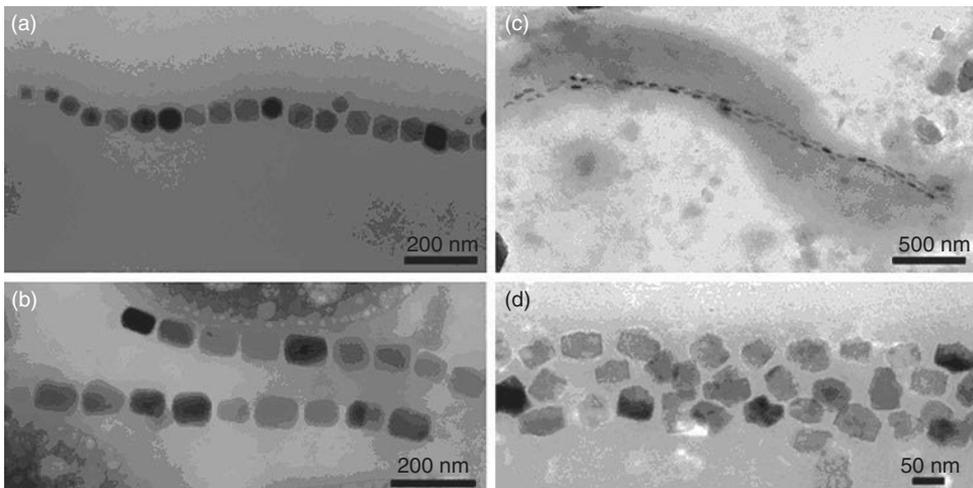


Fig. 11.1. Transmission electron micrograph (TEM) images of magnetotactic bacteria and magnetosomes from environmental samples. (a) High-magnification TEM image of cuboctahedral magnetite crystals; (b) high-magnification TEM image of elongated pseudo-prismatic magnetite crystals; (c) TEM image of two chains of bullet- or tooth-shaped magnetite crystals; and (d) high-magnification TEM image of greigite crystals.

end and one flat end (flat-top shape; fts) and those with two pointed ends (double-triangular shape; dts), which appear as two isosceles triangles sharing a common base (Lefèvre *et al.*, 2011c). In the dts crystals, both projected triangles appear to have the same width, although one triangle is longer than the other in mature crystals. In general, high-resolution transmission electron microscopy and selected area electron diffraction studies show clearly that magnetite particles within magnetotactic bacteria are of relatively high structural perfection and have been used to determine their idealized crystal morphologies (Matsuda *et al.*, 1983; Mann *et al.*, 1984a,b, 1987a,b; Meldrum *et al.*, 1993a,b).

Whereas the equilibrium, cuboctahedral form of magnetite occurs in inorganically formed magnetites (Palache *et al.*, 1944), the presence of elongated-prismatic and elongated-anisotropic habits in magnetosome crystals implies anisotropic growth conditions, e.g. a temperature gradient, a chemical concentration gradient or an anisotropic ion flux (Mann and Frankel, 1989). These latter crystals grow at a different rate in one direction than in another, that is, growth of these crystals is not centrosymmetric. This aspect of magnetosome particle morphology has been used to distinguish magnetosome magnetite ('magnetofossils', discussed in a later section) from detrital or magnetite produced by biologically induced mineralization (by anaerobic dissimilatory iron-reducing bacteria), determined using electron microscopy of magnetic extracts from sediments (e.g. Petersen *et al.*, 1986; Stolz *et al.*, 1986, 1990; Chang and Kirschvink, 1989; Chang *et al.*, 1989; Stolz, 1993).

The magnetosome phospholipid bilayer membrane is the structure that is the locus of control of the biom mineralization of the magnetosome crystals in magnetotactic bacteria (Schüler, 2008). This membrane is composed of fatty acids, glycolipids, sulfolipids, phospholipids and proteins (Gorby *et al.*, 1988; Grünberg *et al.*, 2004). There is both chemical and physical evidence that the magnetosome membrane is derived from the cytoplasmic membrane (Komeili *et al.*, 2006; Tanaka *et al.*, 2006). The magnetosome membrane contains proteins that are found in this structure only and not in any other cell fraction (Bazylinski

and Frankel, 2004; Schüler, 2008). To date, 30–40 proteins have been isolated from the magnetosome membrane of *magnetospirillum* species (e.g. Grünberg *et al.*, 2004). These proteins are known as the Mam (magnetosome membrane) or Mms (magnetic particle membrane specific) proteins and are encoded by their respective *mam* and *mms* genes. In many magnetotactic bacteria, the *mam* and *mms* genes have been found to be clustered as several operons within a so-called magnetosome genomic island (Murat *et al.*, 2010; Lohße *et al.*, 2011). Identifying the function of the magnetosome membrane proteins is the key to understanding magnetosome biom mineralization. These putative functions include magnetosome vesicle/invagination formation, iron uptake into the cell and/or the magnetosome vesicle/invagination, crystal nucleation and biom mineralization of magnetite, and the arrangement of the magnetosomes in the chain motif.

Magnetic and Mineral Properties of Magnetosomes

The size of magnetosome crystals displays a very narrow size range, from about 35 to 120 nm, and size distribution is not log normal and has a sharp cut-off at the high end (Devouard *et al.*, 1998; Frankel *et al.*, 1998). This narrow size range is directly related to the magnetic properties of the particles, as magnetite and greigite particles in this size range are stable single magnetic domains (Butler and Banerjee, 1975; Ricci and Kirschvink, 1992; Frankel and Moskowitz, 2003). Smaller, superparamagnetic particles are not useful to the bacteria, as these crystals do not have stable, remanent magnetization at ambient temperature and would not contribute to a permanent cellular magnetic dipole. Domain walls form in larger particles, in turn forming multiple domains and thereby also reducing the remanent magnetization. However, exceptionally large magnetite magnetosomes observed in some uncultured cocci have dimensions well above the theoretically determined size limits of single-domain magnetite (Farina *et al.*, 1994; Spring

et al., 1998; McCartney *et al.*, 2001; Lins *et al.*, 2005). None the less, as evidenced by magnetic holography in the transmission electron microscope, even these large crystals behave as single magnetic domains when present in the cell in a chain configuration, where they are magnetized by neighbouring crystals (McCartney *et al.*, 2001).

In cells of almost all known magnetotactic bacteria, magnetosomes are usually positioned as one or more chains that traverse the long axis of the cell (Bazylinski and Moskowitz, 1997; Frankel and Moskowitz, 2003) (Fig. 11.1). In this chain arrangement, the magnetic dipole moment of the cell is at its maximum because magnetic interactions between the magnetosomes cause each magnetosome moment to orient spontaneously, parallel to the others along the chain axis by minimizing the magnetostatic energy (Frankel, 1984; Frankel and Moskowitz, 2003). The total magnetic dipole moment of the chain and the cell is thus the algebraic sum of the moments of the individual crystals in the chain. This has been confirmed repeatedly using a number of techniques, including direct magnetic measurements (Penninga *et al.*, 1995), magnetic force microscopy (Proksch *et al.*, 1995; Suzuki *et al.*, 1998) and electron holography (Dunin-Borkowski *et al.*, 1998, 2001). The significance of this is that the chain of magnetosomes in a magnetotactic bacterium functions like a single magnetic dipole rather than as a collection of individual dipoles and causes the cell to behave similarly. Magnetotaxis results from this magnetic dipole imparted by the chain of magnetosomes, which cause the cell to align passively along geomagnetic field lines while it swims (Frankel, 1984; Frankel and Moskowitz, 2003). Living cells are neither attracted nor pulled toward either geomagnetic pole, and dead cells, like living cells, also align along geomagnetic field lines but do not swim. Magnetosomes must be anchored in place within the cell to function as described in the next section. If they were free-floating in the cell, they would probably clump, causing a significant reduction in the cellular dipole moment. This is accomplished by dedicated cytoskeletal structures and close attachment to the inner cell membrane (Komeili *et al.*, 2006; Scheffel *et al.*,

2006). In addition to biological control, assembly of magnetosome chains involves magnetostatic interaction, and magnetic 'docking' to stable magnetic single-domain particles is a key mechanism for building the functional cellular magnetic dipole (Faivre *et al.*, 2010).

Function and Mechanism of Magnetotaxis

Due to their cellular magnetic dipole moment, cells of magnetotactic bacteria experience a torque in magnetic fields, causing the cells to align passively along magnetic field lines as they swim, the definition of magnetotaxis (Blakemore, 1975; Frankel and Blakemore, 1980). Although several models and ideas have been proposed on how this benefits magnetotactic bacteria in nature, in the most accepted model, magnetotaxis aids cells to be more efficient at chemotaxis, i.e. at finding an optimal position in vertical chemical and redox gradients (Frankel *et al.*, 1997, 2007).

In the original model (Blakemore, 1975, 1982), it was assumed that all magnetotactic bacteria were microaerophilic and had a polar preference in their swimming direction. Magnetotaxis was thought to help guide cells to deeper, less oxygenated regions of aquatic habitats by swimming downward. Once cells reached their preferred microhabitat (e.g. surface sediments), they would stop swimming and attach to sediment particles until environmental conditions such as oxygen concentration changed. This idea was supported strongly by the observation that magnetotactic bacteria were primarily north-seeking in the northern hemisphere and south-seeking in the southern hemisphere. Cells of either polarity swim downward in their respective hemispheres, along the earth's inclined geomagnetic field lines (Blakemore *et al.*, 1980). However, this model did not explain a number of later important observations, most involving the presence of polar magnetotactic bacteria at the OAI in the water columns of natural habitats, as well as in culture medium (Frankel *et al.*, 1997, 2007; Simmons *et al.*, 2004; Moskowitz *et al.*, 2008). According to the original model, cells of polar magnetotactic bacteria in these situations should continue to

swim downward until they reach the sediment or the bottom of the culture tube.

Two forms of magnetotaxis are now recognized (Frankel *et al.*, 1997), which are called more appropriately magneto-aerotaxis because the magnetotactic bacteria examined in this study were not only magnetotactic but also strongly aerotactic as microaerophiles. In the first form, axial magneto-aerotaxis, cells lack a polar preference in their swimming direction and use the magnetic field as an axis while swimming in both directions under oxic conditions. In contrast, in polar magneto-aerotaxis, cells have a polar preference in their swimming direction under oxic conditions (e.g. north-seeking cells are north seeking in the northern hemisphere under oxic conditions). Both types of cells form microaerophilic bands of cells in semi-solid oxygen gradient media and can swim in the opposite direction. This reversal in swimming direction appears to be based on chemical/redox taxis, although they appear to use a different taxis mechanism (Frankel *et al.*, 1997, 2007). Regardless of whether cells use axial or polar magneto-aerotaxis, once cells are aligned along the earth's inclined magnetic field lines, their search for an optimal position in a vertical chemical/redox gradient is reduced from a three-dimensional search (for non-magnetotactic bacteria such as *Escherichia coli*) to one of a single dimension, thereby increasing the efficiency of chemotaxis (in this case aerotaxis) (Frankel *et al.*, 1997, 2007).

Although this model of magneto-aerotaxis appears to fit well for many magnetotactic bacteria, particularly microaerophiles that require sulfide as an electron donor and oxygen as a terminal electron acceptor (the OAI is the only location for a bacterium to access both compounds in many environments), it does not explain the behaviour of other magnetotactic bacteria. For example, it does not explain the presence of large numbers of south-seeking polar magnetotactic bacteria in natural habitats of the northern hemisphere (Simmons *et al.*, 2006; Shapiro *et al.*, 2011). In the model of polar magnetotaxis described here, these organisms presumably would continue to swim southward/upward towards oxygen-rich surface waters in the northern hemisphere and die, and thus be

selected against. In addition, why do cells need magnetosomes for navigation when there are many obligately microaerophilic, non-magnetotactic bacteria at the OAI? It seems logical that there are physiological reasons for magnetosome biomineralization (e.g. energy conservation through iron reduction and/or iron oxidation (Guerin and Blakemore, 1992); detoxification of free iron ions in the cell; decomposition of toxic oxygen radicals produced during respiration, such as hydrogen peroxide (Blakemore, 1982; Guo *et al.*, 2012a)) but, to date, any convincing physiological link has yet to be found.

Biomining of Magnetosomes

The species-specific crystal morphologies and the consistent narrow size range of intracellular magnetosome particles (Devouard *et al.*, 1998) represent typical characteristics of a biologically controlled mineralization and are clear indications that the magnetotactic bacteria exert a high degree of control over the biomineralization processes involved in magnetosome synthesis (Bazyliński and Frankel, 2003). The biomineralization process of the bacterial magnetosome is a complex process composed of several steps that occur simultaneously within the cell and has only been studied thus far in *Magnetospirillum* species that biomineralize cuboctahedral crystals of magnetite.

The first of these involves invagination of the cell membrane and the possible formation of a magnetosome membrane vesicle through the pinching off of the cell membrane, an important question that currently remains unresolved (Komeili *et al.*, 2006; Katzmann *et al.*, 2010). A number of Mam proteins appear to play roles in magnetosome invagination/vesicle formation. For example, MamB, MamI, MamL and MamQ are essential for the formation of the magnetosome membrane in *Magnetospirillum magneticum* (Murat *et al.*, 2010). Some may not only be involved in the formation of the magnetosome membrane but also in the bending and shaping of the structure (Komeili, 2012). The MamA protein, whose gene is present in the genomes of all magnetotactic bacteria

examined (Okuda *et al.*, 1996; Grünberg *et al.*, 2001; Komeili *et al.*, 2004; Matsunaga *et al.*, 2005; Nakazawa *et al.*, 2009; Schübbe *et al.*, 2009; Abreu *et al.*, 2011; Lefèvre *et al.*, 2011b), displays high amino acid sequence similarity to proteins of the tetratricopeptide repeat (TPR) protein family (Okuda *et al.*, 1996). MamA may be important in protein–protein interactions that might occur in magnetosome synthesis and construction of the magnetosome chain (Okuda *et al.*, 1996; Okuda and Fukumori, 2001). Because multiple copies of TPRs are known to form scaffolds within proteins to mediate protein–protein interactions and to coordinate the assembly of proteins into multi-subunit complexes (Ponting and Phillips, 1996), MamA might serve as a scaffolding protein to coordinate the assembly of oligomeric protein complexes (Zeytuni *et al.*, 2011).

Iron uptake by the cell is required for magnetosome synthesis and is surely occurring continually as long as it is available. Cells of cultured magnetotactic bacteria are extremely proficient at iron uptake, as they have been shown to consist of greater than 3% iron on a dry weight basis, a value several orders of magnitude over non-magnetotactic bacterial species (Blakemore, 1982; Schüler and Baeuerlein, 1998; Heyen and Schüler, 2003). In addition, iron uptake for magnetite synthesis appears to occur relatively quickly (Schüler and Baeuerlein, 1997, 1998; Heyen and Schüler, 2003). Both Fe(II) and Fe(III) are taken up by cells of magnetotactic bacteria for magnetite synthesis, although not necessarily simultaneously (Schüler and Baeuerlein, 1998; Suzuki *et al.*, 2006; Matsunaga and Arakaki, 2007). How iron is taken up by magnetotactic bacteria for magnetosome synthesis is unknown, but it would seem that there are multiple mechanisms for this in a single bacterium, as have been found in other non-magnetotactic bacteria (Paoletti and Blakemore, 1986; Calugay *et al.*, 2003; Dubbels *et al.*, 2004). While several studies implicate siderophores, low molecular weight iron chelators (Neilands, 1984, 1995), in iron uptake by some magnetotactic bacteria (Paoletti and Blakemore, 1986; Calugay *et al.*, 2003; Dubbels *et al.*, 2004), a specific role in magnetosome synthesis for these compounds

has not yet been established. In one magnetotactic bacterium, siderophores were formed only when iron was depleted in the growth medium (Calugay *et al.*, 2003) and thus were probably not involved in magnetite synthesis. Results from other studies implicate a copper-dependent iron uptake system in iron uptake by *Magnetovibrio blakemorei* (Dubbels *et al.*, 2004) and an accessory role for the ferrous iron transport protein B gene (*feoB1*) in magnetosome formation in *Magnetospirillum gryphiswaldense* (Rong *et al.*, 2008).

Specific magnetosome membrane proteins appear to be involved in transporting and confining iron in the magnetosome invagination/vesicle. While the MagA protein was the first protein thought to be important in iron transport to the magnetosome in *M. magneticum* (Nakamura *et al.*, 1995a), more recent evidence shows that MagA may not to be involved in magnetosome synthesis (Uebe *et al.*, 2012). The homologous proteins MamB and MamM (and MamV in some magnetotactic bacteria) are cation-diffusion-facilitator-transporters that have been shown to facilitate the influx or efflux of cadmium, iron and zinc (Paulsen *et al.*, 1997; Grass *et al.*, 2005; Haney *et al.*, 2005) and are currently thought to transport iron to the magnetosome membrane vesicle/invagination (Grünberg *et al.*, 2001). The genes for these proteins are present in the genomes of all magnetotactic bacteria examined thus far, while the gene for the third homologous protein, MamV, is only present in *Magnetospirillum magnetotacticum* and *M. magneticum* (Grünberg *et al.*, 2001; Matsunaga *et al.*, 2005; Nakazawa *et al.*, 2009; Schübbe *et al.*, 2009; Abreu *et al.*, 2011). Suzuki *et al.* (2006) showed that, during magnetite biomineralization, genes encoding ferrous iron transporter proteins were upregulated, whereas genes encoding ferric iron transporter proteins were downregulated in magnetotactic bacteria. Interestingly, there was no change in the expression patterns of the cation-diffusion-facilitator-transporter proteins, MamB and MamM. Uebe *et al.* (2011) recently demonstrated that MamB and MamM formed heterodimers and interacted with other magnetosome proteins, suggesting that magnetosome formation was a complex process that likely involved the coordinated interactions

of many different proteins and genes. It has been shown in *M. gryphiswaldense* that the Fur (ferric uptake regulator) protein is involved in global iron homeostasis, probably by balancing the competing requirements for iron in essential biochemical reactions (e.g. haem synthesis) and magnetite biomineralization (Uebe *et al.*, 2010; Qi *et al.*, 2012).

Finally, there is nucleation, controlled biomineralization and maturation of the magnetite crystal within the magnetosome invagination/vesicle. It was once suggested that magnetite precipitation occurred through the reduction of hydrated ferric oxide(s) (Frankel *et al.*, 1979, 1983; Schüler and Baeuerlein, 1998), although this now seems unlikely because cells of *M. gryphiswaldense* shifted from iron-limited to iron-sufficient conditions showed no delay in magnetite production (Heyen and Schüler, 2003). This finding implies that there are no mineral precursors to magnetite in this organism (Heyen and Schüler, 2003; Faivre *et al.*, 2007) during biomineralization or that they are unstable and transform to magnetite extremely quickly. A ferritin-like protein was found in the membrane fraction of cells of *M. gryphiswaldense* during biomineralization (Faivre *et al.*, 2007). Ferritins are ubiquitous intracellular proteins that store iron and release it in a controlled fashion when needed by the cell (Theil, 1987). Faivre *et al.* (2007) postulated that iron contained within the ferritin-like protein co-precipitated soluble ferrous iron to form magnetite crystals in the cell membrane, which were then transported into the magnetosome invagination/vesicle.

Specific magnetosome proteins may be involved in magnetosome magnetite crystal maturation. The cytosolic protein, FtsZ, is a ubiquitous tubulin-like protein in bacteria, which polymerizes into an oligomeric structure that forms the initial ring at mid-cell and has an essential role in cytokinesis (Errington *et al.*, 2003). A similar gene, called *ftsZ*-like, is present in the magnetosome gene island of *Magnetospirillum*. Like FtsZ, the FtsZ-like protein is able to form filaments *in vitro* that are GTP dependent (Ding *et al.*, 2010). When the *ftsZ*-like gene was deleted in *M. gryphiswaldense*, cell division was unaffected but the magnetite crystals were significantly

smaller than those of the wild type, resulting in non-magnetotactic cells (Ding *et al.*, 2010). In a similar experiment with *M. magneticum*, there was no detectable change in the magnetite crystals (Murat *et al.*, 2010).

