Rational Design of Self-Assembled Nanostructures Based on Polymers Synthesized via Aqueous Reversible Addition-Fragmentation Chain Transfer Polymerization

Stacey Kirkland York
University of Southern Mississippi

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RATIONAL DESIGN OF SELF-ASSEMBLED NANOSTRUCTURES BASED ON POLYMERS SYNTHESIZED VIA AQUEOUS REVERSIBLE ADDITION-FRAGMENTATION CHAIN TRANSFER POLYMERIZATION

by

Stacey Kirkland York

Abstract of a Dissertation Submitted to the Graduate School of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2010
ABSTRACT

RATIONAL DESIGN OF SELF-ASSEMBLED NANOSTRUCTURES BASED ON POLYMERS SYNTHESIZED VIA AQUEOUS REVERSIBLE ADDITION-FRAGMENTATION CHAIN TRANSFER POLYMERIZATION

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Recent advances in reversible addition-fragmentation chain transfer (RAFT) polymerization have allowed the rational, bottom-up design of biorelevant assemblies. Utilizing foresight, polymers can be tailored to self-assemble into nano-, micro-, and macroscopic structures. Given the size scale on which rationally-designed polymers can be tailored, they hold significant promise in the biomedical field. For example, nanoscale materials can be designed to carry small-molecule and gene therapeutics while macroscopic structures can be tailored for cell growth scaffolds. The design process begins by selecting monomers, chain transfer agents, and reaction conditions which will yield the desired polymer architecture and composition. The work herein builds on these concepts and may be divided into three sections.

In the first section, the synthesis of narrowly dispersed, temperature-responsive BAB block copolymers capable of forming physical gels under physiological conditions is described. A difunctional trithiocarbonate was utilized in the aqueous reversible addition fragmentation chain transfer (RAFT) polymerization of the BAB block copolymer, allowing a two-step synthetic approach to obtain a triblock copolymer with symmetrical outer blocks. The outer B blocks of the triblock copolymers consist of
poly(N-isopropylacrylamide) (P(NIPAM)) and the inner A block consists of either poly(acrylamide) (P(AM)) or poly(N,N-dimethylacrylamide) (P(DMA)). The copolymers form reversible physical gels above the phase transition temperature of P(NIPAM) at concentrations as low as 7.5 wt% copolymer. Mechanical properties similar to that of collagen, a naturally occurring polypeptide used as a three dimensional in vitro cell growth scaffold, have been achieved. The mechanical properties of the gels as a function of solvent, polymer concentration, and inner block length are discussed. Structural information about the gels was obtained through pulsed field gradient NMR experiments, confocal microscopy, and small angle x-ray microscopy.

In the second section, the reversible formation of ordered physical gels from stimuli-responsive ABA [A=P(DMA), B= P(NIPAM))] triblock copolymers is investigated utilizing dynamic light scattering, small angle x-ray scattering, and low-shear rheometry. As the temperature is increased above the phase transition temperature of the P(NIPAM) segment, triblock copolymers under a critical molecular weight are capable of packing into body-centered cubic arrays. Rheometric tests indicate that the storage moduli of the gels at 50 ºC are inversely related to the molecular weight of the polymer. In addition, cyclic heating of polymer solutions demonstrates the fast, reversible nature of the physical gelation.

In the third section, the facile synthesis of polymer-stabilized Au nanoparticles (AuNPs) capable of forming neutral, sterically stable complexes with small interfering RNA (siRNA) is reported. The amine-containing cationic block of poly(N-2-hydroxypropyl methacrylamide-block-N-[3-(dimethylamino)propyl] methacrylamide) was utilized to promote the in situ reduction of Au³⁺ (NaAuCl₄) in solution to Au⁰ (Au
nanoparticles). Subsequently, this nanostructure was utilized to bind siRNA while the nonimmunogenic, hydrophilic block provided steric stabilization. Significant protection against nuclease was demonstrated by enzymatic tests while gene down-regulation experiments indicated successful delivery of siRNA to cancerous cells.
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<td>A549</td>
<td>human lung cancer</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilino-1-naphthalenesulfonic acid</td>
</tr>
<tr>
<td>APMA</td>
<td>N-3-aminopropyl methacrylamide</td>
</tr>
<tr>
<td>ATRP</td>
<td>atom transfer radical polymerization</td>
</tr>
<tr>
<td>AuNPs</td>
<td>Au nanoparticles</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CMP</td>
<td>2-(1-carboxy-1-methylethylsulfanylthiocarbonylsulfanyl)-2-methylpropionic acid</td>
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<tr>
<td>CTA</td>
<td>chain transfer agent</td>
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<tr>
<td>CTP</td>
<td>4-Cyanopentanoic acid dithiobenzoate</td>
</tr>
<tr>
<td>CVF</td>
<td>critical volume fraction</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine-3</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DMA</td>
<td>N,N-dimethylacrylamide</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>N-2-(diethylamino)methyl methacrylate</td>
</tr>
<tr>
<td>DMAPMA</td>
<td>N-3-dimethylaminopropyl methacrylamide</td>
</tr>
<tr>
<td>DEAEMA</td>
<td>N-2-(diethylamino)ethyl methacrylate</td>
</tr>
<tr>
<td>DEGA</td>
<td>di(ethylene glycol)ethyl ether acrylates</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DPAEMA</td>
<td>N-2-(diisopropylamino)ethyl methacrylate</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeability and retention</td>
</tr>
<tr>
<td>FRP</td>
<td>free radical polymerization</td>
</tr>
<tr>
<td>GLuc</td>
<td>Gaussia Luciferase</td>
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<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
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<tr>
<td>HbC</td>
<td>hydrophilic-(b)-cationic</td>
</tr>
<tr>
<td>HbS</td>
<td>hydrophilic-(b)-stimuli-responsive</td>
</tr>
<tr>
<td>HELA</td>
<td>human cervical cancer</td>
</tr>
<tr>
<td>HEMA</td>
<td>N-2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HPMA</td>
<td>N-2-hydroxypropyl methacrylamide</td>
</tr>
<tr>
<td>IMP</td>
<td>iniferter-mediated polymerization</td>
</tr>
<tr>
<td>IPEC</td>
<td>interpolyelectrolyte complexes</td>
</tr>
<tr>
<td>KB</td>
<td>human epidermal cancer</td>
</tr>
<tr>
<td>MADIX</td>
<td>macromolecular design via the interchange of xanthates</td>
</tr>
<tr>
<td>MPC</td>
<td>methacryloyloxyethyl phosphorylcholine</td>
</tr>
<tr>
<td>MPS</td>
<td>mononuclear phagocytic system</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAS</td>
<td>N-acryloxysuccinimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NIPAM</td>
<td>N-isopropyl acrylamide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NMS</td>
<td>N-methacryloxysuccinimide</td>
</tr>
</tbody>
</table>
NT  nucleotide
NVP  \textit{N}-vinyl pyrrolidone
PDI  polydispersity Index
PDSM  pyridyl disulfide ethyl methacrylate
PEG  poly(ethylene glycol)
PEO  poly(ethylene oxide)
PHD  poly(2-hydroxyethyl methacrylate-s-2-(diethylamino)methyl methacrylate)
RAFT  reversible addition-fragmentation chain transfer
RISC  ribonucleic acid induced silencing complex
RNA  ribonucleic acid
RNAi  ribonucleic acid interference
SAXS  small angle x-ray scattering
SFRP  stable free radical polymerization
siRNA  small interfering ribonucleic acid
SH-ONs  thiolated oligonucleotides
SKOV3  human ovarian cancer
ssRNA  single-stranded ribonucleic acid
V-501  4,4′-azobis(4-cyanopentanoic acid)
VA-044  2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride
CHAPTER 1

