Chapter 2

Literature Review
2.1. INTRODUCTION

Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. The word “Nano” is a greek word synonymous to dwarf meaning extremely small. Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. The properties of materials change as their size approaches the nanoscale. Nanomaterials often show unique and considerable change in physical, chemical and biological properties compared to their macro scaled counterparts [1]. For bulk materials larger than one micrometer, the percentage of atoms at the surface is minuscule relative to the total number of atoms of the material. For example, the bending of bulk copper (wire, ribbon, etc.) occurs with movement of copper atoms/clusters at about the 50nm scale. Copper nanoparticles smaller than 50nm are considered super hard materials that do not exhibit the same malleability and ductility as bulk copper. Ferroelectric materials smaller than 10nm can switch their magnetization direction using room temperature thermal energy, thus making them useless for memory storage. Suspensions of nanoparticles are possible because the interaction of the particle surface with the solvent is strong enough to overcome differences in density, which usually result in a material either sinking or floating in a liquid. Nanoparticles often have unexpected visible properties because they are small enough to confine their electrons and produce quantum effects. For example, gold nanoparticles appear deep red to black in solution. Nanoparticles have a very high surface area to volume ratio and this provides a tremendous driving force for diffusion, especially at elevated temperatures. The large surface area to volume ratio also reduces the incipient melting temperature of nanoparticles. Generally, metal nanoparticles can be prepared and stabilized by physical and chemical methods; the chemical approach, such as chemical reduction, electrochemical techniques, and photochemical reduction is most widely used [2,3]. Number of approaches are available for the synthesis of
nanoparticles, such as reduction of metal salt in aqueous phase [4], microemulsion approach [5], sol-gel technique [6], hydrothermal technique [7], green approach [8] etc.

Studies have shown that the size, morphology, stability, physical and chemical properties of the metal nanoparticles are strongly influenced by the experimental conditions, the kinetics of interaction of metal ions with reducing agents and adsorption processes of stabilizing agent with metal nanoparticles [9,10]. Hence, the design of a synthesis method in which the size, morphology, stability and properties are controlled has become a major field of interest [11]. Synthesis of noble metal nanoparticles for applications such as catalysis, electronics, optics, environmental, and biotechnology is an area of constant interest [12-18]. Gold, silver, and copper have been used mostly for the synthesis of stable dispersions of nanoparticles, which are useful in areas such as photography, catalysis, biological labeling, photonics, optoelectronics and surface-enhanced Raman scattering (SERS) detection [19,20]. Functionalized, biocompatible and inert nanomaterials (for example: gold and silica) have potential applications in cancer diagnosis and therapy [10, 21-25].

**Nanomedicine** is the medical application of nanomaterials [26]. Nanomedicine ranges from the medical applications of nanomaterials to nanoelectronic biosensors, and even possible future applications of molecular nanotechnology. Current problems for nanomedicine involve understanding the issues related to toxicity and environmental impact of nanoscale materials. Nanomedicine seeks to deliver a valuable set of research tools and clinically useful devices in the near future [27,28]. The commercial applications in the pharmaceutical industry may include: advanced drug delivery systems, new therapies and in vivo imaging [29]. Neuro-electronic interfaces and other nanoelectronics-based sensors are another active goal of research. Further down the line, the speculative field of molecular nanotechnology believes that cell repair machines could revolutionize medicine and the medical field. Two forms of nanomedicine that have already been tested in mice are using gold nanoshells to help diagnose and treat cancer and using liposomes as vaccine adjuvants and as vehicles for drug transport [30]. Similarly drug detoxification is also another application for nanomedicine which has shown promising results in rats [31]. A benefit of using nanoscale materials for medical technologies is that smaller devices are less invasive
and can possibly be implanted inside the body. These devices are faster and more sensitive than typical drug delivery [32]. Nanomedical approaches to drug delivery center on developing nanoscale particles or molecules to improve drug bioavailability. Bioavailability refers to the presence of drug molecules where they are needed in the body and where they will do the most good. Drug delivery focuses on maximizing bioavailability both at specific places in the body and over a period of time. This can potentially be achieved by molecular targeting using nanoengineered devices [33,34]. It is all about targeting the molecules and delivering drugs with cell precision. More than $65 billion are wasted each year due to poor bioavailability. In vivo imaging is another area where tools and devices are being developed. Using nanoparticle contrast agents, images such as ultrasound and MRI have a favorable distribution and improved contrast. The new methods of nanoengineered materials that are being developed might be effective in treating illnesses and diseases such as cancer. What scientists will be able to achieve in the future is beyond current imagination. This might be accomplished by self assembled biocompatible nano-devices that will detect, evaluate, treat and report to the clinical doctor automatically. Drug delivery systems, lipid- or polymer-based nanoparticles can be designed to improve the pharmacological and therapeutic properties of drugs [35].

2.2. PREPARATION OF ULTRAFINE PARTICLES USING VARIOUS TECHNIQUES

It has been reported that the use of powders consisting of unagglomerated submicron sized spherical particles with narrow size distribution as starting materials in the processing of ceramics and metal particles, give rise to an important reduction of scattering time and temperature and improves considerably the mechanical properties of final products [36,37]. In order to produce fine powders, several techniques have been developed.

2.2.1. Evaporation Condensation

In this technique the bulk sample is heated to liquification in vacuum. An inert gas is passed over it and the gas carries some of the evaporated sample along with it. The gas
may then be forced through a precipitator, or exposed to a cooled substrate, in order to obtain the microparticles. Chemical reaction may occur almost incidentally in the course of the evaporation condensation process, or it may be necessary to make special provisions for such a reaction.

Using the first method, Amick et al. [38], have prepared fine metal oxide by striking an arc in hydrogen atmosphere. Examples of special surface hydroxylation of the fine powders by control of water content of atmosphere in which condensation occurs [39,40] or in the provision of a specific reactive atmosphere in which vapor-vapor reaction may take place. By means of vapor-vapor reaction wide range of materials such as metal, metalloids, oxides, carbides, borides, nitrides, silicides, sulfides, have been prepared [41]. Other reactions falling into this category may be of the type:

\[
\text{vapor} + \text{vapor} \rightarrow \text{solid} + \text{vapor}
\]

Depending on the reaction condition (temperature, rate, concentration of reactants, condensation, conditions etc.) the product can take the form of platelet, whiskers, amorphous deposit or fine powders. To produce a fine powder, multiple nucleation and restricted particle growth are necessary. This can be obtained by quenching, conditions of concentration, gas flow etc., and the rate of provision of nuclei are such that the particle growth is naturally restricted. An example of such natural control of growth is the hydrolytic decomposition of volatile alkoxides of titanium, zirconium etc [42]. Many of the fine powders produced on an industrial scale result from vapor phase reactions. Such materials are argon black, titanium dioxide, zinc antimony oxides.

### 2.2.2. Sol-gel Technique

The sol-gel method involves the conversion of a sol (a fluid colloidal suspension of a solid and a liquid) to a gel (a semi rigid colloidal dispersion of a solid in a liquid). In this technique, first, the concentrated suspension of a metallic oxide or hydroxide is formed (Sol). The sol is then dehydrated by evaporation or solvent extraction, resulting in formation of colloidal suspension (Gel). Controlled heating in autoclave or in vacuum, dries the gelated material. This converts the gel to finely divided metal oxide powder, with particles size in the range of 0.03 to 0.1µm. This method can produce
extremely homogeneous mixtures of two or more components because the mixing of ingredients takes place at the atomic level in a liquid rather than in the solid state. A wide range of pure and mixed oxides may thereby be produced with controlled particle size and the composition. Krishnakutty et al. [43] have prepared titanium isopropoxide with an alcoholic solution of water. The titania hydrosol thus prepared, was gelled by drying and then calcined to give TiO$_2$ particles of 6 nm.

All the above methods produce particles of larger size with high polydispersity. Dr. Richard L. Axelbaum at Washington University in St Louis has developed a patented technology that makes nanoparticles smaller, faster, purer and cheaper [44]. It is based on the sodium reduction of metal halides such as boron trichloride and titanium tetrachloride, in a 4 ft long reactor with a 3-inch flame. This produces nanoparticles of various metals and ceramics, which are 10-60nm in size.

2.2.3. Precipitation

With this process, in order to produce a fine powder, a large number of nuclei must be formed and growth should be restricted to keep the particles small. Rate of nucleation and growth vary widely in reactions in which a precipitate is formed and consequently there are wide differences in the effects of conditions (for example, concentration or degree of mixing) on the particle size of the final precipitate. After precipitation is complete, there is still change in the particles size distribution in the precipitate which can be attributed the greatest solubility of the finer particles, the so-called ‘Ostwald ripening’. Because of this, the coarse particles grow at the expense of the finer [45]. In addition normal recrystallization occurs and leads to cement together of the precipitate particles [46]. The extent to which this later event occurs is dependent on the absolute solubility, since it involves the deposition of materials at necks between particles as a result of exchange between the surface of solid and solution. Absolute solubility, in turn is affected by change of pH, presence of common ions or neutral salts etc. To some extent, also, the size of the precipitated particles is also dependent on these factors. This has been explained for zinc sulfide precipitates [47].

One of the important advantages of precipitation process for the preparation of fine powders lies in their ability to prepare double oxides following simultaneous
precipitation of the component hydrated oxides, oxalates etc. However, it is possible that the preparative conditions (e.g. pH, concentration, rate of mixing, adsorption) may preclude quantitative stoichiometric and simultaneous precipitation and prevent the formation of compounds and solid solutions of mixtures.

Precipitation processes prepare much of the silica used industrially. We get hydrated silica from this process, which is then dried to get pure silica. Hydrated finely divided silicates of calcium, aluminium etc. are also prepared by precipitation [48]. It has been found possible to prepare essentially monodisperse silica spheres, larger than about 0.05µm by hydrolysis of alkyl silicate and subsequent condensation of silicic acid and alcoholic solutions [49]. Titanium dioxide is also prepared by precipitation [50]. In this method a solution of titanyl sulphate is hydrolyzed at elevated temperatures. Seeding is employed, primarily to obtain the required rutile form of the oxide. The precipitate is initially amorphous, but crystallizes on ageing. Important factors in the precipitation include the quantity of seed, the activity, concentration of the solution and the rate of heating.

2.2.4. Thermal Decomposition

If the thermal decomposition takes the form:

\[ \text{A}_{\text{solid}} \rightarrow \text{B}_{\text{liquid}} + \text{C}_{\text{gas}} \]

The formation of the new phase B results from local structural fluctuations in the lattice of A which produce conditions favorable for the formation of a nucleus of B. Such sites are situated at regions of disorder e.g. vacancy, interstitial impurity clusters etc. In most decomposition the molecular volume of the product is less than that of the reactants. Because of this volume change, both reactants and product crystal become strained, and this strain cannot be relieved until the critical shear stress in the neighborhood of the nucleus is exceeded. Thus, there is a strain energy involved in the growth of a nucleus upto, and especially beyond, it’s critical radius. Typically, the interfacial strain energy is of the order of 1 Kcal/mole and this is sufficient to account for the slow growth of small nuclei, which leads to the formation of a fine powder [51]. For a particular chemical decomposition the rate of formation of nuclei is governed, in part, by imperfection in
the crystal lattice of reactant A. In some instances the stereochemical configuration of the imperfection, seems to be important. For example, in the decomposition of calcium carbonate, the reactivity of the solid is enhanced only at certain dislocation [52]. Growth, in turn, may vary with the particle size and the method of preparation of reactant A. Thus, the formation of anhydrous barium sulfate from the monohydrate is much more rapid when the monohydrate is in the form of small crystals, than when the monohydrate crystals are large. This is due to surface nucleation followed by preferential growth along the surface of grain boundaries (where strain energy is less) prior to penetration of the crystallites [53]. Study of decomposition of oxy compounds; show that the oxide product has a definite orientational relationship to the starting materials. Thus magnesia prepared from needles of magnesium carbonate trihydrate, retains the needle shape in spite of about 70 percent reduction in volume [41]. This does not mean that, in order to prepare a fine powder by thermal decomposition, the starting material must be in the form of fine powder. The macroscopic form of the product is no indication of its microstructures. Decomposition of magnesium hydroxide in either massive form, or as submicrometer hexagonal platelets, produces magnesia of the same order of surface area [54].

### 2.2.5. Aerosols Technique

This method essentially consists of the generation of an aerosol of a liquid metallic compound, which is further hydrolyzed to form corresponding metal oxide. Since such droplet acts as a separate container, this technique as a rule produces spherical particles, the size of which can be controlled by adjusting the experimental conditions. Marico et. al. [55] have prepared spherical titanium dioxide by hydrolysis of aerosol consisting of liquid titanium (IV) compounds. The droplets were nucleated with AgCl in a falling film generator and subsequently hydrolyzed in chambers kept at different temperatures. When titanium (1M ethoxide) was used as starting material, they obtained TiO$_2$ particles with model diameter ranging between 0.06 and 0.6 µm. They have varied the size of particles by changing the temperature of the falling film, generator, the flow rate of carrier gas, and AgCl nuclei concentration. Kaczmarek et. al. [56], have also used aerosol technique to prepare ultrafine barium ferrite particles. This technique was
employed to prepare particles ultrafine barium ferrite particles. This technique was employed to prepare particles of narrow size distribution from various ceramic oxides such as $\text{Al}_2\text{O}_3$ [57], $\text{SiO}_2$ [58], $\text{SnO}_2$ [59], mixed titanium silicon and titanium aluminium oxide [60].

