Chapter 5
Synthesis of Nanomaterials—III (Biological Methods)

5.1 Introduction

In his, very famous speech delivered in 1959, before the scientists of American Physical Society, Nobel Laureate Richard Feynman asked the scientists to derive the inspiration from *Mother Nature* to make the things smaller and see the advantages of making things smaller. Indeed the biological world, animal kingdom and plants make optimum use of materials and space. Nature indeed makes use of small spaces and corresponding confinement to synthesize inorganic materials or minerals abundantly found in earth's crust. It uses insoluble, complex organic molecules as a reactor in which nucleation and growth of complex and hierarchical structures of inorganic materials takes place by reactions of organic soluble molecules. When we think of biological world, we normally think of delicate, temperature sensitive, carboneous or organic materials like leaves, roots, stems, cells, tissues and skin. Inorganic materials are also produced in biological systems. Bones, teeth, shells, nanomagnets inside the bodies of some bacteria and birds are some examples. Inorganic materials inside organic matter or organisms are known as biocomposites or biominerals. A variety of mechanically strong or weak, rigid or flexible, porous or nonporous, thick or thin materials either organic or inorganic in small or large quantities are abundantly produced in contact with live cells. These materials exhibit a wide variety in their functions like providing support to body, allow body movements and in general carry out various essential body functions. Table 5.1 gives a list of some biominerals produced or observed in the biological systems. The list is not at all complete and many more minerals are observed to be synthesized in nature under different environmental conditions.

Many of the materials synthesized by microorganisms, animals and plants in nature can indeed be synthesized using them in laboratories even on large scale. This is considered to be a very attractive possibility so as to have *eco-friendly* or so-called *green synthesis*. Further the ability of bio-world to synthesize materials of variety of shapes and sizes is quite unique and often difficult to mimic, though not impossible.

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Table 5.1 Some of the biominerals produced by the biological world

Minerals	Formula
Arsanate	
Orpiment	As_2S_3
Carbonates	
Calcite	$CaCO_3$
Mg-Calcite	$(Mg_xCa_{1-x})CO_3$
Monohydrocalcite	$CaCO_3.H_2O$
Hydrocerrussite	$Pb_3(CO_3)_2(OH)_2$
Amorphous calcium carbonate (various forms)	$CaCO_3.H_2O$
Chlorides	
Athcamite	$Cu_2Cl(OH)_3$
Fluorides	
Fluorite	CaF_2
Hieratite	K_2SiF_6
Hydrated silica	
Amorphous silica	$SiO_2.nH_2O$
Hydroxides and hydrous oxides	
Goethite	α-FeOOH
Lepidocrocite	γ-FeOOH
Ferrihydrite	$5Fe_2O_3.9H_2O$
Brinessite	$Na_4Mn_{14}O_{27}.9H_2O$
Metals	
Sulfur	S
Organic crystals	
Uric acid	$C_5H_4N_4O_3$
Cu tartarate	$C_4H_4CaO_6$
Guanine	$C_5H_3(NH_2)N_4O$
Oxides	
Magnetite	Fe_3O_4
Amorphous iron oxide	Fe_2O_3
Amorphous manganese oxide	Mn_3O_4
Phosphates	
Octacalcium phosphate	$Cu_8H_2(PO_4)_6$
Francolite	$Ca_{10}(PO_4)F_2$
Virianite	$Fe^{2+}{}_3\ (PO_4)_2.8H_2O$
Amorphous calcium pyrophosphate	$Ca_2P_2O_7.2H_2O$
Sulphates	
Gypsum	$CaSO_4.2H_2O$
Barite	$BaSO_4$
Jarosite	$KFe_3{}^{+3}(SO_4)_2(OH)_6$

(continued)

Table 5.1 (continued)

Minerals	Formula
Sulphides	
Pyrite	FeS_2
Hydrotroilite	$FeS.nH_2O$
Sphalerite	ZnS
Wurtzite	ZnS
Galena	PbS
Acanthite	As_2S

