CHAPTER II

LITERATURE REVIEW
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2.1 INTRODUCTION

Nanotechnology emerges from the physical, chemical, biological and engineering sciences where novel techniques are being developed to probe and manipulate single atoms and molecules. In nanotechnology, a nanoparticle \((10^{-9}\text{ m})\) is defined as a small object that behaves as a whole unit in terms of its transport and properties.\(^1\) The science and engineering of nanosystems is one of the most challenging and fastest growing sectors of nanotechnology. Since the birth of nanotechnology, it has never been a single field technology. It is more preferably called nanotechnologies, as refers to a set of methods and approaches in physics and chemistry, engineering fields, biological and medical areas.\(^2\)

Early history: The concept of nanotechnology though considered to be a modern science has its history dating to as back as the 9th century. Nanoparticles of gold and silver were used by the artisans of mesopotamia to generate a glittering effect to pots. The first scientific description of the properties of nanoparticles was provided in 1857 by Michael Faraday in his famous paper Experimental relations of gold (and other metals) to light (Faraday, 1857).\(^3\) In 1959, Richard Feynman gave a talk describing molecular machines built with atomic precision. This was considered the first talk on nanotechnology. This was entitled ‘There’s plenty of space at the bottom’.

The 1950’s and 1960’s saw the world turning its focus towards the use of nanoparticles in the field of drug delivery. One of the pioneers in this field was Professor Peter Paul Speiser at the ETH (swiss Federal Institute of Technology) in Zurich. His research group at first investigated polyacrylic beads for oral administration and then focused on microcapsules and in the late 1960s developed the first nanoparticles for drug delivery purposes and for vaccines.\(^4\)
The nano-revolution conceptually started in the early 1980’s with the first paper on nanotechnology being published in 1981 by K. Eric Drexler\textsuperscript{5} of Space Systems Laboratory, Massachusetts Institute of Technology. This was entitled as an approach to the development of general capabilities for molecular manipulation.

With gradual advancements such as the invention of techniques like TEM, AFM, DLS etc., nanotechnology today has reached a stage where it is considered as the future to all technologies.\textsuperscript{6}

### 2.2 STRATEGIES USED TO SYNTHESIZE NANOPARTICLES

Previously nanoparticles were produced only by physical and chemical methods. Basically there are two approaches for nanoparticle synthesis namely the bottom up approach and the top down approach.\textsuperscript{7,8} In the top down approach, larger scale materials are grinded to the nanometer scale to increase the surface area to volume aspect ratio for more reactivity.

The bottom up approach is a process that builds towards larger and more complex systems by starting at the molecular level and maintaining precise control of molecular structure. As the opposite to top-down fabrication technologies, bottom-up methods refer to a set of technologies which fabricate by stacking materials on top of a base substrate.

Some of the commonly used physical and chemical methods for synthesis include:

- Chemical reduction, which is the reduction of an ionic salt in an appropriate medium in the presence of some stabilising agents. Some of the commonly used reducing agents are sodium borohydride, hydrazine hydrate, potassium auro chlorate and sodium citrate.

- Solvothermal synthesis, which is a versatile low temperature route in which polar solvents under pressure (high) and at temperatures above their boiling points are used. Under solvothermal conditions, the solubility of reactants increases significantly, enabling reaction to take place at lower temperature.\textsuperscript{9}
Chapter II

- Sol-gel technique, which is a wet chemical technique used for the fabrication of metal oxides from a chemical solution which acts as a precursor for integrated network (gel) of discrete particles or polymers. The precursor sol can be either deposited on the substrate to form a film, cast into a suitable container with desired shape or used to synthesize powders.\textsuperscript{10}

- Laser ablation, which is the process of removing material from a solid surface by irradiating with a laser beam. At low laser flux, the material is heated by absorbed laser energy and evaporates or sublimes. At higher flux, the material is converted to plasma. The depth over which laser energy is absorbed and the amount of material removed by single laser pulse depends on the material’s optical properties and the laser wavelength. Carbon nanotubes can be produced by this method.\textsuperscript{11}

- Inert gas condensation, where different metals are evaporated in separate crucibles inside an ultra high vacuum chamber filled with helium or argon gas at typical pressure of few 100 pascals. As a result of inter atomic collisions with gas atoms in chamber, the evaporated metal atoms lose their kinetic energy and condense in the form of small crystals which accumulate on liquid nitrogen filled cold finger. e.g., gold nanoparticles have been synthesized from gold wires.\textsuperscript{12}

- Nanoparticles can be broadly grouped into two categories: namely organic\textsuperscript{13} and inorganic\textsuperscript{14} nanoparticles. Organic nanoparticles includes polymeric nanoparticles, carbon nanoparticles (fullerenes)\textsuperscript{15,16} etc while inorganic nanoparticles includes magnetic nanoparticles\textsuperscript{17}, noble metal nanoparticles\textsuperscript{18} (like gold and silver) and semiconductor nanoparticles\textsuperscript{19} (like titanium dioxide and zinc oxide). They are present as an aerosol (mostly solid or liquid phase in air), a suspension (mostly solid in liquids) or an emulsion (two liquid phases).

In the presence of chemical agents (surfactants), the surface and interfacial properties may be modified. Indirectly such agents can stabilise against coagulation or aggregation by conserving particle charge and by modifying the outmost layer of the particle.
Chapter II

2.2.1 Microemulsion mediated nanoparticle synthesis:

The development of nanotechnology depends strongly on the advances in nanoparticle preparation. Nowadays, there are a number of technologies available for nanoparticle synthesis, from the gas phase techniques such as laser evaporation (Gaertner & Lyditin, 1994)\(^{20}\), to the liquid phase techniques such as coprecipitation from homogeneous solutions and sol-gel reactions\(^{21}\), solvothermal processes (Gautam et al. 2002)\(^{22}\), sonochemical and cavitation processing (Suslick et al.)\(^{23}\), and surfactant and polymer-templated synthesis (Holmberg, 2004).\(^{24}\) Amongst the surfactant-based approaches, the microemulsion reaction method is one of the most used techniques for the preparation of very small and nearly monodispersed nanoparticles. In 1894, first Emil Fisher has introduced the superamolecular interactions. Since then, there has been a growing interest in the research on organized surfactant assemblies that include micelles and microemulsions, vesicles, self-assembled monolayer and supramolecular hosts such as zeolites. Microemulsions are obtained from the mixtures of oil, water (brine) and a surfactant (an amphiphile molecule). In most cases, the addition of a cosurfactant (mainly small chain alcohol) is required to ensure the stability of the microemulsion. A microemulsion consists of two phases (i) dispersed phase (the phase broken into fine droplets) and (ii) continuous phase (the liquid surrounding the droplets).\(^{25}\) Depending on the proportion of components and the hydrophilic-lipophilic balance (HLB)\(^{26}\) and the concentration of the surfactant used, the formation of microdroplets can be in the form of oil swollen micelles dispersed in water as oil-in-water (o/w) microemulsion or water swollen micelles dispersed in oil as for water-in-oil (w/o) microemulsion also called reverse microemulsion.\(^{27}\) For a given overall composition, one can obtain: an oil-in-water microemulsion in equilibrium with an excess oil phase (Winsor I), a water-in-oil microemulsion in equilibrium with an excess water-phase (Winsor II), and a microemulsion in equilibrium with both water and oil excess phases (Winsor III). Winsor I microemulsions consist of spherical micelles of surfactant and co-surfactant dispersed in water and filled with oil. Reciprocally, Winsor II microemulsions consist of micelles dispersed in oil and filled with water. Winsor III microemulsions are generally pictured as bicontinuous media where oil and water domains are separated by aggregated surfactant.\(^{28}\)
Microemulsions are thermodynamically stable, and they remain clear indefinitely. They form spontaneously when the following four components are mixed in specific proportions: water, oil, surfactant, and co-surfactant (generally a low molecular weight alcohol). The presence of a cosurfactant is critical in reducing the interfacial tension between the droplets and the continuous phase to near zero. In the absence of cosurfactant at the droplet interface, the emulsions become milky and unstable owing to the creation of much larger droplets. Three factors characterize a microemulsion: transparency (optical isotropy), droplet size (6–80 nm) and stability (thermodynamic). Microemulsion method was invented as an effective process of preparing nanoparticles in the 1980s. Microdroplets, in which reactants are dissolved, act as nanoreactors. By controlling the molar ratio of the mixture oil/water/surfactant, it is possible to predetermine the size and shape of those droplets and, as a consequence, to tailor the size and shape of the final product.