### 2.2.6. Green and Aqueous Approach

**Green chemistry**, also called sustainable chemistry, is a philosophy of chemical research and engineering that encourages the design of products and processes that minimize the use and generation of hazardous substances [61]. Green chemistry seeks to reduce and prevent pollution at its source. It aims to avoid problems before they happen. As a chemical philosophy, green chemistry applies to organic chemistry, inorganic chemistry, biochemistry, analytical chemistry, and even physical chemistry. The focus is on minimizing the hazard and maximizing the efficiency of chemical. It is distinct from environmental chemistry which focuses on chemical phenomena in the environment. In 2005 Ryōji Noyori identified three key developments in green chemistry: use of supercritical carbon dioxide as green solvent, aqueous hydrogen peroxide for clean oxidations and the use of hydrogen in asymmetric synthesis [62]. The term *green chemistry* was coined by Paul Anastas in 1991. Paul Anastas and John C. Warner developed 12 principles of green chemistry [63], which help to explain what the definition means in practice. The principle covers concepts such as:

- the design of processes to maximize the amount of raw material that ends up in the product;
- the use of safe, environment-benign substances, including solvents, whenever possible;
- the design of energy efficient processes;
- the best form of waste disposal: not to create it in the first place.

Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment [64,65]. This increasing demand must be accompanied by “green” synthesis methods. In the global efforts to reduce generated hazardous waste, “green” chemistry and chemical
processes are progressively integrating with modern developments in science and industry. Implementation of these sustainable processes should adopt the fundamental principles of green chemistry [66-70]. These principles are geared to guide in minimizing the use of unsafe products and maximizing the efficiency of chemical processes. Hence, any synthetic route or chemical process should address these principles by using environmentally benign solvents and nontoxic chemicals [66].

Nowadays, synthesis of metal nanoparticles via green approach is gaining much attention. Metal nanoparticles have been synthesized very quickly and efficiently by using natural available reducing agents rather than using harmful organic chemicals. Synthesis of metal nanoparticles via green approach have been reported by using green tea extract, coffee powder, fruit extract, caffeine, fruit pulp, [71-74] etc. A green tea extract is an herbal derivative from green tea leaves, containing antioxidant ingredients – mainly green tea catechins (GTC). They can also be called green tea polyphenols (GTP or GTPs). The category includes epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), of which, EGCG accounts for more than 40% of the total content. The cardinal antioxidative ingredient in the green tea extract is green tea catechins (GTC), which comprise four major epicatechin derivatives; namely, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Other components include three kinds of flavonoids, known as kaempferol, quercetin, and myricetin.

2.3. MICROEMULSION

Microemulsions are clear, thermodynamically stable, isotropic mixtures of oil, water and surfactant (sometimes in combination with a cosurfactant). The aqueous phase may contain salt(s) and/or other ingredients, and the “oil” may actually be a complex mixture of different hydrocarbons and olefins. In contrast to ordinary emulsions, microemulsions form upon simple mixing of the components and do not require the high shear conditions generally used in the formation of ordinary emulsions. The three basic types of microemulsions are direct (oil dispersed in water, o/w), reversed (water dispersed in oil, w/o) and bicontinuous.
A micelle is an aggregate of surfactant molecules dispersed in liquid (water). A typical micelle in aqueous solution forms an aggregate with the hydrophilic “head” regions in contact with surrounding solvent, sequestering the hydrophobic tail regions in the micelle centre (Figure 2.1 (a)). This type of micelle is known as a normal phase micelle (oil-in-water micelle). On the other hand, reverse micelle are defined as: In a non-polar solvent, exposure of the hydrophobic tail to the surrounding solvent, giving rise to a water-in-oil system. In this case the hydrophilic heads are in the micelles core and the hydrophobic tail is away from the center and in contact with the organic solvent (Figure 2.1 (b)).

Micelles are approximately spherical in shape. Other phases, including shapes such as ellipsoids, cylinders and bilayers are also possible. The shape and size of a micelle is a function of the molecular geometry of its surfactant molecules and solution conditions such as surfactant concentration, temperature, pH, and ionic strength. The process of forming micelles is known as micellisation and forms part of the phase behavior of many lipids according to their polymorphism. Micelles only form when the concentration of surfactant is greater than the critical micelle concentration (CMC) and the temperature of the system is greater than the critical micelle temperature or Krafft temperature. In water, the hydrophobic effect is the driving force for micelle formation, despite the fact that assembling surfactant molecules together reduces their entropy. At very low concentrations, only monomers are present in true solution. As the concentration is increased, a point is reached at which the unfavorable entropy
considerations, derived from the hydrophobic end of the molecule, become dominant. At this point, the hydrocarbon chains of a portion of the surfactant must be sequestered away from the water and hence, micelles start forming. Broadly speaking, above the CMC, the entropic penalty of assembling the surfactant molecules is less than the entropic penalty of caging the surfactant monomers with water molecules. Also important are enthalpic considerations, such as the electrostatic interactions that occur between the charged parts of surfactants.

### 2.3.1. Preparation of Ultrafine Nanoparticles using Microemulsion Approach

A wide range of techniques have been developed for the preparation of nanomaterials. Nowadays, there are a number of technologies available for nanoparticles synthesis, physical methods such as mechanical milling [75] and inert gas condensation [76], along with chemical methods such as chemical reduction [77], photochemical reduction [78], electrodeposition [79], hydrothermal [80], gas phase techniques such as laser evaporation [81], sputtering, laser pyrolysis, flame atomization and flame spray pyrolysis [82] etc., to the liquid phase techniques such as coprecipitation from homogeneous solutions and sol-gel reactions [83,84], solvothermal processes [85], sonochemical and cavitation processing [86], and surfactant and polymer-templated synthesis [87], but among all these methods, the microemulsion technique has been demonstrated as a very versatile and reproducible method that allows to control over the nanoparticle size and yields with a narrow particle size distribution [88]. This method offers a series of advantages with respect to other methods, namely the use of simple equipment, the possibility to prepare a great variety of materials with a high degree of particle size and composition control, the formation of nanoparticles with often crystalline structure and high specific surface area and the use of soft conditions of synthesis near ambient temperature and pressure.

Microemulsion not only act as microreactors for hosting the reaction but also as steric stabilizer to inhibit the aggregation of polymeric reacting species during the reaction period. As a result, inorganic precipitation in w/o micro-emulsion usually generates colloidal particles of nanometer range (less than 100nm), which are much smaller than those synthesized in normal aqueous solutions. Indeed, micro-emulsion synthesis routes
have been used to make nanoparticles of numerous materials, including metals, metal oxide, metal boride, metal sulfide, metal halide, and metal carbonate [89].

A large number of different nano-materials have been synthesized in water-in-oil microemulsions or reverse micelles. Particle growth has shown to be strongly dependent on intermicellar exchange rates. The resultant particle size appears to be dependent on five dominant parameters [90]:

- solvent type
- surfactant/co-surfactant type
- concentration of the reagents
- ionic additives
- composition via [water]:[surfactant] ratio, $W_o$

The traditional method is based on water-in-oil microemulsions (W/O), and it has been used for the preparation of metallic and other inorganic nanoparticles since the beginning of the 1980’s [91]. The main strategy for the synthesis of metallic nanoparticles in W/O microemulsions consists in mixing two micro-emulsions, one containing the metallic precursor and another one the precipitating agent. Upon mixing, both reactants will contact each other due to droplets collisions and coalescence, and they will react to form precipitates of nanometric size. This precipitate will be confined to the interior of microemulsion droplets [92]. Numerous investigations have been published about the use of W/O microemulsions for the preparation of a variety of nanomaterials, such as metallic and bimetallic nanoparticles, single metal oxide as well as mixed oxides, quantum dots and even complex ceramic materials [91,93-98]. Materials synthesized in w/o microemulsions exhibit unique surface properties; for example, nano-catalysts prepared by this method show better performance (activity, selectivity) than those prepared by other methods [99].

The preparation procedure of metallic nanoparticles in W/O microemulsion commonly consists of mixing of two microemulsions containing metal salt and a reducing agent [100], as shown in Figure 2.2.
Figure 2.2: Schematic illustration of nanoparticles preparation using microemulsion techniques: Particle formation steps. $k_{\text{chem}}$ is the rate constant for chemical reaction, $k_{\text{ex}}$ is the rate constant for intermicellar exchange dynamics, $k_n$ is the rate constant for nucleation and $k_g$ is the rate constant for particle growth.

After mixing two microemulsions, the exchange of reactants between micelles takes place during the collisions of water droplets as a result of Brownian motion, the attractive van der Waals forces and repulsive osmotic and elastic forces between reverse micelles. Successful collisions lead to coalescence, fusion and efficient mixing of the reactants. The reaction between solubilizates results in the formation of metal nuclei. Bönnemann et al. [101] reported that at the initial stage of the nucleation, metal salt is reduced to give zerovalent metal atoms, which can collide with further metal ions, metal atoms, or clusters to form an irreversible seed of stable metal nuclei. Growth then occurs around this nucleation point where successful collision occurs between a reverse micelle carrying a nucleus and another one carrying the product monomers with the arrival of more reactants due to intermicellar exchange. The nucleation reaction and particle growth take place within the micelles. The size and morphology of as-prepared
nanoparticles depend on the size and shape of the nanodroplets and the type of the surfactant, whose molecules are attached on the surface of the particles to stabilize and protect them against further growth.

Nanoparticles have been synthesized employing this method for a variety of novel applications; typical examples include catalysts for fuel cells [102,103], food applications [104], nanoprobes for fluorescent bioassays [105], novel nanofluids [106] and uses in dechlorinating chlorinated olefins [107]. Other preparations have employed novel biocompatible microemulsions [108], environmentally safe systems [109,110] including high-efficiency silicone surfactants [111,112]. More complex syntheses have also been reported which include mixed Co/Ag [113] nanoparticles, microemulsions containing monomer and initiator to form nanoparticles constrained inside a polymer matrix [114] and nanotube-containing microemulsions for generating carbon nanotube/polyaniline composites.

AOT (Sodium 2-ethylhexylsulfosuccinate) based systems are amongst the best characterized systems, and it has been found that the size of the inverse or reverse microemulsion droplets formed by this type of systems increases linearly with the amount of water added to the system [115] and can increase from 4nm to 18nm with 0.1M sodium AOT surfactant (water/AOT/isooctane). AOT based systems are probably the most used for the synthesis of inorganic nanoparticles in w/o microemulsions for two reasons: good control of droplet size and the large microemulsion regions found in water/AOT/alkane systems, which give rise to a great deal of compositions available for nanoparticle synthesis. Systems based on cetyltrimethylammonium bromide (CTAB) usually combine this surfactant with alcohols such as hexanol as the oil phase. This alcohol act as co-surfactant, adsorbing at the oil/water interface along with the surfactant. The microemulsion region of water/CTAB/hexanol system is relatively narrow, however, when shorter alcohols such as butanol are added as cosurfactant, the microemulsion regions are considerably enlarged [116].

Tin dioxide nanoparticles were prepared from SnCl₄.5H₂O using the microemulsion system with cetyltrimethyl ammoniumbromide (CTAB), 1-butanol, and isooctane [117].
CeO$_2$ nanoparticles were synthesized by the thermal decomposition of cerium oxalate precursors. The precursor oxalate was synthesized by the reverse micellar (microemulsion) route with CTAB as the surfactant, the cerium oxalate precursor was synthesized using two different microemulsions, one containing 0.1M cerium nitrate hexahydrate and the other containing 0.1M ammonium oxalate solution. Similarly, ZrO$_2$ nanoparticles were also obtained from the decomposition of zirconium oxalate [117].

A wide variety of nanosize particles of Cd$_{1-x}$Mn$_x$S (a magnetic semiconductor) [118], Cd$_{1-x}$Zn$_x$S [119], Ag$_2$Se [120], Cu$_2$S, CuS [121] and AgCl [122] were prepared. Similarly Fe-Cu alloys [123] with super paramagnetic properties or alkythiol covered Ag and Ag$_2$S particles, which can be self assembled at surfaces, have been prepared [124,125]. Hydrolysis of Ti(iPrO)$_4$ or Si(OEt)$_4$ in water in oil microemulsion yield nanometer sized TiO$_2$ [126] or SiO$_2$ [127], respectively. Ayyub et. al. [128] have synthesized Fe$_2$O$_3$ using three components microemulsion consisting of sorbitant monoleate as the surfactant, 2-ethyl hexanol as the oil and an aqueous Fe(NO$_3$)$_3$ solution as the water pool. The precipitation was effected by adding NH$_4$OH to the microemulsion and the resultant powder was calcined at 250°C. They have also studied the effect of ferric ion concentration in water pool on the particle size and the nature (phase) of Fe$_2$O$_3$. It was found that increasing the Fe(NO$_3$)$_3$ concentration in the aqueous precursor solution from 0.312% to 20% resulted in an increase in the particle size of the final product from 5 to 80nm. The formation hollow silica [129] and hollow gold nanoparticles [130] have been reported by using reverse micelle approach (AOT/water/hexane).

Wongwailikhit et al. [131] prepared iron (III) oxide, Fe$_2$O$_3$ using W/O microemulsion by mixing the required amount of H$_2$O in a stock solution of AOT in n-heptane. They obtained spherical, monodisperse nanoparticles with diameter of about 50nm.

Sarkar et al. [132] prepared pure monodispersed zinc oxide nanoparticles of different shapes. Microemulsion was composed of cyclohexane, Triton X-100 as surfactant, hexanol as cosurfactant and aqueous solution of zinc nitrate or ammonium hydroxide/sodium hydroxide complex. The nanoparticles were separated by centrifuging and dried at 50°C for 12 hr.
Sanchez-Dominguez et al. [133] prepared Pt, Pd and Rh nanoparticles by an oil-in-water microemulsion reaction method. The microemulsion containing metal precursor (Pt-COD, Pd-AAc, Rh-COCl) was prepared by mixing appropriate amounts of surfactant, cosurfactant(s), oil phase and deionized water. The used systems: water/Tween 80/Span 20/1,2-hexanodiol/ethyl oleate (System A); water/Brij 96V/butyl-S-lactate (System B) and water/Synperonic 10/5/isoctane (System C). Then to the solutions, under vigorous stirring at 25°C, a small amount of an aqueous solution of sodium borohydride was added.