INTRODUCTION

Rational Design of Biorelevant Assemblies

Recent advances in controlled radical polymerization (CRP) have allowed the rational bottom-up design of biorelevant assemblies. Utilizing foresight, polymer block length and side-chain functionality can be tailored to allow self-assembly under specific conditions. As seen in Figure I-1, the judicious choice of monomer and polymer block lengths can lead to a nanoassembly capable of inducing microscopic and macroscopic responses. Given their high potential impact, the design of polymers for biomedical applications, such as the theranostic nanocarrier (top) and temperature-responsive gel (bottom) in Figure I-1, is of significant interest.

The design process begins by selecting monomers (Figures I-2, I-3), architecture, and composition which will have the requisite properties for the desired application. Of particular interest are polymers which assemble into nanostructures capable of producing microscopic and macroscopic change. For example, the theranostic nanocarrier illustrated in Figure I-1 (top) is assembled via electrostatic interactions between gene therapeutic (yellow) and the polymeric stabilizing ligands (blue/red) coordinated to the Au surface (pink). By simultaneously delivering the diagnostic gold nanoparticle and gene therapeutic to cancer cells, real-time monitoring of treatment progression is realized. The temperature-responsive gel (Figure I-1, bottom) forms as a result of the change in hydrophilicity of the “light grey” block. In dilute solution micelles form via the hydrophobic association of temperature-responsive blocks from multiple chains. Gelation of the polymer solution occurs (macroscopic response) once a critical concentration of
micelles is reached. By controlling polymer block length, important gel properties, such as gelation temperature and modulus, can be targeted. In the subsequent sections background information on self-assembling block copolymers, cell growth scaffolds, gold nanoparticles, and gene delivery vehicles will be presented to lay the foundation for the rationale of the thesis work.

Figure I-1. The bottom-up rational design of a theranostic nanocarrier (top) and temperature-responsive gel (bottom). The top nanoassembly is capable of delivering gene therapeutics to cancerous tissue resulting in suppression of tumor growth. The bottom depiction represents a system which is forms macroscopic structures in response to changes in temperature.
Figure 1-2. A selection of neutral (M1-M4), temperature-responsive (M5-M8), and activated monomers (M9-M12) copolymerized via CRP techniques.
Figure I-3. A selection of cationic (M13-M20), anionic (M21-M25), and zwitterionic (M26-M27) copolymerized via CRP techniques. Counterions are not shown.
Self-Assembled Block Copolymers

Hydrophilic-\textit{b}-hydrophobic copolymers can self-assemble into advanced architectures when dissolved in a solvent selective for one block. However, direct dissolution into a selective-solvent often produces large, inhomogeneous aggregates or precipitation, and thus an alternate route must be used to obtain self-assembled structures. An often-used alternative involves the dissolution of the polymer in a common solvent (i.e. dioxane/water mixture) and subsequent dialysis against a selective solvent (i.e. water). However this process is tedious and can often take several days to accomplish.

In order to quickly and reversibly form self-assembled structures in aqueous solution, a large number of research groups have focused on hydrophilic-\textit{b}-stimuli-responsive (\textit{HbS}) copolymers.\textsuperscript{1,2} In contrast with hydrophilic-\textit{b}-hydrophobic copolymers which require indirect dissolution methods, \textit{HbS} copolymers can be dissolved directly in water. Stimuli commonly used to induce changes include pH, salt, and temperature. Most stimuli-responsive block copolymers have been synthesized by RAFT or ATRP polymerization due to versatility in monomer selection and mild reaction conditions these techniques offer.

