

CHAPTER 1

INTRODUCTION

In this chapter we first provide a brief introduction to nanoparticles and their use for biomedical applications. Organically modified silica nanoparticles and gold nanorods that have been used for the work described in the thesis are discussed in more detail. The basic excited state processes of fluorophores and how these processes (absorption and fluorescence) can get affected in the presence of nanoparticles are also discussed. This is followed by the experimental methodology and a brief outline of the thesis.

1.1 Introduction and historical background

‘Nano’, derived from Greek ‘nanos’, meaning dwarf, is a much talked about word of present times. However, it is pertinent to note that the use of nanotechnology has been pursued since centuries back. For instance: various Ayurvedic medications had been using nanoparticles (NPs) in different formulations such as in ‘bhasmas’, as smokes of some medicinal herbs, and are known in Indian sub continent since seventh century AD to treat various ailments [1, 2]. Also, over a thousand of years ago artists created beautiful glass works using colloidal gold and silver particles, for example creating red and yellow colours in the stained glass windows of medieval churches [3] etc. However, the science behind these technologies was not known. According to the literature, the vision towards nanoscience and nanotechnology was first given in a talk entitled “There’s Plenty of Room at the Bottom” by physicist Sir Richard Feynman at an American physical society

meeting at the California institute of technology, on December 29, 1959 which stimulated people for the first time to pay attention to the question of fabricating materials and devices at atomic/molecular scale [4].

The term ‘nanotechnology’ was first defined by Professor Norio Taniguchi in 1974 in a paper as ‘Nano-technology, mainly consists of the processing of, separation, consolidation and deformation of materials by one atom or by one molecule’ [5]. This needed to investigate the nanomaterials with respect to size, size distribution, shape and structure evolution and manipulate them. Although, the first transmission electron microscope (TEM) had been built in 1931, with the development of the scanning tunnelling microscope (STM), the atomic force microscope (AFM) etc. in 1980s the age of modern nanotechnology began. In 1981 K. Eric Drexler developed and popularized the concept of nanotechnology and founded the field of molecular nanotechnology [6]. His book ‘Engines of Creation: The Coming Era of Nanotechnology’ is considered to be the first book on the topic of nanotechnology. The observations made by him set a benchmark in the relationship between biology and nanotechnology which visualizes several cellular organelles working at nanoscales as devices and machines. He regarded cell biology as source of inspiration to synthesize model nanodevices with artificial materials for nanomedicine [7]. In the early 1980s the birth of cluster science led to the discovery of fullerenes (in 1986) and carbon nanotubes a few years later. Contemporary to this, the synthesis and properties of semiconductor nanocrystals and metal oxide nanoparticles were studied. During the past three decades an exponential growth of activities have been witnessed worldwide in developing nanoparticles with various materials, following top-down or bottom-up approaches, in order to understand the new science as well as hope for their potential applications in multidisciplinary areas due to their especial properties.

1.2 Properties of nanoparticles

NPs, due to their small size, get especial properties which are very different from bulk. Reduction in size causes modification of characteristics of the materials along with the emergence of phenomenon such as quantum size confinement in semiconductors, localized surface plasmon resonance in metals, superparamagnetism in magnetic materials, etc. Large surface to volume ratio, due to nm size, leads to prominence of interfacial effects: increased reactivity, catalytic properties, lubricant properties, surface hardness of the coated surfaces, sensitivity to environment, etc. Surface charge properties and small size also lead to stable particle suspensions. Therefore, by controlling the size, shape, composition and surface properties, NPs with enhanced and novel, physical, chemical and biological properties can be obtained. Presently, NPs are being synthesized using various types of organic, inorganic, polymeric materials as well as their combinations, which are finding applications in various areas of science and technology including biomedical field.

1.3 Biomedical Applications

Nanotechnology has shown a considerable promise to generate, manipulate and deploy nanoparticles so as to provide new possibilities for advanced bio-analytical tools and both ex-vivo and in-vivo applications [8, 9, 10]. Gold nanoparticle based immunoassay for pregnancy test is the well known ex-vivo diagnostic application which is being routinely used and kits are commercially available. Because of their small size, NPs can reach even the micro capillaries easily through blood and can therefore be useful for in-vivo diagnostic as well as drug delivery applications [11]. Also, these can be designed so as to solubilise certain dyes/drugs, which are of limited use due to their poor solubility in aqueous medium, and to minimize their high toxicity to the vital organs (such as liver, heart, kidney), in vivo degradation and short circulation times by coating and functionalizing them [12].

For in-vivo application it is important to understand the response of a body to NPs, which depends on various factors including their size. For example, small NPs get easily cleared through renal filtration, whereas NPs of size larger than 20 nm can avoid it which prolongs their circulation time [13]. The circulation time can be further increased by making their surface hydrophilic or coating them with molecules such as polyethylene glycol (PEG) in order to avoid detection by immune system(i.e., reticuloendothelial system, or RES). The process of enhanced permeability and retention (EPR) effect, due to the leaky tumour blood vasculature and poor lymphatic drainage, leads to their retention, for example, in the tumour tissue, to a higher extent as compared to the normal tissue (passive targeting) [14]. In addition, these can be functionalized easily with ligands against tumour markers or antibody against antigen for site specific targeting, where these markers are over expressed (active targeting). This can avoid the side effects of the drugs, which are carried by these NPs, making their use more effective, thereby minimizing the quantity used and hence the cost. A variety of nanoparticles such as : gold, silica, quantum dots, magnetic NPs, liposome, dendrimers, polymeric NPs and many more, are being synthesized and investigated for their potential applications in biomedical imaging, diagnosis as well as therapy which includes drug delivery [13, 15].

