Chapter-1
Review of Literature
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Introduction

The term nanoparticle is used to describe a wide variety of materials of submicron size. An internationally accepted definition for nanoparticles does not exist. It is used differently, according to the context of material being described. According to a recent definition suggested by British Standards Institution “Nanoparticles are the particles with one or more dimensions at the nanoscale.” They have defined the nanoscale as dimensions of the order of 100 nm or less. It is critical length scale at which certain novel size related properties develop and the materials start behaving differently than the molecules or bulk material (PAS71 2005). Here novel properties refer to optical magnetic and electrical properties which typically appear in materials below 100 nm size. However, many a times, besides “strictly nano” (1-100 nm) all submicron colloidal particles i.e. particles with at least one dimension in the scale of 1-1000 nm, also called mesoscale, are referred as nanoparticles, to include organic polymers and vesicles widely used in the area of drug delivery (Leary et al. 2005, Gelperina et al. 2005). At this scale the properties of the matter are different from atomic or molecular properties which are governed by laws of quantum mechanics, or the properties of bulk materials determined by laws of classical physics. So the dimension of 1-100 nm may be considered as an intermediate state between atomic or molecular state and bulk state where materials exhibit some unexpected and unusual new properties which can not be defined by classical laws of physics (Hochella, Jr. et al. 2008). It is these unusual properties which have attracted immense attention of researchers from almost every field science including biology and medicine. Investigation of novel properties of nanoparticles and their application has become very active area of research.

Historical Perspective

Nanoparticles have been in use in pottery and medicine since ancient times. Historical evidences suggest that Gold nanoparticles were used as drug by Chinese during 2500 BC. Red colloidal gold is still in use under the name of Swarna Bhasma and Makaradhwaja” in traditional medicine system of India called Ayurveda, which dates back to 1st millennium BC (Bhattacharya & Mukherjee 2008). Recent scientific study of the a vessel of Roman period (4th century AD) called “Lycurgus Cup,” kept
in British Museum, London shows the use of Nanoparticles of Gold-Silver alloy for its decoration (Freestone et al. 2007). Similarly, churches of Middle Ages used gold in colloidal state trapped within the matrix of glass to make aesthetically pleasant ruby coloured glasses of different hues and colours (due to the formation of nanoparticle of different sizes). In 16th Century Europe an aqueous form of colloidal gold called “Aurum Potabile (drinkable gold)” was thought to have curative properties for many diseases (Caseri 2000). In 1857 Michael Faraday described methods for synthesis of stable aqueous dispersions and optical properties of gold nanoparticles (Faraday 1857). In 1915, in his famous book “The World of Neglected Dimensions”, Wolfgang Ostwald recognized colloidal particles as unique state of matter, whose particles “are so small that they can no longer be recognized microscopically, while they are still too large to be called molecules.” However the credit of realising the enormous potential of nanoparticles and their possible implications in different fields is given to Richard P. Feynman. In his classical lecture in 1959 at California Institute of Technology (Caltech) during Annual meeting of the American Physical Society Feynman has stated:

“........I would like to describe a field, in which little has been done, but in which an enormous amount can be done in principle. This field is not quite the same as the others in that it will not tell us much of fundamental physics (in the sense of, What are the strange particles?)………

........ In the year 2000, when they look back at this age, they will wonder why it was not until the year 1960 that anybody began seriously to move in this direction…..

When we get to the very, very small world---say circuits of seven atoms---we have a lot of new things that would happen that represent completely new opportunities for design. Atoms on a small scale behave like nothing on a large scale, for they satisfy the laws of quantum mechanics. So, as we go down and fiddle around with the atoms down there, we are working with different laws, and we can expect to do different things............
The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big ……..

The problems of chemistry and biology can be greatly helped if our ability to see what we are doing, and to do things on an atomic level, is ultimately developed---a development which I think cannot be avoided.”

There is Plenty of Room at Bottom (Feynman 1959).

Because of such brilliant foresight and visionary thinking about the properties of nanoparticle and their future implications, Feynman is often referred as Father of the field of nanoparticle research (Erren 2007). Later Eric Drexler Published His book “Engines of Creation: The Coming Era of Nanotechnology” which brought Feynman’s vision to a broader audience (Wilsdon 2004). It gave a detailed view of concepts, its potentials and even dangers if it is misused.

However the, availability of suitable methods for synthesis of nanoparticles of uniform size and techniques for their characterization was a limiting step in realization of Feynman’s dream till early nineties. During this period work in groups of Turkovich, Frens, Stöber, Iijima, Bawendi and others has resulted into development of synthetic methods to produce uniform nanostructures of gold, silica, carbon, cadmium etc. with sizes in the range of 1 to 100 nm (Jaiswal & Simon 2004). During the same period development of technique like Atomic Force Microscopy, Scanning Tunneling Electron Microscopy, Dynamic light scattering have enabled detailed study and manipulation of materials at nanoscale. This has set the stage for a burst of research activities involving study, manipulation and application of nanoparticles. Initially nanomaterials research was mainly focussed in the area of materials science, mainly in development of microelectronic and optoelectronic devices. However, in last 10 years, the dramatic size dependent optical, electronic, magnetic, and mechanical properties of nanoparticles have attracted attention of researchers from almost every science including biology and medicine (Tan et al. 2004).
A Survey of Synthesis, Properties and Biological Applications of Important Nanoparticles

The high aspect ratio and resultant special properties exhibited by matter at nanoscale has been a great attraction for development and study of nanoparticles from every possible material. In order to study and exploit the enormous potential provided by “nanoscale”, every possible building block for development of nanoparticles is being explored. The design and synthesis and surface modification of nanomaterials with novel properties has become an exciting area of research. Considering that primary limit is only the size range of 1-1000 nm, there is virtually no limit of the possible ways, for fabrication of nanoparticles. Indeed, almost limitless types of nanoparticles of different shape, size and surface properties are being developed from a range of materials of inorganic, organic, biological or hybrid nature. Availability of new methods of fabrication and tools for characterization and manipulation has resulted in a variety of innovative application of nanoparticles (Rao & Cheetham 2001). In last ten years, the nanomaterials research which was initially restricted to materials science has seen cross-disciplinary expansions to almost every field of science including biology and medicine.