Ag/Cu bimetallic nanocatalysts supported on reticulate-like γ-alumina were prepared by a microemulsion method using N₂H₄.H₂O as the reducing agent. The catalysts were activated by calcination followed with hydrogen reduction at 873K, and the properties were confirmed using various characterization techniques. Ag-Cu bimetallic nanoparticles supported on γ-alumina showed better catalytic activity on the epoxidation of styrene as compared with the corresponding monometallic silver or copper [134].

2.4. SILICA

Silicon dioxide, also known as silica (from the Latin silex), is an oxide of silicon with the chemical formula SiO₂. It has been known for its hardness since ancient times. Silica is most commonly found in nature as sand or quartz, as well as in the cell walls of diatoms. In the majority of silicates, the Si atom shows tetrahedral coordination, with 4 oxygen atoms surrounding a central Si atom (as shown in Figure 2.3). The most common example is seen in the quartz crystalline form of silica SiO₂. In each of the most thermodynamically stable crystalline forms of silica, on average, all 4 of the vertices (or oxygen atoms) of the SiO₄ tetrahedra are shared with others, yielding the net chemical formula: SiO₂. SiO₂ has a number of distinct crystalline forms (polymorphs) in addition to amorphous forms. With the exception of stishovite and fibrous silica, all of the crystalline forms involve tetrahedral SiO₄ units linked together by shared vertices in different arrangements.
Mesoporous silica is a form of silicon dioxide (SiO₂), commonly known as silica. A mesoporous material is a material containing pores with diameters between 2 and 50nm. Porous materials are classified into several kinds according to their size. According to IUPAC notation, microporous materials have pore diameters of less than 2nm and macroporous materials have pore diameters of greater than 50nm; the mesoporous category thus lies in the middle. The most common types of mesoporous nanoparticles are MCM-41 and SBA-15. These are solid materials, which are comprised of a honeycomb-like porous structure with hundreds of empty channels [135].

The synthesis of mesoporous materials has been of great interest due to an ever-expanding list of uses, ranging from chemical sensors to drug delivery [136]. In the past decade, many mesoporous materials have been developed. Research continues on the particles, which have applications in catalysis, drug delivery, cancer therapy and imaging. One of the well-developed mesoporous materials is silica. Recently, mesoporous silica nanoparticles (MSN) have been intensively explored in materials research due to their unique properties, such as high surface areas, large pore volumes, tunable pore sizes with a narrow distribution and tunable particle diameters [137,138]. Silica is attractive because it is chemically inert, thermally stable, harmless and inexpensive [139]. Many preparation methods of mesoporous silica materials with various morphologies from thin film, sphere, fiber, bulk form, such as the MCM series [140] and the SBA series [141], have been reported. In these synthetic reactions the organic template-driven synthesis process is commonly used [142]. Although the
previously reported methods are feasible for industry, they have several disadvantages: (i) use of harmful chemicals (e.g. ammonia [143,144] or \( \text{N}_2\text{H}_4 \) [145] as a catalyst) that can be a problem for bio-applications and (ii) the preparation of spherical particles with controllable size less than 100nm in diameter. A procedure for producing mesoporous silica was patented around 1970. It went almost unnoticed and was reproduced in 1997. Mesoporous silica nanoparticles (MSNs) were independently synthesized in 1990 by researchers in Japan. These were later produced also at Mobil Corporation laboratories and named Mobil Crystalline Materials, or MCM-41. Six years later, silica nanoparticles with much larger 4.6 to 30nm pores were produced at the University of California, Santa Barbara. The material was named Santa Barbara Amorphous type material, or SBA-15. These particles also have a hexagonal array of pores. The researchers who invented these types of particles planned to use them as molecular sieves. Today, mesoporous silica nanoparticles have many applications in medicine, biosensors, and imaging.

MSN of various types and exhibiting different properties have previously been synthesized via different methods [146-156]. In MSN synthesis, various chemical materials, such as sodium silicate, tetramethyl ammonium silicate and tetraethyl orthosilicate etc. are used as silica precursors. Quat-ernary ammonium surfactants are used as structure-directing agents under a wide range of pH and temperature conditions [157,158]. By liquid crystal templating (LCT) mechanism, in aqueous solution, surfactant micelles organize into an ordered array of hexagonal micellar rods that form a liquid template. Hydrolysis of the silica precursor yields the silica nucleus which enters the template. Inorganic silica walls are formed upon condensation of the silicate seeds [159]. The template is subsequently removed by either solvent extraction using alcohols (methanol or ethanol) or by calcination at high temperatures. Calcination is a key step for obtaining accessible meso-pores, large surface areas and pore volumes. During calcination, condensation of silanol groups (Si-OH) into siloxane (Si-O-Si) bridges takes place, thus consolidating the mesoporous structure [160].

Silica-based ordered mesoporous materials combine advantages of both silica and mesoporous materials [161]. The versatility of silica chemistry allows for facile integration with other materials, including metal nanoparticles, fluorescent molecules,
or rare earth elements [162-165]. Mesoporous materials provide large surface area, high pore volume, and uniform pore size distributions [166]. Combining aforementioned advantages in both bulk and nanosized materials offers characteristics that can be used in a range of applications [166,167]. Soon after the discovery in the early 1990’s [168,169], scientists have focused on broadening the functionalities of mesoporous silica, such as incorporating or attaching organic molecules [166,170]. Mesoporous silica nanoparticles (MSNs) have been drawing attention from researchers in medicine, in particular, as they could potentially be used as cargo (drug) delivery vehicles, imaging probes, or theranostic materials [171-174]. Size and stability of MSNs in physiological media as well as surface properties are crucial in aforementioned applications [175,176]. Furthermore, the pore structure and geometry of MSNs also determine their final use [177]. These features will influence uptake and release rates of adsorbents as diffusion rates are geometry-dependent [178,179]. Width of pore entrance and pore/cavity size will limit the size of guest molecules to be carried. Finally, the chemical functional groups present at the surface will contribute to adsorption–desorption affinity between adsorbate and adsorbent [178,179]. Carefully tuning all of these features is likely a key to success in an application of interest. There are various reports focusing on the study of surface functionalization and structure control of MSNs [180-184]. The structure of mesoporous silica is generally controlled by the interaction between surfactant micelles and silica species. The geometry of surfactant micelles is known mainly to determine the morphology of mesoporous materials [167]. Organosilanes are used to introduce covalently linked surface functionality either by post-synthetic grafting or by co-condensation [185]. The latter route provides uniform surface modification but can affect shape and morphology of final products at the same time [180,181]. Use of a high amount of co-silane precursor often results in loss of hierarchical structure [180,186]. This loss of structure is because organosilane molecules can disrupt the packing of surfactants and/or alter the geometry of structure-directing micelles, which in return affects the assemblage between silica-micelle complexes [180,181]. Miscibility of the silica precursors is also reported to play a role in directing the structure [183]. Incorporating a desired amount of organosilane into MSNs without rupturing their periodicity is thus a challenge. Recently, Atluri et al. [186] reported the synthesis of micrometersized mesoporous silica materials by means
of co-condensation between tetraethyl orthosilicate (TEOS) and 3-aminopropyl triethoxysilane (APTES) yielding a cubic $Pm\bar{3}n$ structure. By varying the molar ratio between cationic surfactant (hexadecyltrimethylammonium bromide; CTAB) and APTES, structures of resulting materials changed from hexagonal to mesocage cubic with $Pm\bar{3}n$ symmetry. This work opened the possibility of the direct synthesis of cubic MSNs containing organic moieties. Zhu et al. [187] demonstrated the biocompatibility, the high structural stability and chemical versatility of silica using silica-coated semiconductor quantum dots. Furthermore, the use of silica particles has been shown in mice. Popat et. al. [188] used fluorescent labeled silica particles at a low dose of 20 mg kg$^{-1}$, the particles were cleared from the mice body through the renal system. Additionally, the researchers noted that MSN are non-toxic at relatively low doses.

MSN with controlled properties can be extended to a wide range of applications, such as catalysts supports, drug delivery, adsorption and separation of proteins, cell imaging, cell labeling and enzyme adsorption and immobilization [189]. More specifically, the potential utilization of MSN materials in medical and pharmaceutical drug delivery systems is well documented [190-198]. Radin et al. [199] research findings showed that MSN could be used to store and gradually release antibiotics and other drugs since they posses abundant pore surface Si-OH bonds for loading and releasing drug molecules. Moreover, the use of nanoparticles as drug carriers can enhance penetration of therapeutic drugs into the brain [200] and prolong blood circulation time of drugs [201]. The suitability of MSN for important biotechnological and biomedical applications is also due to their small size which allows a facile endocytosis by living animal and plant cells without any significant cytotoxicity [202]. Small and tunable pore sizes allow the adjustment of loading of different drug molecules and to study the kinetics of drug release with high precision. High surface areas and large pore volumes allows high loading, while the unique porous structure renders the ‘no-leaking’ capability of loaded drug molecules [188,199].

Researchers are incessantly engaged in efforts to come up with facile methods for the synthesis of MSN with controlled properties mostly with smaller particle sizes, tunable pore diameters, high surface area and high pore volumes. The use of auxiliary reagents,
such as pore expanders and/or use of surfactants with varying chain lengths are the commonly used techniques for tuning MSN properties.

2.5. GOLD NANOPARTICLES

Although gold is the subject of one of the most ancient themes of investigation in science, its renaissance now leads to an exponentially increasing number of publications, especially in the context of emerging nanoscience and nanotechnology with nanoparticles and self-assembled monolayers (SAMs). AuNPs are the stable metal nanoparticles, and they present fascinating aspects such as their assembly of multiple types involving materials science, the behavior of the individual particles, size-related electronic, magnetic and optical properties (quantum size effect) and their applications in catalysis and biology [203]. The reputation of soluble gold until the Middle Ages was to disclose fabulous curative powers for various diseases, such as heart and venereal problems, dysentery, epilepsy, and tumors, and for diagnosis of syphilis [203].

In 1857, Faraday reported the formation of deep red solutions of colloidal gold by reduction of an aqueous solution of chloroaurate (AuCl₄⁻) using phosphorus in CS₂ (a two-phase system). He investigated the optical properties of thin films prepared from dried colloidal solutions and observed reversible color changes of the films upon mechanical compression (from bluish-purple to green upon pressurizing) [204]. The term “colloid” (from the French, colle) was coined shortly thereafter by Graham, in 1861 [205]. Although the major use of gold colloids in medicine in the Middle Ages was perhaps for the diagnosis of syphilis, a method which remained in use until the 20th century [206-208]. In the 20th century, various methods for the preparation of gold colloids were reported and reviewed [208-214].

Recently, several methods have been developed to synthesize gold nanoparticles with different sizes and shapes, such as spherical gold nanoparticles [215], gold nanorods [216], gold nanoshells [217] and gold nanocages [218]. To date, many methods including chemical reduction of metal salts, photolysis or radiolysis of metal salts, ultrasonic reduction of metal salts, and displacement of ligands from organometallic compounds have been used to prepare gold NPs. In situ chemical reduction of tetrachloroauric acid (HAuCl₄) precursor is perhaps the most popular route for
synthesizing gold NPs. Reducing agents such as sodium or potassium borohydride, hydrazine, ascorbic acid, nitric/hydrochloric acid, and dimethyl formamide are commonly used in the reduction of metal ions. When a reducing agent is added to the solution containing the metal salt, the metal ions are reduced and metallic solid particles are nucleated. Because NPs tend to be unstable in solution, special precautions have to be taken to avoid their aggregation or precipitation. The most common strategy is to employ surfactants or some other capping agents, which not only prevents aggregation but also results in functionalized and stabilized metal particles.

Among the conventional methods of synthesis of spherical gold nanoparticles by reduction of gold(III), the most popular one has been that using citrate reduction of gold chloroaurate (HAuCl₄) in water, which was introduced by Turkevitch in 1951. Gold nanoshells were first engineered by Halas and co-workers with directly depositing gold onto silica colloidal spheres. In a typical process, uniform silica spheres were first synthesized using the Stöber method.

The size-dependent optical, magnetic, electronic and catalytic properties of metal nanoparticles have attracted significant attention in the recent years. While the chemical composition of a nanoparticle is important, even more important are the morphology (size and shape) and its surface properties. Metal nanoparticles, particularly gold, are being considered in wide ranging applications such as photonics, information storage, electronic and optical detection systems, therapeutics, diagnostics, photovoltaics, and catalysis. Gold NPs are well known for their surface plasmon resonance (SPR) properties, which originate from collective oscillation of their conduction electrons in response to optical excitation. SPR is an optical phenomenon arising from the interaction between the conduction electrons in a metal and the electromagnetic field. These nanoparticles strongly enhance the scattering and absorption of the electromagnetic field which leads to several applications such as surface enhanced Raman scattering (SERS), bio-imaging contrast enhancement agents and photothermal therapy. The SPR frequency of gold NPs has been shown to depend on particle size, shape, dielectric properties, surface modification, and refractive index of the surrounding medium. For some novel metals such as Au, Ag and Cu nanoparticles, the SPR peaks are in the visible region [219]. At SPR frequency, these nanoparticles
strongly enhance the scattering and absorption of the electromagnetic field. The SPR highly depends on the geometry of the nanoparticles. For the spherical gold nanoparticles with a diameter 40-100nm, SPR peaks are centered at around 550nm. For the gold nanorods, the SPR peaks are split into two parts; one is located in the visible light region and the other one can be shifted into near infrared region. Figure 2.4 shows a typical absorption spectrum of gold nanorods. The surface plasmon absorption of gold nanorods have two bands: a strong long wavelength band in the near infrared region due to the longitudinal oscillation of electrons and a weak short wavelength band in the visible region around 520nm due to the transverse electronic oscillation. For the gold nanoshells and gold nanocages, the SPR peaks can be tuned in the range of 400nm-1000nm by controlling the shell thickness.

Figure 2.4: Typical surface plasmon absorption spectrum of gold nanorods. The strong long wavelength band in the near infrared region around 800nm is due to the longitudinal oscillation of electrons and the weak short wavelength band in the visible region around 520nm is due to the transverse electronic oscillation.