Conventionally, biomedical research and clinical diagnostic applications based on optical imaging mostly use organic dyes as the fluorescence markers. However, these have certain drawbacks, such as, these require lasers at different excitation wavelengths, their fluorescence gets bleached under long exposure time and also there is lack of discrimination when multiple dyes are used. NP based diagnostics and imaging is being currently explored to overcome these drawbacks. For example, there is a major advancement to use fluorescent NPs such as quantum dots (QDs) for imaging. Stable, sharp and size tunable fluorescence and wide absorption band of QDs enable them as ideal fluorophores for cell imaging and diagnostics [16]. However, toxicity due to QDs is an issue, arising due to leakage of heavy atoms, which limits their use for in vivo

applications [17]. Other NPs systems, such as dye doped silica and gold NPs, are also being actively explored for imaging and diagnostics [18, 19, 20].

For drug delivery applications, a range of organic systems (e.g., micelles, liposome, polymeric NPs, dendrimers, etc.) have been investigated [21]. For example, liposome and albumin-based delivery of drugs such as doxorubicin and paclitaxel have already been approved and functionalized liposome and polymer based drug delivery system is in phase I trial [22, 23, 24]. Although, these organic systems are biocompatible and biodegradable, their limitations include poor thermal and biochemical stability, rapid elimination due to their small size (in case of micelles and dendrimers) and opsonisation (adsorption by blood protein markers on the surface of nanocarriers and their elimination by immune system). Oxide based NPs such as silica, alumina, titania etc., have been shown to be biocompatible, stable and “stealthy” (i.e., avoid detection by immune system due to their hydrophilic surface) and therefore, show a great promise in many medical and pharmaceutical applications [25, 26]. Different porous silica based nanostructures including mesoporous and organically modified silica (ORMOSIL) nanoparticles are being actively explored to encapsulate, conjugate or entrap dyes/drugs in their porous matrix and are being investigated for imaging as well as drug delivery applications [27, 28].

While silica NPs are transparent, metal NPs such as silver, gold, copper show localized surface plasmon resonance (LSPR) in the visible to NIR region [18, 29], which lead to large enhancement (5-6 orders of magnitude as compared to organic dyes) of extinction coefficients (scattering + absorption coefficients) and large surface electric field at the metal NP sharp edges [30]. LSPR strongly depends on the metal type (: silver, gold, copper etc.), size, shape and composition of the NP, dielectric constant of the environment and the nanoparticles-surface effects. Any changes at the surface, e.g. covering the surface with some material, strongly influence the resonance properties. These properties make these particles exclusive agents for applications such as sensing

(due to plasmon peak shift caused by the change in surface refractive index), surface enhanced Raman scattering (SERS) of the adsorbed molecule (due to enhanced surface electric field at NP sharp edges), imaging (due to their large scattering coefficients) as well as for photothermal therapy (using their large absorption coefficients) [18, 19]. Among metal NPs, gold NPs have been reported to be inert and non-toxic.

Due to their versatile physical and chemical properties and biocompatibility, amorphous silica and gold nanostructures are being extensively explored for biomedical diagnostic and therapeutic applications [18, 20].

1.4 Silica NP and their applications:

Silica (SiO_2) is one of the most commonly encountered oxide materials in our life. It can exist in amorphous (vitreous silica) or in crystalline form and is transparent over the whole visible region and beyond (~200-2000 nm). Fused silica, the high purity amorphous form, is a non-toxic compound and is already being used as food additive and as carrier material in pharmaceutical tablets. Various amorphous silica based nanostructures including colloidal, mesoporous and ORMOSIL are being used and explored for their promising potential as drug/dye carrier [31]. Further advantages of silica nanoparticles, as mentioned earlier, are their inherent hydrophilicity due to the presence of charged groups (silanol) at their surfaces. This decreases their clearance by the immune system, increasing their circulation time in blood and insensitivity to microbial attack as compared to the organic NPs such as liposome, polymeric NP, etc.

These nanostructures can be conveniently synthesized by sol-gel methods by employing a variety of commercially available silica precursors. The physical characteristics including the density, pore size and structure of the silica matrix can be tailored by controlling the sol-gel reaction kinetics [32]. Two methods: Stober and micro-emulsion are typically followed for sol-gel based silica NP synthesis [33]. Stober method is based on hydrolysis and subsequent polymerization condensation of the silica

precursors such as tetraethyl etoxysilane, in the presence of ammonium hydroxide as catalyst at ambient temperature, to produce electrostatically stabilized NPs in which dye/drug molecules can be either encapsulated or covalently attached [34]. Although, this method can generate NPs of tens to hundreds of nm in size, the particle size may not be uniform and also modifications of the particle surface are not easy.