The first successful pH-responsive block copolymers synthesized by aqueous RAFT polymerization were reported in 2001 by our laboratory.\textsuperscript{3} A poly(sodium styrene sulfonate) (PSS) (\textsuperscript{M22}) macroCTA was successfully chain extended with sodium 4-vinylbenzoic acid (VBA) (\textsuperscript{M21}) yielding a pH-responsive block copolymer. At low pH, the carboxylic acid groups of PVBA are protonated rendering the PVBA block hydrophobic and thus leading to micellization. Because pH is utilized to induce micellization, association is fully reversible in aqueous solution. The reversible
micellization monitored by dynamic light scattering (DLS) indicated individual block copolymer chains (unimers) with hydrodynamic diameters of 8 nm at high pH and micelles at low pH with hydrodynamic diameters of 38 nm. Other pH-responsive block copolymers synthesized by RAFT include poly(sodium 2-acrylamido-2-methylpropanesulfonate-\textit{b}-sodium3-acrylamido-3-methylbutanoate) (PAMPS-\textit{b}-PAMBA) (M25-\textit{b}-M24),\textsuperscript{4,5} poly(N,N-dimethylacrylamide-\textit{b}-N,N-dimethylbenzylvinylamine) (PDMA-\textit{b}-PDBVA) (M2-\textit{b}-M18),\textsuperscript{6} poly(2-vinylpyridine-\textit{b}-4-vinylpyridine) (P2VP-\textit{b}-P4VP) (M19-\textit{b}-M20),\textsuperscript{7} and poly(N,N-dimethylacrylamide-\textit{b}-[N-isopropylacrylamide-s-N-acryloylvaline]) (PDMA-\textit{b}-[PNIPAM-s-PAVAL]) (M19-\textit{b}-(M5-s-M23)).\textsuperscript{8}

A number of thermoresponsive polymers have been studied as potential pharmaceutical delivery agents. Most thermoresponsive polymers are synthesized from \textit{N}-alkyl acrylamides, the most researched being P(NIPAM) (M19) since it possesses a lower critical solution temperature (LCST) of 32 °C, close to physiological temperature, 37 °C. Above the LCST, P(NIPAM) becomes hydrophobic, a result of a gain in entropy due to the disruption of the water shell associated with the isopropyl groups of NIPAM. For detailed information, the reader is directed to Appendix I and to the work of Winnik and coworkers\textsuperscript{9-12} who have published extensively on the behavior of conventionally and RAFT-polymerized P(NIPAM). Other thermoresponsive polymers include poly(\textit{N}-n-propylacrylamide) (PnP) (M6), poly(\textit{N}-acryloylpiperidine) (PNAP) (M7), and poly(\textit{N}-acryloylpyrrolidine) (PNAPy) (M8).

In 2000, Ganachaud and coworkers\textsuperscript{13} reported the RAFT polymerization of NIPAM in 1,4-dioxane. Subsequently a large number of reports of RAFT polymerization
of this monomer in organic media with various CTAs have appeared.\textsuperscript{14} Using an appropriate CTA and initiator, we reported the first room temperature RAFT polymerization of NIPAM directly in water, utilizing a diazo initiator and either a monofunctional or difunctional chain transfer agent.\textsuperscript{15} Significantly, this procedure allows polymerization at temperatures below the LCST of P(NIPAM) and thus prevents polymer aggregation. In addition we have prepared di- and triblock copolymers by first polymerizing the hydrophilic monomer DMA (M\textsubscript{2}) and subsequently chain extending the resulting macroCTA with NIPAM (M\textsubscript{5}).\textsuperscript{15} ABA triblocks and AB diblocks were synthesized in two steps using a difunctional and monofunctional CTA, respectively.\textsuperscript{15} The micellization behavior of the block copolymers was studied via DLS and static light scattering (SLS). It was found that as the NIPAM block length increased, the critical micelle temperature (CMT) decreased.

Oupicky and coworkers\textsuperscript{16} reported the temperature-mediated association behavior of heterobifunctional copolymers in aqueous solution. Poly(ethylene glycol)-\textit{b}-P(NIPAM) (PEG-\textit{b}-M\textsubscript{5}) block copolymers were synthesized via organic RAFT polymerization utilizing a PEGylated CTA. After the successful polymerization of NIPAM, the thiocarbonylthio moiety of the CTA was reduced using excess hexylamine and the resulting thiol was then conjugated to maleimide derivatized biotin. The binding efficiency of biotin with avidin was monitored at temperatures below and above the LCST of the block copolymer. Above the LCST, it was found that biotin is less accessible to interact with avidin, while below the LCST biotin is readily available. It is noteworthy that such temperature-responsive systems allow the selective presentation of ligands that may be beneficial in biomedical applications. Several other research groups
including Yusa and Morshima,\textsuperscript{17} Liu and Perrier,\textsuperscript{18} and Voit et al.\textsuperscript{19} have reported P(NIPAM)-based block copolymers for temperature controlled assembly.

While temperature and pH-responsive micelles have received significant attention due to their ability to sequester and deliver small molecule hydrophobic drugs, a wide range of opportunities exist for other self-assembled structures. For example, physical gels can be formed through intermicellar bridging between adjacent micelles. In the subsequent section, the potential application of physical gels as cell growth platforms is described.