Due to the strong surface plasmon absorption, gold nanoparticles offer great potential in photothermal therapy applications. It has been found that the strong absorbed radiation is converted efficiently into heat on a picosecond time domain due to electron-phonon and phonon-phonon processes [220]. Thus, upon the laser irradiation at the surface
plasmon absorption band, the nanoparticles absorb photon energy and then immediately
transfer into heat energy. If the nanoparticles are present in the vicinity of malignant
cells, this heat energy will cause the sharp increase on the local temperature around the
nanoparticles and thus causes the damage of the surrounding cells and this photothermal
therapy can be used for cancer treatment. Thus, gold nanoparticles provide a novel class
of photo-absorber in medical applications. For clinical application of treating cancer in
vivo under the skin, one need to use near infrared (NIR) laser light which has larger
penetration depth. Light in the region between 650-900nm has a penetration depth of at
least several centimeters depending on the types of the tissue [221]. It was stated that
the gold nanorods absorb strongly in the NIR region. This offers the great opportunity
for the nanorods to be used as the photo-absorbers in NIR and for photothermal therapy.

Gold nanoparticles have large surface-to-volume ratio compared to bulk materials, thus
they are attractive to be used as catalysts. Traditionally gold was thought to be inert in
catalysis. But within decades, gold nanoparticles have received wide interests due to the
availability of small size nanoparticles which provides higher percentage of surface
atoms and thus become active for catalysis.

The most reported catalysis is the low-temperature oxidation of carbon oxide [222-
225], propylene epoxidation [226,227], combustion of hydrocarbons [228,229], NOx
reduction [230,231], hydrogenation reactions [232] and some other reactions [233-235].

Since, gold nanoparticles are biocompatible and inert, they can be safely used for
medical applications. The potential applications of mesoporous gold lies in the field of
catalysis, biosensing electrodes, analytical chemistry, solid oxide fuel cells, detection of
biomolecules using ellipsometry, and plasmonics. Nanoshells of gold typically exhibit
much higher optical sensitivity than solid core gold nanoparticles. This feature has been
extensively explored for use in fabricating optical filters, photon energy transport
devices, probes for scanning near-field optical microscopy, active surface for surface-
enhanced Raman and fluorescence scattering, rapid immunoassay, and photothermal
ablation of tumor cells in vivo. But gold nanoparticles, being completely benign
material in the living system, have seldom been explored as carriers for enzyme
therapeutic purposes [130].
2.5.1. Biomedical Applications of Gold Nanoparticles

Gold nanoparticles have been extensively explored for *in vivo* biomedical applications particularly for cancer imaging and therapy. The cancer cell is profoundly abnormal cells which can be observed under simple optical microscope. Cancer cell’s morphology is different from that of a normal cell. Its nucleus is larger and irregular. Generally, gold nanoparticles are regarded as biocompatible and no acute cytotoxicity has been observed so far. The colloidal gold has been safely used to treat rheumatoid arthritis for half a century [236]. Moreover, gold nanoparticles can be easily functionalized with targeting biomolecules through well-established thiol-gold conjugation chemistry.

The applications of gold nanoparticles to cancer nanotechnology originate from their SPR effects. SPR resulted in the enhancement of scattering and absorption of the local electromagnetic field at the metal surface which has made gold nanoparticles as attractive candidates for cancer imaging [237,238] and photothermal therapy agents [239]. For *in vivo* biomedical applications, it required deeper penetration of NIR light. The reason is because the primary absorbers in tissue are water and blood (hemoglobin and oxyhemoglobin) and both are slightly” transparent” in the NIR range [240]. Therefore, by designing gold nanoparticles with SPR peaks in this NIR region, the NIR light is preferentially scattered and absorbed by gold nanoparticles and doesn’t attenuate by the tissue.

2.5.1.1. Gold Nanoparticles as Diagnostic Agents

The development of new and early cancer diagnostic techniques is contributing to an increase in cancer survival rates [241]. Researchers are trying to improve the resolution of the conventional imaging techniques and developing new imaging modalities. The performance of these platforms could be increased through integration with appropriate contrast enhancement agents such as gold nanoparticles. Optical coherence tomography (OCT) [242,243] and surface-enhanced Raman scattering (SERS) [244-246] using gold nanoparticles as agents are promising diagnostic techniques for *in vivo* imaging. These gold nanoparticles possess larger and tunable absorption and scattering cross-section.
Nie and co-workers [238] have demonstrated gold nanoparticles based SERS technique for \textit{in vivo} cancer biomarker detection. The pegylated Raman dye encoded spherical gold nanoparticles were functionalized with ScFv antibody to target EGFR-positive tumors and then the strong enhanced Raman signal detected optically.

\textbf{2.5.1.2. Gold Nanoparticles as Photothermal Agents}

Cancer cells are more sensitive to heat damage than healthy (normal) tissue [242], heat induced cell death is used as a noninvasive cancer treatment method. Several kinds of gold nanoparticles such as spherical gold nanoparticles, gold nanorods and gold nanoshells have been demonstrated that they can be used as photothermal therapy agents. All of them have shown their capability to generate localized heat to induce cancer cell death only in the nearby area of the nanoparticles while limiting the damage to the surrounding health tissues.

Spherical gold nanoparticle has the SPR peak located at approximately 530nm where chromophores also have high absorbance at this wavelength [247]. The overlapping of the absorption peak decreases the efficiency of induced heat to tumor sites. Nevertheless, the red-shifted SPR peak can be achieved with aggregated gold nanoparticles. As the nanoparticles are accumulated on the cancer cells, the absorption peak shift from 530nm to as far as 1000nm [248]. This ensured the near infrared light can easily penetrate the tissue and the majority of light is absorbed by gold nanoparticles, thereby heating the cancer cells. Unfortunately, the red shift is dependent on the concentration of the gold bound to each cell and may not be reproducible or controllable among samples.

Multiple \textit{in vivo} studies have also demonstrated the efficiency of gold nanoshells for the non-invasive treatment of tumors through targeted photothermal ablation [249,250]. O’Neal and co-workers [249] have successfully treated mice inoculated with tumors using this technique. In their experiment, mice were inoculated subcutaneously with colon cancer cells and pegylated gold nanoshells solution was injected into mice via a tail vein. After certain time, gold nanoshells were accumulated on the tumor sites and the NIR light at 808 nm was illuminated. The results show a complete destruction of tumors and all mice were healthy and free of tumors up to 90 days after NIR treatment.
Although gold nanocages have also shown that the SPR peaks can be shifted to cover a spectral region from 400-1200nm by controlling the porosity of the shells [251-253], and the ability of gold nanocages to mediate the photothermal destruction of targeted cancer cells in vitro [254]. However, the details of in vivo studies carried out with gold nanocages have not yet been reported.

2.5.1.3. In vitro Studies on Cytotoxicity of AuNPs

Multiple studies have shown that AuNPs exert their cytotoxicity through the induction of oxidative stress [255]. For example, when exposed to 1.4nm AuNPs, HeLa cervical carcinoma cells exhibited increased reactive oxygen species (ROS) production and oxidative stress, leading to protein and lipid oxidation, severely impaired mitochondrial function and eventually cell death [256]. The same investigators showed that Z-VAD-fmk, a caspase inhibitor was unable to rescue the cells from dying, leading to the conclusion that cells were killed by necrosis. Furthermore, genome-wide mRNA expression analysis verified that treatment with AuNPs caused up-regulation of stress-related and inflammation-related genes and a concomitant decrease in the expression of cell cycle genes. It appears that continual production of endogenous ROS within the cell exhausted the intracellular antioxidant pool and therefore induced irreversible damage that eventually lead to necrosis. Oxidative stress was observed in MRC-5 fetal human lung fibroblast cells following exposure to 20nm AuNPs [257] with concomitant down-regulation of cell cycle genes such as Cyclin B2 and B1 and DNA damage response genes. In a follow-up study, the same investigators observed the presence of autophagy (validated by biochemical and morphological parameters) concurrent with oxidative stress in the lung fibroblasts following uptake of AuNPs [258]. It was also demonstrated that AuNPs treatment led to the up-regulation of antioxidants and expression of stress-response genes and proteins, lending support to the hypothesis that oxidative stress could be a manifestation of AuNPs cytotoxicity.

2.5.1.4. In vivo Studies on Cytotoxicity of AuNPs

In a recent study, blue mussel *Mytilus edulis* was observed to experience oxidative stress within 24 hr of exposure to AuNPs [259], indicating the possible impact of
AuNPs to the ecosystem and aquatic animals. The same investigators also proposed the use of *M. edulis* as an ideal animal model for environmental toxicology studies of NPs. Another *in vivo* study utilized zebrafish embryos to assess the feasibility of AuNPs as probes for embryonic imaging [260]. In this study, the real-time effects of AuNPs on zebrafish embryos were investigated, and results showed that owing to the random diffusion of AuNPs to various parts of the embryo, toxic effects influencing the developmental outcome of the embryo were largely stochastic in nature. Among the 76% of zebrafish embryos that survived, only a minority (2%) of zebrafish embryos exhibited deformities while the remaining 74% developed normally. The authors therefore proposed that given its relatively non-toxic nature, AuNPs could be exploited for *in vivo* imaging applications for embryonic studies.

For mammals, however, there is at present limited information regarding the *in vivo* toxicity of AuNPs. Studies have largely focused on the biodistribution of AuNPs in the body. A rat model study revealed the size-dependent organ distribution of AuNPs following intravenous (iv) administration. For 10nm AuNPs, the distribution was found to be widespread, permeating the blood and organs of the cardio-respiratory system, immune system (such as spleen and thymus) and reproductive system, liver, kidney and brain, whereas larger AuNPs (50, 100 and 250nm) were localized only to the blood, liver and spleen [261]. A similar study conducted using 15, 50, 100 and 200nm AuNPs showed that while the AuNPs with the largest dimension could only accumulate minimally in organs following iv administration into mice, AuNPs with the smallest dimension were detected in all tissues including blood and other organs such as the liver, lung, spleen, kidney, brain, stomach, and heart [262]. The results imply that smaller size AuNPs are more accessible to various tissues in the body and therefore the propensity to cause widespread harm, if any. Cho et al. [263] assessed the *in vivo* toxicity of 13nm AuNPs coated with poly (ethylene) glycol (PEG) in mice and showed that following iv injection of AuNPs, the NPs accumulated in mouse liver and spleen for up to a week, and induced acute inflammation and apoptosis in the liver. The same group of investigators [264] also demonstrated that iv administration of 4 nm or 100 nm PEG-coated AuNPs in mice induced up-regulation of common genes associated with apoptosis, cell cycle, inflammation and metabolic process in liver tissues.
2.5.1.5. AuNPs as Sensors for Probing and Imaging Tumor Cells

AuNPs are good candidates for labelling applications because of their ability to interact strongly with visible light. Upon exposure to light, free electrons in gold atoms are excited to a state of collective oscillation known as surface plasmon resonance (SPR), conferring gold the ability to absorb and scatter visible light [265]. In labelling applications, AuNPs are targeted and accumulated at the site of interest and based on their optical scattering properties, they enable visualization of the region under study. AuNPs may then be detected by any of the following ways: phase contrast optical microscopy, dark field microscopy, photothermal imaging, and photoacoustic imaging [266]. In addition, owing to its high atomic weight, AuNPs remain the preferred label for visualization and immuno-staining at the ultrastructural level using transmission electron microscopy [267]. A crucial step in successful cancer therapy involves early diagnosis. The strong optical scattering properties of AuNPs, coupled with their relative biocompatibility, make them suitable as probes for cancer imaging. Through the conjugation of antibodies specific for antigens over expressed on tumor cells, AuNPs can be directed to tumor cells, thus pinpointing their precise location in the body (Figure 2.5). It has been demonstrated that antibody-conjugated hollow gold nanospheres can be used for the surface-enhanced Raman spectroscopy (SERS) imaging of tumor biomarkers which are over expressed in MCF-7 breast cancer cells [268].

Figure 2.5: Schematic diagram showing the localization of antibody conjugated gold to receptors present on the plasma membrane of cells.
2.5.1.6. AuNPs as Drug Delivery Agents Targeted to Cancer Cells

A prominent application of AuNPs is their use as vehicles for delivery of molecules into cells. AuNPs have been described as “promising nanocarriers for therapeutics” owing to their ease of synthesis and functionalization, relative biocompatibility [269] as well as low toxicity in preliminary assays [270]. However, various factors need to be considered in designing an effective drug delivery system. The properties of AuNPs such as their size, charge and surface chemistry have been shown to affect the uptake of AuNPs into cells as well as their subsequent intracellular fate. In addition, effective drug delivery strategies must take into account the nature of drug-AuNP interaction (covalent/non-covalent binding) as well as the means of drug release following introduction of the drug-AuNP complexes to cells [271]. If AuNPs are used solely as carriers into cells, it is also critical to monitor any toxic effects of residual materials in the cell after delivery; a biodegradable NP vector whose lifespan is limited to the therapeutic window of the drug would be ideal [272]. If the NP vector is cleared from the system once its purpose is reached, it will reduce exposure and limit its toxic effects in the body. In the field of cancer therapy, AuNPs are currently being explored as potential drug delivery agents for the introduction of drugs into tumor cells [273]. Cells are known to take up colloidal AuNPs of various shapes and sizes [274] either by specific (via ligand-receptor interaction) or non-specific means. Earlier it has been shown that AuNPs are being taken up by breast cancer cells in vitro [255]. In order to ensure the specific killing of cancer cells while sparing healthy cells, AuNPs were conjugated with appropriate surface ligands which directed them only to tumor cells (Figure 2.6). Huang et al. [275] have described two methods for tumor targeting: the first involved conjugation of AuNPs to PEG and the second involved conjugation of AuNPs with specific antibodies which bind unique biomarkers expressed on tumor cells. PEG prevented AuNP aggregation and lengthened their retention time in blood. This facilitated the preferential accumulation of AuNPs in tumor cells over healthy cells because of the elevated permeability of poorly differentiated blood vessels around tumors following angiogenesis, as well as the decreased clearance rate caused by the deficit of functional lymphatic vessels in tumors [276]. Using PEG is considered a passive targeting approach, as opposed to the active targeting of tumor cells through the help of specific antibodies. Following cellular uptake, AuNPs are stored in
endosomal/lysosomal vesicles. In order to liberate these AuNPs and introduce the drug which has been delivered into the cell cytoplasm, the NPs need to be modified by the conjugation of membrane translocating sequence-based peptides which enable them to traverse monolayers [277].