Many processes taking place inside the living cells are not understood well yet. Interestingly shape, texture and variety of structures observed in the biological systems, taking place by atomic (ionic) molecular interactions and diffusion are not controlled by thermodynamics but are kinetically driven. Mineral nucleation is controlled by interfacial energies and growth depends upon the control through passivation of the material surface. Solution composition, ionic activities, their transport, availability of suitable growth sites, ion accumulation and transportation are all governing factors in the nucleation and growth of particles. Starting with nanoparticles, hierarchical structures are produced by self assembly which will be discussed in the next chapter. Such structures using nanomaterials also is a topic of great interest and will be discussed under applications of nanoscience to technology. In this chapter we shall restrict only to synthesis of nanomaterials.

Driven by the motivation of understanding biological systems as well as mimicking the nanosynthesis by nature's way, scientists have been using the methods by which inorganic materials are synthesized by using biomaterials like enzymes, DNA and membranes. A variety of metal, semiconductor and insulator nanoparticles or their assemblies (one dimensional, two dimensional and even three dimensional) have been made. There are also attempts to make biocompatible, bioactive or biopassive materials, specially for different medical applications like body implants, drug delivery, cancer therapy and so on. Here we shall discuss only the synthesis part using biological methods.

Synthesis of nanomaterials using biological ingredients can be roughly divided into following three types.

1. Use of microorganisms like fungi, yeasts (eukaryotes) or bacteria, actinomycetes (prokaryotes). Eukaryotes are higher organisms with proper nucleus in their cells and prokaryotes are lower organisms without any nucleus.
2. Use of plant extracts or enzymes.
3. Use of templates like DNA, membranes, viruses and diatoms.

In the following sections we shall outline these methods with some examples (Boxes 5.1, 5.2, and 5.3).

Box 5.1: Biomaterials

Carbon, hydrogen, oxygen and nitrogen are the major constituents (∼95 % by weight) of any living cell, animal or plant. Some other elements like iron, copper, manganese, zinc, selenium etc. are also found to be present in varying amounts (sometimes only as trace elements) in biosystems. All the elements together form various body parts and have to carry out different functions to execute life cycle activities. This involves a rich variety of molecules or materials from nano to macro scale. They are all synthesized in bioenvironment and can self assemble or dissociate; they can recognize some specific molecules or bodies due to their charges or shapes, bind weakly or strongly with other molecules.

Building blocks of living systems can be divided into four classes viz. very small molecules, proteins, nucleic acids and carbohydrates. Small molecules like water (H_2O), oxygen (O_2), nitric oxide (NO) or even slightly bigger molecules like sugars, enzymes, acids are quite important in biosystems. They may be present as energy sources, purifiers or excretions in various parts.

There is a large number of different types of proteins. Proteins form a major part of biological systems. Nails, hair etc. are made up of proteins. They are responsible for numerous life activities.