The novel properties of nanoparticles hold enormous potential for applications in both basic and applied area of research in Biology. Addressing different problems in biology using the nanoparticles has been an active area of research. This merger of nanomaterials research with Biotechnology has given birth to a new discipline called Nanobiotechnology, in which innumerable types of nanoparticles are being constantly developed and investigated for better understanding of biological system as well as development of new products and technologies (Niemeyer 2001, Roco 2003).

Nanobiotechnology is now a burgeoning field, having influence in almost every aspect in biomedical research. Because of the diversity of the field it is almost impossible to review different aspects of every type of nanoparticle. So let us examine few most important nanoparticles which are being studied for applications in biology and medicine.
i) Gold Nanoparticles

Amongst an array of nanomaterials being investigated for applications in biology, Gold nanoparticles (GNP) are probably most extensively studied nanoparticles. In fact, as described in previous section, gold nanoparticles has been in use since ancient times in one or other form, be it in traditional Chinese, Indian or European medicine or for decorative purpose in “Lycurgus Cup.” The work of Faraday has pointed towards specific optical properties of colloidal gold, long before the recognition of changing properties of matter at nanoscale. Development of synthetic protocols for reproducible synthesis of monodisperse gold particles, initially by Turkevich and then by Frens gave a further boost to gold nanoparticle research.

Currently a number of techniques for preparation of gold nanoparticles of various shape and size in both aqueous and organic medium are available (Daniel & Astruc 2004). Most of these protocols employ reduction of gold ions from a gold salt in presence of a capping agent which prevents the aggregation of particles. The most common method of synthesis of gold nanoparticles for application in biology is the one, first described by Turkevitch in 1951 and further developed by Frens in 1973 (Turkevich et al. 1951, Frens 1973). In this method, GNP are obtained by heating a solution of HAuCl4 in presence of Sodium citrate. In this reaction citrate initially acts as reducing agent to convert Au(III) to Au(0) to form particles and then it acts as a capping agent by adsorbing to nanoparticle surface and repelling particles from each other, thereby preventing aggregation. This protocol can be used to prepare spherical sizes from a range of sizes from 16-147 nm (Daniel & Astruc 2004). The temperature, ratio of gold to citrate, and the order of addition of the reagents control the size distribution of gold nanospheres generated by this method (Eustis & El-Sayed 2006). In a slightly modified method, smaller size (Approx. 3.5 nm) of GNP can be prepared by reducing Chloroaurate ions with cold solution of Sodium Borohydrate (NaBH₄) instead of heating in presence citrate ions. In later case, citrate acts as the capping agent only, as it cannot reduce gold salt at low temperature (Jana et al. 2001). The small nanoparticles prepared in this way can be used as seed for preparing particles of higher size or different geometry. Very small nanoparticles (1.5-5.2 nm) with narrow dispersity can be prepared using two phase Burst-Sciffrin method. In these methods the particles are first synthesized by reducing chloroaurate ion in organic medium, in presence of thiols,
xanthates, disulphides, phosphines, amines or carboxylates as capping agent. Then depending upon capping agent the particles can be dried and re-dispersed in other organic solvents or water. This method has been method of choice for synthesis of so called monolayer protected clusters (MPCs) (Daniel & Astruc 2004).

A variety of methods are available for conjugation of ligands and biomolecules with GNP for different biological applications. The gold particles has been directly obtained as biomolecule protected GNP using some peptides, lipids and other ligands as capping agent (Guo & Wang 2007). Passive adsorption of antibodies and proteins with colloidal gold has been in practice since long time. However, most commonly used current methods for specific immobilization of molecules at GNP surface exploit strong affinity of thiols with GNP. A –SH modified biomolecule eg. mercaptoalkylolgonucleotides can be directly chemisorbed to GNP surface by formation of strong Au-S bond. Alternatively GNP can be functionalized using a bifunctional linker having –SH group at one end. Then the desired ligand can be conjugated at the surface of functionalized nanoparticle using a suitable conjugation chemistry for the functional group at other end (e.g. EDC coupling for –COOH group at functionalized GNP and –NH2 group at proteins) (Sperling et al. 2008).