![AuNP carriers conjugated with anticancer drugs and ligands](image)

**Figure 2.6:** Schematic diagram showing AuNP carriers conjugated with anticancer drugs and ligands which are recognized by receptors on the surface of tumor cells.

### 2.5.1.7. AuNPs as Antiangiogenic Agents

Interestingly, AuNPs have been reported to possess antiangiogenic property [278]. The exact mechanism of action is still not clearly understood but it was observed that AuNPs bind preferentially to vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-165 and basic fibroblast growth factor (bFGF) primarily through the heparin-binding domain. This has led researchers to suggest that AuNPs are able to inhibit angiogenesis by preventing the downstream signaling effects of these mitogens on angiogenesis in cancer cells [279].

### 2.6. CORE SHELL NANOPARTICLES

The stable core shell structure of a nanoshell, defines a nanosized dimension consisting of a rigid shell with a nano-sized hollow space inside the shell, which is about 1 to 100nm in size. The contents of this space can be varied while synthesis of nanoparticles. In contrast to other confinements, either they don’t have well defined shape (eg: heterogeneous pores of zeolite matrices) or they are susceptible to leakage and rupture (eg: lipid vesicles). The materials used to construct these particles are
usually inorganic or organic polymers, and the hollow spheres may contain liquid or solid substances (fluorescent dyes, enzymes or drugs).

Over the last decade there have been immense efforts to fabricate core shell colloidal materials with tailored structural, optical and surface properties [280,281]. Investigations have largely been spurred by the applicability of such colloids in modern materials science and for their technological importance. Composite colloids are utilized in the areas of coatings, electronics, catalysis, separations, and diagnostics [280,281]. The creation of core shell colloidal particles is also of interest from a fundamental and academic point of view, especially in the areas of colloid and interface science. They can be utilized as model systems to investigate factors governing colloidal interactions and stabilization [282,283]. The term used to describe the synthesis of core shell particles with defined morphologies and properties can be referred to as particle engineering. This typically involves tailoring the surface properties of particles, often accomplished by coating or encapsulating them within a shell of a preferred material. Core shell nanoparticles are mainly prepared by using these four methods:

1. Particle templating method
2. Phase separation method
3. Micelle and vesicle templating
4. Nozzle reactor method

Core shell nanoparticles are the recent addendum to nanoscience [284-289]. The surface of these particles, being reactive, needs to be protected with appropriate covers and monolayer protection is often used. Different metal oxides of desired thickness are coated on the particles with appropriate chemistry. The core shell geometry causes enhancement in the luminescence of semiconductor nanoparticles [287(a)], chemical and colloidal stabilities [287(b)], charging of metal cores [288(a)] and optimization of magnetic properties [288(b)]. A number of methods have been employed to produce core shell particles, that is, particles that consist of solid or liquid cores surrounded by shells of organic or inorganic materials. These include hetero aggregation (aggregation of oppositely charged particles) [290], polymerization process (e.g. interfacial
polymerization in emulsion, photo polymerization of monomers in two phase aerosol droplets, dispersion/precipitation polymerization) [291] and controlled phase separation of polymers within droplets of oil-in water emulsions [292]. The two main approaches that have been employed to produce shells of various inorganic coatings (silica, yttrium basic carbonates and zirconium hydrous oxide) on microparticles are those employing direct surface reactions and the controlled precipitation of inorganic molecular precursors from solution [293]. For example Ohmori et al. [293(e)] coated spindle shaped hematite particles with silica layer by the hydrolysis of alkoxide tetraethylorthosilicate. Submicrometer size silica spheres have been coated with titania by hydrolysis of titanium dioxide precursors [294]. An alternative approach to the formation of core shell particles is by using sonochemistry. In sonochemical process, the effects of ultrasound, which arise from the formation, growth and implosive collapse of bubbles in liquid (known as acoustic cavitation) have been exploited to prepare a variety of metal, oxide and composite nanoparticles [295].

Several approaches have been used recently to make silica [285], titania [286(a)] and zirconia [286(b)] covered noble metal clusters. The synthesis and characterization of TiO$_2$ covered Ag nanoparticles has been reported by Liz-Merzan et al. [286(a)]. The coating of metal nanoparticles with a thin layer of oxide material makes it possible to control interparticles and particle matrix interaction thereby improving their potential for applications.

In contrast to solid metal, semiconductor or polymeric nanoparticles, used in various research and industrial applications, the chemical and physical properties of these materials are dominated by surface interactions between the shell, its contents and the outside solvent. The shell protects the core substance and alters its chemical or physical stability leading to unique properties which were originally not present. Some of the features unique to core shell nanoparticles of various types are protection of the core material including the doped biomolecules, drugs, proteolytic enzymes, controlled mass diffusion of molecules, enhanced optical and electronic tuning of the assembly.

Gold nanoshells possess physical properties similar to colloidal gold and exhibit strong scattering and absorption effects due to the strong plasmon resonance of the metallic-
dielectric concentric spherical configuration [296,297]. In particular, the optical behaviour of gold nanoshells in the NIR shows scattering and/or absorption cross sections often several times the particle geometric cross section. This is not seen with comparable nanoparticles such as colloidal gold nanoparticles, which show weak optical activity in the NIR spectrum region [297,298]. By varying the relative core size and shell thickness, the peak resonance of gold nanoshells can be dramatically varied across a broad range of the optical spectrum that spans the visible and the NIR spectral regions [299].

Recently the metal nanoparticles have also found use as building blocks to construct core-shell particles and hollow spheres. The properties of these particles are distinctly different from those of their solid counterparts.

2.7. COATING ON NANOPARTICLES TO ENHANCE THEIR PROPERTIES

Particle coating is carried out for a myriad of reasons [280,281]. For example, the shell can alter the charge, functionality, reactivity of the surface, and can enhance the stability and dispersing nature of the nanoparticles. Magnetic, optical, or catalytic properties may be readily imparted to the dispersed colloidal matter depending on the properties of the coating. Encasing colloids in a shell of different composition may also protect the core from extraneous chemical and physical changes [300]. The coating over nanoparticles is done to prevent aggregation and to increase the stability of nanoparticles for their applications in various fields. Coating of colloidal particles with a layer of a different material is used as a means to modify their surface reactivity, chemical, catalytic, optical, or magnetic properties. Optimization of the surface characteristics of particles through coating processes is also of primary importance for the successful application of composite particles.

2.8. HOLLOW NANOPARTICLES

There has been a wide interest in the synthesis of hollow nanoparticles (spheres or capsules) in the micron (> 1000nm), sub-micron (100-1000nm) and nanometer (1-100nm) size ranges. Hollow nanoparticles have many applications in chemistry, biochemistry and material sciences owing to their characteristic macroscopic structure.
These particles often exhibit properties that are substantially different from those of general particles (e.g.: their low density, large specific area, stability and surface permeability) thus making them attractive from both scientific and technological viewpoints. These particles have numerous applications in cosmetics, capsule agents for drug delivery (DDS), catalysis, coatings, composite materials, dyes, inks, artificial cells, fillers, and protection of sensitive agents such as proteins and enzymes [301-304]. Hollow particles of nanometer to micrometer dimensions represent an important class of shape fabricated materials in which the shell of the hollow sphere can be constructed by a variety of materials of high scientific interest and technological importance like magnetic, semi conducting, ceramic, metallic, polymer, composites etc. [301,305].

There are a variety of routes to fabricate a wide range of hollow shells of various compositions. Among the more traditional methods are nozzle reactor processes, emulsion/phase separation processes (often combined with sol-gel process), and sacrificial core technique [304]. Self assembly is an elegant and attractive approach for the preparation of hollow shells. Vesicles [306,307], dendrimers [308,309], and block hollow copolymer spheres [310-312] are all examples of self assembled hollow containers that are promising for the encapsulation of various materials.

Self-assembly phenomenon can be exploited to create a range of versatile and useful hollow capsules. Lipid liposomes and vesicles are a special group of hollow structures that are formed from the phospholipids through self assembly.

The most widely used process of forming the hollow particles is sacrificial core approach. This process entails depositing a coating on the surface of the particles by either the controlled surface precipitation of inorganic molecular precursors from solution or by direct surface reaction [313-316] followed by removal of the core by thermal or chemical means. For example, a procedure has been developed to coat colloidal polystyrene spheres with a smooth and well defined layer of amorphous titanium dioxide [317]. The resulting composite particles are highly monodispersed. The core shell particles were then turned into spherical hollow titania shells by dissolution of the polystyrene cores in suspension (or by calcination of the dried particles in a furnace). Calcination also crystallizes the titania into its anatase form.
Calcination results in spherical hollow shells composed of a dense arrangement of TiO$_2$ (anatase) nanocrystals. Removal of the polystyrene core by calcinations has also been used to make hollow spheres of yttrium [315], zirconia [318] and silica [319].

A more recent approach for the synthesis of hollow nanocapsules is to employ a micrometer or nanometer sized particles as template and growing a shell around them. Dissolution of the template particles often yields a structurally intact hollow capsule. Although this approach is reminiscent of the sacrificial core method, the nanoparticles are first trapped and aligned in membrane pores by vacuum filtration rather than coated while in aqueous solution. The nanoparticles are employed as templates for polymer nucleation and growth. Polymerization of a conducting polymer around the nanoparticles results in polymer coated particles and following dissolution of the core particles, hollow polymer nanocapsules are obtained. For example, to prepare hollow nanoscopic polypyrrole and poly (N-methyl pyrrole) capsule is prepared by employing gold nanoparticles as template for polymer nucleation and growth [320]. Etching the gold leaves a structurally intact hollow polymer capsules with a shell thickness governed by polymerization time and a hollow core diameter dictated by the diameter of the template particles. These templates could be employed to deliver guest molecules into the capsule core. For example, ligands attached to the gold surface prior to poly (N-methylpyrrole) formation remained trapped inside the hollow capsules following polymer formation and gold etching.

Porous hollow silica nanoparticles (PHSNP) with an average diameter of 60-70nm and wall thickness of approximately 10nm were synthesized [321] by using calcium carbonate nanoparticles as inorganic template followed by dissolution of CaCO$_3$ in dilute HCl solution. The synthesized PHSNP were then employed as drug carriers to investigate in vitro release behavior of cefradine in stimulated body fluid. The amount of cefradine entrapped inside the carrier was analyzed by UV-spectroscopy and TG study. Cefradine release profile from PHSNP followed a three-stage pattern and exhibited a delayed release effect.

Another approach for the preparation of hollow microspheres involves the direct synthesis of intact inorganic shells around soft templates such as vesicles and emulsion
droplets. For example, hollow titania microspheres ranging from 100 nm to a few micrometer in diameter were synthesized using surfactant stabilized non aqueous emulsion droplets [322]. Well-defined micron sized hollow spheres with amorphous titania walls typically 50nm thick were prepared by addition of water to formamide dispersions of hexadecane droplets containing titanium ethoxide. In contrast, addition of titanium ethoxide to formamide water droplets dispersed in hexadecane produced hollow spheres of amorphous titania only 100nm in diameter. In both the cases, hydrolysis/condensation reaction at the formamide/oil interface gave rise to intact shells that could have uses as low density pigments, self repairing coatings, photoactive storage/release agents, as well as compartmentalized structures in nanotechnology.

Micrometer sized hollow silica particles were synthesized by sol gel reaction in water in oil emulsion [323] with polyethylene glycol (PEG) and hydroxypropyl cellulose (HPC) as reaction matrix. To stabilize the emulsion structure, HPC was added into the oil phase and PEG was added to the water phase. HPC was influenced by the formation of spherical shape and PEG had an important role in the formation of hollow structure. Dense or hollow structures of the particles were prepared depending upon the viscosity of the internal phase and the different molecular behavior of polymers (PEG or PVP); the other factors effect the size and size distribution of the particles.

Sharma et al. [129] synthesized hollow silica nanoparticles encapsulating HRP by using water-in-oil microemulsion approach (AOT/water/hexane). They have demonstrated a much softer method of synthesizing HRP-doped silica nanoparticles, by leaching out silver chloride template using dilute ammonia solution, from the pores of silica nanoparticles.

2.8.1. Hollow Gold Nanoparticles

Hollow gold nanoparticles with porous shell structure can best be used as carriers for therapeutic compounds in vivo. This is more particularly important when therapeutic compounds are enzymes. Enzymes can be used as agents for specific degradation of unwanted metabolites, including toxic compounds in diseased individuals. Exogenous enzymes being antigenic in nature, it is desirable that the enzyme should be entrapped permanently within the biocompatible and non-antigenic carriers such as nanoparticles.
made of suitable ceramic and polymeric materials, and the undesirable metabolites should reach the enzyme through the porous shells of the carriers. The metabolism of excessive urea accumulated in the liver or kidney by the enzyme urease, or the reduction of blood L-asparaginase concentration to a minimum level by using L-asparaginase are expected to be best examples of possible applications of enzyme therapy using nanoparticles as enzyme carriers. However, biodegradable nanoparticles cannot be used for such a purpose, as these carrier materials, after degradation, would enable the entrapped enzyme to come out in the body system causing immunogenic reaction.