MamC (also known as Mms12 and Mms13), MamD (Mms7), MamF, MamG (Mms5) and Mms6 are thought to be important in magnetite crystal maturation and in controlling the shape of the magnetite crystal (Arakaki *et al.*, 2003; Grünberg *et al.*, 2004; Fukuda *et al.*, 2006; Taoka *et al.*, 2006). MamC, MamD and MamG appear to be the most abundant proteins in the magnetosome membrane and make up for about 35% of all proteins present in this structure (Scheffel *et al.*, 2008). Mms6 is an amphiphilic protein consisting of an N-terminal leucine-glycine-rich hydrophobic region and a C-terminal hydrophilic region containing many acidic amino acids (Arakaki *et al.*, 2003; Prozorov *et al.*, 2007). It has been shown to bind iron and control the morphology of magnetite crystals precipitated *in vitro* (Arakaki *et al.*, 2003; Prozorov *et al.*, 2007).

Applications of Magnetotactic Bacteria and Magnetosomes

Cells of magnetite-producing magnetotactic bacteria and their unique membrane-bounded magnetosomes and magnetosome crystals have novel magnetic, physical and optical properties that have been exploited in a variety of scientific, commercial and other applications. Magnetite magnetosomes have proven to be superior to chemically produced magnetite nanoparticles in the same types of applications, due partially to the presence of the magnetosome membrane. While the number of applications and patents involving magnetotactic bacteria appears to be ever increasing, a major problem is the mass culture of these organisms and the subsequent efficient harvesting of magnetosomes. However, there has been significant progress in this area in the last decade.

Considering that the amount of magnetic materials from magnetotactic bacteria required for most applications is relatively high, obtaining higher yields of magnetotactic

bacterial cells and magnetosomes from cultures poses a significant challenge. In order to produce enough cells, magnetosomes and magnetite crystals for these applications, cells must be grown in mass culture, where conditions for growth and magnetite synthesis must be optimized (Fig. 11.2). In almost all cases, the focus of these studies always involves modification of growth media and conditions under which cultures are incubated. *Magnetospirillum* species have been the only magnetotactic bacteria used in published studies. In some cases, it is difficult to compare yields directly, as some studies focus on magnetosomes and it is unclear whether magnetosome membranes are included in the yield values.

Early studies involving the mass culture of magnetotactic bacteria used two strategies: scaling up of batch cultures or growing cells in a fermenter. Unfortunately, almost all studies involved the use of *Magnetospirillum* species, which all biomineralized cuboctahedral crystals of magnetite (Bazylinski and Frankel, 2004). The problem with this is that many magnetotactic bacteria synthesize elongated prismatic crystals, which might be more advantageous than cuboctahedra in specific applications but have not been tested because of the lack of growth experiments on these latter organisms.

In an early study, cells of *M. magneticum* were grown in a 1000 l fermenter, resulting in a magnetosome yield of 2.6 mg l⁻¹ of culture (Matsunaga *et al.*, 1990). Culture optimization experiments were later conducted in fed-batch cultures of the same organism but did not result in a higher yield of cells or magnetosomes (Matsunaga *et al.*, 1996, 2000a). A recombinant *M. magneticum* strain harbouring the plasmid pEML was grown in a pH-regulated fed-batch culture system where the addition of fresh nutrients was feedback controlled as a function of the pH of the culture (Yang *et al.*, 2001a). Here, the magnetosome yield was maximized by adjusting the rate of addition of the major iron source, ferric quinate at 15.4 mg min⁻¹, resulting in a magnetosome yield of 7.5 mg l⁻¹. Different iron sources and the addition of various nutrients and chemical reducing agents (e.g. L-cysteine, yeast extract, polypeptone) were also later shown to have

significant effects on magnetosome yield by *M. magneticum* grown in fed-batch culture (Yang *et al.*, 2001b).

Improved, more precise control over the growth of *Magnetospirillum* species was achieved using an oxygen-controlled fermenter (Heyen and Schüler, 2003; Lang and Schüler, 2006). Three species were grown using this method, *M. gryphiswaldense*, *M. magnetotacticum* and *M. magneticum*, and magnetite yields of 6.3, 3.3 and 2.0 mg l⁻¹ day⁻¹ were obtained from each species, respectively (Heyen and Schüler, 2003). Using a similar type of fermenter, except that dissolved oxygen was controlled to an optimal level using the change of cell growth rate rather than from a direct measurement from the sensitive oxygen electrode, Sun *et al.* (2008) obtained a cell density of OD₅₆₅ of 7.24 for *M. gryphiswaldense* after 60 h of culture. The cell yield (dry weight) was 2.17 g l⁻¹ and the yield of magnetosomes (dry weight) was 41.7 mg l⁻¹ and 16.7 mg l⁻¹ day⁻¹. By decreasing the amount of carbon and electron source (lactate) in the same fermenter, Liu *et al.* (2010) later reported growth and magnetosome yields of OD_{565nm} of 12 and 55.49 mg l⁻¹ day⁻¹, respectively, after 36 h of culture, again using *M. gryphiswaldense*. Recently, it was shown that iron-chelating agents such as rhodamine B and EDTA stimulated growth and magnetosome production (Alphandéry *et al.*, 2012a).

There are a number of reports describing the purification of magnetite magnetosomes from magnetotactic bacteria, all involving magnetic separation techniques (Gorby *et al.*, 1988; Bazylinski *et al.*, 1994). Washing magnetosomes tends to be a relatively tedious task, although there is one report of a more rapid procedure for magnetosome purification (Guo *et al.*, 2011).

Living and dead magnetotactic bacterial cells have been shown to be useful in numerous medical, magnetic and environmental applications. Magnetotactic bacterial cells are excellent means of cell sorting and separation because they can be manipulated easily using magnetic techniques that exploit their magnetic behaviour. Because of this, the use of magnetotactic bacteria in the uptake and remediation of heavy metals and radionucleotides from wastewater is possible

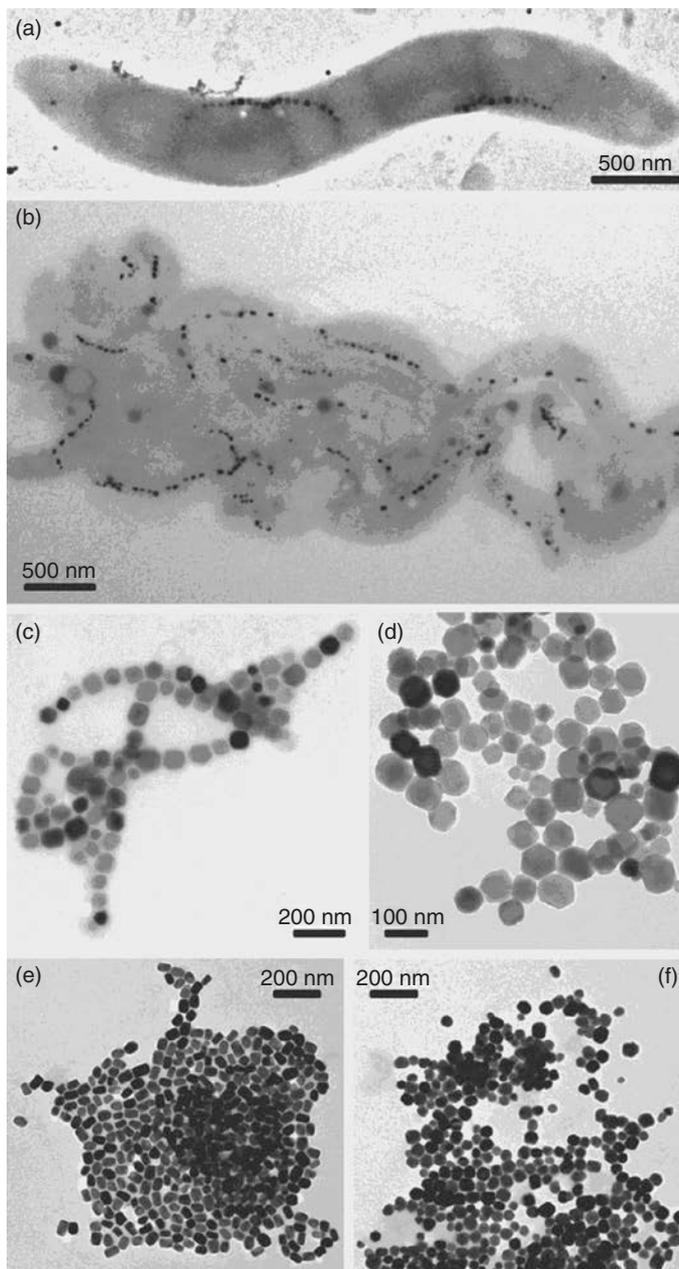


Fig. 11.2. Transmission electron micrograph (TEM) images of cultured magnetotactic bacteria and their extracted magnetosomes. (a and b) TEM images of a cell of *Magnetospirillum magneticum* strain AMB-1 and an aggregate of cells of *Magnetovibrio blakemorei* strain MV-1 grown in high yield in a bioreactor, respectively. (c) Purified magnetosomes of *Magnetococcus marinus* strain MC-1 negatively stained with uranyl acetate. Magnetosome membranes are represented by the electron-lucent layer surrounding each crystal. Note the presence of chains. (d–f) Purified magnetosomes of strains MC-1, MV-1 and AMB-1 after treatment with 1% sodium dodecyl sulfate (SDS). Note the absence of magnetosome membrane on crystals and the absence of chains.

and has been investigated in some detail (Bahaj *et al.*, 1993, 1998a,b,c; Arakaki *et al.*, 2002). Granulocytes and monocytes, after phagocytizing magnetotactic bacterial cells, can also be easily separated magnetically (Matsunaga *et al.*, 1989).

Recently, magnetotactic bacteria were shown to be promising biosorbents for heavy metals (Zhou *et al.*, 2012). Cells of polar magnetotactic bacteria have been used to determine south magnetic poles in meteorites and rocks containing fine-grained (<1 mm) magnetic minerals (Funaki *et al.*, 1989, 1992) and for non-destructive magnetic domain analysis on soft magnetic materials (Harasko *et al.*, 1993, 1995). Ma *et al.* (2012) used the magnetotactic bacterium strain MO-1 (Lefèvre *et al.*, 2009) as microrobots in a microfluidic chip and showed that it was possible to transport 2 µm diameter microbeads efficiently. This study illustrates possible uses of magnetotactic bacteria in chemical analyses and medical diagnoses using biochips, as well as in nano/microscale transport.

Magnetosomes contain single magnetic domain crystals that have useful magnetic and physical properties. Like magnetotactic bacterial cells, magnetosomes can be used for cell separation (Kuhara *et al.*, 2004). Importantly, the phospholipid magnetosome membrane that surrounds the magnetic crystals allows for the attachment of a number of biological molecules including proteins and nucleic acids on their surfaces. Exploiting this characteristic of magnetosomes, magnetite magnetosomes have been used in the immobilization of enzymes (Matsunaga and Kamiya, 1987) and in the formation of magnetic antibodies useful in various fluoroimmunoassays (Matsunaga *et al.*, 1990) involving the detection of allergens (Nakamura and Matsunaga, 1993) and squamous cell carcinoma cells (Matsunaga, 1991) and the quantification of immunoglobulin G (Nakamura *et al.*, 1991). Fluorescein isothiocyanate-conjugated monoclonal antibody immobilized on magnetosomes has also been used in the detection and removal of cells of *E. coli* (Nakamura *et al.*, 1993). Magnetite magnetosomes have been used to detect single-nucleotide polymorphism based on a fluorescence resonance energy transfer (FRET) technique in which

double-stranded labelled DNA synthesized by PCR and immobilized to the magnetosomes hybridizes to target DNA and a fluorescence signal is detected (Nakayama *et al.*, 2003; Ota *et al.*, 2003; Tanaka *et al.*, 2003; Yoshino *et al.*, 2003; Maruyama *et al.*, 2004).

Magnetosomes have been used to detect biomolecular interactions in medical and diagnostic analyses. Biotin and other molecules attached to a monolayer-modified substrate can be detected by streptavidin immobilized to magnetosomes using magnetic force microscopy (Arakaki *et al.*, 2004). For instance, streptavidin-modified magnetosomes have been used for the immobilization of biotin-modified antibodies (Amemiya *et al.*, 2005). Other biomedical applications include the use of magnetosomes in drug delivery after attachment of the drug in question (Matsunaga *et al.*, 1997) or as a non-viral gene delivery system (Xiang *et al.*, 2007a).

Specific magnetosome membrane proteins as anchor molecules have been used as protein displays for the assembly of foreign proteins on the surface of magnetite magnetosomes (Nakamura *et al.*, 1995a,b; Matsunaga and Takeyama, 1998; Matsunaga *et al.*, 2000b, 2002; Okamura *et al.*, 2001; Arakaki *et al.*, 2003; Yoshino and Matsunaga, 2005, 2006) that were fused to the chemiluminescent protein luciferase (Matsunaga *et al.*, 2000a, 2002; Yoshino and Matsunaga, 2006) to determine the stability of the anchor proteins. The most stable of these proteins among those tested was Mms13 (MamC, Mam12), since this fusion resulted in 400–1000 times the luminescence activity observed for Mms16 or MagA fusions (Yoshino and Matsunaga, 2006). The MamC protein has also been used as an anchor for a paraoxonase on the surface of magnetosomes, causing them to have phosphohydrolase activity effective in the degradation of ethyl-paraoxon, an organophosphate pesticide (Ginet *et al.*, 2011). The result was the production of functionalized magnetic nanoparticles efficient as a reusable nanobiocatalyst for pesticide bioremediation in contaminated effluents (Ginet *et al.*, 2011). Oligomeric proteins have also been expressed by genetic fusion to the MamC protein in *M. gryphiswaldense* (Ohuchi and Schüler, 2009).

Although proteins fused with the magnetosome membrane anchor protein Mms13 have been demonstrated to be an effective and stable method for the display of functional proteins on magnetosomes as described in the previous paragraph (Yoshino and Matsunaga, 2006), its use for the expression of some human proteins is relatively low (Kanetsuki *et al.*, 2012). To improve the human protein expression level on magnetosomes, a mutant strain of *M. magneticum* was constructed with a deleted native *mms13* gene. This strain synthesized magnetosomes with significantly improved expression of two human proteins, thyroid-stimulating hormone receptor (TSHR) and the class II major histocompatibility complex (MHC II) molecules (Kanetsuki *et al.*, 2012). Thus, it seems that strains that do not show high levels of specific protein expression can be genetically modified to become more effective in this regard.

Magnetosomes are useful in the isolation of nucleic acids. Magnetosomes, modified using compounds such as hyperbranched polyamidoamine dendrimers or amino silanes, have been used for the extraction of DNA (Yoza *et al.*, 2002, 2003a,b). mRNA can also be isolated efficiently using oligo(dT)-modified magnetosomes (Sode *et al.*, 1993).

Chemically produced magnetic nanocrystals have been shown to be useful in a number of medical applications. The specificity, affinity and binding capacity of magnetic nanoparticles depend on their size, form, dispersion and surface chemistry. When conjugated to antibodies, they have been shown to enhance magnetic resonance imaging (MRI) sensitivity for detection of cancer markers compared with other types of probes currently available (Lee *et al.*, 2007) and of acute brain inflammation in multiple sclerosis (McAteer *et al.*, 2007). Functional antibody fragments ('nanobodies') have also been coupled *in vivo* by genetic fusion to magnetosomes (Pollithy *et al.*, 2011). These types of conjugated magnetic nanoparticles can also be linked to genes or drugs and could be used as carriers of these molecules for targeted therapy of tumours (Chertok *et al.*, 2007; Zhao *et al.*, 2010; Guo *et al.*, 2012b; Tang *et al.*, 2012b). Magnetosomes may be useful as carriers of genes or drugs for cancer

therapy or other diseases (Sun *et al.*, 2007; Tang *et al.*, 2012b).

Interestingly, when magnetic nanoparticles are in the presence of an oscillating (alternating) magnetic field, heat is produced (Duguet *et al.*, 2006; Gloeckl *et al.*, 2006; Dutz *et al.*, 2007a,b), presumably as a result of hysteresis losses (Hergt *et al.*, 1998). This characteristic led to the suggestion that magnetic nanoparticles might thus be effective in the destruction or elimination of tumours through hyperthermia or thermoablation (Hilger *et al.*, 2001, 2005; Ito *et al.*, 2006; Ciofani *et al.*, 2009). Bacterial magnetite magnetosomes have also been shown to possess this characteristic (Hergt *et al.*, 2005, 2006; Liu *et al.*, 2012), even when the magnetite has oxidized to maghemite (Alphandéry *et al.*, 2011, 2012b). Chemically synthesized greigite particles of 50–100 nm were also shown to be useful in hyperthermia therapy against cancerous cells of a human lung adenocarcinoma epithelial cell line (Chang *et al.*, 2011). The first axenic culture of a greigite-producing magnetotactic bacterium, designated strain BW-1, has been reported recently (Lefèvre *et al.*, 2011b) and it will be interesting to investigate whether greigite magnetosome crystals biomineralized by this organism are useful for this and other applications.

Initial toxicity studies suggest that purified, sterilized magnetosomes from magnetotactic bacteria are non-toxic for mouse fibroblasts *in vitro* (Xiang *et al.*, 2007b). Recently, it was shown that extracted chains of magnetosomes administered directly within xenografted breast tumours of a mouse progressively left the tumours during the 14 days following their administration and were then eliminated in large proportion in the faeces (Alphandéry *et al.*, 2011). Purified magnetosomes from *M. gryphiswaldense* were labelled with the fluorescence dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin perchlorate and injected into the tail vein of nude mice, where they were tracked using a whole-body fluorescence imaging system for a relatively long period (Tang *et al.*, 2012a). The magnetosome membranes did not appear to decompose in the body in the duration of this study, another desirable quality to the use of bacterial magnetosomes.

The discovery of magnetotactic bacteria has had a major impact on geology, particularly in the areas of palaeontology and palaeomagnetism (Bazylinski and Moskowitz, 1997). Indeed, it seems that magnetotactic bacteria may be among the oldest prokaryotes found in the fossil record. When magnetite-producing magnetotactic bacteria die and lyse, their magnetosome crystals are released into the surrounding environment, where they sometimes persist or undergo dissolution (e.g. through iron(III) reduction) and/or transformation into other minerals (e.g. iron sulfides) (Vali and Kirschvink, 1989; Bazylinski and Moskowitz, 1997), depending on environmental conditions. Where these crystals persist, they are referred to as 'magnetofossils' that have been used as evidence for the past presence of magnetotactic bacteria in sediments dated to about 2 billion years ago (Chang and Kirschvink, 1989). However, the small size of magnetosome magnetite crystals makes them particularly susceptible to relatively rapid dissolution in reducing environments because of their large surface to volume ratio (Abrajevitch and Kodama, 2011). For example, magnetosome magnetite crystals are not likely to be preserved in systems where sediment accumulation and reductive diagenesis are coupled (Vali and Kirschvink, 1989; Snowball, 1994; Abrajevitch and Kodama, 2011). However, in habitats where the crystals persist for some time, magnetotactic bacterial magnetite has been shown to be a significant, sometimes the primary, carrier of magnetic remanence in some oceanic and lake sediments (Snowball, 1994; Oldfield and Wu, 2000; Snowball *et al.*, 2002; Kim *et al.*, 2005; Just *et al.*, 2012). This fine-grained magnetic material recorded the earth's magnetic field at the time it was deposited. Using isotopic dating and other technologies, investigators were able to determine approximately when sediments were deposited and track changes in the magnetic field, which in turn provided information about the origin of the geomagnetic field and properties of the deep earth, history of plate motions and magnetic reversals, colonization of magnetotactic bacteria and even magnetic proxy records of palaeoenvironments and palaeoclimate (Verosub and Roberts, 1995;

Evans and Heller, 2003; Paasche and Løvlie, 2011; Roberts *et al.*, 2011, 2012; Kind *et al.*, 2012; Larrasoana *et al.*, 2012). Very little is known regarding the deposition and significance of greigite in sediments, although magnetotactic bacterial greigite appears to have been recovered from such habitats (Snowball and Thompson, 1988; Snowball, 1991; Preisinger *et al.*, 2012).

Magnetotactic bacteria have also had a major impact on the field of astrobiology. Putative magnetite magnetofossils have been found in the Martian meteorite ALH84001 (Thomas-Keprta *et al.*, 2000, 2001, 2002; Clemett *et al.*, 2002). The presence and interpretation of these crystals in Martian meteorite ALH84001 have evoked great controversy and debate. If the magnetite crystals are indeed biogenic, the implication is that bacterial life existed on ancient Mars (McKay *et al.*, 1996; Thomas-Keprta *et al.*, 2000, 2001, 2002; Buseck *et al.*, 2001; Clemett *et al.*, 2002; Weiss *et al.*, 2004). In turn, this debate has led to a number of criteria being used to distinguish biogenic magnetite from inorganically produced magnetite (Thomas-Keprta *et al.*, 2000; Arato *et al.*, 2005; Kopp and Kirschvink, 2008; Jimenez-Lopez *et al.*, 2010; Gehring *et al.*, 2011; Kind *et al.*, 2011).