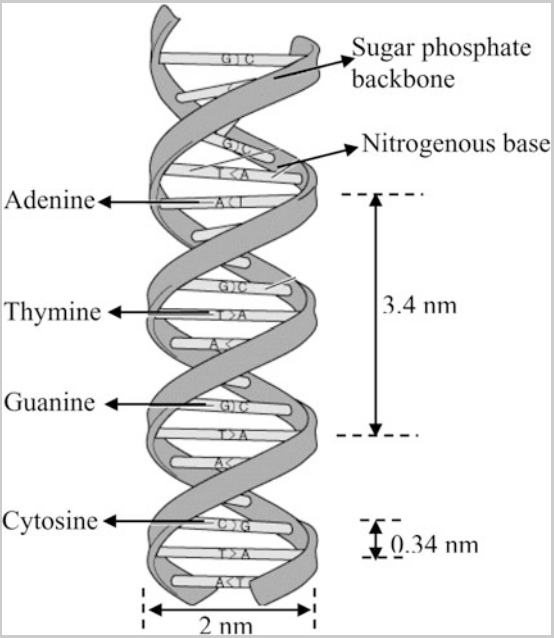
DNA and RNA form nucleic acids. They are major constituents of proteins. They are genetically coded and are, therefore, most important as nature's messengers of life from one generation to the next. DNA is a long chain molecule composed of a backbone of sugars and phosphates. There are four different small planer molecules viz. adenine (A), thymine (T), guanine (G) and cytosine (C). They are usually denoted by the capital letters in the brackets. These molecules have a peculiarity that A can pair only with C and G can pair only with T. Any strand can therefore be coded with a long or short sequence of molecules like ACGGT, AGCTT and even keep on repeating the sequence. This gives a unique identification to DNA. Two complementary strands of DNA bind to form a double helix as illustrated in Fig. 5.1. Length of a DNA can be as short as few nanometres and as long as few micrometres. There is a large variety of shapes too. There are circular, branched, T-shaped, Y-shaped DNA molecules.

Carbohydrates are macromolecules as important as proteins for living organisms. They are basically sugars or polysaccharides of long chain lengths.

(continued)

Box 5.1 (continued)

Fig. 5.1 Double helix of DNA



Box 5.2: Diatoms

Diatoms are beautiful algae found in marine or any moist environment. They are unicellular, forming amorphous silica shell with living part inside. Diatoms can be ~20–200 μm long. Silicic acid [$Si(OH)_4$] is absorbed inside the cell, which is bonded to cofactor so that polycondensation is avoided. Golgi bodies in cell store silicic acid. Silica transport vesicles. Vesicles are phospholipid bilayers which can trap some solution or water and combine to form silica deposition vesicles (SDV), mineralized part of organism. Silicic acid condense in SDV and grow rapidly. It forms bottom part of new cell wall. Forms of silica shells are decided by genetic factors and are influenced by the organic patterns inside. A large variety of diatoms exist. Dead diatoms are deposited on ocean beds (Fig. 5.2).

(continued)

Box 5.2 (continued)

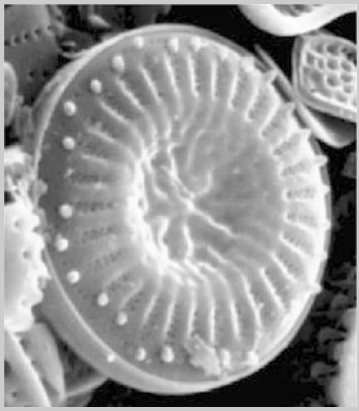


Fig. 5.2 Scanning Electron Microscope (SEM) image of a diatom

Box 5.3: Molecular Recognition

Response of living systems to surrounding depends upon molecular recognition. For example, smell, allergy, diseases etc. are due to molecular recognition. Molecules recognize each other due to their opposite charges or fitting shapes. Insects like ants even attract others by excretion of some molecules like pheromones.

5.2 Synthesis Using Microorganisms

Microorganisms are the organisms that can be observed under a microscope, such as bacteria, fungi or yeasts. Some bacteria are quite useful and are used in the processing of cheese, curds, bread, alcohol and vaccines. Some are harmful and responsible for spoiling food or causing diseases.

Microorganisms are capable of interacting with metals coming in contact with them through their cells and form nanoparticles. In Fig. 5.3a prokaryotic and (Fig. 5.3b) eukaryotic cells are illustrated respectively. The cell-metal interactions are in general difficult to understand due to complexity of cells themselves. It is, however, well known that certain microorganisms are capable of separating metal ions. This is widely used to either recover precious metals or detoxify water. However use of microorganisms in deliberate synthesis of metal, semiconductor or insulator nanoparticles is relatively new area and will be discussed now with some examples.