\[ \text{Figure 1.1: A typical UV/Visible spectrum of Gold nanoparticles. Inset: Different sizes of Gold nanoparticles.} \]
Because of Au, being a heavy element gold nanoparticles form sharp electron micrographs with excellent contrast. In fact, because of its electron density, availability in a range of size, ease in synthesis, versatility in adsorption to macromolecules and its ability to be measured morphometrically GNP conjugated to antibodies or other specific ligands serves an excellent cytochemical marker in Electron Microscopy (EM). Actually colloidal gold has been in use in EM long before term nanoparticles came in fashion (Bendayan 2001). However, most popular property of the GNP for biomedical applications is its unusual optical properties which arise due to localized Surface Plasmon resonance (SPR). The SPR in GNP arises due to resonance of collective oscillation of electron cloud of 6S electrons of conduction band at surface (surface plasmon) of particles and electromagnetic field of incoming light. Because of SPR, GNP show heavy absorption of visible light at 520 nm. This gives brilliant red colour to GNP which varies according to their size (Figure 1.1). The resonance frequency (absorption maxima) depends upon the shape size and the dielectric constant of the particle and medium. The absorption maxima of GNP show characteristic shifts upon changes in environment at particles surface and change in inter-particle distance. A change in local environment due to binding or conformational changes at particle surface and thereby change in electron density results into resonance frequency. The resonance frequency also shifts dramatically with change in inter-particle distance. These two characteristics form the basis for probably most popular applications of GNP in detection of biomolecules (Sperling et al. 2008). Measurable shifts in SPR spectra of GNP has been observed upon pH induced conformational changes in yeast iso-1-cytochrome c adsorbed at its surface (Chah et al. 2005). A GNP based protease assay with nanomolar sensitivity using C- and N-terminal cysteiny derivative of peptide substrate specific to target protease has been reported (Guarise et al. 2006). Lactose conjugated GNP havs been shown to undergo visible colour changes upon binding with lectins. The colour change was reversible and dependent upon lectin concentration (Otsuka et al. 2001). Using same principle competitive colorimetric assay for detection of protein protein interaction has been poposed. Visible colorimetric changes have been observed for interaction of Thyroglobulin with Concanavalin-A and other lectins using mannose modified GNP. Even binding constants could be calculated based on the wave length shifts (Tsai et al. 2005). Pioneering work from Mirkin’s group has resulted into a number of applications of SPR properties of GNP conjugated with oligo-nucleotides for detection of DNA, RNA and proteins (Nam et al. 2003, Han et al. 2006, Sperling et al. 2008).
Owing to its high absorbance spectra gold nanoparticles can quench many fluorophores and act like an acceptor in a Förster Resonance Energy Transfer (FRET) pair. GNPs have been proven to be better than organic quenchers with higher quenching efficiency and over greater distances. Using a Oligo conjugated GNP and a Cy5 labeled oligo probes Mirkin’s group has reported a “Nano-flare” which could be used detect desired RNA species inside cell (Seferos et al. 2007). Molecular beacons having GNP as quencher has been shown to be 8 fold more efficient in detecting a single base DNA mismatch than other fluorophore labelled molecular beacons (Dubertret et al. 2001). Because of very high curvature, Raman Scattering of molecules closed to GNP surface is greatly enhanced. This is called Surface Enhanced Raman Scattering (SERS). This property can be used to detect the biomolecules potentially at single molecule level. Recently SERS effect produced by GNP through Raman active reporters has been demonstrated for both in vivo and ex-vivo detection of different analytes and proteins, even beyond pico-molar sensitivity (Kneipp et al. 2008).

Gold nanoparticles are naturally taken up by many cell types and do not cause any detectable cytotoxicity. This fact has been extensively exploited for delivery of DNA inside cells. Antisense oligonucleotide conjugated GNP has been shown to be spontaneously taken up by cells and bring down the expression level of target genes (Rosi et al. 2006). Cationic polymer agents show improved transfection efficiency when conjugated to GNP (Thomas & Klibanov 2003). Because of the versatility in surface modification of GNP with specific ligands GNP based transfection agents also have potential of targeted gene delivery. After excitation with a suitable light source GNP get heated up and dissipate heat to its immediate environment. This property is called hyperthermia. With careful design and excitation with a suitable light source heat produced by hyperthermia in GNP is sufficient enough to produce localized heating and killing a cell or manipulating tissue environment. Together with a variety of available method for selective targeting, hyperthermia has been used for selective killing of cancerous cells both in vitro and in vivo, either directly by heating or by localized release of anti cancerous molecules trapped in hydrogels (Everts 2007).

**ii) Iron oxide nanoparticles**

Nanoparticles from iron oxide have gained attention of researchers because of their extra ordinary magnetic and optical properties. Initially research efforts for applications of iron nanoparticles (INP) were limited to development of high density
magnetic storage media primarily because of lack of sufficient surface chemistry. However, recent development at synthetic front has enabled numerous application of INP in biosensing, protein separation and purification, imaging, drug delivery, diagnostics etc (Gu et al. 2006).

There are three most common approaches for synthesis of magnetic iron oxide (Fe$_3$O$_4$ and γFe$_2$O$_3$) nanoparticles: (1). The classical co-precipitation of aqueous Fe$^{2+}$/Fe$^{3+}$ ions in presence of an alkali hydroxide or ammonia solution; (2) thermal decomposition of an iron complex under high pressure and (3) Sonolysis of organo metallic complexes (Cornell & Schwertmann 1996). The particles synthesized with these methods tend to aggregate through non-covalent interactions. This can be avoided by using stabilizers. Many surfactants and other organic compounds with specific functional groups have been developed for this purpose (Lu et al. 2007). Among these, water soluble stabilizers like PEG, polyvinyl alcohol, polyamines etc. are especially useful for synthesis of INP for biomedical applications. The stabilizers can be incorporated at the time of synthesis itself or during a coating step after the synthesis of particles. The stabilizers present in medium at the time of synthesis of INP prevent particle coalescence during formation. The particles size can also be controlled by varying stabilizer concentration (Si et al. 2004). Specific biomolecular ligands can be conjugated to INP surface using specific functionality of stabilizers, which in turn can be used for selective targeting to specific cells, tissues or organs.