Using modified Stober's method i.e., microemulsion based hydrolysis-condensation method, silica nanoparticles (SiNPs) with controllable size and pore size have been prepared [32]. This method enables the production of nanoparticles with relatively more homogeneous size and drug/dye distribution which is important for using them in biological systems. For example, photostable and targetable SiNP were developed by controlled hydrolysis of tetraethyl orthosilicate (TEOS) using microemulsion method. These dye doped SiNPs showed good photostability with respect to bare dye or dye doped in latex based fluorescent particles and were effectively used for imaging leukaemia cells [35]. Mesoporous silica NPs (MSN) have been prepared with different tetra alkoxy ortho silicates as silica precursors to give NPs with variable and controllable pore size (~ 2-30 nm) using surfactant such as cetyltrimethylammonium bromide (CTAB) and other polymers under controlled reaction conditions, such as pH, temperature etc. These have been used to encapsulate drugs/dyes/macro molecules such as enzymes, genetic materials, etc, depending on the pore size and morphology and showed controlled release of the encapsulated molecules (such as cancer drugs) [36]. For example, in a study hollow MSN carrying fluorescein isothiocyanate (FITC, which served as a model drug) were shown to release the drug in a controlled fashion [37]. In this study, FITC encapsulated in silica nanocapsules was shown to be released over the period of a week, when soaked in an inorganic medium whereas a burst release within few hours was observed when these NPs were soaked in an organic solution indicating controlled release. Similarly, another study showed the release of anticancer drug, doxorubicin, over a period of 20 days in a constant rate [31]. Furthermore, MSN were able to enhance the

delivery of the hydrophobic anticancer drug paclitaxel to pancreatic cancer cells and allowed a targeted delivery of a chemotherapeutic agent methotrexate (MTX). In this study, while free MTX caused apoptosis in cancer as well as in healthy cells, MTX bound to MSN, in contrast, induced cell death only in cancer cells but not in normal tissue [38, 39]. These studies show the promise MSN offer as carriers, as well as for controlled drug delivery for in vivo applications, just like polymeric NP with the advantage that these do not get degraded with the external environment and are biocompatible. In addition, a variety of organically modified silica precursors available in market, provide an opportunity to make silica NPs with desired functional groups such as thiol/hydroxyl/carboxyl/amino, which can be further attached them with targeting molecules. These NPs do not show swelling or porosity changes with alteration in pH or environment which may be very important with regard to the path of delivery, for example oral application [40, 41]. These have also shown enhanced blood stability along with sustained release of anti-tumor agents and therefore, show good potential for cancer treatment [42, 43]. ORMOSIL nanoparticles, prepared using organically modified silica precursors, show amphiphilic characteristics. While these have hydrophobic porous core, they also show rich surfaces chemistry. Therefore, these particles provide an ideal platform for fabrication of multimodal imaging probes and at the same time can be used for drug encapsulation/conjugation for delivery, gene therapy or photodynamic therapy [20].

1.4.1 Organically modified silica (ORMOSIL) nanoparticles

ORMOSIL NPs are the silica NPs prepared using organically modified silica precursors, having both organic and inorganic properties (hybrid type), as starting materials. In these, trifunctional alkoxy silanes, $R'Si(OR)_3$ are the most common precursors to introduce organic groups, where the organic moiety (R') is attached through a stable Si-C bond. A variety of such silanes are commercially available. The advantage of these NPs is that

their surface could be further modified for their biocompatibility and targetability and the NPs may slowly fragment through biochemical decomposition of the Si-C bond, which could help the removal of these particles from the body [44]. The presence of organic group also imparts flexibility, to some degree, to the otherwise rigid silica matrix, which is expected to enhance the stability of these particles in the aqueous systems against settling down [45].

Various trifunctional alkoxy silanes precursors with different organic moieties have been used to prepare a variety of ORMOSIL NPs by different research groups for their studies. For example, ORMOSIL NPs using n-octyl triethoxy silane as silica precursor were prepared following both micellar and reverse micellar approach to generate spherical, nearly monodisperse NPs with diameters below 100 nm. These NPs were used to study and compare leaching of hydrophobic or hydrophilic molecules (entrapped in their hydrophobic or hydrophilic regions). In comparison to micelles, prepared using Triton X-100, the leach was found to be extremely slow indicating controlled release [44].

ORMOSIL NPs using organically modified siloxanes, vinyltriethoxysilane (VTES) and aminopropyltriethoxysilane (APTS) were prepared in the micellar core of surfactant Aerosol OT to result in amphiphilic NPs [45]. These NPs had porous hydrophobic core whereas their surface had the negatively charged silanol groups as well as the positively charged aminopropyl groups. Folic acid functionalized ORMOSIL NP, doped with hydrophobic two photon fluorescence dye, was used to target folate receptor over expressing HeLa cells. ORMOSIL NPs were also used as gene carriers [46]. Kneuer et al showed that plasmids bound to amine-modified silica nanoparticles were completely protected from enzymatic digestion in human epithelial cancer cells [43]. Furthermore, Paras N Prasad's group at the university at Buffalo showed that ORMOSIL could be effectively used to introduce genes into neuronal cells in vivo. The same group along with other groups showed the properties of silica nanoparticles as oral drug carrier [47, 48].

ORMOSIL NP were conjugated with the well-known positron emission tomographic (PET) imaging probe Iodine-124 and NIR fluorophore DY776 in a study by Rajiva Kumar et al. These NPs could allow bioimaging independent of tissue-depth, as well as accurate quantification of accumulation of nanoparticles in various major organs *in vivo*. The biodistribution of these NPs by NIR optical and radiolabelling studies suggested accumulation of the NPs in the major RES organs in mice. The clearance studies of the injected nanoparticles indicated that almost 100% of the nanoparticles were effectively cleared out of the animal via the hepatobiliary excretion, without any sign of organ toxicity. These studies showed the use and safety of these ORMOSIL nanoparticles for diagnostic and therapeutic applications. Their results also demonstrated the use of these ORMOSIL nanoparticles as promising carriers for safe *in vivo* applications [28].

