BIOCOMPATIBLE HYBRID NANOMATERIALS INVOLVING POLYMERS AND HYDROGELS INTERFACED WITH PHOSPHORESCENT COMPLEXES AND TOXIN-FREE METALLIC NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

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Marpu, Sreekar B.  *Biocompatible Hybrid Nanomaterials Involving Polymers and Hydrogels Interfaced with Phosphorescent Complexes and Toxin-Free Metallic Nanoparticles for Biomedical Applications*. Doctor of Philosophy (Materials Science and Engineering), August 2011, 359 pp., 23 tables, 160 illustrations, 247 chapter references.

The major topics discussed are all relevant to interfacing brightly phosphorescent and non-luminescent coinage metal complexes of [Ag(I) and Au(I)] with biopolymers and thermoresponsive gels for making hybrid nanomaterials with an explanation on syntheses, characterization and their significance in biomedical fields. Experimental results and ongoing work on determining outreaching consequences of these hybrid nanomaterials for various biomedical applications like cancer therapy, bio-imaging and antibacterial abilities are described. In vitro and in vivo studies have been performed on majority of the discussed hybrid nanomaterials and determined that the cytotoxicity or antibacterial activity are comparatively superior when compared to analogues in literature. Consequential differences are noticed in photoluminescence enhancement from hybrid phosphorescent hydrogels, phosphorescent complex ability to physically crosslink, Au(I) sulfides tendency to form NIR (near-infrared) absorbing AuNPs compared to any similar work in literature.

Syntheses of these hybrid nanomaterials has been thoroughly investigated and it is determined that either metallic nanoparticles syntheses or syntheses of phosphorescent hydrogels can be carried in single step without involving any hazardous reducing agents or crosslinkers or stabilizers that are commonly employed during
multiple step syntheses protocols for syntheses of similar materials in literature. These
astounding results that have been discovered within studies of hybrid nanomaterials are
an asset to applications ranging from materials development to health science and will
have striking effect on environmental and green chemistry approaches.
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Sreekar B. Marpu
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CHAPTER 1

INTRODUCTION

1.1 Overview of Dissertation

The following work represents part of the work that I performed as a graduate student at the University of North Texas. Some of this work has been published and some are in process of submission to the scientific journals. One of the published papers\(^1\) and another submitted manuscripts are represented in two of the chapters of the dissertation, whereas other papers and patent applications are not included. Making hydrogel nanoparticles from different thermosensitive polymers like NIPA (N-isopropyl acrylamide) and PEG (polyethylene glycol) and understanding their phase transition behavior during formation of stimuli sensitive colloidal crystals and investigating their applications related to drug delivery, biosensors and microfluid channels is the major area of research of one my sub groups guided by Dr. Zhibing Hu.\(^2\) My other sub group guided by Dr. Mohammad Omary\(^3,4\) has broad research interests in syntheses and characterization of mainly \(d^{10}\) and \(d^8\) complexes of metal ions such as Au(I) and Pt(II) respectively, for extensive applications ranging from organic light emitting diodes (OLEDs) to gas storage and bio-imaging. Major investigations discussed in this dissertation involve developing hybrid materials by interfacing materials from both labs to understand unexplored novel properties of these hybrid systems that would further enhance applications of both material types. Phosphorescent hydrogels or polymeric nanoparticles produced using a metal-based phosphor as physical crosslinker, for example, is never been reported. Such new hybrid composite materials have strong
potential to improve sensor and bioimaging applications of luminescent polymeric nanoparticles. Work discussed in this dissertation initially started with introducing water soluble phosphorescent Au(I) complex \((\text{AuP})^5 \text{Na}_8[\text{Au(TPPTS)}_3]\), \((\text{TPPTS}=\text{tris}(3,3',3''-\text{trisulfonatophenyl})\text{phosphine})\) in to PNIPAM (poly-N-isopropyl acrylamide) based hydrogels which is discussed extensively in chapter 2. Investigation on doping gold phosphor (AuP) in to different forms of hydrogels followed by functional group, pH and concentration-dependent photoluminescence analysis of hybrid systems in different physical forms detailed. Unusual phosphorescence enhancement by more than two orders of magnitude from hybrid microgels was momentous result. Later in chapter 3, phenomenon of physical inverse thermo-reversible gelation in hybrid PNIPAM-co-allylamine microgels is realized using the same gold phosphor (AuP). During this process, in situ physical crosslinking property of AuP discovered while forming chitosan and NIPA based polyelectrolyte complex phosphorescent microgel nanoparticles in complete absence of any extra crosslinker. This highlights AuP as potential candidate to act as phosphorescent physical crosslinker with positively charged soft polymer materials. Physical crosslinking ability of AuP not only results in formation of polymeric nanoparticles but also encourage using AuP as contrast agent. It is hypothesized and experimentally proven that, phenomenon of physical gelation and formation of phosphorescent polymeric microgel nanoparticles in presence of gold phosphor is due to strong electrostatic interactions between microgels or polymers and AuP. Rigidochromic tunable emission shift predicted due to T-shape excited state distortion of \(\text{AuP}^6\) was conceived within hybrid hydrogel also even within phosphorescent chitosan
microgels both at body temperature and at room temperature as discussed in chapter 2 and 3 respectively. This is the first experimental demonstration of such emission tunability in soft polymer systems using Au(I) based phosphorescent complex. Combined work from both chapters 3 and 4 filed under a patent at UNT. Taking a step ahead amalgamating non-luminescent Au(I) sulfides with various PNIPAM based hydrogels and with long list of thermosensitive, pH sensitive, biologically and environmentally benign polymers unveiled a facile technique for in situ formation of size, shape, SPR (surface plasmon resonance) tunable gold nanostructures. chapter 4 outlines detailed experimental investigations about the novel syntheses technique involved during formation of these AuNPs (gold nanoparticles). The toxin free nature of AuNPs confirmed from both in vitro (MTT) and in vivo (zebrafish) cytotoxicity assays conducted in comparison with analogues samples available in the market. High cell viability and survival rates forward our AuNPs as promising candidates especially for biomedical/health science applications. This entire work is also filed in a patent with UNT patent office. As a further expansion of chapter 4, following similar photochemical syntheses protocol both visible and near-infrared absorption tunable silver nanoparticles are stabilized within chitosan and poly(acrylic acid) polymers. Positive results from antipathogenic studies ensured their usage for further biomedical applications in various fields. Simple variations in experimental conditions allowed tuning in size and SPR in both AuNPs and AgNPs as detailed in chapter both 4 and 5 respectively. All the collaborators involved or assisted in experimental work or data analysis are accredited.
The following sections of this introductory chapter represents some background material of each of the dissertation topics where a) previous work on each subsequent topic is discussed, and b) how the work presented in the dissertation provides unexplored methodology or a better feasible alternative compared to existing literature that would enhance the application range of hybrid materials.

1.2 Brightly-Phosphorescent, Environmentally-Responsive Hydrogels Containing a Water-Soluble Three-Coordinate Gold(I) Complex

Hydrogels or microgels are simply polymer networks that are made mostly from physical or chemical crosslinking of organic polymer chains which may absorb from 10%-20% to hundred times their dry weight in water.\(^9\),\(^10\) Crosslinking provides dimensional structural stability and high water content brings fluid like properties. This particular intermediate physical behavior makes hydrogels extensively studied for number of applications.\(^9\),\(^10\) Use of thermo-sensitive sensitive polymers like PNIPAM (poly-N-isopropyl acrylamide) has resulted in environmental sensitive hydrogels that can simulate biological tissues or swell and collapse\(^11\),\(^12\) in response to many different kinds of external stimuli like temperature, pH, ionic strength, electrical and magnetic\(^1\),\(^2\) making them potential candidates especially for biological and biomedical applications. Phosphorescent materials with long lifetimes and higher luminescence quantum yields are highly desired for many materials and biomedical applications.\(^13\) Understanding electronic structure and guiding luminescent gold (I) complexes for opto-electronic applications has been major contribution from one of my subgroups.\(^14\),\(^15\) Luminescent Au(I) complexes exist in two, three and tetra coordinate geometry, while metal-centered luminescence is believed to be absent in 4–coordinate tetrahedral
complexes.\textsuperscript{14} Three-coordinate complexes such as $[\text{Au}(\text{PR}_3)_3]^+$ and $[\text{Au}(\text{PR}_3)_2\text{X}]$, (R=aryl or alkyl, X=halide) containing gold(I) in a trigonal planar geometry, have been shown to exhibit luminescence both in the solid state and in non-aqueous solvents.\textsuperscript{14b} But only after Corey and Khan postulated singlet $^1\Delta_g \text{O}_2$ quenching as mechanism of action of gold drugs Fackler et al.\textsuperscript{16} synthesized water soluble Au(I) luminescent complexes using water soluble TPPTS and TPA (1,3,5-Triaza-7-Phospha-Adamantane) ligands.\textsuperscript{5} Understanding electronic structure of gold(I) complex in biologically relevant media was a main motivation for the syntheses of these complexes. In the detailed study by Fackler et al. one of these phosphine based complexes, $[\text{Au(TPPTS)}_3]^{8^-}$, was shown to be stable in aqueous solution with emission quantum yield that is actually higher than that of low dielectric constant organic solvents like acetone.\textsuperscript{5} Energy difference from the spin allowed absorption and the emission along with long lifetimes imply emission is phosphorescence from triplet-excited state. The author’s work in this dissertation has diversely utilized $[\text{Au(TPPTS)}_3]^{8^-}$ (AuP) for its high stability and water solubility in understanding unprecedented changes in both photophysical and stimuli sensitive properties of hybrid gels obtained by fusion of AuP with PNIPAM gels. Phase transition studies aimed at employing these PNIPAM nanogels for drug delivery applications are demonstrated in detail by Hu et al. previously.\textsuperscript{2,17} Luminescent materials like organic dyes, semiconductor nanoparticles (QD’s) or luminescent transitional metal complexes (Tb(III) or Eu(III)) are loaded in to polymers or gels for enhancing functions of these hybrid materials in variety of fields like biolabeling, biosensors, genetic fluorescence detection and photonic crystals formation.\textsuperscript{18,19,20} In chapter 2, a simple methodology
has been followed for formation of phosphorescent PNIPAM based hydrogels in different physical forms along with demonstration of phosphorescence enhancement. Loading of AuP in to PNIPAM microgels was observed to be selective to pH of the medium and co-monomer of the microgel. Once loaded, photoluminescence properties of AuP are completely retained in all hybrid gels forms. Systemic emission tuning demonstrated by Omary et al.\textsuperscript{6} in similar Au(I) phosphine complexes using extended quantum mechanical calculations was realized in these real PNIPAM hybrid microgel systems close to human body temperature (~37 °C). Fluorescence enhancement, a typical tool used for sensing applications that is mostly achieved in presence of plasmonic nanoparticles, but scarcely observed in presence of micelles is demonstrated with perfection.\textsuperscript{1} Photoluminescence enhancement discovered in these hybrid microgels surpasses all micelle-based PL enhancements and stands outstanding being phosphorescence in nature compared to any known literature work to date.

1.3 In Situ Synthesis and Inverse Thermoreversible Gelation of Biocompatible Linear Polymers by Au(I) Phosphors Toward Biological Imaging and Heavy Metal Sensing Applications

Chitosan (CS) is a naturally occurring benign biodegradable positively charged polysaccharide.\textsuperscript{21,22} Extraordinary biocompatibility, biodegradability, bioadhesive, nontoxic, antifungal properties and presence of abundant amine and hydroxyl groups makes CS polymer highly desirable in various fields ranging from pharmaceuticals, tissue engineering, water treatment, nutrition to drug delivery.\textsuperscript{21,22} Free amine groups of CS are highly pH sensitive and exhibit strong affinity towards negatively charged polymers or polyanionic molecules. This behavior has been exploited for long time in
making CS nanoparticles or microparticles for various drug delivery applications. Applications of these chitosan nanoparticles (CHNPS) for bioimaging, diagnostics imaging of cancer cells, labeling of stem cells, and imaging of pathogenic cells is also under investigation. In view of huge uproar for polymeric luminescent nanoparticles, a recent review has listed important features for a successful nanoparticle based optical imaging agent as (a) in vitro and in vivo stability, (b) resistance to metabolic disintegration and non-toxicity, (c) high quantum yield and high absorbance (d) sufficient dispersability in the biological environment, (e) non-toxic nature of contrast agent. Based on the requirements it is hypothesized that stabilizing imaging agents in aqueous solution encapsulated with polymeric materials that can form nanoparticles and possess reactive functional groups for further bio-conjugation would be highly desirable hybrid nanostructures. For the above stated reasons CHNPs are strongly pursued for fabrication in to various bio-nanocomposites. In spite of extended promising applications for luminescent chitosan nanoparticles, imparting luminescence in to CHNPS is brought only by doping quantum dots (like CdSe, CdTe) or organic dyes like FITC (fluorescein isothiocyanate), or by addition of lanthanide based chelates. Some of the disadvantages of above-mentioned luminophores are listed in chapter 3 exclusively. Overriding the existing methods an unprecedented phenomenon of in situ physical crosslinking triggering formation of size tunable phosphorescent chitosan microgel nanoparticles is discovered in chapter 3. This phenomenon of in situ physical crosslinking when applied to thermosensitive PNIPA polymers, they show formation of thermosensitive phosphorescent PNIPAM nanoparticles. Formation in both
cases was attributed due to strong electrostatic interactions between polyanionic AuP and positively charged polymer systems. These electrostatic interactions were further evaluated within positively charged PNIPAM-co-allylamine microgels to disclose inverse thermoreversible gelation using a phosphorescent molecule for the first time.

Physical gels are always preferred over gels formed by chemical crosslinking due to non-involvement of toxic crosslinkers and also due to retained sensitivity of polymers. Calcium alginate gels are superior and foremost example of physical gelation with ability to form microspheres at room temperature in absence of any chemical crosslinkers or surfactants. Gelation under influence of change in pH or change in temperature is highly preferred for employing hydrogels directly for tissue engineering or for microencapsulation studies. Temperature sensitive phase transition behavior of poly(NIPAM) microgels is shown to reversibly or irreversibly floculate in to aggregates in presence of high concentration of simple salts like NaCl. Free flowing colloidal aqueous solutions aggregate in to semisolid gels above gelation temperature. Literature survey shows soft polymers exhibit thermoreversible gelation under three different scenarios: Physical interactions between different block copolymers, oppositely charged polymer units, or lastly the presence of simple polyelectrolytes. Demonstration of inverse thermoreversible gelation in aqueous medium illustrated in chapter 3 is first of its kind with involvement of a transitional metal based phosphorescent molecule acting as gelating and emitting center both in sol and gel forms. Within scope of chapter 3, chitosan polymer was employed to in situ synthesize and stabilize very important class of color tunable aqueous sensitive
phosphorescent complexes. Cyclic trinuclear pyrazole (Pz) and triazole (Tz) based d^{10} complexes are famous for their sensitive emission color tunability with respective to chain length of trimer complex or due to presence of heavy metal ion or due to pH. Formation, photophysical properties and applications of these phosphorescent trimers was well demonstrated previously from our group (Omary et al.) in non-aqueous medium.\textsuperscript{33,34} Encouragement by their strong, environmental sensitive phosphorescence features have intrigued us to investigate their formation in complete aqueous medium, for extending their applications in to biomedical arena. In this regard using Pz’ (modified pyrazole) ligand, Au(I) cyclic trimer complexes are synthesized and stabilized in chitosan polymer with complete retention of environmental sensitive photophysical properties. Emission color tunability (red/green/blue) was achieved both by changes in pH and due to presence of heavy metal (Ag^{+1}/Pb^{+2}/ Tl^{+1}) ions. Such results exhibit strong potential for these polymer stabilized phosphorescent complexes as optical sensors for detecting changes in pH or trace presence of heavy toxic metal ions in water.

1.4 In Situ Syntheses of Toxin Free Spherical and Anisotropic Gold Nanoparticles Stabilized within Biocompatible Polymers

Nanoparticles broadly defined as particles having one or more dimensions of the order of 100 nm or less. Due to their unique physical, chemical and biological properties, nanoparticles are of considerable interest in a wide range of applications.\textsuperscript{35,36,37} The unique properties of nanoparticles that differentiate them from the corresponding bulk materials arise from their large surface to volume ratio, surface energy and spatial confinement. Even though these unique properties develop under
the length scale of about 100 nm, particles smaller than 10 nm are even more interesting for molecular behavior.\textsuperscript{35,37} Specifically noble metal nanoparticles (Au or Ag or Cu) can be easily differentiated from other metal (Tin), metal oxide (Fe\textsubscript{2}O\textsubscript{3}) or semiconductor (CdSe, ZnO) nanoparticles due to their strong surface plasmon resonance (SPR) which is sensitive to size and shape of nanoparticles along with dielectric constant\textsuperscript{37} of the medium. Among noble metal nanoparticles, AuNPs have attracted exclusive interest for their broad applications in sensing, imaging, delivery, diagnostics and therapy initiating numerous synthetic approaches for making stable gold nanoparticles in different medium.\textsuperscript{35} In spite of tremendous potential for AuNPs in biomedical industry, conventional syntheses techniques are mostly restricted to usage of highly acidic HAuCl\textsubscript{4} precursor in presence of a strong reducing agent like NaBH\textsubscript{4} (sodium borohydride) to obtain size tunable AuNPs. However, considering applications of gold nanoparticles in biomedicine or health science, where the pH value is close to neutral, the challenge of dispersing them in aqueous solution at neutral pH still exists along with impurities from reducing agents and surfactants need to be addressed. Through many studies on different cell lines, it has been shown that different size AuNPs by themselves are non-toxic but toxicity was generally associated with HAuCl\textsubscript{4}, stabilizers, surfactants or reducing agents intricately involved during syntheses.\textsuperscript{38,39} HAuCl\textsubscript{4}, a prevalent starting precursor used in >99\% syntheses reports is found to be toxic by itself.\textsuperscript{38} However little is rendered to circumvent this problem. Toxin-free protocols that do not involve any of the toxic chemicals to make size tunable AuNPs in wide range of environmental sensitive or biocompatible stabilizers is always desirable.\textsuperscript{40}
Both positively and negatively charged AuNPs are synthesized easily by simple variation of polymers or stabilizers. In case of non-spherical or anisotropic NIR (near-infrared) absorbing AuNPs synthesized primarily for their stronger biomedical usage, issue of biocompatibility is more severe. CTAB (cetyltrimethyl ammonium bromide) a strong surfactant known for disrupting bio-membranes\textsuperscript{41} is directly involved in stabilization process. Numerous studies have demonstrated cytotoxicity of CTAB even in rats,\textsuperscript{41b} but again very little is done in designing protocols that completely avoid usage of CTAB during syntheses. However, toxicity of CTAB is anticipated to be minimized by capping or ligand exchange using biocompatible polymers like PEG.\textsuperscript{41} In contrast to existing literature on syntheses of AuNPs, the instant methodology described in chapter 4 provides a facile synthesis technique for producing size tunable gold colloidal nanoparticles using Au(I) sulfide complexes such as Au(THT)Cl or Au(Me\textsubscript{2}S)Cl as starting precursors in complete aqueous medium. Under photolysis, thermolysis, sonolysis or ambient reaction conditions reduction of Au(I) to Au(0) is effectively achieved in complete absence of reducing agents compared to reduction of Au(III) to Au(0) in presence of chemical reducing agents from prior art works. chapter 4 demonstrates tunable plasmon absorption capability across visible and NIR regions in complete absence of potentially harmful chemicals like CTAB, NaBH\textsubscript{4}, or BDAC (benzyl dodecyl ammonium chloride). Employing protocol discovered in chapter 4, colloidal gold is formed within biologically benign polymers like Chitosan (CS), Agarose, PAA (polyacrylic acid), PVA (polyvinyl alcohol), HPC (hydroxypropyl cellulose) and Alginic acid and also within positively and negatively charged “smart” thermo-responsive
PNIPAM hydrogels. Formation and stabilization of different size, shape AuNPs completely rely on thermo or photo sensitivity of Au(I) sulfides and stabilizing features of gels or polymers. Such a wide usage of polymers or microgels for making size tunable AuNPs by facile single step methodology is first of its kind. Our prediction about non-toxin nature of our gold nanostructures is validated by performing in vitro (MTT assay) and in vivo (zebrafish) studies on these AuNPs in comparison with analogues purchased from market. Concentration of samples and controls was monitored from absorbance and ICP-MS (ion couple plasma-mass spectroscopy) data to ensure proper comparison.

1.5 A Simple Photochemical Route for Syntheses of Spherical and Anisotropic Silver Nanoparticles Stabilized within Biologically Benign Polymers and their Antipathogenic Properties

Silver nanoparticles are extensively studied like gold nanoparticles for their strong optic, electronic and biomedical applications.\textsuperscript{42,43} Compared to AuNPs, nanosilver is known to possess antibacterial properties and utilized throughout history because of its oligodynamic nature (toxic effect of metal ions on living cells like fungi, bacteria, viruses, prokaryotes and eukaryotes). Silver sulfadiazine considered as the gold standard drug for topical treatment of burn patients.\textsuperscript{43} Recently silver nanoparticles have been shown to possess anti-inflammatory properties, which can accelerate wound healing process similar to silver sulfadiazine.\textsuperscript{42,43} Silver is still in use clinically and resurgent interest in nanosilver has emerged due to rampant of antibiotic resistance bacteria.\textsuperscript{43} For example, textile manufacturers have started incorporating nanosilver into fabric for socks, nanosilver is also being impregnated in to different surfaces like
plastics, food containers, refrigerators and chopping boards to exploit antibacterial activity and inhibit microbial growth for long time.$^{43}$ Extensive research is also underway in medical industry to utilize potential antibacterial properties of AgNPs by impregnating nanosilver into surface coatings (neurological shunts and venous catheters), bone cement, implants and surgical threads.$^{43,42}$ In spite of huge activity, potential toxicity of silver salts to humans has severely limited usage of silver ions [argyria: disease due to prolonged contact of silver salts or silver ions] which is currently being overcome by utilizing nanosilver.$^{43}$ Known for large surface area, nanosilver is expected to have greater effect on bacteria compared to silver ions and most importantly, it has been shown to possess low toxicity to humans. Emerging biomedical applications of nanosilver rely on efficient syntheses routes that can make silver nanoparticles within broad range of biocompatible stabilizers nullifying any environmental or biological toxic concerns.$^{40}$

Because of its long existence, numerous syntheses techniques are listed in literature for making silver nanoparticles of different sizes and shapes. However, in chapter 5 a facile technique introduced results in formation of different size and shape AgNPs in different physical forms of polymers (solutions, gels, films). Like in situ stabilized nanosilver doped chitosan gels can enhance potentiality of wound dressing materials compared to similar wound dressing materials made from chitosan-polylactate polymers obtained by doping NaBH$_4$ reduced or sodium citrate reduced AgNPs.$^{44}$ Photochemical technique described in chapter 5 achieves different shape; size AgNPs by simple variation of regular glass material instead of using any special designed filters.$^{45}$
Similar to AuNPs research, NIR absorbing silver nanoparticles are strongly advocated for photothermal therapy, imaging and architectural applications but synthesized mostly by following multiple step seed-mediated growth techniques starting from NaBH$_4$ reduced silver nanoseeds. NIR absorbing silver nanoparticles realized in chapter 5 in complete biocompatible medium is first ever report of a single step protocol in complete absence of any special reducing agent. Strongly know for antibacterial property, demonstration of antibacterial activity within wide range of bacteria or microorganism is very much limited to nanospheres only. Very little or no confirmed data is available to explain how size, shape affects bacterial activity of AgNPs because of contradictory reports on size related issues. Here for the first time we demonstrated antipathogenic activity of NIR absorbing AgNPs in two different plant pathogens. Finally yet importantly, film-forming ability of chitosan was exploited to make highly stable nanosilver films for strong commercial applications.

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CHAPTER 2*

BRIGHTLY-PHOSPHORESCENT, ENVIRONMENTALLY-RESPONSIVE HYDROGELS CONTAINING A WATER-SOLUBLE THREE-COORDIANTE GOLD(I) COMPLEX

2.1 Introduction

Hydrogels are composed of hydrophilic organic polymer networks crosslinked physically or chemically. Some hydrogels exhibit stimulus-sensitive behavior that leads to undergoing conformational changes in response to various external variables such as pH, temperature, electric, magnetic and/or optical stimuli. Such materials have been called “smart” or “intelligent” hydrogels because the aforementioned behavior allows them to be useful for various biomedical applications such as site-specific and controlled drug delivery, tunable optics and biosensors, encapsulation of cells, molecular imaging, immobilization of cells, protein assays, separation and wastewater remediation, and in vitro tissue formation. Poly-N-isopropylacrylamide (PNIPAM) represents the most extensively-studied stimulus-sensitive polymer hydrogel material.

Studies on hydrogels already covered a broad domain in materials science; little attention has been paid, however, to hydrogels as hosts for versatile molecular luminophores. Though studies of ligand-lanthanide luminescent molecules in connection with hydrogels as host matrixes are known, similar studies using a transitional metal-based luminescent complex are yet unexplored, perhaps due to limited pursuit of watersoluble and stable systems that exhibit sufficiently bright phosphorescence at

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physiological conditions. Given the quantum leap that phosphorescent transition metal complexes have caused in photonic applications such as organic light-emitting diodes, it is important to pursue applications that take advantage of their remarkable photophysical properties by seeking strategies that enable their introduction to biocompatible media such as hydrogels. The work herein represent our first effort to fill this gap by introducing the water-soluble phosphorescent transition metal compound Na₈[Au(TPPTS)₃] into the polymer network of PNIPAM. (figure 2.1) Phosphorescent hydrogels can enable experiments such as tracking diffusional processes, monitoring phase transformations of entangled biopolymers, and sensing environmental stimuli. The conformational changes in response to external stimuli typically lead the hydrogels to undergo a reversible volume phase transition between a swollen state and a collapsed state. In their swollen state, hydrogels are usually transparent and colorless. Advances in instrumentation technology have rendered luminescence spectroscopy a very versatile and powerful yet readily accessible tool for the characterization of polymers and hydrogel systems. In order to track the gel swelling behavior or to monitor the location of gel particles in cells, fluorescent dyes or quantum dots are typically used so that the hydrogels are distinguished from the surrounding biological environment. In this process, fluorescent labels have some disadvantages that include short nanosecond lifetimes that may coincide with any biological background emission (auto-fluorescence), self-quenching effects, high photo-bleaching and weak fluorescence intensity at physiological pH. Some of these disadvantages are predicted to be overcome by different emission enhancement mechanisms such as the
enhancement in presence of either plasmonic colloidal metal nanoparticles\textsuperscript{22b} or a micelle medium.\textsuperscript{22d} Next-generation biological and chemical sensors are aimed to be developed from luminescent systems employing these mechanisms of enhancement. Though there is considerable literature work on explaining the mechanism of metal colloid-based enhancement with different fluorophores\textsuperscript{22b,c} little is known about the exact mechanism of fluorescence enhancement mechanism in presence of micelles. Fluorescence enhancement in presence of a micelle medium is predicted to result from a combination of various factors that include enhanced stability or solubility of the fluorophore and/or decrease in non-radiative decay of the fluorophore in presence of micelle medium; however, a definitive mechanism about fluorescence or phosphorescence enhancement in presence of micelle or scattering polymeric colloidal medium is not well-documented.

There are few examples in the literature explaining the effect of PNIPAM or other polymer-based systems on the luminescent properties of lanthanide complexes. Work by Huang and coworkers\textsuperscript{18} detailed interactions of Tb(III) with PNIPAM-co-styrene microspheres. McCoy and coworkers reported the incorporation of Eu(III)-based complexes in methylmethacrylate:hydroxyethylmethacrylate (MMA:HEMA) hydrogels.\textsuperscript{18} The Wang group showed the formation of PNIPAM polymer chain incorporating a Tb(III) complex in the solid state, leading to emission enhancement of Tb(III) in PNIPAM-Tb(III) systems due to effective intramolecular energy transfer from the PNIPAM polymer chain to the Tb(III) centers.\textsuperscript{18a} In this paper, we show that the phosphorescence of a three-coordinate gold(I) phosphor can be enhanced by more
than an order of magnitude compared to the situation in a plain aqueous medium at different temperatures. While Raman scattering enhancement is well-documented for detection of small molecules at very low concentrations,\textsuperscript{22c} the work herein paves the way to a wider scope of research by the simpler and more widely accessible method of steady-state photoluminescence spectroscopy to enable scattering-stimulated enhanced detection of molecules at very low concentrations.

\[
[Au(TPPTS)_3]^8^-. 
\]

\[\begin{array}{c}
\text{PNIPAM} \\
\end{array}\]

Figure 2.1 Structures of [Au(TPPTS)_3]^8^- (AuP) and thermosensitive polymer (NIPAM: N-isopropyl acrylamide).

Thus, we demonstrate the loading of a highly-stable water-soluble polyanionic three-coordinate gold(I) complex, [Au(TPPTS)_3]^8^-, into stimulus-responsive PNIPAM hydrogel colloidal particles in aqueous medium without sacrificing the environmental sensitivity of the hydrogel while simultaneously enhancing the Au-centered phosphorescence. The selection of three-coordinate Au(I) complexes as phosphors for this project is inspired by the work of Fackler, Assefa, and co-workers, who reported
that some of these complexes can maintain their luminescence even in aqueous solution,\textsuperscript{23} the work fits within the realm of our own interest in this class of complexes toward understanding their excited-state structure and the consequent impacts on photonic applications.\textsuperscript{24}

Figure 2.2 Schematic illustration for formation of PNIPAM microgels. [NIPA: N-isopropylacrylamide monomer, BIS: \(N,N'\)-methylene bis-acrylamide (chemical crosslinker), SDS: Sodium dodecyl sulfate (surfactant), KPS: Potassium persulfate (reaction initiator)]. Day light picture of PNIPAM-\textit{co}-allylamine microgel crystals.
Figure 2.3 Structure of different chemicals and illustration of reaction mechanism during formation of PNIPAM microgels (NIPA: N-isopropylacrylamide monomer, BIS: N,N methylene bis-acrylamide, SDS: Sodium dodecyl sulfate, KPS: Potassium persulfate).

2.2 Experimental Section

2.2.1 General Procedures

General syntheses were carried out under vacuum atmosphere or under purified argon using standard schlenk techniques. Millipore water was used for all workout techniques. Glassware was oven-dried at 150 °C overnight.
2.2.2 Physical Measurements

Luminescence measurements were carried out for purified materials. Steady-state luminescence spectra were acquired with a PTI QuantaMaster Model QM-4 scanning spectrofluorometer. The excitation and emission spectra were corrected for the wavelength-dependent lamp intensity and detector response, respectively. Lifetime data were obtained using a xenon arc flash lamp and phosphorescence detector. For quantum yield measurements relative method was adopted using quinine sulfate as standard. Liquid nitrogen was used for low temperature PL studies. Absorption spectra were acquired with a Perkin-Elmer Lambda 900 double-beam UV/VIS/NIR spectrophotometer for solutions of gold phosphor and turbidity ($\alpha$) of the microgel dispersions was measured vs. wavelength using a diode array UV-Visible spectrometer (Agilent 8453). The hydrodynamic radius of each sample was measured by dynamic light scattering (DLS). The details of the LLS instrumentation and theories have been described elsewhere. All measurements were done with scattering angle detection at 90°. The temperature of the samples was controlled by a circulation water bath (Brinkmann Lauda Super RM-6) to within ± 0.02 °C. The samples for all the dynamic light scattering analysis were prepared by homogenization followed by dilution with millipore water. Each sample was measured 3 times and the mean radius was reported. Zeta potential was measured on a zetasizer nano ZS (Malvern instruments) loading samples into maintenance-free cells. $^1$H and $^{31}$P{$^1$H} NMR spectra were recorded in D$_2$O at ambient temperature on a Varian spectrometer operating at 300 MHz for proton spectra. The chemical shifts in the $^{31}$P{$^1$H} NMR spectra are reported relative to 85% H$_3$PO$_4$ in D$_2$O.
2.2.3 Synthesis of Na₈[Au(TPPTS)₃] (AuP)

The phosphorescent gold compound Na₈[Au(TPPTS)₃] (figure 2.1) was synthesized by adding three equivalents of Na₉TPPTS to Au(tetrahydrothiophene)Cl, based on the procedure described in literature.²³ Briefly Au(THT)Cl (0.1 g, 3.1 X 10⁻⁴ mol) was dissolved in 10 ml of CH₂Cl₂ and the solution is stirred for 15 minutes before addition of TPPTS (0.5 g, 9.4 × 10⁻⁴ mol) along with 10 ml of water. After 5 hrs the biphasic mixture was filtered under vacuum. Final white powder was obtained by removal of solvents. The powder was re-dissolved in water to remove any suspension. The purity of the complex was ascertained via $^1$H and $^{31}$P{$^1$H} NMR, IR, electronic absorption and emission, and time-resolved luminescence spectroscopic data compared to the literature. Na₈[Au(TPPTS)₃]: $^{31}$P{$^1$H} NMR (D₂O): $\delta$ 43.5 ppm. Table 2.1 contains NMR data for AuP samples made using TPPTS ligands brought from two different companies (Strem chemicals and J&K chemicals). AuP made using TPPTS brought from J&K chemicals exhibited better purity confirmed from weak or insignificant TPPTS oxide peak.

2.2.4 Syntheses of PNIPAM Microgels

The hydrogel nanoparticles were made (figure 2.2 & 2.3) using the free radical precipitation polymerization method.⁸ Thus, 3.8 g of the N-isopropylacrylamide monomer, 0.2 g of either allylamine or acrylic acid (for PNIPAM-co-allylamine or PNIPAM-co-acrylic acid, respectively), 0.066 g of BIS, and 0.08 g of sodium dodecyl sulfate = “SDS” were dissolved in 245 g of distilled water.
Table 2.1 Structural characterization and photoluminescence properties of Na₈[Au(TPPTS)₃] (AuP) used in comparison with literature.

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Literature</th>
<th>Result</th>
</tr>
</thead>
<tbody>
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<td>UV-vis solution</td>
<td>2 abs max: 270 nm &amp; 280 nm</td>
<td>271 nm &amp; 280 nm</td>
</tr>
<tr>
<td>Steady state photoluminescence solution (RT)</td>
<td>513 Emi, 293 Exc</td>
<td>525 Emi, 285 Exc</td>
</tr>
<tr>
<td>Steady state photoluminescence solid (RT)</td>
<td>494 Emi</td>
<td>500 Emi and 350 Exc</td>
</tr>
<tr>
<td>Lifetime solution at RT</td>
<td>~1.9 µS</td>
<td>~ 2.04 ± 0.02 µS</td>
</tr>
<tr>
<td>$^{31}$P {$^{1}$H} NMR In D₂O</td>
<td>45.5 and 43.5 ppm ([Au(TPPTS)₂]$^5$ and [Au(TPPTS)₃]$^8$)</td>
<td>J&amp;K TPPTS: -6.02 and 34.2 ppm (Very weak) AuP: 45.03 and 42.13 ppm. (insignificant at 34.45 ppm) Strem TPPTS: -5.81 ppm and 34.5 (strong signal); AuP: 45.44 and 34.75 almost equal signals.</td>
</tr>
</tbody>
</table>

The solution was stirred under nitrogen gas atmosphere for 40 min and then placed into a 60 °C hot bath. A 5.0 mL solution of potassium persulfate = “KPS” (0.166 g) was then added to initiate the radical polymerization. The reaction was continued for 5 h under nitrogen atmosphere at 60 °C. The resultant PNIPAM-co-allylamine or PNIPAM-co-acrylic acid hydrogel colloidal particles were dialyzed against water for 1 week at room temperature to remove any unreacted small molecules. After further purification by ultra centrifugation, microgel crystals are formed by allowing the dispersions to settle at room temperature.
2.2.5 Syntheses of Gold Phosphor (AuP) Doped Hybrid PNIPAM Microgels

Loading of the phosphor was carried out by stirring 10 mL of a 1.5 wt% aqueous solution of the relevant PNIPAM microgel and 3 mL of a 0.001 M aqueous solution of Na₈[Au(TPPTS)₃] for 48 hours under argon atmosphere, followed by centrifugation at 25000 rpm for 3 hours. After every one hour of centrifugation the supernatant was discarded and the sediment washed with millipore water. After three washes, the microgel obtained was incubated in DI water at around 22 °C for 2 days, allowing the formation of Au phosphor-loaded PNIPAM-co-allylamine or PNIPAM-co-acrylic acid microgel colloids or colloidal crystals.

2.2.6 Syntheses of Phosphorescent Crystalline PNIPAM Microgel Networks

Crystalline luminescent microgels in water obtained after incubation were crosslinked using di-glutaraldehyde as described elsewhere.⁸ Phosphorescent PNIPAM-co-allylamine crystalline microgel was added to di-glutaraldehyde at neutral pH followed by incubation at 4 °C for chemical crosslinking. After 48 hours, the fully crosslinked crystalline microgel network was removed and washed twice before storing in fresh millipore water, resulting in formation of stable phosphorescent PNIPAM-co-allylamine engineered hydrogel crystal networks.

2.2.7 Formation of Phosphorescent Lyophilized PNIPAM Microgels

The dispersion of crystalline arrays of phosphorescent microgels was freeze-dried. After 7 days, the freeze-dried microgel was characterized by luminescence spectroscopy at room temperature, and then re-dispersed in fresh millipore water to check the loading stability of the gold phosphor. The re-dispersion was carried out by
dipping the freeze-dried microgel into fresh millipore water and then waiting for 24 hours to check the photoluminescence stability of the re-dispersed phosphorescent gel.

2.3 Results

2.3.1 Characterization of PNIPAM Microgel Dispersions

Formation of PNIPAM microgels was characterized from measuring the turbidity and average hydrodynamic radius of microgels. The size and distribution for typical PNIPAM-co-allylamine nanoparticles were shown in figure 2.4. In figure 2.4A, the PNIPAM-co-allylamine nanoparticles were narrowly distributed with average hydrodynamic radii (R_h) around 110 nm. The formation of PNIPAM nanoparticles may be understood in terms of polymerization of NIPA monomer and resulting in crosslinked polymer chains in presence of BIS (N,N, methylene bis-acrylamide) as chemical crosslinker.

![Graph](image)

Figure 2.4 A) Hydrodynamic radius (R_h) distribution of PNIPAM-co-allylamine microgel dispersions. B) Turbidity of a typical PNIPAM-co-allylamine microgel dispersions at room temperature (inset shows picture of microgel dispersions).
The dispersions with varying polymer concentrations exhibit iridescent colors which indicate that the particles self-assemble into an orderly arrangement. (Inset picture in figure 2.4A) The color observed in the dispersions is due to diffraction from ordered colloidal arrays with a lattice spacing on the order of the wavelength of visible light according to the Bragg law: \( 2n dsin\theta = m\lambda \), where \( n \) is the mean refractive index of the dispersion, \( \theta \) the diffraction angle, \( d \) the lattice spacing, \( m \) the diffraction order, and \( \lambda \) the wavelength of diffracted light. Figure 2.4B shows the turbidity of the PNIPAM-co-allylamine nanoparticle dispersions as a function of wavelength.\(^8\)

2.3.2 Spectroscopic Characterization of \( \text{Na}_8[\text{Au(TPPTS)}_3] \) (AuP)

\( \text{Na}_8[\text{Au(TPPTS)}_3] \) comprises a highly water soluble three-coordinate Au(I) anionic complex that exhibits phosphorescence at room temperature both in aqueous solution and the solid state. According to the literature, \( \text{Na}_8[\text{Au(TPPTS)}_3] \) exhibits a broad emission band maximum at 494 nm in the solid state at room temperature, whereas at 77 K the emission intensity increases and undergoes a blue shift in peak maximum to 486 nm. In aqueous solution, the \([\text{Au(TPPTS)}_3]^{8-}\) emission is red-shifted with unsymmetrical broad emission band centering approximately at 515 nm. The absorption and photoluminescence spectra we obtained for the gold phosphor samples we synthesized were such that an aqueous solution of 0.001 M \([\text{Au(TPPTS)}_3]^{8-}\) exhibits a strong emission maximum at 525 nm and excitation maximum at 292 nm. The broad green emission band in solution is blue-shifted to turquoise-blue in the solid state with unsymmetrical broad emission at 500 nm with excitation maximum at 356 nm. The
photophysical properties observed for our samples synthesized are in overall good agreement with those reported by Assefa et al\textsuperscript{23} as observed in figure 2.5 and 2.6.

2.3.3 Luminescence Spectra for Different Forms of AuP Loaded Hydrogels

The gold phosphor has been successfully loaded into three forms of PNIPAM-co-allylamine hydrogel systems: microgels in water, chemically-crosslinked bulk hydrogel, and lyophilized freeze-dried xerogel. Schematic representation (figure 2.7) of loading AuP in to PNIPAM microgels resulting in phosphorescent hybrid gels is shown. Figure 2.8 shows that these different forms of hydrogel systems after incorporating the gold phosphor exhibit similar photoluminescence spectra to those for the gold phosphor itself in aqueous solution or the solid state. These results suggests that the phosphor molecule retains its optical properties in its native form even in presence of different hydrogel environments, which would be very attractive for different biological applications as most of the fluorescent dyes are expected to be sensitive to changes in microenvironment.\textsuperscript{22} Gold Phosphor loaded PNIPAM-\textit{co}-allylamine microgel dispersion (figure 2.9A) and the bulk hydrogel (figure 2.9B) exhibit broad unsymmetrical green emission with $\lambda_{\text{max}}$ near 525 nm, mirroring the emission of an aqueous solution of the phosphor.
Figure 2.5  Steady state photoluminescence (solid & aqueous solution) and electronic absorption spectra (aqueous solution) of Na₈[Au(TPPTS)₃] (AuP).
Figure 2.6  Time-resolved photoluminescence decay for a typical AuP solution at room temperature measured using xenon flash lamp excitation (337 nm) and N₂ laser (337 nm). The decay analysis included deconvolution of the pulsed excitation signal using the Felix software for the PTI instrument (inset shows lifetime values and other parameters).
Figure 2.7 $^{31}$P{$^1$H} NMR spectra in D$_2$O for the Na$_8$[Au(TPPTS)$_3$] sample prepared in this work (A) vs. the free TPPTS ligand (B). Note the main broad peaks at 45.04 & 42.14 ppm for the Na$_8$[Au(TPPTS)$_3$] complex concomitant with low intensities of peaks due to free ligand, and the low intensity of the TPPTS oxide peak at 34.2 ppm in the spectrum of the free ligand (purchased as 95% purity).
Figure 2.8 Loading of gold phosphor in to PNIPAM-co-allylamine microgels based on electrostatic interactions between positively charged PNIPAM microgel and polyanionic gold phosphor.
Figure 2.9  Steady state photoluminescence spectra of different forms of phosphorescent hydrogels at room temperature. A) Microgels. B) Chemically crosslinked bulk hydrogel. C) Freeze dried xerogel. D) Redispersion of luminescent xerogel in water (picture taken at $\lambda_{252}$ nm UV exposure).
Figure 2.10 Schematic illustrations for formation of chemically crosslinked phosphorescent bulk hydrogel network from phosphorescent PNIPAM-co-allylamine microgels.
Figure 2.11 Monitoring time dependent changes in photoluminescence emission from phosphorescent PNIPAM-co-allylamine microgels for four days (instrumental parameters are kept constant; sample is subjected to centrifugation for 5-10 minutes before testing).

Formation of bulk phosphorescent hydrogels by chemical crosslinking of phosphorescent PNIPAM-co-allylamine microgels is shown in figure 2.10. An increase in emission intensity accompanied with a blue shift to 490 nm is observed in the lyophilized freeze-dried xerogel (figure 2.9C) at room temperature. The characteristic broad unsymmetrical emission bands from the gold phosphor are retained with minor shifts. Previous studies based on quantum mechanical computations illustrated the feasibility of systematic tuning of emission by controlling steric bulk in very similar three-coordinate Au(I) complexes, suggesting a Jahn-Teller distortion from a trigonal-
planar ground state toward a T-shaped phosphorescent excited state. PNIPAM microgels and bulk hydrogels in their swollen form in aqueous solution allows for a relatively unencumbered distortion toward the T-shape geometry of the phosphorescent state, whereas the freeze-dried xerogel form entails a significant constraint to such a large molecular distortion. The underlying “luminescence rigidochromism” phenomenon in such three-coordinate d^{10} systems is addressed at a fundamental level elsewhere for water-insoluble complexes; the phenomenon is well-known for other classes of phosphorescent molecules, including their use to monitor sol-gel-xerogel and other setting transformations in inorganic silicates.

2.3.4 Testing Stability of AuP Loaded PNIPAM Phosphorescent Microgels

Stability of phosphorescent PNIPAM-co-allylamine microgels was also tested with respective to time to ascertain their ability to retain photoluminescence properties. Luminophores are notorious for their quenching abilities due to surrounding environmental influences. Photoluminescence of stable phosphorescent PNIPAM-co-allylamine microgels is tested after every 24 hours for four days by storing them at room temperature and light. Care is taken to keep all the instrumental parameters constant for comparing PL intensities. Retained photoluminescence for monitored time (4 days) convincingly demonstrates stability of phosphorescent microgels (figure 2.11) with minor variations in emission peak profile due to complexity of microgel matrix. The difference in PL intensity is attributed to variations in instrumental parameters on different days.
2.3.5 Influence of AuP on Thermosensitive Properties of PNIPAM Microgels

PNIPAM-co-allylamine microgels in water with polymer concentrations within 1.5 to 5.0 wt% can self-assemble into colloidal arrays with bright iridescent patterns at room temperature discussed elsewhere in detail. Even after loading the gold phosphor, hybrid microgels at a concentration of about 2.0 wt% self-assemble into a temperature sensitive ordered crystalline arrangement (figure 2.12A). Because of the Bragg diffraction from the crystalline phase at 21°C, the UV-visible spectrum exhibits a sharp attenuation peak measured with UV-visible spectroscopy. At an elevated temperature (24°C), both the peak height and peak wavelength decrease with time, indicating melting of crystalline structures (figure 2.12A). The shift in the sharp Bragg diffraction peak and its disappearance upon temperature increase in the hybrid phosphorescent microgels here is remarkably similar to the behavior with the unloaded PNIPAM microgel systems. This result signifies that the presence of gold phosphor does not result in sacrificing either formation or stimuli responsiveness of PNIPAM microgel crystals. The magnitude of electrostatic attraction or repulsion is considered a key factor in controlling the dispersion mechanism of colloids in general. Loading the gold phosphor into PNIPAM-co-allylamine microgel dispersions due to electrostatic interactions in the aqueous medium is explained based on changes in dynamic light scattering data, zeta potential values, pH and functional group-dependent luminescence data obtained from various microgels studied at room temperature.
Figure 2.12 A) Changes in turbidity vs wavelength for crystalline phosphorescent PNIPAM-co-allylamine microgel dispersions at two different temperatures (a: at 21 °C; b: at 24 °C; c: at 24 °C after 5 minutes; a: at 24 °C after 10 minutes). B) Changes in dynamic light scattering data for PNIPAM-co-allylamine microgel before and after loading the gold phosphor.