INP serve excellent contrast agent for magnetic resonance imaging (MRI). The ability of INP as MRI contrast agent, together with potential for selective targeting has resulted into wide range of studies for potential applications in MRI based imaging and diagnostics. Several antibodies and other ligands has been conjugated to INP and tested for MRI imaging of tumours (Laurent et al. 2008). Recently, simultaneous MRI imaging and destruction of breast cancer cells was demonstrated using targeted delivery of INP, entrapped into PLGA (Poly-D, L-lactide-co-glycolide) nanoparticles containing anticancer drug Doxurubicin and antibody Herceptin1. Fluorophore conjugated INP called magnetofluorescent nanoparticles have also been developed for multimodal optical and MRI imaging (Mrinmoy De et al. 2008). Using INP conjugated with cell surface receptor specific ligands, a modified cellular Enzyme Linked Immunosorbent Assay (ELISA) called Cellular Magnetic Linked Immunosorbent Assay (C-MALISA) has also been developed (Burtea et al. 2005).
Small INP are magnetized in presence of an external magnetic field. This property has been used for magnetic separation of proteins. Xu et. al. have successfully demonstrated affinity purification of recombinant His-Tag proteins using Ni-NTA terminated INP (Xu et al. 2004). They have incubated Ni-NTA terminated INP with lysate of recombinant His-tag protein (Figure 1.2). The protein binds with INP because high affinity of His-Tag with Ni-NTA present at its surface and can be pulled out easily using a magnet. Use of magnet also makes washing and elution of recombinant proteins easier than traditional Ni-NTA resin columns. Moreover, because of its high surface to volume ratio, fast movement and good dispersability this system should perform better than traditional micron sized particles and should show better binding rate and binding capacity. High binding rate should also reduce non-specificity, because due to quick binding of target molecule, availability of unoccupied area for nonspecific adsorption will be reduced (Gu et al. 2006).

![Figure 1.2: Purification of His-tag proteins using Ni-NTA terminated INP](image)

(a) Optical images of a magnet attracting 6c and 6c-6xHis-GFP; (b) surface-modified magnetic nanoparticles selectively binding to histidine-tagged proteins in a cell lysate; (c) SDS/PAGE analysis of the cell lysate (lane 1), the fraction (lane 2) washed off a commercial Ni\(^{2+}\)–NTA column; the fraction washed with the freshly made 6c using imidazole solution (10 mM, lane 3; 80 mM, lane 4; 500 mM, lane 5); fractions washed off the reused 6c using imidazole solution (10 mM, lane 6; 20 mM, lane 7; 500 mM, lane 8); and (d) the cell lysate (lane 1), the molecular weight marker (lane 8), the fractions washed from the freshly made 9b (lanes 2 and 3), boiled 9b (lanes 4 and 5), and the commercial HiTrap affinity column (lanes 6 and 9), and the concentrations of imidazole are 10 mM (lanes 2, 4, and 6) and 500 mM (lanes 3, 5, 9). 6c: A NTA terminated FePt magnetic nanoparticle; 9b: NTA terminated Core Shell magnetic nanoparticle. 

*(Reproduced with permission from Gu et. al. 2006; © Royal Society of Chemistry, UK)*
The magnetic property of INP also holds potential for applications in drug and gene delivery. A number of studies have focused on development of biocompatible INP with positively charged surfaces for binding to and delivery of DNA inside cell. Often suitably modified INP itself is able to transfect DNA in many cell types. Application of strong magnetic pulses has been shown to promote transfection levels (Dobson 2006). Magnetic INP conjugated with anticancer drug Methotrexate, which can kill many cancerous cells overexpressing folate receptor has shown higher accumulation of INP and release of methotrexate and killing of cultured breast and cervical cancer cells (Kohler et al. 2005). Alexiou et al. has shown that accumulation of INP upon application of magnetic field at tumour location (Alexiou et al. 2006). Like GNP and other metal nanoparticles INP also show hyperthermia. Iron nanoparticles targeted to tumours can be used for simultaneous MRI imaging and destruction by heating. Ability of INPs to be targeted to tumours, MRI contrast agent, delivery of anticancer drugs into tumour cells and hyperthermia makes them ideal candidate for cancer management. An INP conjugated to a tumour specific ligand and a anticancer drug will be a wonderful chemotherapy strategy having ability for simultaneous MRI detection and multimodal destruction of tumour by chemotherapeutic agent as well as a heating by a LASER.

iii) Semiconductor

Quantum Dots

Semiconductor quantum dots are probably first nanomaterials to be adopted for biological applications. Quantum Dots (QD) are small nanocrystals of semiconductor compounds (eg. CdSe, CdTe, CdS, ZnSe, InP and InS) which have got importance because of their excellent fluorescence properties. Owing to their small size, semiconductor QD can exhibit a rare quantum mechanical phenomenon called quantum confinement. This gives rise to many important features of the excitation and emission spectra of QD, distinct from conventional organic fluorophores (Chan & Nie 1998). Notably, QD exhibit high quantum yield, are resistant to photobleaching, broad excitation spectrum and narrow
emission spectrum. The QD can be excited for hours without any significant loss in photostability making them fluorophore of choice for experiments involving recording of fluorescence for longer periods. The narrow emission spectrum of QD depends upon the size and constituent elements. Depending upon requirement, QD with different sizes and composition having emission spectra ranging from UV to far red region are available. Moreover, the broad excitation range allows excitation of multiple QDs using same wavelength which will emit light at different wavelength depending upon their size and composition. These unique optical properties of QD make them excellent candidates for multiplex assays, enabling simultaneous probing of different molecules or cells labelled QDs of with different colour, using single excitation source. Because of their unique features QD are considered next generation fluorescence marker with potential of revolutionalizing the fields of diagnostics and imaging (Bruchez, Jr. et al. 1998, Cai et al. 2007).