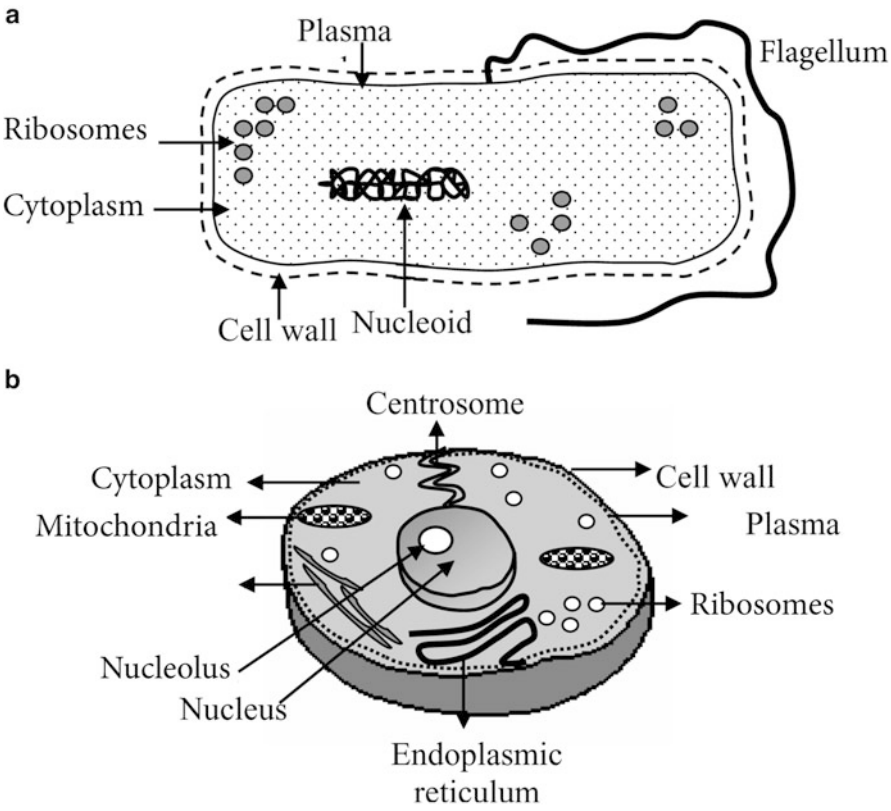


Fig. 5.3 (**a**) Prokaryotic and (**b**) Eukaryotic cells

Some microorganisms produce hydrogen sulphide (H_2S). It can oxidize organic matter forming sulphate, which in turn acts like an electron acceptor for metabolism. This H_2S can, in presence of metal salt, convert metal ions into metal sulphide, which deposits extracellularly.

In some cases, metal ions from a metal salt enter the cell body. The metal ions are then converted into a non-toxic or less toxic form and covered with certain proteins in order to protect the remainder of the cell from toxic environment.

Certain microorganisms are capable of secreting some polymeric materials like polysaccharides. They have some phosphate, hydroxyl and carboxyl anionic groups which complex with metal ions and bind extracellularly.

Cells are also capable of reacting with metals or ions by processes like oxidation, reduction, methylation, demethylation etc.

Different processes of metal-microorganism interactions are schematically shown in Fig. 5.4.

We shall have now some examples of metal-microorganism interactions.