\textit{Cell Growth Platforms}

For many years researchers have cultured cells in order to synthesize biological products, study metabolic pathways, and test drug efficacy.\textsuperscript{20} One of the major culture media is the petri dish, a shallow polystyrene dish rendered hydrophilic through plasma treatment or other methods.\textsuperscript{21, 22} The petri dish is often used because it is cheap, convenient, and established throughout the literature. Despite its advantages, research suggests that cells grown in a two dimensional environment often exhibit anomalous behavior and therefore result in incongruous results between \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{23-28}

While the use of petri dishes remains widespread, there is significant research aiming to develop new three dimensional cell growth platforms. These new materials aim to replicate the three dimensional environment that surrounds cells in tissues, called the extracellular matrix (ECM). The major structural component of the ECM, collagen, has a rod-like structure with a length of approximately 300 nm and a diameter of 1.5 nm.\textsuperscript{29, 30} The mechanical strength, porosity, and chemical functionality of the
extracellular matrix provide an environment conducive to cell growth. Mechanical strength can govern such processes as cell differentiation, migration, organization, and morphology.\textsuperscript{31} Porosity can dictate cell migration and the diffusion of nutrients through the matrix.\textsuperscript{32} With few exceptions, cells must be anchored to a surface to maintain normal cell functions. Once anchorage is lost, cells undergo programmed cell death known as apoptosis. While cells can naturally adhere to certain materials through nonspecific interactions, ECM adhesion ligands may be incorporated into the material to facilitate anchoring of specific cells.\textsuperscript{33}

A number of natural and synthetic-based three dimensional matrices which address the above issues have been developed.\textsuperscript{33-35} Biologically-derived materials are the most common; however, problems with immunogenicity, batch consistency, and gelation procedure have prompted the continued research on synthetic scaffolds.\textsuperscript{36} Synthetic polymeric gels are an attractive alternative to naturally-derived materials because of the wide variety of available functional monomers. For example, Anseth and coworkers have photopolymerized ethylene glycol-based monomers to obtain gels with tunable mechanical properties and pore sizes.\textsuperscript{37} In addition, cell-binding domains were incorporated into these networks in order to increase cell viability. Though covalently cross-linked gels have been successfully used as three dimensional cell growth platforms, the irreversible nature of the network prevents the removal of viable cells. While not an issue for all cell growth experiments, in certain situations it would be advantageous to remove viable cells/tissue from the matrix for further studies. Thus researchers have begun investigating systems which reversibly gel in response to a mild stimulus.
**BAB Physical Gels.** Physical gels from BAB triblock copolymers, where the A block is permanently hydrophilic and the B blocks have stimuli-dependent hydrophilicity, have been investigated for potential cell growth platforms because of the noncovalent, reversible nature of the cross-links (Scheme I-1). Typically, a temperature or pH sensitive B block is incorporated for cell growth applications. As illustrated in Scheme I-1, the morphology depends on both the stimulus and polymer concentration. The triblock copolymer exists as unimers when both polymer components are soluble. As a stimulus is applied, the outer B block is rendered hydrophobic and either flower-like micelles or gels can form. At low concentrations, micelle formation dominates whereas at moderate to high concentrations of polymer, physical cross-links can form when the polymer is placed in a solvent selective for the inner block. Physical networks may also form through the entangling of adjacent micelle coronas.  

48, 49
Scheme I-1. The reversible formation of self-assembled structures in aqueous solution from BAB triblock copolymers where the B block is stimuli-responsive and the A block is permanently hydrophilic. At low polymer concentration flower-like micelles dominate whereas at high polymer concentration physical networks form as the result of B blocks of a single unimer residing in different hydrophobic cores.

The formation of a physical network is dependent on the relative populations of “loops” and “bridges”.50, 51 The inner block forms a bridge when the outer blocks of a polymer are incorporated into different hydrophobic domains, while a loop forms when both blocks of a chain are incorporated into the same hydrophobic domain. Loop formation is limited by the entropically unfavorable conformational constraints that are imposed on the system as the inner block attempts to back fold on itself.46, 52-54

Bridge formation may also be entropically unfavorable if the inner block of the polymer chain must adopt a stretched conformation in order to span between hydrophobic domains. Thus, bridging is not favorable at low polymer concentrations. Another structural possibility, dangling chain ends, is limited by the increase in interfacial free
energy which occurs as the chain end is placed in an incompatible solvent.\(^{46, 51}\) As previously reported, exchange rates between unimers and micellar structures decrease as the hydrophobicity of the lipophilic block is increased.\(^{55-57}\) Using Monte Carlo simulations, the formation of loops, dangling chain ends, and bridges in symmetric BAB triblock copolymers was studied by Mattice \(et\) \(al\).\(^{48}\) The results indicate that the existence of dangling chain ends in physical gels should decrease as the solvent becomes more incompatible with the outer block.\(^{48}\) Further theoretical treatments on the mechanism and driving forces behind the physical gelation of associating polymers may be found in the literature.\(^{58-65}\)

Until recently, research in this area had been limited by techniques available to produce well-defined amphiphilic triblock copolymers. The advent of controlled/\('\)living\(')\ free radical techniques offers new routes to obtain polymers of complex architecture with a wide range of monomers.\(^{66-72}\) In addition, CRP provides narrowly dispersed polymers of controlled molecular weight. The ability to control the molecular weight of individual block lengths while maintaining narrow polydispersity affords unique opportunities to study structure-property relationships of (co)polymers. For this reason, polymers synthesized via controlled/\('\)living\(')\ free radical techniques may have numerous high impact applications in the biomedical field.