A number of synthetic approaches for QD have been explored. Most common and successful methods involve synthesis in organic solvents. QD of CdTe, CdSe, and CdS can be obtained by injecting liquid precursors (eg Dimethyl cadmium or Cadmium oxide and Selinium for CdTe) into hot (300°C) co-ordinating organic solvents. The size of crystals depends of temperature, time of reaction and amount of limiting reagent. Usually the resultant crystal are coated with a shell of higher band gap material like ZnS and CdS (Michalet et al. 2005). This gives the so called name “Core Shell” nanoparticles to QDs (Figure 1.3). The shell coating improves the fluorescence yield as well as provides protection against oxidation to core. The QD synthesized through these methods are usually not soluble into water, a prerequisite for most biological applications. This limitation has been overcome by functionalization of QD surface by a hydrophilic ligand or encapsulating into a thick organic or inorganic coating. The coat improves colloidal stability, insulated deterioration of QD surface in aqueous media. It also as provides points for attachment of specific ligands for different biological applications.(Medintz et al. 2005) The biomolecules can be attached to QD surface by a partial ligand exchange. Alternatively they can be covalently linked by taking advantage of functional groups provided in surface coating.

The development of surface chemistry for conjugation of biomolecules has dramatically increased the utility of unique optical properties of QD for applications
in biology. Streptavidin coated QD have been used to label biotinylated protein present at cell surface (Howarth et al. 2005). The extraordinary photostability, unique optical properties together with ability to conjugate antibodies have led extensive application of QD in fluorescence immuno labelling and imaging applications (Sukhanova et al. 2004, Gao et al. 2004, Howarth et al. 2005, Pinaud et al. 2006). Jaiswal et al. have demonstrated long term multi colour imaging of live cells upto 12 days, using multi colour quantum dots. The cells were labelled by endocytic uptake of QD or using QD conjugated to antibodies against cell surface markers (Jaiswal et al. 2003). Because of high quantum yield, QDs along with organic donors has been used as FRET pair in a number of applications. An inhibition assay for biomolecules using GNP and QD as FRET pair has been demonstrated for determining avidin concentration in sample solution (Oh et al. 2005). Because of the size dependent emission spectra QD has been used to determine the optimal size of nanoparticles for renal clearance (Choi et al. 2007). So et al. have used energy of luciferase catalysis to create a QD based self illuminating in vivo imaging system. As this system does not require any external energy source for excitation, the problems of opacity and autofluorescence are greatly reduced (So et al. 2006). Using streptavidin coated QD and biotin tagged phage detection of up to 10 bacterial cells in artificial samples has been reported (Edgar et al. 2006). Surface modified QD has been used as a multipurpose vector delivery of siRNA, real time tracking and ultrastructural localization (Yezhelyev et al. 2008).

Clearly, QD have appeared to be a boon for many fluorescence based applications in biology. However, semiconductors are made from toxic heavy metals like cadmium which can be toxic potential. Recent research has shown that QD are toxic for cells because of release of Cd\textsuperscript{2+} ions inside cells. This poses a big limitation for their in vivo imaging applications. Photoblinking is another limitation which reduces their applications requiring single molecule detection (Alivisatos et al. 2005).

iv) Silica Nanoparticles

Among an array of inorganic nanomaterials, silica nanoparticles (SiNP) have made their own niche because of easy synthesis and availability of rich surface chemistry. SiNP are generally synthesized by hydrolysis followed by condensation polymerisation reaction of Tetraethyl orthosilicate (TEOS) catalyzed by liquid
ammonia in following two ways. The first method called sol-gel method or Stöber synthesis involves ammonia induced hydrolysis of TEOS in presence of water into ethanol solution under constant stirring or sonication. The particle size can be controlled by changing ammonia and water content (Rossi et al. 2005). In second method the polymerisation reaction takes place in confined environment, into “reverse micelles” formed by emulsification of a surfactant into a hydrophobic solvent (Figure 1.4). The hydrophilic core of reverse micelle acts as nanoreactor for the polymerisation reaction. The size of nanoreactor and thereby the size of the particles can be changed by changing the water to surfactant ratio (Bagwe et al. 2004). Usually the particles prepared using these methods are functionalized by coating with a shell of silica using functionalized alkoxysilanes. Depending upon the silane used, the compact shell provides desired functional groups which can be used for conjugation of biomolecules.

![Diagram](image)

**Figure: 1.4: Synthesis of dye doped SiNP by Reverse micelle method.** Upper Panel: Polymerization reaction of TEOS in presence of NH4OH. Lower Panel: Formation of DDS in reverse micelle. Dye and TEOS get confined inside the hydrophilic core of reverse micelle which acts as a “nanoreactor” for polymerisation of TEOS to form spherical silica matrix in which dye molecules get trapped. (Adopted with permission from Tan et. al 2004 © Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc.)

Silica is a chemically inert and optically transparent material which makes it an excellent matrix for encapsulation of molecules sensitive to change in environment. The transparent nature of SiNP provides one of the most widely used application as a matrix for entrapment of fluorescent organic dyes. The fluorescent dyes can be encapsulated into silica matrix by doping the TEOS with dye during polymerisation reaction. Different dye doped SiNP (DDS) has been synthesized from
both Stöber and Reverse Micelle methods described above (Santra et al. 2001, Rossi et al. 2005). However because of lack of covalent bonding, dye molecules tend to leach out over a period of time. This problem has been overcome in a recently reported core shell SiNP in which a core is synthesized by an alkoxy silane covalently attached to dye molecule and then coated with a tight silica shell for protection from solvent interaction and to provide functional group for conjugation of ligands and biomolecules (Ow et al. 2005).