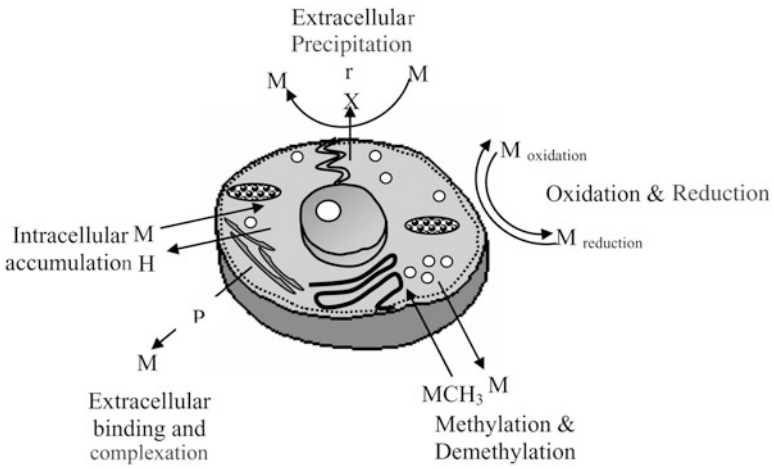


Fig. 5.4 Metal microorganisms interactions

Pseudomonas stutzeri Ag259 bacteria are usually found in silver mines and are capable of accumulating silver inside or outside of their cell walls. Using this fact, these bacterial strains can be challenged with high concentration of silver salt like $AgNO_3$. Numerous silver nanoparticles of different shapes can be intracellularly produced having size <200 nm. Although detailed analysis shows that silver nanoparticles are quite abundant per cell, some silver sulphide particles also are found to be present.

Low concentrations of metal ions (Au^+, Ag^+ etc.) can be converted to metal nanoparticles by *Lactobacillus* strain present in butter milk. By exposing the mixture of two different metal salts to bacteria, it is indeed possible to obtain alloys under certain conditions.

Use of various fungi also has been made to obtain large quantities of metal nanoparticles. For example *Fusarium Oxysporum* challenged with gold or silver salt for approximately three days produces gold or silver particles extracellularly. Extremophilic actinomycete *Thermomonospora* sp. produces gold nanoparticles extracellularly.

When silver metal salt is treated with another fungus *Verticillium* sp., the nanoparticles can be produced intracellularly. The procedure for the synthesis can be briefly described as follows. *Verticillium* sp. extracted from Taxus plant should be placed at 25 °C in potato–dextrose agar slant. Fungus should be grown in Erlenmeyer flask containing MGYP medium composed of 3 % malt extract, 1 % glucose, 0.3 % yeast extract and 0.5 % peptone maintained at ∼25 °C. The flask needs to be shaked for 4 days. After fermentation mycelia separated from culture broth by centrifugation at 10 °C, for ∼20 min need to be washed in water. Harvested mycelial mass should be exposed to $AgNO_3$ solution of 5.5–6.0 pH in Erlenmeyer flask for 3 days while shaking and maintaining the 28 °C temperature. *Verticillium* biomass can be fixed in glutaraldehyde in distilled water by maintaining it at room temperature for 2 h.

After fixation, the centrifugation leads to cell sedimentation rich in silver nanoparticles. Changes in biomass colour from initial yellow to final brown, after exposure to silver salt, is a visual indication of silver nanoparticles formation. Particles can be recovered by washing with some suitable detergent or ultrasonication. Interestingly, *Verticillium* does not die if exposed to $AgNO_3$ solution. Small specks of Ag nanoparticles containing biomass, placed in an agar plate, clearly indicate the growth of *Verticillium* in about eight days. The possible mechanism of nanoparticles formation can be considered as follows. Silver ions from a silver salt possibly get trapped on the surface of the fungal cells perhaps due to electrostatic interaction between positively charged silver ions and negatively charged carboxylic groups in the enzymes present in cell walls of mycelia. The ions after nucleation can grow by further accumulation of silver ions to form nanoparticles.

In a similar way gold nanoparticles can be produced using *Verticillium* sp. However the colour of biomass is from pink to blue depending upon the particle size.

Semiconductor nanoparticles like CdS, ZnS, PbS and many others can be produced using different microbial routes. There are some sulphate reducing bacteria of the family *Desulfobacteriaceae* which can form 2–5 nm ZnS nanoparticles. There are some reports in the literature which discuss such ZnS nanoparticle formation observed in nature.