Previous work by Frisken and co-workers⁸ illustrated a sharp decrease in particle size by introducing charged or ionic components into similar PNIPAM microgel systems. Figure 2.12 B shows the hydrodynamic radius changes in PNIPAM-co-allylamine microgels after loading the gold phosphor, giving rise to a 23% decrease in $R_h$ values, from 118 nm to 92 nm, under identical concentration and experimental conditions. Strong electrostatic interactions between the microgel particles and the gold phosphor also results in improved particle distribution, as clearly manifested by the reduction in the breadth of the DLS peak of the microgel dispersion after loading the gold phosphor figure 2.12B.
The changes in hydrodynamic radius emphasize charge component interactions between the gold phosphor and PNIPAM-co-allylamine microgel particles as observed elsewhere in similar microgel particles. Examination of zeta potential values also confirms the same conclusion. Thus, the +25.1 mV zeta potential value for the positively charged microgel at pH 5.0 before loading, which is attributed to protonated allylamine groups of microgel dispersion decreases to +5.3 mV immediately after adding the gold phosphor. This decrease in zeta potential values in hybrid microgels clearly indicates adsorption of negatively charged phosphorescent complexes onto positively charged microgel spheres. Based on these results, we assume strong polyelectrolyte interactions between PNIPAM-co-allylamine microgel and the gold phosphor. Figure 2.8 illustrates that the anionic Au(I) complex can effectively screen the interaction between positive charges on the microgel and lead to size reduction of the microgel. It is noted that other factors may also contribute the reduction of the particle size. For example, for charged colloids the diffusion coefficient is larger at lower ionic strengths, which corresponds to smaller size. The crystallinity of PNIPAM microgel crystals can be lost by shaking the dispersion at room temperature or heating the colloidal crystals just above room temperature. To stabilize the colloidal structure, the crystalline arrays of gold phosphor-loaded microgel particles were covalently-crosslinked with di-glutaraldehyde following a published procedure. The resulting hybrid bulk hydrogel network (figure 2.10) not only has a stable colloidal crystalline structure but also retains the broad green emission peak unchanged at 525 nm (figure 2.9B). It is noted that the fabrication of the hydrogel network form involves heating, slow cooling,
followed by soaking the sample for two days in fresh millipore water to remove excess unreacted di-glutaraldehyde. If the gold phosphor were not well-entrapped within the matrix of the microgel, the so formed network of microgel particles would not have retained any emission because the gold phosphor would have leached away during the process of soaking and water exchange. This suggests the presence of a strong electrostatic attraction between the PNIPAM-co-allylamine microgel and the gold phosphor. To our knowledge, this is the first example of a luminescent crystalline network that has been successfully obtained in aqueous medium.

Photoluminescence spectra recorded at room temperature for the lyophilized sample (figure 2.9C) show a broad turquoise emission with maximum at 481 nm, similar to that for the gold phosphor solid. These spectra, however, are significantly different from those of the hybrid microgel aqueous samples with a significant blue shift. The lyophilized hybrid microgel (figure 2.9D) can be re-dispersed into fresh millipore water, re-generating the green emission characteristic of the aqueous microgel. If [Au(TPPTS)₃]⁸⁻ complexes were not strongly adsorbed or loaded into the freeze-dried form of the microgel, the heterogeneous physical mixture on re-dispersing into fresh millipore water would have exhibited green emission uniformly from the entire aqueous solution. However, only the re-swollen portion dispersed at the top exhibits green emission with no emission observed from the supernatant. These findings demonstrate stable loading of the gold phosphor into PNIPAM-co-allylamine microgels both in the solid and solution phase.
Figure 2.13 Steady state photoluminescence spectra for pH and functional group dependent loading of AuP into PNIPAM microgels. a) For allylamine at pH 4.0 b) For allylamine at pH 9.0 c) For acrylic acid at pH 4.0 d) For acrylic acid at pH 9.0

2.3.6 pH and Functional Group Dependence of AuP Loading

Guided by the steady state photoluminescence data, loading of the gold phosphor into PNIPAM microgels is determined to depend on both the identity of the co-monomer and the pH of the microgel solution. Loading studies were conducted for PNIPAM microgels with allylamine and acrylic acid co-monomer functionalities at acidic and basic pH values. Figure 2.13 shows steady-state photoluminescence data for the gold phosphor loaded under these variations. The pH of the PNIPAM-co-allylamine
microgel dispersion was adjusted from acidic values within 5.5-4.0 to basic values near 9.0 by addition of suitable volumes of 0.1 M acetic acid and 0.1 M ammonium hydroxide solutions, respectively, to the microgel dispersion. The efficiency of the gold phosphor loading can be readily determined by contrasting the photoluminescence intensity of supernatants vs sediments in each of the four samples studied. The most efficient loading is observed in PNIPAM-co-allylamine microgels at pH 4.0, whereas very little or no loading is observed in PNIPAM-co-acrylic acid microgels at pH 9.0 (figure 2.13). PNIPAM-co-allylamine microgels at pH 4.0 exhibit efficient loading of the gold phosphor. But for the same system at pH 9.0, the PL data indicate incomplete or inefficient loading. In the case of acrylic acid-based microgels at pH 9.0, on the other hand, all the gold phosphor was retained in the supernatant while the sediment did not exhibit any emission (figure 2.13), indicating 0% loading. At pH 4.0, the same acrylic acid microgel shows < 20% emission intensity from the sediment of the microgel while most of the gold phosphor was still in the supernatant without being loaded. Among the four samples studied, only PNIPAM-co-allylamine microgels at acidic pH showed efficient loading of the Au phosphor, demonstrating the importance of pH and nature of the co-monomer. Attachment of the gold phosphor to the surface of the microgel is, therefore, attributed to electrostatic interactions between the anionic sulfonate groups of the gold phosphor and the allylammonium groups of the co-monomer in the PNIPAM-co-allylamine microgel. For the PNIPAM-co-acrylic acid microgel, the repulsive interaction between negatively charged gold phosphor and negatively charged carboxyl group in aqueous medium at pH 9.0 results in inefficient loading. The results also negate
possible alternative mechanisms such as coordination of neutral amine groups from the PNIPAM-co-allylamine microgel or anionic carboxylate groups from the PNIPAM-co-acrylic acid microgel at basic pH. Both the stability of the three-coordinate geometry and softness of the Au(I) center are likely reasons for the insignificance of such possible coordination mechanisms. The strength of electrostatic interactions at different pH can be readily predicted based on pKₐ values of different interacting functional moieties. The pKₐ of allylamine and acrylic acid are 9.69 and 4.25, respectively, whereas the pKₐ of benzene sulfonate is -6.5. Thus, one would predict that electrostatic interactions between the -NH₃⁺ allylammonium groups amply dispersed at the surface of each microgel nanoparticle and the nine dissociated -SO₃⁻ phenylsulfonate groups of each gold complex would result in efficient loading of the gold phosphor into the PNIPAM-co-allylamine microgel particles under both acidic and neutral pH.

A previous report by Gong et al. showed the entrapment of photoluminescent quantum dot (QD) nanocrystals into PNIPAM microspheres due to centrifugation force and weak hydrogen bonding between thioglycerol-capped CdTe nanocrystals and PNIPAM microspheres;²⁷ the stable fluorescent microspheres were observed after centrifugation only in the sediment compared to the supernatant of the sample. Uniform size of the nanocrystals and aggregation were controlling factors for loading such QD nanocrystals into PNIPAM microspheres. However, the present results suggest that such uniformity and aggregation problems can be avoided upon loading polyelectrolytic molecular phosphors like Na₈[Au(TPPTS)₃] into PNIPAM microspheres. Photoluminescence was reported in lanthanide-doped hydrogel systems due to energy
transfer from PNIPAM-\textit{co}-styrene to Tb(III)$^{18b}$ or MMA:HEMA to Eu(III)$^{18}$ phosphorescent centers, whereas the systems herein do not contain a chromophore in the hydrogel host as the transition metal phosphorescent center contains chromophoric ligands already. The Eu(III)-doped MMA:HEMA hydrogel films exhibited pH-dependent photoluminescence due to populating the lanthanide excited state by sensitization. Phosphorescent transition metals have been incorporated in gel soft materials besides hydrogels, e.g., as described in the work of Dunn and Zink for Re(I) complexes in orthosilicate sol-gel systems.$^{26}$ Aida and co-workers for Ag$^+$ adducts of trinuclear Au(I) complexes with 4-(3,5-dioctadecyloxybenzyl)-3,5-dimethylpyrazole organogels,$^{28}$ and Yam and co-workers for Pt(II) alkynyl complexes in 2,6-bis($N$-dodecylbenzimidazol-2'-yl)pyridine.$^{29}$ We are also unaware of any precedents of hydrogel environments entrapping molecular or semiconductor species to exhibit photoluminescence in such a broad range of gel forms and/or responsiveness to the range of experimental variations that we report in this work.
Figure 2.14 Comparing photoluminescence enhancements in hybrid microgels at room temperature as a function of gold phosphor concentration. The spectra shown are before and after addition of 0.1 mL of a 2% solution of PNIPAM-co-allylamine microgel to a 2.5 mL aqueous solution of the gold phosphor at pH 5.5. A) [Au] = 3× 10^{-6} M. B) [Au] = 5× 10^{-6} M. C) [Au] = 1× 10^{-5} M. D) [Au] = 5× 10^{-5} M. (All samples are monitored using same excitation wavelength).
Figure 2.15 Photoluminescence enhancement of Au phosphor with respective to PNIPAM-co-allylamine microgel concentration. \([\text{AuP}] = \sim 1 \times 10^{-6} \text{ M (2.50 mL)}\) and microgel wt\% \(\sim 2.0\) at pH 5.5 and room temperature. (Left: Titration data; Right: Stern-Volmer graph).

Figure 2.16 Photoluminescence enhancement of freshly made AuP with respective to PNIPAM-co-allylamine microgel concentration. \([\text{AuP}] = 1 \times 10^{-5} \text{ M (3.0 mL)}\) and 3.5 wt\% microgel at pH 5.5 and room temperature. (Left: Titration data; Right: Stern-Volmer graph. Inset shows difference in luminescence intensity between bulk AuP solution and hybrid microgel solution).
Figure 2.17 A) pH dependent photoluminescence enhancement in hybrid microgels as a function of microgel concentration, on titration of a $1 \times 10^{-6}$ M gold phosphor aqueous solution by addition of $x$ mL quantities of 2.0 wt% PNIPAM-co-allylamine microgel at pH 9.0 and room temperature. B) Stern Volmer graph for the same; the PL enhancement ratio $I/I_0$ is not corrected for dilution or inner-filter effects that contribute to the enhancement drop at higher gel volumes.

2.3.7 Photoluminescence Enhancement of Gold phosphor in Presence of PNIPAM-co-allylamine Microgels

One can attain significant photoluminescence (PL) enhancement in multiple fashions upon incorporation of the gold phosphor in all hydrogel forms in this work. A dramatic manifestation is noticed upon titrating the gold phosphor with PNIPAM-co-allylamine microgels. The extent of PL enhancement can be varied by controlling the gold phosphor concentration, temperature above or below the volume phase transition temperature of the microgel, pH, etc. Titration experiments at various gold phosphor concentrations while keeping the concentration of the microgel constant are shown in figure 2.14 while the effect of the microgel concentration is shown in figure 2.13. The
highest ratio of PL enhancement is attained at the lowest concentration of gold phosphor tested. In this set of experiments, we attain a 4-fold, 9-fold, 14-fold, 17-fold, and 50-fold PL enhancement upon decreasing the gold phosphor concentration along the direction $5 \times 10^{-5}$ M, $1 \times 10^{-5}$ M, $5 \times 10^{-6}$ M, $3 \times 10^{-6}$ M, and $1 \times 10^{-6}$ M, respectively.

Table 2.2 Photoluminescence enhancement and lifetime values for hybrid microgels as a function of AuP concentration (purging with inert gas (N2) or lack thereof). The data were obtained upon titration of a freshly synthesized AuP (3.00 mL) with ~3.5 wt% microgel at pH 5.5 and room temperature. The enhancement values shown are typically attained after addition of 50-60 μL and do not account for the small dilution factor. (NA: could not record)

<table>
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<th>AuP</th>
<th>10⁻³ M</th>
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<tr>
<td>PL</td>
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<td>5.5 times</td>
<td>62.8 after 36 hrs:37.4 times</td>
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<td>Γ/μS</td>
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</tr>
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</tr>
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<td>2.79±0.10</td>
<td>2.92±0.09</td>
<td>2.78±0.12</td>
</tr>
<tr>
<td>(hybrid)</td>
<td></td>
<td></td>
<td></td>
<td>3.48±0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.38±0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.75±0.23</td>
</tr>
</tbody>
</table>
Figure 2.18 Time-resolved photoluminescence decay of a typical photoluminescence enhancement AuP solution with and without microgel using xenon flash lamp excitation (Exc 285 nm) and a gated microsecond detector. The decay analysis included deconvolution of the pulsed excitation signal using the felix software for the PTI instrument. (Ar purged [Au] = 5 × 10^{-5} M).

Here we propose that, at a fixed microgel concentration, only a certain number of phosphorescent molecules will be loaded or are present in the vicinity of the microgel surface so as to contribute to the PL enhancement. The number of these molecules will not proportionally increase with an increase in concentration of the gold phosphor as concentration of microgel is fixed. So at higher concentration of gold phosphor, molecules contributing to PL enhancement within the microgel will not increase compared to the number of phosphor molecules in the control or plain aqueous
solution. This results in a decrease in the ratio of emission intensity enhancement at higher concentrations of the gold phosphor within the microgel environment, as shown. Titration experiments were also performed at basic (pH 9.0) conditions (figure 2.17); those attained smaller PL enhancement, as expected in view of the results of the previous section. The microgel concentration affects the PL enhancement in all the cases. For example, figure 2.15 shows gradual PL enhancements up to 70-fold on addition of 0.2 mL of microgel while further microgel aliquots lead to slow decrease or a plateau; the inner-filter effect becomes relevant at higher gel concentrations. A freshly synthesized sample of the Au phosphor was titrated with smaller quantities of microgel to diminish the inner-filter effect while increasing the PL enhancement up to 157-fold (figure 2.16).

These results signify that, at a fixed concentration of gold phosphor, the highest PL enhancement is attained at a critical concentration of microgel, which is very similar to the micellar effect on fluorescence enhancement as noticed by Takeuchi. The collection of experiments we performed suggests delicate interplay between the critical microgel concentration, concentration of the gold phosphor, pH, temperature, ionic strength, sample freshness, molecular weight and refractive index of polymer gel, etc. Although exhaustive were not performed but quantitative studies to maximize the relative or absolute PL enhancement under all conditions are detailed. All these data along with life times are tabulated in table 2.2. Time resolved photoluminescence decay for a typical PL enhancement sample with and without microgel is shown in figure 2.18.
Figure 2.19 Photoluminescence enhancement of the hybrid PNIPAM-co-allylamine microgels upon heating from room temperature (RT) to 37 °C, contrasted with quenching for aqueous bulk gold phosphor.

An interesting situation is shown in figure 2.19 in which a blue shift in emission maximum, from 532 nm to 515 nm, concomitant with emission enhancement, is seen upon heating microgels from ambient temperature to around 37 °C. The luminescence data in figure 2.19 suggest that the physical state of heated hybrid microgel sample is intermediate between solution form and the lyophilized dried form. This result is consistent with the luminescence rigidochromism phenomenon discussed above for three-coordinate Au (I) complexes. Thus, the Jahn-Teller T-shape distortion in the phosphorescent excited state from the trigonal ground state geometry becomes more
greatly hampered as one proceeds from the fluid solution, to the more viscous heated gel, and then to the lyophilized dried gel, leading to greater blue shift in that direction. As shown in the temperature-dependent titrations in figure 2.19, heating the gold phosphor microgel system to 37 °C results in not only a 620 cm⁻¹ blue shift in emission maximum but also approximately 50% PL enhancement. This thermally-induced PL enhancement is fully reversible, as shown by three heating/cooling cycles in figure 2.19. The PL enhancement on heating is at least partially ascribed to the change of the microenvironment from hydrophilic to hydrophobic as PNIPAM is heated above its lower critical solution temperature (LCST, which is about 34 °C). The PL enhancement is likely further assisted by an increase of the refractive index of the microgel when the temperatures is higher than the LCST, as observed previously in III-V semiconductor QD- or ZnO-embedded PNIPAM microgels.²⁹ The control study for an aqueous solution of the gold phosphor in absence of the PNIPAM microgel leads to quenching upon heating from ambient temperature to 37 °C, as expected due to increased non-radiative decay by multiphonon de-excitation to the ground state. This result is promising for possible utilization of the phosphorescent hybrid systems here for live bioimaging applications, for which a strong PL signal at the physiological temperature of 37 °C is critically important.

Notable literature precedents included the work of Li et al. in which a 4% PL enhancement was reported from different size quantum dots embedded in thermosensitive PNIPAM gels at room temperature.²¹ These authors attained nearly 10-fold quenching upon heating the PNIPAM-QD hybrid gels to near body temperature,
unlike the dramatic enhancement we obtain in this work (figure 2.19). Zhou and co-workers observed a 1.72-fold PL enhancement for terbium citrate upon binding to silver nanoparticles in solution, attributed to electric field enhancement around terbium from the electron plasmon resonance of silver nanoparticles.\textsuperscript{22} Takeuchi reported more significant PL enhancements of 8-20x for dansyl amino acid probes in presence of different surfactants due to micellar effects.\textsuperscript{22} The 1-2 order-of-magnitude PL enhancement seen herein in aqueous hydrogel media is rivaled only in purely organic media, which are usually less susceptible to quenching than aqueous media; e.g., a 40-fold PL enhancement for pyrene was reported upon embedding the fluorophore in hydrophobic polystyrene (toluene solution).\textsuperscript{21} As for the origin of PL sensitization, there are several mechanisms that have been invoked in prior literature precedents. Various groups reported that when a fluorophore is positioned within a restricted space provided by micelles or metallic nanoparticles, the electronic absorption and fluorescence spectra often change due to the effect of the microenvironment on fluorophores.\textsuperscript{22} Increase in structural rigidity accompanied by decrease in accessibility to the surrounding aqueous medium in presence of micelles decreases the non-radiative deactivation, resulting in fluorescence enhancement in fluorophores positioned within the microenvironment of micelles or vesicles.\textsuperscript{22} In case of fluorophores localized close to metallic nanoparticles, fluorescence enhancement is explained as being due to intensified electromagnetic field from electronic plasmons, resulting in increased excitation rate or radiative rates.\textsuperscript{22} Finally, an increase in the refractive index of the microgel was invoked to explain PL enhancement in PNIPAM microgels embedded with
III-V semiconductor quantum dots or ZnO nanoparticles. These different explanations are all relevant to the PL enhancement observed herein. PNIPAM hydrogels indeed offer a micelle environment because they contain both hydrophilic and hydrophobic parts, thus limiting the quenching by water molecules compared to analogous aqueous solutions of the gold phosphor that do not contain the hydrogel. Both the electrostatic interactions (between the anionic gold complex and cationic ammonium groups of the hydrogel) and vander Waals or other hydrophobic interactions (between hydrocarbon and other non-polar parts of the phosphor and PNIPAM moieties) act to reduce water quenching and thus the rate of non-radiative decay. In addition to the drastic PL enhancements seen in figures 2.14 to 2.16, we have observed an increase of the phosphorescence lifetimes (from 1.4 μs to 2.6 μs in a typical example, table 2.2) for solutions that exhibit an order-of-magnitude PL intensity enhancement on addition of PNIPAM hydrogels to the aqueous solutions of the gold phosphor. The magnitude of the lifetime increase is less than the corresponding intensity increase, suggesting that suppressed non-radiative deactivation via multiphonon relaxation to the ground state is not solely responsible for the PL enhancement see. Therefore, gel scattering must play at least some role in the PL sensitization, consistent with multiple literature precedents for other classes of emitters.
Figure 2.20 Absolute quantum yield data of gold phosphor solution and gold phosphor doped hybrid PNIPAM-co-allylamine microgels at room temperature. QS: Quinine sulfate, AuP: Gold phosphor, AuP/gel: Gold phosphor doped hybrid microgel. A) Absorption spectra. B) Photoluminescence spectra.

Table 2.3 Table summarizing quantum yield calculations for AuP and hybrid phosphorescent microgel at two different excitation wavelengths against standard fluorophore. QS: Quinine sulfate standard; AuP: Na₈[Au(TPPTS)₃]; Gel: Hybrid phosphorescent PNIPAM-co-allylamine gel. In QY column values enclosed for hybrid are obtained by including turbidity of PNIPAM gels for calculations. (Refractive index for water is taken as 1.33 and for gels taken as 1.34; S/A: Scattering/Absorbance ratio was found to be 1.41 and 2.00 at 275 nm and 285 nm excitation wavelengths respectively).

<table>
<thead>
<tr>
<th>Exi λₙₘ (nm)</th>
<th>Emission (Peak area)</th>
<th>Peak Absorbance</th>
<th>Quantum Yield (QY)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QS</td>
<td>AuP</td>
<td>Hybrid</td>
</tr>
<tr>
<td>275</td>
<td>1.48 E+08</td>
<td>1.02 E+07</td>
<td>1.06 E+08</td>
</tr>
<tr>
<td>285</td>
<td>2.08 E+08</td>
<td>7.40 E+06</td>
<td>8.14 E+07</td>
</tr>
</tbody>
</table>
2.3.8 Relative Photoluminescence Quantum Yield (QY)

Luminescence quantum yield (Φ) gives efficiency of luminescence process (fluorescence or phosphorescence). It is generally defined as the ratio of the number of photons emitted to the number of photons absorbed during excitation and de-excitation process of a luminophore.

\[
\Phi_{(PL)} = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}}
\]

The maximum photoluminescence quantum yield would be 1 (100 %) when all photons absorbed results in photoemission. The most common method for determining quantum yield of a luminophore is comparison with standard of known quantum yield. Quinine sulfate is one of the common standard fluorophores. Fackler and co-workers have determined.\(^{23}\) Relative luminescence quantum yield of \([Au(TPPTS)\_3]^{-}\) as \(\Phi=0.042\), measuring against quinine sulfate (\(\Phi=0.56\)). Relative quantum yield was calculated using equation

\[
\Phi_{(SP)} = \Phi_{(st)} \left( \frac{I_{sp}}{I_{st}} \right) \left( \frac{A_{st}}{A_{sp}} \right) \left( \frac{\eta^2_{sp}}{\eta^2_{st}} \right)
\]

Where sp= sample, st= standard, I the integrated areas of the corrected emission spectra, A is the absorbance value at the excitation wavelength of sample and standard and \(\eta\) is the refractive index of solvents respectively.

Figure 2.20 shows PL emission spectra and absorption spectra of all samples used during QY calculations. Absorbance and emission are recorded exactly for the
same samples under constant experimental conditions. Emission spectra of QS, bulk AuP solution and hybrid gel are recorded for two different excitation wavelengths (275 nm & 285 nm). For QY calculations, two values of absorbance are considered for hybrid gel, one absorbance of bulk AuP solution itself and other value is change in absorbance due to turbidity of microgel. Peak integrated area, absorbance and quantum yield values for [Au(TPPTS)₃]Na₈ are tabulated in table 2.3. For hybrid gels two different QY values are reported based on two different absorbance values. QY value for bulk AuP solution is in good agreement with literature which underlines legitimacy of these results. Increase in relative quantum yield upon PL emission enhancement from hybrid microgel sample was not proportional to increase in emission intensity of hybrid microgels due to interference from turbidity of microgel dispersions which originate mainly from absorption, scattering and interference property of microgels.⁸ Though calculations were performed involving or excluding microgel turbidity but it was difficult to subtract real scattering value from absorbance of hybrid gels. So further detailed investigation is required to correlate PL enhancement and QY of hybrid microgels at different concentrations of AuP and microgel in similar lines with PL enhancement studies.

2.4 Conclusions

A simple route for syntheses of stimuli-sensitive phosphorescent hydrogel microspheres by incorporating a transition metal coordination compound has been discovered, Na₈[Au(TPPTS)₃], as phosphor into PNIPAM microgels. The resulting hybrid material exhibited sensitized Au-centered emission compared to that of the gold
complex in water both above and below the volume phase transition temperature of the microgel. The resulting phosphorescent microspheres showed decreased size and PL enhancement with particularly high phosphorescence sensitization at physiological pH and temperature. The results show strong dependency of the phosphor loading on pH and nature of functional group to maximize the interactions between the PNIPAM microgel host and the gold phosphor guest. These results encourage us to develop new classes of water-soluble transition metal complexes which can serve multiple functions to act as phosphorescent physical crosslinkers of various biopolymers, biological labeling reagents for imaging, and/or optical sensors for various biological and environmental applications.

2.5 References


CHAPTER 3 

IN SITU SYNTHESIS AND INVERSE THERMOREVERSIBLE GELATION OF BIOCOMPATIBLE LINEAR POLYMERS BY Au(I) PHOSPHORS TOWARDS BIOLOGICAL IMAGING AND HEAVY METAL SENSING APPLICATIONS

3.1 Introduction

Luminescent nanomaterials represent one of the widely developing fields in nanomedicine and biology for their tendency to probe and gain information about molecular environment relying on sensitivity of luminophore. Especially syntheses of polymer coated luminescent nanoparticles are extensively pursued these days for their brightness, photo-stability and robust platform features for targeting, biodetection, optical imaging, and sensing and delivery capabilities. On encapsulation, each nanoparticle can accommodate thousands of luminophores, which enhance sensitivity, less affected by the external environment and photobleaching. These features have drawn strong surge in syntheses of luminescent polymeric nanoparticles. Nanoparticles especially from biocompatible polymers with controllable size are of great importance because of their non-toxic significance and technological applications. Despite the huge potential, for luminescent polymeric nanoparticles idea of luminescent crosslinkers is still underdeveloped and engineering phosphors doped polymeric nanostructures that have an edge over fluorescent nanostructures have met with limited success especially due to limited availability of stable water soluble brightly phosphorescent molecules. Though fluorescence based detection using regular organic dyes is relatively simple but most of conventional fluorescent labels have some disadvantages.

Parts of this chapter is submitted to Adv. Mat.
like nanosecond lifetimes which coincide with many biological background (auto-fluorescence) which can be overcome by using long decay times and large Stokes shift features of transitional metal-based phosphors.\textsuperscript{1} Though different transition metal based luminescent systems, notable for their intensive emission and long lifetimes have already shown to differentiate autofluorescence (nanoseconds lifetimes) for biological labeling, biomolecular crosslinking and for detection studies,\textsuperscript{1d,e} but never before any transitional metal based phosphor has been directly employed as crosslinker for formation of phosphorescent chitosan or NIPA microgel nanoparticles. Popularly three common approaches are followed for making polymeric luminescent nanoparticles. One is capping existing luminescent nanocrystals (Quantum dots or Lanthanide based nanoparticles) with polymers.\textsuperscript{1,4} In the second approach, luminophores are directly doped in to existing polymeric microspheres. In third case organic dye (like FITC: fluorescein isothiocyanate) labeled polymer is chemically crosslinked.\textsuperscript{1,4} However in all the above cases leakage of heavy metal ions, non-biocompatibility, aggregation tendency, surface sensitivity, syntheses involving mostly organic solvents or high temperature treatments, absence of surface ligands for further conjugation, photostability and nanosecond lifetimes are crucial issues needs to be addressed for enhancing utility of these materials.\textsuperscript{1a,b,d,2g,4a}

Au(I) mononuclear and multinuclear luminescent compounds are well documented for their intriguing photophysical properties and applications.\textsuperscript{5} But water solubility considered to be impeding factor for broad utilization of these luminescent gold(I) compounds for biological sciences has been overcome with development of
phosphine ligands. TPPTS (tris(3,3',3''-trisulfonatophenyl) phosphine) is a very famous water soluble ligand widely used in making transitional metal complexes of Ruthenium, Rhodium and Palladium for biphasic catalysis. Using TPPTS ligand Fackler and co-workers were able to synthesize first water soluble phosphorescent Au(I) complex with bright metal centered tunable emission exhibiting microseconds lifetimes both in solid and in aqueous solution. Proteins and polymers are routinely tagged by organic dyes like FITC to study drug release and phase transition properties of microspheres and polymers. But as organic dyes are known to suffer from drawbacks, so in previous and present chapter, we were trying to exploit interesting emissive properties of [Au(TPPTS)_3]Na_8 (AuP) for integrating with thermosensitive and pH sensitive polymers or hydrogel systems in order to demonstrate Au(I) phosphors ability for prospective biological or polymer associated applications. To the best of our knowledge until know only three main categories of phosphorescent molecules are explored for such polymer or biomolecular based studies. Eu(III) or Tb(III) based rare earth chelates by doping in to PMMA matrix systems, Pt or Pd based porphyrins as protein probes and the last group belongs to Ruthenium, Rhenium, Osmium and Iridium based complexes for labeling biomolecules like oligonucleotides, proteins and other derivatives. But in all the above cited cases luminescent molecules are either used as direct probes or proposed as potential biological immunoassay agents. Nevertheless, no prior studies have utilized these luminophores as crosslinkers for in situ formation of phosphorescent polymeric microgels or nanoparticles.
Figure 3.1  Structure of chitosan polymer (CS).

Chitosan (CS) a naturally occurring biodegradable and second most abundant or most abundant positively charged polysaccharide. Free amine groups of CS polymer have strong affinity for negatively charged polymers or polyanionic molecules. Physically or chemically, crosslinked chitosan nanoparticles exhibit numerous biomedical applications, mostly due to extraordinary biocompatibility, biodegradability, bioadhesive, nontoxic and antifungal properties of chitosan (CS) polymer itself. Regular chemical crosslinkers like glutaraldehyde or glyoxal were initially employed for making CHNPS but because owing to their physiological toxicity different negatively charged polymers like methacrylic acid, polyacrylic acid or polyanionic molecules like tripolyphosphate (TPP), dextran sulfate (DS) are being preferred. Many approaches like spray drying, coacervation/precipitation have been demonstrated to prepare CS micro and nanoparticles, but formation of chitosan hydrogels or nanoparticles by polyelectrolyte complexation (PEC) is an interesting alternative to crosslink polymer chains by electrostatic interactions. As PEC is generally more biocompatible, cationic nature of chitosan has been conveniently exploited for development of PEC based microspheres and nanoparticles. Aside from its complexation with negatively charged polymers, an
interesting property of chitosan is its ability to gel upon contact with special polyanions, a process referred to as ‘ionotropic gelation’. This gelation process is due to the formation of inter and intra crosslinkages between or within polymer chains, mediated by the polyanions. More recently, chitosan NPs have been developed based on the ionotropic gelation of chitosan with tripolyphosphate (TPP).³ This simple and straightforward technique involves the addition of an alkaline phase (pH = 7–9) containing TPP into an acidic phase (pH = 4–6) containing chitosan. NPs are formed immediately upon mixing of the two phases through inter and intra molecular linkages created between TPP phosphates and chitosan amino groups.

So engineering nanostructures from CS polymer under most benign and facile conditions is highly desirable in order to redeem all the above-mentioned intrinsic biocompatible properties of CS polymer. Recent advances have stretched applications of these chitosan nanoparticles (CHNPs) for various bioimaging purposes such as diagnostic imaging of cancer cells, labeling of stem cells, and imaging of pathogenic cells.² In spite of extended promising applications for luminescent chitosan nanoparticles in diagnosis and labeling, but to the best of our knowledge imparting luminescence in to CHNPs is brought only by doping quantum dots or by chemical modification of fluorescent organic dye molecules as discussed previously. Therefore in situ syntheses of luminescent chitosan nanostructures using a phosphorescent polyanionic system that can physically crosslink and impart luminescence is unprecedented and significant forward step in the direction of making highly stable
phosphorescent chitosan nanostructures under most benign and lucid conditions that would find applications in different biomedical scenarios.

Interest in microgels has grown rapidly over last 20 years because of their ability to exhibit drastic changes in properties in response to environmental stimuli combined with easy preparation and potential applications. When a polyelectrolyte is combined with multivalent ions of opposite charge, it may form a physical gel commonly known as “ionotropic hydrogel”. Calcium alginate is an example of this type of hydrogel employed for cell encapsulation studies. Mixture of aqueous polymer solutions are known to exhibit thermoreversible gelation (that is gelation upon heating) above gelation temperature, this ability is highly sought after because thermoreversible gels can be used as injectable scaffolds for delivering medicine or encapsulating cells. Inverse thermoreversible gelation using triblock copolymers (pluronic or poloxamer) or degradable triblock copolymers is well known. Thermosensitive Poly(NIPAM) microgels that shrink and swell in response to changes in temperature have ability to flocculate in to aggregates in presence of high concentration electrolytes. Benne and co-workers have reported unusual behavior of irreversible aggregation or macroscopic gels formation when poly(PNIPAM)-co-vinyl laurate microgel particles are heated in an electrolyte solution above 40°C. Whereas Zhu and co-workers have reported aggregation behavior from block copolymer microgels of poly(NIPAM) and Poly (ethylene glycol). Recently inverse thermoreversible gelation has been reported from our group based on PNIPAM-PAAc (positively charged PNIPAM microgels and polyacrylic acid polymer) IPN microgels. Gan and co-workers have reported in situ gelation using
(P(NIPAM-HEMA)) based microspheres as building blocks in presence of CaCl$_2$.\textsuperscript{9b} To the best of our knowledge, either in the above cited cases or from well known literature works physical gelation or aggregation or flocculation behavior among PNIPAM systems is brought by physical interactions between two or more oppositely charged polymer systems or due to presence of high concentrations of electrolytes like NaCl or NaNO$_3$ or CaCl$_2$ (~0.08M) only. But here for the first time we are reporting completely inverse thermoreversible physical gelation or aggregation behavior of phosphorescent PNIPAM-co-allylamine microgels at low concentration of both microgels (2wt\%) and gold phosphor (0.005 mM) in complete absence of any other common electrolyte. Intrigued from above literature background on CHNPs and inverse thermoreversible PNIPAM gels, chapter 3 presents a facile synthetic approach to make size tunable and pH sensitive phosphorescent chitosan microgel nanoparticles (PCHNP). Physical gelation within soft PNIPAM microgels is also demonstrated using the same gold phosphor (AuP) which acts as both physical crosslinker and light emitter in both cases. Along with detail study using chitosan polymer, ability of AuP to act as phosphorescent physical crosslinker is reconfirmed using a temperature sensitive positively charged PNIPA-co-RCONH$_2$ polymer.

3.2 Experimental Section

3.2.1 General Procedures

Exact procedure was followed as described in chapter 2. (Please see section 2.2.1 for details).
3.2.2 Physical Measurements

Please refer to section 2.2.2 in chapter 2 for other instrumentation details. FTIR spectra were collected using PerkinElmer spectrum one FTIR spectrophotometer, equipped with both transmission and ATR modes. Samples made using KBr pallets. $^1$H-NMR spectra were collected using a broadband two channels 400 MHz spectrometer with four nuclear ($^1$H/$^19$F/$^{31}$P/$^{13}$C) capabilities.

3.2.3 Electron Microscopy

The size, morphology, dispersity and elemental analysis of different silver nanoparticle samples was performed using high resolution analytical transmission electron microscope (HR-TEM). An FEI Co. Tecnai G2 F20 S-Twin 200keV field-emission Scanning Transmission Electron Microscope (STEM) was used. A 1nm STEM probe allows for an imaging resolution of 0.19 nm, and a high angle annular dark field detector (HAADF) allows for Z-contrast imaging in STEM mode at high resolution. High resolution analytical capabilities are provided on the TF20 STEM. It is equipped with an EDAX energy dispersive x-ray spectrometer (EDS), a gatan tridiem parallel electron energy loss spectrometer (EELS) with a 2k x 2k CCD for energy filtered imaging and high rate spectrum imaging EELS. Unless otherwise mentioned all images are collected in bright field mode. Scanning electron microscopy (SEM) images are collected using a FEI Nova 200 NanoLab (A dual column ultra-high resolution field emission scanning electron microscope). Nanoscale chemical analysis may be performed with an EDAX energy dispersive X-ray spectroscopy (EDS) system with spectrum imaging control. The secondary electron image resolution at the dual beam coincidence point is 1.5 nm at 15
kV. The FIB optics has better than 7 nm resolution at 30 kV. Unless otherwise mentioned all SEM images are collected using in-Lens SE (secondary electrons) detector.

3.2.4 Synthesis of \([\text{Au(TPPS)}_3]\text{Na}_8\)

A detailed syntheses and characterization procedure was listed in chapter 2 (please see section 2.2.3 and 2.3.2).

3.2.5 Formation of Phosphorescent Polyelectrolyte Complex Chitosan Microgel Nanoparticles (PCHNPs) by Simple Dropping Method

Medium molecular weight chitosan polymer was purchased from sigma-aldrich, degree of deacetylation of chitosan was 85%. In a typical procedure, phosphorescent polyelectrolyte microgel nanoparticles (PCHNPs) were prepared by mixing positively charged chitosan polymer with polyanionic AuP complex by simple dropping followed by stirring the solution for few hours. To 5 ml of required weight percentage of chitosan polymer solution, medium molecular weight chitosan dissolved in 1% (w/v) acetic acid solution added drop-by-drop required concentration of AuP with continuous stirring until an opalescent suspension was obtained. In case of excess addition of AuP results in precipitation of polyelectrolyte complex from solution. With extra care, gold phosphor is added to chitosan solution to prevent precipitation. The resultant opalescent solution is first centrifuged @ 15000 rpm followed by filtering to separate any unreacted chitosan polymer. Samples are than sonicated to prevent agglomeration and improve dispersion of particles. Finally, the samples are dialyzed for 1-2 days before taking out for further characterization.
3.2.6 Effect of AuP and Chitosan Polymer Concentration on PCHNPs

Different molar concentrations of AuP (0.5 mM, 1 mM, 5 mM) solutions are prepared separately and then added to fixed weight percentage chitosan polymer to determine effect of AuP concentration on average hydrodynamic radius, zeta potential and stability of PCHNPs. pH is maintained constant either by addition of required amount of 0.1M acetic acid or 0.1M ammonium hydroxide. In order to determine how CS wt% effects formation, stability and size of PCHNPs, two different wt% chitosan solutions (0.05 wt% and 0.1 wt%) are made. Different concentrations of AuP are added to different CS wt% and changes in average radius, zeta potential are tabulated.

3.2.7 Effect of pH and Salt on PCHNPs

Effect of pH on average size, distribution and stability of PCHNPs is determined by varying only pH either by addition of required amount of acid or base but all other parameters like AuP concentration, CS wt% are maintained constant. Effect of presence of salt is tested by direct addition of 0.1M KCl salt to PCHNPs solution.

3.2.8 Syntheses of PNIPA-\textit{co}-RCONH$_2$ Polymer

Polymerization of N-isopropylacrylamide-N-(3-aminopropyl) methacrylamide hydrochloride copolymer (R-\textit{co}-NH$_2$) is carried out by free radical co-polymerization in water using potassium persulfate (KPS) and tetramethylethylenediamine (TEMED). 1.5gm (0.008 molar) of N-isopropylacrylamide monomer and 0.05gm (0.2mmol) of co-monomer, 0.5ml of TEMED were added to 100ml water. The solution was stirred by heating up to 55 °C under nitrogen atmosphere for about 4 hours. At the end of polymerization, solution was cooled down to room temperature and then dialysed for
one week against millipore water. Formation of polymer in solution was confirmed by
heating the solution above phase transition temperature of the polymer (32-33 °C).
Formation of cloudy opalescent turbid solution due to precipitation of the polymer on
heating above its phase transition temperature confirms polymer formation.

3.2.9 Formation Phosphorescent PNIPA-co-RCONH$_2$ Nanoparticles

2 ml of 0.5% (wt/v) of PNIPA polymer solution was taken in a clean 10 ml
beaker the solution was first homogenized by stirring for few minutes, Poly(N-isopropyl
acrylamide) nanoparticles are synthesized by drop by drop addition of 0.001 molar gold
phosphor solution in to PNIPAM polymer solution at room temperature followed by
stirring. Formation of polymeric nanoparticles is confirmed as colorless solution turns in
to opalescent color. At this stage, addition of gold phosphor is stopped and the
nanoparticles solution is stirred continuously for one more hour and then kept in dialysis
tube against millipore water.

3.2.10 Syntheses of PNIPAM-co-allylamine Microgels

For syntheses and characterization, please refer to chapter 2, sections 2.2.4 and
2.3.3 respectively.

3.2.11 Inverse Thermo-reversible Gelation of PNIPAM-co-allylamine Microgels

PNIPAM-co-allylamine microgel particles between 1 wt% to 2.5 wt% are tested
for inverse thermoreversible gelation using different concentrations of gold phosphor. In
a standard procedure, 3ml of above PNIPAM-co-allylamine microgel particles is taken
and diluted for required weight percentage in a culture tube. 1ml of required
concentration of gold phosphor is added and then mixed thoroughly at room
temperature. Solution is then heated to 37 °C (±1 °C). Gelation or aggregation is observed instantly in few minutes visualized by increased viscosity and verified by turning the culture tube upside down. After few minutes, the gelation sample is cooled back to room temperature and the phenomenon of gelation is tested 3 cycles for reversibility and reproducibility. A strong irreversible gel is formed at very high concentration of microgel (> 4wt%) and gold phosphor (> 0.01 molar) combination which is similar to chemically crosslinked bulk hydrogel as shown in chapter 3.

3.2.12 In Situ Syntheses of Cyclic Trinuclear Au(I)-Pz’ Complex within Chitosan Polymer (Pz’=[3,5-(COOH) (CH₃)Pz]) modified Pz ligand is first dissolved in hot methanol, followed by addition of required moles of Au(THT)Cl. After 24 hours of stirring at room temperature the solution is centrifuged and the unreacted material is discarded. The sample is then subjected to dialysis.

3.3 Results

3.3.1 Photoluminescent Properties of AuP

Detailed photoluminescence characterization of gold phosphor described in chapter-2 (section 2.3.2).

3.3.2 Formation of Phosphorescent Polyelectrolyte Complex Microgel Nanoparticles (PCHNPs)

As illustrated in figure 3.2, electrostatic interactions between positively charged protonated amine groups of CS polymer and negatively charged anionic sulfonate groups of AuP allows for physical crosslinking and formation of phosphorescent polyelectrolyte complex microgels. This mechanism is similar to ionic gelation or
polyelectrolyte complexation mechanism known in literature using polyanionic molecules like TPP (tripolyphosphate) or DS (dextran sulfate). \(^{12}\) Chitosan (CS) with a pKa of \(\sim 6.5\) is polycationic. When dissolved in dilute mineral acids presents –NH\(_3^+\) sites. Either this positively charged CS polymer has been shown to exhibit strong affinity with negatively charged polymers like PAA (polyacrylic acid) or with poly anions (TPP or DS) to form CS based nanoparticles and microspheres. \(^{12}\) Extensively utilized physical crosslinker TPP contains five anionic sites per tripolyphosphate molecule. \(^{12c,d}\) Nine anionic sites in trigonal planar AuP complex\(^{5d}\) can favor more crosslinking points while phosphorescence becomes added advantage using proposed physical crosslinker. Here
tuning of PCHNPs size and stability is completely controlled by varying concentrations of both AuP and CS which is very similar to existing reports in literature with other well known physical crosslinkers also. Different studies in literature have shown how concentrations of both polymer and crosslinker along with pH of the medium can affect average size and stability of CHNPs. Superscript 12 Effect of these three governing factors was studied in detail and results are tabulated in table 3.1.

3.3.3 Effect of AuP Concentration on Tuning Average Size of Phosphorescent Polyelectrolyte Complex Chitosan Microgels (PCHNPs)

In general at fixed pH and wt% of CS polymer, as AuP concentration increases PCHNPs average size decreases accompanied by decrease in zeta potential values. As seen from table 3.1, at 0.05 CS wt% and pH-3.0, a steady decrease in mean average hydrodynamic radius ($R_h$) from 384 ± 15 nm to 108 ± 6 nm was noticed accompanied by decreased PDI (polydispersity index) along with decreased FWHM (full-width half max) values of DLS curves (figure 3.3) with stepwise increase in AuP concentration from 0.5 mM to 5 mM. An enhanced electrostatic interaction at higher concentration of AuP makes more compact matrix microgel particles with better dispersity. The DLS curves for the tabulated $R_h$ values are shown in figure 3.3 and 3.4 respectively.

Decrease in zeta potential values from +53.8 ± 5.9 mv (for chitosan polymer itself) to +39.5 ± 4.5 mv (for 108 ± 6 nm sample) is expected due to neutralization or decrease in surface positive charge of CS polymer due to interactions with polyanionic AuP during formation of PCHNPs.
Table 3.1 Interdependency of CS wt%, AuP concentration and pH on average hydrodynamic radius ($R_h$) and distribution of phosphorescent chitosan microgel nanoparticles (ZP: zeta potential; PDI: polydispersity index; FWHM: full-width half-max of dynamic light scattering curves).