There are few reports of greigite magnetotactic bacterial magnetofossils, probably partially because greigite is metastable (has limited stability) and is very susceptible to dissolution and transformation to other minerals under oxidizing and highly reduced conditions (Rickard and Luther, 2007). One investigation describes putative greigite magnetofossils in Pliocene claystones (dated from between 5.3 to 2.6 million years ago) from the Carpathian foredeep of Romania (Vasiliev *et al.*, 2008).

Conclusions

After 40 years of scientific studies, research on magnetotactic bacteria remains a fascinating and exciting field, with serious impacts not just in microbiology but also in a number of diverse research fields including geology, mineralogy and biomineralization, crystallography, chemistry, biochemistry, physics,

limnology and oceanography, and astrobiology. It is clear that the bacterial magnetosome and its magnetic properties have been refined and optimized in the course of evolution by the organisms that synthesize them, by controlling the chemical composition, size and morphology of the magnetosome crystal, as well as their arrangement within the cell. Thus, it is difficult, if not impossible, to reproduce chemically what evolution has improved and seemingly attempted to perfect over billions of years. Thanks to relatively recent progress in the development of genetic systems, in genome sequencing and the isolation in culture of new species of magnetotactic bacteria, we predict that the next significant discoveries regarding magnetotactic bacteria will involve the determination of specific functions of magnetosome proteins. In turn, once functions of specific magnetosome proteins have been determined, we also expect that it will be possible to take advantage of the proteins involved in magnetosome biomineralization to produce similar particles

synthetically. The minimum set of genes necessary for magnetosome formation has already been determined (Murat *et al.*, 2010; Lohße *et al.*, 2011) and several laboratories are now competing in order to express this set of genes in a non-magnetotactic bacterium such as *E. coli*. The optimization of magnetosome production to high yields, one of the main focuses of laboratories working with magnetotactic bacteria, should facilitate the discovery of many more creative commercial and scientific applications for them.

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12 Interactions Between Plant-produced Nanoparticles and Antibiotics as a Way of Coping with Bacterial Resistance

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Introduction

Nowadays, multidrug-resistant bacteria have become common among agents of different infectious processes (Davies and Davies, 2010; Kalan and Wright, 2011). It has recently been revealed that genes for antibiotic resistance existed long before the discovery of antibiotics (D'Costa *et al.*, 2011). Constant use of antibiotics creates favourable conditions for selection and multiplication of the resistant microorganisms carrying such genes. The high prevalence of antibiotic resistance and the rapid increase in its levels is a matter of great concern. An illustration of this is the 39% prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) among invasive isolates in Mediterranean countries (Johnson, 2011) and the 2.5-fold increase in the number of infection-related hospitalizations with antibiotic resistance (Mainous *et al.*, 2011). Resistance has emerged in all classes of antibiotics, leading to a continuous need for the production of new drugs. However, only a few new antibiotics have been discovered during the past 40 years, and one of the

reasons for this decline is the difficulty in identifying new chemical substances that are effective and non-toxic (Kalan and Wright, 2011).

The antibacterial properties of metals, especially silver, have been known for many centuries. The continuous growth of resistance levels to antibiotics, along with the production of metals in nano-scale size, have renewed interest in the properties of this type of antimicrobials. One of the promising methods in coping with bacterial resistance is the application of synergistic activity between different antimicrobial agents, especially between antibiotics and non-antibiotics (Kalan and Wright, 2011). Such synergistic combinations may be composed of antibiotics and bacteriophages (Hagens *et al.*, 2006; Bedi *et al.*, 2009; Lu and Collins, 2009), antibiotics and plant extracts, essential oils or their components (Lorenzi *et al.*, 2009; Aiyegoro *et al.*, 2011), antibiotics with quorum-sensing inhibitors (Brackman *et al.*, 2011) and antibiotics with metallic nanoparticles.

Metallic nanoparticles have an advantage in their smaller size, which enhances their biological activity due to the increase in the contact

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area of a metal with a microorganism (Pal *et al.*, 2007; Rai *et al.*, 2012). However, the toxicity of metallic nanoparticles restricts their broad clinical application as antibacterial agents. This problem can be solved by combining nanoparticles with antibiotics. The synergistic interactions between the components of these combinations not only reduce the toxicity of both agents towards human cells, by decreasing the requirement for high dosages, but also enhance their bactericidal activity. Combinations of antibiotics and nanoparticles also restore the ability of antibiotics to destroy bacteria that have acquired antibiotic resistance.

This chapter reviews the available literature on the interaction between metallic nanoparticles and antibiotics, with particular emphasis on plant-produced nanoparticles, summarizes the experimental methods used in various studies on nanoparticle–antibiotic combinations and formulates areas of future research.

Methods for Studying Interactions Between Nanoparticles and Antibiotics

Assessment of the interactions between two antimicrobial agents is performed classically by diffusion methods, the chequerboard method by building isoboles and by time–kill curves (Verma, 2007). The disk diffusion method is considered as the least informative because it does not allow assessment of the concentration- and time-dependent interaction of the agents and, furthermore, there are no strict criteria to define synergy, additive effect and antagonism in this method. But, at the same time, it is the most easy to perform, produces demonstrative visual results and is commonly used, especially for first-time and screening studies (Table 12.1).

In general, the disk diffusion method is performed by placing an amount of antibiotic, nanoparticles and antibiotic–nanoparticle mixture on to paper disks, which are then placed on the surface of inoculated medium. Likewise, in a well diffusion modification, instead of paper disks tested agents are dropped into wells made in the medium. After

incubation, the diameters of the zones of inhibition of bacterial growth are assessed and then the zones around the combination are compared with the zones around the agents alone. However, in this method there are some nuances, which vary between studies.

Various concentrations of antibiotics and nanoparticles are used in different studies. Geoprincy *et al.* (2011) impregnated disks with 10 µg of silver nanoparticles (for nanoparticles alone), 10 µg of antibiotics (amoxicillin, chloramphenicol, erythromycin or rifamycin – for antibiotic alone) and 10 µg of nanoparticle with 0.5 µg of antibiotic. Bonde (2011) and Bonde *et al.* (2012) used commercial antibiotic disks (Hi-Media, India), which, to study the combined effect, were impregnated with 20 µl of silver nanoparticles in a concentration of 3.35×10^8 particles ml⁻¹. Ghosh *et al.* (2012), to study the combined effect, impregnated disks containing 500 µg of antibiotic with 30 µg of silver nanoparticles.

In assessing the effect of combinations, there is also no consensus between researchers. In some studies, increased zone size is calculated as the difference between two zones (Roy *et al.*, 2010; Thati *et al.*, 2010), while in others interactions are assessed either by the per cent of increase (Fayaz *et al.*, 2009; Banoee *et al.*, 2010) or by the fold increase (Shahverdi *et al.*, 2007; Birla *et al.*, 2009; Bonde, 2011; Bonde *et al.*, 2012; Ghosh *et al.*, 2012).

These mild technical and calculation disparities between the methods of assessing data lead to difficulties in comparing the results of different studies and therefore require further standardization.

Dilution methods generally assume the preparation of serial dilutions of antibiotic and nanoparticles, either in liquid or solid medium (broth dilution or agar dilution methods, respectively), with determination of minimal inhibitory concentration (MIC). The broth dilution method also allows determining the minimal bactericidal concentration (MBC) by withdrawing some of the liquid from each culture tube or well and seeding it on fresh solid medium. However, in spite of the fact that these methods may provide more information than the disk diffusion method, they are still not very common in studies of antibiotic–nanoparticle combinations.

Table 12.1. Methods used in the study of interactions between nanoparticles and antibiotics.

Method	Measurement	Advantages	Disadvantages	References
Diffusion methods	Diameter of inhibition zone	<ol style="list-style-type: none"> 1. Technical simplicity and economy of time. 2. Possibility of using commercial disks impregnated with antibiotics, which enhances accuracy of antibiotic dosing. 3. Photographing inhibition zones provides visually demonstrable results. 	<ol style="list-style-type: none"> 1. There are no criteria to distinguish between synergistic and indifferent interactions. 2. Differences in the level of diffusion of agents into the medium may distort results significantly. 3. It is not possible to differentiate between bacteriostatic and bactericidal effects. 4. Concentration-dependent interactions can be studied only approximately by placing different doses of agents on the disks. 5. Time-dependent interactions are not studied. 	Shahverdi <i>et al.</i> , 2007; Birla <i>et al.</i> , 2009; Burygin <i>et al.</i> , 2009; Fayaz <i>et al.</i> , 2009; Banoee <i>et al.</i> , 2010; Roy <i>et al.</i> , 2010; Thati <i>et al.</i> , 2010; Thirumurugan <i>et al.</i> , 2010; Bonde, 2011; Bonde <i>et al.</i> , 2012; Ghosh <i>et al.</i> , 2012
Dilution methods (broth or agar dilution)	Minimal inhibitory concentration, minimal bactericidal concentration	<ol style="list-style-type: none"> 1. Bacteriostatic and bactericidal effects are distinguished. 2. Cultivation of microorganisms in the presence of sub-inhibitory concentrations allows assessment of the formation of resistance mutants. 	<ol style="list-style-type: none"> 1. Concentration-dependent and time-dependent interactions are not studied or can be assessed only approximately. 2. In the microdilution method, nanoparticles may aggregate with bacteria and make it impossible to assess changes in optical density objectively. 	Li <i>et al.</i> , 2005; Burygin <i>et al.</i> , 2009; Zhao <i>et al.</i> , 2010; Ahmad <i>et al.</i> , 2011
Chequerboard	Fractional inhibitory concentration (FIC) index	<ol style="list-style-type: none"> 1. Concentration-dependent interactions between agents are assessed. 2. Differentiation between synergy, indifference and antagonism is possible. 	<ol style="list-style-type: none"> 1. Rather laborious and time-consuming technique. 2. Calculation of FIC indexes assumes a linear dose–response curve, which hides any other variants of dose-dependent interactions. 3. Time-dependent interactions are not assessed. 	Ruden <i>et al.</i> , 2009
Time–kill curves	Colony-forming units	<ol style="list-style-type: none"> 1. Assessment of time-dependent interactions between agents. 	<ol style="list-style-type: none"> 1. The most cumbersome and laborious technique. 2. Only a limited number of combinations can be evaluated in a study. 	Gu <i>et al.</i> , 2003; Li <i>et al.</i> , 2005; Perni <i>et al.</i> , 2009

More advanced information about antibiotic–nanoparticle interactions is provided by the checkerboard method, which allows the study of combinations of different concentrations of nanoparticles and antibiotics. There is a strict definition for synergy, indifference and antagonism through the calculation of fractional inhibitory concentration (FIC) index values, which gives this method advantages over the disk diffusion method. This method is widely used in the study of antibiotic combinations but has been used in only a limited number of studies dealing with nanoparticles (Ruden *et al.*, 2009) and has still not yet been applied to plant-produced nanoparticles. There are also some limitations in the checkerboard assay: it is laborious and does not evaluate bactericidal activity. Besides, there is an assumption about linear dose–response curves for both agents during the calculation of FIC indexes. When a dose–response is not linear, assessment of the results is less obvious. The significant limitation of the disk diffusion and checkerboard methods is that they only provide the opportunity to study time-independent interactions because their results are assessed at a single time interval (Verma, 2007).

Study of time-dependent nanoparticle–antibiotic interactions can be performed in two ways. In the microtube dilution method, measuring the optical density of culture wells in dynamics provides some preliminary information about the dynamic interactions. Theoretically, the same may be also performed in the checkerboard method. More precisely, these interactions can be studied by time–kill curves. This method is widely used in the study of antibiotic interactions; however, it is more cumbersome and laborious and there is only a small number of studies in which time–kill curves have been used to assess the antibacterial effect of nanoparticles alone (Pal *et al.*, 2007; Rezaei-Zarchi *et al.*, 2010) and nanoparticle–antibiotic combinations (Li *et al.*, 2005). Using time–kill curves to evaluate interactions between nanoparticles and antibiotics will provide further information on the time-dependent activity and stability of antibacterial effects. This method is also useful in studying the potential of such combinations for selecting resistant mutants.

Combinations of Plant-produced Silver Nanoparticles and Antibiotics

In spite of a rapid increase in the number of publications devoted to the antibacterial properties of nanoparticles, articles assessing nanoparticle–antibiotic combinations are not abundant. Most studies on nanoparticle–antibiotic combinations cover either chemically produced nanoparticles (Li *et al.*, 2005) or nanoparticles produced biosynthetically using fungi (Birla *et al.*, 2009; Fayaz *et al.*, 2009; Bawaskar *et al.*, 2010; Gade and Rai, 2011; Raheman *et al.*, 2011) or bacteria (Shahverdi *et al.*, 2007). All published studies on such combinations exploit silver nanoparticles only. Furthermore, there is a noticeable lack of studies on the interactions of plant-produced nanoparticles with antibiotics. However, the plant synthesis of nanoparticles has advantages in eliminating the elaborate process of maintaining microbial cultures (Shankar *et al.*, 2004) and therefore requires greater attention.

In the study of Thirumurugan *et al.* (2010), silver nanoparticles were synthesized by exposing silver nitrate solution to *Artocarpus heterophyllus* plant leaf extracts. The synthesis of silver nanoparticles was confirmed by the yellowish-brown colour of the solution, characterized by ultraviolet-visible (UV-Vis) spectrophotometer. The interactions between the synthesized silver nanoparticles and various antibiotics were assessed by the disk diffusion method against two Gram-negative bacteria, *Salmonella paratyphi* and *Klebsiella pneumoniae*. The results obtained demonstrated that against *S. paratyphi* there was an increase in the activity of vancomycin, amoxyclav and tetracycline in the presence of silver nanoparticles: the diameters of the inhibition zones for vancomycin increased from 8 mm without nanoparticles to 19 mm in their presence; for amoxyclav, the diameters increased from 8 to 18 mm, respectively; and for tetracycline, the diameters increased from 19 to 27 mm, respectively. Against *K. pneumoniae*, there was a remarkable increase in the activity of norfloxacin, ciprofloxacin and erythromycin: the diameters of the inhibition zones increased from 17 to 29 mm, respectively, for norfloxacin and from 19 to 29 mm, respectively, for both ciprofloxacin and erythromycin.

Ahmad *et al.* (2011) produced silver nanoparticles using extract of the entire plant of *Desmodium triflorum*, and they studied the activity of nanoparticles alone and in combination with gentamicin against three bacteria, Gram-positive *Staphylococcus epidermidis*, Gram-negative *Escherichia coli* and spore-producing *Bacillus subtilis*. In general, the activity of combinations increased approximately two times compared with the activity of agents alone.

Geoprincy *et al.* (2011) synthesized silver nanoparticles using tea extract (*Camellia sinensis*) and they studied the activity of the produced nanoparticles against four clinical isolates (*Bacillus cereus*, *B. subtilis*, *K. pneumoniae* and *Vibrio cholerae*) in combination with amoxicillin, chloramphenicol, erythromycin and rifamycin. Enlargement of inhibition zones was noticed in most antibiotics; however, the effect was not very pronounced. The enhancing effect was a little higher against *Bacillus* spp. than against two other species (*K. pneumoniae* and *V. cholerae*).

Bonde (2011) reported the synthesis of silver nanoparticles using leaf extract of *Foeniculum vulgare* (fennel, saunf) for the first time and also studied their activity in combination with gentamicin, oxacillin, vancomycin, ampicillin and amoxicillin against two reference strains, *S. aureus* and *E. coli*. An enhancing effect was observed in all combinations. In general, the enhancing effect was higher against *E. coli*, with the best effect shown in the combination of vancomycin and silver nanoparticles (the increase in fold area was 5.76). Against *S. aureus*, the highest enhancing effect was noticed in gentamicin, with an increase in fold area of 2.6. The beneficial interactions observed between the silver nanoparticles and various antibiotics may be explained by the plasmolysis and separation of the bacterial cytoplasm from the cell wall, inhibiting cell synthesis and inducing metabolic disturbances (Song *et al.*, 2006; Bonde, 2011).

The synthesis of silver nanoparticles from leaf extract of *Murraya koenigii* (Indian curry leaf tree) was first reported by Bonde *et al.* (2012). The authors studied the activity of synthesized nanoparticles in combination with gentamicin, ampicillin, tetracycline and streptomycin against three bacterial strains, *S. aureus*, *E. coli* and *Pseudomonas aeruginosa*.

An enhancing effect was present in all combinations. In the combination with gentamicin, ampicillin and streptomycin, the effect was more pronounced against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), especially significant in gentamicin (the increase in fold area was 4.06 and 1.11 against *E. coli* and *P. aeruginosa*, respectively). At the same time, in the combination of silver nanoparticles with tetracycline, the effect was more noticeable against Gram-positive *S. aureus*, with an increase in fold area of 2.16.

In a recent study, silver nanoparticles were synthesized using *Dioscorea bulbifera* tuber extract (Ghosh *et al.*, 2012). The activity of these nanoparticles was evaluated against 14 bacterial strains in combination with 22 antibiotics of different groups (aminoglycosides, penicillins, cephalosporins, carbapenems, cyclic peptides, glycopeptides, macrolides, quinolones, rifamycin, tetracyclines and others) by the disk diffusion method. The effect of the combinations varied widely, not only between groups of antibiotics but also within them; however, mainly synergy was detected. Even *Acinetobacter baumannii*, commonly possessing a high level of resistance to antibiotics, showed susceptibility to some combinations, particularly to combinations with penicillins. The authors explained that the observed enhancing effect of the combinations by an increase in the local concentration of an antibiotic at the cell membrane was due to the bonding reaction between the antibiotics and silver nanoparticles by chelation. Silver nanoparticles act as a drug carrier and transport antibiotics to the cell surface. The selective ability of the silver nanoparticles to bind the sulfur-containing proteins of the bacterial cell membrane increases its permeability, thus allowing the antibiotic to penetrate the cell (Ghosh *et al.*, 2012).

Summarizing the above-mentioned studies, plant-produced silver nanoparticles have demonstrated the ability to enhance the activity of different groups of antibiotics, particularly: beta-lactams (amoxicillin/clavulanic acid) and glycopeptides (vancomycin), which inhibit cell wall synthesis in bacteria; tetracyclines (tetracycline) and macrolides (erythromycin), which inhibit protein synthesis; and fluoroquinolones (ciprofloxacin and norfloxacin), which interfere with DNA synthesis.

Better understanding of the general principles of nanoparticle–antibiotic interactions can be achieved by comparing the results obtained from different researches (Table 12.2). Most studies have demonstrated the enhancing influence of silver nanoparticles on the activity of many antibiotics, especially on the activity of beta-lactams, independent of the method of synthesis. Moreover, in general, an enhancing effect was more noticeable against Gram-negative bacteria than against Gram-positive bacteria.

Mechanism of Interactions Between Nanoparticles and Antibiotics

Several explanations can be proposed for the enhancing effect of antibiotics and silver nanoparticles (Fig. 12.1). Differences in the mechanism of action of nanoparticles and antibiotics could enhance the activity of their combination, i.e. if a bacterium is resistant to one of the agents, another may kill it in a different way (Li *et al.*, 2005). For example, in bacterial strains without resistance to amoxicillin, the enhanced effect of amoxicillin–silver nanoparticle combinations can be explained by the binding reaction between the amoxicillin and the silver nanoparticles. Hydroxy and amido groups in the amoxicillin molecule react easily with the nanoparticles by chelation; this leads to the formation of a nanosilver core surrounded by amoxicillin molecules. The concentration of antimicrobial agents increases at the site of contact with bacterium and causes more destruction. Because silver chelation prevents DNA from unwinding, nanosilver's antimicrobial groups lead to more dramatic DNA damage.

Another explanation given by Li *et al.* (2005) is based on the role of nanosilver as an antibiotic carrier. In contrast to hydrophilic amoxicillin, nanosilver is hydrophobic and interacts easily with a bacterial membrane composed of hydrophobic phospholipids and glycoproteins. Thus, amoxicillin is transported easily to the cell surface and acts more pronouncedly.