Bacteria *Klebsilla pneumoniae*, which is a pathogen, can be used to synthesize CdS nanoparticles. When $[Cd(NO_3)_2]$ salt is mixed in a solution containing bacteria and solution is shaked for about one day at ∼38 °C, the CdS nanoparticles in the size range ∼5–200 nm can be formed.

CdS nanoparticles with narrow size distribution can be synthesized using the yeasts like *Candida glabrata* and *Schizosaccharomyces pombe*. A nitrogen-rich medium containing 2 % tryptone, 1 % yeast extract and 2 % glucose (pH 5.6) is used to grow the *Schizosaccharomyces pombe*. When challenged with cadmium sulphate solution after about 12–13 h of growth, after 36 h cells rich with CdS get formed. Solution can be centrifuged and frozen at −20 °C. The frozen cells can be thawed at 4 °C for 2–4 h. The thawed solution can be centrifuged again so that the cell debris precipitates and supernatant contains CdS nanoparticles. The particles are covered by proteins which can be removed by heating at 80 °C for few minutes. Process of CdS nanoparticles formation can be understood as follows. When the cells are exposed to cadmium salt, a series of reactions takes place. Cadmium ions are toxic to the cells and in order to reduce this effect they need to coat them with some protective coating of proteins available to them. To begin, an enzyme phytochelatin synthase gets released to synthesize phytochelatin with structure $(Glu\text{-}Cys)_n$–Gly with n ∼2–6. A low molecular weight phytochelatin–Cd complex is formed. An ATP binding cassette (ABC) type vacuolar membrane protein HMT1 transports Cd complex across the membrane. When inside the vacuole, Cd ions receive sulphur ions forming large molecular weight phytochelatin–CdS complex. These are nothing but the CdS nanoparticles.

Similarly it is possible to synthesize PbS by challenging *Torulopsis species* with lead salt like $PbNO_3$.

Silver nanoparticles can be synthesized using MKY3 yeast strain which is isolated from garden soil. This is capable of tolerating ∼0.8 mM $AgNO_3$. Thus it produces silver nanoparticles extracellularly with negligible amount of intracellular particles. MKY3 can be inoculated in Erlenmeyer flask in growth medium having yeast extract (1 %), tryptone (2 %) and glucose (2 %). Solution pH should be ∼5.6. Flask should be shaked at 30 °C for eight hours and then silver nitrate solution can be poured into it. Flask shaking should be continued for one day in dark. Cells can be then recovered by centrifugation. The cell-free medium contains silver nanoparticles which can be recovered by freeze-thaw technique in which advantage is taken of different thawing temperatures of different ingredients. Thus the solution separated from cells is poured upto the brim in a polycarbonate bottle and frozen to −20 °C. It is then thawed to 0 °C. At −8 °C, there is a swelling of the medium. At this temperature silver particles get pushed upwards and can be collected in a sample cup. The procedure results into silver nanoparticles formation with size less than ∼20 nm. A possible mechanism for silver nanoparticles formation may be as follows. When highly reactive silver ions from silver nitrate solution come in contact with yeast cells, the cells secrete some chemicals donating electron to metal ion. Electron donor group along with biomolecules control the accumulation of silver leading to nanoparticles formation.

5.3 Synthesis Using Plant Extracts

Use of plants in synthesis of nanoparticles is quite novel leading to truly *green* chemistry that technologists are looking for. However, compared to the use of microorganisms to produce nanoparticles, use of plant extracts is relatively less investigated. There are few examples which suggest that plant extracts can be used in nanoparticles synthesis. For example it has been reported that live alfalfa plants are found to produce gold nanoparticles from solids.

Leaves of geranium plant (*pelargonium graveolens*) have also been used to synthesize nanoparticles of gold. It should be mentioned that there is also a plant associated fungus which can produce compounds such as taxol and gibberellins. There is an exchange of intergenic genetics between fungus and plant. However, the nanoparticles produced by fungus and leaves have quite different shapes and size distributions. Nanoparticles obtained using *Colletotrichum* sp. fungus related to geranium plant has a wide distribution of sizes and particles are mostly spherical. On the other hand geranium leaves produce rod and disk shaped nanoparticles.