Kumar et al. [130] reported the synthesis hollow gold nanoparticles of size less than 100nm in diameter using reverse micelle system. They prepared hollow nanoshells of gold entrapping an enzyme, horseradish peroxidase (HRP), in the cavity of the nanoshell by leaching out silver chloride (AgCl) from Au_{shell}AgCl_{core} nanoparticles with dilute ammonia solution. This soft-chemical method for the preparation of hollow gold particles allows the entrapped enzyme to remain active inside it.

2.9. MODE OF ATTACHMENT OF ENZYME

There are number of methods available for the immobilization of enzymes. Using combinations of these basic approaches one can devise additional or modified immobilization techniques.

2.9.1. Ionic Bonding

Ionic binding is influenced by many of the same factors that influence physical adsorption. Carriers most commonly used for ionic bonding includes polysaccharides and a variety of synthetic polymers having ion exchange groups attached. Binding of the enzyme is usually carried out under mild conditions. Generally ionic bonding causes little or no conformational changes in the protein and yield immobilized enzymes of rather high activity. Ionic bonding specifically for enzyme immobilization was first reported by Mitz [324]. He immobilized the enzyme catalase by binding it to diethylaminoethanol (DEAE) cellulose. Since then a variety of enzymes have been immobilized by ionic binding.
2.9.2. Covalent Binding

This method is the most widespread and involves the formation of covalent bonds between the enzyme and the support material. The groups participating in the bond formation are the amino acid residues on the enzyme and the functional groups on the support. The selection of conditions for immobilization by covalent binding is more difficult than in other carrier binding methods. The reaction conditions required are relatively complicated and not usually mild [325]. The bond formation with the support matrix should involve only functional groups of the enzyme that are not essential for its catalytic action and thus active site should be unaffected by the various reagents used. Another requirement for good enzyme coupling is the physical adsorption of the enzyme on the substrate prior to covalent coupling. A large number of support materials are available commercially such as polysaccharide polymers (alginate, chitosan, agarose, cellulose, dextran, starch, proteins (silk, gelatin, collagen), porous silica, synthetic polymers (polyacrylamide, polyamides, methacrylates), and inorganic minerals (clay, Kieselgur, bentonite). The wide variety of binding reactions and a plethora of matrices with functional groups capable of covalent coupling or susceptible to being activated to give such groups, make this a generally applicable method of immobilization. Most of the important methods have been reviewed and described in detail by Mosbach [326] and Zaborsky [327].

2.9.3. Physical Adsorption

Immobilization by surface adsorption is the simplest of all techniques. The forces involved are a combination of ionic, van der Waals, hydrogen bonding, and hydrophobic interactions. Even though each of these forces are weak compared to covalent binding, put together, they are sufficient to achieve a strong enough adhesion for practical systems. Since the existing non-covalent interaction between the enzymes and support is utilized, no chemical modifications are required and little damage is done to enzymes via this technique. The procedure consists of mixing together the enzymes and a support material with favorable adsorption properties under suitable conditions of pH and ionic strength for a certain period of incubation, followed by washing steps to remove any excess unbound molecules. Commonly used supports are alumina, glass,
mesoporous silica, titania, collagen, derivatives of carbon (activated carbon, charcoal) and lectin. Thus, this technique is simple, low-cost, quick to yield the final product, and allows for facile regeneration of the support. On the other hand, weak and non-specific binding leads to desorption of enzymes under high ionic strength or drastic pH and temperature conditions. This desorption can also be caused during a reaction, either when the conformation of the enzyme changes, or during accumulation of the product. Physical working conditions such as agitation, high flow rates and particle-particle abrasion can also lead to desorption of the enzymes. Under conditions where the substrate or the product is charged and interacts with the support, the desorption gets aggravated. The diffuse layer of substrate and product electrostatically bound to the enzyme and support can also lead to local pH inhomogeneities which may be an important consideration for enzymes with specific pH requirements. These constraints make the general applicability of the physical adsorption technique quite low.

2.9.4. Encapsulation

Encapsulation may be considered as trapping the enzyme molecules in tiny containers whose shell walls are semi-permeable. The large proteins cannot leak out but at the same time smaller substrate molecules can readily diffuse inside the shell, react with the enzyme, and the products can diffuse out. A variety of materials have been used to synthesize capsules in the size range of 10-100nm. These capsules, due to their small size and hence larger surface area become especially useful for therapeutics, where they can be administered to the body intravenously or through dermal absorption. Encapsulation of enzymes can be achieved by coacervation (or phase separation), interfacial polymerization, liquid drying, or within thermodynamically self-assembled hollow structures such as liposomes and vesicles [328]. Liposomes in general are not physically stable in high ionic strength solutions or even when they are taken out of solution conditions and dried. This makes it mandatory to crosslink their shell, which is a non-trivial process. Recently, Caruso et. al. reported a new technique wherein oppositely charged polyelectrolytes were sequentially adsorbed onto enzyme crystals by a layer-by-layer technique. This technique, even though appears quite general, is time intensive and leads to physically unstable spheres. Compared to other techniques, the synthesis conditions involved in microencapsulation are relatively mild. However,
challenges with diffusion limitations across the semipermeable membranes due to stagnant boundary layer formation, both outside and inside the capsule, are yet to be surmounted.

2.9.5. Physical Entrapment

Immobilization by entrapment differs from adsorption and covalent binding in that the enzyme molecules are free in solution but greatly restricted in movement by the lattice structure of the matrix. The pore sizes of the solid matrix is such to ensure that the structure is tight enough to prevent leakage of the enzyme, yet at the same time allow free movement of the substrate and product. Inevitably, the support will act as a mass transfer barrier, and although can lead to serious implications, it can have useful advantages since harmful cells and proteases are prevented from interaction with the immobilized enzyme. The general methods of entrapment are by gelation of macromolecules (e.g., polyacrylamide) and precipitation from an immiscible solvent (e.g., polystyrene) [329]. Sol-gel techniques (mainly with silicate matrix in the form of xerogels and aerogels) have seen tremendous growth recently owing to the bridging of well known chemistry of ceramics and other inorganic materials on one hand with the current interest in bioactive materials on the other. Enzyme entrapment in mesoporous silica [330] and supports made from biomimetic polymers/silica [331] have also been investigated. However, the issues with material brittleness, narrow pore structure imposing diffusion limitations and leaking of the enzymes remain with this technique. In addition, there is a likelihood of physical adsorption of the enzyme on the support material.

2.9.6. Crosslinking

Enzymes can be cross-linked by a variety of bifunctional cross-linking agents that produce covalent bonds. The activity of such products also depend on pH, nature of the coupling reagent, enzyme and reagent concentration, and to the great extent, on the size of the enzyme used. Cross-linking is devoid of any support and relies on coupling the individual enzyme molecules to form macroscopic aggregates. This can be achieved by chemical techniques (covalent cross-linking by glutaraldehyde or toluene diisocyanate)
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or physical methods (flocculation). Typical cross-linking agents include glutaraldehyde, isocyanates, bis-diazibenidine, bifunctional alkylation agents, bifunctional imidates, bifunctional isothiocyanates. The functional groups on the enzymes, which participate in the reaction, include ε-amino groups of lysine, phenolic groups of tyrosine and phenylalanine, sulphhydryl groups of cysteine and imidazole groups of histidine. The toxicity of chemical reagents becomes a limiting factor at industrial scales. Physical methods such as flocculation are well known in the biotechnology arena. Flocculating agents such as polyamines, polystyrene sulfonates and various phosphates have been used extensively and well characterized. Physical or chemical crosslinking is rarely used as the only means of immobilization due to their lack of mechanical properties and poor stability.

### 2.10. ENZYMES IN THE CAVITY OF REVERSE MICELLES

The reverse micellar droplets are composed of aqueous cores which are surrounded by a surfactant monolayer and these droplets are dispersed in oil. The incorporation of enzymes and proteins into reverse micellar droplets has opened up a new approach to the modelling of biochemical processes of the cell. Enhanced catalytic activity of the enzymes entrapped into an AOT reverse micellar droplet is an interesting feature in micellar enzymology [332]. The enzyme entrapped in the aqueous core of the reverse micelles was found to have an activity which is comparable to the activity in aqueous buffer. The diffusion of the reverse micellar droplets as a result of their Brownian motion and their coalescence/decoalescence phenomena are responsible for direct interactions between the entrapped enzymes and substrate molecules causing enzymatic reaction in the micellar system and the products diffusing away from it. The reverse micellar droplets are in constant Brownian motion and move erratically and independently in the dispersed oil phase. During this process the droplets undergo collision and form ‘transient droplet dimers’ through collision process within which the intermixing of electrolytes takes place. The average lifetime of these transient droplet dimers is about a hundred to a thousand times longer than the exchange rate of the electrolytes (small molecules) during the decalescence process. Therefore, the enzyme and substrate molecules have sufficient time to undergo catalytic reaction in the transient dimers of the droplets before they are decoalesced. It has been noticed that the
catalytic activity of enzymes are highly specific to the size of the aqueous core of the droplet, chemical composition of the micelle [333], pH of the aqueous core [334] and temperature of the system. Reverse micelles with solubilized enzymes and proteins are characterized by structural and functional properties similar to that of enzymes in a cell [335]. At a certain temperature called the percolation threshold temperature, there exists a strong inter-droplet interaction among the droplets with the formation of infinite clusters [336]. The enzyme molecules hosted by some droplets may interact with the empty aqueous core of other droplets creating a situation such that the idea of conformational stability of the enzyme entrapped into the matrix of the reverse micellar droplet is partly lost. It may be predicted that if the inter-droplet interaction in the micellar system is reduced, by varying the temperature, composition and physico-chemical characteristics of the reverse micelles, the enzyme molecule present in the droplet remains isolated and may retain its active conformation even at elevated temperatures [337].

The use of reverse micelles to solubilize enzymes in organic solvents has attracted considerable interest in the past decade. The reverse micellar environment provides an aqueous phase for hydrophilic enzymes, an interface for surface active enzymes and an organic phase for hydrophobic substrates or products. It has been well established that enzymes can be incorporated into reverse micelles while retaining their activity. Several theoretical models for enzyme kinetics in reverse micelles, reviewed by Bru et. al. [338], have been proposed. In the first approximation they can be divided into diffusional and non-diffusional models [338]. Diffusional models consider that diffusion effectively controls the enzymic reactions which occur inside reverse micelles. Three mechanisms are proposed for the exchange of solubilizates between reverse micelles. In the case of hydrophilic molecules, the formation of transient dimer is postulated [339]. For AOT as a surfactant, exchange occurs with a second-order rate constant of $10^6$-$10^8$ M$^{-1}$ s$^{-1}$, indicating that one collision in 100-10000 results in content exchange [339]. Exchange of amphiphilic or interface-bound molecules may occur during an encounter between droplets without fusion [338]. In the case of molecules that are distributed to any great extent in both water and organic solvent, intermicellar transfer may occur through the organic phase [338]. The non-diffusional models assume
that the flow of substrate to the enzyme in reverse micelles is not limited by mass transfer, because the enzymic reaction is slow compared with the exchange of solutes between water pools. Most of the kinetic studies of enzymes in reverse micelles have been done under steady state conditions. In some cases the $k_{\text{cat}}$ was found to exceed the aqueous value (so called ‘superactivity’) [340]. There are, however, few papers reporting the influence of micellar microenvironment on the individual rate constants of enzymatic reaction [341,342]. An outstanding ‘superactivity’ was found, among others, for heme enzymes, catalase [343] and horseradish peroxidase (HRP) [344-348]. For the latter the dependence of the $k_{\text{cat}}$ on $W_o$ (the molar water to surfactant concentration ratio) has been shown to be bell-shaped and the $k_{\text{cat}}$ value obtained at optimum $W_o$ was about 100 times higher in reverse micelles than in water [347-349].

The specificities of enzyme catalysts promise improvements in many applications, but the short lifetimes of enzymes presently limit their usefulness. Improvements in enzyme stability can enable further practical applications. It can reduce the required amount of enzymes for various applications, prolong the lifetime of enzyme, increase the potential for enzyme reuse, increase its stability or retain their activity for longer time, maintains the good signal of biosensors and prevention of enzyme from denaturation.

Immobilization might improve enzyme properties because substrate specificity might be enhanced and the effect of inhibitors might be reduced. However, many methods of immobilization and entrapment cause significant structural deformation of the enzyme, leading to reduction in activity. Significant optimization of the immobilization method is therefore often required and factors such as stability might be sacrificed in favour of increased loading capacity.

Proteases, which irreversibly inactivate enzymes via proteolysis are often present during in vitro and in vivo biocatalysis reactions involving biological fluids, cellular extracts or implantable biosensors and devices. Mostly enzymes encapsulated in inert matrices would not be accessible to proteolytic digestion due to the steric hindrance against the penetration of proteases into crosslinked matrix.

Scientists have recently developed a new enzyme composite of nanometer scale that we call “single-enzyme nanoparticles (SENs)”.

In a form of SENs, each enzyme molecule
is surrounded with a porous composite organic/inorganic network of less than a few nanometers thick. This approach represents a new type of enzyme-containing nanostructure.

Reduction in the size of enzyme-carrier materials can generally improve the efficiency of immobilized enzymes [350]. In the case of surface attachment, smaller particles can provide a larger surface area for the attachment of enzymes, leading to higher enzyme loading per unit mass of particles [351]. In the case of enzyme immobilization into porous materials, much reduced mass-transfer resistance is expected for smaller porous particles owing to the shortened diffusional path of substrates when compared to large-sized porous materials. There have been extensive studies on the use of micrometer-sized particles for the enzyme immobilization [352-354]. Recently, a growing interest has been shown in using nanoparticles as carriers for enzyme immobilization [351, 355-359].