<table>
<thead>
<tr>
<th>Wt% CS</th>
<th>pH (ZP of CS)</th>
<th>AuP (mM)</th>
<th>0.1</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.0 (+62.5 ± 6.6mv)</td>
<td>518 ±19</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5.0 (+45.4 ± 5.4 mv)</td>
<td>544 ±21</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>352 ±16</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>210 ±10</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>384 ±15</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>219 ± 9</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>108 ± 6</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>420 ±17</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>246 ±13</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Mean $R_h$ (nm)</td>
<td></td>
<td>ppt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ZP (mv)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDI</td>
<td>0.32</td>
<td>0.18</td>
<td>0.08</td>
<td>0.31</td>
</tr>
<tr>
<td>FWHM (nm)</td>
<td>183</td>
<td>135</td>
<td>40</td>
<td>261</td>
</tr>
</tbody>
</table>
Figure 3.3  Dynamic light scattering spectra representing changes in average size and distribution of hydrodynamic radius ($R_h$) of PCHNPs made at pH 3.0.

Figure 3.4  Dynamic light scattering spectra representing changes in average and distribution of hydrodynamic radius ($R_h$) of PCHNPs made at pH 5.0.
Figure 3.5  TEM images of PCHNPs made using 0.05 wt% CS at pH 3.0. A) 7 mM AuP, average size: 39.6 nm ± 9.4 nm (inset shows individual particle). B) 1 mM AuP, average size: 133 nm ± 29 nm. B’) TEM image of individual particle.
Figure 3.6 Dynamic light scattering spectrum and TEM images of PCHNPs made using 5 mM AuP, 0.1 CS wt% at pH 3.0. A) Average hydrodynamic radius ($R_h$) and distribution of PCHNPs ($R_h$) (inset shows zeta potential and FWHM). B) TEM images of PCHNPs. C) TEM images of individual PCHNPs (average size: 167 nm ± 15.7 nm).
Figure 3.7  Dynamic light scattering spectrum and TEM images of PCHNPs made using 5 mM AuP, 0.05 CS wt% and pH 3.0. A) Average hydrodynamic radius ($R_h$) and distribution of PCHNPs (inset shows zeta potential and FWHM). B) TEM images of PCHNPs. C) TEM images of individual PCHNPs (average size: 80 nm ± 8.7 nm).
Figure 3.8 Dynamic light scattering spectrum and TEM images of PCHNPs made using 5 mM AuP, 0.1 CS wt% at pH 5.0. A) Average hydrodynamic radius ($R_h$) and distribution of PCHNPs (inset shows zeta potential and FWHM). B) TEM images of PCHNPs (red circle denotes aggregation). C) TEM images of individual PCHNPs (average size: 267 nm ± 37 nm).

This mechanism of tuning average size with respective to crosslinker concentration is well documented. He et al.\textsuperscript{12g} have explained how increasing in concentration of anionic crosslinker would decrease average size and zeta potential based on valency of polyanion.\textsuperscript{12g} Therefore we assume a rapid decrease in average $R_h$ size and zeta potential at higher concentrations of AuP as noticed from table-1, fig 3.3 \\ & 3.4 is due to availability of more anionic sulfonate groups for crosslinking. Comparing TEM images from figure 3.5, cohesively demonstrates tuning size of PCHNPs with respective to changes only in AuP concentration anyway between 50 nm to 150 nm. These TEM images demonstrate significance of AuP concentration for tuning size of PCHNPs when all other experimental parameters are kept constant. Obtaining PCHNP smaller than 100 nm (figure 3.5) in aqueous medium itself is very crucial, as most of
literature reports especially using physical crosslinkers are restricted to sizes ranging between 175 to 600 nm with only few exceptions due to intrinsic properties of CS polymer itself.\textsuperscript{12j} Above certain concentration of AuP, PCHNPs precipitates out of aqueous medium due to non-availability of \(-\text{NH}_3^+\) sites for crosslinking. Some very small particles ranging between 30-50 nm are also obtained on precipitation (figure 3.5) at a step higher concentration of AuP (7mM) than mentioned in table 3.1 but stability is sacrificed. Precipitation behavior is common with polyelectrolyte complex CHNPs at higher concentrations of crosslinkers even with regular polyanionic systems\textsuperscript{12h} due to non-availability of protonated \(-\text{NH}_3^+\) groups that render solubility to CS. It is interpreted that obtaining monodispersed CS microgel particles less than 50 nm is still very difficult due to intrinsic self-adhesive nature of CS polymer that induces particle-particle association\textsuperscript{2b,c} combined with higher molecular weight of natural polysaccharide.

3.3.4 Effect of CS Polymer wt\% on Average Size of PCHNPs

Another important factor that affects average size of PCHNPs is CS polymer wt\%. Guided from table 3.1, average hydrodynamic radius (\(R_h\)) of PCHNPs decreases with decrease in CS wt\% by keeping all other parameters constant, lower CS wt\% induces smaller average size (\(R_h\)) particles due to smaller aggregation tendency. Comparing TEM images from figure 3.6B & 3.7B a clear difference in size and stability is noticed at different wt\% of CS polymer. Lower wt\% (0.05) of CS results in formation of PCHNPs with average size 80 nm ± 8.7 nm (figure 3.7), while under similar experimental conditions PCHNPs with average size 167 nm ± 15 nm are formed using 0.1 wt\% of CS. DLS data also shows similar difference in average radius, (table 3.1) mean average \(R_h\)
of 108 ± 6 nm is noticed with 0.05 wt% CS polymer compared to 169 ± 9 nm average radius with 0.1 wt%. It is well explained by He et al. and\textsuperscript{12f,g} Wei et al. that at lower concentration of CS polymer, number of polymer chains dispersed in solution will be less resulting in smaller size particles due to smaller aggregation tendency. Below 0.05 wt% of CS though smaller particles would form but again stability is sacrificed due to insufficient surface charge indicative from zeta potential values. So, these results conclude that a critical concentration or optimal ratio of CS polymer and AuP are required for balancing electrostatic interactions to form stable PCHNPs in solution when pH is maintained constant.

3.3.5 Effect of pH on Average Size and Stability of PCHNPs

pH is an intrinsic sensitive property of CS polymer based on which many chitosan drug delivery vehicles are designed and plays governing role during formation, tuning average size and determining stability of CHNPs as shown by many research groups previously.\textsuperscript{12} Un-modified CS polymer is soluble only in acidic solutions below pH \textasciitilde 6.5. A clear decrease in zeta potential of CS polymer itself with increase in pH (table-1) is due to deprotonation of -NH\textsubscript{3}\textsuperscript{+} groups as shown by Kaloti et al.\textsuperscript{12c} Mattoso et al.\textsuperscript{12j} explained how pH affects average size by demonstrating a clear increase in size of CHNPs with pH. Deprotonation at higher pH results increase in average \( R_h \) of polymer matrix due to decreased electrostatic interactions or due to decreased solubility. A clear effect of pH on size and distribution of PCHNPs can be noticed by comparing data from figure 3.6 and 3.8 where samples are made at same concentration of CS (0.1wt%) and at same (5mM) AuP concentration but at different pH. TEM images from figure 3.6
shows well distributed PCHNPs made at pH 3.0 exhibit average size 167 nm ± 15 nm where as TEM images from figure 3.8 corresponding to sample made at pH 5.0 shows particles with average size 267 nm ± 37 nm. This difference in size and distribution can also be correlated to average radius ($R_h$) data and FWHM of DLS curves (table 3.1). CS wt% was also found to play determinant role especially governing stability of PCHNPs at higher pH as noticed by comparing figure 3.7 and 3.9. Precipitation or agglomoration behavior is noticed from TEM images in figure 3.9 when PCHNPs are made at lower concentration of CS (0.05 wt%) compared to stable particles in figure 3.7 where PCHNPs are made at comparatively higher CS wt% (0.1). Again this difference in stability is due to difference in surface charge at different pH. Comparing (table-1) mean average size ($R_h$) at 0.1 wt% CS polymer at three different AuP concentrations show 5%, 14% and 20% difference in mean $R_h$ at pH 5.0 and pH 3.0 respectively. These results suggest strong interdependency of size and stability on pH of the medium due to significant difference in surface charge of CS polymer at different pH and at different wt% as indicated from zeta potential values (table 3.1).

3.3.6 Effect of Salt on Average Size and Distribution of PCHNPs

It is well known that presence of salt leads to decrease in zeta potential due to electrostatic shielding at higher ionic strengths, which can eventually suppress electrostatic interactions as explained from electrokinetic theory. Presence of 0.1M KCl salt results in broad distribution of PCHNPs with reduced zeta potential value, compared to sample that is synthesized in absence of KCl under identical conditions.
(figure 3.10). This difference is presumably due to decreased electrostatic interactions between CS and AuP owing to screening effect of salt. Presence of salt in

Figure 3.9 TEM images of PCHNPs made using 5 mM AuP, 0.05 CS wt% at pH 5.0. A) Represents TEM images of agglomerated PCHNPs. B) Represents TEM images of individual particles.

Table 3.2 Effect of addition of salt (KCl) on zeta potential and ionic conductivity of PCHNP solution.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta potential (mv)</th>
<th>Conductivity (mS cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With KCl</td>
<td>28.2 ± 3.6</td>
<td>3.12</td>
</tr>
<tr>
<td>Without KCl</td>
<td>35.3 ± 3.9</td>
<td>0.19</td>
</tr>
</tbody>
</table>
the system can be confirmed from higher ionic conductivity value (table 3.2). This simple experiment concludes that average size, stability and distribution of PCHNPs are readily tuned by altering electrostatic interactions between CS polymer and AuP with strong interdependency on pH of the medium, wt% of CS polymer, AuP concentration and Ionic strength of the medium as discussed in detail in above sections. SEM images of PCHNPs after addition of KCl salt is shown in figure 3.11 which contain large aggregated particles as expected from DLS data.

3.3.7 Analysis of Individual Particles Size and Morphology by Electron Microscopy

TEM and SEM samples are prepared directly by simple cast drop method on SEM and TEM grids respectively avoiding usage of any contrast agents as enough contrast is
obtained from AuP itself. Non-uniform amorphous structures indicate polymeric nature of particles. Accumulation of AuP molecules inside PCHNPs matrix are

Figure 3.11 SEM images of PCHNPs in presence of KCl salt (agglomeration of particles is in good agreement with DLS data shown in figure 3.10).
Figure 3.12 EDAX spectrum of a typical PCHNP sample (inset shows changes in morphology of PCHNP due to high-energy electron exposure).

seen as dark spots at higher magnification in some samples. On comparing with light scattering data, sizes of individual particles from TEM were always smaller as expected due to their strong microgel behavior. For example, figure 3.6 shows individual particle size with average size 167 nm ± 15.7 nm using 0.1 wt% of CS polymer at pH 3.0 and 5mM AuP concentration. But the DLS spectra for the same sample represents mean average radius ($R_h$) of 175 nm. While altering CS wt%, particles ranging between 80 nm ± 8.7 nm (fig 3.7B) are obtained where as DLS spectra for the same sample represents mean average radius ($R_h$) of 108 nm (figure 3.7A). These TEM images strongly confirm formation, tunability and stability but owing to microgel behavior of PCHNPs, exact correlation between DLS data and TEM size was not possible. As described earlier, much discrepancy\textsuperscript{13} between DLS and TEM sizes is due to fact that
DLS measures the equivalent-hydrodynamic sphere size in aqueous solution where as TEM measures actual physical size for dried samples. Definite decrease in size from TEM is due to loss of water along with minimizing clustering or assembly tendency of particles on drying compared to DLS. EDX (Electron Dispersive X-ray Spectroscopy) analysis plays vital role in acquiring elemental composition information especially in nanostructure materials. EDX spectra collected on a typical PCHNP (figure 3.12) showed obvious energy peaks from carbon, nitrogen, phosphorus, sulphur and gold, which are in good agreement with Energy dispersive X-ray lines of those atoms.\textsuperscript{13b} Inset picture (figure 3.12) shows degradation of PCHNP on exposure to electron beam during EDX analysis which strongly confirms polymeric nature of PCHNPs. Strong saturated peaks from Cu are invariably seen from Cu grid. Clear distinctive energy peaks from sulphur, phosphorus and gold authenticate entrapment of AuP molecules in the CS polymer matrix of PCHNPs as there is no other alternative source for these elements to show their presence in the sample except from AuP itself. Stability of AuP is also cross-examined under strong photolysis conditions that show no decomposition of AuP in to gold metal or gold nanoparticles, which signifies stability of AuP, while indicating Au energy peaks observed during EDX analysis are solely from AuP in molecular form. More experiments are in progress to correlate exact sizes from TEM and DLS results.

3.3.8 Photoluminescent Properties of PCHNPs

Another significant outcome of this work is complete retention of photoluminescence (PL) properties accompanied by unusual PL enhancement after formation of PCHNPs as noticed in our work on hybrid PNIPAM microgels.\textsuperscript{14} Figure 3.13
shows photophysical properties of PCHNPs in comparison with AuP bulk solution. 8-10 fold emission enhancement noticed from PCHNPs solution compared with bulk AuP solution is explained due to combination of colloidal scattering accompanied by decrease in water accessibility to AuP within colloidal polymer matrix. Unaltered photophysical properties verify preservance of AuP in its native form as well as signify interactions behind formation of PCHNPs as purely electrostatic. This is very significant as emission from most conventional organic dyes or QD’s are notorious for their sensitive or quenching behavior with changes in surface chemistry and microenvironment of the medium\(^2\). Looking at inset pictures from figure 3.14 (AuP: PCHNP) for two samples containing same concentration of AuP clearly depicts visual difference in emission intensity due to PL enhancement. Lifetime values (table 3.3) and photoluminescence decay (figure 3.15) for AuP bulk solution and PCHNPs solution does not exhibit much variation as expected from PL enhancement work demonstrated for hybrid microgels in chapter-2. Though enhancement noticed here is order of magnitude lower but still significant enough to distinguish from bulk AuP solution. In addition to PL enhancement from colloidal PCHNPs in aqueous medium, characteristic broad emission peak (figure 3.14) at ~530 nm undergoes 20 nm blue shift with distinctive changes in emission color due to agglomeration or precipitation of PCHNPs at RT itself (inset 3.14 PCHNPs ppt). This is similar to reversible systemic emission tuning demonstrated in chapter 2 within phosphorescent PNIPAM microgels above phase transition temperature (~34 °C). Taking a step ahead, in PCHNPs intermediate emission due to T-shaped distortion of AuP is realized at RT only when polymer matrix has become rigid on
precipitation or aggregation, coupled with aggregation of phosphor molecules resulting in intermediate emission (~508 nm) compared to bulk solution (~528 nm) or solid (~500 nm). Lifetime values for AuP in different physical states (AuP bulk solution, PCHNPs, PCHNPs ppt) are listed in figure 3.14. All the above data comfortably demonstrates not only retention of photophysical properties of AuP in different forms of CS matrix but also elucidates AuP PL sensitivity to changes in matrix of polymer medium.

3.3.9 Photoluminescent Chitosan Films

Chitosan films and membranes usage in dialysis, contact lenses, dressings, encapsulation of mammal cells and other optical, bio-photonic applications is well documented. This film forming ability of CS has been utilized to demonstrate a facile way for formation of highly stable phosphorescent films (figure 3.14 inset PCH film) which are stable for more than an year retaining strong emission along with microseconds lifetime (figure 3.16). This very simple drop cast technique for formation of highly stable luminescent films can be further explored in combination with many other transitional metals based luminescent materials especially sensitive to oxygen or environment for biological, biophotonic and food applications.
Figure 3.13 Photoluminescence and absorption spectra of bulk AuP and PCHNPs solution. a, b, c) Represents absorption, excitation and emission spectra of AuP bulk solution. a', b', c') Represents absorption, excitation and emission spectra of PCHNPs solution. (Exc $\lambda_{285\text{ nm}}$, Emi $\lambda_{530\text{ nm}}$). d) Absorption spectra of chitosan polymer.

Table 3.3 Photoluminescence lifetime decay values of bulk AuP and PCHNPs aqueous solution. Decay for the same is shown in figure 3.15 (the data were acquired using a xenon flash lamp excitation at 285 nm and a gated microsecond detector, emission monitored at 530 nm).

<table>
<thead>
<tr>
<th>Samples</th>
<th>LT (µ sec)</th>
<th>Chi$^2$ / Durbin Watson</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuP/H$_2$O</td>
<td>1.54</td>
<td>0.9345/1.744</td>
</tr>
<tr>
<td>PCHNPs</td>
<td>2.19</td>
<td>0.905/1.961</td>
</tr>
</tbody>
</table>
Figure 3.14 Changes in photoluminescence spectrum of PCHNP samples under different physical conditions. (PCHNPs solution, PCHNPs precipitate, PCH: CS film doped with AuP). Pictures of corresponding samples taken under UV excitation and lifetime values of each individual sample are enclosed.
Figure 3.15 Time resolved photoluminescence decay of AuP and PCHNPs in aqueous solution. The data were acquired using a xenon flash lamp excitation (Exc λ\textsubscript{285 nm}) and a gated microsecond detector (Emi λ\textsubscript{530 nm}).

3.3.10 FTIR and \textsuperscript{1}H-NMR of PCHNPs

Retention of AuP with in CS matrix after dialysis was confirmed from FTIR and \textsuperscript{1}H-NMR results.\textsuperscript{16,6a} FTIR absorbance was recorded using KBr pallet. While \textsuperscript{1}H-NMR was recorded in D\textsubscript{2}O solvent. Through FTIR analysis of CS, AuP and PCHNP samples (figure 3.17, table 3.4) interactions between CS and AuP are elucidated. FTIR absorption spectra shows no major absorbance changes in PCHNPs sample compared to CS polymer or AuP itself. Only minor shifts and narrowed peaks are noticed from PCHNPs in FTIR absorption spectra compared to CS or AuP. In figure 3.17 CS spectrum presents
characteristic peaks at 1076 cm$^{-1}$ associated with C-O-C vibration, 1424 cm$^{-1}$ relates to C-N stretch while 1553 cm$^{-1}$ and 1639 cm$^{-1}$ reflects amide I and C-O stretch respectively which are in good agreement with literature. AuP absorption spectra (figure 3.17) with characteristic peaks at 1196 cm$^{-1}$, 1038 cm$^{-1}$ and 624 cm$^{-1}$.

Figure 3.16 Time resolved photoluminescence decay of AuP doped CS film. The data were acquired using a xenon flash lamp excitation (Exc $\lambda_{305\text{ nm}}$) and a gated microsecond detector (Emi $\lambda_{500\text{ nm}}$).
Figure 3.17 FTIR spectra recorded for dried samples of AuP, CS polymer and PCHNPs by making pallets using KBr salt. (AuP: Gold phosphor; CS: Chitosan polymer; PCHNPs: Phosphorescent chitosan microgel nanoparticles). Inset shows zoom of active region from 1750 cm\(^{-1}\) to 500 cm\(^{-1}\).
Table 3.4  List of FTIR active frequencies and proton NMR chemical shifts of CS polymer, AuP and PCHNPs samples in comparison with literature (PCHNPs are first ever reported, literature values are not available).

<table>
<thead>
<tr>
<th>FTIR (cm⁻¹)</th>
<th>FTIR (wave number) cm⁻¹</th>
<th>H-NMR δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AuP</strong></td>
<td>sulfonyl (νSO) TPPTS: 1196 1040 and 626</td>
<td>sulfonyl (νSO) TPPTS: 207/1196, 1040, 628.</td>
</tr>
<tr>
<td><strong>PCHNPs</strong></td>
<td>Minor shifts: 1553 (amine) shifted to 1560; 626 (νSO) shifted to 621; 1040 (νSO) shifted to 1035.</td>
<td>3.70, 3.53, 2.98, 1.86 7.80, 7.6 (Coincide with signals from both CS and AuP)</td>
</tr>
</tbody>
</table>

Reflects exactly the sulfonyl (νSO) stretch of TPPTS ligand. Absorption spectrum of PCHNPs sample did not present any new peaks as such because of non-modification of either CS or AuP chemically after formation of PCHNPs. Existence of distinguished peaks of TPPTS ligand with minor shifts and increased intensity in PCHNPs sample confirms entrapment and retainement of AuP in colloidal PCHNPs even after dialysis due to strong polyelectrolyte interactions between CS polymer and AuP molecule. ¹H-NMR chemical shifts listed in figure 3.18 for CS polymer and AuP are in good agreement with reports in literature exhibiting all characteristic signature chemical shifts. Entrapment of
AuP within CS matrix is confirmed for one more time from retained proton chemical shifts of both CS and AuP in PCHNPs sample.

![Proton NMR spectra](image)

Figure 3.18 Proton NMR spectra for AuP, CS polymer and PCHNPs in D$_2$O solvent (AuP: Gold phosphor; CS: Chitosan polymer; PCHNP: Phosphorescent chitosan microgel nanoparticles).

3.3.11 Formation of Phosphorescent PNIPA-$
\text{co}$-RCONH$_2$-AuP Nanoparticles

Enormous work is found in literature for making PNIPAM based nanoparticles for various applications due to thermosensitivity of NIPA polymer. Most of the syntheses techniques for making NIPA nanoparticles employ chemical crosslinkers liker BIS (N,N, methylene bisacrylamide) for formation of PNIPAM nanoparticles. In order to take complete advantage of thermosensitive NIPA polymer for release applications, physical crosslinking methods are devoted for making PNIPAM nanoparticles but are less popular mainly due to lack of sufficient surface charge for crosslinking.$^{3,8}$ In view of extensive applications of PNIPAM nanoparticles, extended work has been performed to determine cytotoxicity of PNIPAM nanoparticles by many groups on different cell lines. From these
works it has been concluded that PNIPAM nanoparticles are less or non toxic or biocompatible compared to NIPA monomer. Surface modification due to crosslinking has been predicted for non-toxicity of PNIPAM nanoparticles compared to NIPA monomer.\(^{17}\) So, preparing PNIPAM based nanoparticles in absence of any other chemical crosslinkers or surfactant is always desirable which avoids additional toxicity concerns. Polyelectrolyte interactions realized between positively charged chitosan and AuP is further tested to synthesize nanoparticles from positively charged thermosensitive Poly(N-isopropyl acrylamide) co-polymer. As seen in case of chitosan, in order to generate stable and strong polyelectrolyte interactions between positively charged co-polymer of PNIPAM chain and negatively charged gold phosphor in aqueous solution, a critical concentration of both polymer and gold phosphor are required. Figure 3.19 shows formation of size tunable phosphorescent PNIPAM nanoparticles by simply varying molar concentration of gold phosphor at constant concentration of NIPA polymer. Average hydrodynamic radius of PNIPAM nanoparticles was shown tunable anywhere between 260 nm to 40 nm. At AuP concentration more than 10 mmoles, particles with average radius \(R_h\) smaller than 130 nm with better distribution are obtained. While with AuP concentration less than 10 mmoles results in formation of broad size distribution particles with average hydrodynamic radius larger than 150 nm.
Figure 3.19 Light scattering data for phosphorescent PNIPA-α-RCONH₂ nanoparticles formed using AuP as physical crosslinker. Changes in average hydrodynamic radius ($R_h$) and distribution with respect to AuP concentration (enclosed values represent AuP molar concentration).

This is very similar to results obtained using CS polymer where strong polyelectrolyte interactions at higher concentration of AuP results in smaller size particles with narrow size distribution. In case of PNIPAM polymer formation of very small particles (< 50 nm) is predicted due to two interdependent factors. Low surface charge of PNIPAM polymer demands higher concentration of AuP whereas low molecular weight of PNIPAM polymer initiates formation of particles smaller than 50 nm compared to natural positively charged polysaccharide. Using 20 mmoles of AuP results in formation of very small ($R_h=38$ nm) narrowly dispersed PNIPAM nanoparticles.
Comparing scattering intensities and particle distribution of polymeric particles with respect to polymer precursor (figure 3.19) under similar experimental conditions confirms formation of polymeric microparticles with gold phosphor acting as physical crosslinker. Formation of polymeric nanoparticles is re-confirmed from light scattering data collected on a different light scattering instrument for a typical sample before and after addition of AuP (figure 3.20). Retained phosphorescence emission (figure 3.21) from nanoparticles solution at room temperature even after dialysis (3000 MCO tube) confirms formation of physically crosslinked phosphorescent PNIPAM nanoparticles.

Figure 3.20 Light scattering data reconfirming formation of phosphorescent PNIPA-co-RCONH₂-AuP nanoparticles for a typical sample using a different light scattering instrument. A) Polymer by itself. B) After addition of AuP (Dr.Petros in chemistry assisted in collecting the data).
Figure 3.21 Photoluminescence spectrum of phosphorescent PNIPAR-\textit{co}-RCONH$_2$ nanoparticles solution at room temperature (Exc $\lambda_{305}$\,nm, Emi $\lambda_{522}$\,nm).

Thermosensitivity of physically crosslinked phosphorescent PNIPA-\textit{co}-RCONH$_2$ nanoparticles is tested by measuring the changes in hydrodynamic radius of particles above and below phase transition temperature of PNIPA polymer ($\sim$33 $^\circ$C). From figure 3.22 it is observed that on increasing temperature of nanoparticles solution, average hydrodynamic radius exhibits continuous increase. Starting with particles having average hydrodynamic radius ($R_h$) of 126 nm at room temperature (24 $^\circ$C) an increase in average size is noticed at every step with increase in temperature. Above phase transition temperature (32-33 $^\circ$C) of NIPA polymer, particles with average radius >330 nm are obtained with broad particle distribution. These temperature dependent changes in hydrodynamic radius ($R_h$) of phosphorescent PNIPAM nanoparticles first verify retained temperature sensitivity but increase in size is due to aggregation of polymeric
particles at higher temperatures. PNIPAM nanoparticles are expected to shrink in size with increase in temperature, but exact opposite behavior noticed in presence of AuP indicates strong electrostatic interactions between adjacent PNIPAM nanoparticles results in increased size due to aggregation when NIPA polymer starts precipitating above phase transition temperature.

![Graph showing dynamic light scattering data for temperature dependent changes in average hydrodynamic radius (R_h) and distribution of phosphorescent PNIPAR-co-CONH₂ nanoparticles.](image)

Figure 3.22 Dynamic light scattering data for temperature dependent changes in average hydrodynamic radius (R_h) and distribution of phosphorescent PNIPAR-co-CONH₂ nanoparticles.

3.3.12 Temperature Induced Inverse Thermo-reversible Gelation in AuP Loaded PNIPAM-co-allylamine Microgels

After realizing effect of polyelectrolyte interactions between positively charged polymers (Chitosan and PNIPA-co-RCONH₂) and polyanionic AuP combined with effect of temperature dependent aggregation on PNIPA nanoparticles, an attempt was made to combine both these features of electrostatic interactions and temperature sensitivity.
within hybrid PNIPAM-co-allylamine microgels (AuP doped microgels). As explained in chapter 2, formation of phosphorescent microgels in different physical forms is solely based on electrostatic interactions between positively charged amine groups of PNIPAM-co-allylamine microgels and polyanionic AuP. But, combined effect of wt% of microgel, AuP concentration and temperature on physical properties of hybrid microgel was not studied elaborately in chapter 2. Polymer solutions with physical thermoreversible gelation ability are highly sought after because they are preferred as injectable scaffolds for delivering medicine or encapsulating cells. Literature works cited in introduction for chapter 3, have inspired us because in the cited cases as well as from other known literature works, physical gelation or aggregation or flocculation behavior in PNIPAM and other block co-polymers is attained only due to physical interactions between oppositely charged polymer systems or in presence of simple polyelectrolytes. But, inverse thermoreversible gelation in PNIPAM polymers using a phosphorescent molecular system was never reported before.

After testing different concentration combinations of both PNIPAM-co-allylamine microgels and AuP, concentrations that govern inverse thermo-reversible gelation are determined and summarized in table 3.5. A quick look at the changes in average hydrodynamic radius upon heating the hybrid PNIPAM-co-allylamine microgel dispersion solution above LCST (> 33 °C), shows sudden increases from nanometer size in the sol to micron size aggregates in the gel state indicating onset of gelation or aggregation. From figure 3.23 it is very clear that this sudden aggregation of microgel nanoparticles above LCST is strongly dependent on AuP concentration. Keeping microgel wt%
constant, weak gelation (figure 3.23) is indicated with abrupt 7 times increase in hydrodynamic radius using 0.001 M AuP concentration compared to more than 18 times increase in average hydrodynamic radius ($R_h$) in case of strong gelation at 0.005 M AuP concentration. However no gelation from concentration $=/< 0.0001$ M AuP corroborates that aggregation or gelation is facilitated only above certain concentrations of AuP. This phenomenon of gelation is comfortably demonstrated at higher concentrations (> 1.5 wt%) of microgel by physically inverting test the tube (figure 3.24). It is noted from table 3.5, at microgel concentration lower than 1.5 wt% gelation cannot be observed physically even on addition of highest tested concentration of AuP (0.005 molar) but whereas with 2 wt% of microgel, gelation can be tuned from weak to strong by varying concentration of AuP. Beyond 3 wt% of microgel, a strong physical aggregation is observed which is not reversible for few hours. Repeating the heating and cooling cycles for three times this process of gelation is found to be synchronously reversible between sol-gel states with temperature. So process of inverse thermo reversible gelation is found to be tunable from point of no gelation to strong gelation by balancing concentrations of both AuP and PNIPAM-co-allylamine microgels dispersions as sown in fig 3.24 & table 3.5. The insight mechanism of gelation is investigated in detailed from temperature dependent dynamic light scattering data produced (table 3.6 & fig 3.25) at different concentrations of AuP and at different temperatures by keeping wt% of microgel constant. A hydrodynamic radius change in un-doped control microgels was also monitored to contrast presence and absence of AuP. This data provides valuable insight to onset of gelation (what temperature does gelation start), growth of cluster
sizes with respective to AuP concentration and differentiates significance of presence or absence of AuP. As expected (table 3.6) all the control (undoped) and no gelation samples exhibited customary decrease in particle size representing no gelation either due to absence of AuP or due to lack of required concentration of AuP for initiating gelation. While sample with highest AuP concentration exhibited highest change in average hydrodynamic radius (figure 3.25, table 3.6) due to strongest interactions possible between AuP and PNIPAM microgels.

Table 3.5  Summarizes molar concentrations of AuP and wt % of PNIPAM-co-allylamine microgel dispersions required to exhibit inverse thermo-reversible gelation.

<table>
<thead>
<tr>
<th>AuP Molarity</th>
<th>Microgel wt%</th>
<th>1.5%</th>
<th>2.0%</th>
<th>&gt;3wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 M</td>
<td>Weak</td>
<td>YES</td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>0.0001 M</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
</tbody>
</table>

Though mechanism of inverse thermoreversible gelation in PNIPAM-co-allylamine/AuP system is not understood completely but predicted due to both Vanderwal interactions between neighboring PNIPAM-co-allylamine microgels above LCST and strong electrostatic interactions between protonated –NH$_3^+$ groups of co-monomer allylamine of the microgel and anionic –SO$_3^-$ of AuP ligand during precipitation of PNIPAM polymer above phase transition temperature. Similar mechanism was proposed by
Figure 3.23 Sudden changes in average hydrodynamic radius ($R_h$) of PNIPAM-co-allylamine microgel dispersion samples during gelation using various concentrations of AuP. All microgel samples are diluted equally starting with 2wt % solution.

Figure 3.24 Demonstration of physical inverse thermo-reversible gelation using PNIPAM-co-allylamine microgels at various concentration combinations. A) 2 wt% of microgel: 0.0001M AuP. B) 2 wt% of microgel: 0.005 M AuP. C) 2 wt% of microgel: 0.001 M AuP.
Figure 3.25 Temperature dependent average hydrodynamic radius ($R_h$) of PNIPAM-co-allylamine microgels due to inverse thermo-reversible gelation. Black line: Control microgel without addition of AuP; Red line: Strong gelation sample due to presence of AuP (inset shows change in $R_h$ beyond 34 °C).

Benee et al.\textsuperscript{11a} for irreversible and reversible flocculates formed from PNIPAM microgels in presence of different electrolyte solutions. In another similar work Saunders et al.\textsuperscript{11b,c} have demonstrated effect of electrolyte NaNO$_3$ concentration on PNIPAM graft copolymer aggregates formation. From understanding above literature works, we assume dominant attractive electrostatic interactions between positively charged PNIPAM-co-allylamine microgels and negatively charged anionic AuP play significant role
for aggregation or gelation. The above assumption is experimentally evident with formation of larger macroscopic aggregates ($R = 1870$ nm) at higher concentration of polyanionic AuP (figure 3.25, table 3.6). Gelation temperature is directly related to LCST (lower critical solution temperature) of PNIPAM-co-allylamine microgels in either weak or strong gelation samples. This is because the thermo sensitive property of PNIPAM microgel does remain intact even in presence of AuP as explained in chapter 2.\textsuperscript{14} The significance of electrostatic interactions for initiating gelation in hybrid microgels is verified with PNIPAM-co-acrylic acid microgels and as expected there was no gelation noticed due to absence of any strong electrostatic interactions between

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>Strong Gelation (0.005 M AuP) ($R_h$ nm)</th>
<th>Weak Gelation (0.001 M AuP) ($R_h$ nm)</th>
<th>No Gelation (0.0001 M AuP) ($R_h$ nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hybrid</td>
<td>Control</td>
</tr>
<tr>
<td>21</td>
<td>118</td>
<td>90</td>
<td>117</td>
</tr>
<tr>
<td>24</td>
<td>109</td>
<td>87</td>
<td>115</td>
</tr>
<tr>
<td>27</td>
<td>107</td>
<td>84</td>
<td>108</td>
</tr>
<tr>
<td>30</td>
<td>102</td>
<td>66</td>
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</tr>
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<td>32</td>
<td>99</td>
<td>58</td>
<td>97</td>
</tr>
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<td>34</td>
<td>77</td>
<td>95</td>
<td>78</td>
</tr>
<tr>
<td>36</td>
<td>57</td>
<td>1870</td>
<td>54</td>
</tr>
</tbody>
</table>
Figure 3.26 Temperature dependent photoluminescence changes in hybrid PNIPAM-\textit{co}-allylamine microgel dispersions. \textbf{Sol}: Hybrid microgel solution at room temperature. \textbf{Gel}: Hybrid microgel solution on gelation at 37 °C (Exc $\lambda_{300}$ nm).

Figure 3.27 Pictures of hybrid PNIPAM-\textit{co}-allylamine microgel samples at different stages. \textbf{Sol}: Solution in day light; \textbf{Sol$^1$}: Solution under UV light; \textbf{Gel}: On gelation after heating to 37 °C in day light; \textbf{Gel$^1$}: On gelation after heating to 37 °C in UV light.

PNIPAM-\textit{co}-acrylic acid and AuP molecules. Extensive studies to determine any small variations in LCST of hybrid microgels at wide concentration ranges of AuP still need to
be perceived. Hybrid PNIPAM-co-allylamine-AuP system not only exhibit inverse thermoreversible gelation behavior but also exhibits retained photoluminescent properties of AuP accompanied by emission blue shift as noticed in case of PCHNPs (figure 3.26) on gelation. Retained broad unsymmetrical green emission peak at ~525 nm in sol state undergoes ~620 cm⁻¹ turquoise blue shift on gelation in hybrid microgels. Pictures in figure 3.27 show emission blue shift behavior compared to sol state during gelation.

Many luminescent complexes have been studied as molecular probes and luminescent sensors, especially some luminescent transitional metal-based complexes were doped specifically in sol-gel materials to gain information about rigidity of the matrix. Like Iha and co-workers studied luminescence rigidochromism using Rhenium (I) carbonyl complexes in different matrixes,¹⁸ while Zink et al. elaborately explained changes in PL properties of different luminophores in sol-gel systems.¹⁸ᵃ,ᵇ Lastly emission color tunability was perfectly demonstrated from Au(I) Pz’ based organogels by Aida et al.¹⁸ᶜ However, tunability in emission using a gold phosphor based complex within water soluble soft materials like PNIPAM or chitosan microgels is unprecedented. This novel phenomenon of temperature-induced gelation accompanied by reversible emission shifts can open a new door for fundamental study of phase behavior in positively charged soft matter systems by doping AuP and using luminescence spectroscopy as monitoring tool. So, we predict luminescence spectroscopy that is routinely employed for detecting phase transformations in polymers and gels in presence of different chromophores¹⁸ᵈ can utilize this emission shift behavior to detect
changes in rigidity and microenvironment of polymer or gel matrices using similar gold based luminophores that have ability to exhibit rigidochromism especially in soft materials like hydrogels or polymers solutions. Lastly demonstration of biological activity with similar TPPTS based Au compounds by Laguna and Contel et al.\textsuperscript{19} separately, advocates strongly for extended usage of these AuP based hybrid nanoparticles for medicinal applications.

3.3.13 In Situ Formation of Cyclic Trinuclear Au(I) Pyrazolate Complex Sensors in Aqueous Medium Stabilized within Chitosan Polymer

Importance of cyclic trinuclear d\textsuperscript{10} transitional complexes for their significant acid-base chemistry, metalloaromaticity, metallophilic bonding, supramolecular assemblies, M-M-bonded excimers and host-guest chemistry properties has been thoroughly investigated by many groups.\textsuperscript{20} Fascinating luminescent properties like luminescence thermochromism, (emission shift with temperature) luminescence solvatochromism, (emission shift with solvents) luminescence rigidochromism (emission shift with rigidity) are reported earlier by Omary et al.\textsuperscript{20a,b} from Cu\textsuperscript{I} pyrazolates. Later the same group has also explained the role of coinage metal on solid state packing, photophysics and acid-base properties in cyclic trimeric Cu\textsuperscript{I}, Ag\textsuperscript{I}, Au\textsuperscript{I} complexes using highly fluorinated pyrazolate ligands.\textsuperscript{20a} A year later Omary and co-workers have again shown formation and interesting aurophilically bonded dimer-of-trimer units from gold(I) triazolate trimers.\textsuperscript{20b} These new class Au(I) trinuclear trimers were first of its kind based on triazolate bridging ligands which exhibit multiple phosphorescence bands in both the solid state and solution along with pH dependent reversible quenching in
Figure 3.28 A) Structure of substituted pyrazole (Pz’) and schematic formation of cyclic trinuclear Au(I) Pz’ trimer complex due to Au-Au aurophillic interactions. B) X-ray crystal structure of fluorinated Pz’ based Au(I) cyclic trimers packing to illustrate chain formation ability of σ10 trinuclear cyclic trimers due to aurophilic interactions.20a (The ligand Pz’ for this work was provided by Dr. Yang from Omary group).
Figure 3.29 pH dependent photoluminescence emission spectra of cyclic trinuclear Au(I) Pz’ trimer solution at room temperature. A) Addition of 0.1M CH$_3$COOH to trimer solution initially at pH 6.3. B) Addition of 0.1M NH$_4$OH to trimer solution initially at pH 3.0.
solution. With abundant work on syntheses and characterization of different $d^{10}$ cyclic trimers already undertaken by many of previous and present group members, this fascinating work driven by promising photonic and sensor applications was also extended in to aqueous based systems as part of utilizing biocompatible polymers like chitosan for interfacing with phosphorescent molecular systems. In situ syntheses of Au(I) Pz’ cyclic trimers within chitosan polymer is also included as part of chapter 3.

Figure 3.28, shows schematic illustration for formation of cyclic gold (I) modified pyrazolate trimer (CAuPz’T) in aqueous solution. As these cyclic trimers are expected to exhibit multiple phosphorescence bands either due to difference in trimer chain length (dimmers of trimer or trimers of trimer) or due to carboxylic acid modified pyrazole ligand, strong pH sensitive emission is observed from CAuPz’T aqueous solution at room temperature. As seen in figure 3.29A formation of trimer molecules in aqueous solution is confirmed from multiple phosphorescence emission colors, followed by demonstration of retained sensitive photophysical properties even when reaction is performed in aqueous medium. Importance of polymer presence is tested by performing the exact same reaction in absence of polymer in aqueous medium which resulted in complete decomposition of Au(THT)Cl without any emission. As noticed in figure 3.29, trimer solution exhibits two distinguished emission peaks depending on pH of solution. If the reaction is carried out in presence of acidic medium ($pH \sim 3.0$) by addition of acetic acid, strong blue to turquoise emission solution with peak maximum ranging between 450 nm to 500 nm is obtained. (figure 3.29A) Minor variations in pH and molar concentrations of both pyrazole ligand and Au(THT)Cl determine turquoise or blue
emission. But under similar molar concentrations if the reaction is carried at pH > 6.5 (addition of NH₄OH) only a strong red emission solution with peak maximum ~690 nm is obtained. Remarkable pH dependent reversible quenching is shown in figure 3.29.

Titrating red emission (~685 nm) solution by step wise addition of 20 microliters of acid (acetic acid), results in quenching of 685 nm peak accompanied with increase in intensity of turquoise emission peak at ~500 nm. However reversible addition of NH₄OH (figure 3.29B) retains red emission (~ 680 nm) peak with high reproducibility. Excitation spectra (not included in figures) also exhibits similar shift with respective to pH, shorter excitation wavelength (~265 nm) corresponding to red emission undergoes red shift to λ > 300 nm with rise of turquoise emission and vice-versa. Though exact mechanism for pH dependent emission is not clear but it is expected in similar lines with cyclic trinuclear Au(I) triazole phosphors known before. COOH substituted pyrazole ligand makes it more pH sensitive. Figure 3.9 A and B shows very clearly that emission shift is not sudden change from red to turquoise or vice-versa. Intermediate emission consisting of both phosphorescence peaks highlights presence of chains of cyclic molecules in solution that undergo transformations with respective to addition of acid or base.

Previous works by Omary et al. has shown the ability of these cyclic trimer chains to exhibit sensitive emission with entrapment of heavy metal ions also. There are many previous studies using physically or chemically modified chitosan polymer for heavy metal ion uptake based on chelation on sorption properties of chitosan polymer.
Figure 3.30 Changes in photoluminescence emission spectra of cyclic trinuclear Au(I) Pz’ trimer solution due to addition of different metal salts (changes in excitation spectra are shown in black solid lines).
Figure 3.31 Demonstrating heavy metal ion sensitivity using cyclic trinuclear Au(I) Pz’ trimer solution. Initially blue shift in 685 nm emission peak due to addition of Ag\(^+\) and Pb\(^{2+}\) salts and later reversibility is exhibited by addition of EDTA. (Solid blue and green lines represent shift due to addition of Pb\(^{2+}\) and Ag\(^+\) ions respectively, scatterer blue and green line represent reversibility due to addition of EDTA).
Mechanism of uptake monitoring is always limited to Uv-spectroscopy, Mass spectroscopy, Infrared spectroscopy, Potentiometry or Calorimetric titrations. But here for the first time optical based sensors tethered to chitosan polymer are convincingly utilized to demonstrate heavy metal ion uptake (Ag$^+$, Tl$^{+1}$ and Pb$^{+2}$) which can be easily monitored by large changes in photophysical properties before and after entrapment.
This entrapment behavior of heavy metal ions is also found to be reversible. Figure 3.30 shows clear emission shift when the red emitting trimer solution is added with either AgNO$_3$ or Tl(NO$_3$)$_3$ or Pb(NO$_3$)$_2$ salts. Thallium (Tl$^{+1}$) exhibits highest emission blue shift from 685 nm to 460 nm followed by Pb$^{+2}$ (685 nm to 491 nm) and Ag$^{+}$ (685 nm to 508 nm). Reversibility of this emission shift due to presence of heavy metal ions was studied using EDTA (ethylenediaminetetraacetic acid).$^{22}$ EDTA is a well known hexadentate chelating ligand popular for absorbing heavy metal ions like Pb$^{+2}$ from water. On addition of EDTA to heavy metal entrapped CAuPz’T solution, blue shifted emission is completely reversed retaining strong red emission peak. Both Ag$^{+}$ and Pb$^{+2}$ entrapped CAuPz’T solutions exhibited synchronously tunable emission color between red and turquoise in presence and absence of EDTA respectively (figure 3.31). Modified chitosan based polymers under trade names of chitochel and chitoplex$^{21b}$ are already manufactured to adsorb toxic chemicals like mercury, lead from drinking water. As extraction of heavy toxic metal ion Pb$^{+2}$ was already shown, sensitivity of CAuPz’T systems to toxic metal mercury (Hg$^{+2}$) was demonstrated by simple titration performed by addition of Hg$^{+2}$ salt to red emissive cyclic trimer solution (figure 3.32). Strong sensitivity of trimer emission to presence of Hg$^{+2}$ ions was displayed from quenching of red emission. Control experiment with addition of same volume of millipore water does exhibit retained red emission peak suggesting that emission quenching is due to sensitivity of cyclic trimer to presence of Hg$^{+2}$ ions. Results from this simple titration experiment encourages employing these phosphorescent chitosan polymer based cyclic
Figure 3.33 Different heavy metal ions entrapped cyclic trinuclear Au(I) Pz’ trimer chitosan films both under day light and under UV exposure.

This trimer system as optical sensor to detect heavy or toxic metals like Ag⁺/ Pb⁺²/Tl⁺¹/ Hg⁺² in water simply by monitoring changes in emissive properties.

Film forming ability of chitosan polymer has been exploited again to make films of chitosan doped CAuPz’T solutions before and after addition of Pb⁺² and Ag⁺ salts. These pH and heavy metal ion sensitive phosphorescent chitosan films encourage their easy usage as optical sensors. We assume these aqueous sensitive cyclic Au(I) Pz’ trimers that are favorably synthesized in organic phase are found to exhibit stable strong emission with retained sensitive photophysical properties even in aqueous medium due to tethering of these cyclic molecules easily on to chitosan polymer backbone which promotes stability to these otherwise water sensitive complexes.
Figure 3.34 Photoluminescence spectra of solution obtained by mixing individually doped Ag⁺/Pb²⁺/Tl⁺ cyclic trinuclear Au(I) Pz’ trimer solutions (inset shows picture of the sample along with lifetime value for the same solution).