Armes \(et\) \(al\). have synthesized a number of BAB triblock copolymers via atom transfer radical polymerization (ATRP) that physically gel in response to changes in pH or temperature.\(^{38, 41-43, 45}\) Several of the copolymer gels showed promise in applications such as cell growth scaffolds\(^{42}\) and controlled release substrates.\(^{43}\) For example, polymers containing either 2-(diisopropylamino)ethyl methacrylate (DPAEMA, \(M_{17}\)) or 2-
(diethylamino)ethyl methacrylate (DEAEMA, M16) pH-responsive B blocks and methacryloyloxyethyl phosphorylcholine (MPC, M27) a permanently hydrophilic A block have been synthesized. The P(DPAEMA-b-MPC-b-DPAEMA) block copolymer forms a free standing gel when the pH is increased above 7.4. While the MPC block confers biocompatibility to the system, cell viability substantially decreases as pH deviates from physiological pH (~7.4). Thus, gels formed at pH values higher than 7.4 are not practical. There are similar practical considerations for temperature-responsive gels which require the gel to be stable at physiological temperatures (37 °C) while reverting to the sol state under cell-friendly conditions.

Extensive research has been conducted on NIPAM-based polymers (M5) for biomedical applications such as cell growth scaffolds due to the lower critical solution temperature it exhibits at 32 °C (See Appendix I). While P(NIPAM) has been widely used in chemically cross-linked polymer gels, the development of reaction conditions for the controlled polymerization of the monomer opened the door to a new class of temperature-responsive physical assemblies. In 2000, Ganachaud and coworkers reported the RAFT polymerization of NIPAM. Subsequent work by Convertine and coworkers led to the synthesis of P(NIPAM) homo- and block copolymers at room temperature in dimethylformamide and water.

In 2005 Armes and coworkers synthesized a series of P(NIPAM-b-MPC-b-NIPAM) (M5-b-M27-b-M5) copolymers via ATRP. However, gel permeation chromatography (GPC) analyses were not conducted and thus definitive control of the polymerization could not be stated. In order to determine whether the material could gel, tube inversion and rheometric tests were performed. An 8 wt% solution increased in
viscosity from 0.1 Pa·s$^{-1}$ to 100 Pa·s$^{-1}$ as the temperature was increased from 20 to 50 ⁰C. Tube inversion test indicated the material was capable of forming free standing gels at elevated temperatures. While these materials have shown promise for 3-D cell growth applications, the ability to fully characterize the polymers restricts the usefulness because of the inability to determine batch-to-batch consistency and to draw useful conclusions on the effect of block lengths on material properties.

In 2009 Sumerlin and coworkers reported the synthesis of temperature and redox responsive hydrogels based on P(NIPAM-$b$-DMA-$b$-NIPAM) (M$_5$-$b$-M$_2$-$b$-M$_5$) and P(NIPAM-$b$-(di(ethylene glycol)ethyl ether acrylate)-$b$-NIPAM) (PDEGA). A symmetrical difunctional chain transfer agent was utilized in the synthesis of the polymers, providing a redox-sensitive trithiocarbonate core. Physically cross-linked networks formed due to intermicellar bridging of polymer chains (Scheme I-2). Subsequently, a primary amine containing small molecule was introduced resulting in dissolution of the gel due to cleavage of bridges. Introduction of an oxidizing agent allowed reformation of a gel through the cross-linking of micelles via disulfide linkages. While rheometric tests were not conducted on the materials and thus conclusions could not be drawn about the difference in mechanical properties of trithiocarbonate and disulfide-based gels, the report demonstrates the utility of the chain transfer agent in the reversible formation of micellar gels. It should be noted that samples with short inner blocks such as P(NIPAM$_{102}$-$b$-DMA$_{123}$-$b$-NIPAM$_{102}$) (M$_n$=35300 g mol$^{-1}$) did not form gels unless greater than 50 wt% polymer was added. Conversely, polymers with substantially longer inner blocks (P(NIPAM$_{102}$-$b$-DMA$_{824}$-$b$-NIPAM$_{102}$) were able to form gels at approximately 10 wt% polymer.
Scheme I-2. Reversible formation of physically cross-linked redox sensitive gel based on either P(NIPAM-b-DMA-b-NIPAM) or P(NIPAM-b-PDEGA-b-NIPAM). Structures apply when the temperature is greater than the LCST.\textsuperscript{75}
While the previously described examples demonstrate the potential utility of BAB polymers as cell growth scaffolds, these studies either completely lacked rheological testing or only had minimal studies. Furthermore, it is difficult to draw conclusions about the applicability of the *in vitro* use of these materials because DI H\textsubscript{2}O was utilized as a solvent in the majority of cases. Because cells are supported in a saline environment and it is known that salt affects the behavior of P(NIPAM) and polyzwitterions like PMPC, testing in salt solutions is needed to draw meaningful conclusions.