Differences in the activity of combinations against Gram-positive and Gram-negative bacteria can be explained by the variations in cell wall structure and by more difficult penetration of the Gram-positive cell

wall (Birla *et al.*, 2009; Fayaz *et al.*, 2009; Bonde, 2011; Bonde *et al.*, 2012). Beta-lactams destroy the cell wall and increase its penetration by silver nanoparticles.

Banoee *et al.* (2010) revealed the enhancing effect of another type of nanoparticle, ZnO, on the antimicrobial activity of ciprofloxacin, and proposed that the mechanism for this was by the inhibition of antibiotic efflux pumps. Efflux pump systems are responsible for resistance to many antibacterial agents such as fluoroquinolones, macrolides, aminoglycosides, tetracyclines and other antibiotics in both Gram-positive and Gram-negative bacteria (Hooper, 2005). Therefore, the explanation proposed can also be generalized towards different nanoparticles and antibiotics. Nanoparticles may interfere with the pumping activity of NorA protein, inducing faster electron transfer kinetics in its active site. This protein mediates the active efflux of hydrophilic fluoroquinolones from the bacterial cell and thus provides resistance to the bacterium. This in turn leads to an inhibition of the antibacterial activity of ciprofloxacin. Nanoparticle interference with NorA protein restores the action of ciprofloxacin. Another suggested mechanism is explained by the interactions of ZnO nanoparticles with the membrane Omf protein, which is associated with permeation of the cell membrane by fluoroquinolones. By influencing the Omf protein, ZnO nanoparticles enhance ciprofloxacin absorption into the cell.

Future Prospects

Plant synthesis of nanoparticles is economically feasible, ecologically friendly and less laborious compared with other biosynthetic methods because it does not require maintaining microbial cultures. However, more extensive studies are necessary on combinations between plant-produced nanoparticles and antibiotics. Most published studies are devoted to either nanoparticles produced chemically or nanoparticles produced biosynthetically using fungi or bacteria. Future studies should be directed towards study of the interactions between plant-produced nanoparticles and antibiotics.

Table 12.2. Studies on combinations of plant-produced silver nanoparticles and antibiotics.

Plant used for biosynthesis	Nanoparticle size (nm)	Test bacteria	Method of study of interactions	Antibiotics in combination	Observed effect in the presence of nanoparticles	References
<i>Artocarpus heterophyllus</i> leaf extract	Not measured	<i>Salmonella paratyphi</i> , <i>Klebsiella pneumoniae</i>	Disk diffusion	Norfloxacin, ciprofloxacin, kanamycin, vancomycin, erythromycin, nitrofurantoin, methicillin, amoxyclav, tetracycline, nalidixic acid	The activities of vancomycin, amoxyclav and tetracycline increased against <i>S. paratyphi</i> . Norfloxacin, ciprofloxacin and erythromycin antibacterial activities increased against <i>K. pneumoniae</i> .	Thirumurugan <i>et al.</i> , 2010
<i>Desmodium triflorum</i> entire plant extract	5–20	<i>Staphylococcus epidermidis</i> , <i>Escherichia coli</i> , <i>Bacillus subtilis</i>	Broth dilution	Gentamycin	Approximately twofold increase in the combination activity.	Ahmad <i>et al.</i> , 2011
Tea extract (<i>Camellia sinensis</i>)	5	Clinical isolates of <i>Bacillus cereus</i> , <i>B. subtilis</i> , <i>K. pneumoniae</i> , <i>Vibrio cholera</i>	Disk diffusion, bioautography (agar over layer method) proposed by authors	Amoxicillin, chloramphenicol, erythromycin and rifamycin	Enhancement of antibacterial activity of all studied antibiotics, with the most pronounced effect against <i>Bacillus</i> spp.	Geoprincy <i>et al.</i> , 2011
<i>Foeniculum vulgare</i> leaf extract	18–83	<i>Staphylococcus aureus</i> , <i>E. coli</i>	Disk diffusion	Gentamicin, oxacillin, vancomycin, ampicillin and amoxicillin	Enhancement of antibacterial activity of all studied antibiotics, with the most pronounced effect in the combination with vancomycin against <i>E. coli</i> and with gentamycin against <i>S. aureus</i> .	Bonde, 2011

<i>Murraya koenigii</i> leaf extract	40–80	<i>E. coli</i> , <i>S. aureus</i> , <i>Pseudomonas aeruginosa</i>	Disk diffusion	Gentamicin, ampicillin, tetracycline, streptomycin	Enhancing effect in all combinations. More pronounced enhancing effect with gentamicin, ampicillin and streptomycin against <i>E. coli</i> and <i>P. aeruginosa</i> ; and with tetracycline against <i>S. aureus</i> .	Bonde <i>et al.</i> , 2012
<i>Dioscorea bulbifera</i> tuber extract	8–20	<i>Acinetobacter baumannii</i> , <i>Enterobacter cloacae</i> , <i>E. coli</i> , <i>Haemophilus influenzae</i> , <i>K. pneumoniae</i> , <i>Neisseria mucoza</i> , <i>Proteus mirabilis</i> , <i>P. aeruginosa</i> , <i>Serratia odorifera</i> , <i>Vibrio parahaemolyticus</i> , <i>B. subtilis</i> , <i>Paenibacillus korensis</i> , <i>S. aureus</i>	Disk diffusion	22 antibiotics of different groups	Effect was mainly synergistic, with the most noticeable synergy against <i>P. aeruginosa</i> , <i>E. coli</i> and <i>A. baumannii</i> .	Ghosh <i>et al.</i> , 2012

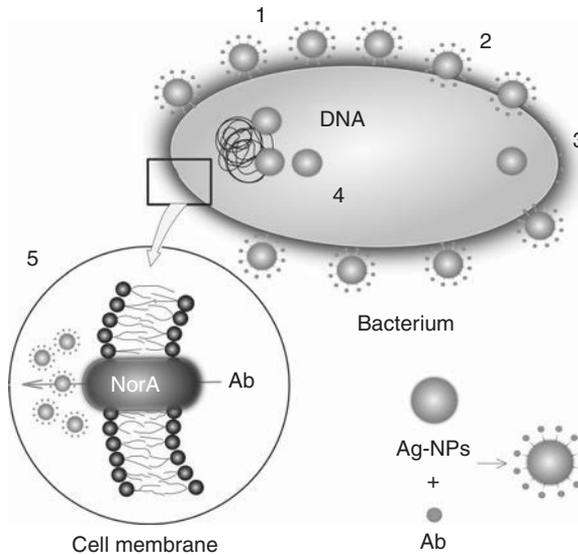


Fig. 12.1. Mechanism of interactions between antibiotics and nanoparticles. During the formation of an antibiotic–silver nanoparticle complex, the bonding reaction between the antibiotic and the nanoparticles increases antibiotic concentration at the bacterial surface (1); the approach of the hydrophobic antibiotic to the bacterial cell is facilitated by the silver nanoparticles, which act as a carrier for an antibiotic (2); beta-lactam or glycopeptide antibiotics destroy the bacterial cell wall and enhance its permeability for silver nanoparticles (3); silver nanoparticles prevent the unwinding of DNA (4); nanoparticles of different types may inhibit efflux pumps, which reduces antibiotic efflux from the cell and restores its activity (5). Ab = antibiotic; Ag-NPs = silver nanoparticles; NorA = efflux pump protein. (Figure is based on the data of Li *et al.*, 2005; Fayaz *et al.*, 2009; Banoe *et al.*, 2010).

Although some articles have proposed possible explanations of the synergistic effect, it is not yet fully understood. Researchers generally associate synergistic effect with the impairing activity of antibiotics on cell walls and more significant accumulation of nanoparticles inside bacterial cells. It has also been discussed that the combined effects of antibiotics with nanoparticles are more pronounced on Gram-negative bacteria than on Gram-positive bacteria, because the cell wall of the Gram-negative bacteria is thinner. However, some studies have demonstrated that the combination of nanoparticles with antibiotics that inhibit the protein synthesis of bacteria also has high antibacterial efficacy. There is a need for thorough studies with a better understanding of the mechanism of interaction between nanoparticles and the different groups of antibiotics.

Future studies could also be focused on pharmaceutical formulations containing nanoparticles and antibiotics, and on testing

their toxicity towards mammalian cells. Moreover, studies on the interactions between metallic nanoparticles and antibacterial agents are devoted mainly to conventional antibiotics, but combinations of nanoparticles with antiseptics and bacteriophages also require greater attention.

Conclusions

In spite of the different methods proposed to combat microbial resistance, the high prevalence of multidrug-resistant bacteria indicates an urgent need for new approaches to cope with this problem. The high biological and chemical activity of metallic nanoparticles makes them promising agents in antibacterial treatment. Current wide clinical usage of metallic nanoparticles is restricted because of their toxicity; however, combined application of nanoparticles with antibiotics may overcome this problem.

The combined use of antibiotics with metallic nanoparticles can restore the activity of some previously effective antibiotics, for example some penicillins, and can also slow the emergence of resistance to presently highly effective antibiotics. Likewise, combining nanoparticles with antibiotics or other antimicrobial agents is promising in reducing the toxicity of both components towards mammalian cells.

The majority of research is focused on the study of interactions between antibiotics and silver nanoparticles, and many combinations have shown a promising enhancing effect *in vitro*, mainly with beta-lactams (ampicillin, amoxicillin) and glycopeptides (vancomycin). These effects can be explained by the increased permeability of the cell wall to nanoparticles as a result of antibiotic action. The enhancing activity of non-beta-lactam antibiotics can be explained by the inhibition of antibiotic efflux in the presence of nanoparticles and merits further investigation.

In summary, the results of studies generally indicate that combinations of metallic nanoparticles with antimicrobial agents have significant potential to cope with drug resistance in bacteria. The phytosynthesis of nanoparticles has advantages over other methods of synthesis, and therefore more investigations related to this subject are necessary to study the effect of plant-produced nanoparticle–antibiotic combinations against different bacteria, and to study the combinations between plant-produced nanoparticles and different classes of antimicrobial agents.

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13 Nanostructured Particles from Coccolithophores – An Undiscovered Resource for Applications

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Introduction

In the beginning of the 21st century, when nanotechnologies and nanomaterials were an established and undisputed part of both scientific and everyday life, nature remained the most ingenious source of knowledge and ideas. This is confirmed by the constant interest and advances in the bioinspiration-related fields involving bioinorganic (nano)materials and structured/hybrid smart biomaterials. The philosophy behind this bioinspiration involves biological concepts, mechanisms, functions and design features as the basis of new synthetic materials, characterized by advanced structures, properties and functions. The ultimate achievement is not simply to recreate natural biological structures or to utilize already existing structures but to use the knowledge in order to generate new guiding principles and ideas, and finally to revolutionize the corresponding field of application.

The 'learning from nature' approach has historically undergone significant evolution, and despite the initial variable success, has paved the way towards the current practical, real-world contributions. One of the first documented attempts to solve engineering problems by imitating nature belonged to

Leonardo da Vinci. He studied birds in order to create a 'flying machine', described in his work *Codex on the Flight of Birds* (Pedretti and Frost, 2001). His ideas were developed over the centuries and were finally perfected successfully by the Wright brothers.

Natural mechanisms for seed dispersion were the models facilitating the invention of the versatile and commercially successful Velcro hook-and-loop fasteners by the Swiss engineer, George de Mestral. Bioinspiration also enabled the development of cat-eye reflectors, gecko-like fasteners and bonding systems, self-cleaning lotus leaf-like surfaces, low-weight wood-like composite materials, butterfly-like anti-reflective coatings and opal-like-effect pigments, self-repair plastics, bionic medical implants and even tissue repairs (Xia and Jiang, 2008).

Preliminary estimations of the economic impact of biomimetics reveal that although this field is just emerging, it represents US\$300 billion annually of US gross domestic product (GDP) and could account for 1.6 million jobs and about US\$1.0 trillion of GDP by the year of 2025 (Anon., 2010), turning 'green' into 'gold'.

Beyond the realms of biomimetics or the use of biological principles as inspiration for

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design or functions, the idea to use microorganisms to produce spatially structured materials for attractive technical applications emerged more than a century ago. The incorporation of nitroglycerine in diatomite mediated the invention of dynamite in 1867 by Alfred Nobel (Nobel, 1868) and has changed the world ever since.

Diatoms are a perfect example for the direct application of inorganic structures with microbial origin, based on their properties and functions. The potential and the advantages of microorganisms as a source of new materials for particle technology are barely known, and this field is still in the initial stages of its development. Microorganisms, with their highly effective synthetic capacities, not only deliver unique, ready-to-use spatial inorganic structures but also can produce the components of high-value hybrid materials or inspire novel synthetic approaches. It is an assignment and challenge for bioprocess engineering and particle technology to transfer the knowledge gathered and to develop technologies based on the production of attractive (nano)structured materials with microorganisms.

This chapter approaches probably one of the most fascinating incarnations of the process of biomineralization; that is, the formation of calcium carbonate microplates (coccoliths) by the group of calcifying phytoplankton designated as coccolithophores. The focus here is less on the biological, physiological or ecological aspect and more towards the cultivation and production challenges, the material characteristics and the possible emerging applications. Coccolithophores are extremely interesting and attractive objects to study. On the one hand they combine efficient biological systems, which are the algae, and on the other hand they form biominerals with unique structures. That makes them a treasure to material scientists and a challenge for biomimetic applications. By analogy with biomineralization, which stands as a bridge between the organic and inorganic world, this chapter extends a bridge of knowledge between biotechnology and materials science. It gives an idea of the strain-dependent diversity of coccolith structures; outlines the

factors and conditions that favour the growth of coccolithophores in both environmental and laboratory situations, together with the equipment requirements for future mass production; and summarizes the properties of the calcite nanostructured particles with regard to their technical applications.

The Strain-specific Structural Diversity of Coccoliths

Taxonomy of coccolithophores

Coccolithophores are a group of marine calcifying algae, which belong to the division Haptophyta (Jordan and Chamberlain, 1997). The distinctive feature of coccolithophores is the formation of remarkable biomineral structures – an outer covering of mineralized scales known as coccoliths. Haptophytes dominate phytoplankton populations in nearly all marine environments worldwide and form extensive blooms, clearly and easily visible from outer space. Geological evidence suggests that this ecological abundance has been present for at least the last 200 million years, leaving well-documented fossil records in the form of huge chalk deposits such as the spectacular white cliffs of Dover, UK, or their analogue on the island of Rügen, Germany (Mann, 2001). The dimensions of these algae blooms nowadays raise interest regarding their impact on marine ecosystems, sulfur and carbon cycles, and define them as a major global climate player, operating the organic carbon pump (Rost and Riebesell, 2004). Additionally, coccolithophore algae are responsible for half of the existing global CaCO_3 production (Purdie and Finch, 1994; Buitenhuis *et al.*, 1999).

Understanding the genesis and the causality for the synthesis of coccoliths is the first step towards defining their prime function and, more importantly, towards their introduction for new biomimetic applications or for upgrading existing materials. On the way towards revealing the origin of coccoliths, a useful milestone is consideration of the diversity of their structures and forms, which are strain specific and unique.

Most haptophytes occur as marine coastal or open oceanic planktic forms and only as an exception are they found in fresh-water (Thomsen *et al.*, 1994). Their size ranges from 2 μm to macroscopic colonies (Thomsen, 1986). Haptophytes may occur as non-motile single cells (many coccolithophores), as non-motile colonies of single cells embedded in mucilage, as motile single cells (most non-coccolithophores and some coccolithophores, e.g. *Syracosphaera*) or colonial flagellates (Saez *et al.*, 2004). This variety of form and morphology impedes the correct classification of haptophytes. Additionally, many of them have alternate morphotypes that presumably are alternate stages in a haplo-diploid life cycle (Billard, 1994; Green *et al.*, 1996).

The standard classification of modern haptophytes as suggested by Parke and Green (Parke and Dixon, 1976) distinguished four orders: Cocosphaerales (coccolith bearing), Prymnesiales (non-coccolith-bearing haptophytes), Isochrysidales (with some coccolith-bearing genera) and Pavlovales (with flagella of unequal lengths). Almost two decades later, it was postulated that only the order Pavlovales had unambiguously well-differentiated group characteristics and the other three orders were united into the single subclass Prymnesiophycidae in the order Prymnesiales (Green and Jordan, 1994). According to recent phylogenies of the Haptophyta, based on new molecular data, the orders Pavlovales and Prymnesiales have been raised to class level (classes Pavlovophyceae and Prymnesiophyceae) (Edvardsen *et al.*, 2000). Class Pavlovophyceae contains three major groups divided by differences in their morphological and pigment characteristics, including *Exanthemachrysis* and *Rebecca*. The group containing *Pavlova* spp. is further divided into two clades, one containing 'true' *Pavlova* and the other containing *Diacronema* (Van Lenning *et al.*, 2003). The class Prymnesiophyceae is collective for several major clades, classified by phylogenetic reconstruction based on nucleotide sequences of the 18S rRNA (Saez *et al.*, 2004): Clade A – Phaeocystales, with several colonial species; Clade B – Prymnesiales, containing non-motile and flagellate, scale- and/or coccolith-covered cells; Clade C – Isochrysidales; and

Clade D – Coccolithales, containing diverse sets of coccolithophores. Additionally, Clade E and Clade F are differentiated. They originate from DNA sequences isolated from bulk genomic DNA, purified from filtered water samples, and until now their morphology remains undiscovered and unknown (Moon-Van der Staay *et al.*, 2000).

The identification of haptophyte algae to species level relies exclusively on scale and coccolith morphology. In general, the molecular data support and confirm systematic classification based on traditional morphological information, which raises confidence that there are still many novel yet undescribed coccolithophores in the world's oceans (Saez *et al.*, 2004).

Coccolith structure and the extracellular architecture of the calcifying algae

The taxonomy of coccolithophores is based in general on the morphological description of the single coccoliths, which are elements of the entire extracellular architecture of the algae. Some coccolithophores have one or more underlying layers of non-mineralized scales in addition to the outer layer of coccoliths. The mineralized and non-mineralized scale layers are referred to as coccospheres (Marsh, 1999a). The shape of the coccosphere can vary considerably and may be spherical or ovoid to ellipsoidal in form, or display elaborate modified coccolith appendages. As intact coccospheres are an exception, coccoliths remain the most reliable source for taxonomic classifications.

Coccolithophores are capable of arranging the coccoliths in one or more distinct layers, or theca. A coccosphere that consists of a single layer of coccoliths is regarded as monothecate. Two discrete layers are characteristic for the dithecate, where coccoliths are divided into endo- and exothecate. Coccospheres with two or more layers of coccoliths are multilayered, with no differentiation in endo- and exotheca. For example, *Emiliania huxleyi* has two or more layers with no coccolith differentiation and is classified as multilayered (Young *et al.*, 1997).

Based on morphological criteria, the modern variety of coccolithophores has been divided into about 200 species (Jordan and Kleijne, 1994). According to other authors, the number of calcifying microalgae is over 300 (Jordan and Green, 1994). This number is most likely too high, as increasing evidence suggests that most, if not all, coccolithophores exhibit haplo-diploid life cycles, with the expression of two or more completely different coccolith forms during different stages (Geisen *et al.*, 2002).

The calcite (CaCO_3) crystals that form coccoliths are attached to an underlying oval-shaped organic base plate. Presumably, these base plates are always present during the initial stages of coccolith formation, although they are missing in mature coccoliths in some species (Marsh, 1999a). Coccoliths are divided into heterococcoliths and holococcoliths, based on calcite crystal morphology. Holococcoliths have crystals, characterized by rhombohedral or prismatic crystal growth, that is typical for the inorganic sources of calcite. The haploid phase of the coccolithophore life cycle is characterized by these holococcoliths, which lack radial symmetry and consist of numerous (several hundred to a few thousand) small calcite crystals, c.0.1 μm in size (Young and Henriksen, 2003). On the other hand, heterococcoliths have elaborate and complex shapes that are species specific and are not present in the inorganic sources of the mineral (Marsh, 1999a). They characterize the diploid phase and have a limited number (typically below 100). In addition to holo- and heterococcoliths, there is a third minor group, designated as nannoliths. These calcareous structures lack the typical features of the other two groups but display features generic to coccolith structure and are presumably formed by a different mode of biomineralization (Young *et al.*, 1999). Considering the existence of holo- and heterococcoliths and the estimated number of holococcolith-bearing species (about 70), the number of existing coccolithophore species appears to be about 130 (Geisen *et al.*, 2002).