Formation of stable films from more than one cyclic trimer solution elucidates that CAuPz’T formation is taking place on polymer matrix backbone being retained in films (figure 3.33). Intrigued from three emission colors (red, green, blue) these solutions doped with different metal ions were mixed in equal proportions, which resulted in a solution that has broad emission ranging from 480 nm to 680 nm. Though
Figure 3.35 Time resolved photoluminescence decay of trinuclear Au(I) Pz’ trimer solutions both at pH 6.3 and 3.0 (inset shows pictures of samples along with lifetime value for the same solutions). Red emission sample: Corresponds to pH 6.3; Turquoise emission sample: Corresponds to pH 3.0 (data was acquired using xenon flashlamp excitation (337 nm, 290 nm) and a gated microseconds detector. (Dr.El-bjeirami assisted in collecting lifetime data)

emissive color of the sample mixture is not exactly white but exhibits broad peak extending in to red, green and blue wavelength regions (figure 3.34). Time resolved photoluminescence decay data and microseconds lifetime values from individual red and turquoise emissive trimer solutions at pH 6.5 and 3.0 (figure 3.35) and for the heavy
metal ions entrapped mixture solution (figure 3.34) confirms emission is phosphorescence in all these systems under different conditions. Phosphorescent color tunable compounds emissive at room temperature are recognized as very important class of phosphorescent materials due to their limited availability. Such phosphorescent materials with reversible emission capability are considered as interesting materials for sensors and display applications.\textsuperscript{18c} A phosphorescent based thermoreversible organogel with capability to switch emission color was demonstrated by Aida and coworkers from trinuclear Au(I) pyrazolate complex in hexane medium.\textsuperscript{18c} However this is the first ever report that shows formation of color tunable phosphorescent trinuclear Au(I) Pz’ complexes in complete aqueous medium which allows to take complete advantage from these extensively studied phosphorescent systems for biological, biomedical and sensor applications.

3.4 Conclusions

Ability of highly stable, water soluble phosphorescent Au(I) complex that was earlier (chapter 2) used to form phosphorescent hydrogels was adopted again in this chapter to realize its physical crosslinking ability with oppositely charged polymers and PNIPAM microgels. A simple polyelectrolyte complexation (PEC) approach was used to synthesize highly water soluble PCHNPs (phosphorescent chitosan nanoaprticles) using the same phosphorescent molecule which acts as both physical crosslinker and light emitter. Size and stability of PCHNPs are controlled by balancing concentrations of both CS and AuP indicating importance of electrostatic interactions. Both TEM and DLS analysis are used in evaluating size, distribution and
morphology of PCHNPs at different concentrations of AuP and CS polymer. By following the DLS and TEM data critical concentrations of AuP and CS that result in formation of small and stable particles at different pH is evaluated. This allows for facile syntheses of different size luminescent chitosan nanoparticles in a single step. With not much detailed work, same physical crosslinking ability was reconfirmed using PNIPAR-co-NH$_2$ polymer where NIPA polymer chains are crosslinked in to nanoparticles. Owing to retained emission, PL enhancement and microseconds lifetime the ability of AuP not only as physical crosslinker but also as promising contrast agent within polymer matrices is explained. This can facilitate AuP usage as promising phosphorescent physical crosslinker with broader domain of positively charged amine based polymers and biomolecules. After looking at these results, detailed studies on PNIPAM-co-allylamine microgels exposed ability of AuP to instigate inverse thermo-reversible physical gealtion/aggregation within PNIPAM-co-allylamine microgels. Retained photoluminescence properties of AuP in both sol and gel forms signifies stability of AuP in presence of different matrix systems. Systemic emission tuning exhibited by AuP within these different soft material systems based on rigidity of the matrix support its practicable usage as optical indicator for sensing changes in matrix rigidity of polymer systems. Concentrations of PNIPAM-co-allylamine microgels, AuP are observed to be determining factors for tuning strength of inverse thermo-reversible gelation process. Phenomenon of physical gelation is easily visualized from simply inverting the test tube and also from quantitative changes in hydrodynamic radius during gelation. Light scattering and PL data cellarily
demonstrates preserved intrinsic features of both polymer systems and AuP. In a different scenario chitosan matrix has been shown to act as tempalte to form aqueous sensitive trinuclear Au(I) phosphorescent complexes in complete aqueous medium. Color tunability with respective to pH and heavy metal entrapment is completely retained in polymer supported phosphorescent complexes as expected from their original counterpart in organic phase. This particular investigation entails ability of chitosan polymer to carry reactions with other wise aqueous sensitive systems.

3.5 References


CHAPTER 4

IN SITU SYNTHESSES OF TOXIN FREE SPHERICAL AND ANISOTROPIC GOLD NANOPARTICLES STABILIZED WITHIN BIOMpatible POLYMERS AND THERMORESPONSIVE HYDROGELS

4.1 Introduction

Interest in nanoscale materials arises from the fact that at this length scale properties that vary depending on size of material are not exclusively effect of scaling but also strongly get influenced depending on nature and shape of nanomaterials.¹ For example in semiconductor nanoparticles famously referred as “Quantum dots” (CdSe, CdTe) property change at nanoscale is brought due to quantum confinement of electron motion. Below size of Bhors radius or when De-Broglie wavelength of valence electrons is of the same order as the size of particle, free mobile electrons becomes more confined and the particle behaves as zero dimensional quantum dot.¹a This quantum confinement of electrons results in quantization of energy levels similar to molecular systems, because of which band gap emission in such systems is observed to shift through the entire visible region depending on size of quantum dot. Smaller the size more is emission blue shift as shown in figure 4.1.¹b This significant quantum confinement of the electrons has been explored for many materials and biological applications.¹b When it comes to metals, electrons are least confined as there is no separation between conduction and valence bands. In noble metals like Au (gold), Ag (silver), Cu (copper) or Pt (platinum) decrease in size below electron mean free path (the distance electron travels between scattering collisions with lattice centers) or Bhors radius, gives rise to intense absorption or scattering due to coherent oscillation of free
surface electrons in conduction band. In classical picture plasmons (electron gas) can be described as an oscillation of free electron density against the fixed positive metal core (figure 4.2A). Electromagnetic radiation in resonance with oscillations of these free surface electrons result in surface plasmon absorption (SPA) or surface plasmon resonance (SPR) or also called as localized surface plasmon resonance (LSPR) since it is localized at the surface of metal (figure-4.2A).

Figure 4.1 Shows tuning of photoluminescence for CdSe quantum dots with respective to change in size.\textsuperscript{1b}

This strong absorption or resonance condition is sensitive to size, shape of the nanoparticles and pH, dielectric constant of the surrounding medium resulting in vivid colors and tunable SPR\textsuperscript{1c,d} (figure 4.2, 4.3). Even long before scientific enthusiasm there are many examples showing usage of these brilliant color materials as pigments throughout Europe in stained glass windows of cathedrals and by the Chinese in coloring vases and other ornaments such as those contained in the glass of the famous Lycurgus Cup\textsuperscript{2,3} without much understanding about their origin. As the shape or size of
the nanoparticle changes, the surface geometry changes, and a shift in the electric field density on the surface causes shift in SPA (figure 4.2B). These coherent collective oscillations of electrons that induce large surface electric fields make the absorption cross section of these nanoparticles orders of magnitude stronger than the organic dyes.\textsuperscript{1b,d,3} For instance when monodispersed spherical gold nanoparticles (~10-25 nm) in solution are excited by electromagnetic radiation it results in surface plasmon absorption with extinction spectrum peak around 520 nm.\textsuperscript{1,3} Using Maxwell’s equations, Gustav Mie’s \textsuperscript{3} has been able to explain origin of SPR, vivid colors and shift in SPR that colloidal gold solutions display depending on size, shape or dielectric constant of the medium. In contrast to only small changes noticed in SPA or color with respective to

Figure 4.2  A) Origin of SPR (surface plasmon resonance) due to collective oscillation of surface plasmons.\textsuperscript{1d} B) Sensitivity of SPA (surface plasmon absorption) with respective to size and shape of AuNPs.\textsuperscript{1}
different sizes of spherical gold nanoparticles, the optical properties of anisotropic (non-spherical) nanoparticles can change dramatically and result in high sensitive nanostructures.\textsuperscript{1b,2} According to Mie theory, frequency of the plasmon absorption band varies from spherical to non-spherical nanoparticles (nanospheres, nanoshells, nanocages, nanorods, nanoprisms, nanopods, dogbones, cubes) as seen in figure 4.3.\textsuperscript{4} Multiple absorption bands exhibited by anisotropic particles are correlated with their multiple axes. For example among two plasmon resonances of nanorods longitudinal plasmon resonance seen at longer wavelength (< 600 nm) is more sensitive to aspect ratio (longitudinal: transverse length) (figure-4.2 and 4.3). The transverse surface plasmon resonance does not depend on the aspect ratio and is at the same wavelength as the plasmon resonance of nanospheres (~525 nm).\textsuperscript{1,2} Edges, corners, width and thickness in nanoprisms, nanoplates, nanocubes, and nanododecahedral structures dictate tuning of SPR.\textsuperscript{2,5} Another important class of gold nanoparticles that are widely studied for their strong SPR tunability are gold nanoshells, composed of dielectric cores (silica) coated with thin gold layers (few nanometers). Nanoshells allow tuning of the SPR across the visible and infrared region with slight variations in size ratio of core to shell.\textsuperscript{5c} Though works of Halas and Naomi have verified promising features of nanoshells for biomedical applications, chapter 4 does not involve any contrasting studies with nanoshells because of their bimetallic (silicon-Au) feature.
Figure 4.3 Vivid colors that arise due to different shapes/sizes of different plasmonic nanoparticles. a) Au nanospheres. b)Au nanorods. c) Ag nanoprisms. d) Alloy (Au-Ag) nanoparticles. e) Au nanorods with increasing aspect ratio. f) Ag nanoprisms with different lateral sizes.2

This chapter describes in detail about a new invention pertaining to facile syntheses of AuNPs of various sizes and shapes in wide range of environmentally sensitive and biocompatible polymers/gels which are expected to have wider range of applications (figure 4.4) in view of their non-toxin nature and environmental/biological compatibility. In order to validate our claim, effort has been made to contrast with all the popular existing methods in literature regarding preparation of different shape/ size gold nanoparticles so that the new syntheses protocol can be appreciated. As it is not within scope of this dissertation to describe all existing techniques in detail a quick
overview of commonly applied syntheses techniques and applications are tabulated. Because this chapter is mostly concerned with detailing a new invention on syntheses of AuNPs, mechanism of nucleation or growth of different nanostructures are not discussed in detail.

Figure 4.4  A) Lists out optical properties and related applications of AuNPs. B) A simple pie chart representation of different biomedical applications of AuNPs.
Before going into details about syntheses of colloidal gold nanoparticles it is important to understand significance of stabilizers. Due to high surface energy nanoparticles at short interparticle distances tend to attract and coagulate or agglomerate resulting in loss of characteristic optical properties. Stabilization or counteraction is generally achieved by steric or electrostatic stabilization by special molecules or solvents. Even in non-polar medium like organic solvents, stabilizing molecules act as strong adsorbents and prevent agglomeration. The use of metallic nanoparticles as structural and functional units for all the applications depends on functionality of these stabilizing units. Among different ligands, thiol based ligands are highly recommended for their strong affinity to bind with gold due to soft character of both Au and S. Other sulfur based ligands like xanthates, disulfides, have been used to stabilize AuNPs. Non sulfur ligands like phosphines, phosphine oxides, amines and carboxylate ligands are also known to stabilize AuNPs. With special interest for these nanoparticles for biomedical applications, this chapter is mainly focused on stimuli sensitive and biologically benign polymers and gels as stabilizing units in order to avail synergetic advantages of both stimuli sensitive templates and AuNPs.

Generally, there are two approaches for making metal sols, “Bottom-up” and “Top-Down” approach. All the synthetic strategies described in this chapter either for AuNPs or silver nanoparticles in later chapters are “Bottom-Up” approaches. In this approach a metal salt precursor is reduced into metal nanoparticles predominantly by employing a chemical reducing or by thermolysis or photolysis or radiolysis or solvalolysis. “Top-down” approach is based on diminishing process of bulk metal in to metal
nanoparticles either by photo lithography or electron beam lithography. “Top-Down” approach can reach only upper end of nanometric regime and suffer from poor dispersity, cost and complex techniques. Whereas “Bottom-Up” approach allows for preparation of monodispersed defect free, crystalline nanoparticles through facile techniques in different media. 7b

Table 4.1  Different syntheses techniques known in literature for making spherical AuNPs.

<table>
<thead>
<tr>
<th>Syntheses techniques for making gold nanospheres</th>
<th>Comments (HAuCl₄ as universal precursor)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Reduction</td>
<td>NaBH₄ as reducing agent in aqueous medium, hydrazine for organic solvents. Mostly thiol capped or citrate capped. ³,⁸a</td>
<td>³, ⁸</td>
</tr>
<tr>
<td>Laser Ablation</td>
<td>Using pulsed laser, pulverization of solid target. ⁸a,⁹ (gold film as precursor)</td>
<td>⁸a, ⁹</td>
</tr>
<tr>
<td>Microwave heating</td>
<td>Uniform heating promotes dispersity, in presence or absence of reducing agents. ⁸a,¹⁰ (HAuCl₄ precursor)</td>
<td>⁸a, ¹⁰A, B, C</td>
</tr>
<tr>
<td>Photochemical syntheses</td>
<td>Using AuCl₄⁻ precursor, different polymers used as stabilizers. In absence/presence of reducing agents. Very rarely using Au(I) precursors in organic solvents by Vogler and Omary et al separately. ⁸a,¹¹,¹²</td>
<td>⁸a, ¹¹A, B, C, D, ¹² A, B, C, D</td>
</tr>
<tr>
<td>Electrochemical method or Electrode depositions</td>
<td>Using HAuCl₄ as precursor, different kinds of stabilizers, polymers or surfactants. ⁸a,¹³ Size and stability controlled by varying electric potential parameters.</td>
<td>⁸a, ¹³ A, B, C</td>
</tr>
<tr>
<td>Sonochemical method</td>
<td>Using HAuCl₄ precursor, different kinds of stabilizers. Size and distribution controlled by ultrasound frequency. ⁸a,¹⁴</td>
<td>⁸a, ¹⁴ A, B, C, D</td>
</tr>
<tr>
<td>Radiolytic method</td>
<td>Using AuCl₄ as precursor, strength and time of γ-radiation controls size. ⁸a,¹⁵</td>
<td>⁸a, ¹⁵A, ¹⁵B, C</td>
</tr>
</tbody>
</table>

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Metal colloid science has begun with making of gold sols by Michael Faraday in 1857, where Faraday has prepared red solutions of colloidal gold by reduction of aqueous solution of chloroaurate (AuCl₄⁻) using phosphorus in CS₂ (carbon disulfide) as reducing agent. The term colloid was coined shortly thereafter by Graham in 1861.³ Over the years many methods for preparation of gold colloids were reported but extensive studies by Turkevich and Brust el al. separately has refined understanding of nucleation, growth and agglomeration of gold sols.³ Turkevich’s reduction of Au(III) compound HAuCl₄, either in presence of NaBH₄ or in presence of hot sodium citrate solution is still most popular.³ In this method size and dispersity of colloidal gold is controlled by varying concentrations of both chloroauric acid and sodium citrate or NaBH₄. Here sodium citrate acts as both reducing and stabilizing agent but monodispersity is sacrificed with increase in size.⁸ Later two phase method developed by Burst³ involve dissolving HAuCl₄ in water and subsequently transporting into toluene by means of tetraoctylammonium bromide (TOAB), which acts as a phase transfer agent. The toluene solution is then mixed and thoroughly stirred together with an aqueous solution of sodium borohydride, in the presence of thioalkanes or aminoalkanes, which readily bind to the Au nanoparticles. Very small particles ranging between 5-10 nm obtained by this method are stabilized by thiols or amines. But this method is limited to non-aqueous systems which would limit its applications.³,a,⁸ Brust biphasic method was applied to develop famous Schmid’s method which resulted in highly monodispersed gold clusters or very small (1-5 nm) monodispersed AuNPs.³ [Au₅₅(PPh₃)₁₂Cl₆], [Au₁₀₁(PPh₃)₁₃₁Cl₆] clusters are formed by dissolving HAuCl₄ and
N(C₈H₁₂)Br mixture in water-toulene mixture to which PPh₃ and NaBH₄ are added. But this method suffers with solubility issues associated with starting precursors and other intermediates.³ With advent of technology, many different syntheses approaches are in rise, so some popular syntheses techniques for making spherical gold nanoparticles are summarized in table-4.1. Among all chemical syntheses techniques universal precursor is HAuCl₄, a quick Scifinder search with same limiters hits only one article using Au(I) precursor compared to 430 articles using HAuCl₄. Toxicity and other issues related to using HAuCl₄ precursor are discussed in detail in many literature reports previously.⁶ To the best of our knowledge only less than 5 reports exist in literature that employ Au(I) precursors to make AuNPs but however all these reports are severely limited to organic solvents only (see references in table-1).

From table 4.1, it is clear that chemical methods using reducing agents are popularly preferred for convenience and simplicity but concentration of reductants, solvent miscibility, environmental and biological hazards are all determining and controlling factors.¹⁶ Electrochemical methods avoids usage of reducing agents but more predominantly applied for producing anisotropic structures due to involvement of specialized templates that restrict facile syntheses under normal conditions.⁸,¹³ Radiolytic and photochemical methods have aroused much attention for preparation of size-controlled colloidal gold particles due to several advantages like, (i) controlled reduction of precursor ions in complete absence of any special reducing agent; (ii) radiation is absorbed regardless of the presence of light-absorbing solutes or precursors; (iii) reduction is uniformly performed in the solution, and the rate of
reaction can be determined easily. But in point of practical applications, photochemical method is more competitive without the needs of specific instrumentation in contrast to radiolytic methods (see table 4.1 for references).

Though detailed applications of gold nanospheres are not discussed but a quick look at table 4.2, highlights usage of gold nanospheres significantly for biomedical usage compared to materials applications. With this regard and also considering environmental/biological hazards of reducing agents and stabilizers or organic solvents, AuNPs synthesized within environmental sensitive and benign polymers or gel templates has attracted special attention compared to using strong surfactants (table 4.3). Employing hydrogels as stabilizing templates in syntheses of metallic nanoparticles is very fascinating and expanding research area driven by potential applications and interesting science associated with these hybrid systems. Of particular interest is the conjugation of gold nanoparticles with “smart” stimuli sensitive polymers such as poly-N-isopropylacrylamide (PNIPAM). Table 4.3 shows, two common approaches to prepare Au nanoparticle–PNIPAm hybrid composites or in general polymer hybrid composites. Both approaches employ HAuCl₄ as starting precursor in presence of regular reducing agents. In one approach citrate stabilized or prior formed AuNPs are placed in to hydrogels/polymers matrices and second method involves formation of AuNPs within hydrogels and polymers by using a reducing agent. Both these methods take advantage of temperature sensitivity of PNIPAM polymer to study changes in thermosensitivity of hybrid hydrogels as sensors and delivery agents (table 4.3).
Table 4.2  Applications listed in literature for spherical AuNPs.

<table>
<thead>
<tr>
<th>Some important applications of AuNPs (nanospheres)</th>
<th>Syntheses protocol and comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calorimetric detection of polynucleotides, DNA assay, organizing Au nanoclusters on DNA, nanoplasmonic molecular ruler</td>
<td>Using citrate or phosphine stabilized AuNPs and modifying them using –SH or amine based oligonucleotides. Calorimetric complementary detection or size change of nucleic acids due to attachment to AuNPs helps detection and analysis. (^{17})</td>
<td>17A, B, 26</td>
</tr>
<tr>
<td>Sensing biomolecules like glucose</td>
<td>Using conductive polymer polyaniline stabilized AuNPs, due to efficient electroactivity. (^{18})</td>
<td>18, 26</td>
</tr>
<tr>
<td>SERS (surface enhanced Raman scattering) using AuNPs stabilized by different polymers or biomolecules</td>
<td>For probing different analytes like biomolecules or DNA or Nucleic acids or differentiating tumor cells. (^{19})</td>
<td>19 A, B ,C</td>
</tr>
<tr>
<td>Fluorescence quenching</td>
<td>Distance dependent or due to FRET (fluorescence resonance energy transfer) detect analyte concentration. (^{20})</td>
<td>20 A, B, 26</td>
</tr>
<tr>
<td>Sensors</td>
<td>Presence of analyte detected by changes in SPR or color. (^{21})</td>
<td>21A, B</td>
</tr>
<tr>
<td>Heat source</td>
<td>Hyperthermia (to kill cancer cells), optical triggered opening of polymeric capsules or melting of DNA or protein bonds, photoacoustic tomography and photothermal imaging. (^{22,1d})</td>
<td>22A, B ,C, D, 1D</td>
</tr>
<tr>
<td>Bioconjugation, Detection, Contrast agents, Tracking (X-ray computed tomography)</td>
<td>Using antibody labeled AuNPs (binding to EFGR), detecting cancer cells by different optical imaging techniques. (^{23})</td>
<td>23 A, B,</td>
</tr>
<tr>
<td>Immunostaining</td>
<td>Visualization of cellular organelles by simple optical microscopy. (^{24})</td>
<td>24</td>
</tr>
<tr>
<td>Delivery</td>
<td>Delivery of genes, DNA, nucleotides or biomolecules either by specific or nonspecific uptake (site specific receptors). (^{25,26})</td>
<td>25A, B, C, 26</td>
</tr>
<tr>
<td>Catalysis</td>
<td>Mostly prepared by physical deposition techniques but deposition techniques found to have less control over size. (^{27})</td>
<td>27A, B, C,D</td>
</tr>
</tbody>
</table>
Table 4.3  Different syntheses techniques known in literature for making spherical AuNPs stabilized within different hydrogels and polymers.

<table>
<thead>
<tr>
<th>Brief chemical syntheses protocol of gold nanospheres stabilized within polymers and gels</th>
<th>Remarks (AuCl$_4^-$ as universal precursor)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>THPC/PNIPAM-acrylic acid gel</td>
<td>Tetrakis(hydroxymethyl)phosphonium chloride as reducing agent (thermoreponsive AuNPs).$^{28}$</td>
<td>28</td>
</tr>
<tr>
<td>Citrate stabilized AuNPs/PNIAPM-amine gels</td>
<td>Au core PNIPAM gels (thermoreponsive AuNPs).$^{29}$</td>
<td>29</td>
</tr>
<tr>
<td>PNIPAM gel/ NaBH$_4$</td>
<td>Thermoresponsive AuNPs$^{30}$</td>
<td>30</td>
</tr>
<tr>
<td>Citrate reduced AuNPs/PNIPAM-amine gels or –SH terminated PNIPAM</td>
<td>Gold coated hydrogels for optical driven drug delivery device, thermoresponsive AuNPs.$^{31}$</td>
<td>31 A, B, C</td>
</tr>
<tr>
<td>PNIPAM-core shell/NaBH$_4$</td>
<td>Making functional building blocks of complex tunable optical materials.$^{32}$</td>
<td>32 A, B</td>
</tr>
<tr>
<td>PEG-$b$-PVP-$b$-PNIPAM/NaBH$_4$</td>
<td>Polymer Shell-Au@core.$^{33}$</td>
<td>33</td>
</tr>
<tr>
<td>C$_{14}$-SH, PS-SH, or PEO-SH (lithium triethylborohydride/THF)</td>
<td>High grafting polymer coated AuNPs.$^{34}$</td>
<td>34</td>
</tr>
<tr>
<td>Allylmercaptan/NaBH$_4$/PNIPAM</td>
<td>Electrical properties are thermo-switchable.$^{35}$</td>
<td>35</td>
</tr>
<tr>
<td>Modified Polyacrylic acid/heat</td>
<td>Polymer stabilized single step syntheses.$^{36}$</td>
<td>36</td>
</tr>
<tr>
<td>PAMAM dendrimers/NaBH$_4$</td>
<td>Organic-Inorganic hybrids (catalytic applications).$^{37}$</td>
<td>37 A, B</td>
</tr>
<tr>
<td>Agarose/P(CH$_2$NHCH$_3$COOH)$_3$</td>
<td>P(CH$_2$NHCH$_3$COOH)$_3$ as reducing agent; for detection of micromolar concentrations of DNA nucleosides using SERS.$^{38}$</td>
<td>38</td>
</tr>
<tr>
<td>Polyglycidyl-PNIPAM/NaBH$_4$</td>
<td>Thermoresponsive nanoreactors, catalysis in aqueous medium.$^{39}$</td>
<td>39</td>
</tr>
<tr>
<td>N-acetylated chitosan/NaBH$_4$/ Heat</td>
<td>For gene delivery, for transmucosal delivery of insulin.$^{40}$</td>
<td>40 A, B</td>
</tr>
</tbody>
</table>
Table 4.4  Applications of NIR (near-infrared) absorbing or scattering anisotropic AuNPs known in literature (excluding gold nanoshells).

<table>
<thead>
<tr>
<th>Applications of NIR absorbing anisotropic AuNPs</th>
<th>Comments and brief description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiating tumor cells from normal cells by molecular imaging. (dark field, optical, two photon, optoacoustic and photoacoustic tomography)</td>
<td>Dark filed optical imaging of cancer cells (functionalized AuNRs by coating with anti-EFGR (epidermal growth factor receptor) antibodies). Detection of bioconjugated cancer cells by enhanced Raman signals, detection of tumors in deep tissue by two-photon enhanced luminescence. Imaging deep tissue to differentiate tumor cells, early detection of tumor cells, deep tissue imaging. 41, 42</td>
<td>41, 42</td>
</tr>
<tr>
<td>Detection, Sensing</td>
<td>Detection of H1gG (human immunoglobulin G antibodies), fluorescent AuNRs as optical biosensors and for DNA analysis. Raman based intracellular biosensing, refractometric biomolecular sensors, sensing based on Raman enhanced signals. 41, 42</td>
<td>41, 42</td>
</tr>
<tr>
<td>Delivery</td>
<td>Multifunctional nanorods for gene deliver, release of plasmids DNA (by laser irradiation). 43</td>
<td>43</td>
</tr>
<tr>
<td>Hyperthermia or Plasmonic photothermal therapy</td>
<td>AuNRs functionalized with specific targeting molecules followed by irradiation to generate heat that kills localized tumor cells (membrane blebbing). Targeting macrophage cell lines. 43, 44, 45</td>
<td>43, 44, 45</td>
</tr>
</tbody>
</table>

Compared to spherical AuNPs anisotropic particles with strong sensitivity to size/shape and environment has attracted special interest specifically in biomedical arena due to their strong tendency to absorb light in the NIR region. One of the highly anticipated applications of NIR (near-infrared) absorbing AuNPs is Photothermal therapy (PTT). It exploits the fact that resonance of these anisotropic nanoparticles can...
be tuned to the infrared “water window” (700 nm-900 nm) wavelength region where biological tissue is highly transparent. So when nanoparticles are injected into the bloodstream and accumulated at tumor sites by antigen antibody interactions, they can heat their local environment when irradiated with laser light whose wavelength coincides with the SPR wavelength of the nanoparticles. Adjacent healthy tissue without feded nanoparticles is unaffected by the laser light alone, but cancer cells in the direct vicinity of the nanoparticles undergo hyperthermia and get destroyed, resulting in drug-free tumor death. Critical requisite for PPTT (plasmonic photothermal therapy) is gold nanoparticles with strong absorption cross section, leading to efficient photothermal heating in the direct vicinity of the nanoparticle in a physiologically compatible wavelength range. Other property of NIR plasmon structures important to consider is light scattering, which does not lead to local heating but does allow to easily locating the presence of nanoparticles and hence is a highly desirable property for providing contrast in bio-imaging applications (table 4.4). Some other detailed biomedical applications of anisotropic particles are listed in table 4.4. In contrast to artless single step spherical nanoparticles syntheses, syntheses of anisotropic particles are more complicated involving multiple step protocols. Complete control on size and shape of anisotropic particles is possible by template based “Top-Down” approach however these approaches are not as popular as chemical reduction techniques for involvement of horrendous effort in making milligrams of sample and also requiring a suitable stabilizing template to redisperse. Of known methods, wet chemical seed-mediated protocol (table 4.5 and table 4.6) is famous for making shape desirable gold
### Table 4.5 Different chemical reduction techniques known in literature for making anisotropic AuNPs (excluding photochemical approach).

<table>
<thead>
<tr>
<th>Wet chemical techniques for making anisotropic AuNPs</th>
<th>Syntheses description and comments (HAuCl₄ as universal precursor)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed mediated growth technique for making AuNRs (gold nanorods)</td>
<td>Au seeds from reduction of HAuCl₄ in presence of NaBH₄ followed by addition of seeds to growth solution of CTAB and ascorbic acid with small quantities of AgNO₃. Yield depends on exact ratio of Ag⁺/ascorbic acid/CTAB (3 step protocol).</td>
<td>46 A, B, C, D</td>
</tr>
<tr>
<td>Seed mediated growth of nanoprisms</td>
<td>A three step method involving formation of seeds and growth solutions by reduction of HAuCl₄ in presence of CTAB/ascorbic acid/NaBH₄/NaOH.</td>
<td>46</td>
</tr>
<tr>
<td>Mixture of triangular and hexagonal plates by thermolysis</td>
<td>Mixing heating solutions of HAuCl₄ and sodium citrate added to hot solution of CTAB and HAuCl₄ solution. (Mixture of spheres, triangles, polygonal structures, not good NIR absorption peak).</td>
<td>47 A, B</td>
</tr>
<tr>
<td>Size controllable syntheses by thermolysis</td>
<td>HAuCl₄/ Sodium citrate/ PVP (polyvinyl pyrolidone) by heating. NIR absorbance tuned by ratio of PVP/Au ratio. Sensitive to precursor’s ratio.</td>
<td>48</td>
</tr>
<tr>
<td>Syntheses of gold nanorods by ultrasonication</td>
<td>Ultrasonic irradiation/ HAuCl₄/ CTAB/ascorbic acid/AgNO₃. Ultrasonication helps reduction of Au⁺³. CTAB and Ag⁺ ions promote growth of rods. pH&lt; 3 more NIR absorbing species. pH&gt;7 more spheres.</td>
<td>49</td>
</tr>
<tr>
<td>Microwave assisted syntheses of anisotropic gold nanoparticles</td>
<td>HAuCl₄/ Tetradecylammonium bromide (TOAB)/acetone/sodium citrate. Microwave heating results in formation of seed and TOAB acts as templating surfactant (mixture of rods and spheres).</td>
<td>50</td>
</tr>
</tbody>
</table>

Nanostructures. Vast majority of syntheses techniques involving making of anisotropic or shape controlled AuNPs rely on seed mediated growth technique. Shape control has
been shown to be achieved by two step process; in the first step small monodispersed spherical seed particles are produced by chemical reduction. Second step follows addition of more gold ions and different reducing agents along with shape templating surfactants or polymers. For example, Murphy group showed high yield syntheses

Table 4.6 Photochemical syntheses protocols known in literature for making anisotropic AuNPs.

<table>
<thead>
<tr>
<th>Photochemical syntheses of anisotropic AuNPs</th>
<th>Syntheses protocol and comments (HAuCl₄ as universal precursor)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape controlled syntheses of AuNPs</td>
<td>30 W Hg lamp (λ=253 nm)/ AuCl₄⁻/ PVA as capping agent, 48 hrs exposure. PVA concentration decided spheres or other shapes (no NIR peaks).</td>
<td>51</td>
</tr>
<tr>
<td>NIR absorbing AuNPs</td>
<td>8 W Hg lamp (λ=306 nm)/ AuCl₄⁻/ sodium oxalate/ 120 minutes. NIR peaks &gt; 700 nm tuned with concentration of sodium oxalate, NIR peaks absorbance lower than spherical SPR peak (mixture of spheres and different shapes). (oxalates are toxic to humans (Wikipedia))</td>
<td>52</td>
</tr>
<tr>
<td>Mixture of rods and spheres by UV irradiation using cationic micelle template</td>
<td>200 W Hg lamp (λ=253.7 nm)/ AuCl₄⁻/ Hexadecyltrimethyl ammonium chloride. AuCl₄⁻ concentration dictates ratio of spheres or rods (no strong NIR peak, mixture of rods and spheres).</td>
<td>53</td>
</tr>
<tr>
<td>Photochemical syntheses of gold nanorods</td>
<td>420 µW/cm² Hg lamp (λ=254 nm) is light source. Hexadecyltrimethyl ammonium chloride/ Tetracdecltrimethyl ammonium bromide as growth surfactants/ AuCl₄⁻/ AgNO₃/ cyclohexane/ acetone. Tuning in NIR peaks with Ag⁺ ion concentration. Good control over shape.</td>
<td>54</td>
</tr>
</tbody>
</table>
of nanorods stabilized in CTAB surfactant starting with citrate capped NaBH₄ reduced seed particles in absence of silver nitrate and later investigated effect of silver nitrate on aspect ratio of nanorods and also other shapes. In contrary Nikoobakht and El-Sayed produced aspect ratio tunable nanorods using CTAB stabilized seeds in presence of BDAC. Triangular prisms were produced by Mirkin et al. by using citrate capped seeds in a saturated solution of CTAB where as Yun et al. employed similar method for producing nanoplates by using PVP (poly (vinyl pyrolidone) instead of CTAB. Later on many research groups have tuned nanoprisms thickness and length by varying pH or ionic strength of medium.

Table 4.7 A comparison of popular literature protocols with proposed invented protocol for syntheses of spherical and non-spherical AuNPs

<table>
<thead>
<tr>
<th>Method</th>
<th>For spherical AuNP’s</th>
<th>For anisotropic particles</th>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>HAuCl₄</td>
<td>HAuCl₄</td>
<td>Precursor</td>
</tr>
<tr>
<td></td>
<td>NaBH₄ or hydrazine</td>
<td>NaBH₄, ascorbic acid, silver nitrate (2-3 step protocol)</td>
<td>Reducing agent</td>
</tr>
<tr>
<td></td>
<td>CTAB, hydrogels, polymers</td>
<td>CTAB, BDAC, Hydrogels</td>
<td>Stabilizing agents</td>
</tr>
<tr>
<td>Method described</td>
<td>Au (THT)Cl/ Au(Me₂S)Cl</td>
<td>Au(Me₂S)Cl (1 step protocol)</td>
<td>Precursor</td>
</tr>
<tr>
<td></td>
<td>Light or heat or ambient</td>
<td>Light or heat</td>
<td>Reducing agent</td>
</tr>
<tr>
<td></td>
<td>Biocompatible polymers like chitosan, PAA, PVA, alginic acid and hydrogels.</td>
<td>Hydrogels/ chitosan polymer</td>
<td>Stabilizing agent</td>
</tr>
</tbody>
</table>
In summary, usage of strong toxic surfactants like CTAB in aqueous medium or PVP in organic medium has been found to be critically unavoidable for making high impact NIR absorbing gold nanostructures whatever experimental approach is followed. Toxic effect of CTAB and other reducing agents involved in the above described protocols is discussed in detail in later sections. Considering biological and environmental concerns, for ideal usage of MNPs (metal nanoparticles) for biological activity and to adopt “green chemistry” principles following conditions needs to be satisfied: (i) they must be formed in an aqueous solution; (ii) they must be free of toxic impurities on the surface; (iii) they must contain reactive chemical groups to

![Figure 4.5A](image-url)
simplify subsequent attachment to biomolecules; (iv) A green method for preparation should pass evaluation in four primary aspects: the solvent, the precursor, the stabilizer and the reducing agent. Keeping in view of above listed requirements a unfamiliar combination of Au(I) starting precursor in combination with biopolymers and hydrogels for making size/shape tunable AuNPs is detailed in this chapter. A generalized overview (table 4.7) of conventional literature protocols for syntheses of both spherical and non-spherical AuNPs highlights proposed protocol in this chapter as comparatively non-toxic and biologically or environmentally friendly approach.

Using Au(I) compounds for syntheses of AuNPs is rarely been explored in organic media or completely unexplored in aqueous medium due to decomposition tendency of these Au(I) compounds. Explaining the light sensitivity of Au(I) and Au(III) compounds, Vogler group detailed photolysis mechanism of different Au(I) systems in organic solvents. These studies though explained mechanism of reduction of Au(I) to Au(0) during photolysis of Au(CO)Cl (gold carbonyl chloride) in dichloromethane but did not focus on stabilizing colloidal Au(0). Significant leap in this direction was taken by our group by demonstrating formation of AuNPs starting from Au(I) isonitriles and carbonyls by simple photolysis under ambient conditions in organic medium along with explaining mechanism of photolysis in LAu(I)X compounds (figure 4.5A). Addition to these reports Lu et al. described formation of colloidal gold by dissolving Au(I) halides (AuCl, AuBr) in chloroform in presence of alklyamines by heating to 60°C while reduction of Au(I) phosphine complexes such as [AuCl(PR₃)] in presence of B₂H₆ reducing agent has been shown to produce Au₅₅ clusters. Careful examination of above
literature reveals unexplored area of stabilizing AuNPs in aqueous media using Au(I) starting precursors. Chapter 4 includes a detailed study about formation of AuNPs using Au(I) sulfide precursors. Au(THT)Cl or Au(Me₂S)Cl⁵⁵ are both temperature and moisture sensitive compounds, labile THT or Me₂S ligands make it easy to form AuNPs even under room light. This photodecomposition of (figure 4.5A,B) Au(I) sulfide compounds makes them very desirable starting precursors for syntheses of Au nanoparticles by employing appropriate stabilizers in complete absence of any reducing agent. Much work has been published in literature on using Au(THT)Cl or Au(Me₂S)Cl as starting precursors for syntheses of Au(I) and Au(III) compounds⁵⁶ concerned to different applications. Significance of this proposed methodology lies in fact that reduction of Au(III) to Au(0) involves two electrons whereas from Au(I) to Au(0) involves single electron making it feasible in absence of special reducing agents. Understanding these significant parameters, this is the first time we are proposing a method to form stable colloidal gold nanoparticles of various sizes and shapes in aqueous solution by using Au(I) sulfides in wide range of polymers and gels. Our novel preparative method for gold nanoparticles rely on reductive elimination of labile ligand (THT or Me₂S) simultaneously followed by stabilization of growing gold nuclei within polymer/hydrogels matrices.

4.2 Experimental section

4.2.1 General Procedures

All glassware was cleaned with aqua regia and then with strong base (saturated KOH in isopropyl alcohol) before use. THT and Me₂S ligands are brought from Sigma-
Aldrich and used as received. Chemicals and polymers required are brought from Polysciences.

Table 4.8  ICP-MS (Inductively coupled plasma mass spectroscopy) instrument parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Parameters (L/min)</td>
<td></td>
</tr>
<tr>
<td>A) Plasma flow</td>
<td>17.5</td>
</tr>
<tr>
<td>B) Auxiliary flow</td>
<td>1.65</td>
</tr>
<tr>
<td>C) Sheath gas</td>
<td>0.17</td>
</tr>
<tr>
<td>D) Nebulizer flow</td>
<td>0.95</td>
</tr>
<tr>
<td>Torch alignment (mm)</td>
<td></td>
</tr>
<tr>
<td>Sampling depth</td>
<td>5.0</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Stabilization delay (s)</td>
<td>0</td>
</tr>
</tbody>
</table>

4.2.2 Physical Measurements

Please see section 2.2.2 in chapter 2 for spectroscopy and light scattering instrumentation and 3.2.3 in chapter 3 for electron microscope details. ICP-MS (Inductively coupled plasma mass spectroscopy) data was collected using Varian 820 MS system in collaboration with Dr. Verbeck in chemistry. Details of ICP-MS instrument set up are shown in table. Utilizing ICP-MS for quantitative analysis of AuNPs is discussed elsewhere.\textsuperscript{57a}

4.2.3 Photolysis

Medium pressure, mercury-vapor lamp (Ace Glass) lodged in to photochemistry box with facilities for stirring and temperature control was used. Of total energy radiated from Hg lamp, approximately 40-48% is in the ultraviolet portion of the spectrum, 40-43% in the visible, the balance in the infrared region.
4.2.4 Syntheses

4.2.4.1 Syntheses of Au(THT)Cl or Au(Me₂S)Cl

Au(THT)Cl or Au(Me₂S)Cl was freshly prepared following literature procedure. Briefly 0.9 gm of gold was first dissolved in 3:1 ratio of 9ml HNO₃: HCl mixture by simple stirring at 65 °C. After few hours 1.5 ml of HCl is added step wise for 3 hours, followed by cooling the reaction mixture with addition of either 1ml of THT ligand or Me₂S ligand. Addition is carried drop wise until a clear white precipitate is formed. The white precipitate is first washed with water: ethanol mixture than dried with diethyl ether. Purified and dried Au(I) compound are stored in dark at 4 °C. Detailed syntheses and characterization of these Au(I) sulfides described elsewhere.⁵⁵

4.2.4.2 Syntheses of PNIPAM Based Microgels

Syntheses of various PNIPAm based microgels using allylamine or acrylic acid as co-monomers are described in chapter 2 sections 2.2.4.

4.2.4.3 Different Polymers

All the other polymers like chitosan, polyacrylic acid (PAA), alginic acid, hydroxypropyl cellulose (HPC), polyvinyl alcohol (PVA), agarose are brought as powders and dissolved in aqueous medium with varied wt% and used directly.

4.2.4.4 Syntheses of PNIPAM Microgels Stabilized Spherical AuNPs

To require wt% of homogenized microgel dispersion solution (0.5 to 2 wt%), sufficient quantity of Au(THT)Cl solid (2mg -5mg) was added directly followed by stirring at room temperature. The stirred solution is either transferred in to ES-Quartz cuvette for photolysis by maintaining temperature of photochemistry set at 24 °C or
subjected to heating above LCST of the PNIPAM microgels (~35 °C). For testing effect of ambient conditions same solution was transferred in to regular borosilicate glass and then subjected to stirring at room temperature and light. Reactions are stopped when initial colorless solution turns in to red or violet or brown color depending on reaction conditions. Similar procedure is followed for making AuNPs stabilized with in PNIPAM-co-acrylic acid microgels. For calculating photochemistry quantum yield, change in absorption spectra at specific time intervals was recorded starting from time zero to end of reaction is recorded. In order to clearly understand long term effect of agglomeration, same batch of samples are stored under ambient conditions and tested by measuring absorbance changes after few days.

4.2.4.5 Syntheses of Different Polymers Stabilized Spherical AuNPs

Aqueous solutions of different polymers (CS, PAA, PVA, agarose, HPC, alginic acid) are individually homogenized at the beginning of reaction. To the required wt% of polymer solutions sufficient quantity of Au(THT)Cl or Au(Me₂S)Cl is added followed by either heating (~37 °C) or by photolysis of solutions at room temperature.

4.2.4.6 Syntheses of PNIPAM-co-allylamine Stabilized Anisotropic AuNPs

Syntheses of anisotropic AuNPs within PNIPAM-co-allylamine microgels is carried in a similar way as syntheses of spherical AuNPs in microgels except for using Au(Me₂S)Cl instead of Au(THT)Cl. Reaction solution was subjected to photolysis in regular orthoborosilicate glass by maintaining the temperature between 0 °C to 5 °C or reaction was initiated by heating the reaction mixture between 35 °C to 40 °C and than maintained at 0 °C to 5 °C. Final reaction mixture is centrifuged to separate isotropic
spherical particles from anisotropic particles. Supernatant and sediment are collected and tested separately.

4.2.4.7 Syntheses of Chitosan Stabilized Anisotropic AuNPs

Initially pH of chitosan polymer solution was adjusted to pH 3.0 by addition of acetic acid, to more than 0.5 wt% of CS polymer required quantity of Au(Me₂SCl) (5 mg to 7 mg) was added. The solution was homogenized and later subjected to photolysis using orthoborosilicate glass by maintaining reaction temperature at 0 °C to 5 °C.

4.2.4.8 Syntheses of AuNPs Loaded PNIPAM Microgel Crystals

After synthesizing the AuNPs loaded PNIPAM microgels following procedure listed in section 4.2.3.4, the hybrid microgels dispersion was concentrated by centrifugation to about 2wt%. The concentrated hybrid microgel dispersions are centrifuged at low rpm (> 5000) for few hours than left undisturbed for more than 24 hours at room temperature. After 24 hours colloidal crystals of hybrid AuNPs doped PNIPAM microgels are formed.

4.2.4.9 In Vitro Test Using Chitosan Stabilized Spherical and Anisotropic AuNPs by MTT Assay

Samples are dialyzed to ensure removal of undesired side products formed during reaction. Both spherical and anisotropic chitosan stabilized AuNPs are subjected to dialysis using 3000 MCO dialysis tubing. Absorbance of samples is tested before and after dialysis. Samples pH is adjusted by addition of required amount of 10 X PBS (phosphate buffered saline) solutions. Changes in absorbance were also noted after addition of PBS. Analogues samples were bought from BBI international (20nm size...
AuNPs stabilized in citrate). NIR absorbing anisotropic gold nanoparticles were bought from Nanopartz (CTAB stabilized Nanorods and PEG-amine stabilized gold nanorods). Concentration of spherical and anisotropic AuNPs was adjusted by quantitative calibration of absorbance for all samples. Porcine proximal tubule cell lines (LLC-PK1) are used for MTT assay following NCL-NCI protocol (Nanotechnology characterization laboratory from National Cancer Institute). In brief, APAP (acetaminophen) and Triton X are used as positive controls. Series of 1:4 dilutions were made (2-7) using PBS diluted NPs. 0.1 mL of each dilution was added to wells of a 96-well plate (10,000 cells per well), which made a total volume of 0.2 mL/well. One plate was loaded with your NPs and one plate was loaded with the control NPs. NPs and cells incubated at 37 °C for 24 hours. MTT assay was run after removing media. Absorbance measured at 580 nm and corrected using absorbance measurements at a reference wavelength (670 nm). Since the MTT assay is based on an absorption measurements, controls containing only cells and nanoparticles were run (no MTT) to make sure they do not interfere with the measurement (their effect is indeed negligible). Reproducibility of the results was ensured by performing MTT assay for more than one time on all samples.