*ABA physical gels.* In contrast with BAB block copolymers which form gels due to intermicellar bridging, the architecture of AB diblock and ABA triblock copolymers require an alternate mode of gelation. Several research groups have determined that AB and ABA block copolymers form physical gels through the packing of micelles (Scheme I-3).\textsuperscript{76-85} As illustrated in Scheme I-3, star-like micelles form at low concentration and ordered or disordered gels form at high concentrations of polymer. Several groups have demonstrated the importance of the PEO block length and polymer concentration on the ordering and gelation of such systems.\textsuperscript{77, 79, 86} For example, face-centered cubic (FCC) gels require a critical volume fraction (CVF) of 0.74 while body-centered (BCC) gels require a (CVF) of 0.68.\textsuperscript{87} Furthermore, Hamley and coworkers reported that crew-cut micelles were more likely to form FCC gels while hairy micelles were more likely to form BCC gels.\textsuperscript{88} As an empirical relationship, Hamley found that FCC lattices formed at low concentration of polymer when the ratio of the water-soluble block to organic-soluble block (W:O) was $< 10$. Conversely, BCC lattices formed at high concentrations or at low concentrations when W:O $> 10$.\textsuperscript{87}
Historically, most micellar gels have been based on poly(ethylene oxide) (PEO) diblock copolymers. For example, Jérôme et al. synthesized the CBA triblock copolymer poly(styrene)-b-poly(2-vinylpyridine)-b-PEO via anionic polymerization. The poly(2-vinylpyridine) block was utilized as a pH-responsive block capable of altering gel properties. Given the hydrophobicity of the polystyrene block, gels could not be formed directly in H₂O, but rather required polymer dissolution in DMF and subsequent dilution with H₂O to induce gelation. At acidic pH values, hard gels were formed (1.4 kPa) while soft, free-flowing gels were formed under basic pH conditions (0.65 kPa).

Although most biomedically relevant ABA gels contain PEO, recent advancements in CRP techniques have provided a route for synthesizing a diverse set of stimuli-responsive vinyl-based polymers. For example, Loo et al. prepared a series of triblock copolymers comprised of poly(styrene) outer blocks and a poly(2-
hydroxyethyl methacrylate-s-DMAEMA) (M₄-s-M₁₅) inner block.⁹² The triblock copolymers formed lamellar morphologies in the solid state. When swollen in water, the copolymers maintained a lamellar nanoscopic morphology, but lost macroscopic integrity, thus indicating susceptibility to dilution effects.⁹² Additionally, diblock copolymers comprised of PHD and polystyrene capable of forming an array of morphologies (disordered, lamellar, and hexagonally packed cylinders) in response to temperature were synthesized.⁹³ Swelling characteristics could be tuned by adjusting the DMAEMA content.⁹³ Although these materials are interesting, gelation does not occur in biologically relevant conditions.

As described in the above sections, gel properties are highly dependent on monomer selection, block length, and architectures. Thus the demonstrated ability to control these requisite properties through the bottom-up design of polymers utilizing the RAFT technique would represent significant progress over presently available procedures. Likewise, the rational design of polymers has significant potential to advance other areas of biomedical research, such as gene delivery.

**Gene Delivery Vehicles**

The *modus operandus* of small interfering RNA (siRNA), down-regulation of a target messenger RNA via the RNA interference (RNAi) pathway, provides a promising route for treatment of numerous diseases.⁹⁴,⁹⁵ In the RNAi pathway (Scheme I-4), a double-stranded RNA (or single-stranded RNA which back-folds on itself to form a “hairpin” duplex) is cleaved into the appropriately sized siRNA effector molecule (usually 21-23 base pairs) by an enzyme known as DICER. Following cleavage by DICER, the antisense strand of siRNA is delivered to an assembly of endoribonucleases
to form a complex known as RISC (RNA-induced silencing complex). RISC containing the antisense strand of siRNA binds with a messenger RNA (mRNA) containing a complementary sequence and ultimately cleaves the targeted gene. Thus down-regulation of virtually any protein may be achieved by designing and delivering a specific siRNA sequence to target a specific gene. However, inefficient delivery, cell specificity, and limited stability of siRNA has limited the potential of this gene therapeutic.\textsuperscript{96}

Scheme I-4. General mechanism for siRNA-mediated gene silencing via the RNA interference pathway. A double-stranded RNA is cleaved by an enzyme known as DICER. The cleaved antisense strand forms a RNA-induced silencing complex (RISC) which is able to bind and cleave the complementary target mRNA. Cleavage of the mRNA results in the down-regulation of protein expression.

Given the promise of this therapeutic, a significant emphasis has been placed on the development of oligonucleotide carriers. While viral carriers offer promise, concerns over toxicity, cell specificity, and manufacturing costs have prompted the investigation of non-viral carriers.\textsuperscript{97-100} Non-viral systems have been conventionally divided into two general categories, liposome and polymer-based carriers, however inorganic
nanoparticles are emerging as a useful class of vehicles. Though liposomes meet several of the prerequisites for effective delivery, deficiencies such as partitioning, physiological instability, and suboptimal pharmokinetics have precluded their widespread use.

A number of polymer-based vehicles have been explored for delivery-based applications including polymer prodrugs, micelles, and nanoparticles. These carriers are designed to target genes as effectively as viral vectors, while avoiding non-specific toxicity and immunogenicity issues. In addition, polymeric carriers must overcome several extra- and intracellular barriers, as delineated in Table I-1. Overcoming extracellular barriers ensures the gene and carrier have sufficient time, stability, and specificity to reach the intended tissue. Additionally, the circulation half-life can be increased by designing a carrier with a neutral surface charge and by increasing the molecular weight to above the renal threshold (30-50 kDa). Equally critical is the ability to overcome intracellular barriers, which permits the gene to be delivered to the cell and released from the carrier. The development of novel polymers that can efficiently overcome extra- and intracellular barriers has been heavily pursued by the research community.
Table I-1. Extra- and Intracellular Barriers Encountered by Gene Delivery Vehicles.