Recently ORMOSIL doped with dye IR-820 were used for imaging of mice brain by J. Qain et al. These NP were used to target the subcutaneously xenografted tumour of mouse for long time observations. They also used ORMOSIL NP encapsulated with photophrin IX, a PDT drug, for two photon excited cytotoxicity towards tumour cells [49, 50]. Interestingly, ORMOSIL NPs were used as carrier of hydrophobic photosensitizer such 2-divinyl-2-(1-hexyloxyethyl) pyropheophorbide entrapped in their core [51]. The idea was that although these particles do not release the entrapped drugs, their porous matrix is permeable to the molecular as well as singlet oxygen which is required to achieve the photodynamic therapeutic action (Photodynamic therapy (PDT) is a process where the photosensitizer preferentially accumulates in the tumour tissue and when excited with appropriate light, generates reactive oxygen species which are cytotoxic). Significant damage to tumour cells impregnated with these NPs upon irradiation with light of wavelength 650 nm was shown. All these results show that ORMOSIL NPs are versatile carriers of dyes/drugs for imaging as well as therapeutic applications.

1.5 Metal NPs

Noble metal nanoparticles show localized surface plasmon (LSP). These are charge density oscillations (due to the presence of conduction band electrons) confined to metal NPs and nanostructures. Excitation of LSP in the presence of the electromagnetic field at frequency where resonance occurs, leads to coherent charge density oscillations which manifest into strong light scattering, intense absorption bands and enhancement of local electric fields. Particularly in metals such as silver and gold, the 'd' electrons are free to travel through the material. As the NP size is much smaller than the wavelength of light, it can set up standing resonance condition resulting in displacement of this sea of conduction band electrons with respect to the positively charged ions that form the metallic lattice as shown in Fig.1.1.

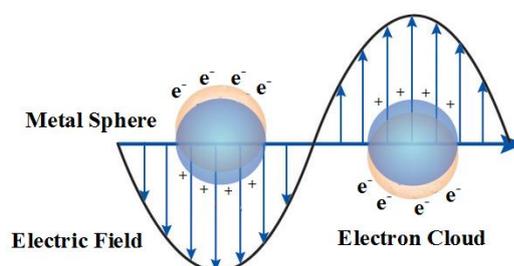


Figure 1.1 Plasmon resonance in a metallic NP excited by a light wave. Recreated from ref.52

The resulting electric dipole on the particle produces a restoring force, which determines the finite eigen frequency of the surface polarization. Amplitude of oscillation reaches maximum i.e., resonance takes place when this frequency matches with the applied field frequency, and the condition is called localized surface plasmon resonance. The resonance condition is determined from absorption and scattering spectroscopy. The position, the shape and the amplitude of the surface plasmon band strongly depends on factors: dielectric constant of the NP and its surrounding medium, on their size and size distribution, shape and the composition. Also, the electronic interaction between the

stabilizing ligands and the NP which alters the electron density of the NP alters the position of the SPR [53]. Hence, NP can be considered as a harmonic oscillator, which is driven by a light wave and damped by some losses such as ohmic loss (which leads to production of heat) and radiative (scattering) loss. For small spherical particles the scattering, extinction and absorption cross-sections are given in Rayleigh limit as follows [54]:

$$C_{scat} = \frac{3}{2\pi} \left[\frac{\omega}{c} \right]^4 \varepsilon_{diel}^2 V^2 \frac{(\varepsilon_r - \varepsilon_{diel})^2 + (\varepsilon_i)^2}{(\varepsilon_r + 2\varepsilon_{diel})^2 + (\varepsilon_i)^2} \quad \dots\dots\dots 1)$$

$$C_{ext} = 9 \left[\frac{\omega}{c} \right] (\varepsilon_{diel})^{\frac{3}{2}} V \frac{(\varepsilon_i)}{(\varepsilon_r + 2\varepsilon_{diel})^2 + (\varepsilon_i)^2} \quad \dots\dots\dots 2)$$

$$C_{ext} = C_{abs} + C_{scatt} \quad \dots\dots\dots 3)$$

Here ε_r and ε_i are the real and imaginary parts of the dielectric constant of the metal and ε_{diel} is the dielectric constant of the environment around, ω and c are the light frequency and velocity and V is the volume of the NP. C_{ext} is the summation of the absorption and scattering cross-section, related to extinction coefficient as [55]:

$$\epsilon \text{ (M}^{-1}\text{cm}^{-1}\text{)} = 10^{-3} N_0 C_{ext}(\text{cm}^2)/2.303.$$

In equation 1 and 2, denominator is minimum, i.e., resonance occurs, for $\varepsilon_r + 2\varepsilon_{diel} = 0$. In case of noble metals this happens in the visible region, as real part of dielectric constant is negative and the imaginary part is ~ 0 . SPR position is determined by this real part of the dielectric constant of the metal while the imaginary part determines the bandwidth. These equations also show that while the scattering cross-section depends quadratically on the volume, the extinction cross-section depends only linearly. Therefore, for very small particles (~ 10 nm) the extinction is dominated by absorption and it is very hard to see any

scattered light whereas as the size increases (~80 nm) scattering starts dominating over absorption. This suggests that smaller NPs can be used for absorption based applications such as biosensing, photothermal therapy of cancer, etc. whereas larger size metal NPs can be utilized for biological imaging and labeling [56]. Different metal nanospheres, for example, Ag (diameter=20 nm), Au (12 nm), Cu (12 nm) exhibit plasmon absorption bands with maxima at 410, 520, and 564 nm respectively while platinum, palladium have weak and broad band in the UV region [57].