4.2.4.10 In Vivo Test Using Chitosan Stabilized Spherical and Anisotropic AuNPs by Direct Intravenous Injection in to Zebrafish

Samples are purified exactly as stated for in vitro studies. Zebrafish (Danio rerio) used as animal model for toxicity tests. 5 microliters of sample solution was intravenously injected in to adult zebra fish and survival of the fish with respective to control fish (injected with PBS) were monitored for 48 hours.
4.3 Results

4.3.1 Formation of Spherical AuNPs Stabilized in PNIPAM Based Microgel Dispersions

Gold (I) sulfide complexes undergo photochemical decomposition in aqueous medium to give agglomerated metallic gold as shown in figure 4.5 Absorption spectrum and TEM images obtained from the decomposed solution of Au(I) indicate ability of Au(I) sulfide precursors to form stable AuNPs if proper stabilizing conditions are enforced. This decomposing tendency of Au(I) to Au(0) avoids usage of any special reducing agents. So we predicted if the same reaction is carried in presence of stabilizing matrices, (polymers or gels) Au colloidal nanoparticles would be produced.

![Absorption spectrum of Au(Me₂S)Cl in water at room temperature.](image)

Figure 4.5B Absorption spectrum of Au(Me₂S)Cl in water at room temperature (inset shows TEM image for decomposed solution). Red circle indicates formation of AuNPs due to decomposition of Au(I). Inset shows vial containing precipitate solution after 2 minutes of mixing Au(I) in water.
with ease. Our prediction was validated by insitu formation and stabilization of AuNPs within different PNIPAM microgels under different experimental conditions (figure 4.7). The size, stability and SPR of colloidal AuNPs are tuned based on concentrations of starting reactants and experimental conditions (photochemical or ambient). Wavelengths from Hg lamp were not selective during irradiation, which avoids usage of any special band pass filters. Schematic mechanism of stabilization of AuNPs within PNIPAM microgels is illustrated in figure 4.6 that shows AuNPs are physically entrapped within gels. As Au(I) sulfides are described as sensitive to heat and light, effect of both heat and light on formation and stability of AuNPs can be understood

![Diagram](image_url)

Figure 4.6 Schematic illustration of stabilization of AuNPs within PNIPAM microgels under different conditions.
from Table 4.9. However, heating alone can result in formation of small spherical AuNPs within PNIPAM microgels but detailed experimental discussion is avoided for simplicity and also because of insignificant difference in SPR and color of samples produced either due to photolysis or due to thermolysis. Sensitivity of light during formation of AuNPs within PNIPAM microgels is understood by following time dependent electronic absorption spectrum and from SEM and TEM images of AuNPs stabilized in PNIPAM hydrogels both under photolysis and under ambient light conditions (Figure 4.7). Although the hydrodynamic radius of all hydrogel templates examined in these experiments were about the same size, the gold nanoparticle core or distribution grew larger when reaction was carried out at ambient room light compared to sample irradiated by Hg lamp. Time dependent UV-vis absorption spectrum shows that at the beginning of reaction there is no absorbance peak from reactant mixture (PNIPAM gels + Au(I) sulfide) either due to dominant scattering from microgels or due to absence of any chromophore.

Table 4.9  Effect of light and heat during formation of AuNPs in PNIPAM microgels. (A, B, C, D: Samples containing equal concentrations of PNIPAM microgels and Au(I) precursor).

<table>
<thead>
<tr>
<th>Samples</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>NO</td>
<td>NO</td>
<td>YES (ambient)</td>
<td>YES (hv)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>~ 35 °C</td>
<td>RT</td>
<td>RT</td>
</tr>
<tr>
<td>Reaction Time</td>
<td>20-24 hrs</td>
<td>20-30 min</td>
<td>3-4 hrs</td>
<td>45-55 min</td>
</tr>
<tr>
<td>Final Product</td>
<td>Not clear</td>
<td>Size &lt;20 nm, narrow distribution</td>
<td>Broad distribution, size range~20-50 nm</td>
<td>Narrow distribution, size &lt; 20 nm.</td>
</tr>
</tbody>
</table>
Figure 4.7 Formation of AuNPs within PNIPAM-co-allylamine microgels under both photolysis and ambient light conditions (A= ambient; B= Photolysis). A & B) Time dependent absorption spectra under ambient and photolysis condition respectively (inset shows pictures taken in day light). A\(^1\) & B\(^1\) FE-SEM images. A\(^2\) HR-TEM images 20 nm±7 nm. B\(^2\) HR-TEM images 14 nm±3 nm. (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
On irradiation, a peak around 550 nm is evolved from both samples dependent on time of reaction. Photolysis samples show faster evolution and growth of SPR peak maximum at 534 nm (figure 4.7B) compared to sample exposed to ambient light which has SPR peak maximum at 572 nm (figure 4.7A). These initial results highlight light sensitive of Au(THT)Cl. Photolysis samples shows narrow distribution whereas sample exposed to ambient light shows red shifted SPR peak with broad distribution. FE-SEM images show clear entrapment of AuNPs with in hydrogel matrices, close observation in some FE-SEM images reveal presence of more than one AuNPs within single microgel particles. This is in exact agreement with the schematic illustration shown in figure 4.6. Similar results of AuNPs template hydrogel particles were shown previously by Lee et al.\textsuperscript{28} but FE-SEM images shown in the report were not as distinctive as seen in figure 4.7 or 4.8. In any case Lee et al. stabilized AuNPs using HAuCl\textsubscript{4} precursor in presence of THPC (tetrakis hydroxymethyl phosphonium chloride) reducing agent. SEM and TEM images of two samples (figure 4.7) are in good agreement with absorbance data, where photolysis sample showed smaller size particles and better distribution. More aggregation tendency is noticed from figure 4.7A\textsuperscript{1} which represents samples synthesized under ambient light. TEM images revealing individual sizes also exhibit similar pattern. Figure 4.7A\textsuperscript{2} for ambient light exposed sample shows broad size ranging between 15-30 nm where as figure 4.7B\textsuperscript{2} which represents photolysis sample shows average size of 14 ± 3 nm. Effect of Au(I) precursor concentration on size and distribution are exclusively shown for photolysis samples (figure 4.8).
Figure 4.8  Formation of AuNPs by photolysis of Au(THT)Cl within PNIPAM-co-allylamine microgels at different concentrations of Au(I). A) Normalized absorption spectra at different concentrations of Au(THT)Cl. B & C) HR-TEM image of 1mg of Au(I) sample (~7 nm). D) FE-SEM image for 3mg Au(I) sample. (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
Figure 4.9  Formation of AuNPs by thermolysis of Au(THT)Cl within PNIPAM-co-allylamine microgels A) Time dependent absorption spectrum. B) Representative HR-TEM images of the sample. (~10 nm ± 4nm)
Sample with highest concentration of Au(I) exhibited more red shifted SPR peak maximum but individual FE-SEM and HR-TEM images does not exhibit much deviation in size or shape (compare figure 4.7 and 4.8). Except for sizes less than 10 nm are observed corresponding to blue shifted SPR peaks at lowest Au(I) concentration. TEM images and absorption spectra from figure 4.7 and 4.7 concludes two important points, One is formation of stable AuNPs within PNIPAM microgels both at ambient and photolysis conditions and other point is recognizing photolysis as favorable condition for formation of highly monodispersed particles even smaller than 5 nm. After understanding effect of light, direct effect of heat is quickly explored just to verify if heating can alone result in AuNPs with in PNIPAM microgels. Presence of particles smaller than size 20 nm (figure 4.9) and time dependent evolution of SPR peak comfortably demonstrate formation of AuNPs within soft template PNIPAM-co-allylamine microgels by heating alone also.

After demonstrating formation of AuNPs within PNIPAM-co-allylamine microgels (positively charged), we tried another variation by employing PNIPAM-co-acrylic acid based microgels. Photolysis of Au(I) precursor in presence of PNIPAM-co-acrylic acid microgels also resulted in formation of stable narrowly dispersed spherical AuNPs with average size less than 20 nm. Figure 4.9 shows Au(THT)Cl concentration dependent absorption spectra along with FSEM and HRTEM AuNPs stabilized in PNIPAM-co-acrylic acid microgels. Absorption spectra (figure 4.9A) do not show much difference in SPR peak maximum for small variation in concentration of Au(I). This is in good agreement with HRTEM images collected for 5mg and 1mg of Au(THT)Cl samples. Under similar
experimental conditions, HR-TEM images do not show drastic difference in average size of AuNPs produced from either 5mg Au(I) containing sample or 1mg Au(I) sample. Both samples contain AuNPs with average size less than 20 nm. This data from PNIPAM-co-acrylic acid hybrid samples show versatility of our method or ability of our method to form AuNPs within both positively charged and negatively charged PNIPAM microspheres under similar experimental conditions. However physical observations and photolysis quantum yield calculations (figure 4.10 and 4.11) show very clearly that negatively charged microgels exhibit higher quantum yield (for 5 mg and 1.5 mg of Au(I), Φ =0.1 and 0.04 respectively) compared to positively charged microgels (for 5 mg and 1.5 mg of Au(I), Φ =0.07 and 0.01 respectively) at different concentrations of Au(I) precursor. Time dependent optical absorbance for photolysis quantum yield calculations is shown in figure 4.10 and 4.11. Zeta potential (ζ) values re-confirm surface charge of both kinds of AuNPs. Zeta potential of PNIPAM-co-acrylic acid stabilized AuNPs = -30 ± 4.5 mv; ζ of PNIPAM-co-allylamine stabilized AuNPs = +28 ± 3.9 mv respectively. This is promising strategy to produce both positively and negatively charged PNIPAM-Au hybrid particles employing light sensitive reduction technique in absence of any special reducing agents or surfactants to retain environmental or thermosensitivity of PNIPAM gels. However, chemical reduction carried out in presence of special reducing agents is also apparently simple process, but presence of excess or undesired chemical reagents can affect final product, which would drastically effect many anticipated biomedical applications. Conducting control experiments using HAuCl₄ under similar experimental conditions using both PNIPAM-co-allylamine and PNIPAM-
co-acrylic acid microgels did not yield any colloidal gold nanoparticles unless NaBH₄ is added. These results self explain that using Au(I) sulfide precursor avoids usage of any special reducing agent.

Figure 4.10 Formation of AuNPs during photolysis of Au(THT)Cl within PNIPAM-co-acrylic acid microgels. A) Absorption spectra at different concentrations of Au(THT)Cl. B) FE-SEM images for 5mg Au(THT)Cl sample. C) HR-TEM image for 5mg Au(THT)Cl sample. D) HR-TEM image for 1mg Au(THT)Cl. (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
Figure 4.11 Photolysis quantum yield during formation of AuNPs stabilized in PNIPAM-co-allylamine microgels at two different concentrations of Au(I). A) 5 mg. B) 1.5 mg (inset shows Absorbance vs Time curves; QY = Quantum yield (Φ)) (Dr. El-bjeirami assisted in analyzing the data)
Figure 4.12 Photolysis quantum yield during formation of AuNPs stabilized in PNIPAM-co-acrylic acid microgels at two different concentrations of Au(I). A) 5 mg. B) 1.5 mg (inset shows Absorbance vs Time curves; QY = Quantum yield (Φ)).
4.3.2 Formation of AuNPs Loaded Hybrid PNIPAM Microgel Crystals

Spherical PNIPAM microgel nanoparticles are famous for formation of environmentally sensitive colloidal crystals, and used extensively for the study of phase transitions and fabrication of photonic applications. Hydrogels with environmentally sensitive colors are made from PNIPAM based gels by embedding different co-monomers or other polymeric particles like polystyrene or silica particles but never before AuNPs doped hybrid colloidal crystals are reported in literature. When concentrated to 2-3 wt%, AuNPs loaded PNIPAM microgel dispersions are subjected to slow cooling, formation of lustrous colloidal crystals are shown in figure 4.13. Both acrylic acid and allylamine based hybrid microgels exhibited ability to form colloidal crystals. Turbidity measurements of these hybrid colloidal crystals show characteristic Brags diffraction peak due to colloidal crystals similar to undoped PNIPAM crystalline array and SPR peak from AuNPs. Retainement of both Braggs diffraction along with SPR peak in hybrid colloidal array signifies retention of characteristic traits of both AuNPs and PNIPAM microgels. This could enhance applications of PNIPAM colloidal crystals.
Figure 4.13 Formation of AuNPs loaded PNIPAM microgel crystals. Inset shows pictures taken under day light. A, B: Pictures before forming crystals; A¹ and B¹ after forming crystals. Red color of the crystals is due to loading of AuNPs in to PNIPAM microgels (A= PNIPAM-co-acrylic acid; B= PNIPAM-co-allylamine).

Temperature sensitivity of hybrid microgels (AuNPs loaded PNIPAM microgels) is demonstrated by measuring changes in absorbance and hydrodynamic radius ($R_h$) of typical hybrid microgel dispersions. From figure 4.14A, it is observed that hydrodynamic radius changes from both unloaded PNIPAM microgels and hybrid microgels show similar sensitivity to temperature. Between 32-33 °C changes in $R_h$ is maximum indicating phase transition temperature (LCST) of PNIPAM microgel particles or hybrid microgels. At any given temperature, hydrodynamic radius ($R_h$) of thermosensitive AuNPs (hybrid microgels) is smaller than unloaded PNIPAM microgel dispersions due to physical interactions between Au core and microgel matrix that shrinks size of
microspheres. This unchanged temperature sensitivity even after doping AuNPs resembles with temperature sensitive AuNPs reported in literature from similar PNIPAM templates. But again thermosensitive hybrid microgels cited in literature are obtained by reduction of Au(III) with reducing agents. SPR peak of AuNPs loaded within environmental sensitive PNIPAM microgel (figure 4.14B) exhibits temperature sensitivity demonstrating importance of hydrogels template that incites thermosensitivity to AuNPs. Both temperature dependent absorption spectra and dynamic light scattering data (figure 4.14) confirm formation of thermosensitive AuNPs. Though, few previous works in literature have already shown formation of AuNPs doped PNIPAM hydrogels, like Langer et al. have embedded NaBH₄ reduced AuNPs in to thermoresponsive PNIPAM by using thiol (–SH) terminated crosslinkers for their affinity to Au⁺³. Gold core PNIPAM nanogels are synthesized by Lyon et al. starting with citrate stabilized AuNPs. Thiol terminated NaBH₄ reduced AuNPs are tethered to PNIPAM polymers by Li and co-workers to produce thermoresponsive AuNPs. All these works abide to common technique of using HAuCl₄ as starting precursor which compels usage of a chemical reducing agent to convert Au(III) to Au(0). Photolysis or ambient experimental methodology detailed in this chapter in complete absence of any reducing agent is first of its kind demonstrating insitu formation of AuNPs within thermosensitive PNIPAM gels and its thermoresponsive properties.
Figure 4.14 Temperature sensitivity of a typical AuNPs loaded PNIPAM microgels. A) Changes in hydrodynamic radius of hybrid and unloaded PNIPAM microgels. B) Changes in absorbance of hybrid PNIPAM microgels on heating and cooling cycle.
Formation of Spherical AuNPs Stabilized in Different Polymers, Surfactants and Proteins

Encapsulating metal nanoparticles into polymer matrices was found to be an effective way for enhancing functions of both polymers and metal nanoparticles. Especially conjugation of polymers to AuNPs and using them as templates allows building structural and functional units useful for many optoelectronic and biological applications.60 Different water soluble polymers have been tested for their ability to stabilize AuNPs using our methodology to verify versatility of our technique and to demonstrate feasibility of using Au(I) precursors with more than one kind of stabilizing templates. Figure 4.15 illustrates schematically stabilization of AuNPs with different polymers.
polymers and also lists out wide range of polymers or surfactants that are employed as stabilizing templates. Figure 4.16 shows electronic absorption spectra for different polymer stabilized AuNPs either by thermolysis or photolysis. In order to minimize the confusion by elaborating all different modes of reactions (ambient, photolysis, thermolysis) with all different polymers or surfactants, results with only single reaction condition is shown (figure 4.16 and 4.17) to demonstrate feasibility of proposed protocol. Visible colors, evolution of SPR peaks and TEM images strongly confirm formation of AuNPs within this wide range of polymers or surfactants. TEM images show AuNPs with size ranging anywhere between 5 to 50 nm using different polymers. Though an exact correlation between size and polymers used is not attained but polymers with acidic functional groups (COOH) exhibited not only narrow SPR peaks but also contain better monodispersed smaller size particles. As alginic acid shows, a narrow SPR peak at 525 nm and TEM images for the same sample has particles with average size between 5nm to 10 nm. While sodium dodecyl sulfate (SDS), a negatively charged surfactant exhibits narrow SPR peak maximum at 528 nm and particles with average size between 5 nm to 10 nm. In contrast HPC (hydroxypropylcellulose) and PVA (polyvinyl alcohol) which have only hydroxyl functional groups exhibits broader SPR peaks along with average size of particles ranging between 30-40 nm. Exact tuning of size and distribution by varying Au(I) concentration, polymer concentration and experimental conditions are under investigation. Polymers have been selected based on their significance for biological applications or environmental sensitivity. Like pH
sensitive polyacrylic acid (PAA) is widely used in household and personal care products like diapers, moisturizers and in making superporous hydrogels.\textsuperscript{61}

![Absorption spectrum and pictures of gold nanoparticle samples stabilized in variety of polymer templates. A) Pictures taken under day light [SDS: Sodium dodecylsulfonate; Alg: Alginic acid; PVA: Poly(vinyl alcohol); HPC: Hydroxypropyl cellulose; NIPA: N-isopropylacrylamide; CS: Chitosan]. B) Representative absorption spectra for AuNPs prepared under different conditions (H: Heating; P: Photolysis).]
Figure 4.17 Representative HR-TEM images for AuNPs stabilized in different polymers. 
A) SDS surfactant (average size: 10-15 nm); B) Agarose polymer (average size: 10-15 nm); C) HPC polymer (average size: 30-40 nm); D) NIPA polymer (average size: 10-15 nm); E) Alginic acid polymer (average size: 5-10 nm); F) PVA polymer (average size: 15-20 nm); G and I) BSA (average size: 15-20 nm); H) PAA polymer (average size: 20-30 nm). (Dr. S. Park & Dr. M. Kim at UTD collected some of the above TEM images and some of them are collected at UNT-CART)
Figure 4.18 Comparing photolysis and ambient conditions for formation of AuNPs stabilized in PNIPA-NH₂ polymer. A) Absorption spectra showing shift in SPR peak for two conditions. B, B₁) FE-SEM and HR-TEM images for ambient sample. C, C₁) FE-SEM and TEM images for photolysis sample. (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
Figure 4.19 Temperature dependent studies of thermoresponsive AuNPs stabilized in NIPA-NH$_2$ polymer by photolysis. A) Changes in absorption spectra above and below LCST (~32 °C) of NIPA polymer. B) Changes in hydrodynamic radius of NIPA-NH$_2$ stabilized AuNPs at different temperatures.
Binding and coating capabilities of PVA is efficiently utilized in food industry.\textsuperscript{62} While Agar is a gelatinous polysaccharide derived from agarophyte red algae. Its solidifying property at 32–40 °C is employed for many biological assays.\textsuperscript{63} Hydroxypropyl cellulose is derivative of cellulose which is soluble both in water and many other organic solvents typically used in tablet binding and tablet coatings also used as ophthalmic protectant and lubricant.\textsuperscript{64} One of the most important nutrients in cell culture and different biochemical application is BSA (Bovine serum albumin).\textsuperscript{65} In existing literature only one or two few reports exist for making AuNPs using some of these above mentioned polymers however HPC was never used before as template. Even among those few works prevalent condition is usage of HAuCl\textsubscript{4} as precursor and reduced by either photoinitiators or chemical reducing agents\textsuperscript{66,38} which dissociate methodology proposed in this chapter from those protocols in literature. Other than above mentioned results, some detailed studies are performed using selective polymers for their significance in different biological and biomedical applications. Like chitosan was investigated for its strong biocompatibility, NIPA for its thermo responsiveness, PAA and Alginic acid for their pH sensitivity.

4.3.3.1 PNIPA-co-amine Polymer Stabilized AuNPs

Effect of light on formation of AuNPs with in PNIPA-co-RCONH\textsubscript{2} polymer is studied to conclude similar observation as in case of PNIPAM gels. At constant concentration of Au(THT)Cl and NIPA polymer sample exposed to ambient light exhibits broader and red shifted SPR peak (figure 4.18), FE-SEM and TEM images conclude better dispersed AuNPs by photolysis and higher aggregation tendency from samples
exposed to ambient room light. Close comparison of TEM images for photolysis samples made using polymer and microgels show better distribution from microgels compared to polymers (compare figure 4.7 and 4.18). We assume this difference is due to difference in matrix of polymers and gels. Again as NIPA polymer is famous for its thermoresponsive nature, sensitivity of PNIPA polymer stabilized AuNPs to temperature was studied in detail. Figure 4.18, which shows changes in SPR peak and hydrodynamic radius of PNIPA stabilized AuNPs above and below LCST of NIPA polymer (~32 °C) elaborates thermosensitivity of hybrid AuNPs. Close examination of figure 4.19 shows that with increase in temperature the peak maximum of SPR peak (~538 nm) does not shift much but the baseline at longer wavelength rises due to increase in tendency for aggregation above phase transition temperature. This result can be contrasted when compared with PNIPAM microgels (figure 4.7B) as we expect mechanism of stabilization is different for polymers compared to gels. As illustrated in figure 4.15, polymer surface is tethering to gold core by physical interactions between gold core and functional groups of polymer chains. So in case of NIPA polymer above phase transition temperature, when it starts precipitating out of aqueous medium absence of stabilizing template favor precipitation of AuNPs. But complete retainement of SPR peak on cooling back to room temperature was anticipated as NIPA polymer becomes soluble on cooling regenerating template for stabilization. Temperature dependent changes in hydrodynamic radius ($R_h$) of NIPA polymer stabilized AuNPs also illustrates strong temperature sensitivity. As noticed from figure 4.19B, broad light scattering peak with average hydrodynamic radius of 45 nm at 22 °C undergoes red shift continuously with
increase in temperature. Increase in average hydrodynamic radius could be due to aggregation of polymer stabilized AuNPs on heating. Again when compared with temperature dependent hydrodynamic radius changes of microgel stabilized AuNPs (figure 4.14A), polymer stabilized AuNPs undergo large variation in hydrodynamic radius due to difference in mechanism of stabilization. In case of microgels as AuNPs are entrapped within matrix of microgel, change in radius of hybrid particles is proportional to chemically crosslinked microgel but in case of polymer stabilized AuNPs, un-crosslinked polymer chains aggregate or dissociate with respective to temperature which results in shift of hydrodynamic radius with change in temperature. In a different case if chitosan polymer stabilized AuNPs are tested for temperature sensitivity, no change in size or aggregation is noticed due to insensitivity of chitosan polymer to temperature.

4.3.3.2 Polycrylic acid Polymer Stabilized AuNPs

After experimenting with a thermosensitive polymer, formation and sensitivity of AuNPs employing a pH sensitive polymer (PAA) has been investigated in detail. Figure 4.20A first shows formation and stabilization of AuNPs within PAA polymer by photolysis. Effect of Au(I) concentration on SPR and size of AuNPs is clarified from electronic absorption data and TEM images. At lower concentration of Au(I), broad SPR peak maximum is noticed at 550 nm while at higher Au(I) concentration a similar SPR peak accompanied by broad shoulder around 700 nm is noticed. From existing literature it is well know that SPR shifts are mostly observed either due change in size, distribution or shape of nanoparticles when other reaction parameters like pH, ionic
strength, dielectric constant of medium are maintained constant. At high concentration of Au(I), mixture of different sizes, are noticed from TEM images (figure 4.20 D). Some particles sizes are 15-25 nm while few other particles have sizes around 75-100 nm.

Figure 4.20 Formation and stabilization of AuNPs using PAA as template. A) Changes in electronic absorption spectra of AuNPs with respective to Au(I) concentration during photolysis. B) TEM images of 3 mg Au(I) concentration sample. C) TEM images of 7 mg Au(I) sample. D) Dynamic light scattering data for both samples. (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
Close observation also reveals that some of these particles have non-spherical morphology which is in good agreement with broad SPR peak around 700 nm in absorption spectrum. Difference in size of particles at different concentrations of Au(I) is also demonstrated from dynamic light scattering data (figure 4.20E). In order to understand effect of pH sensitivity on PAA stabilized AuNPs changes in electronic absorbance and hydrodynamic radius for two samples at different pH is studied. Changes in optical absorbance (figure 4.21) for low concentration Au(I) sample does not exhibit changes in SPR peak at different pH (figure 4.19) while sample containing high concentration of Au(I) exhibits strong variation in SPR peak (figure 4.22A) indicating sensitivity of non-spherical particles compared to spherical particles. Light scattering data also verifies sensitivity of PAA stabilized AuNPs in different buffer medium (figure 4.22 B). Because of sensitivity of PAA (pka ~4.5) to pH both SPR peak and light scattering peaks exhibit clear changes. Though dynamic light scattering data indicates aggregation of particles but retained SPR at pH 7.0 signify stability of AuNPs stabilized in PAA at pH 7.0 which is very important for any biological applications. These temperature and pH dependent results from different polymers containing AuNPs stabilized in similar manner strongly illustrate and confirm that sensitivity of AuNPs stabilized in polymers rely strongly on environmental responsiveness of polymers. Like if the polymer or stabilizing template is pH sensitive than AuNPs will be pH sensitive and if polymer or stabilizer is temperature sensitive than it will result in formation of thermoresponsive AuNPs.
Figure 4.21 Demonstration of pH sensitivity for PAA stabilized AuNPs. A) Changes in absorption spectra monitored at different pH.

4.3.4 Film Forming Ability of AuNPs

Though numerous advantages are discussed for stabilizing AuNPs in aqueous medium for bio-conjugation with respect to many biomedical applications, but strongest drawback of aqueous stabilized nanoparticles is inability to form powders that can be readily redispersed in to solutions\textsuperscript{7c} for long term storage or commercial applications. So we tried to overcome this problem by exploiting film forming ability of gels/polymers used in this study. As most of the polymers used in this study (chitosan, polyacrylic acid, PNIPA) are well known for their film forming ability. By simple drop-casting of polymers
or gels stabilized AuNPs on a clean glass slide resulted in formation of AuNPs films that can be re-dispersed easily in to solution based on solubility of polymers or gels.

Figure 4.22 Demonstration of pH sensitivity for PAA stabilized AuNPs containing 7mg of Au(I). A) Changes in optical absorbance monitored at different pH by addition of different buffers. B) Changes in hydrodynamic radius monitored for same samples.
Figure 4.23 Powder and film forming ability of AuNPs stabilized in polymers and gels. A: AuNPs doped PNIPAM-\textit{co}-allylamine microgel powder, made in summer 2008. B: AuNPs doped chitosan polymer film made in fall 2009.

Figure 4.23 shows AuNPs doped PNIPAM-\textit{co}-allylamine microgel and chitosan films. These powders or films exhibit forever stability even when stored under ambient light or temperature. This property adds strong commercial applications for the proposed syntheses protocol.

4.3.5 Formation of NIR (near-infrared) Absorbing Anisotropic AuNPs

Compared to spherical AuNPs, Anisotropic AuNPs with strong efficiency to scatter or absorb light in the NIR region are highly desirable with ability to absorb light and rapidly convert it into heat via a series of photophysical processes.\textsuperscript{44,45} If rate of absorption is controlled by controlling optical absorbance cross section area and laser pulse, plasmonic excitations can result in photothermal heating of nanoparticles and surrounding medium. Then heat can selectively melt membrane of tumor cells (membrane blebbing). Such a photothermal therapeutic method capable of destroying cancerous cells is already demonstrated in vitro and in vivo studies are in progress.\textsuperscript{44,45}

After substantiate and confirmative work on syntheses of gold nanospheres in different
polymers or gels by facile techniques; we shifted our attention to more challenging and intriguing process of making anisotropic or NIR absorbing gold nanostructures within environmental sensitive polymers by single step method. Major intuition behind extending our syntheses protocol for anisotropic or NIR absorbing AuNPs was absence of any direct single step syntheses protocols to make biocompatible or thermo-responsive anisotropic AuNPs in existing literature (for verification see table 4.5 and 4.6). Only Au(Me₂S)Cl is employed as starting precursor in view of more labile Me₂S group and also primarily due to fact that Me₂S boils at 37 °C. Me₂S (dimethyl sulfide) is also known as biological sulfur compound noticed in vegetables (cabbage, beetroot, maize) seafood and also used as a food flavoring substance. Biological presence of this ligand encouraged us to ensure that all the components involved in syntheses of anisotropic NIR absorbing AuNPs are completely biocompatible. Both thermolysis and photolysis methods are applied for syntheses. These biocompatible anisotropic gold nanoparticles are tested both in vitro and in vivo in comparison with analogues AuNPs available in the market for evaluating their cytotoxicity in cell lines and zebra fish.

4.3.5.1 Formation of NIR Absorbing AuNPs within PNIPAM-co-allylamine Microgels by Photolysis

In-order to understand effect of different reaction conditions and to determine favorable experimental condition, (ambient, photolysis, thermolysis) a set of three samples containing same concentration of Au(Me₂S)Cl and PNIPAM-co-allylamine microgels are evaluated under different reaction conditions (figure 4.24). Reactions are initiated by irradiating with Hg lamp or subjected to heating (~36 °C) or carried in
complete ambient condition. In case of thermolysis, just after 15-20 minutes once the sample solution undergoes initial color change the reaction temperature is lowered and maintained between 0-5 °C and during photolysis also reaction temperatures are monitored between 0-5 °C. While in case of ambient condition, reactions are performed completely under room fluorescent lights and room temperature. Figure 4.24 shows very clearly that except for ambient conditions, samples initiated either through heating or photolysis produce AuNPs in solution that has strong NIR absorbance. This simple test confirms effect of light and heat on formation of NIR absorbing AuNPs. Formation, stabilization and tuning is explained by photolysis process first than thermolysis process is discussed. Monitoring time dependent optical absorbance changes (figure 4.25A) during photolysis of Au(Me₂S)Cl-PNIPAM-co-allylamine microgel solution shows evolution of NIR absorbing SPR peaks with respective to time. At the beginning of reaction no specific peaks are noticed from reaction mixture of Au(I) and PNIPAM microgel, higher absorbance baseline at shorter wavelength region is accounted due to scattering from microgel medium. After 15-20 minutes of irradiation, evolution of broad SPR peak ~550 nm indicates initial formation of spherical particles. As the illumination time increases, the absorption band at ~550 nm remains unchanged, while there is new peak arising in the NIR region with peak maximum around ~800 nm. Furthermore, the intensity of the broad absorption band in the NIR region (>700 nm) increases with irradiation time. After 70 to 90 minutes when shifting of strong NIR SPR peak halts or when reaction solution is saturated the reaction is
Figure 4.24 Difference in optical absorbance of AuNPs stabilized in PNIPAM-co-allylamine microgels using Au(Me₂S)Cl under three different conditions by maintaining same concentration of Au(I) precursor and microgel for all three samples.
Figure 4.25 Formation and tuning of NIR absorbing AuNPs in PNIPAM-co-allylamine microgels by photochemistry. A) Time dependent changes in absorbance for 9mg Au(I) sample during photolysis. B) Difference in NIR absorbance of samples made at different Au(I) and PNIPAM-co-allylamine microgel concentrations (absorption spectra of sediment samples).
Figure 4.26 Demonstrating changes in absorption spectra during centrifugation for a typical NIR absorbing AuNPs sample stabilized in PNIPAM-co-allylamine microgel (sample made using 9 mg of Au(Me₂S)Cl₂ by photolysis).

is stopped to retain stable anisotropic particles. Perfect stability of samples is demonstrated from unchanged SPR peaks even after one week of syntheses. Quick look at the time dependent absorption spectrum indicate that initially reaction starts with formation of spherical particles (SPR peak ~550 nm) that fuse and grow in to anisotropic structures with irradiation (rise of NIR peak). Due to minor variations in concentration of Au(I) or PNIPAM gels, though samples with NIR absorbing AuNPs are formed but in some cases NIR peaks are seen only as shoulder which indicates low ratio of non-spherical particles. This issue was simply resolved by employing centrifugation
technique. Recording changes in optical absorbance for a typical NIR AuNPs sample (figure 4.26) produced by photolysis shows very distinctive changes in optical absorbance before and after centrifugation signifying increase in ratio of anisotropic particles after centrifugation. Comparing absorption spectra of sediment and final reaction solution interprets ability to separate spherical particles if required because final supernatant solution (figure 4.26 supernatant_2) does clearly exhibit only single SPR peak corresponding to spherical AuNPs.

In order to evaluate effect of Au(I) concentration on formation and tuning of NIR absorbing AuNP samples different concentration combinations of Au(I) precursor and PNIPAM microgels were tested. Figure 4.25 B, represents absorption spectra of AuNPs obtained at various concentrations of Au(I) precursor which shows very clearly concentration effect on tuning NIR absorbance. Using lower than 5 mg (0.018 mmoles) of Au(Me₂S)Cl AuNPs with only visible absorbance are produced, so in both photolysis and thermolysis minimum of 5mg Au(I) precursor is used to obtain NIR absorbing AuNPs. This concentration of Au is low compared to using two portions of 2.5×10⁻⁴M HAuCl₄, 0.2M CTAB, 0.1M NaBH₄, in case of seed mediated growth methods.⁴⁴,⁴⁵,⁴⁶ 0.1wt% of PNIPAM-co-allylamine microgels is found minimum concentration required for stabilization again this is also very low concentration of PNIPAM polymer compared to 0.2M CTAB. With increasing concentration of Au(I) precursor, a clear red shift in absorption spectrum is noticed with peak maximum shifting anywhere from 650 nm to 800 nm (figure 4.25B). A clear shoulder around 550 nm consistently seen from all samples irrespective of concentration of Au(I) precursor is justified either due to
presence of spherical AuNPs or due to sharp edges or transverse plasmon absorptions of rods/ triangles as expected from literature.\textsuperscript{45,43} The second absorption band appearing at wavelength >650 nm in the near infrared (NIR) region indicates the presence of non-spherical AuNPs.\textsuperscript{1d,5b,45} Furthermore, this broad absorption band shifts
to a longer wavelength (> 800 nm) at higher Au(I) precursor concentration. As protocol described in this section for making NIR absorbing AuNPs is not completely selective for formation of single anisotropic shapes (rods or triangles or prisms) due to absence of any kind of special growth controllers that are commonly employed in seed mediated protocols (table-4.5, 4.7).

Though NIR optical absorbance peaks are in good agreement with literature for presence of mixture of shapes, we substantiate our assumption by performing TEM analysis on more than one sample. TEM images from sample prepared using 5 mg of Au(I) precursor (figure 4.27) shows particles with average size of 30 nm ± 7 nm, but careful observation of any individual particle from this sample set shows non-spherical morphology. Some very clear pyramidal structures are also noticed in the same sample. Fine edges and anisotropic morphology is always linked to more than single SPR peak due to different modes of plasmon oscillations in anisotropic structures.\textsuperscript{5b} These TEM structures strongly confer with 650 nm SPR peak maximum for the sample seen in optical absorption spectra, however our assumption of formation of large anisotropic structures (rods or prisms or triangles) is realized after examining TEM images of sample made using 9mg Au(I) for more than one time (figure 4.28 & 4.29). Mixture of rods, prisms, triangles noticed from TEM images are in good agreement with our assumption and broad SPR peaks.
Figure 4.28 HR-TEM images of NIR absorbing anisotropic sample stabilized in PNIPAM-co-allylamine microgels by photolysis (9mg of Au(Me₂S)Cl sample). (Dr. S. Park and Dr. M. Kim at UTD collected TEM, SEM images)
Figure 4.29 HR-TEM images demonstrating reproducibility of making NIR absorbing anisotropic AuNPs stabilized in PNIPAM-co-allylamine microgels by photolysis. (Sample made using 9mg of Au(Me₂S)Cl for reproducibility)
A: Sediment solution. B: Supernatant solution
Figure 4.30 Formation and tuning of NIR absorbing AuNPs in PNIPAM-co-allylamine microgels by thermochemistry. A) Time dependent optical absorption spectra changes for a typical sample. B) Effect of Au(I) concentration on tuning NIR absorbance at 1.5 wt% of PNIPAM-co-allylamine microgels.
Figure 4.31 TEM images of NIR absorbing anisotropic sample stabilized in PNIPAM-co-allylamine microgels by thermolysis (sample made using 5mg of Au(Me₂S)Cl sediment solution).
Figure 4.32 TEM images of NIR absorbing anisotropic sample stabilized in PNIPAM-co-allylamine microgels by thermolysis (sample made using 9mg of Au(Me₂S)Cl). A: Initial solution. B: Supernatant. C, D: Sediment.
As noticed in literature\textsuperscript{46} SPR peaks in NIR region are shown tunable by tuning aspect ratio of gold rods by Murphy\textsuperscript{43} and El-Sayed\textsuperscript{45} groups separately, while Mirkin and co-workers have related SPR tuning with width or thickness or size of nanoprisms and nanotriangles.\textsuperscript{46b} But in these cases or other controlled syntheses protocols involve special reducing agents or strong surfactants that control growth of anisotropic structures. However, SPR peak broadness is always affiliated to mixture of different shapes or sizes of AuNPs.\textsuperscript{16e} As our primary motive was developing a single step biocompatible protocol for making strong NIR absorbing anisotropic AuNPs in a highly reproducible method, looking at TEM images for two different samples made on different times following same experimental protocol confirms formation of anisotropic structures and reproducibility of proposed method (figure 4.28 and 4.29). We assume perfect control to get single shape particles was not achieved due to absence of any kind of special shape controlling surfactants in the reaction mixture as compared to existing literature protocols. Work is in progress to gain complete control over growth of anisotropic structures by minor variations in the protocol.

4.3.5.2 Formation of NIR Absorbing AuNPs within PNIPAM-\textit{co}-allylamine Microgels by Thermolysis

Our methodology for making anisotropic AuNPs was also confirmed by preparing samples by thermolysis, as many literature works have already shown that following exactly similar syntheses protocols either by heating or photochemical process anisotropic gold nanoparticles can be produced.\textsuperscript{47,48} Though light sensitivity of Au(I) sulfide complexes was well explained by Vogler et al.\textsuperscript{12b} but encouraged by spherical AuNPs formation by thermolysis, syntheses of NIR absorbing particles was also
attempted. Following exactly similar procedure like photolysis except for initiating the reaction by heating (~36 °C) resulted in formation of NIR absorbing colloidal gold. Evolution and growth of NIR peaks is very similar to photolysis (figure 4.30A), except for much broader NIR peaks. Even in case of thermolysis, effect of Au(I) precursor concentration on tuning NIR absorbance was same (figure 4.30B). TEM analysis of two samples (5 mg and 9 mg Au(I) samples) confirmed presence of anisotropic structures (Rods or triangles). Likewise in photolysis reaction, sample made using lower concentration of Au(I) precursor (5 mg/0.018 mmoles) contained particles (figure 4.31) with average size larger than 30 nm, but even here careful examination of any single particle showed non-spherical morphology and some small isolated rods and prisms. Few differences are noticed both from absorption spectra and TEM images between photolysis and thermolysis samples. Thermolysis samples have broader NIR SPR peaks compared to photolysis samples under same concentrations of starting materials. Other contrast between photolysis and thermolysis was presence of some unusual shapes (figure 4.32, tailed hexagons, tailed polygons) specifically from thermolysis samples and we assume these unusual larger size particles cause broader NIR SPR peaks in absorption spectrum. Exact mechanism of formation of these unusual structures is still unknown, but we predict it could be due to slow uncontrolled fusion of spherical and anisotropic structures in absence of any growth controlling surfactants.

Importance of centrifugation (figure 4.33) to enhance concentration of NIR absorbing particles is demonstrated by comparing changes in optical absorbance before and after centrifugation for few sets of samples prepared by photolysis and thermolysis.
In all samples, centrifugation always results in enhancing ratio of anisotropic particles in solution. This is very important especially for our uncontrolled method of syntheses. We also tried to confirm our assumption of increase in NIR absorbing AuNPs ratio after centrifugation by comparing TEM images collected from samples at different stages of centrifugation.

Figure 4.33 Effect of centrifugation to increase NIR absorbance ratio of anisotropic AuNPs samples stabilized in PNIPAM-co-allylamine microgels by photolysis and thermolysis. (X/dotted line = Initial solution; X\(^1\)/Solid line = Sediment after centrifugation). A, B: Thermolysis samples; C, D: Photolysis samples.
Figure 4.34 HAADF (High angle annular dark field image) TEM images and EDX spectrum of AuNPs. A) TEM images of specific particles on which EDX analysis was performed. B) HAADF images of the same. C) Typical EDX spectrum showing elemental composition of anisotropic particles to confirm presence of Au. (Au EDX signals: Au: 9.711 ev; 2.123 ev, S: 2.308 ev, Cl: 2.633 ev) (Dr.Dericks at UNT-CART assisted in collecting TEM images)

For a photolysis sample (figure 4.28) good difference is noticed in TEM images from supernatant and initial solutions. Though sediment was not analyzed, supernatant has
more or only spherical shape particles compared to mixture of shapes in initial solution.

Another complete demonstration of centrifugation effect can be noticed from a thermolysis sample. Figure 4.32 show more of spherical particles from supernatant solution compared to reaction solution and some increase in ratio of anisotropic particles is observed by comparing reaction solution (figure 4.32A), with sediment (figure 4.32 C). Though TEM images show some differences before and after centrifugation but as particles before and after are dispersed in same medium or solvent. So, we assume it is practically not possible to completely separate spherical and anisotropic particles. Hence, TEM images do not exhibit complete separation between initial and sediment solutions as noticed from electronic absorption spectrum. We think this could be due to fact that while performing TEM analysis on sediment sample we could not isolate a specific region where only anisotropic particles can be identified.

4.3.6 EDX (Energy Dispersive X-ray Spectroscopy) to Confirm Formation of Anisotropic Gold Nanostructures.

The final confirmation on formation of anisotropic gold nanostructures was obtained from EDX analysis on more than single anisotropic gold particles (figure 4.34). Au peak positions are in good agreement as expected, these peaks not only confirm elemental composition of nanoparticle but helps to understand if there are any contaminations from other elements during formation of nanoparticles. As the starting precursor contains sulfur, absence of any sulfur signal in EDX spectrum symbolize nanostructures observed in TEM or SEM are purely made from Au but not any mixture of Au-S particles. This would also indicate clear loss of Me₂S ligand. Strong signals from
C, O, Cu, Si, are expected either from TEM grid or due to contamination during sample preparation or from polymer microgels.

4.3.7 Syntheses of NIR Absorbing AuNPs in PNIPAM-co-allylamine Gels by Sonolysis

After successful formation of NIR absorbing anisotropic AuNPs within PNIPAM-co-allylamine microgels, investigating other facile techniques resulted in formation and stabilization of anisotropic AuNPs by Sonochemical method also. Preliminary results both from electronic absorbance data and SEM images confirm formation of stable anisotropic AuNPs (figure 4.35). Time dependent absorbance changes with respective to sonolysis time show exactly similar mechanism of formation as seen in photolysis or thermolysis. Further work need to be carried out to investigate effect of energy of ultrasound waves and Au(I) concentration on formation of anisotropic AuNPs by sonochemical method in detail. Here now it is very important to mention importance of positively charged PNIPAM microgels for formation of NIR absorbing anisotropic AuNPs. Under any experimental condition mentioned above (photolysis or thermolysis or sonochemical) using PNIPAM-co-acrylic acid microgels (negatively charged) does not result in any NIR absorbing AuNPs. This signifies importance of surface charge for stabilizing anisotropic particles. Even from literature it is very clear that only positively charged surfactant (CTAB) was directly utilized as stabilizing template for producing anisotropic AuNPs by different mechanisms (table 4.5). To the best of our knowledge, no single reproducible method is known for directly making anisotropic gold nanoparticles in negatively charged polymers/surfactants.
Figure 4.35 Formation of NIR absorbing AuNPs in PNIPAM-co-allylamine microgels by sonochemical method. A) Time dependent changes in absorption spectra for sample containing 7 mg Au(I) precursor. B) FE-SEM images for 45 minutes solution.
4.3.8 Testing Stability of NIR Absorbing AuNPs stabilized in PNIPAM-co-allylamine Microgels with Time and in Presence of Salt (NaCl)

Though most of the TEM images collected from NIR absorbing samples are atleast after one week of syntheses, however high stability of these samples is verified by comparing absorption spectra at different time intervals. Figure 4.36 shows changes in absorption spectra for some randomly selected anisotropic AuNPs samples stabilized in PNIPAM-co-allylamine microgels under different conditions. Most of these samples are tested for their stability after 3-6 months of preparation and some are tested after 18 -24 months. This simple test strongly verifies stability of NIR absorbing samples produced by protocol described in this chapter. In view of proposed biomedical applications of these AuNPs stability of these nanostructures was also tested in presence of salt (NaCl) which is very significant forward step for utilizing these nanostructures in biological medium where stability of nanoparticles in presence of salts is necessary criteria. Extraordinary stability is demonstrated from one such sample (figure 4.36D). Absorption spectra and SEM images (figure-4.37) of AuNPs stabilized in PNIPAM-co-allylamine microgels in presence of 0.1M NaCl salt signifies stability of anisotropic structures even on altering ionic strength of the medium. Along with spherical particles and anisotropic structures, at very low magnification micron size salt particles are also noticed from SEM images.
Figure 4.36 Testing stability of some NIR absorbing AuNPs stabilized in PNIPAM-co-allylamine microgels with respect to changes in optical absorbance with time. (Black line: Day of syntheses; Redline: After time gap) A) Photolysis sample after 3 weeks. B) Thermolysis sample after 3 weeks. C) Photolysis sample after 1 year. D) NaCl stabilized sample after @ 2 years.
Figure 4.37 Absorption spectra and FE-SEM images of NIR absorbing AuNPs synthesized in PNIPAM-co-allylamine microgels by photolysis using Au(THT)Cl as precursor in presence of 0.1M NaCl (presence of micron size salt particles is also seen in SEM images).
Figure 4.38 Stabilization of AuNRs by formation of bi-layer of CTAB molecules (inset shows chemical structure of CTAB).\textsuperscript{43}

Except for electrochemical deposition methods that uses hard templates like alumina or polycarbonates for making NIR absorbing/anisotropic gold nanostructures, all other syntheses techniques use CTAB at some stage during formation of anisotropic nanostructures.\textsuperscript{45} With high significance of NIR absorbing gold nanostructures for biological and biomedical applications examining toxicity of different chemicals involved in syntheses has gained much attention recently.\textsuperscript{45} CTAB is a strong positive charged surfactant known for degradation of biomembranes, peptides and found determinant to
human cells by many research groups\textsuperscript{44,45,68} and it is evidently present in solutions after syntheses due to electrostatic interactions with AuNPs (figure 4.38).