<table>
<thead>
<tr>
<th>Extracellular Barriers</th>
<th>Intracellular Barriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum stability</td>
<td>Endosomal release</td>
</tr>
<tr>
<td>Sufficient circulation half-life</td>
<td>Cytoplasmic transport</td>
</tr>
<tr>
<td>Specific cellular binding</td>
<td></td>
</tr>
</tbody>
</table>

Carriers can reach the intended delivery site either passively or actively. In passive targeting of cancer tissue, carriers rely on the enhanced permeability and retention (EPR) effect.\textsuperscript{111-118} The EPR effect (Scheme I-5) is a consequence of the poorly developed vasculature and lymphatic systems found in cancerous tissue. Enhanced permeation occurs because capillaries around tumors are “leaky” and allow macromolecules to escape from the vasculature to the interstitium while retention results from the inability of tumor tissue to remove macromolecules due to poor lymphatic drainage.\textsuperscript{111,119} Because normal tissue does not permit vascular permeation of macromolecules and is able to sufficiently drain macromolecules through the lymphatic system, accumulation of macromolecules occurs primarily at the tumor site.
Scheme I-5. Anatomical characteristics of normal and tumor tissue with respect to the enhanced permeation and retention effect.

Actively-targeted systems rely on a moiety which recognizes specific cellular surface features, resulting in the preferential association of the gene carrier with the desired cell. As illustrated in Figure I-4, actively targeted polymeric carriers have a targeting moiety and therapeutic material conjugated to a water-soluble polymeric carrier. Although Ringsdorf originally proposed this model in the 1970s, synthetic limitations at the time precluded design of such carriers. While the model in Figure I-4 depicts chain-end conjugation of the targeting moiety, functionalization via the side-chain is also a viable route. Common targeting moieties include folic acid, peptides, proteins, carbohydrates, aptamers, and antibodies. In addition, research indicates that actively targeted carriers are taken into the cell more efficiently than polymeric carriers lacking
targeting ligands.\textsuperscript{119, 122, 123} Recently, the McCormick group reported the synthesis of an actively-targeted siRNA prodrug. Briefly, a statistical copolymer of HPMA (M\textsubscript{3}) and APMA (M\textsubscript{14}) was synthesized by RAFT polymerization.\textsuperscript{124} A fraction of the primary amine groups from APMA was converted to activated disulfides and subsequently reacted with a thiolated RNA. The remaining APMA groups were reacted with an NHS-activated folic acid derivative. The resulting polymer could release siRNA under reducing conditions as demonstrated by gel electrophoresis studies.

\textbf{Figure I-4.} Actively-targeted polymeric prodrug consisting of a hydrophilic macromolecule with bioconjugated therapeutic and targeting moieties.

\textit{Interpolyelectrolyte complexes.} While covalent linkages can be employed to conjugate siRNA to the side-chain and end-group of polymers,\textsuperscript{125} oligonucleotides are commonly complexed via electrostatic interactions to polymeric carriers. These polymer-oligonucleotide systems, known as interpolyelectrolyte complexes (IPECs), provide protection against degradative enzymes while increasing the circulation half-life.\textsuperscript{126, 127} Charge ratio plays an important role in the stability and transfection efficiency of the IPEC and is described by an N/P ratio, where N and P respectively describe the nitrogen content from the polycation and the phosphate content from the oligonucleotide. Negatively charged complexes (N/P < 1) experience electrostatic repulsion with the anionically charged cell membrane and thus are not readily internalized. Conversely,
positively charged complexes (N/P > 1) are strongly attracted to cell membranes, and thus lack cell specificity, leading to uptake of the IPEC in both healthy and diseased tissue. Charged complexes may also adsorb serum proteins resulting in clearance by phagocytic cells.\textsuperscript{128-131} Neutral complexes (N/P=1) circumvent these problems but often have poor solubility in aqueous solution due to charge neutralization, thus limiting applications in biological systems.

Hydrophilic-\textit{b}-cationic (H\textit{b}C) copolymers offer an attractive alternative to cationic homopolymers because neutral complexes can be achieved while retaining water-solubility.\textsuperscript{1,126,128,129,132-134} Additionally, the hydrophilic block sterically stabilizes the complex. Although cationic-\textit{b}-hydrophilic copolymers can be synthesized by utilizing macroinitiators, these processes generally lead to ill-defined molecular weights and broad polydispersity indices (PDIs). Thus, a controlled polymerization technique, such as RAFT, provides an attractive route to obtain polymeric gene carriers.\textsuperscript{1}

In 2006 our group reported the controlled synthesis of poly(N-2-hydroxypropyl methacrylamide) (PHPMA, \textbf{M3}),\textsuperscript{135} a nonimmunogenic biocompatible polymer, and the subsequent chain extension with a cationic monomer, N-3-dimethylaminopropyl methacrylamide (DMAPMA, \textbf{M13}). Neutral, water-soluble complexes were formed with a 43-nucleotide small interfering ribonucleic acid (siRNA).\textsuperscript{136} Because RAFT polymerization allows precise control over block length, the effect of HPMA and DMAPMA degree of polymerization (DP) on complex stability could be studied. DMAPMA block length appeared to be the major contributing factor in protecting siRNA from enzymatic degradation.\textsuperscript{136} Because the absorbance at 260 nm of mononucleotides is greater than that of oligonucleotides, degradation of siRNA can be easily monitored by
UV-Vis spectroscopy. As shown in Figure I-5, unbound siRNA rapidly degrades in the
presence of nucleases while the siRNA complexed with P(HPMA$_{258}$-$b$-DMAPMA$_{23}$)
(M$_3$-$b$-M$_{13}$) remains stable. The initial increase in absorbance of the polymer/siRNA
system is attributed to the presence of a small amount of unbound siRNA.

![Graph](image)

**Figure I-5.** Enzymatic degradation of small interfering ribonucleic acid with RNase A in
the presence and absence of P(HPMA$_{258}$-$b$-DMAPMA$_{23}$) (M$_3$-$b$-M$_{13}$).