Silica has been widely used as an inert and stable matrix for enzyme immobilization [360] owing to its high specific surface areas and controllable pore diameters, which can be tailored to the dimension of a specific enzyme: that is, microporous (< 2nm pore size), mesoporous (2-50nm pore size) or macroporous (> 50nm pore size) silica. Because most enzymes are of the order of 3 to 6 nm in diameter, mesoporous materials are most commonly used [361,362]. Enzyme immobilization in MCM-41 was first reported by Diaz and Balkus, and it was found that the enzyme immobilization was dependent on the molecular size of enzymes. Especially, large enzymes, such as horseradish peroxidase (spherical molecular diameter: 4.6nm), ended up with a poor enzyme adsorption into MCM-41 (pore size: 4nm). Generally, MCM-type materials are successful in hosting small enzymes, but they are restrictive as a host of enzymes due to their too small pore size for large enzyme molecules. The developments of SBA-15 (pore size: 5-13nm) and meso cellular foam (MCF, pore size 15-40nm) have solved this critical problem with MCM-type materials, and made these mesoporous materials more suitable for enzyme immobilization.

The inorganic particles have a number of advantages over organic ones in vivo applications in the sense that (i) these are not subjected to microbial attack, (ii) there is no swelling or porosity change occurring in these particles with the change of pH, (iii)
these materials can be prepared at low temperature so that the enzyme activity can be retained, (iv) some of these inorganic materials are nontoxic and highly biocompatible and (v) these particles containing enzymes exhibit excellent storage stability of enzymes. Ultrafine silica nanoparticles doped with enzymes ([\(^{125}\)I] tyraminylulin (mol wt~5 kD)), FITC-dextran (mol wt~19.6 kD), and horse radish peroxidase (mol wt~44 kD)) has been prepared by using an aqueous core of reverse micellar droplets as the nanoreactor [363]. Immobilization of enzymes in nanoparticles has received much attention in the construction of biosensors [364-368]. Especially, gold nanoparticles are favorable candidates for the immobilization of enzymes because amine groups and cysteine residues in the enzymes are known to bind strongly with gold colloids [369]. Previously it has been shown [368,370,371] that enzymes immobilized on gold nanoparticles can maintain their enzymatic and electrochemical activity for a considerable long time [372].

Catalytic activity of solubilized enzymes is exhaustively discussed. One of the most striking effects is superactivity of the entrapped enzyme (studied in terms of pH independent values of \(k_{\text{cat}}\), which are free of trivial effects of the pH shift and a possible increase in substrate concentration inside micelles). Concerning the activity of enzymes in reverse micellar systems, the following generalizations are possible: (1) Enzyme maintains activity comparable to that found in aqueous solution. (2) There is no significant change in the kinetic behavior and a Michaelis Mentens behavior has been observed in the reverse micelles just as in aqueous solution. (3) The maximum activity in reverse micelles is not found at the maximum water content but rather at low \(W_0\) values. (4) Enzymes in reverse micelles are able to accept water soluble substrate but also water insoluble ones, namely those which are directly solubilized in the hydrocarbons and (5) the stability of enzymes is generally comparable to that in water, being greater at small \(W_0\) values and generally small at larger \(W_0\) values.

2.11. MAGNETIC RESONANCE IMAGING

2.11.1. Principle of MRI

MRI is a relatively recent technology; the first studies on a living human being were published in 1977 [373]. Basically, MR imaging is the visualization of hydrogen atoms
in free water and in organic macromolecules (i.e., lipids and proteins). In simple terms, this technology is based on the response of the body’s hydrogen atoms (protons) to a magnetic field that receives a pulse from a radio-frequency (RF) transmitter. First, the magnetic field causes the hydrogen atoms, which behave like bar magnets, to align themselves along the magnetic field. They are not stationary and they don’t align themselves perfectly in this external magnetic field; they continue to rotate in place (in a cone-shaped fashion). The frequency of this rotation, termed precession, is dependent on the strength of the magnetic field. An applied radio-frequency pulse adjusted to the same frequency of procession alters the alignment of the protons in this magnetic field from a longitudinal to a perpendicular (or transverse) axis. This alteration can be measured. After the RF pulse, the hydrogen atoms recover back to their original state. This recovery in both the longitudinal and transverse magnification is termed relaxation. The time it takes after an RF pulse for the tissue to recover to its original longitudinal magnetic field is termed $T_1$ (or spin-lattice relaxation). The time it takes after an RF pulse for the relaxation of the transverse magnetization to dissipate is termed $T_2$ (or spin-spin relaxation). $T_1$ and $T_2$ values vary depending on the tissue type; for example, water has a relatively long $T_1$ and $T_2$ compared to fat, which has a relatively short $T_1$ and $T_2$. The RF pulse-induced alterations in transverse and longitudinal magnetic fields are detected by an antenna system housed within the scanner and converted into an image. By applying specifically designed sequences of RF pulses, the MR operator can vary the image to emphasize the characteristics of a particular tissue [374]. The appearance of $T_1$- and $T_2$– weighted images will differ depending on the tissue type being studied [375]. Under most conditions, tissue proton spin density and the longitudinal ($T_1$) and transverse ($T_2$) relaxation times are usually high enough to provide sufficient contrast for quality MR images; however, some pathological conditions do not display specific enough changes in relaxation times to differentiate them from surrounding healthy tissue [375]. In these cases, MRI contrast agents that alter the local relaxation times of tissue have been shown to improve detection of pathological tissue. This combination of MRI and contrast agents has improved our ability to visualize a variety of disease states, including inflammation (arthritis), tumor angiogenesis, atherosclerosis, and multiple sclerosis.
2.11.2. Contrast Agent in MRI

**MRI contrast agents** are a group of contrast media used to improve the visibility of internal body structures in magnetic resonance imaging (MRI). The most commonly used compounds for contrast enhancement are gadolinium-based. MRI contrast agents alter the relaxation times of atoms within body tissues where they are present after oral or intravenous administration. In MRI scanners sections of the body are exposed to a very strong magnetic field, a radiofrequency pulse is applied causing some atoms (including those in contrast agents) to spin and then relax after the pulse stops. This relaxation emits energy which is detected by the scanner and is mathematically converted into an image. The MR image can be weighted in different ways giving a higher or lower signal. Most clinically used MRI contrast agents work through shortening the $T_1$ relaxation time of protons located nearby. $T_1$ shortens with an increase in rate of stimulated emission from high energy states (spin anti-aligned with the main field) to low energy states (spin aligned). Thermal vibration of the strongly magnetic metal ions in the contrast agent creates oscillating electromagnetic fields at frequencies corresponding to the energy difference between the spin states (via $E = h\nu$), resulting in the requisite stimulation. MRI contrast agents may be administered by injection into the blood stream or orally, depending on the subject of interest. Oral administration is well suited to G.I. tract scans, while intravascular administration proves more useful for most other scans. A variety of agents of both types enhance scans routinely. MRI contrast agents can be classified in many ways including by their:

1. chemical composition
2. administration route
3. magnetic properties
4. effect on the image
5. metal center’s presence and nature
6. biodistribution and applications:
   a. Extracellular fluid agents (also known as intravenous contrast agents)
   b. Blood pool agents (also known as intravascular contrast agents)
   c. Organ specific agents (i.e. gastrointestinal contrast agents and hepatobiliary contrast agents)
d. Active targeting/cell labeling agents (i.e. tumor-specific agents)
e. Responsive (also known as smart or bio-activated) agents
f. pH-sensitive agents

2.11.3. Different Types of Contrast Agents

2.11.3.1. Gadolinium (Gd): Paramagnetic

Figure 2.7: Effect of contrast agent on images: Defect of the blood–brain barrier after stroke shown in MRI. T₁-weighted images, left image without contrast medium and right image with contrast medium administration.

Gadolinium(III) based contrast MRI contrast agents (in a complex) are the most commonly used for enhancement of vessels in MR angiography or for brain tumor enhancement associated with the degradation of the blood–brain barrier. Gadolinium MRI contrast agents have proved safer than the iodinated contrast agents used in X-ray radiography or computed tomography. Anaphylactoid reactions are rare, occurring in approx. 0.03–0.1%. As a free solubilized aqueous ion, gadolinium (III) is highly toxic, but was generally regarded as safe when administered as a chelated compound. The carrier molecule compounds can be classified by whether they are macro-cyclic or have linear geometry and whether they are ionic or not. Cyclical ionic Gd(III) compounds are considered the least likely to release the Gd(III) ion, and hence the safest.

2.11.3.2. Iron Oxide: Superparamagnetic

Two types of iron oxide contrast agents exist: superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO). These contrast agents consist of
suspended colloids of iron oxide nanoparticles and when injected during imaging reduce the T\textsubscript{2} signals of absorbing tissues. SPIO and USPIO contrast agents have been used successfully in some instances for liver tumor enhancement. The common oral iron oxide contrast agent used is Lumirem/Gastromark.

2.11.3.3. Iron Platinum: Superparamagnetic

Superparamagnetic iron platinum particles (SIPPs) have been reported and had significantly better T\textsubscript{2} relaxivities compared with the more common iron oxide nanoparticles. SIPPs were also encapsulated with phospholipids to create multifunctional SIPP stealth immunomicelles that specifically targeted human prostate cancer cells. These are, however, investigational agents which have not yet been tried in humans. In a recent study multifunctional SIPP micelles were synthesized and conjugated to a monoclonal antibody against prostate-specific membrane antigen. The complex specifically targeted human prostate cancer cells in vitro, and these results suggest that SIPPs may have a role in the future as tumor-specific contrast agents.

2.11.3.4. Manganese: Paramagnetic

Unlike the other well-studied iron oxide-based nanoparticles, research on Mn-based nanoparticles is at a relatively early stage. Manganese chelates such as Mn-DPDP enhance the T\textsubscript{1} signal and have been used for the detection of liver lesions. The chelate dissociates in vivo into manganese and DPDP where the former is absorbed intracellularly and excreted in bile while the latter is eliminated via the renal filtration. Manganese ions (Mn\textsuperscript{2+}) are often used as a contrast agent in animal studies usually referred to as MEMRI (Manganese Enhanced MRI). Due to the ability of Mn\textsuperscript{2+} to enter cells through Calcium Ca\textsuperscript{2+} channels Mn\textsuperscript{2+} can e.g. be used for functional brain imaging.

MRI contrast agents mainly includes; dextran coated superparamagnetic iron oxide nanoparticle, which possesses a very large transverse relaxivity (r\textsubscript{2}) of water proton of 100-200 s\textsuperscript{-1} m M\textsuperscript{-1} (T\textsubscript{2} MRI contrast agent) [376]. The other is the paramagnetic Gd(III)-chelate that possesses a longitudinal relaxivity (r\textsubscript{1}) of water proton of 3-5 s\textsuperscript{-1} mM\textsuperscript{-1} (T\textsubscript{1} MRI contrast agent) [377]. Nowadays, the latter is prevailing in clinical use because it
can be generally used for all organs, whereas the former is liver-specific due to its large particle diameter [378, 379]. An important drawback of iron oxide nanoparticles is that they produce a negative signal on proton-based MR imaging and this is more difficult to detect than a positive signal [380]. Organic molecules capable of linking to paramagnetic chelates include dendrimers [381], nanomicelles [382], liposomes [383], viral capsids [384], and even natural products such as functionalized low density lipoprotein particles [385]. Chelating the gadolinium with a strong macrocyclic chelate greatly reduces the chance of toxicity due to the release of free gadolinium ion. This strategy has been the basis of a number of dual MR/optical agents thought to be potentially translatable to the clinic. For instance, dendrimers with multiple Gd–DTPA chelates have been proposed as viable MR agents. Toxicity may be further reduced with the use of nano-sized agents, which are small enough to be excreted by the urinary system and thus, theoretically, have better safety. Gd(III) ion itself has been known to be the best metal ion in the periodic table which can be used as a T₁ MRI contrast agent. First of all, it possesses seven unpaired 4f electrons (⁸S⁷/₂), giving a large electron magnetic moment. No other metal ions possess unpaired electrons more than this. Furthermore, these electrons solely yield the S state (no angular) electron magnetic moment, and thus, the Gd(III) ion can very efficiently induce the longitudinal relaxation of a water proton.

The MRI technique has recently adopted nanoparticle probes to enhance the sensitivity of its measurements [386]. Nanoparticles have also been used to improve the image quality and have significantly increased the sensitivity of cancer detection [387]. Many kinds of nanoparticles have been developed for MRI, which include magnetic gold nanocomposites [388], biodegradable nanoparticles [389], and smart drug-loaded polymer gold nanoshells [390]. Nano-sized colloidal metal oxides have recently been synthesized. Among them, gadolinium oxide (Gd₂O₃) nanoparticles (GONPs) are receiving attention as candidate multi-functional contrast agents that can be targeted towards a specific cell by attaching an antigen or an antibody to them [391]. Gadolinium is a paramagnetic material that has been used as the core of contrast agents such as gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) for T₁ imaging in MRI. The Gd-DTPA has low toxicity but it is easily removed by renal excretion due to
its low molecular weight. Therefore, a new gadolinium composite that has the form of a nano-sized colloidal nanoparticle has been required. The gadolinium composite can also be a good THz contrast agent because gadolinium significantly absorbs electromagnetic waves [392].

Gadolinium is a paramagnetic trivalent lanthanide cation (Gd$^{3+}$) that is highly toxic as a free ion because it competes with calcium. Because gadolinium has a higher binding affinity for calcium-binding enzymes, it displaces calcium and alters all biological processes catalyzed by these enzymes [393]. Free gadolinium ions interfere with a variety of calcium-related enzymes, including Ca$^{2+}$-activated Mg-adenosine triphosphatase (ATPase) dehydrogenases, kinases, glutathione S-transferases, and aldolase, due to its noncompetitive inhibition of Ca$^{2+}$ binding [394]. Free gadolinium also interferes with calcium channels to block physiological pathways that rely on Ca$^{2+}$ influx (i.e., neural transmission and coagulation) [394]. When bound (or chelated) to an organic ligand, gadolinium is generally regarded as safe for use as an MRI contrast agent. Chelation also improves water solubility [395]. Cyclical ionic Gd(III) compounds are considered the least likely to release the Gd(III) ion, and hence the safest.