Important parameter necessary to understand how the surfactant forms a specific structure spontaneously is the surfactant parameter or “packing parameter”, defined as (v/al), where v is the volume of the surfactant molecule and l is the characteristic length of the hydrophobic section, a the surface area of the aggregate per surfactant molecule. The v parameter is viewed as the volume occupied by an alkyl chain of n carbon atoms, and is deduced from the empirical volume additivity rule of Traube. The length of the hydrophobic section (l) is defined as the maximum possible extension of a hydrocarbon chain, and also is a function of the number of carbon atoms. The following expressions are useful to calculate the values of v and l.

\[
v = 27.4 + 26.9 \, n_c \, (\, \text{Å}^3/\text{chain}), \]
\[
l = 1.5 + 1.265 \, n_c \, (\, \text{Å}).
\]

Small values of the packing parameter imply highly curved aggregates such as in spherical micelles \(((v/al) < 0.33)\) and infinite cylinders \([0.33 < (v/al) < 0.5)\). When \((v/al)\) is close to unity, planar bilayers usually form: inverted cylinders and micelles are obtained for \((v/al) > 1\).
The main strategy for the synthesis of nanoparticles in W/O microemulsions consists in mixing two microemulsions, 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. 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 (Boutonnet et al., 1982; Destrée & Nagy, 2006; Eastoe et al. 2006; Holmberg, 2004; López-Quintela et al. 2004; Pileni 2003).

2.2.1.1 Parameters influencing nanoparticle synthesis:

1. Aqueous solution concentration
2. Reagent concentration
3. Surfactant and co-surfactant
4. Solvent
5. Electrolytes
6. Microemulsion structure

The size of the reverse micelle greatly influences the size of the resulting nanoparticles. An interesting parameter to be determined in order to characterize a reverse micelle as regards to its size is the radius of its water droplet ($R_w$). This parameter is dependent on the water to surfactant molar ratio ($w$) used to prepare the micellar solution.

$$w = [\text{H}_2\text{O}] / [\text{surfactant}]$$

Microemulsion is a dynamic system because of the Brownian motion of the water droplets. When two droplets collide, they fuse and interchange reactants. This
phenomenon is called intermicellar exchange and is strongly dependent on the elasticity of the surfactant film. It is thought that the micellar exchange process is characterized by an activation energy (E_a or energy barrier), which is affected by the flexibility or rigidity of the surfactant layer, in addition to diffusion processes (Fletcher et al., 1987).\textsuperscript{39, 40, 41}

There have been a number of studies dealing with the theoretical aspects of nanoparticle formation by the microemulsion reaction method. Most of these studies use the Monte Carlo method. The studies carried out in the last few years are focused on several aspects: kinetics of nanoparticle formation (de Dios et al., 2009)\textsuperscript{42}, formation of bimetallic nanoparticles (Tojo et al., 2009; Angelescu et al., 2010)\textsuperscript{43, 44}, droplet exchange (Niemann & Sundmacher, 2010)\textsuperscript{45}, cluster coalescence (Kuriyedath et al., 2010)\textsuperscript{46}, and core-shell nanoparticle formation (Viswanadh et al., 2007)\textsuperscript{47}. Kinetics of nanoparticle formation in microemulsion was studied for the Ag and Au nanoparticles using Monte Carlo simulations by de Dios et al. (de Dios et al., 2009).\textsuperscript{42} It was shown that, although the material interdroplet exchange depends primarily on the flexibility of surfactant film, a slow reaction rate leads to a more effective material interdroplet exchange for a given microemulsion. Two factors contribute to this result. Firstly, a slow reaction implies that autocatalytic growth takes place for a longer period of time, because there are available reactants. If the reaction is faster, the reactants are almost exhausted at early stages of the process. As a consequence, autocatalytic growth is only possible at the beginning. Secondly, a slow reaction rate implies the continuous production of seed nuclei, which can be exchanged between micelles due to their small size, allowing the coagulation of two nanoparticles. This exchange only takes place at early stages of the synthesis. Both factors, autocatalysis and ripening, favor the slow growth of the biggest nanoparticles leading to the production of larger particles when the reaction is slower.\textsuperscript{48} This method offers a series of advantages\textsuperscript{49} 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 nano-particles with often crystalline structure and high specific surface area and the use of soft conditions of synthesis, near ambient temperature and pressure. Spherical powder with uniform
dimensional distribution and good dispersibility has been obtained by such method via adjusting the size of reactors and other reaction conditions, such as Cu, PZT, Bi, and BaTiO₃. Qiu et al. took xylol/Tween 80/zirconium (yttrium) nitrate aqueous solution to form the microemulsion system. 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 (Boutonnet et al. 2008).

### 2.2.2 Novel approaches for the extraction of nanoparticles from reaction mixtures:

Often, the nanoparticles formed in a microemulsion are so well dispersed in the reaction media that some solvent has to be added in order to destabilize the microemulsion, which causes desorption of surfactant from the particles and makes their separation by centrifugation or filtration easier. Sometimes the nanoparticles end up so agglomerated that it is difficult to re-disperse them.

Some novel and straightforward approaches have been proposed for an improved recovery or phase transfer of nanoparticles from microemulsion media. (Eastoe et al. Hollamby & Eastoe, 2009; Myakonkaya et al., 2010, 2011; Nazar et al., 2011; Vesperinas et al., 2007) have proposed three approaches for nanoparticle recovery. One of them is based on the use of a photo destructible surfactant for microemulsion formation and in the final step, irradiation with UV-light induces microemulsion destabilization and hence separation of Au nanoparticles. In another approach, excess water is added at the end of the reaction to the microemulsion containing the nanoparticles, inducing a change in phase behavior and hence microemulsion destabilization, followed by phase separation. Interestingly, by this approach, usually the nanoparticles remain in the oil phase, which can be diluted with organic solvents to form stable nanoparticle dispersions (Nazar et al., 2011). This method shows potential benefits for dispersion, storage, application and recovery of NPs, with the great advantage that it is not necessary to add organic solvents for
nanoparticle separation. In other approach reported by the same group, nanoparticle separation has been achieved by changing the solvent, for example, adding squalene to water/AOT/octane microemulsion containing Au nanoparticles (Myakonkaya et al., 2010)\textsuperscript{61}. Abecassis et al.\textsuperscript{62} have proposed nanoparticle separation by thermally inducing the phase separation of the microemulsion media. This was applied to the synthesis of Au NPs, which upon destabilization remained preferentially in the oil phase.

2.3 RECENT ADVANCES IN THE USE OF MICROEMULSIONS AS CONFINED REACTION MEDIA FOR THE SYNTHESIS OF INORGANIC NANOPARTICLES

There have been a number of advances in different aspects of the synthesis of nanoparticles in microemulsions over the last few years. The main ones are: the use of other types of microemulsions for synthesis (o/w and bicontinuous microemulsions)\textsuperscript{63}, the preparation of more complex architectures (core/shell and multishell, hybrid nanocrystals)\textsuperscript{64,65}, the synthesis of more complex ceramics (spinels, perovskites, etc)\textsuperscript{66}, modeling of reactions in microemulsions and novel approaches for the separation of nanoparticles from the reaction mixtures.