A certain level of variety can also be observed among coccoliths that build the coccosphere from a single cell. Often, polar coccoliths differ morphologically from those that cover the body of the cell. Coccoliths

surrounding the flagellar pole are designated as apical coccoliths and those surrounding the opposite pole are designated as antapical coccoliths. Species with coccospheres containing only one morphological type are known as monomorphic, those with coccospheres combining two discrete forms are known as dimorphic and those possessing more than two discrete types are known as polymorphic (Young *et al.*, 1997). The diversity and versatility of coccolithophore species, and particularly of the coccoliths, is extended by the existence of another group, designated as varimorphic. According to Young *et al.* (1997), these species are coccoliths whose size and/or morphology vary according to their position on the coccosphere.

Heterococcoliths, apart from their unique structures, are also the more common type of coccoliths. In general, they consist of a radial array of complex crystal units with variable shapes and sizes, forming an inner central area surrounded by an outer margin or rim. The central area can be completely open or partially closed, with a central opening or protruding spine (Young *et al.*, 1997). Despite the wide range of coccolith shapes, there are three types that summarize the main architecture principles and tendencies. The planoliths exhibit a low, not elevated rim, giving the coccolith a planar appearance; the muroliths are characterized by an elevated, wall-like rim without well-developed shields, creating a bowl shape; and the placoliths demonstrate a rim with two or more well-developed shields.

The formation of coccoliths and their structure has been studied most thoroughly in two placolith-bearing species – *Pleurochrysis carterae* and *E. huxleyi*. In placoliths, which are the most common heterococcoliths, the crystals form a parallel, double disc-shaped structure, radiating from the coccolith rim (Marsh, 1999b). The coccoliths of the species mentioned above are constructed of a ring of single, interlocking crystals with alternating radial (R) and vertical (V) orientation, which is a common structure for most heterococcoliths (Young *et al.*, 1992). The V and R orientations represent the alignment of the crystallographic axes of the crystal units according to the coccolith plane. The parallel, double-disc elements of the mineral ring are known as the distal and proximal shield

elements and the vertical or subvertical structures connecting the shields are designated as tube elements (Young *et al.*, 1997). Apart from the structural principles including R and V units, which are common for most of the heterococcoliths studied, differences occur regarding the terminal orientation and connection between the crystals. For instance, the coccoliths of *P. carterae* are built from four plate-like elements: the distal shield and the outer tube elements, which form the V unit, and the proximal shield and the inner tube elements, which form the R units (Marsh, 1999b). On the other hand, the proximal and distal shield elements in the coccoliths of *E. huxleyi*, together with the inner and outer tube elements, are all derived from the R units (Davis *et al.*, 1995).

The size of coccoliths is another characteristic that varies significantly according to the species' affiliation. The following types of coccoliths are distinguished by their maximum dimension in length: minuscule, <1 μm ; very small, 1–3 μm ; small, 3–5 μm ; medium, 5–8 μm ; large, 8–12 μm ; very large >12 μm (Young *et al.*, 1997).

The formation of coccoliths is a complex and sophisticated biomineralization process that could occur simultaneously or successively. This difference in coccolith synthesis could predetermine the number of coccoliths in a single layer, and the number of layers too (Westbroek *et al.*, 1986). For example, *E. huxleyi*, having only one coccolith vesicle, can produce only one coccolith at a time, resulting in a coccusphere with about 15 coccoliths. Other species, like *P. carterae*, have several coccolith vesicles, each in a different stage of maturation, and produce several coccoliths simultaneously in a rapid process that could cover the entire surface of the cell with coccoliths in about 8 h (van der Wal *et al.*, 1987). As a result, the cells of *P. carterae* are covered with about 200 coccoliths (Westbroek *et al.*, 1986).

The mechanism of coccolith formation involves three fundamental processes – ion accumulation, calcite nucleation and crystal growth. A detailed review of these processes, their relation, driving forces and the leading role of coccolithophores are presented in the chapter 'Synthesis of Nanostructured Calcite Particles in Coccolithophores, Unicellular Algae' by Holtz *et al.* (see Chapter 9, this volume).

A visualization of the variety of unique coccolith structures, forms and sizes demonstrated by different coccolithophore species is presented in Fig. 13.1.

Apart from the possible influence of ecological and environmental factors on coccolith formation, the question regarding the origin of their diversity still remains, and, more specifically, which factors determine the geometry of these elaborate structures. Authors often speculate about the exact reasons and circumstances for this phenomenon, but undoubtedly the potential that these structures possess for attractive applications and bioinspiration remains. The variety of species and forms makes the challenge of the transition from knowledge to practice even greater.

Culturing Coccolithophores

Despite the high specificity of the process, which is related to the unique biomineralization principles, the production of highly structured particles from coccolithophores could also be regarded and investigated as a classic biotechnological process. The success of such a process relies on a perfect combination between upstream processing, including the screening and selection of coccolithophore species that exhibit economic opportunities; bioreaction, representing a balance between process strategy, process environment and equipment; and downstream processing, including product purification and characterization, enabling successful applications.

Selection

The biological object producer always assumes a decisively important position in a biotechnological process. The nature of coccolithophores as very sensitive and demanding microorganisms defines the selection of a strain producer as the crucial stage in the whole process. On one hand, the selected coccolithophores should be easy to cultivate not only in laboratory conditions but also they should express a scale-up potential. On the

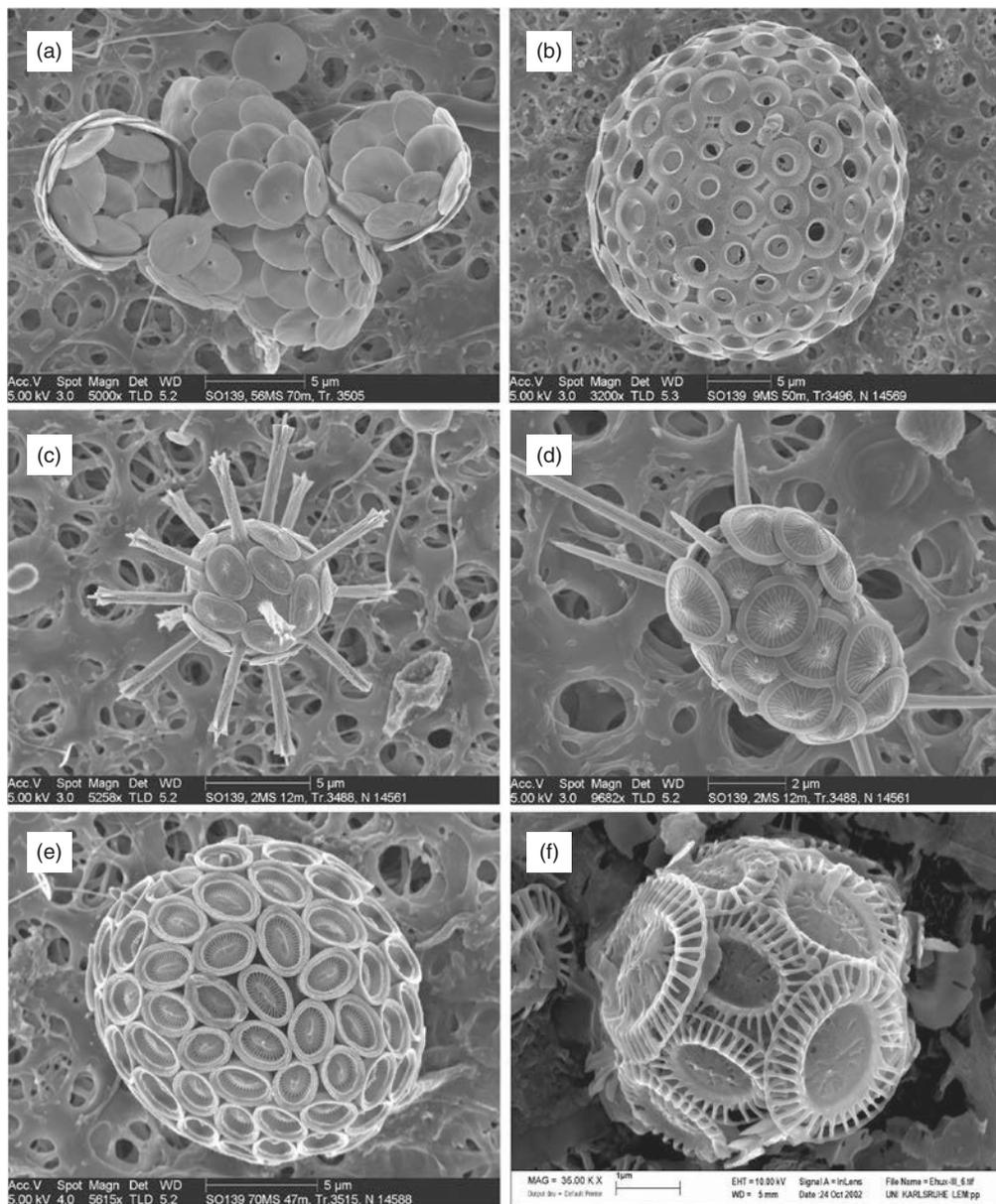


Fig. 13.1. Scanning electron micrographs of six different coccolithophores species. (a) *Oolithothus antillarum*; (b) *Umbilicosphaera sibogae*; (c) *Rhodosphaera clavigera*; (d) *Acanthoica janchenii*; (e) *Coronosphaera mediterranea*; (f) *E. huxleyi*. (Images (a–e) are the property of Dr Harald Andruleit, Federal Institute for Geosciences and Natural Resources, Hannover, Germany; image (f) is the property of the Institute of Bioprocess Engineering, Karlsruhe Institute of Technology, Germany.)

other hand, the species should possess the desired characteristics and calcification specifics that make them attractive for industrial cultivation and future applications.

Similar to many other biotechnology branches and product synthesis, the ultimate source of microorganisms used in bioprocesses is the environment. As oceans cover roughly

70% of the earth's surface, the opportunities for the isolation of species should be vast. Is that really so and is this abundance also of practical significance? Indeed, coccolithophores are widely distributed in temperate and tropical oceans, being the dominant members of the phytoplankton in some areas (Jeffrey and Allen, 1964). The biogeographic distribution of coccolithophores in the world's oceans and seas has been studied intensively by scientists over the past 50 years, by investigating both plankton and sediments (Young, 1994). The first detailed documentation of the coccolithophore populations in the Atlantic Ocean belongs to McIntyre and Bé (1967). Based on the movement of distinct water masses, they defined five different coccolithophore zones: subarctic, temperate, subtropical, tropical and subantarctic. Analogical studies, examining the horizontal and vertical distribution of coccolithophores, identified similar zones in the Pacific Ocean: subarctic, transitional, central, equatorial and subantarctic (Okada and Honjo, 1973). The biggest diversity of coccolithophores is reported in the subtropical ocean areas (Hulburt, 1963), while the polar waters are characterized with significantly lower diversity (McIntyre, 1967).

The general habitat for coccolithophores, as for most phytoplankton, is the photic zone, providing the sunlight necessary for photosynthesis. Certain species such as *E. huxleyi* can form massive seasonal blooms, visible from space (Holligan *et al.*, 1983). Coccolithophore algae blooms have been also observed in inland saline lakes – for example *Pleurochrysis pseudoroscoffensis* in the Salton Sea, California (Reifel *et al.*, 2001).

E. huxleyi is an extremely cosmopolitan coccolithophore species found in all oceans, except in the Arctic Ocean and the high-latitude Southern Ocean (Jordan and Kleijne, 1994). The first reported observations of *E. huxleyi* blooms were conducted around fjords on the western coast of Norway (Birkenes and Braarud, 1952; Berge, 1962) and could cover very large areas, for instance at least 200,000 km² in the Eastern Bering Sea (Sukhanova and Flint, 1998) or 250,000 km² in the North Atlantic south of Iceland (Holligan *et al.*, 1993a). Cell concentrations vary between blooms and depend on

the stage of the bloom. Sometimes, bright waters contain only light-scattering coccoliths left over from a cell population that has mostly died, as light scattering appears both from detached coccoliths and coccoliths in coccospheres (Tyrrell and Merico, 2004). The abundance and the cosmopolitan character of *E. huxleyi* are two of the reasons why these algae are the most investigated and described in detail. The prominence of *E. huxleyi* is also supported by the fact that it is one of the world's major calcite producers and is relatively easy to culture and manipulate under laboratory conditions (Paasche, 2002). Understanding the biomineralization processes in *E. huxleyi*, being a unicellular eukaryote, and its genetic and physiological regulation also has an impact on fields such as biomedicine and materials science (Read and Wahlund, 2007). The German Botanical Society (Deutsche Botanische Gesellschaft, DBG, eV) recognized the significance and global impact of *E. huxleyi* by proclaiming this microalga as Alga of the Year 2009 (Press Release, <http://www.dbg-phykologie.de/pages/22PressRelaseAlgaeYear2009.html>).

Despite the great abundance and variety of coccolithophores, biomineralization studies have been narrowed naturally to a limited number of species that are maintained easily in laboratory culture. Regarding significance and data collected, *E. huxleyi* is followed by *P. carterae* and *Coccolithus pelagicus* (Marsh, 2004). The *Pleurochrysis* coccoliths are composed of an oval organic base plate with a distal rim of interlocking calcite crystals (Marsh, 2007). These coccoliths are quite small and, from the geological aspect, they are unable to withstand sedimentation and lithification processes, which explains their absence in the fossil record and casts uncertainty regarding their evolutionary history (Young *et al.*, 1994). Regardless of the insignificant dimensions of *Pleurochrysis* crystals, compared to the ones produced by heavy calcifiers like *C. pelagicus*, *Pleurochrysis* is probably the most convenient coccolithophore to work with, since its phenotype is very stable in culture and sustains even drastic laboratory manipulations (Marsh, 2007). *C. pelagicus* is well represented in cold waters and is most common in North Atlantic subpolar and polar water masses

(McIntyre and Bé, 1967). It forms the largest (8–16 μm) and most robust coccoliths of any common species, and, due to its exceptionally high preservation potential, is identified easily both in the form of coccospheres from water column samples and in the form of coccoliths from sediment samples (Cachão and Moita, 2000).

The knowledge based on investigations and analysis gathered over the past 50 years reveals that the complexity of the coccolithophores requires a multidisciplinary research approach in order to generate a fully reliable and high-quality scientific product with an impact on science and society. Several major research projects have demonstrated that approaching a single coccolithophore species and focusing on it from different scientific perspectives represents great potential for understanding this plankton group in general (Quinn *et al.*, 2004). Among others are the GEM (Global *Emiliania* Modelling initiative), EHUX (coccolithophorid dynamics: the European *E. huxleyi* programme) and CODENET (coccolithophorid evolutionary biodiversity and ecology network). The latter has applied a multidisciplinary research philosophy to six key taxa: *Gephyrocapsa* spp., *Calcidiscus leptoporus*, *C. pelagicus*, *Umbilicosphaera sibogae*, *Helicosphaera carteri* and *Syracosphaera pulchra* (Quinn *et al.*, 2004).

The successful selection of a coccolithophore strain with commercial potential is preceded by cultural studies revealing whether the morphological differences are stable in cultures. The consistent exhibition of different morphologies, when two monoclonal cultures are grown under similar conditions, is the basic evidence for genotype predefined morphological differences. Further investigations involve the degree of morphological variation within monoclonal strains grown under varying conditions – estimating the degree of ecophenotypic variation occurring within a single genotype (Geisen *et al.*, 2004). Strain selection is a time- and effort-consuming process that requires knowledge involving modern ecology, geological data and fossil history, biogeographic distribution, and finally determination of the optimal growth conditions of the species in culture and the degree of variability in growth optima between strains. Most of the information regarding

coccolithophores comes from observations on cultured materials, revealing that coccolithophores are much more demanding and difficult to maintain than non-mineralized taxa. The number of coccolithophore species maintained successfully in culture around the world is limited. In 1997, less than ten species were reported (Jordan and Chamberlain, 1997), and in 2004, this number was increased to 35 species (Probert and Houdan, 2004).

Optimal conditions and optimization potential

The inconsistency between the number of coccolithophores inhabiting the world's oceans and the ones grown and maintained successfully in laboratory culture conditions suggests significant optimization potential in the philosophy of culturing coccolithophores. The simplified difference between the ocean and the laboratory supposedly involves the presence of unknown growing factors and environmental conditions that benefit the occurrence of coccolithophores in the form of extensive blooms and their stable geological presence for more than 200 million years. The definition of optimal conditions for each calcifying algae species is a combination between observations of the ecological processes and conditions in the habitat and data generated in laboratory situations.

In an extensive analytical study reviewing the sensitivity of phytoplankton to climate and environmental changes, Rost *et al.* (2008) outline the research priorities and directions for representative laboratory and field investigations. The suggested exploratory principles include mimicking of the environmental conditions as much as possible; the implication of multiple environmental factors (for example testing CO_2/pH effects in combination with other factors); a process-based understanding – empirical relationship between growth conditions and phytoplankton response; diversity in responses – testing more and different species; and since mono-specific laboratory cultures lack interactions within trophic levels, the assessment of community responses by on-deck perturbations is regarded as substantial (Rost *et al.*, 2008).

Let us consider again *E. huxleyi* and the reasons why it is the most useful and preferred experimental marine organism. *E. huxleyi* grows easily in a variety of media under a broad range of cultural conditions – a reflection of its almost ubiquitous geographic presence (Jordan and Chamberlain, 1997). Observations and models reveal that most *E. huxleyi* blooms appear to be induced by or have benefited from a combination of thermal stratification and high irradiance in phosphate-poor environments with an excess of nitrate nitrogen (Paasche, 2002). This generalization is confirmed and broadened by Iglesias-Rodriguez *et al.* (2002) by confining the blooms of *E. huxleyi* to nutrient-depleted temperate and high-latitude oceans with relatively high critical irradiances. As already mentioned, both nature blooms and successful laboratory coccolithophore cultivations with increased coccolith production are a result of a combination of favourable factors. In the following, we will outline the importance and influence of the single factors.

Light

Even without any knowledge regarding coccolithophores, their affiliation to the group of marine algae is enough to indicate the essential, life-dependending importance of light for their growth and existence. Summarizing more than 50 years of field studies of coccolithophore blooms illustrates that all of them are confined to the top 10–20 m layers and they are very rarely deeper than 30 m from the ocean's surface in stable stratified waters (Zondervan, 2007). The first hypotheses that *E. huxleyi* required high light conditions were subsequently supported by abundant examples of irradiance values of more than 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during blooms in shallow mixed layers (Nanninga and Tyrrell, 1996). The versatility of *E. huxleyi* is demonstrated by the fact that this species also grows well under low irradiance, with growth rates ranging between 0.5 day^{-1} at 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ up to 1.1 day^{-1} at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Zondervan *et al.*, 2002) and light saturation at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Nielsen, 1997). Opposite to many phytoplankton species, *E. huxleyi* demonstrates no photoinhibition,

even at very high irradiances of up to 1700–2500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (as a comparison, the irradiance at the sea surface at noon under clear skies equals 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Nanninga and Tyrrell, 1996; Zondervan, 2007). These observations, along with the midsummer occurrence of the blooms, suggest that these algae preferably grow at high irradiance (Baumann *et al.*, 2000). Calcification in coccolithophores is an energy-requiring, active process, which qualifies it also as a light-dependent process. Laboratory and field experiments demonstrated the strong dependency of calcification on irradiance (Paasche, 2002). Zondervan *et al.* (2002) achieved in laboratory conditions a 32% increase of the cellular CaCO_3 content over a photon flux range of 15–150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, while Paasche (1999) reported even doubling of cellular calcite with an increase of irradiance from 8 to 330 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The positive effect of higher irradiance on coccolith formation was also observed in nature, where calcification rates were higher in the surface layers and decreasing in the subsurface layers (van der Wal *et al.*, 1995).

The light requirement in the process of calcification could be universal for all coccolithophores, as suggested by the strong light dependency observed in coccolith formation in *C. pelagicus* and *P. carterae* (Paasche, 2002). Nevertheless, calcification is still less light dependent compared to photosynthesis, saturating at lower irradiance than photosynthesis (van der Wal *et al.*, 1995; Paasche, 2002), with saturation levels for *E. huxleyi* between 50 and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Paasche, 1999). This partially explains the ability of *E. huxleyi* to form coccoliths also in the dark, with calcification rates of about 10–15% of the light-saturated rate (Sekino and Shiraiwa, 1996). All the evidence leads to the conclusion that light intensity is one of the most important factors, but still one of several simultaneously acting factors, favouring coccolith formation.

Nitrate and phosphate

A general perception is that coccolithophores outnumber other phytoplankton as a result of their ability to grow in ocean waters, and respectively in media, with poor nutrient contents.