Figure 4.39 Shows effect of dialysis on optical properties of CTAB stabilized AuNRs from changes in SPR spectrum with respective to time (sample bought from Nanopartz).

In vivo and in vitro studies using CTAB based gold nanostructures are expected to be safe in view of no toxicity anticipated from bound CTAB molecules. Presumably unbound CTAB molecules are separated by centrifugation and dialysis, but CTAB on surface of AuNPs is not statistically bound so always some degree of desorption in to
Table 4.10 All possible ways known in literature for enhancing biocompatibility of anisotropic gold nanoparticles.

<table>
<thead>
<tr>
<th>Proposed biocompatible anisotropic AuNPs in literature</th>
<th>Description of procedure and comments (all protocols use HAuCl₄ as precursor and CTAB surfactant as stabilizer).</th>
<th>References</th>
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<tr>
<td>Au nanorods loaded PNIPAM microgels</td>
<td>Seed-mediated (CTAB stabilized) loaded in to PNIPAM microgels just by stirring followed by centrifugation (demonstrate swelling ratio of NIR absorbing microgels by irradiating with 806 nm NIR lamp).⁴¹,⁴²</td>
<td>41, 42</td>
</tr>
<tr>
<td>PEG-PNIPAM loaded Au nanorods</td>
<td>Seed-mediated (CTAB stabilized) AuNRs loaded in to PNIPAM-PEG microgels (demonstrate swelling ratio of NIR absorbing microgels by irradiating with 806 nm NIR lamp).⁶⁹</td>
<td>69A, B</td>
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<tr>
<td>Chitosan modified AuNRs</td>
<td>Thiol modified chitosan as capping agent for CTAB stabilized AuNRs.⁷⁰</td>
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<tr>
<td>Polymers or polyelectrolyte coated AuNRs</td>
<td>PAA (polyacrylic acid) coated AuNRs produced by seed-mediated growth (CTAB) technique. Deposition of PSS (poly styrene sulfonate) and PDADMAC (poly diallyldimethyl ammonium chloride) to decrease cytotoxicity of CTAB stabilized AuNRs. No proof that all CTAB is removed.⁶⁸</td>
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</tr>
<tr>
<td>PEG-SH modified AuNRs</td>
<td>PEG-SH polymer was mixed with CTAB stabilized AuNRs, displayed very low zeta-potential (between 0.2 to 8.6) indicating instability, no proof that all CTAB is removed.⁶⁹b</td>
<td>69B</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC) modified AuNRs in chloroform</td>
<td>PC modification of CTAB stabilized rods by simple mixing and centrifugation. No proof that all CTAB is removed.⁶⁸</td>
<td>68</td>
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</tbody>
</table>

surrounding medium exists. Complete removal can result in undesired aggregation and is questionable.⁴⁴ In order to closely understand literature arguments about removal of excess CTAB by centrifugation and dialysis we tried with samples brought from
Nanopartz company (figure 4.39). It was clearly observed that any attempt to decrease quantity of CTAB in solution results in agglomeration or precipitation of AuNRs with complete loss of characteristic optical properties. This clearly shows that definite amount of CTAB is necessary for maintaining stability of AuNRs in solution removal of which sacrifices optical properties of AuNRs. Looking at the proposed stabilization mechanism using CTAB surfactant (figure 4.38)\textsuperscript{43} also confirms detainment of large amount of adsorbed CTAB molecules on surface of AuNRs to impart stability in solution. So in view of above skeptical arguments two methods are commonly followed to overcome CTAB cytotoxicity. One is simple surfactant exchange by dispersing AuNRs in to chloroform and exchanging with phosphatidylcholine (PC) and a more common method involves capping CTAB with partially non-ionic, surfactants like PEG.\textsuperscript{45} From table 4.10 it is very much evident that though CTAB stabilized AuNRs are covered/capped with some biocompatible polymers like PEG or biomolecules but usage of CTAB for initial syntheses of anisotropic AuNPs is unavoidable. Polymers like PC, PDADMAC (poly (diallyldimethyl ammonium chloride)), PSS (poly (4-styrene sulfonic acid)), PEG-SH (thiol-polyethylene glycol) are employed to cap CTAB stabilized AuNRs by electrostatic adsorption or by surface coating on existing CTAB molecules by simple exchange (table 4.10). Like for instance to coat a negatively charged polymer like PSS (Polystyrene sulfonate), CTAB stabilized AuNPs are stirred with PSS polymer followed by centrifugation to enhance electrostatic interactions between positively charged CTAB and negatively charged PSS polymer. This gives rise to PSS coated AuNRs.\textsuperscript{68} But leakage of CTAB molecules in solution is always questionable. All these surface coating
techniques rely on electrostatic adsorption by simple stirring or exchanging in organic media where tendency of AuNRs aggregation is very high.\textsuperscript{44} And sometimes after coating, zeta potential of PEG coated AuNRs was found to decrease which indicates aggregation tendency or decrease in stability of AuNRs in solution.\textsuperscript{44} So if a technique that can directly result in NIR absorbing AuNPs stabilized directly within biocompatible polymers that avoids multiple steps and also any reducing agents or surfactants will be always desirable. There are few works mentioned in literature for making chitosan stabilized gold nanospheres (table 4.3) but only one single citation exist\textsuperscript{70} involving usage of chitosan for capping CTAB stabilized AuNRs. So a new facile syntheses protocol that result in strong NIR absorbing AuNPs directly within biologically and environmentally benign chitosan polymer is discussed in detail.

4.3.9 Chitosan Polymer Stabilized AuNPs

Chitosan (CS) a naturally occurring benign biodegradable positively charged polysaccharide has been shown to exhibit strong affinity for stabilizing metallic nanoparticles mostly because of its chelating ability.\textsuperscript{70b} Chitosan has been utilized for numerous biomedical applications, mostly due to extraordinary biocompatibility, biodegradability, and bioadhesive, nontoxic and antifungal properties that are intrinsic to polymer itself.\textsuperscript{70b} Here we demonstrate a straight forward approach to make selectively highly monodispersed nanospheres or polydisperse anisotropic AuNPs by simple variation in pH of the medium.
4.3.9.1 Syntheses of Spherical AuNPs Stabilized in Chitosan

Formation and stabilization of AuNPs within chitosan polymer is found to be highly pH dependent, any pH above 6.0 where most of the amine groups are deprotonated (-NH$_2$) favors formation of spherical AuNPs with narrow SPR peak around 525 nm wavelength region. Figure 4.39, shows that in alkaline medium both photolysis and thermolysis results in formation of spherical AuNPs with average size less than 10 nm. Some very small particles less than 5nm are also observed that signifies ability of this method to produce very small particles. But in acidic medium higher baseline and broad SPR peaks denote agglomeration of particles or elucidate promising ability for formation of NIR absorbing particles. Examining time dependent absorption spectra at different combinations of Au(I) precursor and CS polymer concentrations (figure 4.41 & 4.42) under alkaline pH shows evolution of single visible absorption SPR peak. This validates formation of only spherical particles also confirmed from TEM images. At various concentrations of Au(I) and CS polymer only minor variation (~15%) in photochemical quantum yield ($\Phi$) is noticed. Except for minor shift in SPR wavelength or peak distribution different concentration combinations verifies formation of only spherical particles if pH is maintained above 6.0 (figure 4.41 and 4.42). Formation and stabilization of spherical AuNPs within CS polymer though known previously based on chelating property of Chitosan was achieved using HAuCl$_4$ precursor by employing regular reducing agents or by photolysis.$^{40}$ As noticed in case of PNIPAM gels or different polymers, employing Au(I) precursor avoids usage of reducing agent and also provides ability to make particles particularly smaller than 10 nm under different experimental conditions (photolysis or thermolysis).
Figure 4.40 Formation of spherical AuNPs within chitosan polymer at both alkaline (~8.0) and acidic pH (~3.0) under both photolysis and thermolysis condition. A) Electronic absorption spectra of samples. B) TEM images of photolysis samples. C) TEM images of thermolysis sample.
Figure 4.41 Photolysis quantum yield calculations for chitosan stabilized spherical AuNPs. A) 0.2% (wt/v): 5 mg Au(I); $\Phi_{533\text{ nm}} = 0.264$. B) 0.1% (wt/v): 3 mg Au(I); $\Phi_{533\text{ nm}} = 0.202$
Significant advantage of using Au(Me₂S)Cl as starting precursor enables complete removal of any unreacted Me₂S by simple heating to ~35 °C. This provides facile protocol to make perfect biocompatible AuNPs in chitosan polymer not sacrificing biocompatibility of chitosan polymer. In vitro and in vivo tests validating our assumption is discussed in later sections of this chapter.

4.3.9.2 Formation of NIR Absorbing Anisotropic AuNPs Stabilized in CS Polymer

Conceiving easy formation of NIR absorbing anisotropic AuNPs within positively charged PNIPAM microgels by photochemistry and thermochemistry, CS was also tested for its ability to directly stabilize NIR absorbing AuNPs. This would realize our goal for formation of highly accredited NIR absorbing AuNPs perfectly within biocompatible
medium in complete absence of any harsh chemicals. As figure 4.40, shows small spherical AuNPs are produced at alkaline pH, so combination of acidic pH (2-3) and low temperature (0 °C to 5 °C) are employed for stabilizing NIR absorbing anisotropic gold nanostructures. On irradiating homogenous solution mixture of chitosan polymer and Au(Me₂S)Cl, tunable NIR absorbing AuNPs are formed within CS polymer in 45-60 minutes (figure 4.43). After 10-15 minutes of irradiation strong NIR absorbing particles with peak maximum >1000 nm are formed and with time this broad peak undergoes blue shift to absorbance wavelength peak maximum anywhere between 700 nm-900
nm depending on concentration of chitosan and Au(I) precursor (figure 4.44). Unlike PNIPAM microgels, concentration of CS polymer is playing a crucial role in tuning NIR SPR peak maximum and distribution. It was clearly observed that strong NIR absorbing samples are produced only when concentration of CS polymer is above 0.5% (wt/v). Again minimum concentration of Au(I) precursor was found to be >5 mg. Mechanism of formation and effect of centrifugation is understood from figure 4.43, which shows formation of anisotropic structures using CS as stabilizer is similar to PNIPAM microgels except for broader NIR peaks and unusual precipitation after centrifugation. Even at highest concentration of chitosan polymer, centrifugation at same speed as PNIPAM microgels, all the NIR absorbing particles precipitate out leaving only spherical absorption peaks (figure 4.43-blue solid line). This could be due to fact that microgels provide better surface area (matrix of microgel) for stabilization compared to linear chain chitosan polymers in solution. This centrifugation results explains difference in matrix of chitosan polymer and PNIPAM microgels that plays a crucial role during stabilization. Microgels are three dimensional crosslinked particles with large surface area compared to linear chitosan polymer chains which make microgels as good carriers for holding large anisotropic structures within their matrix compared to CS polymer in solution. Broad NIR SPR peaks can indicate agglomeration or precipitation, so formation of stable anisotropic particles was confirmed from TEM analysis from more than one sample (figure 4.46, 4.47) prepared on different occasions.
Figure 4.44 Effect of both Au(I) and chitosan concentration on formation of NIR absorbing anisotropic AuNPs monitored from absorption spectrum (photolysis at pH 3.0).

Specifically stability of these broad NIR peaks was also ascertained from monitoring time dependent changes in absorption spectrum of the samples stored in fridge at 4 °C (figure 4.45) Perfect stability of these samples was confirmed from unaltered absorption spectrum even after 3 months time. As absorbance wavelength region is broad (~200-300 nm), perfect correlation between shape/size of particles and wavelength of absorbance is not attained as in case of CTAB stabilized AuNRs reported in literature. A general conclusion was drawn analyzing TEM images of two samples A and B (figure 4.46). Sample B exhibiting broad NIR peaks (>800 nm) with weak ~530
nm shoulder and sample A containing narrow SPR peaks (<800 nm) with intense shoulder at ~530 nm shows clear difference in percentage of spherical particles.

Figure 4.45 Demonstrating stability of a typical chitosan polymer stabilized anisotropic AuNPs with respective to time. Changes in absorption spectra are monitored on the day of syntheses and later same sample stored in fridge at 4 °C was tested.
Figure 4.46 TEM images of two different NIR absorbing AuNPs stabilized in chitosan by photolysis at different concentration combinations (A: Corresponds to sample labeled 'A' in figure 4.42; B: Corresponds to sample labeled as 'C' in figure 4.43).
Sample with broad NIR peak (sample B) contains very few or no spherical particles and diagonal length of anisotropic particles is much larger compared to perfect mixture of spherical and polygonal shape particles noticed from sample A. Close examination shows, sample B does not exhibit sharp edges as noticed from any anisotropic particles of sample A. We assume this non-sharp edge anisotropic particles
give rise to broad NIR peaks compared to narrow NIR peaks from sharp edge nanostructures (compare TEM images from figure 4.46 & 4.47). Though exact reasons for stabilization of these anisotropic structures by chitosan or PNIPAM microgels is not understood clearly, but we predict protonated amine groups (-NH$_3^+$) of CS polymer or positively charged PNIPAM microgels favoring stabilization of NIR absorbing gold nanostructures similar to commonly used positively charged CTAB surfactant.

4.3.9.3 Testing Stability of Chitosan Stabilized NIR Absorbing Anisotropic AuNPs

In view of strong biological applications of AuNPs they are required to be introduced in to blood stream/cells at some point. Hence, testing stability of AuNPs at physiological pH and biological relevant media like PBS (phosphate buffer saline) is very significant as change in ionic strength and pH of the medium or presence of electrolytes can result in nanoparticles aggregation via electrostatic screening. This aggregation of NPs will influence both physical and chemical properties of NPs. Main objective of obtaining toxin free biocompatible gold nanostructures was to avail their complete advantages for biological applications, so these gold nanostructures that are initially made at either alkaline or acidic pH are tested for their stability at physiological pH (~6-7). Excess of acid or base used during syntheses was removed by simple dialysis using 3000 MCO tubing, than followed by addition of PBS buffer. Changes in absorption spectra before and after cleanup work for a typical spherical and anisotropic particle samples is shown in figure 4.48. A minor change in absorption spectra indicates strong stability of these chitosan stabilized gold nanostructures in biological relevant medium.
Figure 4.48 Demonstrating stability of chitosan stabilized AuNPs in physiologically relevant PBS buffer. Changes in electronic absorption spectra are recorded before and after addition of PBS buffer. A: Typical anisotropic AuNPs samples (pH before 3.0; after 6.5). B: Typical spherical AuNPs sample (pH before > 7.0; after 6.5).

4.3.10 Applications of Toxin Free Gold Nanostructures

4.3.10.1 Demonstration of Photothermal Driven Volume Phase Transitions (PVPT) Using Gold Nanostructures Loaded Hybrid PNIPAM Microgels and Dye Release Studies

Polymer microgels that exhibit temperature sensitive volume phase transitions are applied for promising applications in materials science and drug delivery.\(^{31}\) In particular photothermal modulated volume phase transitions brought by incorporating gold nanoparticles within polymer hosts have received considerable attention in view of
their potential usage as “smart” materials and “switchable” devices.\textsuperscript{41,42} To induce phase transitions, photosensitive moieties like dyes or metallic nanoparticles are embedded in a thermally reversible polymer matrices and irradiated at resonance.

![Irradiance curve for quartz tungsten halogen (QTH) lamp (100 W)](image)

Figure 4.49 Irradiance curve for quartz tungsten halogen (QTH) lamp (100 W) used as radiation source to demonstrate photothermal volume phase transition and dye release studies (taken from Newport website, model 6333).

wavelengths of photosensitive materials. On irradiation light energy is converted to heat through non-radiative relaxation causes hydrogel heating and volume phase transition.\textsuperscript{41,42} As for application of thermoresponsive gels as drug delivery carriers or for photothermal therapy it is vital that the photosensitive species strongly absorb in the “water window” spectral range, that is, at 800 nm < $\lambda$< 1200 nm. To the best of our
knowledge only two groups demonstrated such a volume phase transition by using NIR sensitive nanomaterials. One is Halas and West group who demonstrated photothermal phase transition and drug delivery using SiO$_2$ core Au shell optically active nanostructures$^{42}$ and the other by Kamacheva group who embedded CTAB stabilized gold nanorods within PNIPAM microgels.$^{42}$

Here for the first time in situ stabilized NIR sensitive gold nanostructures are employed for demonstrating photothermally driven phase transition in PNIPAM-co-allylamine microgels. Broad absorption of our AuNP samples (700-1200 nm) encourages for using cheaply available light sources (broad wavelength lamps) compared to expensive wavelength specific laser diodes. In situ stabilized non-CTAB based monometallic AuNPs are used for the first time to demonstrate optically driven shrinkage in PNIPAM microgels. This would encourage direct usage of such opto/thermosensitive gold nanostructures in any biomedical applications without need of any extra capping agents. Photothermal volume phase transition (PTVPT) is demonstrated using anisotropic gold nanostructures loaded PNIPAM-co-allylamine microgels, absorption spectrum and SEM image of hybrid microgel samples used for both PTVPT and dye release studies are shown in figure 4.50. After determining LCST (33°C-34°C) of undoped microgels and hybrid microgels, samples are maintained at 34°C using circulating water flow. A 100 W quartz tungsten halogen lamp with broad irradiance profile has been used as source of irradiation. Irradiance curve for the lamp is shown in figure 4.49, which appear appropriate for our broad NIR absorbing samples. Lamp output under experimental conditions was measured using a thermopile and
Figure 4.50 Absorption spectra and electron microscopy images of NIR sensitive AuNP samples used for different studies. I) A: Hybrid CS polymer used for temperature change studies. B: Hybrid gel used for volume phase transition studies and temperature change studies. C: Hybrid gel used for dye release studies. II) Representative FE-SEM and HR-TEM images of sample B and C (representative HR-TEM images for sample A are shown in figure 4.44 A).
determined to be between 0.13 W to 0.137 W. Combination of converging lens and 500 nm cutoff filters were used to focus the radiation from lamp on to sample. PTVPT experiments are conducted by placing samples inside sample holder of dynamic light scattering instrument (AVL-5000, Germany) than irradiated with the NIR lamp. Samples are not removed until finish of experiment. A constant water flow maintains temperature of sample constant and a fan was operated to avoid any heating from the lamp. Control microgel which does not contain any AuNPs was also studied under exactly same conditions on same day. Each cycle was operated with time interval of 30 minutes which allows the microgel samples to stabilize to room temperature. Figure 4.52A & B demonstrates the reduction or changes in average hydrodynamic radius of both hybrid PNIPAM microgel and control undoped microgels during two cycles of exposure. Anisotropic gold nanostructures containing PNIPAM microgels exhibited 17% ± 4.2 and 29.5 % ± 0.7 decreases in average hydrodynamic radius after 30 minutes and 60 minutes of exposure respectively. By in contrast very
Figure 4.52 A) Dynamic light scattering data representing changes in average hydrodynamic radius ($R_h$) of hybrid microgels (NIR sensitive AuNPs loaded PNIPAM-co-allylamine microgel) and control gel (microgel without AuNPs) at different times of exposure (recorded for two heating and cooling cycles to check reversibility of phase transition). B) Same data plotted for clarity.
insignificant decrease (2.7%) is noticed from undoped control microgels for same exposure time. “Off and On” cycles demonstrate reproducibility and retainement of anisotropic structures during volume phase transition studies. This simple reproducible experiment proves concept of conversion of light energy to heat using optically sensitive AuNPs. In order to reconfirm this photothermally driven volume phase transition in hybrid microgels, concept of drug release using these NIR sensitive hybrid microgels is demonstrated by loading a Pt(II) based phosphorescent molecule (Pt-POP) in to these hybrid PNIPAM microgels. Absorption spectra of hybrid and control microgels after loading dye (Pt-POP) exhibits UV transition band ~380 nm (figure 4.53) which indicates presence of dye molecule both in hybrid and control microgel particles. Retention of AuNPs and dye within microgel after stirring and centrifugation workouts was confirmed from broad absorption spectra of (figure 4.53) hybrid microgels samples. For demonstrating photothermal release of dye molecule or photothermal volume phase transition (PTVPT), both the microgel samples are taken in to dialysis tube and then subjected to irradiation maintained at ~35 °C. Release of dye molecule from both hybrid and control samples is monitored by recording the absorbance and photoluminescence of water solvent in which dialysis tube is immersed during irradiation (see figure 4.49 for schematic illustration of release experiment). Absorption spectra of water solvent corresponding to hybrid sample exhibits strong ~380 nm UV transition corresponding to release of dye molecule, whereas a weak optical absorbance from water solvent corresponding to control sample indicates no or insignificant release of dye molecules (figure 4.54).
Figure 4.53 Absorption spectra of Pt-POP (dye) doped hybrid PNIPAM microgels and control microgel samples used during dye release studies. (Inset zoom absorption spectrum validates retention of AuNPs). Optical absorption spectra of samples recorded after stirring, centrifugation and redispersing. (Pt-POP provided by Nisa Satumtira graduate student in Omary group)

Distinctive difference in absorption spectra (figure 4.54B) clearly demonstrate release of (Pt-POP) dye loaded in to hybrid microgel is promoted due to photothermal volume phase transition driven by optical sensitivity of anisotropic gold nanostructures where as absence of any gold nanostructures in control gel sample (figure 4.54A) does not assist in shrinking and does not release dye molecules. As Pt-POP exhibits strong green emission, significant difference in photoluminescence intensity also shows the difference in amount of Pt-POP released during these studies (figure 4.55). This study
not only demonstrates release of dye molecule (Pt-POP) due to photothermal volume phase transition of hybrid gels but also reconfirm shrinking within PNIPAM microgels driven by photoactive gold nanostructures. After demonstrating the concept of PTVPT and dye release using NIR optically sensitive gold nanostructures, it becomes very important to determine exact change in temperature of gold nanoparticles solution on irradiation. It is understood that light activated therapies to eradicate diseased cells and tissues (tumors) in non-invasive manner involve usage of exogenous agents with large absorption cross section. These non-invasive therapies rely on photothermolysis or optical hyperthermia (conversion of absorbed light in to heat by non-radiative mechanism) capabilities of these exogenous agents. Photothermal agents are expected to be most effective if absorb strongly at NIR frequencies.\textsuperscript{71}
Figure 4.54 Time dependent absorption spectra of water solvent during dye release studies. A: From (Pt-POP) dye doped hybrid PNIPAM microgel sample. B: From (Pt-POP) dye doped control PNIPAM microgel sample.
Figure 4.55 Photoluminescence spectra of water solvent during dye release studies. Both hybrid microgel sample solvent and control microgel sample solvent were measured at same time and under similar instrumental parameters.

4.3.10.2 Determining Change in Temperature of NIR Absorbing AuNPs Samples on Irradiation

Heat induced cell injury is characterized by phenotypic responses such as membrane blebbing, depolymerization of cytoskeletal filaments, thermal inactivation of membrane proteins and mitochondria which are resolved at sub-cellular level by targeting optically active nanoparticles. All these sub-cellular level changes depend on local changes in temperature caused due to hyperthermia.71 Cheng group has demonstrated gold nanorods mediated tumor cell death by compromising membrane
integrity in malignant KB cells. CTAB stabilized AuNRs are functionalized with folic acid, KB tumor cells incubated with folate functionalized AuNRs are scanned with a tightly focused continous-wave (CW) laser beam tuned to plasmon resonance at 765 nm.$^{71}$ Halas and co-workers photodestructed breast carcinoma cells and tumors in mice using silica core/gold shell particles using a CW NIR laser. Later El-Sayed et al. demonstrated PPTT (plasmonic photothermal therapy) of human oral cancer cells using AuNRs photoirradiated by CW Ti-Sapphire NIR laser and determined cancer cells require half the laser energy (10 W cm$^{-2}$) to be photothermally damaged compared to normal cells (20 W cm$^{-2}$).$^{22b,44,72}$ Cortie and co-workers calculated photothermal heat generation by laser irradiation of CTAB stabilized nanorods in murine macrophage cells. Using 30 J cm$^{-2}$ laser strength, effective temperature increase on cell surface was determined to be in the order of 10 °C.$^{72}$

Changes in temperature of controls and gold nanoparticles containing samples on irradiation with QTH lamp are recorded. Three cycles of changes in temperature are tabulated with standard deviation for each sample before and after exposure (table 4.11). Constant temperature water bath was used to maintain equilibrium, after each cycle one hour of lapse time is provided for samples to attain equilibrium. Exact same volumes of solutions are used. Electronic absorbance of both CS and PNIPAM microgel stabilized AuNPs samples used for the study is shown in figure 4.48 A. After fifteen minutes of exposure, hybrid PNIPAM microgels recorded 2.7 °C increase in temperature compared to 1.1 °C increase from control microgel sample, while hybrid chitosan polymer solution recorded 3.3 °C increase compared to 1.2 °C by undoped polymer.
Though these minor changes in temperature might not be sufficient enough for causing cell death or hyperthermia but experiments are in progress with different NIR absorbing AuNPs at different concentrations to get best enhancement in temperature that would be sufficient to kill tumor cells or for hyperthermia.

Table 4.11 Changes in temperature of AuNPs doped PNIPAM-allylamine microgels and chitosan polymer solutions on irradiation with QTH lamp.

<table>
<thead>
<tr>
<th>Control Microgel</th>
<th>Hybrid Microgel (AuNPs loaded microgel)</th>
<th>Time of exposure (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial (T °C)</strong></td>
<td><strong>Final (T °C)</strong></td>
<td><strong>Initial (T °C)</strong></td>
</tr>
<tr>
<td>22.3 ± 0.84</td>
<td>23.4 ± 0.25</td>
<td>22.8 ± 0.4</td>
</tr>
<tr>
<td>21.9 ± 0.9</td>
<td>23.1 ± 0.1</td>
<td>22.1 ± 1.3</td>
</tr>
</tbody>
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4.3.11 In Vitro and In Vivo Toxicity Tests in Comparison with Analogues in the Market

4.3.11.1 MTT Assay

Biocompatibility, biodistribution, biodegradation, inflammation and interference with cells and normal functioning of organs and other factors will determine the toxicity of engineered inorganic nanoparticles and extend of their usage. With increase in use of nanomaterials in increasing quantities in consumer products, biology and medicine their presence and impact on environment and health has gained much attention. Recent developments have utilized biokinetic behavior of NPs for many applications. Like fluorescent nanoparticles for labeling cellular components, use of luminescent or
magnetic particles as contrast agents, targeted delivery or conjugation to biomolecules for biodetection. Size and shape of nanomaterials plays significant role depending on applications, for example objects smaller than 12 nm are expected to cross blood-brain carrier and particles with sizes \( \sim 30 \) nm are shown to endocytosed by cells compared to other sizes.\(^7\) But potential toxicity of these nanomaterials cannot be outweighed so for any further development and applications a key issue is assessment of potential toxicity of nanoparticles. Though numerous scientific studies and reports are published with goal of understanding interactions and effect of size, shape and surface chemistry of NPs on cells and environment but no simple conclusions are available. This is mainly due to variability of parameters such as physical, chemical properties of NPs, cell types used, dosing parameters and biochemical assays used. Major issues pertaining to toxicity of NPs are less understood as majority of investigations are focused on in vitro with less effort concentrated on in vivo or real situations.\(^7\)

When it comes to gold nanomaterials, bulk gold is well known to be safe and chemically inert, and some gold based compounds (Auranofin, Tauredon) have been used in clinic as ant-inflammatory agents to treat rheumatoid arthritis. Radioactive gold microparticles have been used in local radioisotope cancer therapy.\(^6\) Ultimately many applications require introduction of AuNPs in to blood stream of an organism and hence today most common study is assessing toxicity or biocompatibility of AuNPs. Most common is dose-dependent in vitro viability assays on cultured cells, looking for cell survival and proliferation. Concentration of dosage is not emphasized as many drugs that are beneficial at lower dose are known to be toxic at higher doses and also
concentration of particles and number of cells varies widely across different research
groups. Of many assays used to understand cellular impact of a drug LDH (Lactate
dehydrogenase) and MTT (Methylthiazole Tetrazolium) are commonly followed with
MTT assay considered the “gold standard” for cytotoxicity assessment. MTT is a
colorimetric assay that measures the enzymatic activity of cellular mitochondria. The
MTT dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow
tetrazole compound that gets reduced to purple formazan in living cells in presence of
mitochondrial reductase. The absorbance of this colored solution can be quantified by
measuring at certain wavelength (500-600 nm) that quantifies cellular activity. In case
of AuNPs general components that cause toxicity could arise from gold core, surface
bound ligands or left over chemicals during syntheses and thus evaluating all these
components is essential to understand origin of toxicity. The major advantage of
proposed protocol compared to analogues available in the market is usage of
biocompatible polymers like chitosan for stabilization whose biocompatibility and
biodegradability is well established and only other chemicals present during
syntheses is Au(I) precursor. Au(I) sulfides are already in clinical usage for treatment of
rheumatoid arthritis and Me₂S ligand is a characteristic sulfur compound found in
many vegetables and fishes. It can be easily removed by simple dialysis or heating
(37°C). So different AuNPs produced by this protocol are expected to be completely
non-toxic and our assumption has been validated by doing MTT assay on Porcine
proximal tubule cells (LLC-PK1) following protocol designed by NCL-NCI (Nanotechnology
characterization laboratory-National cancer institute) using both
spherical and anisotropic particles in perfect comparison with analogues available in the market.

MTT assay was performed using chitosan stabilized nanospheres and anisotropic particles in comparison with citrate stabilized nanospheres (20 nm particles from BBI International), CTAB stabilized AuNRs and PEG-NH$_2$ modified AuNRs (from Nanopartz). MTT assay with each sample was reproduced more than twice. As it becomes impractical to compare molar concentrations of Au from different samples due to complexities involved, (cannot assume starting precursor concentration as molar concentration of AuNPs) so we compared electronic absorption spectra and ICP-MS data. Though optical characterization techniques (AFM) or electron microscopy techniques (SEM and TEM) can evaluate size, shape, morphology, surface texture, distribution but cannot be used for analytical quantification,$^{57b}$ so ICP-MS that can compare relative Au content in solutions directly is utilized supported by absorbance data. Samples exhibiting equal absorbance (absorbance intensity, peak height and peak position) and relatively similar Au content (from ICP-MS data) are compared during cytotoxicity evaluation studies. As our main objective is only to demonstrate toxin free nature of our AuNPs, exact gold concentration or LD$_{50}$/LC$_{50}$ (lethal dosage or lethal concentration of a drug or toxic substance required to kill half of the population) is not determined. Figure 4.54, shows absorption spectra and relative gold content (C/S) of spherical gold nanoparticles used for MTT assay. Relative Au content was measured
Figure 4.56 A) Absorption spectra and B) ICP-MS data for spherical gold nanoparticles used for MTT assay. (Citrate stabilized AuNPs from BBI international company). Required amount of PBS is added to attain physiological pH (~7.0). (Aron Hart, Dr. G. Verbeck in chemistry provided assistance for collecting ICP-MS data)
Figure 4.57 MTT assay results for spherical gold nanoparticles after 24 hrs and 48 hrs respectively. Omary NPs (Dark red solid column): Chitosan stabilized Au nanospheres; Commercial NPs (Dark red solid column): Au nanospheres from BBI international company-20 nm. Green solid column: APAP= acetaminophen; Triton X = cell lysing agent. (Data collected and analyzed by Dr. R. Petros in chemistry)
based on C/S value obtained from direct samples without addition of any strong acids (HNO₃) to avoid any tampering of AuNPs. C/S counts are determined for three duplicate samples after running millipore water as control before running every sample due to high sensitivity of ICP-MS. From C/S values relative Au content of chitosan stabilized samples is found higher compared to citrate stabilized AuNPs (BBI company) which is in good agreement based on optical absorbance of samples (figure 4.56A,B). MTT assay was performed using Porcine Proximal Tubule cells with APAP (acetaminophen) and Triton-X as positive controls. Both 24 hrs and 48 hrs cell viability profile (figure 4.57) data meets acceptance criteria (APAP cell viability should be within 15 % to 75% and difference in sample and positive control variation within 50%). In first and second dilution 50% and 20% better cell viability is noticed from chitosan stabilized AuNPs compared to citrate stabilized AuNPs with higher Au content. At higher dilutions both samples exhibited similar cell viability. These cell viability results suggests gold nanospheres produced by following proposed protocol in this chapter are completely cell viable (non-toxic) even without following any through purification techniques due to non-involvement of any toxic chemicals during syntheses. These results encourage for strong commercial applications of these gold nanospheres because of two advantages. 1) Does not involve any strong purification techniques as needed for NaBH₄ reduced particles 2) Positively charged particles are seldom made directly due to involvement of sodium citrate (negatively charged stabilizer) for making best biocompatible particles known in the market. There are some contradictory results about toxicity of gold nanospheres in literature based on size and charge of stabilizer. Like, Connor et al.
showed AuNPs of different sizes (4, 12, 18 nm) made using citrate, cysteine, glucose, biotin to be “non-toxic” in presence of leukemia cell line based on MTT assay. While 1.4 nm nanospheres triggered necrosis, mitochondrial damage and oxidative stress and interestingly no evidence of cellular damage was found from 15 nm nanospheres. In another study Patra el al. found 13 nm citrate capped AuNPs toxic to human carcinoma lung cell line but not to human liver carcinoma cell lines at same dosage. All these conflicting results arise due to many factors discussed earlier and hint at urgent need for a single universal protocol specifically to evaluate toxicity of AuNPs. Thorough investigations are currently underway by many research groups to understand minute details of AuNPs interactions with different cell lines to evaluate their biological pharmacokinetic response.

Compared to contradictory toxicity results from spherical AuNPs, CTAB surfactant is universally shown to be cytotoxic. Two different chitosan stabilized anisotropic AuNPs synthesized using different concentrations of Au(I) precursor are tested against CTAB stabilized AuNRs. First set of MTT assay results with absorption spectra of samples are shown in figure 4.58A. Reproducible results were obtained from same set of samples performed on different occasions (figure 4.59). A general overview of MTT assay (figure 4.58B & 4.59) results show up to dilution six, more than 75% difference is noticed between two samples (CS stabilized anisotropic gold nanoparticles and CTAB stabilized AuNRs) both at 24 hrs and 48 hrs time interval. CTAB stabilized samples displayed strong cytotoxicity with less than 10% cell viability until dilution three as expected due to abundant quantity of CTAB molecules in solution. Beyond dilution six
Figure 4.58 A) Absorption spectra of samples used for MTT assay B) MTT assay results for 48 hrs study with anisotropic AuNPs (Omary NPs: Chitosan stabilized anisotropic AuNPs; Commercial NPs: CTAB stabilized AuNRs from Nanopartz). (Data collected and analyzed by Dr. R. Petros in chemistry)
Figure 4.59 Reproducing MTT assay with anisotropic particles. MTT assay results for 24 hrs and 48 hrs study (Omary NPs: Chitosan stabilized anisotropic AuNPs; Commercial NPs: CTAB stabilized AuNRs from Nanopartz). (Data collected and analyzed by Dr. R. Petros in chemistry)
Figure 4.60 A) Absorption spectra of samples used for MTT assay B) ICP-MS data to shows relative Au content in three samples used. (CH-Au: Chitosan stabilized anisotropic AuNPs; CTAB-Au: AuNRs stabilized in CTAB; PEG-Au: PEG coated CTAB stabilized AuNRs from Nanopartz) PBS is added to all samples to attain physiological pH (~7.0). (Aron Hart, Dr. G. Verbeck in chemistry provided assistance in collecting IPC-MS data)
Figure 4.61 48 hours MTT assay results with anisotropic particles (Omary NPs: Chitosan stabilized anisotropic AuNPs; CTAB: CTAB stabilized AuNRs from Nanopartz; PEG-NH$_2$: CTAB stabilized AuNRs capped with PEG-NH$_2$ polymer from Nanopartz). (Data collected and analyzed by Dr. R. Petros in chemistry)

even CTAB stabilized AuNRs exhibited cell survival similar to CS stabilized AuNPs which indirectly indicates toxicity of CTAB at higher concentrations or explain dilution effect. From understanding dilution process detailed in experimental section it will be easy to understand that beyond dilution 4 or 5 there would be technically no AuNPs or stabilizer present. A reproducible MTT assay result strongly confirms cell viability or non-toxic nature of anisotropic AuNPs stabilized in chitosan.
Taking a step further, as explained earlier in previous section PEGylating is the most common approach for overcoming CTAB toxicity of these AuNRs or anisotropic particles. So MTT assay was also performed on chitosan stabilized anisotropic gold nanostructures in comparison with PEGylated AuNRs from Nanopartz. Relative Au content of samples is compared from optical absorbance and ICP-MS data (figure 4.60). Higher content of Au from CS stabilized samples is expected due to broad absorbance profile and higher integrated peak area of the CS sample compared to PEGylated and CTAB samples (figure 4.60A). Though Au content of CS sample is higher, cell count after 48 hours from CS stabilized gold samples was five to six times higher compared to CTAB or PEG stabilized AuNRs up to dilution four. Though at all dilutions PEGylated samples exhibited better cell viability compared to CTAB stabilized samples as expected based on literature works and biocompatibility of PEG however only beyond dilution four PEGylated AuNRS exhibited similar cell viability compared to chitosan stabilized AuNPs (figure 4.61). These results from MTT assay clearly signify role of CTAB in inducing cytotoxicity to AuNP samples. Cell death at lower dilutions from PEGylated samples could be due to intricate presence of CTAB surfactant within PEGylated samples also. More than 5 times cell viability from CS stabilized samples highlights significance of following a direct toxin free syntheses protocol to improve cell viability compared to capping or exchanging of a toxic surfactant with biocompatible polymers after syntheses.
4.3.11.2 In Vivo Toxicity Studies on Zebrafish (Dino rero)

Toxicity may be caused by a compound interacting with molecular target that is distinct from the target selected for therapeutic benefit, defined as off-target effect. So many drugs developed by the pharmaceutical industry fall in clinical trials because of unanticipated toxic side effects. Drugs are usually tested for in vivo toxicity in mammalian models, such as mouse, rats and dogs.\textsuperscript{58} Toxicity testing is a time-consuming and expensive process; from last few years many investigators have begun to explore the use of zebrafish (Dino rero) as an alternative model for toxicity testing because they are small, easy to care for, inexpensive to maintain and produce large numbers of transparent embryos that develop outside of the mother. Zebrafish model are being preferred due to many similar aspects of development and functions compared to vertebrate biology including development and function of cardiovascular system, central nervous system and digestive system. Rapid testing of large number of drugs is possible using zebra fish for ability to culture large number of zebrafish in small space.\textsuperscript{75} National Institute of Environmental Health Sciences (NIEHS) in the United States and the Institute for Environment and Sustainability (IES) in Europe support the zebrafish as one model organism to study environmental toxicity.\textsuperscript{75}

Considering all the above advantages of zebrafish and recognizing prime significance of in vivo toxicity tests we have for the first time adopted adult zebrafish as model for in-vivo toxicity tests on different size, shape AuNPs. 5 microliters (µl) of all in-vitro studied samples (chitosan stabilized gold nanospheres, citrate stabilized gold nanospheres, chitosan stabilized anisotropic AuNPs, CTAB stabilized AuNRs, PEG-NH\textsubscript{2}
stabilized AuNRs) were intravenously injected into adult zebrafish and the survival rate of fish was monitored for either 24 hours or 48 hours.

Figure 4.62 In vivo toxicity tests in zebrafish. A) Absorption spectra of two different sets of AuNPs used (mm cuvette). Concentration B > A ~ 30 times. B) Zebrafish survival data after 24 hours. (N=12) High concentration of citrate stabilized AuNPs not available for comparison. (CS polymer concentration compared with red bar concentration). (Studies conducted in collaboration with Dr. Jagadeeshwaran in biology, data collected by Y. Radhakrishnan)
12 duplicates were monitored with spherical AuNP samples where as 24 duplicates were monitored in case of anisotropic samples due to significant importance of NIR absorbing anisotropic particles A) validate our hypotheses for producing first ever toxin free anisotropic particles using chitosan as stabilizer in single step. B) Due to known toxicity of CTAB used in literature. pH of the samples was adjusted by addition of required amount of 1% PBS solution.

Different concentrations of spherical AuNPs stabilized in chitosan are tested in comparison with citrate stabilized AuNPs from BBI. Fish survival data for different concentrations of AuNPs tested is included. As the concentration of citrate stabilized samples was limited, only chitosan stabilized AuNPs were tested at higher concentrations. Different concentrations of chitosan polymer by itself were not tested as biocompatibility of chitosan is well established and also because the main motive was to test biocompatibility of chitosan stabilized AuNPs but not chitosan by itself. Concentration of CS tested was anyway twice the concentration regularly used for syntheses. Figure 4.62A shows absorption spectra of both CS and citrate stabilized samples used for intravenous injection. From figure 4.62B, it is clear that at low concentration of Au (black bars) CS stabilized AuNPs exhibited 100% survival (12 fish survived) similar to PBS and citrate stabilized samples exhibited 91.6% survival rate (1 fish died). But when concentration of chitosan stabilized AuNPs was increased (based on peak absorbance) almost 30 times (red bars), survival rate was >75% (more than 9 fish survived) and beyond this concentration injection of AuNPs in to blood was not possible. For low AuNP concentration samples, relative Au content of both CS and
citrate stabilized samples can be compared to samples used for MTT assay as both samples belong to same batch. These results very comfortably elucidate non-toxic nature of AuNPs stabilized in CS, ~25% death noticed at very high Au concentration is expected as above certain concentration any drug/molecule is expected to be toxic. Similar intravenous injections were carried out for N=24 duplicates more than one time testing anisotropic samples (CS stabilized AuNPs, CTAB-AuNPs, PEG-AuNPs). Absorption spectra of samples used for these studies have been shown in the figure 4.63A and figure 4.63B shows the integrated peak area which signifies presence of high Au content from CS sample compared to analogues purchased also evident from relative C/S Au count of ICP-MS data (figure 4.64A).

Among all the samples (figure 4.64B) tested, CTAB polymer by itself containing less than half the actual concentration (~0.05 M) regularly required for syntheses (0.2M) of AuNRs as in literature exhibited highest death rate (50% death) within 24 hours and 100% death rate after 48 hours. While CTAB stabilized AuNPs though contain relatively lowest Au content but still exhibited highest toxicity rates among all AuNP samples. About 40% death rate was noticed after 24 hours and 75% death was observed after 48 hours from fish injected with CTAB stabilized AuNPs. However as expected from MTT assay results PEG-NH$_2$ polymer capped AuNRs exhibited better survival rate compared to CTAB stabilized samples. Though CS stabilized anisotropic AuNPs contain relatively more Au content compared to PEG stabilized AuNRs, but fish injected with CS-Au sample exhibited better survival rate compared to any other Au
containing samples. These results are in good correlation with in vitro (MTT) assay performed on similar anisotropic samples. Both these in vitro and in vivo tests

Figure 4.63 A) Absorption spectra of three different anisotropic AuNPs used for intravenous injection in to adult zebra fish. B) Absorption spectra peak area calculation after baseline correction (peak area between 600-1200 nm).
Figure 4.64 A) Relative Au content of anisotropic AuNP samples used for in vivo toxicity tests. (C/S count of ICP-MS data; N=4 duplicates) B) Fish survival data after 24 hours. (Studies conducted in collaboration with Dr. Jagadeeshwaran in biology, data collected by Y. Radhakrishnan)
collectively demonstrate high biocompatibility or non-toxic nature of CS stabilized anisotropic AuNPs even at higher Au content compared to commercial analogues available in the market with strong indications of commercial applications to these patented AuNPs. Minor works on controlling aspect ratio of these biocompatible AuNPs is still under investigation to make them best sought out AuNPs.

4.3.12 Catalysis

Catalysts are used in some 90% of all chemical processes worldwide at some stage during production, with majority of catalysts involving nanoparticles < 20 nm deposited on high surface area supports. Importance of nanoparticles to the performance of catalysts has stipulated immense interest in understanding and developing new techniques as integral part of nanoscience today. Gold in bulk scale is chemically inert and due to several reasons \(^{27}\) was not preferred for catalysis. But works of Bond, Sermon and by Paravano gave first hints of Au catalytic capabilities. Later work by Haruta et al. in 1983 demonstrated that once Au is deposited as hemispherical nanoparticles on selected metal oxide supports it can exhibit surprisingly high catalytic activity (CO oxidation) even at 200K. Catalytic activity at 200k is very significant as most of the heterogeneous catalysis takes place at temperatures more than 400k.\(^{27}\)

Many factors like contact of AuNPs with support (spherical or hemispherical), Type of metal oxide supports (\(\text{SiO}_2, \text{Al}_2\text{O}_3, \text{TiO}_2, \text{Fe}_2\text{O}_3, \text{NiO}\) acidic or insulating or semiconductor based), size of AuNPs, number of layers of AuNPs on support, particle shape or perimeter, metal oxidation state contact area and deposition or preparation method are supposed to contribute for AuNPs catalytic activity.\(^{27}\) Not going in to details,
Figure 4.65 A) Absorption spectra of SDS (sodium dodecyl sulfate) stabilized AuNPs by thermolysis of Au(Me₂S)Cl. [**Blue line**: 0.33 mmoles of Au(I); **Red line**: 1.3 mmoles of Au(I)] B) Respective FE-SEM images of AuNPs on Si (silicon) surface after drop cast deposition and washing with millipore water.
Figure 4.66 XPS (X-ray photoelectron spectroscopy) data during purging treatment with atomic ammonia and molecular ammonia plasma on Si supported AuNPs (SEM images of sample shown in figure 4.62). (Conducted in collaboration with Dr. J. Kelber, data collected by Sneha Sen in chemistry)

with immense interest in AuNPs for catalysis preliminary studies were conducted first to deposit AuNPs on to acidic Si surfaces by simple drop-wash deposition method compared to sophisticated techniques in literature (co-precipitation, co-sputtering, deposition-precipitation and gas-phase grafting).
Initial results have also shown presence of dispersed AuNPs on Si surface both from SEM and XPS data (figure 4.65 & 4.66). Au 4f binding energy is expected at 84.0 and 88.0 ev which is perfectly seen from XPS data for AuNPs deposited on SiO$_2$ surface. This data indicates not only purity of AuNPs deposited on SiO$_2$ surface but also ability of AuNPs to be deposited on to Si wafer surface by simple drop cast method which encourages for further investigations to evaluate catalytic ability of AuNPs on Si surfaces.