Synthesis procedure to obtain gold nanoparticles from geranium plant extract is as follows. Finely crushed leaves are put in Erlenmeyer flask and boiled in water just for a minute. Leaves get ruptured and cells release intracellular material. Solution is cooled and decanted. This solution is added to $HAuCl_4$ aqueous solution, when nanoparticles of gold start forming within a minute.

5.4 Use of Proteins, Templates Like DNA, S-Layers etc.

As discussed earlier, various inorganic materials like carbonates ($-CO_3$), phosphates ($-PO_4$) and silicates ($-SiO_4$) are found to be parts of bones, teeth, shells etc. Thus biological systems are capable of integrating with inorganic materials. This has been widely used not only to synthesize nanoparticles but obtain organized arrays, superlattices or hierarchical structures of inorganic materials using biological systems. DNA, S-layers (i.e. surface layers of cell walls of some bacteria) or some membranes have long range periodic order in terms of some molecular groups of their constituents. Therefore on some periodic active sites preformed nanoparticles can be anchored. Alternatively, using certain protocols nanoparticles can be synthesized using DNA and membranes as templates. Such ordered arrays are formed as a result of various interactions that take place between the templates and the particles. Such arrays are known as self assembly and will be discussed in Chap. 6. Here we shall discuss only the nanoparticle synthesis using ferritin (a protein) and DNA to produce nanoparticles which are not self assembled.

Ferritin is a colloidal protein of nanosize. It stores iron in metabolic process and is abundant in animals. It is also capable of forming uniform three dimensional hierarchical architecture. Ferritin proteins in different animals have only upto 14 % difference in the amino acid sequences. There are 24 protein (peptides) subunits in a ferritin, which are arranged in such a way (see Fig. 5.5a) that they create a central cavity (see Fig. 5.5b) of ~6 nm. Diameter of polypeptide shell is 12 nm. Ferritin can accommodate 4,500 Fe iron atoms. They are in Fe^{3+} state as hydrated iron oxide mineral, ferrihydrite. The protein subunits are composed of light as well as heavy chains having dinuclear ferroxide centres. These centres are catalysts for in vitro oxidation of Fe^{2+} ions.

Methods to extract ferritins are quite standard in biology. The ferritin without inorganic matter in its cavity is known as apoferritin and can be used to entrap desired nanomaterial inside the protein cage. Therefore first step is to remove

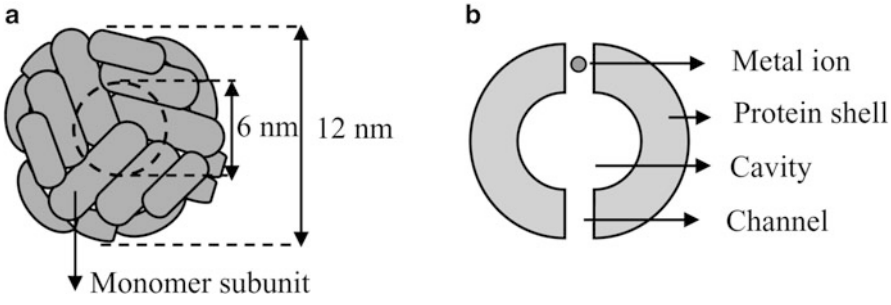


Fig. 5.5 Schematic representation of (**a**) ferritin molecule and (**b**) cavity formed by polypeptide units