1.5.1 Gold NPs

Gold is considered as the most inert among metals. Nanoparticles of gold show properties which make them suitable for use in numerous biological applications. For example spherical gold NPs have been used in vivo, since 1950s, as adjuvant in radiotherapies [58]. Although gold NP of size ~ 2 nm show high catalytic activity and are reported to be toxic [59], larger size gold NP are reported to be biocompatible [53]. These show strong optical extinction peak which can be varied by controlling particle morphology and their surface chemistry allow for easy attachment of the functional groups required for targeting. The current interest in nanotechnology has led to the development of various shapes of gold NPs which include spheres, core-shell, cubes, stars, cages, rods, etc., for biological applications such as imaging, sensing/diagnostics (which are based on change in refractive index, aggregation of NPs, SERS, scattering, two photon luminescence, etc.), tracking, drug delivery, photothermal therapy, etc. [60].

For achieving efficient contrast in imaging/diagnostic and for photo thermal therapeutic applications, it is important to choose nanoparticles of right size and shape. Recently El Sayeed's group [61], calculated and compared absorption and scattering efficiencies and optical resonance wavelengths of gold nanospheres, silica-gold core-shell NPs, and gold nanorods of different sizes using Mie theory and discrete dipole approximation. A systematic quantitative study of the various trends presented by them

showed that by increasing the size of gold nanospheres from 20 to 80 nm, the magnitude of extinction as well as the relative contribution of scattering to the extinction rapidly increases. While nanosphere of size 40 nm showed an absorption cross-section 5 orders higher than conventional absorbing dyes, the magnitude of light scattering by 80 nm gold nanospheres is 5 orders higher than the light emission from strongly fluorescing dyes per particle. The variation in the plasmon peak of nanospheres was from 520 to 550 nm. This is far from the therapeutic window (~650-900 nm), which limits their use for in vivo applications (due to large tissue scattering). In case of gold nanoshells, the optical cross-sections were shown to be comparable to and even higher than the nanospheres. By increasing the ratio of the core-to shell radius, the optical resonance wavelength could be increased upto the near-infrared region, which makes them useful for in vivo applications such as photoacoustic imaging and photothermal therapy [62, 63]. However, controlling the core to shell thickness ratio is practically difficult.

Gold nanorods (AuNR) can be synthesized with aspect ratios (ratio of length to width), which can be conveniently tuned to shift their extinction peak to near infra-red (NIR) spectral region, therapeutic window, allowing deeper penetration of photons in biological tissue. Also, in NIR frequency, AuNR have an order of magnitude higher absorption and scattering coefficients than the nanoshells and nanospheres. Rods also show narrower linewidths due to reduced radiative damping effects leading to higher photothermal conversion efficiency [64]. Therefore, these are being actively investigated for contrast based imaging and PTT [65].

1.5.2 Gold nanorods and Photothermal processes

As the shape of gold NP changes from sphere to rod, the SPR splits into two bands [66]. Oscillation of electrons along the longer axis generates a stronger band in the NIR region (referred to as longitudinal surface plasmon, L-SP), whereas a weak band in the visible ~520 nm along the shorter axis, is similar to nanospheres (referred to as transverse surface

plasmon, T-SP). This transverse band position is almost insensitive to the changes in the rod size whereas the longitudinal band largely shifts from visible to NIR region by increasing the aspect ratio.

While the radiative i.e., enhanced light scattering properties of gold NPs are used for imaging, the absorbed light can be converted into non-radiative processes [67]. Theoretical studies have shown that this is due to the fast phase loss of the coherently excited electrons on femtosecond time scales due to electron-electron collisions leading hot electrons with temperatures $\sim 1000\text{K}$ [55]. These electrons pass the energy to the phonons by electron-phonon interactions on the order of 0.5-1ps, which results in the rise in temp of the lattice \sim few tens of degrees. Subsequently, three processes can occur a) the lattice cools down passing heat to the surrounding medium by phonon-phonon relaxation within $\sim 100\text{ps}$. This can be utilized for sufficient heating of the adsorbed/attached cells using light with wavelength overlapping to the SPR of the NP, b) The lattice heat can lead to particle melting, c) or particle fragmentation depending upon the rate of heating and cooling [68,69]. For applications such as cancer cure, process 'a' has to be dominated which can be realized using continuous wave lasers, whereas the other two processes can be used for drug delivery applications [70]. Among the other structures, gold nanorods convert the electromagnetic radiation efficiently into heat and hence considered to be promising tools in applications such as in vivo photoacoustic imaging [71] tissue soldering [72] drug delivery [73] and photothermal cancer therapy [74].

Several methods had been adopted for the preparation of gold nanorods. In early 1990's rods were prepared by electrochemical reduction of gold into nanoporous aluminium oxide membranes. Although this gave relatively monodisperse structures, the yield was low and the diameter was $>100\text{ nm}$ which showed the optical response dominated by multipolar plasmon resonance modes. Later electrochemical oxidation of gold electrode in the presence of cationic surfactant cetyl trimethylammonium bromide

(CTAB) under ultrasonication was demonstrated to synthesize rods with ~10 nm diameter. These rods exhibited transverse and longitudinal plasmon modes and for the first time verified the gold rod optical theory, proposed by Gans for the scattering and absorption. C. J. Murphy and M. A. El-Sayed groups later demonstrated a colloidal growth method to produce monodisperse gold nanorods with high yield based on seeded growth [18]. In this method ~1.5 nm diameter single crystal seed particles are first produced by reduction of chloroauric acid by borohydride in the presence of CTAB. Aliquot of this seed is then put in the growth medium, which contains Au (I) growth solution, prepared from the mild reduction of chloroauric acid by ascorbate, CTAB and AgNO₃. Using this method, gold nanorods of diameter 10-20 nm and length up to 300 nm had been prepared with relatively high yield. By controlling the growth parameters such as ratio of seed to growth medium, concentration of silver ions, etc. the nanorods of desired and precisely controlled aspect ratio can be obtained. [75,76, 77, 78].