2.4 CHARACTERIZATION TECHNIQUES FOR MICROEMULSIONS

Microemulsions have been characterized by various techniques such as (i) Phase diagrams; the boundaries of different phase regions can be found\textsuperscript{67} (ii) Polarization microscope\textsuperscript{68}; helps to determine whether phases are anisotropic or not (iii) Interfacial tension; Dynamic light scattering\textsuperscript{69}; determines the diffusion coefficients and radius of microdroplets (iv) Small angle X-ray scattering; information about particle shape and size (v) Small angle neutron scattering; information about droplet core and surfactant layer (vi) NMR spectroscopy; measures diffusion coefficients and mobility of various components\textsuperscript{70} (vii) Interfacial Tension; low interfacial tension is the typical
characteristic of microemulsions which helps to determine the area per surfactant or co-surfactant and the radius of droplets (viii) conductivity\(^71\); helps to determine whether the microemulsion is w/o or bicontinuous or o/w type (ix) Viscosity; helps to determine the structure and flow properties\(^72\) (x) Electrical conductivity\(^73\); helps to determine whether the microemulsion is w/o or bicontinuous or o/w type (xi) Classical light scattering; informs the identity of droplets, particle size and interaction between particles and (xii) Electron microscopy\(^74\)

[Source- NanoComposix (Nanotoxicology: Particle Selection)]

2.5 INORGANIC NANOPARTICLES

Over the past few decades, inorganic nanoparticles\(^75\), which exhibit significantly distinct physical, chemical and biological properties from their bulk counterpart's, have elicited much interest. Although inorganic nanoparticles show only moderate transfection efficiencies\(^76\), they possess some advantages over organic nanoparticles. They are not subject to microbial attack, can be easily prepared, often have a low toxicity and exhibit good storage stability. Discoveries in the past decade have demonstrated that the electromagnetic\(^77\), optical\(^78\) and catalytic\(^79\) properties of noble-metal nanoparticles such as gold, silver and platinum are strongly influenced by shape and size\(^80\). The protocols must be robust enough to allow the simple (scalable) production of monodisperse nanoparticles with controlled size, composition and shape. The inorganic nanoparticles provide a robust framework, in which two or more
components can be incorporated to provide multifunctional capabilities. The properties of the inorganic nanoparticles are very different from those of their bulk counterparts for two main reasons: the increased relative surface area\textsuperscript{81} and the quantum confinement effects\textsuperscript{82}. The marked increase in the surface area also increases the nanoparticles chemical reactivity and ability to interact with added functional materials.

Biomedical applications of metal nanoparticles have been dominated by the use of nano bioconjugates that started in 1971 after the discovery of immunogold labeling by Faulk and Taylor.\textsuperscript{83,84,85} Currently metal-based nanoconjugates are used in various biomedical applications such as probes for electron microscopy to visualize cellular components\textsuperscript{86}, drug delivery (vehicle for delivering drugs\textsuperscript{87}, proteins, peptides, plasmids, DNAs, etc), detection, diagnosis and therapy (targeted and non-targeted). However biological properties of bare-metal (naked) nanoparticles have remained largely unexplored. The materials used for biomedical applications include calcium nanoparticles\textsuperscript{88}, iron nanoparticles\textsuperscript{89}, carbon nanotubes\textsuperscript{90}, double hydroxides/clays, silica\textsuperscript{91}, calcium phosphate\textsuperscript{92} and quantum dots.\textsuperscript{93} Among the most promising inorganic nanomaterials being developed are metal, silica, dendrimers, organic-inorganic hybrids and bioinorganic hybrids.

Effective use of nanoparticles in medical and other applications requires precise control and quality assurance throughout the entire manufacturing process, from the design and synthesis of the inorganic nanoparticle to its customisation and monitoring of its full life cycle. Intended uses and fields of application include: drug delivery, nanotoxicology studies\textsuperscript{94}, environmental remediation\textsuperscript{95}, immunology\textsuperscript{96}, catalysis for energy\textsuperscript{97} and control of cell-response.\textsuperscript{98} With nanotechnology in its early stages, this level of control has yet to be widely achieved.
Gold NPs are important in imaging, as drug carriers and for thermotherapy of biological targets. Metal NP contrast agents enhance magnetic resonance imaging and ultrasound results in biomedical applications of in vivo imaging. Hollow and porous inorganic materials have been exploited for drug and gene delivery, diagnostic imaging and photothermal therapy. Silver NPs show improved antimicrobial activity. Silica NPs have been used in drug delivery and gene therapy. Biomolecular inorganic nanohybrids and nanostructured biomaterials have been exploited for targeted imaging and therapy, drug and gene delivery and regenerative medicine. Dendrimers find use as drug or gene carriers, contrast agents and sensors for different metal ions. Various inorganic nanoparticles have been used for drug delivery, magnetic resonance and fluorescence imaging, and cell targeting owing to their unique properties such as large surface area and efficient contrasting effect.

Various applications of nanomaterials based on inorganic nanoparticles to biology and medicine are:
1. Fluorescent biological labels
2. Drug and gene delivery
3. Bio detection of pathogens
4. Detection of DNA and protein
5. Tumor destruction via heating (hyperthermia)
6. Separation and purification of biological molecules and cells
7. MRI contrast enhancement
8. Phagokinetic studies
9. Probing of DNA or protein structures
10. Tissue engineering

Inorganic nanoparticles are promising candidates for biomedical applications but their use in vivo will be very restricted if they are rapidly cleared from blood circulation by the RES. To address this issue, a number of strategies have been developed to coat nanoparticles with polymers to inhibit protein adsorption and uptake by macrophages. In general, these polymer coatings require a component which serves to anchor them to the nanoparticle surface (e.g. through electrostatic interactions or covalent bonds) while another component comprising hydrophilic chains will extend outwards from the surface. For many biomedical applications, particularly for in vivo imaging, inorganic nanoparticles possess good colloidal stability and low toxicity in a biological environment. Although high-quality nanoparticles with uniform size and high crystallinity have been synthesized, they are hydrophobic, and consequently, are not sufficiently stable in aqueous media for successful biomedical applications. Therefore, the surface modification of these inorganic nanoparticles is essential to endow them with hydrophilic properties, so that they can be extensively used for various biomedical applications. Furthermore, surface modification is important for the nanoparticles to impart additional functions, because bioactive materials are conjugated through the reactive groups on the nanoparticle surface. Recently, various surface modification methods have been developed; two representative strategies are ligand exchange with water-dispersible ligands and encapsulation with biocompatible shells. The resulting iron oxide nanoparticles were dispersible in water and successfully used for in vivo MRI. The use of inorganic nanoparticles as probes
to label and track cells in vivo is already a reality. While superparamagnetic nanoparticles\textsuperscript{107} have been the subject of clinical studies involving magnetic resonance imaging, quantum dots and gold nanoparticles are starting to be explored for similar goals in pre-clinical studies involving fluorescence and photo-acoustic imaging. Although exciting results have been obtained from in vivo investigations, there appears to be a general lack of understanding on the effects of physicochemical properties on the labelling efficiency and toxicity of those nanoparticles as well as on their stability in the intracellular microenvironment; essential requirements for using them as probes for cellular tracking.

2.5.1 Magnetic nanoparticles:

Transition metal oxides\textsuperscript{108} constitute one of the most fascinating classes of inorganic solids, as they exhibit a very wide variety of structures, properties, and phenomena. Oxide materials have been employed in many important advanced technology areas due to their many interesting physical properties including magnetic, ferroelectric, superconducting, ionic and electrical conducting characteristics. Nanoparticles of magnetic oxides, including most representative ferrites, have been studied for many years for their applications as magnetic storage media and as ferrofluids. The synthesis of discrete magnetic nanoparticles with sizes ranging from 2 to 20 nm, is of significant importance. Such magnetic nanoparticles could also find applications in ferrofluids\textsuperscript{109}, magnetic refrigeration systems, contrast enhancement in magnetic resonance imaging, magnetic carriers for drug targeting and catalysis. A general review on the synthesis and understanding of nanostructured magnetic materials up to 1996 is given in the article by Leslie-Pelecky and Rieke\textsuperscript{110}.