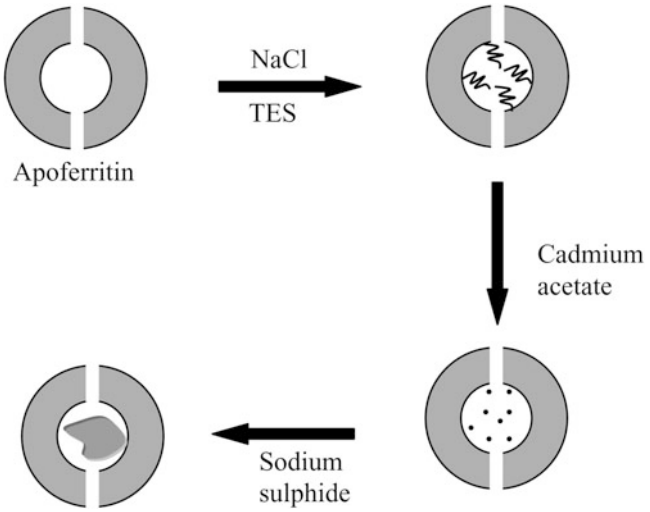


Fig. 5.6 Synthesis of CdS nanoparticles using ferritin

iron from ferritin to form apoferritin and then introduce metal ions to form metal nanoparticles inside the cavity or carry out some controlled reaction with metal ions to make a compound inside the cavity. In any case, ions can be removed or introduced inside the ferritin, through some available channels (see Fig. 5.5).

We shall discuss now the procedure to convert ferritin into apoferritin and how to use it in the synthesis of CdS nanoparticles. Spleen ferritin diluted with sodium acetate buffer should be placed in dialysis bag. A solution of sodium acetate and thioglycolic acid is made in which dialysis bag is kept under nitrogen gas flow for 2–3 h. Solution needs to be replaced from time to time for total 4–5 h. Further dialysis of apoferritin solution should be done against saline for one hour and in refreshed saline for ∼15–20 h. Apoferritin should then be mixed with solution having sodium chloride (NaCl) and N-tris (hydroxymethyl) methyl-2–aminoethanosulphonic acid (TES). Aqueous cadmium acetate is added to this solution and stirred continuously with constant N_2 gas purging. Aqueous solution of sodium sulphide (Na_2S) is added twice with one hour interval.

Process of CdS formation is stepwise (see Fig. 5.6) with Cd loading of 55 atoms per apoferritin colloid taking place in each step. Higher loadings like 110, 165, 220 ... are possible. Due to remarkably constant size of ferritin colloids and apoferritin derived from them, it is possible to obtain nanoparticles of very uniform size.

Besides CdS there are several other examples like controlled iron oxide, manganese, uranyl oxide, cobalt, cobalt-platinum alloy being synthesized inside ferritins.

5.5 Synthesis of Nanoparticles Using DNA

CdS (or other sulphide nanoparticles) nanoparticles can be synthesized using DNA. We have seen in Chap. 4 that organic molecules can cap the surfaces of nanoparticles growing in solutions. Similarly one can use DNA to bind with surface of growing nanoparticles. For example double stranded Salmon Sperm DNA can be sheared to an average size of 500 bp. Cadmium acetate can be added to desired medium like water, dimethylformamide, ethanol, propanol etc. and reaction is carried out in a glass flask with facility to purge the solution and flow with an inert gas like nitrogen. Addition of DNA should be made and then Na_2S can be added dropwise. Depending upon the concentrations of cadmium acetate, sodium chloride and DNA nanoparticles of CdS with sizes less than $\sim$10 nm can be obtained. It is possible to prove the presence of DNA on CdS nanoparticles. It is found that CdS nanoparticles synthesized by this route have cadmium-rich surface. DNA probably bonds through its negatively charged phosphate group to positively charged (Cd^+) nanoparticle surface. The other end of DNA is in fact free to interact with suitable proteins; such particles can be used as sensor of proteins.

We shall not go into more discussion of use of DNA here, but preformed charged nanoparticles can get bonded with phosphate group of DNA and even form organized arrays of nanoparticles.

Further Reading

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