4.4 Conclusions

Thermosensitive hydrogels based on PNIPAM and different polymers are efficiently utilized in making different size, shape and SPR tunable AuNPs as a environmentally and biologically benign route. Size and SPR tunability was achieved very easily controlling different experimental parameters like precursors concentration, pH of the medium and experimental condition. Except for few clues on optimizing experimental parameters for making NIR absorbing anisotropic AuNPs stabilization mechanism is not understood in detail. Therefore, data compiled in this chapter would definitely encourage for further research on understanding mechanism of formation and thereby controlling shapes of anisotropic particles within wide benign polymers and gels which can subdue toxicity concerns. Usage of Au(I) precursors obviate usage of any sort of excess reducing agents either during syntheses of spherical or anisotropic particles which is very favorable from green chemistry aspect. Demonstrating reproducibility in syntheses, viability from both in vitro and in vivo studies projects gold nanoparticle samples synthesized from discovered protocol in this chapter as strong alternative for existing AuNPs in the market.
4.5 References


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5.1 Introduction

Similar to AuNPs work detailed in chapter 4, silver nanoparticles (AgNPs) are also extensively studied plasmonics for their potential applications and scientific interests.\textsuperscript{1,2} Silver nanoparticles are shown to be synthesized through various physical and chemical methods,\textsuperscript{3,4} colloidal processes of surfactant-stabilization in liquid phase remains highly practiced method for its simplicity and easiness for making different sizes and shapes of nanosilver. With some exceptions, most of the chemical synthetic approaches rely on the reduction of water-soluble silver salt precursors, by common reducing agents like boron hydrides, citrates or alcohols.\textsuperscript{5} In contrast to AuNPs, photochemical approach is popular method due to light sensitive property of silver salts. However, for perfect control of size and shape usage of environmentally or biologically hazardous reducing agents or solvents is unavoidable.\textsuperscript{3,4} Increasing interest for minimize usage of toxic chemicals to adopt green chemistry principles encourages new syntheses methods that involve complete absence of reducing agents like borohydrides, hydrazine and organic solvents like dimethyl formamide or other organic compounds associated with environmental or biological toxicity.\textsuperscript{4,5} As presumed by many, important goal of bio-nanotechnology would be stabilization of stimuli sensitive nanostructures under most biologically benign conditions with improved environmental and cell toxicity.\textsuperscript{3,4,5} As discussed in chapter 4, for metallic nanoparticles designed for biological or biomedical
applications, surface chemistry that governs interactions with biological constituents or environment is influenced by stabilizing or capping agents. Investigations on surface modification of metallic nanoparticles with biological polymers showed much improved cytotoxicity and differential targeting of cells. Recent reviews on prospective applications and environmental concerns of AgNPs have summarized their broad usage ranging from consumer products to disinfecting medical devices and home appliances. Apart from it, silver nanoparticles are also widely studied for their superior catalytic activity, surface enhanced Raman spectroscopy (SERS) and for metal enhanced fluorescence (MEF) applications. SERS and MEF considered as powerful tools for high-throughput screening, immunoassays and macromolecular detection dictated by size and SPR of AgNPs. In medical fields, emergence of antibiotic-resistant bacteria has attracted strong attention of silver nanoparticles possessing broad anti-inflammatory, antifungal, antimicrobial and wound healing properties. Because of AgNPs capability to interact with more than one type of cell constituents has gained much prominence in antibiotics research. Release of silver ions that interacts with bacterial mitochondria, DNA or large surface area of nanosilver was associated with mechanism of action of silver nanoparticles against microorganisms. Though silver ions (Ag⁺) at high concentrations are hypothesized to possess side effects in human (argyria: Blue or blue-grey coloration of skin due to extended exposure of silver dust/silver compounds) compared to AgNPs, extensive research is underway to address ambiguities concerned to concentration dependent toxicity arising from AgNPs or Ag⁺ ions.
Like AuNPs, surface plasmon resonance of anisotropic AgNPs can be extended into NIR region (>700 nm), resulting in very significant applications related to high-efficiency solar cells, infrared photo-detectors, heat absorbing optical coatings, sensors and for biomedical fields.\textsuperscript{11,12,13,14} In medical arena, photothermal therapy, photo-acoustic imaging and image guided therapies\textsuperscript{15,16,17,18} have received considerable attention due to development of strong NIR absorbing plasmonic nanoparticles. Minimum absorbance tendency from native tissue in the “water-window” region (800 nm-1300 nm),\textsuperscript{17} has made these NIR absorbing noble metal nanostructures suitable candidates for deep tissue imaging or image guided therapies as discussed in chapter 4. In-spite of huge activity and biomedical applications from AgNPs, it was found; only less than 24% of reported methods completely rely on environmentally friendly techniques for producing less than 20nm size particles which are technically evaluated to be most effective for variety of applications. Seldom, generation of smaller particles using green syntheses techniques is associated to difficulties in controlling size and distribution.\textsuperscript{19} This emphasizes any new syntheses protocol that can make monodispersed, positively and negatively surface charged spherical AgNPs smaller than 20 nm by complete environmentally and biologically benign methods is always desirable.

Compared to different synthetic approaches for making size, shape and SPR tunable AgNPs, photochemical approach remains most exploited and efficient technique due to many advantages over conventional chemical reduction techniques.\textsuperscript{20} Considerable interest shown for light induced formation of size, shape tunable silver nanostructures is for its intriguing mechanism and advantages.\textsuperscript{21} Light sensitive nature
of silver salts used in conjunction with minor variations in light sources, optical band pass filters, excitation wavelengths and pH obtain size and shape tunable silver nanostructures.\textsuperscript{21,22,23,24,25,26,27} Though many research groups have demonstrated benign protocols by employing natural polymers like cellulose or chitosan but were restricted to formation of nanospheres only.\textsuperscript{28,29,30,31,32,33,34} However, these relatively inexpensive and highly stable biopolymers have not been exploited for making anisotropic silver nanoparticles unlike using CTAB (cetyltrimethylammonium bromide) or PVP (poly(vinyl pyrolidone)) or BSPP (Bis(p-sulfonatophenyl) phenylphosphine dehydrate dipotassium salt) stabilizers. To the best of our knowledge all the photo-induced techniques for preparation of NIR sensitive AgNPs reported in literature involve seed-mediated growth protocols starting with NaBH\textsubscript{4} reduced spherical seed particles (5 nm- 10nm) grown in to anisotropic nanostructures stabilized within soft templates like, BSPP, CTAB,\textsuperscript{21,24} PVP,\textsuperscript{21} or sodium dodecylsulfonate.\textsuperscript{25,26} 

Natural polymers like chitosan with special biological and chemical properties are abundant in biomass and easily accessible. Chitosan a derivative of most abundant positively charged polysaccharide chitin is a linear polymer with N-acetyl glucosamine repeating residues.\textsuperscript{35} Unique polycationic, chelating and film forming abilities of chitosan are due to presence of abundant active amino and hydroxyl functional groups.\textsuperscript{35b} CS itself exhibits numerous interesting biological activities generating wide usage in many different fields like medicine, food, pharmaceuticals, nutrition and agriculture.\textsuperscript{35b} It has also been shown to stabilize metallic nanostructures efficiently because of its chelating property.\textsuperscript{35} To overcome environmental concerns and to take complete advantages of
NIR sensitive silver nanostructures, we have designed an efficient simple route amalgamating advantages of light driven technique and stabilizing features of chitosan polymer. Syntheses technique described in this chapter relies on combined factors of light sensitivity of silver nitrate along with pH sensitivity of chitosan polymer. In contrary to existing seed mediated growth techniques, a simple single step solution phase photochemical method for forming NIR sensitive anisotropic silver nanostructures in complete absence of any known reducing agent is described in detail. In-spite of numerous syntheses techniques detailed for preparation of shape and size tunable AgNPs but studies concerned to employing them for antimicrobial studies are mostly limited to using spherical particles only with very few exceptions. Smaller particles are mostly associated with higher antibacterial activity whereas larger (>30 nm) size particles are shown to be exhibit low cytotoxicity in different cell lines. So relation between bacterial activity and cytotoxicity with respective to size or shape of AgNPs is still un-resolved. For the first time anti-pathogenic properties of NIR absorbing AgNPs is demonstrated in this chapter, which should further enhance applications of these NIR sensitive anisotropic AgNPs.

Within the scope of this chapter, synthesis of fluorescent silver nanoparticles has been attempted using famous fluorophore quinine sulfate (QS) as stabilizer. Among metal nanoparticles, fluorescent nanoparticles are of particular interest for sensing and single molecule detection. However, research on fluorescent metal nanoparticles is mostly concentrated on semiconductor based nanoparticles commonly called as “quantum dots”. Though emission from these core-shell or core only semiconductor NPs
can be tuned across visible spectrum but suffer from serious problems related to intrinsic blinking, photobleaching and toxicity that limits their applications to health sciences.\textsuperscript{38} Employing metal enhanced fluorescence (MEF) techniques (emission enhancement from fluorophores when placed in close proximity with plasmonic nanoparticles) some of these drawbacks are shown to overcome due to enhancement of fluorophore emission through localizing a dipole fluorophore in the electric field near a metal nanoparticle.\textsuperscript{38a,c} However achieving MEF in solutions is extremely tricky due to possibility of emission quenching depending on distance between fluorophore and metal nanoparticle. Therefore, studies are in progress to understand MEF in solutions by conjugating dyes to metal nanoparticles that would control distance between nanoparticles and fluorophores.\textsuperscript{38a,c} In contrast to above techniques very small metal nanoclusters (Ag\textsubscript{2}, Ag\textsubscript{3}, Ag\textsubscript{4}) are shown to exhibit well characterized fluorescence with molecular type properties (relative long lifetimes, mirror image relationship between absorbance and emission).\textsuperscript{38b} Dickson and co-workers have reported photoluminescence (PL) from small silver nanoclusters photogenerated by UV-irradiation of silver oxide films. Same group synthesized gold and silver nanodots with size dependent photoluminescence using dendrimers and DNA as templates. In such cases, silver ions are reduced using a reducing agent (NaBH\textsubscript{4}). Later Kumacheva group\textsuperscript{38d} has reported photogeneration of fluorescent silver nanoparticles within PNIPAM-AA-HEA (poly(NIPAM-acrylicacid-2-hydroxy ethylacrylate) microgels. Recently Scalano and co-workers have reported ketly radical stabilized fluorescent silver nanoclusters.\textsuperscript{38b} In-spite of considerable works, strong tendency to aggregate and usage of NaBH\textsubscript{4} remains as
major drawback with these small silver clusters for practical applications. In health sciences quinine is famous for being a naturally occurring alkaloid having antipyretic (fever-reducing), antimalarial, analgesic (pain killing) and anti-inflammatory properties.

Quinine is available with a prescription in the United States and over the counter, and present in very small quantities in tonic water (Wikipedia). The doubly charged cation derived from acidified quinine sulfate solutions is popular standard for measuring quantum yield because of its photostability and immunity to oxygen quenching.\textsuperscript{37a}

In view of above reported works in literature we adopted a simple protocol where a well-known naturally occurring biologically benign fluorophore (QS) is used to stabilize and impart fluorescence to silver nanoparticles in complete absence of any reducing agents. This method not only simplifies procedure for making luminescent nanoparticles but biological significance of quinine would be an added advantage. For any practical applications of metal nanoparticles, stabilizers remain intact to metal core, so we assume fluorescence properties of quinine sulfate stabilizer would enhance applications of AgNPs.

5.2 Experimental Section
5.2.1 Materials

Silver nitrate (AgNO\textsubscript{3}), chitosan (medium molecular weight 85% deacetylated), polyacrylic acid (25000 Mw), ammonium hydroxide (NH\textsubscript{4}OH), acetic acid (CH\textsubscript{3}COOH) and quinine sulfate were brought from Sigma-Aldrich and Polysciences. Used as received without further purification. Water was distilled and subsequently purified to Millipore Milli-Q quality (18.2 MΩ-cm) for all dilution purposes. All glassware used was
cleaned in a bath of freshly prepared aquaregia solution (HCl/HNO₃, 3:1), then rinsed thoroughly with H₂O before use. Medium pressure, mercury-vapor lamp (Ace Glass) lodged in to photochemistry box with facilities for stirring and temperature control was used. Of total energy radiated from Hg lamp, approximately 40-48% is in the ultraviolet portion of the spectrum, 40-43% in the visible, the balance in the infrared region.

5.2.2 Physical Measurements

Please refer to sections 2.2.2 in chapter 2 for electronic spectroscopy details, 3.2.3 for electron microscopy details and 4.2.2 for ICP-MS details. OD (optical density) during bacterial growth was recorded using spectrophotometer (BioMate3) from Thermo Electron Corporation USA. Zeta potential of specific samples was analyzed using zeta nanosizer (Malvern Instruments).

5.2.3 Syntheses of Size Tunable Spherical Silver Nanoparticles within Chitosan (CS) and Polyacrylic acid (PAA) Polymers

In a typical syntheses protocol, to a 5 ml chitosan solution, required volume of 0.1M ammonium hydroxide added drop wise with continuous stirring to raise pH of freshly prepared chitosan solution between 7-8. Now, 1 ml of 0.005 mol.L⁻¹ silver nitrate solutions is added followed by vortexing for few minutes until solution becomes homogenous. This freshly prepared colorless solution is transferred in to ES-quartz (ESQ) glass or orthoborosilicate (OBS) glass and then irradiated using a 450W medium-pressure immersion Hg lamp maintained at ambient temperature. Different sizes of nanospheres are produced by changing concentrations of chitosan polymer and silver nitrate. Visible color of reaction solutions changes from colorless to pale yellow or dark
yellow based on concentrations of chitosan or/and silver nitrate. Exactly similar procedure is followed for making spherical silver nanoparticles stabilized in negatively charged polymer PAA (polyacrylic acid). For calculating photochemistry quantum yield, time dependent absorption spectra for different concentration samples is collected and analyzed. In order to understand long-term effect of agglomeration and stability, samples stored under ambient condition are tested after 3 days for any changes in absorption spectra.

5.2.4 Syntheses of Anisotropic NIR Absorbing Silver Nanoparticles Stabilized in Chitosan Polymer

In a typical procedure, 0.1M acetic acid is added to >0.1wt% chitosan polymer solution until pH of solution reaches 3.0 followed by addition of 25-50 mM silver nitrate solution at constant stirring. Later freshly prepared colorless solution is transferred in to regular borosilicate glass followed by irradiation under Hg lamp maintaining the temperature between 0 °C to 5 °C using an ice bath. The colorless solution was observed to change initially to pink color and then to dark green color indicating completion of reaction.

5.2.5 Syntheses of Spherical and Anisotropic AgNPs Doped Chitosan Films

In a simple procedure, AgNP solutions were drop casted on a clean glass slide and allowed to dry at room temperature. Slow evaporation of solution results in formation of a thin film of silver nanoparticles.
5.2.6 In Situ Formation of Nanosilver Doped Chitosan Gels

Minimum 0.5 wt% of chitosan and 25 mM of Ag\(^+\) concentration is required for formation of AgNPs doped chitosan gels. Simple mixing of polymer and silver nitrate at various concentrations results in gels of varied viscosity. Initially colorless solution becomes viscous immediately after addition of silver salt followed by change in color of gel on exposure to light. Stability of these gels is controlled by varying concentrations of chitosan and silver nitrate.

5.2.7 Bacterial Pathogens and Biological Induction of SAR (Structure Activity Relationship)

Pseudomonas syringae pv. maculicola ES4326 and P. syringae pv. tomato DC3000 with and without the avrRpt2 avirulence gene were propagated at 28°C on King’s B medium containing appropriate antibiotics.\(^{39}\) Pathogen P. syringae pv. tomato DC3000 avrRpt2 is an avirulent pathogen which interacts with resistance gene present in plants and induce defense by up regulating expression of defense related genes while Pseudomonas syringae pv maculicola ES4326 (Psm) is a virulent pathogen which take over the host machinery and normally causes disease in plants. Because of this pathogen is used to check the disease severity in case plants if plants develop resistance.

5.2.8 In Vitro Antimicrobial Efficacy of Silver Nanoparticles

The antimicrobial activities of chemically synthesized silver nanoparticles of different sizes were monitored by measuring the changes in OD (optical density) at fixed OD\(_{600nm}\) of bacteria in 10 ml of liquid King’s B medium. Bacterial cultures are inoculated with fixed quantity of silver nanoparticle representing different stages of
purity along with their respective control and appropriate antibiotics. Bacterial cultures were propagated at 28 °C in an oscillating water bath and the OD$_{600nm}$ recorded at 3hr interval over a 24-hour period. Inhibition of bacterial growth in presence of different types of silver nanoparticles along with respective controls were monitored by monitoring decrease in OD$_{600nm}$ that is true reflection of growth of culture.

5.2.9 Syntheses of Quinine Sulfate Stabilized Fluorescent Silver Nanoparticles

Different concentrations of quinine sulfate ranging between $10^{-5}$ M to $10^{-7}$ M are used. pH of quinine sulfate solution was altered to acidic or alkaline by addition of few drops of 0.1N H$_2$SO$_4$ or 0.1N NH$_4$OH. Silver nanoparticles are produced by simple photolysis of various ratios of AgNO$_3$:QS solutions at room temperature. Colorless quinine sulfate solution turns yellow with time indicates formation of silver nanoparticles.
Figure 5.1  
A) Represents absorption spectrum for two different AgNPs samples (b': 0.5 wt%; 2.5 mM AgNO₃, d': 0.5 wt%; 10 mM AgNO₃) prepared using OBS at pH 8.0 (inset Shows difference in transmittance spectra for ES-Quartz (ESQ) and Orthoborosilicate (OBS) glass materials). B) Shows TEM images for corresponding b' and d' samples.
5.3 Results

5.3.1 Formation of Spherical Silver Nanoparticles

In our syntheses protocol, AgNO₃ subjected to photolysis using medium pressure Hg lamp as light source undergoes reduction from Ag(I) to Ag(0). Affinity of chitosan polymer towards metal ions was known for longtime and being used for stabilizing various metal nanoparticles.²⁸,²⁹ Two different types of common glass materials Orthoboro silicate glass (OBS) and ES-Quartz (ESQ) with recognizable difference in transmittance (figure 5.1) were used during irradiation under alkaline pH (7-8). Though samples irradiated using both glass (ESQ and OBS) materials indicated formation of colloidal silver but a strong recognizable difference is noticed in absorption spectra and TEM images. Detailed mechanism of complex cation [Ag(NH₃)₂]⁺ reduction in alkaline pH by UV irradiation and stabilization of silver nanoparticles by chitosan polysaccharide was already explained in detail elsewhere⁴⁰. Influence of glass material, reaction conditions and effect of precursor concentrations during formation of spherical and anisotropic AgNPs is discussed in following sections.

5.3.1.1 Influence of Glass Material on Formation of Spherical AgNPs

Optical absorption spectroscopy is one of the most powerful, simple techniques for structural characterization of plasmonic nanoparticles. This technique has been extensively utilized as primary characterization tool as noticed even in chapter 4. Samples containing same ratio of chitosan polymer to silver nitrate were irradiated separately in different glass materials (OBS and ESQ) maintaining other parameters like temperature, exposure time and pH constant. Figure 5.1 shows optical absorption
spectrum profile for these samples. Absorption shoulder noticed at \( \sim 280-290 \) nm from initial homogenous mixture of chitosan polymer and silver nitrate is due to Ag-H\(_2\)O or Ag-NH\(_3\)\(^+\) complex as described in literature.\(^{40c}\) On irradiation with time, progression of absorption peak around 400 nm due to localized surface plasmons of spherical silver nanoparticles is observed under both conditions of exposure but with significant difference in base line and peak distribution. Sample irradiated using OBS glass has higher baseline accompanied with broader peak indicating decomposition and aggregation behavior of colloidal particles in to metallic silver. Whereas sample irradiated using ESQ glass exhibited narrow absorption spectrum with peak maximum centered on \( \sim 405 \) nm indicating complete reduction and formation of stable silver nanoparticles in solution. Sensitivity of SPR to nature of solvent, stabilizing agent, dielectric constant or factors related to size and aggregation is explained in literature.\(^{40}\) Therefore, difference in absorption spectra noticed from two samples could only be due to variation in size or aggregation of nanosilver as all other parameters in both the samples are same. When tested at higher concentrations of silver nitrate, sample irradiated using ESQ exhibited red shift in SPR peak from 405 to 440 nm as expected either due to increase in individual size of silver nanospheres or due to larger silver aggregates in solution. However, sample irradiated using OBS glass show more decomposition tendency with steeper increase in absorbance baseline. These results suggest (figure 5.1) formation and stability of silver nanoparticles at alkaline pH is sensitive to nature of glass material. We assume this is due to difference in energy/intensity of light interacting during irradiation depending on nature of glass
material employed. On testing light transmittance of these two glass materials, (inset graph in figure 5.1) exhibit difference in light transmittance between ESQ and OBS glass materials in the ultra-violet region (~280nm-300nm) of the spectrum. Higher percentage of transmission especially in the UV region for ESQ favor stronger interactions with UV light results in complete controlled reduction of Ag\(^+\) salt. Producing stable and narrow dispersed silver nanoparticles compared to sample irradiated using OBS glass where incomplete reduction result in agglomerated micron size silver films or freckles. Mechanism of formation and stabilization of AgNPs in chitosan or in similar matrix was explained\(^{40}\) either based on binding or chelating property of chitosan or due to formation of NH\(_2\) radicals and silver atoms on UV irradiation. Our assumption about aggregation or incomplete reduction of silver salt while using OBS glass is strongly supported from both optical absorption data and HR-TEM images. In figure 5.1B, TEM images corresponding to sample made by using OBS glass material show freckles of silver plates larger than one-micron size corresponding to higher baseline in absorption spectrum due to decomposition of Ag\(^+\) salt in to metallic film or silver aggregates. This is in good agreement with our assumption about intensity/energy of light interacting with sample playing determinant role for stabilizing silver colloids making glass material itself as perfect optical filter to control photochemical reactions.

Since report on photochemical transformation of borohydride reduced silver nanoparticles in to nanoprism by Mirkin and co-workers\(^{21}\) many research groups have exploited light sensitive nature of silver salts by examining role of light in photochemical conversions. Minor modifications based on nature of incident light like excitation
wavelength, power density, light source along with pH of solution were examined for tuning\textsuperscript{21,22,23} size, shape and LSPR of silver nanoparticles. For example, Mirkin group obtained silver nanoprisms with tailorable optical absorbance in NIR region by controlling pH and irradiation source.\textsuperscript{21} Zheng et al described growth of nanodecahedra structures using blue light emitting diode laser (LED) as excitation source,\textsuperscript{23} whereas Calegari et al have shown tuning of borohydride reduced spherical particles in to different sizes and shapes employing single source fluorescent bulb (350 nm < \(\lambda\) < 700 nm) utilizing specially designed optical filters.\textsuperscript{21,23,41} On the other side Bogle et al,\textsuperscript{42} have shown formation of 10-60 nm silver nanospheres varying strength of electron irradiation, whereas Yoksan et al, produced different size particles by varying \(\gamma\)-ray dosage.\textsuperscript{33} Nevertheless, to the best of our knowledge, influence of commonly used glass material on formation and stability of AgNPs was not investigated in such detail. These results emphasize capacity and significance of glass material itself to act as potential optical filters during photochemical conversion, which would be cost effective, compared to special designed filters or light sources.

5.3.1.2 Tuning Size of Spherical AgNPs in Chitosan and Polyacrylic Acid Polymers

At alkaline pH, owing to instability and decomposition tendency of samples prepared using regular OBS glass, ESQ was preferred for tuning size of nanospheres. Tuning size anywhere between 5 nm to 50 nm was achieved by adjusting ratio of chitosan polymer to AgNO\textsubscript{3} concentration. From figure 5.2, evolution of different visible colors accounts for shift in SPR peaks where each color or peak maximum would
indicate specific size or distribution though not definitive. When concentration of chitosan polymer is maintained constant, if AgNO₃ concentration is increased

Figure 5.2 I) Represents visible colors for different AgNPs samples prepared under various concentration ratios (A: AgNO₃ solution, B: 0.5 CS wt% ;2.5 mM AgNO₃, C: 0.1 CS wt% ;2.5 mM AgNO₃, D: 0.5 CS wt% ;10 mM AgNO₃, E: 0.1 CS wt% ;10 mM AgNO₃) prepared using ESQ glass at pH 8.0. II) Shows absorption spectra for same samples (inset shows time dependent absorption spectra for a typical AgNPs sample during irradiation).
Figure 5.3  A) Represents absorption spectra of AgNPs made using 0.5 CS wt%; 2.5 mM AgNO₃. B) HR-TEM TEM images. C) Particle distribution from TEM images. D) Photophysical parameters (inset shows morphology of small particles).
Figure 5.4 A) Represents absorption spectra of AgNPs made using 0.5 CS wt% ; 10 mM AgNO₃. B) TEM TEM images. C) Morphology of individual AgNPs. D) Particle distribution from TEM images. E) Photophysical parameters.
or if concentration of silver salt is maintained constant with decrease in chitosan polymer concentration there is clear red shift along with increase in broadness (FWHM=full-width half-max) and baseline of plasmon peaks (figure 5.2). Increase in size of individual nanoparticles or aggregation of nanoparticles can result in red shift or increasing broadness of plasmon peaks. A definitive correlation and clear understanding about exact size and distribution is attained from HR-TEM images of two different samples containing different ratios of silver salt to chitosan polymer concentration.
Comparing TEM images (figure 5.3 and 5.4) for samples B & D in figure 5.2, red shift and increased FWHM of absorption spectra for sample D compared to sample B is convinced not due to aggregation but due to increase in size of individual particles. Figure 5.3 represents highly monodispersed spherical silver nanoparticles with average size 8.8 nm ± 1.1 nm correlating with sharp SPR peak maximum at 400 nm. On increasing concentration of silver nitrate alone, particles with average size 48 ± 5.6 nm are obtained which correspond to red-shifted 430 nm plasmon peak (figure 5.4). Standard deviation for larger size particles is comparatively higher, reflecting higher FWHM of absorption curve for sample D compared to sample B. However, TEM images (figure 5.3, 5.4) from both these samples confirm spherical morphology. Almost spherical shapes of different size particles are in good agreement with evolution of single plasmon peak during irradiation. Transformation in shape during photochemical process has been shown to result in more than one plasmon peak brought by change in symmetry of nanostructures.21

A representative time dependent absorption spectrum during irradiation for one of these sample solutions is shown in inset figure 5.2. It shows no drastic changes during growth of surface plasmon peak except for increase in absorbance of the peak. According to Mie theory, only a single SPR band is expected in absorption spectra for spherical nanoparticles.1,21,43 This emphasizes formation of only spherical particles from absorption spectrum alone. Convincingly HR-TEM images also strongly support the argument. Broad absorption profile and elevated baseline for sample E (figure 5.2) reveal aggregation/polydispersity, clarified from TEM images (figure 5.5). Clustering of
particles with wide variation in size for sample E is due to insufficient concentration of chitosan for controlled stabilization. Optical absorption data and electron microscopy images from different samples are in good correlation to confirm certain concentration of chitosan polymer to silver salt is necessary for formation of small size, highly monodispersed silver nanospheres. Moreover, beyond this specific concentration, plasmon red shift is noticed either due to increase in size of individual particles or due to aggregation of nanosilver. These results are in good agreement with literature where size of silver nanospheres produced by chemical or photoinduced methods are completely dependent on concentration of both \( \text{Ag}^+ \) and stabilizing agent where a lower concentration of reducing agent or weaker reducing agents have always shown to produce larger size or less dispersed particles and vice-versa.\(^3\)

Stability and capping nature of chitosan matrix has been comprehended by examining dried samples after one week of deposition on TEM grid. Amorphous peel structures with weaker contrast noticed around high contrast silver nanospheres (figure 5.4) not only demonstrate passivation or capping of AgNPs by chitosan polymer but also shows long term stability of nanospheres. Monodispersed particles less than 10 nm are immediate necessity for many applications including catalysis, SERS and MEF. At the other extreme larger size, particles are more favored as they are shown to possess lower cytotoxicity compared to smaller size spherical particles\(^{36}\) making the proposed protocol feasible for producing AgNPs for both purposes.

Zeta potential reveal surface charge of colloidal particles in solution and helps to determine stability of the same. As expected from using chitosan polymer, silver
colloidal solutions represented in figure 5.3 and 5.4 after dialysis against millipore water exhibited +41.5±4.4 mv and +35.5±5.6 mv respectively. 6.3 pH of samples after dialysis, indicate good stability at physiological pH range. It is well known that most of the silver colloids produced by popular methods in literature are negatively charged due to involvement of sodium citrate or ascorbic acid as reducing or stabilizing agents. In view of significance associated to positively charged silver colloidal particles for detecting negatively charged analytes and also due to higher activity reported in case of bacillus bacteria, we assume this would be simple and effective protocol to make different size positively charged AgNPs useful for both biological and materials applications.

In order to demonstrate versatility of our syntheses protocol, we employed poly(acrylic acid) a negatively charged polymer as stabilizer. Figure 5.6, shows absorption spectrum along with HR-TEM images for silver nanospheres synthesized by irradiating homogenized solution of PAA-silver nitrate using ESQ under both acid (3.0) and alkaline pH (8.0). At similar concentrations of polymer and Ag⁺, though quantum yield and rate of photochemical reaction are very similar (φ=0.104 at pH 3.0; φ= 0.105 at pH 5.0) but the difference in absorption mean peak position and distribution reveal effect of pH. PAA as stabilizer also highlights significance of alkaline medium for controlled reduction of Ag⁺, narrow plasmon peak and narrow distribution of particle size at alkaline pH compared to acidic pH. As noticed from HR-TEM images (figure 5.6 A&B) even using PAA polymer, stable silver nanospheres smaller than 10 nm are easily formed along with some very small particles of sizes 2-3 nm. At alkaline pH narrow
absorption spectra with peak maximum at 401 nm contain particles with average size of 4.7±1.7 nm exhibiting high monodispersity where as comparatively broad absorption spectra is corroborated with average size of 9.2±4 nm at acidic pH. Zeta potential of PAA stabilized silver nanospheres as expected show negative surface charge, which imply role of polymer stabilizers in inducing surface charge to AgNPs synthesized under
similar conditions. Measured zeta potential values are -44 ± 4.9 mv and -40 ± 4.2 mv for samples made under acidic and alkaline pH respectively after dialysis. This simple study clearly confirms formation and tunability of spherical AgNPs using both negatively and positively charged polymers for broader applications. Comparing photochemical quantum yields (table 5.1) and from visual observation during irradiation, negatively charged PAA polymer possess better efficiency compared to positively charged chitosan polymer for stabilization of silver nanospheres.

Table 5.1 Summary of photochemical quantum yield (PQY) values for photolysis reactions during formation of AgNPs within chitosan and PAA polymers using different wt% of polymers and molar concentrations of AgNO₃ salt. (Dr. El-bjeirami assisted in analyzing the data)

<table>
<thead>
<tr>
<th>Polymer (stabilizer)</th>
<th>pH</th>
<th>Polymer concentration (wt/v) %</th>
<th>AgNO₃ (concentration mM)</th>
<th>QY (λnm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>8.0</td>
<td>0.1</td>
<td>5</td>
<td>0.051 (420)</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.05</td>
<td>5</td>
<td>0.032 (450)</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.1</td>
<td>2.5</td>
<td>0.021 (420)</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.05</td>
<td>2.5</td>
<td>0.015 (420)</td>
</tr>
<tr>
<td>PAA</td>
<td>3.0</td>
<td>0.1</td>
<td>5</td>
<td>0.104 (455)</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.1</td>
<td>5</td>
<td>0.105 (405)</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>0.1</td>
<td>2</td>
<td>0.037 (425)</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.1</td>
<td>2</td>
<td>0.046 (400)</td>
</tr>
</tbody>
</table>
Figure 5.7  Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized with 0.1 wt% CS, pH 8.0; 5 mM [Ag]. A: Represents time dependent electronic absorbance changes during photolysis. B) Absorbance vs Time curve for QY calculation at 420 nm (QY: 0.051; R² = 0.994).
Figure 5.8 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized with 0.05 wt% CS, pH 8.0; 5 mM [Ag]. A: Represents time dependent absorbance changes during photolysis. B) Absorbance vs Time curve for QY calculation at 450 nm (QY: 0.032; R² = 0.99).
Figure 5.9  Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized with 0.1 wt% CS, pH 8.0; 2.5 mM [Ag]. A: Represents time dependent absorbance changes during photolysis. B) Absorbance vs Time curve for QY calculation at 420 nm (QY: 0.021; $R^2 = 0.962$).
Figure 5.10 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized with 0.05 wt% CS, pH 8.0; 2.5 mM [Ag] A: Represents time dependent absorbance changes during photolysis. B) Absorbance vs Time curve for QY calculation at 420 nm (QY: 0.015; $R^2 = 0.99$).
Figure 5.11 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized within PAA at pH 3.0; 5 mM [Ag]. A) Represents time dependent absorbance changes during photolysis. B) Absorbance vs Time curve for QY calculation at 455 nm (QY: 0.104; $R^2 = 0.987$).
Figure 5.12 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized within PAA at pH 8.0; 5mM [Ag]. A: Time dependent absorbance changes during photolysis. B) Absorbance vs Time for QY calculation at 405 nm (QY: 0.105; R² = 0.979).
Figure 5.13 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized within PAA at pH 3.0; 2mM [Ag]. A: Time dependent absorbance changes during photolysis. B) Absorbance vs Time for QY calculation at 425 nm (QY: 0.037; $R^2 = 0.99$).
Figure 5.14 Determination of photochemical quantum yield (QY) for silver nanoparticles stabilized within PAA at pH 8.0; 2mM [Ag]. A: Time dependent absorbance changes during photolysis. B) Absorbance vs Time for QY calculation at 400 nm (QY: 0.046; $R^2 = 0.974$).
Detailed time dependent absorption spectra and photochemical quantum yield calculations for both chitosan and PAA stabilized AgNPs shown from figure 5.7 to 5.14 respectively. PQY values (table 5.1) explain why negatively charged molecules/stabilizers like trisodium citrate are prevalent for formation of silver nanospheres compared to any positively charged stabilizer.

Figure 5.15 Represents changes in optical absorbance of different AgNPs solutions on day one and day three. (Day 1: Solid black line; Day 3: Black scatterer)  A: 0.5 CS wt%; 2.5 mM AgNO₃, B: 0.3 CS wt%; 2.5 mM AgNO₃, C: 0.5 CS wt%; 5 mM AgNO₃, D: 0.5 CS wt%; 10 mM AgNO₃.
5.3.1.3 Testing Stability of Colloidal Silver Nanoparticle Solutions

Due to light sensitive nature of Ag\(^+\) salts, it is highly recommended to test stability of AgNPs in solution with respective to time. Looking at PQY results it can be understood that not all the initial silver salt is converted in to AgNPs, so there would be some unreacted silver ions in solution that can exhibit continuous activity. To confirm stability of solutions, changes in absorption spectra are monitored with respective to time for the same samples stored under ambient conditions. Figure 5.15 shows changes in optical absorption spectra of randomly selected AgNP solutions monitored on day one and day three. Except for one sample, other samples did not exhibit much variation in absorption spectra that indicates stability of AgNPs.

5.3.2 Formation of Anisotropic Silver Nanostructures Stabilized in Chitosan Polymer

After understanding, translucent effect of pH and glass material on formation of silver nanospheres, a reverse combinatorial effect of pH and glass material has been investigated in detail to see effect of acidic pH (<3.0) in presence of OBS glass on AgNPs formation and stability. Lowering pH of chitosan solution to <3.0 by addition of acetic acid makes chitosan polymer positively charged with plenty of protonated amine groups (-NH\(_3^+\)). Positive surface charge of chitosan polymer is confirmed from zeta-potential measurements (\(\zeta=+65 \pm 5.4 \) mv). Homogenous solution of chitosan polymer and Ag\(^+\) is irradiated by maintaining temperature <5 \(^{\circ}\)C. In few minutes, colorless solution turns in to pink color in contrast with nanosphere samples (figure 5.16). Completion of reaction is indicated, solution changing from pink to dark green color at the end of reaction. Unfolding of broad plasmon absorption peak centered on \(~450-500\)
nm is seen within few minutes of irradiation which undergoes distinguishable red shift with time anywhere between 700 nm-1200 nm dictated by nature of light interactions, concentrations of silver nitrate and chitosan polymer (figure 5.16). Evolution and transformations in absorption spectrum and changes in visible color of samples during irradiation confirm growth of anisotropic structures as more SPR peaks

Figure 5.16 Shows changes in absorption spectra with irradiation time for NIR absorbing AgNPs synthesized using chitosan at pH 3.0 and orthoborosilicate glass combination. I) A= 1wt% CS; 10 mM [Ag]; B= 1wt% CS; 20 mM [Ag]. II) Visible colors of same samples.
are always assigned to decrease in symmetry of nanoparticles. Below certain concentration (> 0.1 wt%) of chitosan polymer or silver nitrate (> 10 mM) strong and clear NIR absorbing samples are not formed. In order to understand amalgamating effect of Ag⁺ and chitosan concentration in combination with effect of irradiation on growth of NIR plasmon absorption peaks, different combinations are studied separately. Primarily two solutions containing varied amounts of Ag⁺ and same concentration of chitosan polymer is irradiated to evaluate effect of Ag⁺ concentration (figure 5.16).

However, both samples exhibited similar growth pattern but distinguishable difference in absorption wavelength and peak position indicate significance of silver concentration. After 15-20 minutes of irradiation, a broad peak centering on 500 nm-550 nm is evolved from sample containing lower concentration of Ag⁺ eventually transforming in to distinguishable peaks centering on ~380 nm, 500 nm, and 745 nm. Whereas sample containing higher concentration of silver (figure 5.16 A) for similar exposure time, start with a clear differentiated absorption spectrum with peak maximums centering on 380 nm, 490 nm and 650 nm undergo greater red shift to ~848 nm (figure 5.16 B). Optical absorption data is substantiated by comparing TEM images of same samples. Intermediated irradiated sample containing lower silver content (5.17 A) shows particles with average sizes larger than 65 nm, closer examination reveals that individual particles show anisotropic morphology away from spherical shape. These electron microscopy images comply with broad 500 nm-550 nm plasmon peaks, sizes larger than 60 nm are reported to exhibit similar plasmon peaks even from calculations.⁴³ In case, of sample with higher silver concentration clear
prismatic shapes (figure 5.17B) with plasmon peaks between 500 nm to 650 nm coincides with SPR of nanoprisms where edge length or thickness of prismatic shapes explains evolution of separate SPR peaks.\textsuperscript{21,41} Plasmon absorbance peak noticed at 385 nm as shoulder from both samples correspond to particles smaller than 5 nm as seen in TEM images (figure 5.17) or due to sharp edges of these anisotropic structures as explained by many previously.\textsuperscript{21}

In the next stage on further exposure with time, lower concentration silver sample contains intermediate polyhedral fusion shapes (figure 5.18 A\textsuperscript{1}), while fully developed worm like structures are dominant from high concentration silver sample (figure 5.18 B\textsuperscript{1}). Very close examination of these worm like structures reveal that they are formed from fusion of uneven rod like structures with polyhedral or spherical shapes. Based on TEM images we assume uncontrolled fusion of polyhedral shapes are resulting in worm like structures with time. Tuning of SPR in the NIR region beyond \(\sim 700\) nm by seed mediated growth techniques were accomplished due to variations in longitudinal length of rods, prisms or nanoplates. So, tuning noticed here (figure 5.16) is first of its kind elucidating formation of NIR sensitive AgNPs by direct single step approach. Difference in longitudinal length/width of these worms like structures facilitates tuning of plasmon peaks in the NIR region.
Figure 5.17 Shows HRTEM images for samples A and B (15-20 minutes irradiation samples) shown in figure 3.8.
Figure 5.18 Shows HRTEM images for samples A¹ and B¹ (30-40 minutes irradiation samples) shown in figure 3.8.
Figure 5.19 Changes in absorption spectra with irradiation time for NIR sensitive AgNPs synthesized using chitosan at pH 3.0 and OBS glass (2wt% CS; 20 mM [Ag]). I) A= 5 minutes irradiation; A¹= 5 minutes irradiation sample stored at 4°C after 24 hours; B= 15 minutes irradiation. C= 30 minutes irradiation (inset shows changes in absorption spectra for A and A¹ samples). II) TEM images of 30 minutes irradiated sample.

Secondly to determine effect of chitosan polymer concentration along with sensitivity of plasmon peaks to light, a typical sample solution containing >2 wt% of chitosan polymer was irradiated continuously (figure 5.19I) until no more changes are noticed in absorption spectrum. Begins with initial broad absorption peak (480 nm-580 nm) within five minutes of exposure, this broad undifferentiated peak starts growing in to red
shifted distinguishable peaks maximum at 384 nm, 500 nm and 625 nm respectively after fifteen minutes irradiation very similar to sample B in figure 5.16. Nevertheless, on further exposure at twenty-five minutes time interval, two clearly separated peaks are observed. One centered around 399 nm resembling either from small particles or from fine edges of anisotropic structures where as strong red shifted plasmon peak maximum centered at ~1000 nm due to longitudinal plasmons of anisotropic worm structures. Strong scattering and red shifted NIR plasmon peak noticed from higher concentration chitosan sample when concentration of Ag⁺ is kept constant elucidates importance of chitosan polymer concentration for tuning NIR plasmon peaks.

These two separate studies verify effect of both chitosan polymer and Ag⁺ concentration on formation of NIR sensitive anisotropic AgNPs. Again, HR-TEM images are used to consolidate our hypotheses. More aggregated, larger and wider size worm structures agree well with our hypotheses that increase in thickness or length of worm like structures results in plasmon red shift (figure 5.19). Here if fraction of the sample solution that was irradiated for 5 minutes was sealed and protected from light by storing at 4 °C, no significant changes are noticed from absorption spectrum of such sealed sample even after 24 hrs, (inset figure 5.19) indicating importance of light interactions for growth of NIR scattering anisotropic structures even after initiation.

Though optical absorption spectra suggests mechanism of formation of NIR absorbing AgNPs is direct single step but, looking at changes in absorption spectra and TEM images collected at different time intervals suggest step-wise growth. we assume at the beginning of irradiation, feeble reduction of Ag⁺ salt inside OBS glass vial (having
weak transmittance in UV region) at low temperatures encourage formation of large anisotropic structures exhibiting broad SPR peaks (450-500 nm), in the later stages uncontrolled fusion of these large anisotropic structures eventually result in strong NIR absorbing worm like structures. In contrast, strong reduction of Ag⁺ salt using ESQ glass in alkaline medium (pH > 7.0) result in formation of spherical nanospheres. However, exact growth mechanism for formation or growth of anisotropic worm like structures is not fully understood but our hypothesis is validated by recording TEM images at different stages of reaction (figure 5.18 & 5.18). Formation of stable NIR absorbing anisotropic structures at pH 3.0, where chitosan polymer is highly positive charged indicates mechanism of stabilization could be similar to positively charged CTAB surfactant prevalent for growing anisotropic gold and silver nanoparticles in literature.21

There are numerous examples for systematic formation of anisotropic silver nanostructures. Caroll and coworkers42b,c reported high yield thermal syntheses of anisotropic AgNPs using sodium citrate, NaBH₄ reducing agents in presence of CTAB surfactant, while Gabriella32d group used PVP, sodium citrate and NaBH₄ to form nanoprisms with controllable thickness at room temperature. Whereas SPR tunability in NIR region beyond 900 nm was achieved by Pietrobon et al. using sodium citrate and NaBH₄ chemical agents.42e But, in all these cases widespread usage of a special reducing agent could not be avoided. However, selective formation or specific control over shape is not achieved in our protocol, but still to the best of our knowledge, this is for the first time a detailed report on direct formation of NIR absorbing AgNPs within
biologically and environmentally benign polymer under complete absence of any known reducing agents is achieved. We assume stabilization of strong NIR absorbing plasmonic silver nanostructures within chitosan polymer would enhance applications of these nanostructures.