This last method is the most common method of preparation of gold nanorods (AuNR) where the gold salt is chemically reduced in the rod-shaped micellar template formed at high concentration of CTAB [18, 76]. CTAB is the structure-directing agent that is used to control gold nanorod shape, and it appears to form a tightly bound cationic bilayer on gold nanoparticles, with the cationic trimethylammonium headgroup exposed to the solvent [79]. However, the high concentration of CTAB employed in AuNR synthesis has raised concerns regarding their toxicity [80]. Efforts have been made to remove the extra CTAB and overcoat the CTAB coated rods with other materials that lead to better biocompatibility and stability of the AuNRs [79]. Different biocompatible polymers have been used for layer by layer coating the Rods [81]. As CTAB coated rods are positively charged these can be easily coated with negatively charged polymers such as polystyrene sulphonate (PSS) which can be further coated with positively charged biocompatible polymers such as poly ally amine hydrochloride (PAH), poly diallyl dimethyl ammonium chloride (PDDAC) etc.

For both diagnostic and therapeutic applications either the NPs themselves can be used or these can be conjugated with dyes/drugs to cause these applications. The conjugation of these dyes/drug molecules with the NPs may be covalent, hydrophobic or electrostatic. However, due to conjugation their properties may change. How the photophysics of these molecules change due to conjugation and whether it affects their biological activity is interesting to study. Dye NPs interaction can be conveniently studied by optical absorption and fluorescence spectroscopy. For this it is important to understand the excited state processes involved upon the excitation of fluorophores.

1.6 Excited state processes

In the presence of electromagnetic field, a dye may absorb depending on its dipole strength. During this excitation, three main processes can be involved: 1) absorption of light energy ($\sim 10^{-15}$ s, associated with an electron transfer to an excited state), 2) this is followed by radiationless decay, either within or from the higher vibrational levels, of the excited states. In biological systems, this vibrational relaxation occurs on a picoseconds time scale and only those chemical processes, with rate constants higher than 10^{12}s^{-1} , compete with vibrational relaxation. Subsequent to excitation, vibrational relaxation is usually complete before electronic relaxation, and 3) fluorescence, i.e., emission with frequency smaller than the excitation frequency. The difference between the wavelength required for excitation and the wavelength of the emitted light is known as the Stokes' shift which corresponds to the radiationless energy loss within the excited state. A representative method for illustrating these electronic processes is the Jablonski diagram.

As shown in Fig.1.2 a molecule initially is in the singlet ground state S_0 . Upon absorption of radiation it goes to excited singlet states S_n , wherefrom through, vibronic relaxation of excited singlet S_1 states it reaches to first excited singlet ground state. From here the molecule can radiatively decay by two basic pathways. Decay from S_1 to S_0 by emission of residual energy as photons may occur called fluorescence. This process

occurs in the states of the same multiplicity and, in terms of quantum mechanics, this is spin allowed process.