This consideration is more than ever valid when it comes to phosphate and nitrate concentrations. Verification of the importance of this factor again proceeds on two levels: it begins with field observations and is confirmed by laboratory experiments. Blooms of *E. huxleyi* almost always occur in areas where nitrate and/or phosphate concentrations are very low (Iglesias-Rodriguez *et al.*, 2002; Tyrrell and Merico, 2004; Zondervan, 2007). In a field study in the Gulf of Maine, Balch *et al.* (1991) established that the ratios of coccoliths to coccolithophores were highest in the centre of the blooms, where the oldest areas with completely exhausted nutrients were. To the contrary, these ratios were lowest in the periphery of the blooms, new areas with fresh nutrients and actively growing cells. In nature, it is more likely that factors other than nutrient concentrations or their ratio, for example light and temperature, induce algae blooms in combination with the above mentioned. Although some authors dispute the association between high N:P ratios (P more limiting than N) and the occurrence of coccolithophore blooms in nature (Lessard *et al.*, 2005), phosphate and nitrate have a clear effect on the regulation of cellular calcification and organic C production (Paasche, 2002).

In culture conditions, studies on the impact of nutrient concentrations on calcification have revealed that reduced levels of both P and N increase the number of coccoliths produced per cell of *E. huxleyi* (Paasche, 1998). P limitation, at lower growth rates, gives rise to higher levels of organic and inorganic C in the cells, and these cells also express the highest coccolith coverage of the cell surface (Riegman *et al.*, 2000). Additionally, P limitation increases the size of the cells of *E. huxleyi* (organic C content) – a phenomenon not observed under N limitation (Riegman *et al.*, 2000; Shiraiwa *et al.*, 2003). The N or P limitation also influences individual coccoliths. N limitation leads to a decrease in the calcium content of a single coccolith, as well as its length, while P limitation demonstrates the opposite effect (Paasche, 1998). In general, coccoliths produced under N limitation appear to be undercalcified, and those produced under P limitation overcalcified (Paasche, 1998).

Silicate

The low levels of silicate are a beneficial factor for the growth of coccolithophores in nature (Tyrrell and Merico, 2004). *E. huxleyi* is a fast-growing species, multiplying more rapidly than most other species. Diatoms, on the other hand, are the fastest growing of all phytoplankton and dominate in environments such as upwellings, river mouths, spring blooms and oceanographic fronts. The prerequisite condition for the diatoms' dominance is sufficient dissolved silicate. In order to compete with diatoms, *E. huxleyi* blooms only in areas with depleted silicate and does not occur in high nutrient, low chlorophyll regions, which are also rich in dissolved silicate throughout the whole year (Fasham *et al.*, 2001).

Carbon dioxide

The chemical reaction for calcification (Eqn 13.1) suggests that the synthesis of calcite coccoliths is followed by the formation of CO₂, available for photosynthesis. The generation of CO₂ from HCO₃⁻ is an advantage for coccolithophores over other phytoplankton when the rate of supply of external CO₂ is limiting for growth (Tyrrell and Merico, 2004).



The dissolved inorganic carbon (DIC) consists of about 90% bicarbonate (HCO₃⁻), 10% carbonate (CO₃²⁻) and less than 1% dissolved carbon dioxide (CO₂(aq)). By the depletion of CO₂(aq), its concentration is compensated for by a quick (within seconds) conversion of HCO₃⁻ to CO₂(aq), and this transition between different dissolved carbon forms provides the necessary CO₂(aq) concentration at all times (Zeebe and Wolf-Gladrow, 2001). A real CO₂(aq) shortage can occur after exhaustion of all DIC or a significant change in pH.

Coccolithophores obtain the carbon necessary for the synthesis of coccoliths from bicarbonate, not from carbonate (Paasche, 2002), but it appears that they are sensitive to carbonate ion concentrations, just like other marine calcifiers such as foraminifera and coral reefs (Tyrrell and Merico, 2004). Coccolithophores in laboratory cultures and

ocean samples subjected to low carbonate saturation state conditions produce malformed coccoliths with declined calcification rates (Riebesell *et al.*, 2000; Rost and Riebesell, 2004).

Recently, issues such as the increasing CO₂ emissions and ocean acidification have put forward the question of the response of coccolithophores, and phytoplankton in general, to the altered ocean chemistry (Rost *et al.*, 2008; Beaufort *et al.*, 2011). Experiments with the morphologically close species *E. huxleyi* and *Gephyrocapsa oceanica* have demonstrated that increased pCO₂ results in reduced calcification on a per-cell basis (Riebesell *et al.*, 2000; Zondervan *et al.*, 2001, 2002). Further experiments on the effects of the elevated CO₂ on three different coccolithophore species reveal little effect on *C. pelagicus* calcification, a CO₂/pH optimum for *C. leptoporus* with a maximum value of present-day CO₂ levels, and decreased calcification with elevated CO₂ for *G. oceanica* (Langer *et al.*, 2006; Mackinder *et al.*, 2011). The conclusion suggests once again that strain affiliation together with growth conditions such as light intensity, temperature, nutrient availability and mixing are the combined factors that determine the responses of coccolithophores to elevated CO₂ (Ridgwell *et al.*, 2009).

Trace metals

Over the years, a multitude of investigations have demonstrated that coccolithophores require metals such as iron (Fe), zinc (Zn), cobalt (Co), selenium (Se), cadmium (Cd) and manganese (Mn) for their metabolism (Muggli and Harrison, 1997; Danbara and Shiraiwa, 1999; Zondervan, 2007). *E. huxleyi* has the ability to replace Co with Zn and vice versa, which is not common for most algae species (Timmermans *et al.*, 2001). This flexibility gives coccolithophores an advantage in populating different ecological niches. It is also extended by the lower Fe requirement, compared with diatoms, allowing coccolithophore distribution in parts of the world's oceans where diatoms are limited by Fe (Muggli and Harrison, 1997). Although some evidence suggests the occurrence of the Zn limitation of coccolithophore growth in the subpolar North Pacific (Crawford *et al.*, 2003),

in general the distribution of total dissolved and free Zn is even less constraining than that of free Fe (Zondervan, 2007). The Zn requirement of *E. huxleyi* with regard to calcification is lower compared to other phytoplankton species (Sunda and Huntsman, 1995; Schulz *et al.*, 2004). Similar observations have been collected regarding the trace metal Mn (Muggli and Harrison, 1996).

Other factors

Several reviews, based on environmental observations, define as necessary conditions for the growth of coccolithophores cold waters with low nutrient concentrations in upwelling areas, near the coast or in shallow seas (Young, 1994; Tyrrell and Merico, 2004). Large blooms usually follow those of diatoms in waters depleted in organic nutrients and stable in terms of vertical mixing (Holligan *et al.*, 1993b). Opposite to many algae, *E. huxleyi* requires thiamine (vitamin B₁) for its growth but does not require vitamin B₁₂, which is a possible reason for the absence of *E. huxleyi* during the first stages of spring blooms (Paasche, 2002).

Neither temperature nor salinity appear to be of great significance for the growth of coccolithophores (Paasche *et al.*, 1996; Paasche, 2002; Tyrrell and Merico, 2004). Although cold stress and low temperatures stimulate cell enlargement and intracellular calcification by the coccolithophores *E. huxleyi* and *G. oceanica* (Sorrosa *et al.*, 2005; Satoh *et al.*, 2009), as other observations suggest (De Bodt *et al.*, 2010), these effects could be due to the secondary effects of temperature, in particular the impact on water stability.

Cultivation media and strategies

In order to make the whole process of coccolith production feasible in laboratory conditions and attractive for industrial implementation, not only a recreation of the environmental factors should be performed but also a rational improvement and optimization, regarding technological aspects like yields, costs, substrate availability, etc., should be the priority task in front of bioprocess engineers. The limited

number of coccolithophore species that are maintained easily in laboratory cultures also reflects the number of popular culture media used. Most phycologists still prefer the application of natural seawater for it is sometimes the only reliable source of natural growing factors and a unique combination of nutrients. After decades of research and observation, scientists have developed media based on natural seawater with selected and verified additives (usually trace elements, vitamins and buffers) in order to achieve reproducible and improved growth kinetics. These are the so-called enriched media. For the growth of coccolithophores and other phytoplankton, the most common and widely used general enriched seawater media are the f/2 medium (Guillard, 1975) and the K medium (Keller *et al.*, 1987). The application of such modified seawater-based media still hides some of the drawbacks of natural seawater, namely supply problems and seasonal variability in quality and composition. For over a century, scientists with different backgrounds have been developing artificial media, and one of the most popular is designated as enriched seawater, artificial water (ESAW), possessing an ionic balance closest to natural seawater (Berges *et al.*, 2001). ESAW medium has also undergone different modifications over the years and its relevance has been verified for almost 100 different microalgae, demonstrating better performance compared to natural seawater in about 80% of cases (Berges *et al.*, 2001). The comparative evaluation of ESAW against natural seawater, including different coccolithophore species, was based on specific growth rates, cell number and cell volume, and maximum fluorescence. Experiments with *E. huxleyi* have demonstrated higher growth rates and final yield in ESAW (Berges *et al.*, 2001).

In processes oriented towards the production of structured particles from coccolithophores, it is essential to achieve a high yield on those particles, i.e. coccoliths. There are two possible approaches to ensure this: to provide a high final biomass yield and to increase the number of coccoliths per cell. The ultimate would be a combination of both.

A condition for the establishment of commercially successful processes with algae is the ability to grow the cultures in a continuous or

semi-continuous operational mode for long periods (Moheimani and Borowitzka, 2006, 2011). Batch and semi-continuous cultivations of coccolithophore species including *E. huxleyi*, *P. carterae* and *G. oceanica* in both open and closed systems show absolutely no correlation or general tendencies. The behaviour of the coccolithophores and their successful growth, or the lack of it, remain exclusively strain specific (Moheimani and Borowitzka, 2006; Moheimani *et al.*, 2011). Interesting exploitation of the concept of semi-continuous cultures with coccolithophores has delivered promising results that could lead to progress towards the industrial production of nanostructured calcite particles from algae (Krumov and Posten, unpublished results). The objective in the investigations of Krumov and Posten was the synthesis of relevant high amounts of coccoliths, a precondition for further characterization and application oriented experiments. The biosynthetic process was regarded as a two-stage process. The first stage aimed at the generation of a high algal biomass by providing optimal conditions. The second stage was directed towards the induction of more active and prolonged coccolith synthesis. The two stages were differentiated by the exchange of the culture media. Experiments in shaking flasks and plate photobioreactors involved the species *E. huxleyi* and *P. carterae*. After an extensive selection, ESAW was defined as an appropriate first-stage medium (growth stimulating). This medium ensured specific growth rates for *E. huxleyi* of 0.7 day^{-1} and maximum cell concentration of $7 \times 10^6 \text{ cells ml}^{-1}$ in shaking flasks and $7 \times 10^5 \text{ cells ml}^{-1}$ in plate photobioreactors, and for *P. carterae* respectively specific growth rates of 0.5 day^{-1} and maximum cell concentration of $1 \times 10^6 \text{ cells ml}^{-1}$ for both shaking flasks and plate photobioreactors. The selection for second-stage medium (coccolith synthesis stimulating) distinguished Red Sea Salt (RSS) (Red Sea Fish Pharm Ltd) as most promising. It is a commercially available medium, originally designed for coral growths in aquariums and containing balanced natural levels of elements and salts, harvested from the Red Sea Reef (Table 13.1). This medium had a stimulating effect on coccolith synthesis; this effect was more explicit in the results obtained from the experiments with *E. huxleyi*.

Table 13.1. The effect of different media on coccolith formation by *Emiliana huxleyi* in shaking flask conditions (Krumov and Posten, unpublished results).

Medium	ESAW	TMSS	TMPR	RSS
CaCO ₃ (g l ⁻¹)	0.56	0.58	0.56	0.62
CaCO ₃ (g cell ⁻¹)	1.06·10 ⁻¹⁰	2.6·10 ⁻⁹	4.62·10 ⁻⁹	4.67·10 ⁻⁹
Coccolith wt (g)	2.12·10 ⁻¹¹	2.34·10 ⁻¹¹	3.21·10 ⁻¹¹	1.48·10 ⁻¹¹
Coccoliths (cell ⁻¹)	18	88	86	143

Notes: Media: ESAW = enriched seawater, artificial water; TMSS = tropic marin sea salt; TMPR = tropic marin pro reef; RSS = red sea salt.

The use of RSS medium only increased the number of coccoliths per cell in *E. huxleyi* from 20 up to 150, related to produced and detached coccoliths per cell. It stimulated active and prolonged (even after 50 days of cultivation) coccolith production and resulted in the formation of calcium carbonate microparticles with ideal structure and form.

The prolonged and increased coccolith production achieved demonstrates a new promising characteristic for the cells of *E. huxleyi*, as opposed to previous perceptions that considered the loss of coccolith production as irreversible (Paasche, 2002). The relatively low final cell concentrations 5×10^5 cells ml⁻¹ from cultivations based only on RSS medium predetermined the innovative implementation of the first stage including ESAW medium. The introduction of a semi-continuous process in plate photobioreactors, with exchange of the medium (from ESAW to RSS), increased cell concentrations, increased coccolith yield by 200% and secured continuous coccolith production (Krumov and Posten, unpublished results). This approach could be effective in large-scale situations, achieving both increases in biomass and coccolith yields, and is facilitated by the existing and constantly improving equipment for efficient algae cultivations.

Technical equipment towards successful larger-scale coccolithophore cultivation

Microalgae biotechnology is one of the newest modern and dynamic developing biotechnology branches. Clear evidence of this is the evolution in commercial microalgal production from relatively unsophisticated open-

air culture systems towards highly efficient closed photobioreactors, a process still in progress with constantly emerging, new revolutionary reactor concepts and geometries. The fundamental starting point for the optimization of photobioprocesses is a detailed understanding of the interaction between the bioreactor in terms of mass and light transfer, as well as microalgae physiology in terms of light and carbon uptake kinetics and dynamics.

What makes algae very attractive and desired research objects is their 5% energy efficiency and their carbon and energy neutrality, defining them as very effective systems allowing the synthesis of products and biomass in large amounts for an achievable cost. Production of microalgae biomass has, up to now, been limited to a few thousand tonnes per year, mostly produced in open ponds. Only a few hundred tonnes are produced in closed photobioreactors (Posten, 2009). The advantages of photobioreactors are quite clear: they offer cultivation under a wide variety of conditions and prevent, to some extent, outcompeting of the production strain by other algae or contamination with undesirable microorganism or grassers. The main benefits of closed bioreactor systems include higher areal productivities and the prevention of water loss by evaporation. After the assessment published by Borowitzka (1999), closed photobioreactors have undergone continual development through a process driven not only by experience but also by targeted engineering. Different geometries and the operating methods developed depend on local conditions, the product to be produced and economic constraints.

The typical photobioreactor is a three-phase system, namely the liquid phase, which

is the medium, the cells, as the solid phase, and a gas phase. Light, which is the unique feature of photoreactors, is a superimposed radiation field, sometimes also regarded as a fourth phase. The design of an adapted photobioreactor requires an understanding of the interaction between the environmental parameters and the biological response.

Despite the fact that the calcifying algae (mostly *E. huxleyi*, *P. carterae* and *G. oceanica*) are widely distributed in the world's oceans, until recently their growth was possible only on a laboratory scale (Moheimani and Borowitzka, 2006). Moheimani and Borowitzka (2006, 2007) have demonstrated the feasibility of larger-scale continuous or semi-continuous processes with coccolithophores. They have investigated the influences of different factors (physical, biotic and operational) on the productivity of outdoor cultures. Here again emerged the general tendency observed for coccolithophores, namely that the species affiliation is the main factor that determines the success of the cultivation processes. Long-term culture of *P. carterae* was successful in an outdoor raceway pond for a period of more than 10 months, demonstrating total areal productivity ranging from 16 to 33.5 g dry wt m⁻² d⁻², with mean production <20 g dry wt m⁻² d⁻², while culture of *E. huxleyi* subjected to the same conditions appeared to be unstable and impossible to maintain as continuous culture (Moheimani and Borowitzka, 2006). Further investigations with *P. carterae* have demonstrated a productivity improvement potential by adjusting the pond depth or the cell density in the pond in order to optimize the availability of light. In this case, the growth inhibition factor was the high concentrations of O₂ in the pond during the day, with inhibition also increasing with increasing temperature. The low morning temperatures, which is one of the problems in outdoor raceway ponds, was handled by heating the ponds in the morning by 3–5°C, increasing productivity by 11–21% (Moheimani and Borowitzka, 2007). Besides low productivity and the appropriate climate requirements, representing some of the limitations of the open pond's algal biomass production, a considerable additional problem is contamination by bacteria, other algae and/or protozoa (Borowitzka, 1999; Moheimani and

Borowitzka, 2006). In some commercial algal open cultures, like, for instance, *Dunaliella salina*, *Spirulina* spp. or *Chlorella* spp., the risk of contamination is not an issue, mainly because of the high salinity, alkalinity or nutrient content of the medium beneficial to the growth of these particular algae species (Borowitzka and Borowitzka, 1990). These algal species are grown in highly selective environments, enabling growth in open-air conditions without significant contamination. Species that do not possess this selective advantage are therefore grown in closed systems. *P. carterae* cultures grown in raceway ponds are characterized by an unusually low degree of contamination, secured by the increase of the pH to about pH 11 during the day and the production of chemical defence compounds by this coccolithophore (Moheimani and Borowitzka, 2006). The effective chemical defence system is regarded as the main reason for the lack of protozoans in the culture. The high toxicity of some coccolithophores, including *P. carterae*, towards other species of microalgae has already been observed (Houdan *et al.*, 2004). This phenomenon is common for Haptophytes and is detected in the final stages of ocean blooms, when stressed and dying cells produce a number of extracellular products (bloom products), which may be harmful to other species (Jordan and Chamberlain, 1997).

As already outlined, not all coccolithophores (also valid for algae in general) are suited for growth in open ponds. Sometimes, the only way to provide the necessary conditions is cultivation in closed photobioreactors. Despite the fact that they isolate the culture from the environment and secure axenic conditions, closed photobioreactors guarantee reproducible results, precisely controlled conditions and multiple parameter regulation, better opportunities for automatization, less evaporation, no unwanted dilution, and independency from climate and the environmental situation. Closed systems are much more sophisticated, a natural evolutionary step in applied microalgology, and are regarded as the future of microalgal mass production. Naturally, they also express limitations, based mostly on high investment and operational costs, which represent challenges for bioprocess designers. The first reports of

the successful implementation of closed systems for the cultivation of coccolithophores only became fact in the past 2 years. Moheimani *et al.* (2011) investigated the applicability of different types of closed photobioreactors for the growth of *E. huxleyi*, *P. carterae* and *G. oceanica*. The results once again confirmed that the behaviour and response of the coccolithophores were exclusively strain specific. The photobioreactors used in the study of Moheimani *et al.* were plate, carboy, airlift and tubular. All the species tested were cultivated successfully in the plate and carboy photobioreactors; only *P. carterae* was adapted for growth in an airlift reactor, and no species sustained the conditions in the tubular photobioreactor (Moheimani *et al.*, 2011).

Flat-plate reactors are surely the ones with the most simple and robust design (Fig. 13.2). Roughly speaking, two sheets have to be glued together to make a flat-plate reactor with any desired light path length (d) in the range from a few millimetres up to 70 mm, resulting in a surface/volume ratio (SVR) = $1/d$ for one single plate and about 50 m^{-1} for practical installations (Posten, 2009). Mixing and CO_2 supply is accomplished

by sparging with CO_2 -enriched air. Agitation only by bubbles seems to be the gentlest way with respect to shear stress to the algae. Flat-plate photobioreactors have been employed for decades, have a potential for scaling up and have shown high productivities for some algae species (Moheimani *et al.*, 2011).

Although not designed for scaling up, carboy photobioreactors are regarded as a gentle system for culturing microalgae and have been employed successfully in culturing a wide range of algae (Moheimani, 2005). The lower shear environment in both plate and carboy photobioreactors, together with the different mixing regimes in carboy photobioreactors, a combination of stirring bar and gentle aeration, justify the better growth of all the coccolithophore species tested in these two photobioreactors (Moheimani *et al.*, 2011).