Magnetic nanoparticles are a type of nanosize material consisted of magnetic elements such as iron, cobalt, nickel, manganese, gadolinium and their alloys, oxide compounds, cation complexes along with polymers etc., which shows ferromagnetism, paramagnetism or even superparamagnetism. The physical and chemical properties of magnetic nanoparticles largely rely on the chemical
components, crystal structures, sizes and shapes and sometimes the source of magnetic nanoparticles synthesized.\textsuperscript{111}

Nanoparticle are submicron moieties (diameters ranging from 1 to 100 nm according to the used term, although there are examples of NPs several hundreds of nanometers in size) made of inorganic or organic materials, which have many novel properties compared with the bulk materials\textsuperscript{112}. On this basis, magnetic NPs have many unique magnetic properties such as superparamagnetic, high coercivity, low Curie temperature, high magnetic susceptibility, etc. Fe-NP contain an iron oxide core (Fe$_3$O$_4$ or $\gamma$-Fe$_2$O$_3$) that is surrounded by a ligand shell which consists of small organic or inorganic molecules, polymers or proteins. These ligand shells are essential for the stabilization of the nanoparticles in physiological media.\textsuperscript{113} Magnetism is highly sensitive to size and temperature because it arises from the collective interaction of atomic magnetic dipoles. When a ferro- or ferrimagnetic magnet is reduced to a critical size, it will change from a state that has multiple magnetic domains to one with only a single domain. Below this critical size, the thermal energy becomes comparable to what is needed for spins to flip, leading to the rapid randomization of the magnetic dipoles. Such NPs are referred to as superparamagnetic because they do not have permanent magnetic moments in the absence of an external field but can quickly respond to an external magnetic field.\textsuperscript{114} Hard (high coercivity) and soft (low coercivity) ferromagnetic materials are characterized by different values of $r_c$: for spherical NPs, the $r_c$ is typically 3–4 nm and over 20 nm for very hard and soft magnetic materials, respectively.\textsuperscript{115} More than 10 types of superparamagnetic NPs are now commercially marketed as contrast agents for MR-imaging. The most commonly used superparamagnetic NPs are those based on iron oxides, generally smaller than 20–30 nm in size. Iron oxide NPs enhance the proton relaxation of specific tissues and can serve as MR contrast agents for clinical diagnosis. Both $\gamma$-Fe$_2$O$_3$ (maghemite) and Fe$_3$O$_4$ (magnetite) NPs have been investigated as MR contrast agents for more than two decades and are already approved for clinical use in Europe. Other types of magnetic NPs have also been prepared and actively explored for MR imaging, including those doped with alternative metals to achieve higher relaxivities.
than the commercially available varieties. Notable examples include doped ferrites such as MnFe$_2$O$_4$, CoFe$_2$O$_4$, NiFe$_2$O$_4$, Gd$_2$O$_3$ and gold-coated cobalt NPs.\textsuperscript{116}

The outlook of such magnetic nanoparticles is very promising because these materials will find many important industrial applications. From a synthetic point of view, there are several interesting research areas worth pursuing. First, we need a generalized synthetic procedure, which can synthesize various monodisperse nanoparticles directly without any further size-selection process. Second, magnetic nanoparticles with various chemical compositions, such as CrO$_2$ and lanthanide-containing materials, should be prepared. Third, more extensive shape-control of magnetic nanoparticles should be investigated. Fourth, a large-area alignment of magnetic nanoparticles is critical in magnetic storage media applications. To improve our understanding of such systems and their applications, a collaborative multi-disciplined effort is anticipated. Magnetic nanoparticles functionalized with biocompatible macromolecules\textsuperscript{117} have potential for biomedical applications, such as magnetic field-assisted, extraction of cells and macromolecules, site-specific drug delivery, gene analysis and magnetic hyperthermia in cancer treatment.\textsuperscript{118,119} The design and synthesis of such nanoparticles have centered on techniques such as sol–gel\textsuperscript{120}, chemical coprecipitation\textsuperscript{121}, microemulsion\textsuperscript{122} etc. In order to implement the practical application, the particles must have combined properties of high magnetic saturation, stability, biocompatibility and interactive functions at the surface. Moreover, the surface of iron oxide NPs could be modified by organic materials or inorganic materials, such as polymers, biomolecules, silica, metals etc. Biosensing strategies based on magnetic nanoparticles have received considerable attention because they offer unique advantages over other techniques. For example, magnetic nanoparticles are inexpensive to produce, physically and chemically stable, biocompatible and environmentally safe.

In addition, biological samples exhibit virtually no magnetic background, and thus highly sensitive measurements can be performed in turbid or otherwise visually obscured samples without further processing. Optical techniques on the other hand are often affected by scattering, absorption, and/or auto-fluorescence within the sample.
To date, numerous methods have been developed to sense biomolecules using magnetic labels. These include techniques that use magnetometers such as SQUID\textsuperscript{123}, magnetoresistive sensors\textsuperscript{124} and Hall sensors\textsuperscript{125} to directly detect magnetic particles.

Another method that has achieved considerable success is based on magnetic resonance (MRI/NMR), which involves using magnetic nanoparticles as proximity sensors to accelerate the relaxation rate of neighboring water molecules.\textsuperscript{126} This technique is analogous to magnetic resonance imaging, which is used to look inside the human body and as such has been dubbed diagnostic magnetic resonance (DMR). For these diverse environmental and biomedical applications it is essential to have large amounts of magnetic nanomaterials stably dispersed in water. However, it is a technological challenge to control size, shape, stability, and dispersibility of NPs in desired solvents. Magnetic iron oxide NPs have a large surface-to-volume ratio and therefore possess high surface energies. Consequently, they tend to aggregate\textsuperscript{127} so as to minimize the surface energies. Moreover, the naked iron oxide NPs have high chemical activity, and are easily oxidized in air (especially magnetite), generally resulting in loss of magnetism and dispersibility. MEÎßNER et al. (2009) observed that dispersions of TiO\textsubscript{2} had a high positive $\zeta$ potential and were stable to agglomeration at acidic conditions and low ionic strength, but that the potential decreased in physiological NaCl, buffer solutions (PBS, HBSS) and cell culture medium (DMEM), and the nanoparticles rapidly agglomerated. As a result of agglomeration and sedimentation, the nanoparticle size distribution in suspension (as determined by DLS) will reveal an increasing tail of smaller size agglomerates with increasing primary particle diameter. Particle concentration is also an important parameter in DLS analysis. High particle concentrations produce an increment in particle diameters due to the effects of the agglomeration. These particle diameters can be increased to degrees beyond the measurement limitations of the instrument ($>10 \mu$m).

An inhibition or a limitation of the agglomeration may be achieved by surface coating of the nanomaterials, leading to electrostatic and steric stabilisation. Therefore,
providing proper surface coating and developing some effective protection strategies to keep the stability\textsuperscript{128} of magnetic iron oxide NPs is very important.

The chemistry of inorganic nanoparticles is highly advanced. In recent years, the search for new materials for use as electrodes in energy storage devices such as supercapacitors (SCs) and batteries has increased greatly mainly due to the demand for power systems with high energy and power densities. Manganese oxides have been synthesized by a variety of techniques in different nanostructures and studied for their properties as electrode materials in two different storage applications, supercapacitors (SCs) and Li-ion batteries.\textsuperscript{129} In the case of pseudocapacitors, various noble and transition-metal oxides such as RuO$_2$, IrO$_2$, NiO, SnO$_2$ and MnO$_2$ were used as electrode materials.\textsuperscript{130} However, the high cost of RuO$_2$ has prompted the research community to focus on other transition-metal oxides such as MnO$_2$, NiO etc., mainly because of the involved cost-effectiveness.

### 2.6 ENZYME ENCAPSULATION IN REVERSE MICELLAR SYSTEM

Reversed micelles are at the present time faced as common organic media to perform biocatalysis. They have been associated to the idea of a microreactor\textsuperscript{131} where the enzyme can be sheltered and protected from solvent detrimental effects.\textsuperscript{132} Many of the important physical and chemical processes in a wide range of fields including biology, geology and chemistry occur at and near aqueous interfaces. Transport of material through a cell membrane, erosion and dissolution of minerals and heterogeneous catalysis are all governed by the dynamic interactions of small molecules with a surface.\textsuperscript{133} Often, this small molecule is water, due to its importance as a part of all biological and many other natural systems. The discipline that emerged in the late 1970s, baptized as Micellar Enzymology by Martinek's group, by using a model system based on the self-organizing properties of amphiphiles in solution; this model system is named reverse micelles. It is hypothesized that this system reliably resembles the microenvironment that enzymes find in the cell: reverse micelles.
consist of micropools of water lined by a monolayer of an amphiphile, all dispersed in an apolar solvent. For a hydrophilic enzyme, which is usually found in the cytosol or the matrix of an organelle, reverse micelles provide the waterpools as a mimetic environment, where the properties of the water resemble the properties of the water closely associated with the cell.\textsuperscript{134}

Since the size of the protein and the size of waterpools are similar, the latter can resemble, better than a bulk buffered aqueous solution, the structured and viscous environment of the cytosol. Enzymes which interact with membranes as either peripheral or integral proteins also find the appropriate environment in reverse micelles, provided by the monolayer of surfactant and its closely associated water. Reverse micelles can host all kinds of substrate molecules whether hydrophilic, hydrophobic or amphiphilic and this is an important advantage over an aqueous medium.