Encapsulation of fluorescent dye into silica matrix provides a pseudo solid environment, sequestered from solvent which prevents its self quenching, solvochrome shifts and photobleaching. As many dye molecules are entrapped in one SiNP, quantum yield is also greatly enhanced. A wide number of dyes can be incorporated to cover whole range of spectrum which is highly advantageous for multiplex assays. Even a dye which is not optimal for a particular solvent can also be used because the optical properties of dye do not depend upon the solvent. Moreover, unlike quantum dots DDS do not suffer the problem of stochastic blinking. Finally availability of various oraganosilicates ensures possibility of creating various functional groups at the surface with ease, which provides flexibility in choosing protocols for conjugation of different biomolecules (Burns et al. 2006a). These features have resulted into wide number of applications of DDS in biology. The streptavidin conjugated DDS has been used for various fluorescence based techniques like fluorescence-linked immunosorbent assay, immunocytochemistry, immunohistochemistry, DNA microarray, and protein microarray. DDS were shown to perform better than organic dye Texas red and even QD (Lian et al. 2004). Organically modified SiNP with a positively charged surface and doped with fluorescent dyes has been used for delivery and real time tracking of nucleic acids and drug molecules inside cells (Roy et al. 2005, Fuller et al. 2008). In an interesting application of fluorescent Core shell SiNP, Weisner’s group developed a ratiometric pH sensor. They synthesized a core shell SiNP with core containing a pH insensitive dye TRITC which served as standard. A pH sensitive dye FITC was conjugated at the surface of shell. The changes in pH could be quantitatively measured by comparing the change in fluorescence of pH sensitive FITC to that of TRITC which is present at core and remains unaffected to pH variations. They have demonstrated the utility of this system in vivo by measuring the pH in various cellular compartments of RBL mast cells (Burns et al. 2006b). Because of being optically transparent, availability
of rich surface chemistry and inert nature, silica has been used for coating other nanoparticles like QD and INP for easy surface modification (Yi et al. 2005). Silica coated INP and DDS conjugated with aptamer has been used for selective collection and detection of cancerous cells (Herr et al. 2006). Application of DDS has also been demonstrated for detection of bacteria up to single cell level (Zhao et al. 2004).

v)  **Carbon Nanotubes**

Carbon nanotubes (CNT) are a form of fullerenes, one of the three allotropic forms of carbon along with graphite and diamond. First described by Iijima in 1991 CNT consist of graphitic sheets arranged into a tubular structure. The sheets can be arranged into a single layer called single walled CNT (SWNT) or multiple concentric layers called multi-walled CNT (MWNT). The diameter of the CNT is typically below 100 nm. However, the length can vary from few nanometres up to several micrometres. The CNT are materials with high surface to volume ratio, ultra light weight, good electrical and thermal conductivity, metallic or semi-metallic behaviour and mechanically tough ordered structure (Sun et al. 2002). Several methods e.g. arc discharge, laser ablation, chemical vapour deposition, etc. have been developed for SWNT and CWNT (Dai 2002).

The CNT had limited biological applications because pristine CNT synthesized by above method are insoluble. However, in last few years several strategies have been developed for making CNT compatible with physiological conditions. For example, surface oxidization of CNT using strong acids can be used to generate carboxylic groups, which increase their dispersibility in aqueous solutions. Alternatively Addition reactions to the CNT external walls can also employed to make them soluble in water. Functionalised CNT can be conjugated to a wide variety of molecules including peptides, proteins, nucleic acids and other therapeutic agents according to the requirement of application (Tasis et al. 2006). The CNT have intrinsic property of absorbing low energy Near Infra-Red (NIR) and radiofrequency radiation. Like metal nanoparticles this induces an increase in local temperature of CNT. This has been used to selectively kill malignant cells without damaging normal cells both in vitro and in vivo (Bianco et al. 2008) CNT conjugated with a folate moiety has been used to selectively kill cancerous cell over expressing folate receptor (Kam et al. 2005). Using Radiofrequency, CNT containing cells residing deeper in
side body into liver has been shown to be killed in rabbit models (Gannon et al. 2007). Polyethylene oxide functionalized CNT conjugated to specific receptors has been used for sensitive and specific electronic detection of biomolecule (Chen et al. 2003). Suitably functionalized CNT can cross cell membrane to enter into cytoplasm and even nucleus. This has been exploited for CNT mediated delivery of physically adsorbed or covalently attached peptides, nucleic acids and drugs in a number of reports (Bianco et al. 2005).

vi) Other Inorganic Nanoparticles

Apart from the nanoparticles discussed above, Inorganic nanoparticles from many other elements have been synthesized either in native form or as composites. According to a recent estimate different forms of nanoparticles of at least 44 elements from periodic table, each with its own novel nanoscale features, are already available commercially and the list is growing (www.etcgroup.org 2008). However, applications of many of these nanomaterials have been limited to materials science. Besides the GNP, INP, QD, SiNP and CNT other nanomaterials worth mentioning for biological applications include nanoparticles of Ag, Ti, Zn, Pt, Pd, Gd, Cu etc.