A higher sensitivity towards shear could explain the lack of growth of *E. huxleyi* and *G. oceanica* in airlifts. The turbulent environment in this type of photobioreactor causes a high level of dead cells among the two coccolithophores mentioned, and the lower specific growth rate and productivity of *P. carterae*



Fig. 13.2. A system of mini flat-plate photobioreactors with LED illumination used for the cultivation of *Emiliania huxleyi* and *Pleurochrysis carterae* in the Institute of Bioprocess Engineering, Karlsruhe Institute of Technology, Germany.

define this type of photobioreactor as not applicable for cultivation of these species (Moheimani *et al.*, 2011).

Tubular reactors consist of transparent tubing arranged in parallel lines coupled by manifolds, the so-called solar collector. The single tubes can be straight: they can follow a meandering course either flat on the ground or ordered in panels or coils. The tubes have diameters of 10 mm to a maximum of 60 mm and lengths of up to several hundred metres. The employment of tubes leads to a quite high surface-to-volume ratio (SVR) over 100 m^{-1} , which is one of the main advantages of this design (Posten, 2009).

A basic requirement for mixing is to prevent the microalgal cells settling. The main sources of cell damage are the increased liquid velocities during mixing and bubble damage in sparged photobioreactors. Shear produced by turbulence or bubble rise from a sparger affects the coccolith layer surrounding the cells and leads to loss of flagellae and eventually cell disruption (Moheimani, 2005), which also explains the lack of coccolithophore growth in the tubular photobioreactor.

The design of photobioreactors on a commercial scale has made good progress in the last decade, enabling the substitution of the ocean with highly defined, controlled and improved conditions. The basic principles have been developed extensively into designs with relatively high efficiencies. Suitable process engineering calculation methods have been published to give a quantitative understanding of mass and light transfer. The rapid increase of interest and investment in algae for the production of biodiesel, biomethane or hydrogen has brought additional considerations and demands to photobioreactor design. Innovative concepts include the use of biodegradable one-way reactors or involve highly structured, intelligent production systems. Even high-tech materials could become cheap when needed in really large amounts. A key parameter for the achievement of this goal is the fine-tuning of the conditions inside the reactor with respect to the kinetics and dynamics of the respective cells (Posten, 2009). Finally, the concerns that mass algae production requires large, valuable land areas could be

neutralized by the utilization of areas for which no other claims exist, for example deserts or brackish water districts.

Calcite – Properties and Opportunities

There is no doubt that the most characteristic feature of coccolithophores is the unique and sophisticated spatial structures that surround their cells – the coccoliths. Although the structures are the ones that first attract the attention of the scientist, not less important is the material that composes the coccoliths – calcite. To know the properties of this material is useful on one hand for a complete understanding of the biomineralization process in the calcifying algae and on the other to create a spectrum of the possible applications of these calcite bioparticles.

Calcium carbonate (CaCO_3) is one of the most abundantly occurring minerals on our planet, building more than 5% of the lithosphere and present mostly in the form of sedimentary rock (Geysant, 2001). Apart from its cosmopolitan geological importance, the presence of CaCO_3 and the formation of biominerals in mammals, birds, crabs, shells, eggshells, etc., have had an evolutionary significance for life on earth.

Calcite is one of the crystal forms of calcium carbonate. There are six calcium carbonate minerals with the same principal composition but different structure: calcite, aragonite, vaterite, calcium carbonate monohydrate, calcium carbonate hexahydrate and amorphous calcium carbonate. From all these polymorphs only calcite and aragonite are thermodynamically stable (Mann, 2001). Calcite is a very commonly occurring mineral with trigonal symmetry. Its crystals and aggregates have a variety of habits with colourless through to a milky white to grey colour or, as a result of impurities, the crystals could obtain a yellowish, pink, red, blue, green, brown or black nuance. In nature, the transparent, colourless form is very rare. In the crystal structure of calcite, Ca^{2+} ions can be replaced by different divalent ions, for instance Ba^{2+} , Co^{2+} and Zn^{2+} (Geysant, 2001).

Magnesium ions are accommodated readily in the calcite lattice; therefore, many biological calcites also contain Mg^{2+} ions up to levels of 30 mol% (Mann, 2001). Calcite exhibits several hundred different forms and is regarded as the most form-abundant mineral. The single crystal surfaces are free to create different shapes, as long as the angle between them always remains at 105 and 75 degrees. The basic rhombohedra form is quite rare among naturally occurring calcite; however, it appears as a slit form of most calcite crystals. Most common are the hexagonal prisms and scalenohedra (Geysant, 2001).

Every non-cubic, transparent mineral is characterized by two refractive indexes and exhibits the phenomenon of double refraction or birefringence. These minerals fragment in two on every pass through a crystal beam of light. Birefringence was first described in 1669 by the Danish scientist, Rasmus Bartholin, by examining calcite in particular. Calcite has an anisotropic crystal structure, which means that certain properties of the crystal depend on the direction from which they are determined. In addition, calcite polarizes the light passing through the crystal, so it oscillates in one direction only, perpendicular to the propagation direction (Geysant, 2001). Practical application of this phenomenon is the Nicol prism – a polarizer, optical device used to produce a polarized beam of light from an unpolarized beam, and used in polarization microscopes.

Every crystal lattice is characterized by a defined spatial configuration of its atoms. Every deformation caused by external force generates tension in the crystal lattice, which eventually leads to the appearance of unusual phenomena. Even pressing calcite crystal together with the fingers is sufficient to create a positive electric charge (Geysant, 2001). In normal conditions, with no impact of external forces, the configuration of the atoms in the crystal solely defines physical properties such as, for instance, hardness. Calcite has a defining Mohs hardness of 3 (for comparison, quartz has 7 and diamond has 10) and a specific gravity of 2.71 g cm^{-3} .

Occasionally, calcite demonstrates phosphorescence and fluorescence. Most of its properties have a physical background, such

as, for example, the blue colour of the crystals, which disappears after grinding the crystals into powder and is substituted by a standard white colour (Geysant, 2001).

For industrial purposes, calcium carbonate is obtained mainly by mining or quarrying, or as a result of chemical precipitation from calcium hydroxide and carbon dioxide, the so-called precipitated calcium carbonate (PCC).

Minerals are essential ingredients in almost all raw materials, substances and consumer goods. Calcium carbonate occupies a central position among all the minerals, as no mineral could be as multifunctional as CaCO_3 , with applications ranging from fillers and paints to medicine and nutrition. Probably the most popular industrial incarnation of calcium carbonate is in the form of filler or coating pigment in the paper industry. A fact surprising to many people could be the mineral content in paper, which reaches up to 40%. The main qualities required from a good filler in the paper industry are low price, white colour, insolubility in water and extremely fine dispersion. Fillers define the abrasion profile, the degree of whiteness, the surface qualities and act as a form-supporting factor. No less important is the role of calcium carbonate as a coating pigment in the finishing and refining stages of paper production, giving the product the desired gloss, structure, elasticity or predefined effects. Annually, more than 10 million t of calcium carbonate products (mainly natural powdered calcium carbonate) are being used in the production of paper (Naydowski *et al.*, 2001).

Calcium carbonate is introduced in the form of natural filler for the rapidly growing plastic and synthetic materials industry and market. Its applications range from form giving or reinforcing agent to special surface modifier. It is an important ingredient, improving the impermeability of packing materials and their heat conductivity. Calcium carbonate is involved in the production of thermoplastics, duroplastics, glues and sealing compounds, dyes and varnishes (Naydowski *et al.*, 2001).

A more traditional application of CaCO_3 , which only demonstrates its versatility, is in the field of agriculture and forestry. CaCO_3 acts as a lime fertilizer and increases the yield

of crops. Additionally, it improves soil structure and, more specifically, its compactness, porosity, field moisture capacity, integrity and stability. Calcium and magnesium are used by many plants and crops as nutrients, stabilizing cell walls, increasing their elasticity and stimulating cell division and elongation. In forestry, liming or the addition of CaCO_3 has a fertilizer effect, mobilizes the nutrients in the soil and accelerates the conversion of surface humus (Naydowski *et al.*, 2001).

Other interesting fields of application for CaCO_3 are in animal feed, ecology, medicine, cosmetics and nutrition. Nowadays, calcium is introduced in medical therapy for remineralization after disturbed calcium metabolism or transmineralization after inflammatory processes, allergic reactions, haemorrhage and disorders of the vegetative nervous system. As a nutrient supplement, it is recommended during pregnancies, lactation and growth. Calcium carbonate is well known in cosmetics and has been used for centuries in powders, make-ups, soaps, talc, depilation creams and toothpastes (Naydowski *et al.*, 2001).

When considering different applications for coccolith particles, an important issue needing consideration is the solubility of calcium carbonate. It depends on three major factors: temperature, pH and CO_2 partial pressure. In general, CaCO_3 is a stable chemical compound with solubility that increases with the increase of CO_2 partial pressure and the decrease of temperature and pH.

It has already been stated that not only the chemical composition but also the form and the structure of the products are of significant importance for their application. Considering that, for many applications, size and size distribution, form and spatial conformation of the particles are essential, the mass introduction of coccoliths, with their unique structures, in various industry branches is only a matter of time.

Next Generation Technology and Applications

If we subscribe to the laws of dialectical materialism, and more precisely the law of the transformation of quantitative into qualitative

changes, today's science is probably standing on the threshold of a significant discovery, a qualitative change, regarding the perception and knowledge of coccolithophores. Indeed, the past 50 years or more have been marked by the cumulative gathering of information, evidence, samples and species. New hypotheses have been proposed, others overruled, models of the calcification mechanisms and the structures themselves have been created, and finally a clearer vision about the taxonomic position of coccolithophores has been established. The industrial cultivation of coccolithophores is still a project that requires maturation, but advances in bioprocess engineering and precisely controlled laboratory cultures have already given the first promising results. Although the conventional biotechnological approach has not revealed its entire potential, other even more sophisticated approaches are about to cross the line between vision and innovation. Today's science allows us to go further, deeper and closer, and to carry out experiments which 20 years ago were only a fantasy. From modern biotechnology emerges a new basis technology – the concept of cell-free processes. It is expected to overcome the limitations of classic biotechnology. For many new products, the limitations are connected directly to the microorganisms that produce them. Undesired by-products, product inhibition, low productivity, incomplete functionality of the product and depletion of optimization potential are some of these limitations. The transfer of the cellular metabolic pathways from the cell into a free solution is a road towards solving many of them. It is easy to imagine the *in vitro* consolidation of corresponding enzymes, building a whole reaction cascade in a single metabolic pathway in order to enable the continuous synthesis of a desired product. Despite constant progress in the field of molecular engineering, there are presently only a limited number of known cell-free processes that contain more than three metabolic steps (Resch *et al.*, 2010). Probably, cell-free processes present more challenges and problems than expected, and then the logical question would be why cell-free biomineralization and why based on calcification in algae? Above all is the uniqueness of biomineralization itself – a process that is a bridge between the organic and inorganic worlds, an intersection where biology meets

materials science and chemistry meets physics. An addition of bioprocess engineering, system biology and modelling to the above-mentioned palette of sciences gives us the necessary tool to conduct cell-free biomineralization.

What make the process of cell-free synthesis of coccoliths possible are the basic knowledge and validated technologies regarding calcification in general, techniques allowing the insertion of synthetic molecules in membranes, supported lipid bilayers, and the *in vitro* technical use of complete vesicles or cell compartments, for example chloroplasts or membrane proteins (Bionda *et al.*, 2010; Breyton *et al.*, 2010). Some preliminary works reveal models of calcium carbonate biomineralization (Mukkamala *et al.*, 2006) and the chemical assembly of coccolith-like structures built from nanocrystalline calcite (Mukkamala and Powell, 2004). Calcite crystals with structure and mechanical properties similar to those of biominerals have recently been formed by the incorporation of copolymer micelles in calcite crystals (Kim *et al.*, 2011).

The introduction of a cell-free approach in the synthesis of artificial coccoliths could open new opportunities regarding product modification, element substitutions and structure manipulations that would allow the production of tailored, highly structure particles with the desired properties corresponding to industry requirements. That would allow calcite nanostructured particles to enter application in high-tech fields such as the semiconductor and laser industry, optics, liquid display industry, efficient drug delivery, ultra-fine surface modifications, high-quality special papers (for example, photo paper), self-cleaning surfaces, etc.

Conclusions

Coccoliths are one of the most amazing demonstrations of nature's resourcefulness when it comes to creating sophisticated structures. Indeed, these fine nanostructured particles are the result of a precise and elaborate biomineralization process perfected over millions of years of evolution. This process in the calcifying algae has been of interest to scientists not just because of the unique structures of the coccoliths or their properties and possible applications. Furthermore, understanding the driving mechanisms of this biomineralization process will facilitate the establishment of novel synthesis pathways, allowing the modification of particles' form and structure or exchange/substitution of elements. The advanced knowledge of coccoliths could motivate their incorporation in hybrid materials or inspire the conception of new structures, principles or functions. The adoption and implementation of these principles in the particle industry for technical purposes is the next progressive step. This process has already begun and the ultimate result will be to reveal fully the practical potential of microorganisms as factories for bio-(nano)-materials. The biosynthetic capacity of coccolithophores as producers of bioparticles and the nature of calcite structures meet the criteria that industry requires for new technologies. These are, above all, profitability, the attractive field of application and the element of novelty, giving advantage in competitive markets. Coccolithophores, with their energy and carbon-neutral characteristics, can be regarded as ideal elements in the sustainable, renewable, alternative and 'green' technologies, a priority in the 21st century.

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14 Applications of Nanoparticles Synthesized by Yeasts: A Green and Eco-friendly Method

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Introduction

Nanotechnology collectively describes technology and science involving nanoscale particles (nanoparticles) that increase the scope of investigating and regulating the interplay at cell level between synthetic materials and biological systems (Sinha *et al.*, 2009). There have been fascinating developments in the area of nanotechnology recently, with numerous methodologies formulated to synthesize nanoparticles of predefined shape and size depending on specific requirements (Sathish *et al.*, 2011).

Metallic nanoparticles have become significant in recent years and have created an impact in the areas of chemical, energy, electronic and biological sciences and medicine. Although such particles can be synthesized by physical, chemical and biological methods, in the past few years the last option has gained importance (Durán *et al.*, 2010a,b,

2011; Rai *et al.*, 2010, 2013; Prasad *et al.*, 2011; Schrofel and Kratsova, 2011; Durán and Marcato, 2012; Gupta *et al.*, 2012). On the other hand, increasing awareness towards green chemistry and biological processes has led to a desire to develop an environment-friendly approach for the synthesis of nanoparticles.

The biological methods of synthesis present some important advantages over other methods, such as higher production, rapidity, lower costs and eco-friendly (Durán *et al.*, 2011). Moreover, physical or chemical methods involve the use of toxic, hazardous and non-environment-friendly chemicals (Durán *et al.*, 2011).

Many studies have been published related to the biogenesis of metallic nanoparticles mainly from bacteria, fungi, plants and algae (Das *et al.*, 2009; Durán *et al.*, 2010a,b, 2011; Rai *et al.*, 2010, 2013; Marcato and Durán, 2011; Prasad *et al.*, 2011; Rai and Durán, 2011;

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Schrofel and Kratsova, 2011; Durán and Marcato, 2012; Durán and Seabra, 2012; Gupta *et al.*, 2012; Seabra and Durán, 2013). To date, there is a continuing search for novel microorganisms to synthesize metallic nanoparticles.

In fact, an increasing concern with yeast is evident from the literature (Mandal *et al.*, 2006; Mohanpuria *et al.*, 2008; Krumov *et al.*, 2009; Li *et al.*, 2009; Sinha *et al.*, 2009; Blanco-Andujar *et al.*, 2010; Gade *et al.*, 2010; Narayanan and Sakthivel, 2010; Thakkar *et al.*, 2010; Krumov and Posten, 2011) and opens a novel and interesting field of research for modern nanobiotechnology, with important applications in different areas of interest.

This chapter focuses on the production of metallic and semiconductor nanoparticles from yeast by green and eco-friendly methods. The possible mechanisms involved in nanoparticle formation and characterization are also discussed, together with the patents registered in this field, and finally future prospects.

Metallic Nanoparticles

Yeast strains are now under specialized exploration to engineer gold nanoparticles. Standardization of the growth and other cellular activities for the controlled synthesis of nanoparticles (NPs) has been achieved (Gericke and Pinches, 2006a,b).

Recently, the synthesis of gold nanoparticles (Au-NPs) using a non-conventional yeast, *Yarrowia lipolytica* NCIM3589, has been studied (Agnihotri *et al.*, 2009). Dispersive spectrum analysis revealed the presence of Au nanocrystals at a diverse range of pH (2, 7 and 9), with an absorbance peak at 540 nm and well associated with the cell wall of *Y. lipolytica*.

Studies are still being carried on to search further for diverse groups of beneficial yeasts. Useful and potential yeast sources fabricating silver NPs (Ag-NPs) have been produced from yeast MKY3 (Kowshik *et al.*, 2003).

Ag-NPs were also synthesized by extract of *Pichia pastoris*. Under acidic conditions, the reaction rate was slow and the Ag-NPs were polydispersed. However, at

pH below 12.5, the reaction rate increased and the distribution of the particle size was narrow. However, when the pH reached 12.8 or over, the Ag-NPs were aggregated. Unfortunately, no mechanistic aspects were discussed (Hong *et al.*, 2009).

Biocatalytic reduction and removal of copper ions in aqueous-organic phase by induction of copper-degrading reductase in catabolic repressed yeast by the addition of an inducer (copper ions) during the growth phase of *Saccharomyces cerevisiae* was studied. Excess of glucose under catabolic repression conditions was used to induce Cyt P450 in the yeast cells. The harvested biomass was lysed using a cell disrupter, and microsomes were obtained. The parameters affecting the biocatalytic reduction were initial substrate concentration, time of reaction and initial pH of the reaction mixture (optimization of the enzyme), and concentration and time of addition of the inducer (yeast growth) (Chandran *et al.*, 2001).

Ag-NPs (45–60 nm) synthesized from *Candida glabrata* showed antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Namasivayam *et al.*, 2011). In another study, Au-NPs and Ag-NPs with face-centred cubic structures in the size range of 50–70 nm and 10–20 nm, respectively, were synthesized by the yeast *Candida guilliermondii*. These biosystems were tested against five pathogenic bacterial strains. The highest efficiency for both Au-NPs and Ag-NPs was observed against *S. aureus* (Mishra *et al.*, 2011).

Baker's yeast, *S. cerevisiae*, was also reported to biosorb and reduce Au⁺³ to elemental Au⁰ in the peptidoglycan layer of the cell wall *in situ* by the aldehyde group present in reducing sugars (Lin *et al.*, 2005). Similarly, another yeast, *Pichia jadinii* (*Candida utilis*), intracellularly formed Au-NPs of spherical, triangular and hexagonal morphologies throughout the cell, mainly in the cytoplasm, of 100 nm in size in 24 h (Gericke and Pinches, 2006a,b).

The tropical marine yeast, *Y. lipolytica* NCIM 3589, also synthesized Au-NPs associated with the cell wall (Pimprikar *et al.*, 2009).

The reduction of Au ions occurred in a pH-dependent manner. When cells were incubated at pH 2.0, it produced hexagonal and triangular Au-NPs due to the nucleation on the cell surfaces giving rise to a golden colour in the visible region at 540 nm at pH 7.0 and pH 9.0, with pink and purple colours and with an average size of 15 nm (Agnihotri *et al.*, 2009). A possible mechanism was proposed for the first time by Strouhal *et al.* (2003), who suggested the important role of metallothioneins in the synthesis of metal NPs. *Pichia capsulata*, among 12 species of marine yeasts, exhibited the highest efficiency in the synthesis of Ag-NPs in culture filtrate (1.5 mM AgNO₃, 0.3% NaCl, pH 6 at 5°C for 24 h) (Subramanian *et al.*, 2010).

In another study, the biosynthesis of Ag-NPs and Au-NPs was investigated using an extremophilic yeast strain isolated from acid mine drainage in Portugal. The extremophilic strain under study was able to grow to a high Ag ion concentration, whereas a low Au ion concentration caused a strong inhibitory effect. A successful route for metal NP synthesis was obtained using yeast biomass. When the washed yeast cells were in contact with Ag or Au solutions, Ag-NPs were smaller than the Au-NPs in diameter. The supernatant-based strategy provided evidence that proteins were released to the medium by the yeasts, which could be responsible for the formation and stabilization of the Ag-NPs, although the involvement of the cell wall seems fundamental for Au-NP synthesis (Mourato *et al.*, 2011).