Many organic substrates are water-insoluble while an aqueous environment is necessary to maintain enzymatic activity. An intriguing solution to this problem is offered by the microheterogeneous system of reverse micelles (Luisi and Laane, 1986).\textsuperscript{135} A number of studies in recent years have focused on solubilizing enzymes in reverse micelles and determining the enzymatic activity (Shield et al., 1986; Levashov et al., 1986; Luisi and Steinmann-Hofmann, 1987; Martinek et al., 1989; Gupte et al., 1995).\textsuperscript{136,137,138,139,140} Methods for recovering the solubilized enzymes from the reverse micelles have also been developed (Carlson and Nagarajan, 1992; Gupta et al., 1994).\textsuperscript{141,142} It seems, therefore, that reverse micelles may be an appropriate model system for biological studies at the molecular level. Indeed, studies such as those on the conformational properties of peptides and proteins\textsuperscript{143}, protein folding\textsuperscript{144}, enzymological studies regarding reactivity and specificity\textsuperscript{145}, limited proteolysis catalysed by proteinases\textsuperscript{146}, and so on, have been carried out in reverse micelles and have yielded substantially different results from those obtained in an aqueous medium. In addition, reverse micelles have opened up the possibility of developing new biotechniques to carry out bioconversions of apolar compounds and to extract proteins from a liquid medium.\textsuperscript{147} Enzymes in general have been microencapsulated
in reversed micelles by three methods: injection (the enzyme solution is added to the surfactant in organic solvent); phase-transfer (from an aqueous phase to a micellar phase) and dissolution (lyophilized enzyme is added to reversed micelles already containing an aqueous phase).\textsuperscript{148}

Thus \( W_0 \) controls the size of the droplets and can be routinely varied from zero to several tens, although the upper limit depends on the particular ternary system being studied.\textsuperscript{149} The most widely used system is that formed by water, an alkane (normally isooctane) and the anionic surfactant dioctyl sodium sulphosuccinate, better known as AOT [Aerosol-OT is a trademark substance from American Cyanamid that contains dioctyl sodium sulphosuccinate as a surface active agent. This compound, even when chemically pure, is usually abbreviated to AOT]. Other systems which have been used in micellar enzymology are hexanol/water/cetyl trimethylammonium bromide (CTAB) and iso-octane/chloroform/water/CTAB\textsuperscript{150} where the surfactant is cationic and cyclohexane/water/TX-100\textsuperscript{151} where the surfactant is non-ionic. One of the most important processes leading to micellar effects on reactions is the solubilization of substrates into the surfactant micelles. It is possible to solubilize water insoluble substances or to increase the solubilities of slightly soluble ones in aqueous micellar solutions.\textsuperscript{152,153} They penetrate towards the hydrocarbon-like cores of the micelles. The pathways and the rates of the reactions in micellar systems depend to a great extent on how deep the solubilized species are located within the micelle. The solubilized molecules interact with the polar head groups of a micelle and penetrate towards the core. They reside in the inner core, outer core, palisade layer or between the polar head groups. Sometimes micellar effect can also be observed as a result of the solubilization of substrates as counterions, i.e. without solubilization, with the substrates not hydrophobic enough to be solubilized in the micellar interior.\textsuperscript{154,155} This depends on different factors affecting the solubilization such as (i) concentration of the surfactant and co-surfactant; generally above C.M.C., the solubility increases with the surfactant and co-surfactant concentrations (ii) structure and chain length of the surfactant; if the solubilization occurs in the hydrophobic portion of the surfactant, this will increase with increase in the size of that group, (iii) nature and structure of the solubilize: molecular size, shape and structure, polarity and polarizability, chain
branching (iv) temperature: in most cases solubilization increases with increase in temperature, (v) nature of the counter ion; in general the solubility increases with increase in size and charge of counter ions, (vi) electrolyte; addition of electrolytes to ionic surfactants usually causes an increase in the C.M.C. and hence an increase in the solubilization capacity. It is also noted that micelle formation is a dynamic equilibrium process and hence solubilization process is also called as dynamic solubilization. The micellar interaction is not static or rigid and consequently a solubilized substrate is relatively mobile. Some studies also indicate that the solubilization is, on an average, uniformly distributed in the micellar interior.

Work carried out by Matzke et al.\textsuperscript{156} compared the three methods of enzyme microencapsulation. The solubilization capacity of the reversed micelles depends on the method used for protein addition. The dissolution of dry enzyme did not lead to appreciable microencapsulation unless the micellar diameter was of similar dimensions or higher than the protein diameter.\textsuperscript{157}

2.6.1 **Advantages of using reverse micellar system:**

1. Reversed micelles have a relatively ordered structure.
2. Reversed micelles form spontaneously, reaching an equilibrium state in a short time.
3. Normal and reversed micelles are recognized as models of biological structures.\textsuperscript{158}
4. Solubilization of both hydrophilic and hydrophobic substrates/products.
5. Low reaction volumes are needed.
6. Synthetic processes are favored due to the shift of thermodynamic equilibrium.
7. Side reactions such as reverse hydrolytic, polymerization and other reactions are hindered.
8. Microbial contamination is minimized.
9. Increased interfacial area of contact (10–100 m\(^2\)/mL).
10. Intermicellar exchange processes are fast.
11. Activity/stability may be improved.
12. Higher temperatures are possible as the thermal stability is often enhanced in low water media.
13. High substrates (products) concentration is possible.
14. Enzyme aggregation is avoided.
15. Solutions are isotropic (optically transparent) and this permits the use of several spectroscopic techniques for structural and monitoring purposes.
16. Rigorous control of the amount of water present.
17. The dimensions of the inner cavity may be easily changed.
18. Easy scale-up.

Furthermore, the dynamic character of reversed micelles gives them flexibility, which is profitable to reactivity, but in contrast affects the ordered structure of reversed micelles and consequently the stability of biopolymers.

2.7 DETERMINANT FACTORS FOR BIOCATALYSIS IN REVERSED MICELLES

The most important variables in a catalytic process are the selection of the catalyst and the reaction conditions. Using the $W_0$ parameter, it is possible to modulate the reversed micelles by influencing in this way the activity/stability of the biocatalyst. A common feature is that normally the optimum $W_0$ corresponds to a micellar size comparable to that of the protein. Martinek et al.\textsuperscript{160} found a direct relation between the effective radii of entrapped enzyme and the corresponding size of the micellar core (regulated by the $W_0$ parameter).\textsuperscript{161} The physical properties of water also depend on $W_0$, like temperature of core water, viscosity, dielectric constant etc. Goto et al.\textsuperscript{162} studied the solubilization of water in AOT reversed micelles classifying the water as immobilized, hydration or free water. Below $W_0$ of 2, only immobilized water exists, whereas in the range 4–10 the water hydrates the AOT polar heads and finally the presence of free water is verified at $W_0$ values above 10. The effects of water content
on rotational isomerism of AOT molecules were also analyzed with the same technique.\textsuperscript{163} Another decisive factor to choose the $W_0$ value is the type of catalyzed reaction. Hydrolytic or synthetic reactions have different needs of water and this also accounts for the overall reversed micellar catalytic system performance.\textsuperscript{164} The substrate concentration in the enzyme microenvironment is greatly influenced by this factor. It appears that the bell-shaped\textsuperscript{165} dependence, of activity upon the magnitude of the water pool, represents a general trend in micellar enzymology.