The metal nanoparticles are commonly synthesized by controlled reduction of their respective salts in aqueous medium. For example, a common method for the synthesis of silver nanoparticles is the reduction of AgNO3 with NaBH4. It is called, Creighton method and produces approx. 10 nm particles of narrow size distribution. Variations of this method can also be adapted for synthesis of nanoparticles from other metals such as Pt, Pd, Cu, Ni, etc. The specific protocols depend on the reduction potential of the source ion (Evanoff & Chumanov 2005). The nanoparticles synthesized using these methods generally tend to aggregate and require a capping agent for stabilization. The capping agent can be directly added into the solution at the time of reduction. The shape and size of nanoparticles depend upon the reaction condition and capping agent used. Alternatively, nanoparticles can be capped with desired molecules after the synthesis to tailor their surface chemistry.
vii) Dendritic Polymers

Dendritic polymers represent two architecturally similar classes of polymers called dendrimeric polymers or simply dendrimers and hyperbranched polymers (Paleos et al. 2007). They are distinguished by highly branched tree-like scaffold from which they derive their name (Greek dendron, meaning “tree”). Dendrimers are highly branched, structurally well defined and monodisperse, globular macromolecules with symmetrical, nanometer-sized architecture which consists of a central core, branching units, and terminal functional groups. Hyperbranched polymers are also similar to dendrimers, having branched scaffold, but in contrast to dendrimers, they are asymmetrical and polydisperse (Figure 1.5). Due to their hyperbranched scaffold, dendritic polymers offer several advantages over linear polymers in terms of solubility, lower cytotoxicity, loading capacity, availability of large number of terminal functional groups for desired medication for biomedical applications. They are being extensively investigated to be used as drug delivery systems, as gene delivery vectors, or as drugs on their own (Stiriba et al. 2002). Biologically active molecules can be encapsulated within dendrimers or physically adsorbed or chemically attached at their surface for delivery inside cells (Jain & Asthana 2007). Dendritic polymers have also shown promise in diagnostics and imaging, tissue repair and sensor applications (Lee et al. 2005).

Figure: 1.5: Schematic representation of dendritic polymers. (Left): A multifunctional dendrimers (Right): A hyperbranched polymer

Dendrimer molecules are composed of multiple perfectly branched monomers which originate radially from a central core. Generally, they can be synthesized by
either of following two different multistep polymerization approaches called divergent and convergent approach. In the divergent approach the dendrimer is synthesised from the core as the starting point and built up step by step according the required degree of branching. In the second, convergent approach synthesis starts from the surface and ends up at the core, where the dendrimer segments (dendrons) are coupled together (Boas & Heegaard 2004). Hyperbranched polymers can be prepared in a one step self polymerisation reaction. The polymerization reactions to prepare hyperbranched polymers can be classified into three categories: A: Polycodensation B: Self condensing viny polymerization and C; Ring opening opening polymerisation (Jikei & Kakimoto 2001). All three approaches can give rise to hyperbranched polymers which are rather imperfectly branched and having higher polydispersity but posses many dendrimers like properties. Moreover, since they can be synthesized in single step, their cost of production is considerably lower. Therefore, hyperbranched polymers are being considered as an alternative to dendrimers in many applications which can tolerate polydispersity to some extent and do not require a very well defined branched architecture (Lee et al. 2005).

viii) Liposomes

Liposomes are spherical soft-matter particles composed of one or more phospholipid bilayers membrane(s), encapsulating a volume of aqueous medium. Liposomes resemble cell membranes in their structure and composition and can be used for encapsulating bioactive molecules for a variety of biological applications (Lasic 1998). Depending upon the desired application a number of procedures are available for preparation of liposomes. In its simplest form liposomes can be prepared by mixing phospholipid in aqueous environment under non shearing condition. Due to their amphiphilic nature, in aqueous environment phospholipids align themselves into bilayers with their hydrophobic tails facing each other and their hydrophilic headgroups facing toward the aqueous medium to minimize energy (Figure 1.6) (Jesorka & Orwar 2008). To further minimize the energy, edges of the lipid bilayer join to form a spherical vesicle called liposome. The charge stability and other surface properties of the liposomes depend upon the phospholipid used for their preparation. The most commonly utilized lipids for preparation of liposomes are natural phospholipids like phosphatidylcholine phosphatidic acid, phosphatidylglycerol,
phosphatidylserine, and phosphatidylethanolamine, Stearylamine etc. (Fahy et al. 2005). However, synthetic lipid with desired functional groups for different purposes like coupling reactions, chelating metal ions, or even multifunctional lipids may be used (Roy et al. 2000, Chikh et al. 2002, Hassane et al. 2006). Liposomes may be prepared in a range of sizes starting from 20 nm to few µm. Moreover depending upon method of preparation and the desired application they may be unilamellar having only one lipid bilayer, multilamellar with many lipid bilayers or even multivesicular having more than one small liposomes encapsulated in a large liposome (Jesorka & Orwar 2008).

![Figure 1.6: Schematic representation of self-assembly process of a liposome from phospholipid molecules.](image)

(a) Amphiphilic lipid molecules with hydrophilic head and hydrophobic tails (b), Phospholipid molecules arrange into lipid bilayer with their hydrophobic tails facing each other and their hydrophilic headgroups facing toward the aqueous medium (c). The edges of the lipid bilayer join to form a spherical liposome. (d).

Apart from the structural characteristics of the lipid molecules themselves, the properties and functionality of liposomes are largely defined by their size and the composition of the four distinct regions highlighted in panel c.