5.3.3 Effect of pH and Ionic Strength on SPR of Spherical and Anisotropic Silver Nanostructures

SPR of plasmonic nanostructures are sensitive to dielectric constant, pH and ionic strength based on sensitivity of stabilizing matrix and surface plasmons to surrounding environment. For all biological applications, stability of nanoparticles against aggregation at different pH and ionic strength is critical factor. Nanoparticles with high surface energy agglomerate in response to change in pH or ionic strength. So, a simple study has been performed to demonstrate effect of pH and salt on stability and sensitivity of both spherical and anisotropic AgNPs (5.20 & 5.21). Though, detailed TEM studies were not performed but monitoring changes in absorption spectrum and correlating with literature helped to understand sensitivity of AgNPs to pH and ionic strength.
Figure 5.20 Shows changes in absorption spectra of both spherical and anisotropic AgNPs stabilized in chitosan polymer with respective to changes in pH. A) Typical spherical AgNPs sample prepared at pH 8.0 followed by addition of acetic acid. B) Typical anisotropic AgNPs sample prepared at pH 3.0 followed by addition of ammonium hydroxide.
Figure 5.21 Shows changes in absorption spectra of both spherical and anisotropic AgNPs stabilized in chitosan polymer with respective to changes in ionic strength of medium. A) Typical spherical AgNPs sample prepared at pH 8.0 followed by addition of 0.1M [KX] salts. D) Typical anisotropic AgNPs sample prepared at pH 3.0 followed by addition of 0.1M [KX] salts (redline indicates transformation of NIR scattering peaks).
Characteristic 410 nm SPR peak of spherical nanospheres is highly stable to change in pH or addition of KX salts even after 24 hours (figure 5.20 A & 5.21 A). Electrostatic repulsions prevent agglomoration of sols, but at sufficient high ionic strength, electrostatically stabilized colloidal particles can coagulate due to electrostatic screening effect of salts.\textsuperscript{43} Significant quenching of 410 nm SPR peak in presence of KI (dielectric constant=5.6) salt is due to higher dielectric constant compared to KCl (4.6) or KBr (4.9) salts. Complete preservance of 410 nm SPR peaks under extreme pH and atleast in presence of two common halide salts encourage usage of these nanospheres for any biological related applications where ionic and pH stability is very significant. But in contrast in case of anisotropic AgNPs, transformation of NIR plasmon peaks indicate possible conversion of anisotropic structures in to nanospheres either due to change in pH or in presence of salts (figure 5.20 B & 5.21 B). Higher sensitivity of anisotropic samples to pH or ionic strength could be due to their high anisotropy. Addition of halide salts inadvertently examines stability of AgNPs in solution, as silver salts are known to precipitate in presence of halide ions. Blue shift in SPR peaks or decrease in NIR absorbance is expected as conversion of NIR scattering particles in to visible nanostructures due to change in pH or ionic strength is already well documented in literature. Like Chen et al. reported similar blue shift in plasmon peaks during pH dependent conversion of nanoprisms,\textsuperscript{45} whereas Xu et al. and Huang et al have separately\textsuperscript{45} found that nanoprisms can be truncated in presence of halide ions with blue shift in SPR peaks accompanied by quenching of NIR peaks. Though exact changes
in morphology of AgNPs due to change in pH or ionic strength is not elaborately investigated but these tests substantiate stability.

5.3.4 Silver Nanostructures Doped Chitosan Films

Silver salts are notorious for their light sensitive properties, photosensitive methods are utilizing this light sensitive property of silver salts to form different size, shape silver nanostructures. Nevertheless, on the other end once these different structures are formed in solution they would continuously undergo changes in size, shape either due to availability of excess reducing agents in solution or due to light exposure. So we thought stability of light sensitive AgNPs can be enhanced either by arresting their growth or activity within a reusable matrix. CS polymer’s film forming ability has been exploited for its advantages for doping Ag nanoparticles.\textsuperscript{28,29,30,34} The synergetic effects of chitosan doped AgNPs films are expected to enhance antibacterial properties and proposed to be used in edible coating and packaging.\textsuperscript{34a} Here both spherical and anisotropic particles are made in to films by simple drop-cast method and tested by running diffuse reflectance (5.23 & 5.24). Two different sample solutions containing different size nanospheres are made in to films (F1,F2) by simple drop cast method (figure 5.22). Diffuse reflectance spectrum from both films (figure 5.22) shows clear red shift from 404 nm to 420 nm and 415 nm to 450 nm respectively in comparison with corresponding solutions (S1 and S2). Red shift in peak maximum in the film form is due to change in inter-particle distance when drop-casted as films compared to solutions.
Figure 5.22 Shows AgNPs entrapped chitosan films. A) Absorption spectra of two different concentration AgNP samples (S1 and S2 represent solutions); (F1 and F2: films from same solutions). B) SEM images of F1 and F2 films (inset shows films F1 and F2 deposited on glass slide).
Figure 5.23 Changes in absorption spectra of a typical AgNP sample at different physical forms. (S: Solution, F: Film from same solution, RF: Re-dispersing same film).

Visible color, absorption and SEM images (figure 5.22 B) confirm retention of nanospheres even in film forms. These films are intrinsically much more stable compared to solutions even under complete ambient conditions due to lack of any further activity of unreacted silver ions or nanosilver compared to solutions. A two week old typical silver nanospheres chitosan film when re-dispersed in aqueous medium (figure 5.23), did not exhibit any major changes in absorption spectra with respective to initial solution used for making films. This facilitates a better way to store these light
sensitive nanosols under ambient conditions with ability to re-disperse and encourage using these film forms for commercial applications.

![Absorption Spectra](image)

**Figure 5.24** Changes in absorption spectra of a typical NIR absorbing AgNP sample at different physical forms. (S: Solution, F: Film from same solution).

5.3.5 In Situ Formation of Silver Nanoparticles Entrapped Chitosan Gels

Chitosan has been shown to possess distinctive property to extract heavy metal ions from water at different pH either due to chelation or due to adsorption by deacetylated amine (-R-NH$_2$) groups. Chitosan polymer is famous to exhibit gelation in contact with oppositely charged polymers or polyanions or chemical crosslinkers, which are known to exhibit wide range of applications. At more than 1wt% of chitosan
polymer addition of 0.01M AgNO$_3$ results in instantaneous increase in viscosity of chitosan polymer solution due to gelation. This phenomenon of chitosan gelation under influence of silver nitrate is followed stepwise (figure 5.25). Colorless non-viscous solution of chitosan polymer initially turns in to colorless viscous gel immediately after addition of silver nitrate, followed by change in physical color ranging from dark yellow to violet depending on concentration of silver nitrate under daylight. Strength of gelation is controlled by both concentration of chitosan and silver nitrate. At constant silver nitrate concentration, (figure 5.25) strength of gelation is shown to be stronger at higher concentration of chitosan polymer while similar effect is noticed if concentration of polymer is maintained constant by varying silver salt content. Though, detailed studies are not conducted but gelation was not noticed for any concentration of polymer < 0.5% (wt/v) of CS or with Ag$^+$ concentration < 5mM. We assume gelation exhibited here is due to strong chelation property of chitosan polymer with Ag$^+$ ions not been document earlier.

However, AgNPs doped chitosan gels are reported earlier but none of them have discussed insitu gelation in complete absence of reducing agents. Pure chitosan based gels have been widely used in many biomedical applications like tissue engineering or wound dressing.$^{34}$ Recently AgNPs doped chitin-based scaffolds or PAM (polyacrlamide) based IPN gels have been developed for wound healing and antibacterial applications.$^{34}$
Figure 5.25 Demonstrates formation of AgNPs doped chitosan gels A) 2wt% CS; 10 mM [Ag]. B) 1wt% CS; 5 mM [Ag]. C) 2wt% CS; 20 mM [Ag].

However, in all the above cases AgNPs are produced by using regular reducing agents like NaBH₄ or sodium citrate, so this is the first time where in situ formation of AgNPs during instantaneous gelation has been observed in complete absence of any extra reducing agent. With remarkable antibacterial and wound healing properties possessed
by nanosilver, we assume these nanosilver-doped in situ hybrid gels made from environmentally benign polymer can be employed for variety of applications like wound dressings or as scaffolds for tissue engineering.

Figure 5.26 Absorption spectra of AgNP samples used for bacterial studies before and after dialysis. A: Spherical AgNPs before dialysis; A\(^1\): Spherical AgNPs after dialysis; B: NIR absorbing AgNPs before dialysis; B\(^1\): NIR absorbing AgNPs after dialysis.

5.3.6 Antipathogenic Studies

Our primary objective was to produce environmentally benign silver nanostructures, but a simple study was carried out primarily to check bactericidal properties of both small spherical and large anisotropic nanostructures incorporated in
chitosan polymer. In order to ascertain and differentiate activity of nanosilver with unreacted silver ions (Ag$^+$), extreme care was taken by dialyzing silver nanoparticle samples against millipore water for 24 hours using 10,000 MWCO dialysis cassettes. Leaching of unreacted silver ions during dialysis was confirmed by precipitation of AgCl due to addition of KCl salt to Millipore water used for dialysis. Eventually white precipitate ensures loss of un-reacted Ag$^+$ ions from colloidal samples during dialysis. This would ensure bacterial activity exhibited by sample solutions is exclusively due to colloidal silver. Silver sulfadiazine and silver salt precursor also tested as controls. Optical absorption of samples used is shown in figure 5.26, which does not show much difference before or after dialysis. Dialysis not only helps purification of samples but also indicates stability of samples. Antibacterial tests were performed using two different plant pathogens, (PV288 and ES2346) using both dialyzed and un-dialyzed silver nanoparticles along with silver nitrate, silver sulfadiazine and chitosan as controls. Relative silver content of all samples have been analyzed using ICP-MS (Inductively coupled plasma-mass spectroscopy) technique in order to assess effect of silver content on antibacterial activity. C/S (counts/second) values from three duplicates for each sample are recorded after perfect baseline correction using millipore water. Figure 5.27, shows difference in relative silver content of all samples, all dialyzed samples have relatively lower silver content compared to same batch of un-dialyzed samples as expected. This shows loss of un-reacted silver ions during dialysis. Lowest C/S for dialyzed silver nitrate precursor (Ag-D) solution compared to highest C/S for same batch of un-dialyzed silver nitrate (UD-Ag) solution explains complete leaching of silver.
ions during dialysis. As to begin with, for syntheses, silver nitrate concentration of NIR samples is higher than nanospheres samples. So, relative Ag C/S values for NIR samples (NIR-D or NIR-UD) are higher compared to nanospheres samples (VIS-D or VIS-UD) (figure 5.27).

Table 5.2  Table shows zeta potential and ionic conductivity values of different nanosilver samples used for antibacterial testing.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Zeta Potential (mv)</th>
<th>Ionic Conductivity (mS.cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>+ 57 ± 4.8</td>
<td>0.039</td>
</tr>
<tr>
<td>Visible-UD</td>
<td>+ 35.6 ± 3.2</td>
<td>1.74</td>
</tr>
<tr>
<td>Visible-D</td>
<td>+ 38.5 ± 4.1</td>
<td>0.067</td>
</tr>
<tr>
<td>NIR-UD</td>
<td>+ 39.2 ± 3.5</td>
<td>2.11</td>
</tr>
<tr>
<td>NIR-D</td>
<td>+ 43.6 ± 4.4</td>
<td>0.297</td>
</tr>
<tr>
<td>AgNO$_3$-UD</td>
<td>NA</td>
<td>2.44</td>
</tr>
<tr>
<td>AgNO$_3$-D</td>
<td>NA</td>
<td>0.057</td>
</tr>
<tr>
<td>AgSD</td>
<td>NA</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Ionic conductivity values of all samples (table 5.2) before and after dialysis is in good agreement with assumption of loss of unreacted silver ions or nitrate ions during dialysis. Ionic conductivity of all dialyzed samples is lower than un-dialyzed samples with NIR samples exhibiting higher ionic conductivity again due to involvement of
higher initial concentration of silver nitrate compared to spherical nanoparticle samples.

Ionic conductivity measurements unintentionally assess purity of samples.

Figure 5.27 Relative silver content of all samples used for antipathogenic studies using ICP-MS. (D = Dialysed samples; UD = Un-dialysed samples; CON: Bacteria control; NIR: Anisotropic AgNPs; VIS: spherical AgNPs; Ag: Silver nitrate solution; CH: Chitosan polymer; AgSD: Un-dialysed silver sulfadiazine solution).
Figure 5.28 Demonstrating antipathogenic efficiency of different nanosilver samples by monitoring changes in OD of PV288 pathogen for 24 hours (a: 0 hr; b: 3 hr; c: 12 hr; d: 24 hr ) (D = Dialysed samples; UD = Un-dialysed samples; CON: Bacteria control; NIR: Anisotropic AgNPs; VIS: Spherical AgNPs; Ag: Silver nitrate solution; CH: Chitosan polymer; AgSD: Un-dialysed silver sulfadiazine solution).
Figure 5.29 Demonstrating antipathogenic efficiency of different nanosilver samples by monitoring changes in OD of ES2346 pathogen for 24 hours (a: 0 hr; b: 3 hr; c: 12 hr; d: 24 hr) (D = Dialysed samples; UD = Un-dialysed samples; CON: Bacteria control; NIR: Anisotropic AgNPs; VIS: Spherical AgNPs; Ag: Silver nitrate solution; CH: Chitosan polymer; AgSD: Un-dialysed silver sulfadiazine solution).

Changes in OD$_{600 \text{ nm}}$ of doped and un-doped bacteria with AgNPs is monitored at different time intervals for 24 hours. Control bacteria sample and chitosan polymer sample containing no silver components recorded highest growth as indicated from OD values. Figure 5.28 and 5.29, implies inhibition of bacterial growth is not proportional to content of silver (Ag$^+$). As both dialysed and undialysed NIR (NIR-D, NIR-UD) samples
show higher antibacterial activity compared to Ag-UD (silver nitrate solution) sample which has relatively 10 times higher silver content.

Figure 5.30 Effect of nanosilver concentration on growth of PV288 pathogen.

VIS-D sample exhibited very similar activity compared to well known topical antibacterial silver compound silver sulfadiazine (Ag-SD) whose relative silver content is comparatively higher. Close observation of only dialyzed samples (figure 5.28 and 5.29) reveal NIR samples exhibiting higher activity compared to spherical NP samples. This could be due to higher silver content in NIR samples or could be due to shape or size effect of anisotropic samples that needs to be studied in detail.
Figure 5.31 Effect of nanosilver concentration on growth of ES2346 pathogen.

Though detailed study is required to address relationship between antimicrobial activity and size or shape of nanosilver, but our quick attempt demonstrates that both nanosilver samples (spherical and anisotropic) produced by our methodology possess antibacterial efficiency. Ag-UD sample that contains highest content of silver exhibits lowest activity compared to any nanosilver sample in both bacteria, this proves that bactericidal effect is solely due to nanosilver but not due to unreacted Ag$^+$ ions which is very significant result by itself. Effect of nanosilver concentration on antibacterial activity has been studied separately using single batch of nanospheres on two different bacteria. Figure 5.30 and 5.31, shows highest inhibition with bacteria culture containing highest concentration of nanosilver. This verifies effect of nanosilver concentration on
antibacterial activity similar to reports in literature.\textsuperscript{7} Comparing OD values from figures 5.30 and 5.31 indicate difference in activity depending on nature of pathogen, using exactly same AgNP sample growth inhibition is different for PV288 and ES2346 pathogens. For ES2346 maximum inhibition is observed at 100 µl of AgNPs only whereas for PV288 pathogen 300 µl exhibits maximum inhibition. This show how antibacterial activity of AgNPs also depends on nature of pathogen/bacteria tested. Many research groups have commendably demonstrated antibacterial efficiency of nanospheres earlier\textsuperscript{36} but there are considerably very few reports addressing ambiguities concerning size, shape effect of AgNPs on antibacterial activity.\textsuperscript{36} Our attempt to demonstrate bactericidal activity using NIR sensitive silver nanostructures is a useful illustration in view of low cytotoxicity and better biocidal properties suggested for larger size or anisotropic particles by some previous literature works.\textsuperscript{36} Strong NIR absorbing efficiency would be an added advantage for applications related to photothermal killing of bacteria as demonstrated using gold nanoparticles.\textsuperscript{46}

5.3.7 Syntheses of Fluorescent Silver Nanoparticles Stabilized within Quinine Sulfate

After realizing syntheses and antibacterial properties of different size shape AgNPs, we have tried synthesizing fluorescent AgNPs that would take us a step further for employing them for many biological or materials applications. Though extensive work needs to be, done but preliminary results were encouraging. Photophysics of quinine sulfate dication (QSD) in H\textsubscript{2}SO\textsubscript{4} by itself is subject of intensive research because of its medical applications and its use as fluorescent standard.\textsuperscript{37b} Acidified quinine sulfate has been used as standard to measure photochemical quantum yield because of
its photostability and immunity to oxygen quenching.\textsuperscript{37a} QS exhibits interesting photophysical properties as emission arises simultaneously from two close lying states. In polar solvents and at different pH, QS is shown to exhibit excitation dependent emission either due to change in heterogeneity of the medium or due to existence of the molecule in two different conformations each of which with its own distinct transitions.\textsuperscript{37} Excitation spectrum of QSD was different from its absorption spectrum and a phenomenon called red shift in emission on excitation at red edge of absorption (REE effect) is noticed in QSD. Chen suggested this effect due to two closely lying states, while Fletcher interpreted due to existence of at least two conformers each with its own electronic transition based on solvent relaxation mode. Excitation dependence of emission in QSD is also reported in sol-gels due to changes in heterogeneity of medium.\textsuperscript{37} Figure 5.32 shows excitation and pH dependent emission spectrum for QSD in water taken from literature. As photoemission of QS is strongly dependent on pH and excitation wavelength, we have started by understanding photophysical properties of QS itself in aqueous medium. H\textsubscript{2}SO\textsubscript{4} or NaOH used to adjust pH of the medium, absorption spectra (figure 5.33A) of QS solution with decrease in molar concentration exhibits blue shift in peak maximum from 346 nm to 290 nm and further blue shifts to 272 nm with increase in pH. This behavior of blue shift in peak maximum with increase in pH is also noticed from PL emission spectra. With pH, both emission and excitation peaks exhibit blue shift followed by decrease in intensity (figure 5.33 B) as noticed in literature (figure 5.32).
Figure 5.32 Photoluminescence properties of QS. A) Excitation dependent emission. B) pH dependent emission.
Figure 5.33 Photophysical properties of quinine sulfate (QS) at different concentrations and pH. A) Absorption spectra of QS solution at different concentrations and at different pH. B) PL spectra of $10^{-3}$ M QS solution at different pH and different excitation wavelengths.
Table 5.3  Summarize photoluminescence properties of quinine sulfate solution (QS) at different molar concentrations and pH of solution.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>pH</th>
<th>Exc max (nm)</th>
<th>Emi max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$ M</td>
<td>&gt; 2.0</td>
<td>390</td>
<td>At 452 only</td>
</tr>
<tr>
<td>$10^{-5}$ M</td>
<td>2.5</td>
<td>350 (320 shoulder)</td>
<td>At 450 only</td>
</tr>
<tr>
<td>$10^{-6}$ M</td>
<td>3.5</td>
<td>350 (320 shoulder)</td>
<td>At 450 only (shoulder@ 390 with 330 Exc)</td>
</tr>
<tr>
<td>$10^{-7}$ M</td>
<td>6.5</td>
<td>280-290</td>
<td>At 395 only (very weak)</td>
</tr>
</tbody>
</table>

From experience in stabilizing spherical AgNPs in chitosan polymer, silver nanoparticles stabilization in QS was also found to be more favorable in alkaline medium compared to acidic medium. Absorption spectra of AgNPs stabilized in QS by photolysis under both acidic and alkaline medium are shown in figure 5.34A which shows very clearly significance of pH for formation of AgNPs in aqueous medium. Comparing PL spectra from figure 5.34 and 5.35 it is noticed that AgNPs stabilized in acidic medium show exact same emission and excitation profile as QS itself, but AgNPs stabilized in alkaline medium exhibits red shift in emission maximum from 450 nm to 475 nm -500 nm depending on concentration of QS used. In contrast, QS solution by itself shows blue shift from 450 nm to 390 nm (figure 5.22) as noticed in literature.
Figure 5.34 Shows absorption spectra and PL. A) Absorption spectra of AgNPs stabilized in QS at different concentrations and at different pH. B) PL spectra of QS stabilized AgNPs (corresponding to sample b at different excitation wavelengths).
Figure 5.35 Shows PL spectra of QS stabilized AgNPs. A) PL spectra of QS and QS stabilized AgNPs (corresponds to sample a in figure 5.31). B) PL spectra of QS and QS stabilized AgNPs (corresponds to sample c in figure 5.31).
QS is known to exhibit edge excitation red shift in emission from 450 nm to ~460 nm by exciting with longer excitation wavelengths (figure 5.22), but this behavior of emission red shift in alkaline medium is not reported before. Though exact details for this emission red shift is still under investigation but it shows that there is some coordination taking place between QS and AgNPs during formation or stabilization which is resulting in emission red shift. Size tunability, emission tunability with respective to size of AgNPs and other related studies regarding formation of fluorescent AgNPs by simple photolysis is under further investigation.

5.4 Conclusions

We have evidently demonstrated a reducing agent less, non-seeded and biopolymer based protocol for producing anisotropic silver nanostructures with high yield and stability by simple photochemical approach. During syntheses of spherical or anisotropic silver nanostructures, formation is determined mainly by nature of light interactions and pH of the medium. By varying concentration of silver nitrate and chitosan polymer, the size and NIR scattering efficiency are effectively controlled. Chitosan polymer only plays an assistant role as capping or stabilizing agent to assist growth of well-dispersed and stable nanostructures in solution phase. These light sensitive nanostructures can be protected by turning them in to light insensitive films that retain physico-chemical features of nanostructures on re-dissolving. AgNPs doped chitosan gels could find better applications in biomedical fields due to complete absence of any reducing agent and for ability to form gels. Antibacterial efficiency shown here could instigate further research in to testing NIR scattering nanostructures that can
have better and enhanced applications in biomedicine compared to spherical particles mainly due to NIR absorbance features as well as low cytotoxicity tagged to larger size particles along with stronger biocidal action associated to anisotropic structures by some studies. Furthermore, since the route devised here is very simple and straightforward, only requirement seems to be selection of proper ratio of chitosan to silver nitrate; this method should therefore be extendable to the syntheses of other metal anisotropic nanostructures for scaled up preparations through very cost effective and environmentally benign protocols. Within scope, fluorescent silver nanoparticles were also synthesized using same protocol using quinine sulfate molecule that has strong blue emission in acidic water.

5.5 References


Laudenslager, M. J.; Schifferman, J. D.; Schauer, C. L. *Biomacromolecules* **2008**, *9*, 2682.


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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Topics discussed in this dissertation can be categorized into two main aspects, primarily started with interfacing environmental or thermosensitive hydrogels like PNIPAM and biodegradable polymers like chitosan with phosphorescent transitional metal based Au(I) complexes. Initial results inspired studying effect of interacting non-luminescent starting precursors like Au(I) sulfides and light sensitive Ag(I) salts with same soft polymer templates. Surpassing the initial results, secondary investigations resulted in evolving unexploited chemical syntheses route for formation of different size, shape tunable gold and silver nanoparticles separately in aqueous medium without aid of any special reducing agents. The following is a summary of the major results and conclusions of this dissertation (sections 6.1) as well as the future directions pertaining to this research that is being pursued (section 6.2 and 6.3).

6.1 Conclusions
6.1.1 Interfacing Soft Polymers and Gels with Phosphorescent Au(I) Complexes

In chapter 2 and 3, detailed systematic study conducted elucidates formation of phosphorescent hydrogels and polymeric phosphorescent nanoparticles separately. chapter 2 exclusively discusses about doping a highly stable water-soluble Au(I) phosphorescent complex Na₈[Au(TPPTS)₃] (AuP)¹ in to PNIPAM hydrogels conveniently. Thermosensitive polymeric hydrogels retained phosphorescence in different physical forms. To our knowledge formation of such broad physical forms of phosphorescent PNIPAM based hydrogels were never demonstrated before in literature. Though there
are some studies of lanthanide-based complexes \(^2\) doped in to PNIPAM polymers but phosphorescence emission was not reported before in various soft polymer forms like microgels, bulk hydrogels and dried xerogels. Strong dependency on pH and nature of co-monomer of the PNIPAM microgels host determine maximum interactions for efficient loading of AuP. Retained phosphorescence signifies stability of phosphor in different polymer matrix systems while fluorophores are widely known to be sensitive to environment. \(^3\) What is outstanding is the resulting hybrid microgels exhibited more than two orders of sensitized Au-centered emission enhancement above and below phase transition temperature of microgels compared to that of the gold complex in water. The hydrodynamic radius \((R_h)\) of PNIPAM microgel nanoparticles that is known to decrease in presence of electrolytes \(^4\) exhibited similar decrease even in presence of AuP at room temperature indicating strong oppositely charged interactions between PNIPAM-\(\text{co-}\)allylamine microgels and polyanionic AuP molecules in aqueous medium. There are few examples in literature explaining emission enhancement from well known organic dyes in presence of polymeric colloidal particles but this is first ever demonstration of phosphorescence enhancement. \(^2\) Quantum yield value \((\phi = 0.042)\) obtained for AuP bulk solution by relative method using quinine sulfate as standard is in perfect agreement with literature indicating purity of Au(I) complex, 10 times increment in quantum yield value was noticed from hybrid PNIPAM gels.

Later in chapter 3, the same AuP was adopted for its physical crosslinking ability with oppositely charged polymers and PNIPAM microgels realized systematically. A simple polyelectrolyte complexation (PEC) approach \(^5\) was used to
synthesize highly water soluble PCHNPs (phosphorescent chitosan microgel nanoparticles) using the same phosphorescent molecule which acted as both physical crosslinker and light emitter. Size and stability of PCHNPs are controlled by balancing concentrations of both CS and AuP indicating importance of electrostatic interactions. Both electron microscopy (TEM, SEM) and dynamic light scattering analysis are used in evaluating size, distribution and morphology of PCHNPs at different concentrations of AuP and CS polymer. This allowed for facile syntheses of different size phosphorescent chitosan nanoparticles in a single step in complete absence of any extra crosslinker. With not much detailed work, similar physical crosslinking ability was reconfirmed using PNIPAR-co-NH$_2$ where NIPA polymer chains are crosslinked in to nanoparticles. Owing to retained emission, PL enhancement and microseconds lifetime the ability of AuP not only as physical crosslinker but also as promising contrast agent within polymer matrices is elucidated. After looking at these results, PNIPAM-co-allylamine microgels exposed ability of AuP to instigate inverse thermoreversible physical gelation within PNIPAM-co-allylamine microgels above phase transition temperature of microgels. Emission tuning expected from AuP due to T-shaped excited state distortion was clearly demonstrated during gelation of hybrid microgels or during precipitation of PCHNPs. Intermediate emission (between solids and bulk solution) expected from calculations in similar Au(I) phosphors is realized here in real systems experimentally. A $\sim$620 cm$^{-1}$ shift is noticed in gels and precipitate forms compared to bulk AuP solution. Concentrations of PNIPAM-co-allylamine microgels and AuP are observed to be
determining factors for tuning strength of inverse thermoreversible gelation. Phenomenon of physical gelation is easily visualized from simply inverting the test tube and quantitative changes during gelation is visualized from changes in hydrodynamic radius before and after gelation. Inspired by biological benign nature of chitosan polymer and its ability to interact with AuP has allowed us to take a step further and use it as template to synthesize aqueous sensitive trinuclear Au(I) phosphorescent complexes in complete aqueous polymer solution. Color tunability with respective to pH and heavy metal entrapment is completely retained in polymer supported phosphorescent trinuclear Au(I) complexes as expected from their original counterparts in organic phase.\textsuperscript{7} Presence of chitosan polymer did not alter sensitive phosphorescence emission properties from trinuclear Au(I) systems useful for sensor applications.

6.1.2 In Situ Formation of Plasmonic Metallic Nanoparticles (Au and Ag) within Soft Polymer Templates

In chapter 4 and 5, formation and stabilization of gold and silver nanoparticles is investigated separately. Chapter 3 detailed in situ formation methodology of gold nanoparticles of different sizes and shapes by photolysis thermolysis and ambient reaction conditions. Photolysis or thermolysis of Au(I) sulfides to form AuNPs was shown in wide range of polymers and gels. HAuCl\textsubscript{4} a common Au(III) precursor was replaced with Au(I) sulfides [Au(THT)Cl or Au(Me\textsubscript{2}S)Cl]\textsuperscript{8} which avoided usage of any reducing agent promoted due to single electron reduction of Au(I) compared to two electron reduction of Au(III) during formation of Au(0) nanoparticles. PNIPAM microgels are initially employed as templates for stabilizing spherical AuNPs. Vivid
visible colors and SPR (surface plasmon resonance) from AuNPs solutions indicated formation of colloidal gold which was confirmed from SEM and TEM images. As expected negatively charged PNIPAM microgels exhibited higher quantum efficiency compared to positively charged microgels. Highly monodispersed spherical AuNPs of sizes anywhere between 5-20 nm are easily produced both by photolysis and thermolysis. Under same reaction parameters, reduction under ambient light and heat resulted in AuNP samples with broad SPR peaks and polydispersed particles of sizes >20 nm. Formation of colloidal crystals from AuNPs doped PNIPAM microgels was significant result never achieved before. Exhibition of bragg diffraction peak\(^9\) and SPR peak together confirm formation of colloidal crystals from hybrid microgels. The same methodology of stabilization when extended to different polymers irrespective of their physical and chemical properties showed stabilization of colloidal gold in complete aqueous medium and in complete absence of any reducing agent. Acid (-COOH) functional group polymers like polyacrylic acid, alginic acid, hydroxy (-OH) functional group polymers like polyvinyl alcohol, hydroxy propyl cellulose, amine (-NH\(_2\)) functional group polymers like chitosan, PNIPAR-co-NH\(_2\), proteins like bovine serum albumin (BSA), surfactants like sodium dodecyl sulfate (SDS) are utilized as templates to show versatility of proposed methodology for easy formation of spherical AuNPs either by photolysis or thermolysis. Formation of colloidal gold in all the above stated polymer solutions is confirmed from absorption spectra and TEM images. Intrinsic environmental sensitive properties of these polymers were retained even after formation of AuNPs.
With significant interest in non-spherical or anisotropic AuNPs in different biomedical applications due to their strong NIR absorbing property and also due to compulsive involvement of toxic surfactants like CTAB (see table 4.4 in chapter 4) for making them has intrigued us to test ability of our protocol to make strong NIR absorbing AuNPs. Again in a single step method anisotropic particles of various shapes (traigles, prisms, rods) are produced by both photolysis and thermolysis of Au(Me₂S)Cl both in PNIPAM-co-allylamine microgels and chitosan polymer seperately. Significance of positively charged poymers for formation of anisotropic particels was realized. Strong NIR absorption spectra and TEM or SEM images confirmed formation of mixture of different shapes of AuNPs whose stability was tested with respective to time. Photothermal volume phase transition as expected due to light sensitive nature of NIR absorbing AuNPs was demonstrated by monitoring shrinking of PNIPAM microsphers loaded with NIR absorbing AuNPs. Control micropsheres does not exhibit any change in average hydrodynamic radius compared to AuNPs loaded microspheres. The same concept was re-confirmed from release studies using a phosphorescent molecule as model dye for release from PNIPAM microgels. Once different size, shape AuNPs were attained, the non-toxin nature of these spherical and non-spherical particles are tested by both in vitro (MTT assay) and in vivo (on zebrafish) assays which demonstrated better viability compared to similar analogues available in the market.

Following up with convincing results on formation and stabilization of AuNPs, formation of silver nanoparticles of different sizes, shapes was also investigated
mainly in chitosan polymer. Photolysis of light sensitive silver nitrate was carried out in presence of chitosan and polyacrylic acid polymers that resulted in formation of different size spherical AgNPs. Strong surface plasmon absorption, physical color and TEM images confirmed formation of spherical AgNPs of various sizes. Dispersity and size of particles was completely controlled by varying concentrations of both chitosan or polyacrylic acid polymers and AgNO₃ in aqueous medium. Though few reports already existed on stabilizing spherical AgNPs in chitosan matrix,¹⁰ but this wide range of sizes was never reported. Taking a step ahead, formation of strong NIR absorbing AgNPs was achieved for the first time completely within a single biodegradable chitosan polymer in single step. A simple non-seeded approach taking advantage of pH sensitivity of CS polymer and light sensitivity of Ag salt was exploited in making worm like silver nanostructures which exhibited strong NIR absorbing capacity. Intermediate photolysis samples exhibited perfect polygonal shapes similar to literature.¹¹ With significance of antibacterial applications¹² of AgNPs, both spherical and non-spherical AgNPs exhibited strong antipathogenic activity on different plant pathogens. Within scope of chapter 5, formation of fluorescent AgNPs using a well known blue fluorophore quinine sulfate as stabilizing agent was also investigated.

6.2 Future Directions
6.2.1 Context

Cancer research is basic research into cancer (uncontrolled tumor growth) in order to identify causes and develop strategies for prevention, diagnosis, treatments
and cure. Cancer research is exciting and spreads across many multidisciplines. Today cancer nanotechnology is emerging as a new field of interdisciplinary research, across the disciplines of biology, chemistry, engineering, and medicine. Novel optical, magnetic and structural properties of different metallic, semiconductor and magnetic nanoparticles that are not noticed in their respective bulk systems are efficiently utilized. When linked with tumor targeting ligands such as monoclonal antibodies, peptides or small molecules, these nanoparticles can be used to target tumor antigens (biomarkers) as well as tumor vasculatures with high affinity and specificity. In the size range of 5–100 nm diameter, nanoparticles also have large surface areas and functional groups for conjugation. Recent advances in nanotechnology have led to development of various nanoparticle probes for molecular, cellular imaging, targeted nanoparticle drugs for cancer therapy, and integrated multifunctional nanodevices for early cancer detection and screening. These developments raise exciting opportunities for various fields towards contribution for eradicating cancer. New technologies are continuously under investigation for developing new hybrid materials for different aspects of cancer nanotechnology. Figure 6.1A shows how nanotechnology can be applied to various fields of cancer research.

Not limiting to cancer research only, experimental results shown in different chapters of this dissertation incline towards improving existing methods of making/ utilizing hybrid materials in various biomedical applications. For example figure 6.1B shows different biomedical fields in which gold nanoparticles can be efficiently
utilized other than cancer research, but presence of unwanted chemicals that needs through purification protocols can be competely avoided by synthesizing AuNPs following protocol designed in chapter 4. Another example is regarding work presented in chapter 3, phosphorescent trinuclear Au(I) complexes can be utilized for abstracting heavy toxic metals like lead or thalium from water. And from the work presented in chapter 5 antipathogenic properties of silver nanoparticles can be efficiently applied for making bacteria resistant silver films as coatings in food packing or making nanosilver doped fabric materials that are in high demand in textile industry.

6.3 Research Plans and Future Directions

6.3.1 Fabrication and Development of Colloidal Based Enhancement Tools Similar to MEF or SERS

Main goal of interdisciplinary investigations detailed in the entire dissertation is taking luminescent and non-luminescent Au(I) or Ag(I) metal complexes and polymer materials together in to real world systems by formation of hybrid materials that would retain all the intrinsic properties of both materials in addition to exhibiting unexplored hybrid properties. High priority is interfacing phosphorescent molecular systems with environmentally sensitive polymers and gels. Fluorescent molecules or dyes have long history as labeling agents with polymers and microspheres. However, broad range applications of these materials especially in
conjunction with biological materials have limitations.\textsuperscript{2} Nanosecond fluorescence lifetime coincides with many biological materials that make fluorescence signals difficult to differentiate. Therefore, techniques are developed in literature in order to enhance signals from fluorophores by taking advantage of plasmonic nanoparticles. But to our understanding MEF (metal enhanced fluorescence) reported from organic dyes in
vicinity of plasmonic nanoparticles is very difficult to achieve in bulk solutions due to possibility of luminescence quenching depending on distance between fluorophore and plasmonic particles. Colloidal scattering based enhancement reported in chapter 2 known before was never studied in detail either in phosphorescent or fluorescent materials. Scientific reasons for colloidal based scattering needs clear understanding in view of more than two orders of emission enhancement reported in chapter 2 from hybrid PNIPAM-co-allylamine microgels. Understanding exact mechanism and parameters can be useful to detect very low concentrations of molecules otherwise undetectable by simple PL spectroscopy. This would be similar to single molecule detection by SERS (surface enhanced Raman spectroscopy) carried in presence of plasmonic scattering systems. In addition to AuP complex studied in chapter 2 and 3 we need to investigate different Au(I) and Pt(II) based luminescent compounds for emulating similar results in different polymer gels. For example, an oxygen sensitive Pt(II) complex investigated by a colleague in Omary group is primarily studied for utilizing its oxygen sensitive properties to differentiate tumor cells from non-tumor cells. If the same phenomenon of scattering based enhancement is duplicated with Pt(II) complexes than it will enhance its environmental sensitive properties. Because Z. V. Vardeny group has shown differentiating cancerous from non-cancerous tissues by scattering based enhancement technique.\textsuperscript{15,16} Low quantum yields of water-soluble phosphorescent materials can also be enhanced by this mechanism. However, strong experimental research is required to understand correlation between nature of colloidal particles, phosphorescent materials, concentration and temperature effects on
enhancement. The major advantage of emission enhancement reported in chapter 2 is being operational or feasible to be carried out by simple PL spectroscopy technique in aqueous solution both at room temperature and close to physiological body temperature.

6.3.2 Development of Phosphorescent and NIR Emissive Polymeric Nanoparticles

Chapter 3 highlights importance of luminescent polymeric nanoparticles in different biomedical arena. Due to major limitations with excitation within UV/visible wavelength regime lot of fluorescent or phosphorescent or quantum dots particles fall short for their direct usage in vivo imaging or real time monitoring of biochemical reactions. NIR emitting lanthanide complexes and luminescence up-conversion materials\(^{17}\) are found to be perfect for resolving these issues. Phosphorescent polymeric nanoparticles discussed in chapter 2 and 3 though have considerable advantages compared to fluorescent dyes and quantum dots however UV excitation limits their applications for in vitro and biochemical assays. Similar methodology employed in chapter 3 can be applied to NIR emitting materials or upconversion lanthanide luminescent materials for making perfect luminescent polymeric nanoparticles that can be directly utilized for in vivo studies. Making such NIR emissive materials from Au and Pt based noble metals would be more advantageous compared to existing lanthanide based systems because some of the Au(I), Au(III) and Pt(II) compounds are already recognized for their potential to target biological sites\(^ {18,19}\) (Cisplatin for cancer therapy and Au sulfides for rheumatoid arthritis).
6.3.3 Development of Optical Sensors Based on Phosphorescent Au(I) Trinuclear Complexes

With strong ability for emission color tunability based on pH or heavy metal entrapment, trinuclear Au(I) cyclic complexes are promising candidates as biosensors. Strong red emission is always desirable for bioimaging studies due to minimum background interference from biological tissue. Syntheses of strongly red and NIR emissive systems with capability to get excited by visible wavelengths (400-500 nm) are strongly perused by few students in Omary group. This is achieved by varying functionality on Pz (pyrazole) and Tz (triazole) ligands. These complexes would be perfectly suitable for making most desirable red or NIR emissive biological sensors that can be interfaced with polymeric materials for extending their usage in to biomedical applications. Investigating singlet oxygen production from these complexes is another prominent area of research.\textsuperscript{20} Complexes like porphyrins that have capability to produce singlet oxygen are already utilized in photodynamic therapy.\textsuperscript{20}

6.3.4 Testing and Utilizing AuNPs for Various Applications

6.3.4.1 Tuning Syntheses of Anisotropic AuNPs

In spite of huge advantages of proposed protocol in chapter 4 for making NIR sensitive anisotropic AuNPs, lack of monodispersity remains as major drawback compared to existing literature works. As explained in chapter 4, absence of any perfect growth controller could be the main reason for non-controlled growth of anisotropic structures. So it becomes very significant to re-visit and try to modulate the syntheses protocol by retaining benign or non-toxic property along with controlling growth of anisotropic gold nanostructures. Negatively charged benign polymers need to be
investigated thoroughly for stabilizing anisotropic AuNPs that would provide more alternatives to select. Sometimes negatively charged polymers are expected to cause less injury to cells compared to positively charged polymers due to negative charge nature of cell membranes. In this regard testing both type of materials with similar Au concentration and similar size particles on different cell lines will provide better understanding.

6.3.4.2 Determining Exact Temperature Increase and Demonstration of Hyperthermia in Different Cancerous Cell Lines

With demonstration of photothermal volume phase transition and release studies using hybrid PNIPAM microgels (AuNPs doped microgels) shown in chapter 4, it becomes imperative to determine exact change in temperature of the medium during NIR irradiation. Though different studies have demonstrated photodestruction of cell lines by employing plasmonic photothermal therapy but controversy pertaining to exact temperature required to kill cancer cells still exists due to conflicting reports on different cell lines. A detailed investigation first on determining exact temperature change followed by demonstration of hyperthermia in human cancerous cell lines is major criteria for real time utility of theses gold nanostructures. Experiments are in line in collaboration with Dr. Petros in chemistry on these two aspects.

6.3.4.3 Designing Gold Nanoparticles Kit for Practical Utility

As explained in chapter 4, due to wide discrepancies in toxicity testing methods followed in literature it is highly impractical to attain any conclusion on toxicity issues pertaining to AuNPs. Various cell lines need to be tested with various sizes of AuNPs.
stabilized in different polymers to determine effect of functional group, size and concentration for perfect understating on toxicity. Due to practical feasibility of our protocol to synthesize different size AuNPs in wide range of polymers, surfactants and proteins easily, an AuNPs kit can be made to evaluate effect of size, charge and functionality in more than one type of cell line for perfect understanding on toxicity related issues.

6.3.4.4 Extending in to Materials Applications

As explained in chapter 4, AuNPs have generated strong interest in catalysis. Though bulk gold was never preferred for catalysis but colloidal gold in sizes less than 5 nm\textsuperscript{23} are highly sought for their ability to act as catalyst centers. Monodispersity, size < 5 nm, nature of substrate\textsuperscript{23} are critical factors that govern application of AuNPs for catalysis. Determining catalytic activity of AuNPs produced by proposed protocol in the dissertation (chapter 4) is being investigated in collaboration with Dr. Kelber in chemistry. Initial XPS and SEM results are promising (chapter 4). Further studies on exactly determining efficiency of AuNPs for catalysis need to be pursued in detail. In conjunction with catalysis, hydrogels are very famous as nanoreactors,\textsuperscript{24} heterogeneous catalysis in aqueous phase is very exciting.\textsuperscript{24,25} Hybrid hydrogels or polymer solutions (AuNPs doped) can be utilized to carry catalysis in aqueous medium. Non-hindrance from extra chemicals like surfactants and reducing agents in solution provides additional advantage for utilizing AuNPs produced by proposed protocol compared to existing AuNPs in literature. Feasibility of more than one type of template to stabilize particles also provides more options.
6.3.4.5 Syntheses of Bimetallic Nanoparticles (Au/Ag, Au/Cu, Cu/Ag)

Bimetallic nanoparticles are expected to surpass monometallic nanoparticles in some aspects due to strong synergic effects from individual counterparts. In bi-metallic nanoparticles not only size but composition also plays a determinant role in dictating optical and electronic properties based on alloy/core-shell type nanostructures. They offer tunability in the sense that the metal composition and NP structure can be varied to optimize properties. One of the most promising areas of research for these bi-metallic nanoparticles is their unique plasmonic properties and much higher catalytic activity than corresponding monometallic counterparts especially at particular molecular ratios of both elements as seen for example in Au:Cu systems\textsuperscript{26}. Though, there are many chemical reduction techniques for making bimetallic nanoparticles but our methodology provides more opportunities to explore different combinations of noble metals due to simple syntheses route that avoids any extra chemicals or purifications. Using light or heat sensitive salts of different noble metals, we can easily produce alloy or core-shell bi-metallic nanoparticles. Our preliminary results shown in figure 6.2 on Au-Ag bimetallic nanoparticles is very encouraging for extending in to different combinations of noble metals.
Figure 6.2  Preliminary results on formation of Au-Ag bimetallic nanoparticles. A) TEM images of bimetallic nanoparticles sample. B) EDAX spectrum for the same.

6.3.5  Utilizing Antibacterial Properties of Nanosilver for Making Bacterial Resistant Fibers and Films

Nanosilver doped bacterial resistant films and fibers are already investigated in food, packing and fiber industry. Nanosilver <20 nm are more actively synthesized silver nanoparticles for their strong ability to exhibit antibacterial properties. However, as discussed in chapter 5, simple photolysis, absence of any reducing agent, stable particles even at physiological pH puts the protocol proposed in chapter 5 ahead of existing literature methods. In case of doping silver nanoparticles in to fabric materials extensive studies, using three different fiber materials (Nylon, Polyurethane and
Polyacrylonitrile) are already being investigated in collaboration with E-spin technologies. Initial studies have shown retention of AgNPs in the fibers even after five hours of hot water wash or washing with regular detergents. This is extremely important as existing reports on fibers deposited with nanosilver never reported washing cycle's before.\textsuperscript{27,28} This methodology can also be applied to coatings, food packing or sterilizing surgical components.\textsuperscript{12}

![Diagram of multifunctional nanodevices](image_url)

**Figure 6.3** Schematic illustration of multifunctional nanodevices predictably useful for detecting, diagnosis and killing cancer cells.

### 6.3.6 Designing Multifunctional Nanodevices

Multifunctional nanoparticles have attracted enhanced interest during recent decades due to their increasing use as biosensors and biomarkers. Such nanomaterials possess a combination of properties that do not exist in single-phase materials. For
example, engineering compact imaging probes with highly integrated modalities is a key focus in bionanotechnology and will have profound impact on molecular diagnostics, imaging and therapeutics. However, combining multiple components on a nanometer scale to create new imaging modalities unavailable from individual components has proven to be challenging. Many such systems like core-shell particles of magnetic oxide and gold are developed to enhance imaging techniques combined with MRI (magnetic resonance imaging) property of iron oxide particles and scattering or photoacoustic properties of gold nanoparticles. Similarly gadolinium based chelates are also coupled with AuNPs for making multifunctional nanoprobes.\textsuperscript{29,30} With significant work on syntheses of stable metal based phosphorescent complexes from Omary group, and environmental sensitive polymer microspheres from Hu group it would not be very difficult to realize syntheses of multifunctional nanoprobes as designed in figure 6.3. Once realized, these multifunctional nanoprobes are expected to have all features to detect, diagnose and kill cancer cells.

6.4 References


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