The $W_0$ concept is usually associated only with the hydration level although it is dependent on another variable, the concentration of surfactant. As an individual component in forming the reversed micellar structure, surfactant was found to play essential roles in the enzyme-containing reversed micellar system. Surfactant concentration may affect the physicochemical properties of the reversed micellar micro-aggregations, and change the solubilization or activity of the enzymes. In respect of enzymatic catalysis, surfactant concentration may even influence the distribution of the substrate. Over a wide range of surfactant concentration, variations of the surfactant concentration only affect the number of micelles, without changing their properties and/ or their size and shape.\textsuperscript{166} The strict limitation of this effect has not been established by far. In addition, surfactants of different natures will bring up multiple influences. The concentrations of surfactants can also affect the amount of solubilized enzymes and their catalytic actions. Another reason of the dependence of the enzyme activity with the surfactant concentration is the substrate distribution.\textsuperscript{167} Surfactant amphiphilic molecule consists of a hydrophobic tail usually formed by alkyl chain and together with a hydrophilic polar headgroup. The surfactants structural characteristics determine their physicochemical properties and their functions. The nuclear magnetic resonance spectrum ($^1\text{H NMR}$) indicated\textsuperscript{168} that most of the reactions take place in the surfactant interface of the micellar system. Therefore, the electrostatic property as well as the molecular structure of the surfactant membrane is very important. The electric charges of surfactant head-groups depend on their ionic structures of molecules. The lipases and $\alpha$-chymotrypsin catalyzed hydrolytic reactions in reversed micellar systems have been extensively investigated\textsuperscript{169,170}. In most cases, it has been observed that the hydrolytic activity is
much higher in AOT reverse micelles than in cationic, non-ionic or zwitterionic-based systems under the same condition. Kuwahara et al.\textsuperscript{171} reported the catalytic actions of hexokinase (HK) in reversed micelles of AOT (anionic), HTAC (cationic) and C12E8 (nonionic) surfactants. Results indicated that catalytic activity of HK in HTAC was 2–3 times higher than that in AOT reversed micellar system.\textsuperscript{172}

It is well known that the behavior of enzymes is dependent on the pH of the solution. Regardless of the pH measurement, the ionogenic groups of the enzyme will be affected by the microenvironment and their ionization state severely influences the interaction with substrate(s) and the inhibition by products.\textsuperscript{173} The pH value for optimum activity greatly depends on the exposed residues of protein especially those close to the active site. The pH may also affect the microencapsulation of protein and usually the pH values below the isoelectric point and low ionic strength improve the uptake of protein.

When solubilizing biopolymers, buffer solutions are often used to constitute the aqueous phase forming the water pools. The presence of electrolytes in the water pools will change the maximum $W_0$ value, usually decreasing it. This may be attributed to the increase of repulsion charges among the surfactant head groups. A similar observation was reported for the extraction of C. viscosum lipase with reversed micelles. The increase of ionic strength of the aqueous phase decreases the protein surfactant interactions, leading to the formation of smaller reversed micelles.\textsuperscript{174}

Several additives\textsuperscript{175} have been tested to improve either the biocatalyst’s activity or the stability or both. The addition of cosurfactants may increase or decrease the droplet size of microemulsions, depending on the chemical structure of the cosurfactant added. The addition of alcohols to micellar solutions of surfactants affects micellar properties such as CMC, ionization degree, micellar molecular mass and micellar dynamics. The important role of cosurfactants was also evidenced\textsuperscript{176} that described the self-replication of micelles by in situ transformation of cosurfactant. The effects of increasing or decreasing the cosurfactant chain length are not straightforward. The
most common situation, when long chain cosurfactants are applied is the hindrance of attractive interactions and the decrease of interfacial thickness, although these cosurfactants may also increase the interfacial thickness when the enhancement of the interactions between the micellar interface and the bulk organic solvent occurs.\textsuperscript{177}

Several correlations have been established between solvents and their properties affecting the enzymes behavior and stability. Laane et al. related the stability of biocatalysts with the logarithm of partition coefficient of the organic solvent in the system octanol/water (log P). In reverse micelles the location of enzymes diminishes the effect of solvents that is much less pronounced than using immobilized preparations.\textsuperscript{178}

Another aspect, much less explored, is the effect of the solvent\textsuperscript{179} on the reaction rate. The physical properties of the solvent are determining to define the substrate concentrations in the enzyme microenvironment. The development of models of substrate distribution explains how overall and effective substrate concentrations can vary and the capacity of the organic solvent to solubilize the substrate(s) may be important to the process. The droplet size and interdroplet interaction are also affected by the solvent and increase with the increment of the chain length of the oil, at a constant $W_0$. Alkanes of small chain length are able to penetrate in the surfactant layer more strongly, by reducing the solubilization of water.\textsuperscript{180}

Another important factor for biocatalysis in general, is the temperature and the reversed micellar systems are not an exception. The influence of temperature\textsuperscript{181} on biocatalysis in reversed micelles is regulated by the same rules as in other aqueous or non-conventional media; herein the activity is improved by raising the temperature up to a certain value, dependent on the enzyme stability. Nevertheless, it should be kept in mind the importance of temperature on the definition of the phase diagrams, namely to attain the L2 phase corresponding to the reversed micelles. High temperature and high droplet concentration lead to percolation, leading to changes of the electrical conductivity and eventually to the formation of bicontinuous structures.
A major concern in enzymatic processes is the protein denaturation. For industrial purposes, it is important to have high activities as well as to keep these activities during the time. Important factors that usually improve the stability in reversed micelles are: 1) low water content/reduction in $W_0$, 2) addition of protective compounds (substrates, ligands, neutral salts, polyols, sugars and polymers), 3) choice of a non-deleterious solvent, and 4) use of pH optimum and correct buffer concentration.

The kinetics of chemical reactions catalyzed by microencapsulated enzymes in reversed micelles usually obey the classical Michaelis-Menten model. Nevertheless, the kinetic constants determined for enzymes in these conditions significantly differ from the ones observed with the same enzyme in aqueous solution.

Taking the example of immobilized enzymes, the kinetic constants may be classified as: intrinsic, inherent and apparent (or effective). The denomination of intrinsic refers to the kinetic constants when they comprise the catalytic constants of the free enzyme and the associated conformational aspects. The inherent constants represent the addition of partition effects to the intrinsic parameters and the apparent constants apply to the situations where besides conformational and partition effects, diffusional limitations also exist.

The catalytic activities are dependent on the size of the micelles, i.e., on the $W_0$ parameter. Consequently, the optimal sized micelles allow the achievement of maximal $k_{cat}$ values. A constant value of $W_0$ corresponds to a distribution of various sizes of micelles, which transforms the $k_{cat}$ in an average value. At lower water content the enzymes often present a lower kinetic affinity for the substrate. When water-insoluble substrates are used, these substrate molecules are localized in the organic phase (oil phase) and to a great extent within the micellar phase. A comparison of the product accumulation curves of the enzymic reactions performed both in aqueous medium and in reverse micelles using similar conditions of pH, temperature and enzyme and substrate concentrations is the first step to be taken in the search for particular aspects of enzymatic reactions in reverse micelles. At
first sight, there is no indication that the reactions in reverse micelles progress any differently from the way in which they progress in aqueous medium; in particular, the reverse micellar medium does not abolish or induce any transition phase, whether lag or burst, that did or did not already exist in the reaction progress curve.

Luisi and co-workers\textsuperscript{187,188} have carried out fast kinetics studies of the hydrolases $\alpha$-chymotrypsin and trypsin by using the stopped-flow technique. In a first study on the trypsin catalysed hydrolysis of benzyl oxycarbonyl-LysO-Np at acidic pH, where deacylation of the acyl-enzyme complex is the rate-limiting step in aqueous solution, they observed that the burst which characterizes this reaction disappeared when the reaction was performed in CTAB/iso-octane/chloroform reverse micelles. Thus they concluded that the rate-limiting step in reverse micelles is no longer the deacylation step but the step namely, the acylation of the free enzyme, and therefore a reverse micellar system is able to bring about a change in the enzyme kinetics by changing the step which is rate-limiting.\textsuperscript{189}

In carrying out this kind of study, it was noted from the very beginning of micellar enzymology that sometimes the reaction rate was higher in reverse micelles than in bulk water, despite the fact that the overall concentrations of enzyme and substrate were the same. Likewise, the pH-independent $k_{\text{cat}}$ was shown to be greater in reverse micelles.\textsuperscript{190} Such a phenomenon was named 'superactivity'.\textsuperscript{191} Meanwhile the inner system of reversed micelles is suggested to be a suitable mimetic environment such as in the living cell. This explains the “superactivity” of enzymes in it. As a result, the problem of extending the lifetime of extracellular enzymes as well as making them best suited for non-native reacting environment was approached.\textsuperscript{192,193}

Usually, micelles were assumed not to be restricted by mass transfer limitations or product inhibition, although recent advances had raise some questions and this concept is not accepted as a rule anymore. The control of water content presents multiple advantages that will be discussed in relation to the stabilization. Regarding the system mixing, agitation is still a need but not at high rates, since in the micellar systems, biocatalysts are already dispersed and the interfacial area of contact is high.
This reduces the detrimental effect of shear stress forces on the enzyme structure. The disadvantages are far less numerous and include: i) denaturing effects of surfactant; ii) low \( W_0 \) values are difficult to obtain for non-lyophilized enzymes and iii) product recovery and enzyme re-use that is still difficult. Applications of reversed micellar systems are also limited since the surfactant in high concentration severely complicates the separation of products and hinders the enzyme recovery.