(Adopted with permission from Jesorka & Orwar 2008 © Annual Reviews, USA)

The liposomal shell can be used to enclose or bind many different classes of substances for both in vitro as well as in vivo delivery. Liposomes has been successfully utilized for the delivery of therapeutic agents like antibacterial, antiviral, and anticancer drugs, as well as hormones, enzymes, and nucleic acids (Lasie et al. 1999, Weissig et al. 2006, Eckstein 2007). In fact, many liposome based commercial formulations are already available for delivery of nucleic acids and drugs (Barenholz 2001). The encapsulation of drug molecules inside liposomes offers several advantages like: increases in the circulation time, protection from degradation by
enzymes, slow and sustained release etc., which are essential prerequisite for good delivery agents. Moreover, with the encapsulation inside liposomes it is possible to deliver even hydrophobic toxic drugs which can not be directly injected (Torchilin 2005). These properties together with ability to tailor the lipids used for liposome has been used to deliver nucleic acids and drugs for targeted delivery to desired cellular compartments, cells, tissues or organs (Torchilin 2006). Liposomes labelled with radio isotopes or MRI contrast agents has also been used for in vivo imaging (Torchilin 1996, Martina et al. 2005, Goins 2008).

**Biocompatibility of Nanoparticles**

Owing to their unique properties nanoparticles are being considered to address many of the issues facing society in the areas of health, agriculture, environment, energy, information technology, education, manufacturing, sustainability, transportation homeland security etc. (Helmus 2006). Keeping enormous potential of nanomaterials in view, their commercial application is still in its infancy (Mazzola 2003). Nevertheless, nanoparticle based products are becoming increasingly common (www.nanotechproject.org/ 2008). While unique properties of nanoparticles offer enormous avenues for potential applications in different fields, their xenobiotic nature raise worries regarding their safety. Concerns have been raised that the very properties of nanomaterials which make them attractive, could cause unforeseen health or environmental hazards (Maynard et al. 2006). The commercial applications of nanoparticles require large scale production, use, and disposal of products containing nanomaterials. This may lead to their appearance in air, water, soil, organisms or the human body posing risk to human health and environment. Moreover, problem to human health could be more serious in biomedical applications of nanoparticles as drug-delivery agents, biosensors, or imaging contrast agents which require deliberate, direct administration of nanoparticles into the body.

There are several reports of nanoparticles being toxic. Large variations in toxic potential and underlying mechanism behind toxicity of different nanomaterials are observed. Because of very small size nanoparticles delivered into body fluids can bypass from circulation accumulate into organs and tissues and even enter inside cell. Whole body studies show that inhalation of nanoparticles and entry via the lungs is followed by rapid translocation to vital organs like the kidney and liver. Once inside the
Inhalation of titanium oxide nanoparticles have been shown to induce lung injury by affecting expression of genes involved in pathways involved in cell cycle, apoptosis, chemokines, and complement cascades (Chen et al. 2006). Redox-active, lipophilic fullerenes cause significant lipid peroxidation in brain and glutathione depletion in gills in an aquatic species largemouth bass. Nanoparticles derived from toxic metals like CdSe and CdSe/ZnS may eventually release toxic ions into the living system (Kirchner et al. 2005). Though nanoparticles of CuO have been shown to be highly toxic, the toxicity could not be explained by release of Cu ions alone (Karlsson et al. 2008). Apart from chemical composition, surface properties of the nanoparticles differ considerably with their shape and size. This may influence the uptake of different nanoparticles. While functionalized nanoparticles may interact specifically with certain biomolecules like proteins or DNA, non-specific interaction with sub-cellular structures may depend upon more general features like charge and shape. For instance, because of higher penetrance facilitated by their shape, carbon nanotubes seem to be more prominent in causing epithelioid granulomas and interstitial inflammation than 14 nm carbon black particles in mice (Lam et al. 2004). Recently, upon comparison of cytotoxicity data on carbon, metal, and semiconductor based nanoparticles Lewinski et al. found that while the causes for the increase in cell death observed at higher concentrations and longer exposure times may be material specific, the generation of reactive oxygen species and the influence of cell internalization of nanoparticles are two common causes which mainly govern nanoparticle toxicity (Lewinski et al. 2008).

Due to extraordinarily large surface-to-size ratios and unique surface properties nanoparticles can specifically adsorb to proteins and biomolecules. In fact, size and surface properties of nanoparticles has been found to play a very significant role in determining the adsorption of proteins at nanoparticle surface (Lundqvist et al. 2008). Due to their ability to adsorb biomolecules, nanoparticles can catalyze biomolecular reactions. While this might be useful for some therapeutic applications, products of these catalyzed reactions may also be toxic themselves or produce damaging free radicals. Moreover, adsorption of endogenous proteins to nanoparticles surface could lead change in the protein confirmation and elicit immune response (Daughton 2004). In this way nanoparticles made up of even chemically inert materials might prove toxic...
because of their surface properties. Moreover, in such cases toxic potential will vary with the variation of surface properties of particles with size.

Therefore, extensive characterization of interactions nanoparticles between biological surfaces, cells and nanoparticles is an essential pre-requisite for nanoparticle based clinical applications. Secondly, environmental effects due to aerial emissions, prolonged presence and accumulation in soil, water and food sources need to be evaluated from an environmental standpoint. Lastly, exposure levels in manufacturing sites need to be standardized for occupational safety. Widespread applications of nanoparticles requires that their potential toxic effects need to be evaluated in much the same way as clinical testing of other chemical or pharmaceutical is undertaken. Moreover, keeping in view, the variation in the properties of nanoparticles with their shape and size, each engineered nanomaterials, even chemically identical but of different in shape and sizes should be evaluated separately and the information should be catalogued like e.g. Chemical Abstract Service (CAS) Registry used for other manufactured chemicals.
Chapter-1

Review of Literature

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Chapter-1

Review of Literature


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