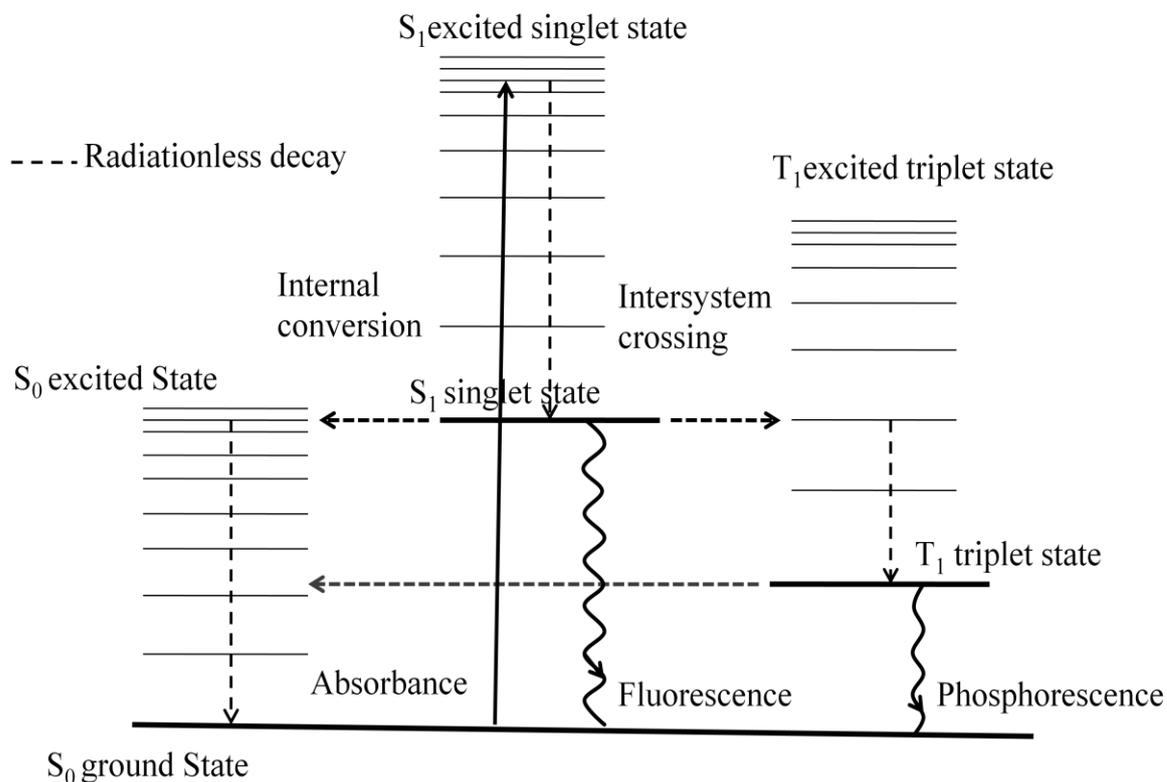


Figure 1.2 Jablonski diagram illustrating electronic excitation processes

The intensity of fluorescence from the excited molecule, although depends on the magnitude of radiative decay rate (k_r), is strongly dependent upon the internal competing processes. The other radiative decay path way, phosphorescence, involves intersystem crossing from S_1 to a triplet state T_1 . This is due to spin-orbit coupling leading to an efficient crossing between states of different multiplicity. As it is a spin forbidden process it has much longer lifetimes as compared to fluorescence. The other nonradiative processes competing with fluorescence include internal conversion to the ground state, charge transfer, energy transfer, the photochemical reactions, photoisomerism, etc. For symmetry allowed transition, radiative decay rate, $k_r \sim 10^9 \text{ s}^{-1}$. In the absence of any other depopulation process, the radiative decay time is $1/k_r \sim 10^{-9} \text{ s}$. This is termed as natural or

mean radiative lifetime, τ_R . In practice, because of the other non-radiative competing processes, as mentioned above, the measured decay time (for complex polyatomic molecules) is less than the mean radiative lifetime. Therefore, the quantum yield of fluorescence is given as follows:

$$\text{Quantum yield, } Q = \frac{k_r}{k_r + k_{nr}} \text{ -----4}$$

$$\text{Life time, } \tau = \frac{1}{k_r + k_{nr}} \text{ -----5}$$

Here k_{nr} is the non-radiative rate.

When light is incident on fluorophores attached to the metallic surface, the electric field associated with the fluorophores may get affected because of the induced electric field caused by the generation of surface plasmon. This effect has the ability to either increase or decrease the effective electric field on the fluorophores [82]. In the presence of metallic NPs, the fluorophores may undergo one of three processes: 1.) energy transfer to the metal that results in fluorescence quenching, 2.) an increase in the intrinsic radiative decay rate of the fluorophores, or 3.) amplification of the incident electric field by the metal, thereby causing an increase in the intensity of the emission [82].

The excited state properties of many organic dyes used for biomedical applications are sensitive to environment properties such as polarity, viscosity, pH, etc. For example 8-anilino-1-naphthalenesulfonate (ANS), 6-p-toluidino-2-naphthalenesulphonate (TNS) are polarity sensitive dyes. In polar medium their excited state decays by charge transfer from donor (anilino and toluidino) groups to the acceptor (sulphonate) group followed by a twist around the donor. This is the dominant non-radiative decay process which leads to significantly low emission quantum yield in polar medium such as water [83]. However, in hydrophobic environment the probability of this process may be significantly suppressed, for example in proteins, where these dyes can

get entrapped in their hydrophobic pockets, leading to an order of magnitude increase in the quantum yield [84]. Similarly some dyes undergo excited state photoisomerism which may also be polarity/viscosity sensitive. For example merocyanine 540, is a well known membrane binding dye (It is also used for PDT) [85]. In polar medium such as water, it exists as mixture of monomer and dimer [86]. While the monomer is weakly fluorescent, the dimer is non-fluorescent [87]. This weak fluorescence is due to the photoisomerization process accounting for the main non-radiative decay pathway for MC540 in the excited state [88]. Binding to membranes, such as liposome, has shown significant enhancement in its emission quantum yield [89]. Several cyanine and fluorescein dyes have been used as pH sensitive dyes, which show emission intensity switching or wavelength shift depending on the ionic strength of the environment [90]. Chlorophyll-a derivative, such as Chlorin p_6 (an amphiphilic photosensitizer) shows pH dependent aggregation [91]. It has three negatively charged carboxylic acid groups at physiological pH. With the increase in the protonation of the environment, it shows a significant decrease in its absorption and fluorescence properties, due to the neutralization of the carboxylic acid groups.

Interaction of dyes with NPs may be either due to electrostatic interaction or hydrophobic interaction or both if the NP itself is amphiphilic in nature. Electrostatic interaction may change the electronic distribution around the donor and acceptor groups of the dye or in the case of hydrophobic interaction, the dye may preferentially get entrapped in the hydrophobic region of the NP thereby restricting its motion in the excited state. Amphiphilic dye may interact in both ways. This interaction may change the excited state properties of these dyes. When dye molecules interact with NPs, depending upon the optical, physical and chemical properties of the nanoparticle, the absorption and fluorescence properties of the conjugated dye may change, which may also affect their efficacy. In case of metal NPs, the interaction between surface plasmon and molecules adsorbed on the surface may greatly affect the excited state properties of molecules and

give rise to interesting phenomenon such as plasmon enhanced fluorescence, fluorescence quenching, surface-enhanced Raman scattering, energy or electron transfer, enhancement of nonlinear optical signals and spin relaxation [92, 93]. The interaction may lead to strong or weak coupling between the wavefunction of the molecules and the plasmon modes which may or may not get perturbed. In addition to fundamental interest, these interactions have potential applications for development of optical devices with functions such as switching, energy transfer and sensing [94]. Also, the adsorption of photosensitizers to plasmonic NPs may enhance the phototoxicity due to a combination of photothermal effect due to plasmonic absorption as well as photodynamic effect due to molecular absorption [95].