A wide variety of oral contrast agents can enhance images of the gastrointestinal tract. They include gadolinium and manganese chelates, or iron salts for T$_1$ signal enhancement. SPIO, barium sulfate, air and clay have been used to lower T$_2$ signal. Natural products with high manganese concentration such as blueberry and green tea can also be used for T$_1$ increasing contrast enhancement.

2.12. CYTOTOXIC EFFECT OF HRP/IAA: A NOVEL CANCER THERAPY

IAA, a plant growth hormone, when oxidised with HRP produces a toxic species that could be used as the basis for a novel cancer therapy [396]. IAA is well tolerated in humans [397] and is not readily oxidised by mammalian peroxidases so that targeting of HRP to a tumour would allow production of the toxic IAA metabolite in the tumour alone, avoiding damage to normal tissues. IAA is a naturally occurring plant growth phytohormone. It has been studied intensively for many years by plant biologists but questions remain as to how it carries out its role. Existing in picomolar levels in plants,
it affects many different growth properties: cell enlargement, division and differentiation, as well as, in some instances, senescence and abscission of leaves [398]. IAA activity is regulated by the control of metabolism through irreversible removal via two different pathways: oxidation by peroxidases leading to decarboxylation and nondecarboxylation reactions forming non-reactive conjugates [399]. The decarboxylation pathway catalysed by HRP has been investigated extensively over the years and is known to be complex. HRP is a widely studied heme-containing peroxidase enzyme existing in its native state in the ferric form. It can oxidise a wide variety of substrates in the presence of hydrogen peroxide catalysing one electron oxidation reactions through its compound I and II forms [400]. The reaction between IAA and HRP has been studied primarily because of interests in plant biochemistry but the mechanism is extremely complex and still not fully elucidated. One key feature in the reaction between IAA and HRP is that hydrogen peroxide is not required to oxidise native HRP to compound I unlike reaction with many other substrates for the enzyme (e.g. phenols).

HRP will oxidise IAA (Figure 2.8, 1) at neutral pH to an indolyl radical cation (Figure 2.8, 2). This cation dissociates to form an indolyl radical (Figure 2.8, 3) with a radical pK\textsubscript{a} of 5.1 for dissociation of the indole N-H group. The radical cation (Figure 2.8, 2) but not the dissociated radical (Figure 2.8, 3) decarboxylates in approximately 40 msec to form a skatoyl radical (Figure 2.8, 4) [401]. This carbon centered radical is very reactive towards oxygen rapidly forming a peroxyl radical (Figure 2.8, 5). The peroxyl radical can decay in two ways. Reduction and protonation form skatole hydroperoxide (Figure 2.8, 8), which reacts further with HRP compound I to form indole-3-carbinol (Figure 2.8, 7) [402]. The hydroperoxide can also decompose non-enzymatically to oxindole-3-carbinol (Figure 2.8, 9) and MOI (Figure 2.8, 10). In addition, combination and elimination by the Russell mechanism, in which two peroxyl radicals combine, form indole-3-aldehyde (Figure 2.8, 6), indole-3 carbinol (Figure 2.8, 7) and singlet oxygen [403], although this may not occur at physiological pH [404]. The reactivity of various indoles with HRP compound I has been shown to be closely related to the reduction potentials of the indolyl radical [405] with an increase in rate constant of approximately 300-fold for a decrease in reduction potential (radical/ground state) of
only 0.1 V. These free radical based mechanisms of the IAA/HRP combination were used as a novel approach for cancer therapy.

**Figure 2.8:** Main reaction pathways involved in oxidative activation of IAA to toxic species [406].

### 2.13. ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

The goal in cancer therapy is to kill tumour cells selectively without harming the normal tissues. The method of selective drug delivery is a two step approach called antibody-directed enzyme prodrug therapy (ADEPT) [407]. In ADEPT, an antibody directed against a tumor-associated antigen is linked to an enzyme and given i.v., resulting in selective accumulation of the enzyme in the tumor. When the discrimination between tumor and normal tissue enzyme levels is sufficient, a prodrug is given through i.v., which is converted to an active cytotoxic drug by the enzyme within the tumor. Selectivity is achieved by the tumor specificity of the antibody and by delaying prodrug administration until there is a large differential between tumor and normal tissue enzyme levels. Drug resistance can be overcome by generating high levels of an alkylating agent in the tumor, and this is achieved through the capacity of each enzyme molecule to convert many molecules of prodrug into drug. ADEPT has
shown antitumor activity in animal tumor models of human chorio carcinoma and colonic and breast carcinoma [408-410].

Figure 2.9: Diagrammatic representation of Antibody Directed Enzyme Prodrug Therapy (ADEPT).

The first pilot-scale clinical trial of ADEPT was carried out at Charing Cross Hospital, London, using an anti-CEA F(ab’)2 antibody conjugated to the bacterial enzyme carboxypeptidase G2 (CPG2) [411]. The antibody used in the first ADEPT clinical trial was of murine origin and the enzyme was bacterial. Host antibodies to both components of the AEC were present in the blood of all non-immuno suppressed patients by day 10 after AEC infusion [412]. Several patients received ciclosporin since it had been shown in rabbits that this could delay the appearance of host antibodies to soluble proteins [413]. The main drawback of ADEPT is the immunogenicity of the Ab-enzyme conjugates which may preclude the administration of repeated doses of the conjugate or we can say that the free enzyme will not retain to the tumor site for a prolong period and therefore repeated dosage of enzyme is necessary so as to form cytotoxic drug in combination with prodrug, within the tumor. To overcome the problem associated with ADEPT, we have used nanoparticles-directed enzyme prodrug therapy.
2.13.1. Other Versions of Directed Enzyme Prodrug Therapy (DEPT)

There are several other strategies to use prodrug/enzyme systems for cancer therapy, including gene-directed enzyme prodrug therapy (GDEPT), virus-directed enzyme prodrug therapy (VDEPT), PDEPT (Polymer-Directed Enzyme Prodrug Therapy), LEAPT (Lectin-directed enzyme-activated prodrug therapy) [414,415], and CDEPT [416] (Clostridial-directed enzyme prodrug therapy).

2.14. DIFFERENT METHODS ADOPTED FOR ANTIBACTERIAL TESTS

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include: Diffusion (Kirby-Bauer and Stokes), Dilution (Minimum Inhibitory Concentration) and Diffusion & Dilution (E-Test method). Antimicrobial susceptibility testing in the clinical laboratory is most often performed using the disc diffusion method. The Kirby-Bauer and Stokes’ methods are usually used for antimicrobial susceptibility testing, with the Kirby-Bauer method being recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 03). The Kirby-Bauer method was originally standardized by Bauer et. al. (the so called Kirby-Bauer method). This method is well documented and standard zones of inhibition have been determined for susceptible and resistant values. The antibacterial characteristics of silver nanoparticles produced have been demonstrating by directly exposing bacteria to colloid silver particles solution.

2.14.1. Metal Nanoparticles as Antibacterial Agents

Human beings are often infected by microorganisms such as bacteria, molds, yeasts, and viruses in the living environment [417]. Research in antibacterial material containing various natural and inorganic substances [418,419] has been intensive. Due to the outbreak of the infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance the pharmaceutical companies and the researchers are searching for new antibacterial agents. In the present scenario nanoscale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties. Bio-nanotechnology has emerged up as integration between biotechnology and
nanotechnology for developing biosynthetic and environmental-friendly technology for synthesis of nanomaterials. The metallic nanoparticles are most promising as they show good antibacterial properties due to their large surface area to volume ratio, which is coming up as the current interest in the researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains [420]. Different types of nanomaterials like copper, zinc, titanium [421], magnesium, gold [422], alginate [423] and silver have come up but silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms [420,424]. Silver nanoparticles (Ag-NPs) have been known to have inhibitory and bactericidal effects [419]. It can be expected that the high specific surface area and high fraction of surface atoms of Ag-NPs will lead to high antimicrobial activity as compared with bulk silver metal [419]. The current investigation supports that use of silver ion or metallic silver as well as silver nanoparticles can be exploited in medicine for burn treatment, dental materials, water treatment, sunscreen lotions, etc. and possess low toxicity to human cells, high thermal stability and low volatility [425].

For centuries silver has been in use for the treatment of burns and chronic wounds. As early as 1000 B.C., silver was used to make water potable [426,427]. Silver nitrate was used in its solid form and was known by different terms like, “Lunar caustic” in English, “Lapis infernale” in Latin and “Pierre infernale” in French [428]. In 1700, silver nitrate was used for the treatment of venereal diseases, fistulae from salivary glands and bone and perianal abscesses [428,429]. In the 19th century granulation tissues were removed using silver nitrate to allow epithelization and promote crust formation on the surface of wounds. Varying concentrations of silver nitrate was used to treat fresh burns [427,428]. In 1881, Carl S.F. Crede cured ophthalmia neonatorum using silver nitrate eye drops. Crede’s son, B. Crede designed silver impregnated dressings for skin grafting [428,429]. In the 1940s, after penicillin was introduced, the use of silver for the treatment of bacterial infections minimized [430-432]. Silver again came in picture in the 1960s when Moyer introduced the use of 0.5% silver nitrate for the treatment of burns. He proposed that this solution does not interfere with epidermal proliferation and possess antibacterial property against Staphylococcus aureus,
Pseudomonas aeruginosa and Escherichia coli [433,434]. In 1968, silver nitrate was combined with sulfonamide to form silver sulfadazine cream, which served as a broad-spectrum antibacterial agent and was used for the treatment of burns. Silver sulfadazine is effective against bacteria like E. coli, S. aureus, Klebsiella sp., Pseudomonas sp. It also possesses some antifungal and antiviral activities [435]. Recently, due to the emergence of antibiotic-resistant bacteria and limitations of the use of antibiotics the clinicians have returned to silver wound dressings containing varying level of silver [432,436].

Klabunde and co-workers [437] demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria. Thus the preparation, characterization, surface modification and functionalization of nanosized inorganic particles open the possibility of formulation of a new generation of bactericidal materials [438]. It is well known that silver ions and silver-based compounds are highly toxic to microorganisms [439,440] showing strong biocidal effects. Thus, silver ions, as an antibacterial component, have been used in the formulation of dental resin composites [441-443] and ion exchange fibers [444] and in coatings of medical devices [445-448].

The antimicrobial activity of silver nanoparticles against *E. coli* was investigated as a model for Gram-negative bacteria. Bacteriological tests were performed in Luria–Bertani (LB) medium on solid agar plates and in liquid systems supplemented with different concentrations of nanosized silver particles. These particles were shown to be an effective bactericide. Scanning and transmission electron microscopy (SEM and TEM) were used to study the biocidal action of this nanoscale material. The results confirmed that the treated *E. coli* cells were damaged, showing formation of “pits” in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial membrane.

### 2.14.2. Effect of Size and Shape on the Antimicrobial Activity of Nanoparticles

The surface plasmon resonance plays a major role in the determination of optical absorption spectra of metal nanoparticles which shifts to a longer wavelength with increase in particle size. The size of the nanoparticle implies that it has a large surface
area to come in contact with the bacterial cells and hence it will have a higher percentage of interaction than bigger particles [449-452]. The nanoparticles smaller than 10 nm interact with bacteria and produce electronic effects which enhance the reactivity of nanoparticles. Thus, it is corroborated that the bactericidal effect of silver nanoparticles is size dependent [451,453]. The antimicrobial efficacy of the nanoparticle depends on their shapes also. This can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles [451]. According to [452] truncated triangular nanoparticles show bacterial inhibition with silver content of 1µg. While, in case of spherical nanoparticles total silver content of 12.5µg is needed. The rod shaped particles need a total of 50 to 100µg of silver content. Thus, the silver nanoparticles with different shapes have different effects on bacterial cell.

Earlier it was shown that the antibacterial properties of differently shaped silver nanoparticles against the gram-negative bacterium *Escherichia coli*, both in liquid systems and on agar plates, have different efficacy [454]. Energy-filtering transmission electron microscopy images revealed considerable changes in the cell membranes upon treatment, resulting in cell death. Truncated triangular silver nanoplates with a {111} lattice plane as the basal plane displayed the strongest biocidal action, compared with spherical and rod-shaped nanoparticles and with Ag\(^+\) (in the form of AgNO\(_3\)). It is proposed that nanoscale size and the presence of a {111} plane combine to promote this biocidal property. These results demonstrate that silver nanoparticles undergo a shape-dependent interaction with the gram-negative organism *E. coli*.

The mode of action of nano-Ag was also found to be similar to that of Ag\(^+\) ions however; the effective concentrations of nano-Ag and Ag\(^+\) ions were at nanomolar and micromolar levels, respectively. Nano-Ag appears to be an efficient physicochemical system conferring antimicrobial silver activities [455].

Silver nanoparticles with different sizes (7, 29 and 89nm) were synthesized using gallic acid in an aqueous chemical reduction method and characterized using TEM, DLS, X-ray diffraction and UV–Vis absorption spectroscopy. The antibacterial activity of the silver nanoparticles was analyzed and it was found that it can be modified with the size of silver nanoparticles. It decreases with an increase of the particle size [456].
Silver nanoparticles with mean diameters of 9, 11, 24 and 30nm were synthesized using hydrazine hydrate and citrate of sodium as a reducing agent. The nanoparticles were characterized by UV/Vis, EDX and TEM. UV/Vis spectra show the characteristic plasmon absorption peak for the silver nanoparticles ranging from 405-418nm. The energy-dispersive spectroscopy (EDX) of the nanoparticles dispersion confirmed the presence of elemental silver signal and no peaks of other impurity were detected. High energy electron diffraction (HEED) confirmed that formation of face-centered-cubic silver nanoparticles. Additionally, the antibacterial activity of the nanoparticulate dispersion was measured by Kirby-Bauer method. The results of this study clearly demonstrated that the colloidal silver nanoparticles inhibited the growth and multiplication of the tested bacteria, including highly multiresistant bacteria such as methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Such high antibacterial activity was observed at very low concentrations of silver (< 6.74 µg/mL) [457].
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