### 2.8 ENZYME HOSTING IN NANOPARTICLES

Nano-size materials have been widely used as support for immobilization of proteins, peptides, enzymes, antibodies and nucleic acids because of their unique properties. Based on the arrangement of drug and polymer matrix, nanoparticles can be classified into two types: nanospheres and nanocapsules. In nanospheres, drugs are either adsorbed or entrapped inside the polymeric matrix. In nanocapsules, drugs are confined to the inner liquid core while the external surface of nanoparticles is covered by the polymeric membrane. The uptake and distribution of nanoparticles depend on its size. Nanoparticles of size ~10 nm are utilized for extended circulation, while ~100 and ~200 nm particles are utilized for passive targeting and intracellular drug delivery respectively. Though nanoparticles have many advantages over other conventional drug delivery systems certain properties like surface hydrophobicity and surface charge needs to be altered so as to increase the uptake of nanoparticles into cells.

Advantages of using nanoparticles as drug delivery vehicles are as follows:

- Longer shelf-stability
- High carrier capacity
- Ability to incorporate hydrophilic and hydrophobic drug molecules
- Can be administered via different routes
- Longer clearance time
- Ability to sustain the release of drug
Chapter II

- Can be utilized for imaging studies
- Increase the bioavailability of drugs
- Targeted delivery of drugs at cellular and nuclear level
- Development of new medicines which are safer
- Prevent the multi-drug resistance mediated efflux of chemotherapeutic agents
- Product life extension

The newly emerging area of inorganic nano-particles entrapping biomolecules has already exhibited its diversity and potential applications in many frontiers of modern material science such as those in sensors, optical materials, biocatalysts, and immunochemistry.\(^{200,201}\) Nanoencapsulation of enzymes by inorganic materials for in vivo use of sustained drug release is also expected to have potentiality in enzyme therapeutics. Enzyme immobilization strategies involve the conjugation of enzyme via covalent attachment, cross-linking, adsorption and entrapment onto hydrophobic or hydrophilic polymeric and inorganic matrixes. The utilization of enzymes as biocatalysts has become an important avenue in chemical and pharmaceutical industries to prepare biochemical products, biosensors and drugs.\(^{202,203}\) Enzymatic reactions are environmentally and user friendly. The catalytic efficiency is high under normal mild reaction conditions. However, most of native enzymes exhibit high reactivity and selectivity only under normal conditions. Under extreme temperatures or pH, enzymes are easily inactivated due to denaturation, either by changes of conformation or other transformations of stereo chemical structure. Thus, native enzymes often suffer severe limitations in broader applications. Moreover, the utilization of natural enzymes has other processing difficulties such as the reuse of enzymes, product contamination and separation. One of the approaches to resolve these difficulties is to immobilize enzymes on solid surfaces which can produce recoverable and stable heterogeneous biocatalysts. There is a long history for enzyme immobilization on solid supports, particularly on inorganic nano-particles. These particles can be prepared at very low temperature so that enzyme activity can be maintained, and they act as excellent storage stabilizers for the enzymes. Immobilized enzymes can be recycled by utilizing the physical or chemical properties of the supporting material. Magnetic nanoparticles
provide advantages as the supporting material for immobilized enzymes over competing materials such as: higher surface area that allows for greater enzyme loading, lower mass transfer resistance, less fouling effect, and selective, nonchemical separation from the reaction mixture by an applied magnetic field. Various surface modifications of magnetic nanoparticles, such as silanization, carbodiimide activation, and PEG or PVA spacing, aid in the binding of single or multienzyme systems to the particles, while cross-linking using glutaraldehyde can also stabilize the attached enzymes. Due to their high specific surface area and easy separation from the reaction medium by the use of a magnet, they have been employed in enzymatic catalysis applications. MNP-enzyme conjugates (MNP-Es) represent a specific class of bio-NP conjugates that are of great interest for biotechnological applications where high catalytic specificity, prolonged reaction time, and in some cases the ability to recycle an expensive biocatalyst is required (Swanson 1999; Alcalde et al. 2006). In addition, magnetic field susceptibility provides a mechanism for efficient recovery of the enzyme complex from reaction products, which is especially important in the pharmaceutical industry where enzyme contamination of the final product can cause detrimental side effects. Xenobiotic chemical degrading enzymes attached to MNPs also hold potential for use in novel nano-remediation technologies that will allow precise delivery (using electromagnetic probes) of the MNP-E conjugate to the contaminant source in locations such as aquifers while enabling recovery and reuse of the MNP-Es. The fate of biomolecules in natural or human-controlled environments (e.g., sewage treatment plants, aquifers, or soils) could also be traced by tagging the biomolecules with MNPs. Many enzymes currently used in biotechnology, including glucose oxidase and peroxidase, have been covalently immobilized to MNPs using several different ligands (Rossi et al. 2004; Kouassi et al. 2005a). Glucose oxidase, an enzyme used in sensors that measure blood glucose, was covalently immobilized to MNPs and found to be stable over a wide range of pH and temperature conditions and to retain activity for three months (Rossi et al. 2004). Another enzyme, cholesterol oxidase, was covalently immobilized for use in clinical applications and sensors (Kouassi et al. 2005b). Lipase, which is important for processing lipids in the food and pharmaceutical industries, was covalently immobilized to nanoparticles by a carbodiimide linkage and shown to maintain
significant activity after one month of storage (Dyal et al. 2003). Trypsin and chymotrypsin have also been stabilized against denaturation and self-digestion at the air–water interface by immobilization on iron and gold nanoparticles (Hansen et al. 2006; Jordan et al. 2006). Streptokinase, which has been used as a therapeutic agent, has been covalently immobilized to MNPs by carbodiimide linkage for use in localized lysis of blood clots in vivo. The magnetic properties of the particle-streptokinase congeners could allow for focusing of the treatment to the exact location where the clot was located, reducing the amount of enzyme required and in turn reducing the risk of eliciting an immune response (Koneracká et al. 2002; Fernandes et al. 2006). It is critical in the preparation protocols for the immobilization of enzymes in confined nanospaces to retain the integrity of tertiary structure and leave the active sites accessible. By immobilizing enzymes, the enzymatic reaction may be carried out under a non-aqueous media for large-scale applications. The solid supports make the enzyme molecules more robust so the catalysts can be reused for several times after easy separation from the reaction media. The above situations demand proper immobilization methods tailored for particular needs.

2.9 DRUG DELIVERY VIA NANO-PARTICLES

Increasing efforts in research and development worldwide in the last decade have been devoted to various inorganic materials such as novel non-viral carriers. Non-specific accumulation into healthy tissues is always a concern for nanoparticle drug delivery systems. Using local sensitization through light or temperature may reduce overall toxicity, but it is expected to damage adjacent healthy tissues as well. Ultimately, inorganic particles may not provide advantages over other types of nanoparticles for systemic targeting of cancer cells because they are not biodegradable, have low payloads and have no controlled release properties. Many inorganic materials, such as calcium phosphate, gold, carbon materials, silicon oxide, iron oxide and layered double hydroxide (LDH), have been studied. Surface modification of inorganic NPs and biomolecules can improve their interaction. The organic molecules used to functionalize inorganic NPs usually have two feature groups: the anchoring group and the charging group. The former anchors itself onto
Chapter II

the NP surface, while the charging group binds with the biomolecules and also impacts the positive charge to the hybrid NPs. Much effort has been put into the surface modification of biocompatible inorganic particles.\(^{218}\) As discussed above, modified NPs as carriers of drugs and biomolecules have several particular advantages in driving loaded drugs to the target site. First, modified NPs with positive charges can drive the particles to approach the normal negatively-charged cell membrane via electrostatic interactions. In addition, inorganic NPs are modified so as to adjust the adhesion of organic/inorganic nanohybrids to the cell membrane. For example, NPs coated with PEG can improve their non-specific cellular uptake. Meanwhile, grafting specific biomolecules onto NPs can provide site-specific delivery into cells. On the other hand, physical targeting is another promising method to drive drug molecules to recognize targeted cells. It is either based on the abnormal environment (e.g., pH, temperature) in a target zone e.g., tumor or inflammation (pH and temperature-sensitive drug carriers) or magnetically targeting\(^{219}\) certain cells by driving drug-loaded biocompatible magnetic nanoparticles (MNPs).