S. cerevisiae, AP22 and CCFY-100 strains were studied for bioaccumulation of Au ions through a low dose of γ -energy. Transmission electron microscopy analysis found that Au³⁺ was reduced *in situ* to Au⁰ intracellularly. The gold nanoparticles gradually moved inward as a function of time from cell wall to cytoplasm to nucleus and finally accumulated in the nucleolus of the cell (Sen *et al.*, 2011).

Au-NPs were prepared by extracellular material of *Hansenula anomala* using Au salt as the precursor and amine-terminated polyamidoamine dendrimer or cysteine as the stabilizers. The ability of as-synthesized Au sol to function as biological ink for producing patterns for the analysis of fingerprints

and to act as an antimicrobial reagent was evaluated. The generality of this toxin-free synthetic approach to other metals was assessed using palladium and silver (Sathish *et al.*, 2011). Au–Ag alloy NPs were biosynthesized by commercial yeast cells and applied to fabricate a sensitive electrochemical vanillin sensor and used in the determination of vanillin from vanilla bean (Zheng *et al.*, 2010).

Semiconductors and Other Nanoparticles

Several reports have described the formation of cadmium sulfide (CdS) NPs from *Schizosaccharomyces pombe* media (Barbas *et al.*, 1992; Mehra *et al.*, 1994; Williams *et al.*, 1996a,b, 2002). The biosynthesis of semiconductors as CdS in yeast has been reported in *Candida glabrata* and *S. pombe* cultured in the presence of Cd salt (Dameron *et al.*, 1989a,b). Dameron and co-workers have suggested a model of the capped peptides associated with the CdS-NPs (Dameron and Winge, 1990a,b; Bae and Mehra, 1998). Kowshik and co-workers (2002a) also reported the intracellular synthesis of CdS-NPs by the *S. pombe* strain (1–1.5 nm size). A low-cost *S. cerevisiae*-mediated biosynthesis of CdS-NPs, the majority having a spherical shape and size between 2.5 and 5.5 nm, has been published (Prasad and Jha, 2010).

Size exclusion chromatography confirmed that Cd was coupled to a protein fraction between 25 and 67 kDa, which corresponded to the theoretical molecular weight of CdS-NPs of 35 kDa coated with phytochelatins (Krumov *et al.*, 2007). In addition to this, *Torulopsis* has been found to be proficient in reducing intracellular Pb²⁺ from biomass with maximum absorption at 330 nm (Kowshik *et al.*, 2002b).

An intracellular synthesis of stable lead sulfide NPs (cubic structure, 2–5 nm size) capped by a sulfur-rich peptide by a marine yeast, *Rhodospiridium diobovatum*, has been reported (Seshadri *et al.*, 2011). Several processes use yeast cells as a template for the synthesis of NPs, as in magnetic Fe₃O₄ mesoporous materials with a size distribution

of 8–14 nm (Zhou *et al.*, 2009a), iron phosphate nanopowders with flake morphologies with a length of 500–600 nm and a width of 200–300 nm (Zhou *et al.*, 2009b), and zinc phosphate (Yan *et al.*, 2009) and cadmium telluride (CdTe) quantum dots (QDs) with a great potential for bioimaging and biolabeling applications (Bao *et al.*, 2010).

S. cerevisiae was also found to produce, at room temperature, spherical-shaped, face-centred cubic unit cell antimony oxide (Sb₂O₃) nanoparticles, 2–10 nm in size, exhibiting semiconductor properties. This was due possibly to the radial tautomerization of membrane-bound quinones or by membrane-bound or cytosolic pH-dependent oxidoreductases (Jha *et al.*, 2009a). Spherical amorphous iron phosphate NPs with a size distribution of 50–200 nm were formed in baker's yeast cells exposed to FeCl₃ solution. In this work, the mechanism of biomineralization was simply discussed using chemical bonds and surface charges (He *et al.*, 2009). Other NPs synthesized by *S. cerevisiae* were spherical extracellular TiO₂-NPs 8–35 nm in size, which suggested that pH as well as partial pressure of gaseous hydrogen (H₂) or redox potential of the culture solution probably played an important role in the process (Jha *et al.*, 2009b).

Mechanisms for the Role of Yeast in Nanoparticle Formation

To date, there is a continued search for novel microorganisms synthesizing NPs. Recently, yeast has been exploited mainly for the synthesis of different kinds of NPs, and different mechanisms of synthesis have been reported. For metallic NP synthesis, microorganisms usually employ NADH and NADH-dependent reductases for metal reduction (Durán *et al.*, 2005; Agnihotri *et al.*, 2009). These reductases may also be involved in the reduction of metals (Agnihotri *et al.*, 2009). In addition, there has been a recent report on the role of another enzyme (protease) in reducing gold salts to NPs (Bharde *et al.*, 2007). Agnihotri *et al.* (2009) reported intracellular synthesis of Au-NPs using *Y. lipolytica*. This yeast is known to

produce proteases, which may also possibly play a role in this synthesis (Agnihotri *et al.*, 2009). Bao *et al.* (2010) demonstrated a simple and efficient biosynthesis method to prepare easily harvested biocompatible CdTe QDs with tunable fluorescence emission using yeast cells (*S. cerevisiae*). The authors confirmed that the CdTe QDs were formed via an extracellular growth and subsequent endocytosis pathway, and had size-tunable optical properties with fluorescence emission from 490 to 560 nm and a cubic zinc blende structure with good crystallinity (Bao *et al.*, 2010). In particular, the CdTe QDs with uniform size (2–3.6 nm) were protein-capped, which made them highly soluble in water, and *in situ* bioimaging in yeast cells indicated that the bio-synthesized QDs had good biocompatibility (Bao *et al.*, 2010). This work proposed that an extracellular growth pathway was responsible for the formation of the protein-capped CdTe QDs and an endocytosis pathway was responsible for the *in situ* bioimaging results. In comparison with other synthesis routes, this strategy allowed the one-step preparation of protein-capped CdTe QDs on a large scale (gram quantities) in aqueous solutions at relatively low temperatures (25–35°C) (Bao *et al.*, 2010). Moreover, the proteins capped on the QDs offered not only high biocompatibility of the QDs but also functional groups for further chemical modifications (Bao *et al.*, 2010).

Chandran *et al.* (2001) have synthesized copper (Cu) NPs from *S. cerevisiae*. This study involved the biocatalytic reduction and removal of Cu ions in aqueous–organic phase by the induction of Cu-degrading reductase in catabolic repressed yeast by the addition of an inducer (copper ions) during the growth phase of *S. cerevisiae* (Chandran *et al.*, 2001). An excess of glucose under catabolic repression conditions was used to induce the Cyt P450 in the yeast cells (Chandran *et al.*, 2001). The harvested biomass was lysed using a cell disrupter and microsomes were obtained (Chandran *et al.*, 2001). The parameters affecting the biocatalytic reduction were initial substrate concentration, time of reaction and initial pH of the reaction mixture (optimization of the enzyme) and the concentration and time of addition of the inducer (yeast growth) (Chandran *et al.*, 2001).

In a recent study, amorphous iron phosphate NPs biomineralized with protein macromolecules in yeast cells were observed, and a possible mechanism of biomineralization has been proposed (He *et al.*, 2009). Yeast cells as a template provide nucleation sites for target materials and induce the oriented growth of iron phosphate (He *et al.*, 2009). The spontaneous formation of iron phosphate NPs in the yeast cells was achieved by immersion of the yeast cells into a solution containing ferric chloride and tertiary sodium phosphate: (i) the increased local concentration of Fe^{3+} caused by the chemical reaction between Fe^{3+} and the hydroxyl and amine group of protein macromolecules in the yeast cells; (ii) the formation of an iron phosphate crystal nucleus in the yeast cells after the addition of the appropriate tertiary sodium phosphate aqueous solution (pH = 5.5); and (iii) the continuing condensation led iron phosphate NPs to assemble on the surface of protein macromolecules in the yeast cells (He *et al.*, 2009). The protein macromolecules during processing were crucial and their role was analysed in depth in order to understand the mechanism responsible for the formation of iron phosphate NPs in yeast cells (He *et al.*, 2009). In the biomineralization, the protein macromolecules in the yeast cells played an important role in providing nucleation sites and immobilized the NPs to establish dispersed NP systems (He *et al.*, 2009).

Prasad *et al.* (2011) proposed a possible mechanism for the biosynthesis of CdS-NPs from *S. cerevisiae*. The membrane-bound (as well as cytosolic) oxidoreductases and quinones might have played an important role in the synthesis. The oxidoreductases were pH sensitive and worked in an opposing manner. At a lower pH value, oxidase became activated, while a higher pH value activated the reductase. Alongside this, a number of simple hydroxy/methoxy derivatives of benzoquinones and toluquinones were elaborated by lower fungi (especially *Penicillium* and *Aspergillus* species). Yeast might be harbouring other such quinones because it belongs to the same class of fungi, thereby facilitating the redox reactions due to its tautomerization (Prasad and Jha, 2010).

The transformation seems to be at the cell membrane level immediately after addition of the CdS solution, which triggers tautomerization of quinones and low pH-sensitive oxidases and makes molecular oxygen available for the transformation. Such a stress-generated response had earlier been suggested in the case of *C. glabrata* cells challenged with cadmium ion in the form of elaboration of an enzyme phytochelatin synthase and a protein HMT-1, which effectively aborted the CdS nanocrystals from the cytosol. Once entered into the cytosol, the CdS might have triggered the family of oxygenases harboured in the endoplasmic reticulum (ER), chiefly meant for cellular-level detoxification through the process of oxidation/oxygenation (Prasad *et al.*, 2011). Making use of the above-mentioned facts, this group had earlier reported the synthesis of antimony trioxide (Sb_2O_3) and TiO_2 -NPs from *S. cerevisiae* through similar mechanisms (Jha *et al.*, 2009a,b).

Sathish *et al.* (2011) synthesized Au-NPs using yeast species *Hansenula anomala*. These studies on *H. anomala* have shown that the isolated membrane fraction of the species contains lactate dehydrogenase (cytochrome b2), NADH ferricyanide reductase, NADPH ferricyanide reductase and cytochrome (Sathish *et al.*, 2011). The presence of electroactive enzymes in the membrane suggests that they can mediate the transfer of electrons generated during metabolism to any electron acceptor (metal ions or electrodes) directly without external mediators in the absence of oxygen (Sathish *et al.*, 2011). This concept has motivated the use of the reducing ability of this yeast to produce Au-NPs (Sathish *et al.*, 2011). To prepare Au-NPs free from cellular material and to develop a preparation procedure that can be scaled up in the next stage, the authors thought of extracting the bioreductants from the yeast cells in order to use them as a reagent for the preparation of Au-NPs (Sathish *et al.*, 2011). Hence, the yeast was allowed to undergo fermentation in the presence of lactate for 2 weeks to produce bioreductants (Sathish *et al.*, 2011). Although the cause of the formation of differently shaped NPs is not clear, this experiment confirms that NADH isolated from the yeast,

H. anomala, is responsible for conversion of the Au salt to Au-NPs (Sathish *et al.*, 2011). In this way, the reducing ability of bioreductants is due to NADH produced during the anaerobic fermentation of lactate by the yeast *H. anomala* (Sathish *et al.*, 2011).

Seshadri *et al.* (2011) reported the intracellular synthesis of stable lead sulfide NPs (semiconductor nanoparticles) by a marine yeast, *R. diobovatum*. The formation of metal sulfides in the cell takes place through two independent steps. One is the chelation of metal ions by phytochelatin, which are induced by the metal, leading to the formation of low-molecular-weight phytochelatin-metal ion complexes in the vacuole (Seshadri *et al.*, 2011). The second step, S²⁻ generation, involves enzymes in the purine biosynthesis pathway and conversion of the phytochelatin-metal complex to a phytochelatin-metal sulfide complex (Seshadri *et al.*, 2011). The lead sulfide NPs synthesized by *R. diobovatum* were most likely capped by peptides like phytochelatin, preventing them from aggregating into bulk crystals. Thus, the thiol compounds can perform the dual function of detoxifying the metal and also capping the metal NPs (Seshadri *et al.*, 2011).

Yan *et al.* (2009) reported the synthesis of zinc phosphate NPs by chemical precipitation using yeast cells. This study demonstrates that hydroxyl groups on cells play a key role in the synthesis of zinc phosphate NPs and, based on the results obtained, yeasts are capable of conjugation with zinc ions from an aqueous solution. The pivotal factor is the hydroxyl groups on biomacromolecules (Yan *et al.*, 2009).

Moreover, extracellular synthesis of Ag-NPs by a silver-tolerant yeast strain MKY3 has been reported (Kowshik *et al.*, 2003). Although the exact mechanism leading to the reduction of silver ions to elemental form needs to be elucidated, it is possible that certain biochemical reducing agents are only excreted by the yeast cells in response to silver 'stress' (Kowshik *et al.*, 2003). It is known that silver cations are highly reactive and tend to bind strongly to electron donor groups containing sulfur, oxygen or nitrogen (Kowshik *et al.*, 2003). Such a binding with

biomolecules like small proteins could restrict the size of the particles (Kowshik *et al.*, 2003).

In another study, magnetic Fe₃O₄ composites with a mesoporous structure have been synthesized by the co-precipitation method, using yeast cells as a template (Zhou *et al.*, 2009a). Also, iron phosphate nanopowders with flake morphologies have been synthesized by the precipitation method, with yeast cells as a biological template, which provide the nucleation sites for regulating the growth of FePO₄-NPs (Zhou *et al.*, 2009a,b). On the basis of the results obtained, the yeast cells play an important role in the synthesis of iron phosphate nanopowders. During the nucleation process, the yeast cells provide a multitude of nucleation centres for iron phosphate. During the calcination process, the organic matters can prevent physical contact with iron phosphate NPs to avoid aggregation. After removing organic matter at 600°C, iron phosphate NPs grow rapidly to the flaky shape (Zhou *et al.*, 2009b).

Mourato *et al.* (2011) reported the biosynthesis of Ag-NPs and Au-NPs using an extremophilic yeast. The results obtained (supernatant-based strategy) provided evidence that proteins were released to the medium by the yeasts, which could be responsible for the formation and stabilization of the Ag-NPs, although the involvement of the cell wall seemed fundamental for Au-NP synthesis because the supernatant was constituted by compounds that had coordinated with the Au ions, and the complex formed had a reduction potential much higher (more negative) than the Ag and Au ions (Ag⁺, Au³⁺, AuCl⁴⁻, AuCl²⁻) (Mourato *et al.*, 2011). The results indicated that Ag-NP and Au-NP formation could take place intracellularly and/or extracellularly (Mourato *et al.*, 2011).

Finally, Ag-NPs have been synthesized extracellularly from *C. glabrata* by Namavayam *et al.* (2011).

Methodology for Nanoparticle Analysis

After the synthesis of nanoparticles by an eco-friendly route, we need several methods and

techniques to characterize them. Each technique is able to give important information about structure, morphology, size and molecular interaction (Chandran *et al.*, 2001; Agnihotri *et al.*, 2009; Zhou *et al.*, 2009a,b; Seshadri *et al.*, 2011). There are many techniques, such as ultraviolet and visible spectroscopy (UV-Vis), X-ray diffraction (XRD), photon correlation spectroscopy (PCS), transmission and scanning electron microscopy (TEM and SEM), atomic force microscopy (AFM), Fourier transformer infrared (FTIR), nitrogen adsorption-desorption isotherms (NADI), thermogravimetric analysis (TGA), differential scanning calorimetric (DSC) and energy dispersive X-ray spectroscopy (EDX). Here, we have described only the important techniques that are frequently used in characterization.

Usually, UV-Vis spectroscopy is used to confirm NP production. This technique is based on surface plasmon resonance (SPR) phenomena that inorganic nanoparticles present. The colour change of a nanoparticle has its origin in collective electron excitation, which arises when an external electromagnetic wave (EM) focuses on the particle, creating an oscillation between the core and the electronic cloud that generates electronic dipoles, quadrupoles and/or multipoles. Each wavelength produces a different oscillation in the electron cloud, which may be resonant or non-resonant. The specific wavelength generates a resonant oscillation, transforming the EM incident radiation into thermal energy; this is the SPR peak. Most studies use UV-Vis to identify the presence and production of nanoparticles (Agnihotri *et al.*, 2009; Das *et al.*, 2009; Sathish *et al.*, 2011). However, the UV-Vis is also able to predict the shape and size of NPs (Noguez, 2007; Sileikaite *et al.*, 2009), unexploited by most work.

Techniques based on the visualization of NPs, such as electron microscopy like TEM and SEM and field emission gun (FEG), are widely used both to estimate NP size and also to study their morphology. The choice of technique depends mainly on the resolution required, as the size of NPs is usually less than 100 nm (Sinha *et al.*, 2009; Li *et al.*, 2011); thus, TEM is the first choice. In some studies (Narayaran *et al.*, 2010), a high-resolution

TEM (HRTEM) microscopy was used. Besides measuring sizes, this technique can provide information on crystallography structure. Another microscopy widely used is AFM, which gives the surface topology of a material. However, it is also possible to study the interaction that occurs on the surface (Das *et al.*, 2009). Meanwhile, electron microscopy is not a cheap approach for simply studying size distribution.

Analyses of size distribution are often carried out by PCS, which uses light scattering and Brownian motion theories. This technique allows the measurement of robust systems in solution and does not require complex preparations. Additionally, it can measure the surface charge of the systems, the Zeta potential (Sinha *et al.*, 2009). Sizes were also estimated by XRD using Scherrer's equation; however, this equation is not used much, as the main use of XRD is concerned with the crystallography matrix (Prasad *et al.*, 2011).

Patents on Eco-Friendly Systems

Table 14.1 shows the patents on the biogenic production of nanometallic nanoparticles by yeast using a dry powder of the microorganisms, where distilled water was added, then boiled and cooled and chlorauric acid added at 50°C for a few minutes. Trigonal gold nanoplates were obtained (5–1000 nm) (CN 101368194) (Li *et al.*, 2009). Sulfur-free nanoparticles from Ag, Au, Pt, Te and Se were obtained in spherical form at high production efficiency (US 20110135932) (Mesler and Ourdane, 2011). A nano-silver colloid from yeast as antibacterial material was prepared (KR 20110100419) (Park *et al.*, 2011). A yeast cell wall solution was used for synthesized CdSe, which was obtained freely after cell lysis with lytic enzymes (CN 10203031) (Xie *et al.*, 2011). A biomimetic approach, where a yeast tRNA was used as an ion-exchange/nucleation site with a polymer and then cadmium ion exchange in the presence of sulfide, produced CdS nanoparticles (US 20026350515) (Lawton and Conroy, 2002).

Table 14.1. Patents on biogenic nanoparticles from yeast.

Publication number	Reference title	Inventors	Publication date
CN 101368194 B	Microorganism reduction preparation method for triangular gold nanoslice	Q. Li (CN), W. Wang (CN), D. Sun (CN), J. Huang (CN), X. Yang <i>et al.</i> (CN)	18 February 2009
US 20110135932 A1	Production of sulfur-free nanoparticles by yeast	Z. Mesler (CA), L. Ourdane (FR)	9 June 2011
KR 20110100419 A	Method for preparing nano-silver colloid for activity of yeast and antibacterial activity	H.O. Park (KR), J.H. Kim (KR), M.K., Jin (KR), K.E. Jung (KR)	14 September 2011
CN 10203031 A	Extracting and purifying method of cadmium selenide quantum dot in yeast cells	Z. Xie (CN), D. Pang (CN), P. Zhang (CN), Y. Li (CN)	27 April 2011
US 20026350515 B1	Biomolecular synthesis of quantum dot composites	C. Lawton (USA), S. Conroy (USA)	26 February 2002

Notes: CA = Canada; CN = China; FR = France; KR = Korea.

Conclusion and Future Prospects

It is clear from this chapter that the production of metallic nanoparticles biogenically by yeast and their applications is an emerging area of nanobiotechnology. For industrial uses, as in other biogenic syntheses by bacteria, fungi, algae and plants, scaled-up production and standardization of toxicity are absolutely necessary in the next few years. Few mechanistic aspects have been published and it is necessary to direct more attention to this area since many challenges need to be overcome in order to improve the industrial applications of this biogenic process. It is curious that only a few patents have been registered. China and Korea appear to be the principal countries producing this innovation in yeast applications. Due to their rich

diversity, yeasts have an innate capability for the synthesis of nanoparticles and they could be regarded as potential biofactories for nanoparticle synthesis. Future research on yeast-mediated biological synthesis of nanoparticles could be of great importance for applications in the areas of chemistry, electronics, medicine and agriculture.

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