1.7 Experimental plan

As discussed in section 1.4, the published reports on ORMOSIL NPs show them as versatile carriers for hydrophobic dyes/photosensitizers (in the hydrophobic core of NP) from both diagnostic as well as therapeutic point of view. However, ORMOSIL NPs prepared with VTES and APTS silica precursors, have amino propyl groups on their surface. As the pK_a of amino group is ~ 9.2 , these amino groups are positively charged at physiological pH and therefore, there is a possibility of electrostatic binding of negatively charged molecules with these amine modified ORMOSIL NP at this pH. As part of the thesis we studied how the photophysics of molecules change when they bind with ORMOSIL NP and how this change affects their functionality.

To investigate the nature of interaction, we have chosen dyes like ANS, TNS and MC540, which are negatively charged polarity sensitive dyes. According to literature, in polar medium, the excited state of ANS and TNS decay non radiatively due to twisted intramolecular charge transfer, whereas MC540 undergo photoisomerism in its excited state, which is the major non-radiative decay channel. Due to these prominent non-radiative decay processes, their fluorescence quantum yield is very poor in aqueous

medium. It has been shown in the literature that binding with proteins, surfactants, membranes and liposome increases their quantum yield. We were therefore interested to study their interaction with ORMOSIL NPs. As MC540 is also a photodynamically active dye, we tested the effect of this dye conjugated with SiNP on the phototoxicity on cancer cell lines.

We also studied the interaction between porphyrin type photosensitizers Cp_6 and PP18 and these SiNPs. Both are interesting photosensitizers because these have significant absorption in the longer wavelength (650-900 nm) where tissue absorb and scatter weakly. Cp_6 is amphiphilic and negatively charged at physiological pH due to the presence of three carboxylic acid groups. Earlier studies have shown that Cp_6 shows pH dependent aggregation behaviour below physiological pH. Also the uptake and efficacy of Cp_6 in cancer cell lines have been shown to be pH dependent. We were therefore interested to study the pH dependent aggregation behaviour of Cp_6 in the presence of SiNP suspended in aqueous medium. The phototoxicity of Cp_6 -SiNP complex on cancer cell lines was also studied. While Cp_6 is amphiphilic, PP18 is hydrophobic and therefore needs a suitable carrier for delivery. The interest to use PP18 for PDT applications is due to its higher extinction coefficient at longer wavelength as compared to Cp_6 , which is desired for deep tissue penetration of light. However, in aqueous medium PP18 converts to Cp_6 due to the hydrolysis of its anhydride ring. We investigated the suitability of SiNPs as carriers of hydrophobic PP18 in aqueous media by monitoring its time dependent conversion to Cp_6 in SiNPs and compared with liposome and polymeric NPs.

As discussed in section 1.5, among the plasmonic NPs gold NPs are biocompatible. Among the different gold nanostructures (spheres, core shell and rods) gold nanorods show an order of magnitude higher extinction coefficient. The more interesting part in gold nanorods is that their L-SP can be easily tuned from visible to NIR by controlling their aspect ratio. We were interested to investigate the effect surface plasmon electric field on the optical (absorption and emission) properties of the dyes

attached to them. To observe the effect of L-SP we used gold nanorods whose L-SP peak position was tuned to and off the absorption maximum of two dyes, MB and NB. We also investigated the spectroscopic properties of a photosensitizer Cp_6 conjugated to gold nanorods. This was motivated by a recent paper Kuo et al., where they have suggested that a combination of photodynamic therapy (PDT) and photothermal therapy (PTT) can be more effective than either PDT or hyperthermia alone and can be easily realised by conjugating NIR absorbing photosensitizers with NIR absorbing AuNRs [95]. This made us interested to study the effect of L-SPR on the photophysical properties of Cp_6 electrostatically conjugated to gold nanorods. As discussed in section 1.5, the CTAB coated gold nanorods are positively charged. However, due to issues regarding the toxicity of CTAB [96] these rods were coated with biocompatible polymer PSS. PSS is a negatively charged polymer and Cp_6 is also negatively charged at physiological pH. For electrostatic binding between Cp_6 and the rods, these rods were again coated with positively charged biocompatible polymers PAH and PDDAC. We investigated interaction of Cp_6 with these coated AuNR of two different aspect ratios, with their L-SP tuned to and away from the Q-band absorption peak of Cp_6 . The effect of L-SP position as well as the nature of coatings on the photophysics of Cp_6 was studied.

1.8 Outline of the thesis

In chapter 2 the methodologies used for the preparation of different NPs, different techniques used for NP characterization and spectroscopic studies along with their basic principles are briefly given. In chapter 3 the photophysical studies on interaction of ANS, TNS and MC540 with ORMOSIL NPs are presented while in chapter 4 we present the spectroscopic investigations on the binding of the photosensitizer Cp_6 with amine modified silica nanoparticles in aqueous media along with the evaluation of photodynamic efficacy of Cp_6 -SiNP complex in colon and oral cancer cell lines. A spectroscopic study on conversion of PP18 to Cp_6 in the presence of liposome, silica and

polymeric nanoparticles is given in chapter 5. Spectroscopic investigations on the binding of Methylene blue and Nile blue to gold nanorods are presented in chapter 6 whereas photophysical properties of Cp_6 bound to gold nanorods are described in chapter 7. Chapter 8 gives the conclusion of the thesis and suggestions for the future research work.