The idea of using magnetic particles as a carrier tool for drug delivery was conceived in the late 1970s by Widder, Senyei and their colleagues. Freeman et al.\(^{220}\) proposed in 1960 that magnetic NPs could be transported through the vascular system and concentrated in a specific part of the body with the aid of a magnetic field. The basic premise of this idea is that therapeutic agents such as targeted chemotherapy or therapeutic nucleic acid molecules are attached to or encapsulated within a magnetic particle core. And this magnetic particle core with a layer of polymer or metal coating enables to be functionalized easily. After being functionalized, the magnetic particle therapeutic agent conjugate is injected or administrated orally into the blood-stream system. Under the guidance of a strongly external magnetic field, the magnetic particle-therapeutic agent composites are guided to the area of the targeted sites and the drugs are delivered.\(^{221}\) Since the idea was proposed by Senyei et al.\(^{222, 223}\) there are a lot of studies on the field of the targeted drug delivery with magnetic guidance. Sun et al.\(^{224}\) used bacterial magnetosomes (BM) as the magnetic-targeted drug carrier and found an antitumor effect of doxorubicin (DOX)-loaded BMs (DBMs) in EMT-6 and HL60 cell lines. Collectively, their studies suggested the therapeutic potential of
Chapter II

DBMs in target-therapy against liver cancer. A cell delivery strategy was investigated by Polyak et al.\textsuperscript{225}

Prior to the use for drug delivery, magnetic microparticles were proposed as contrast agents for localized radiation therapy\textsuperscript{226} and to induce vascular occlusion of the tumors (antiangiogenic therapy)\textsuperscript{227}. Biomacromolecules, such as proteins, lipids and polysaccharides, fluorescent molecules and anticancer drugs, can be conjugated to the surface of nanoparticles. The resulting nanoparticles can not only target human cancers, but also be visualized inside the body by both magnetic resonance (MR) and fluorescence imaging. In addition, these applications need special surface coating of the magnetic particles, which has to be not only nontoxic and biocompatible but also allow a targetable delivery with particle localization in a specific area.\textsuperscript{228} With appropriate surface chemistry, biomolecules can immobilize on iron oxide NPs. In contrast, the major strategy for surface functionalization by biological molecules includes two steps, first synthesizing the small molecules or polymers functionalized NPs, and then coupled to the biomolecules by chemical bond or physical adsorption. Recently, Lee et al\textsuperscript{229} developed a route for conjugating the $\gamma$-Fe$_2$O$_3$ NPs with single strand oligonucleotides. Zhang et al\textsuperscript{230} have reported a microemulsion approach to prepare a human serum albumin (HAS)-coated Fe$_3$O$_4$ magnetic NPs as a radioisotope carrier labeled with $^{188}$Re and explored the optimal labeling conditions with $^{188}$Re. This intelligent carrier can decrease the concentration of the drug at non-target sites, thus reducing side-effects and increase the concentration of the drug at the target site, thus promoting its efficacy.\textsuperscript{231}

2.9.1 There are some disadvantages also of using these nanoparticles like:

a) Involves higher manufacturing costs which may in turn lead to increase in the cost of formulation
b) Involves use of harsh toxic solvents in the preparation process
c) May trigger immune response and allergic reactions
d) Extensive use of poly(vinyl alcohol) as stabilizer may have toxicity issues.

The release mechanism is an essential question in the design of a drug delivery systems, and thus drug loading is not just another ‘functionalisation-challenge’. Drug release may be induced with both environmental factors (e.g. enzymatic action) and properties of the carrier (e.g. temperature-induced release involving alternating magnetic fields).

An alternative targeting approach involves the use of molecules capable of activation following cleavage by tumor- or other tissue-specific enzymes. These compounds have been incorporated into many structures ranging from in vivo imaging constructs (Weissleder et al 1999; Tung et al 2000; McIntyre and Matrisian 2003; Harris et al 2006) to chemically modified drug structures in the form of prodrugs (Suzawa et al 2000, 2002; Liu et al 2003). These studies demonstrate the feasibility of tumor-specific enzyme interaction as a means of modifying the physical structure of delivered drugs and imaging constructs and potentially enables targeted delivery of drug carriers including nanoparticles (NPs).

Conclusively, to take the advantage of inorganic NPs for therapeutic and drug delivery applications a few key aspects must be controlled:

1) The surface of NPs must be tailored to retain the high surface area and reactivity but reduce or minimize the unintentional reaction of NPs with the human body.

2) Extensive biocompatibility and no systemic toxicity to normal cells/tissue at the level of dose administered must be demonstrated.

3) The NPs should stay in the blood for a time long enough for active recognition and uptake by the target organs.

4) The NPs should demonstrate nonspecific accumulation in body and should be able to clear out of the body by normal means.

5) The characteristics of NPs, such as, size, dispersion and surface charge should remain unaltered in the hostile cellular environment.
To demonstrate the above mentioned properties inside the hostile cellular environment, the surface of NPs needs to be protected and/or modified. Bare, uncoated NPs can agglomerate and are cleared (out of the body) by the reticulo-endothelial system (RES) resulting in poor biomedical Properties. Often the surface of NPs is covered and modified with various functional molecules to achieve the desired function. Surface modification of inorganic NPs by biocompatible compounds can tailor the surface properties, such as surface charge, biocompatibility, and solubility. The huge potential of inorganic NPs in therapy, diagnostic imaging, treatment and prevention of diseases has been augmented by the stealth properties provided by the PEG coatings. Smarter design and meticulous chemistry have allowed the vectorization of NPs in conjunction with PEGylation. As the research becomes more multidisciplinary, new synthetic methodologies, conjugating agents and linking molecules will be developed for coating smaller and more diversely shaped NPs leading to efficient encapsulation of the NPs.

2.10 PRODRUG ACTIVATION BY ENZYME, A PROMISING STRATEGY FOR CHEMOTHERAPY

Current cancer treatments include surgical intervention, radiation and chemotherapeutic drugs, which often also kill healthy cells and cause toxicity to the patient. A major problem with the use of many chemotherapeutic agents is their unacceptable damage to normal cells and organs, a narrow therapeutic index, a relatively poor selectivity for neoplastic cells, and multidrug resistance upon prolonged treatment due to up-regulation of efflux pumps, increased glutathione S-transferase expression, and enhanced DNA repair (Lowenthal and Eaton, 1996; Nielsen et al., 1996; Stavrovskaya, 2000). A potential strategy to overcome the limitations of chemotherapeutic agents is the use of prodrugs. Prodrugs of therapeutically active agents have rightfully been receiving increased attention. The term prodrug describes chemicals with little or no pharmacological activity that undergo biotransformation to yield a therapeutically
active metabolite (Albert, 1958). The chemical changes involved in creating prodrugs are usually designed to improve one or more physio-chemical properties that are lacking in the parent drug. Prodrugs are often divided into two groups: 1) prodrugs designed to increase the bioavailability to improve the pharmacokinetics of antitumor agents and 2) prodrugs designed to locally deliver antitumor agents. Currently, delivery methods for an enzyme/prodrug strategy can be divided into two major classes: (a) delivery of genes that encode prodrug-activating enzymes into tumor tissues (GDEPT, VDEPT, etc.); and (b) delivery of active enzymes onto tumor tissues (ADEPT). Although each approach (GDEPT, VDEPT, and ADEPT) has been tested in clinical trials, there are some potential problems using the current delivery systems. The generated HRP encapsulated nano-particles can be used for Indole-3-acetic-acid (IAA) activation by HRP. There is great potential in the immobilization of biomaterials and combination with IAA for enzyme/prodrug cancer therapy.
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Chapter II


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