Further developments by York and coworkers led to the successful design of
targeted siRNA carriers. A three-component polymer was synthesized to contain 1) a
hydrophilic, nonimmunogenic block 2) reactive side functionalities and 3) a
cationic block. Components 1 and 2 were achieved by statistically polymerizing HPMA
(M$_3$) and APMA (M$_{14}$) to form a macroCTA. Chain extension of the macroCTA with
DMAPMA (M$_{13}$) yielded a [P(HPMA-$s$-APMA-$b$-DMAPMA)] carrier which was then
reacted with folic acid activated with N-hydroxysuccinimide. Subsequently, neutral
polymer/siRNA complexes could be selectively delivered to cancerous cell lines over-
expressing folate receptors. The potential of polymeric-based carriers is evidenced by the rapid developments in successful delivery of cells. While polymeric gene delivery vehicles remain a popular choice, promising research has also been conducted with siRNA loaded Au nanoparticles (AuNPs). These vehicles may provide similar stabilization as polymeric carriers, while introducing a diagnostic component.

_Au nanoparticles._ While synthetic and biological polymers are being extensively explored as carriers, a number of research groups\textsuperscript{140-146} have concurrently focused on preformed AuNPs as gene carriers. AuNPs have features which forecast utility in nanomedicine including availability in a wide variety of sizes, facile modification by a ligand-exchange process,\textsuperscript{147-151} and potential as X-ray contrast agents\textsuperscript{152}. Promising work has been reported recently in which preformed AuNPs are modified with thiolated oligonucleotides (SH-ONs) via a ligand exchange process.\textsuperscript{143-146, 153} However, this method requires the use of modified oligonucleotides capable of chelating to the Au surface (i.e. thiol) and conditions which limit the number of unexchanged ligands (excess SH-ONs). Additionally, this method results in a highly anionic surface charge resulting in the nonspecific adsorption of serum proteins (opsonization) and hence clearance by the mononuclear phagocytic system (MPS).\textsuperscript{108, 128-131, 154-157}

Nagaski and coworkers utilized PEG\textsubscript{110-\textit{b}-PDMAEMA\textsubscript{48}} to stabilize preformed AuNPs and subsequently complex DNA, RNA, and SH-RNA.\textsuperscript{105, 146, 158, 159} The PEG block provided a hydrophilic neutral shell while the PDMAEMA (M\textsubscript{15}) provided multiple ligands (tertiary amine) for coordination to the Au surface. This carrier offers two key advantages including the ability to complex unmodified RNA and presentation of a neutral surface due to the PEG shell. However, the PEG\textsubscript{110-\textit{b}-PDMAEMA\textsubscript{48}}-
stabilized AuNPs had low binding capacities, with only ~10% of the oligonucleotide feed capable of complexing to the polymer-stabilized AuNP. The poor binding capacity may be due to a large number of amines coordinated to the Au surface, limiting the number of RNA complexation sites.

In situ formed AuNPs have not yet been utilized as gene carriers; however, these materials have promise since they may circumvent binding capacity limitations reported for preformed AuNPs. Nagaski and Oiishi demonstrated the ability of amine-containing HbC polymers to reduce Au$^{+3}$ to Au$^0$ under mild conditions; however, gene complexation studies were not conducted with these materials and thus comparison with their work on siRNA complexed AuNPs cannot be made. The McCormick and Armes groups have independently demonstrated the use of HbC polymers as dual reductants and stabilizers. For example, Armes and coworkers synthesized a series of P(MPC-\textit{b}-DMAEMA) (M27-\textit{b}-M15) via ATRP which were capable of reducing Au$^{+3}$ to Au$^0$ while stabilizing AuNPs through multivalent interactions between the tertiary amine of DMAEMA and the Au surface. Control studies indicated a P(MPC) homopolymer incapable of reducing Au$^{+3}$ to Au$^0$ while P(DMAEMA) incubated in the presence of Au$^{+3}$ resulted in the formation of AuNPs. Thus, it was concluded the tertiary amine of the P(DMAEMA) was needed for the reduction. Further work conducted by Li and Smith in the McCormick group demonstrated the usefulness of the autoreduction of Au$^{+3}$ in the presence of P(DMAEMA) by “locking” self-assembled block copolymers. As illustrated in Scheme I-6, vesicles can be self-assembled from P(DMAEMA-\textit{b}-NIPAM) (M15-\textit{b}-M5) when heated to 50 °C. Addition of NaAuCl$_4$ resulted in the formation of
AuNP cross-linked vesicles which remained intact after lowering the temperature to 25 °C.

Scheme I-6. Au nanoparticle cross-linking of a self-assembled vesicle comprised of P(DMAEMA-b-NIPAM) (M15-b-M5).\textsuperscript{163}

With the rapidly growing areas of gene delivery, tissue engineering, and other biomedical fields, there is an emerging need for polymers which can be tailored for high performance applications. Recent advancements in RAFT polymerization provide an unprecedented opportunity to design polymers with specific compositions and architectures, allowing the delineation of structure-property relationships. In the subsequent section, fundamental aspects of RAFT polymerization which have allowed this technique to move to the forefront of biomaterial synthetic methods will be described.

Controlled/Living Free Radical Polymerization

Significant technological advancements in controlled/living free radical polymerization (CRP) have allowed for the development of narrowly-dispersed polymers with complex architectures. In contrast to conventional free radical polymerization, the rate of termination (R_t) is suppressed in CRP, allowing polymers of controlled molecular weights and narrow polydispersity to be synthesized.\textsuperscript{165-168} Quirk and
coworkers defined the following experimental criteria for classification of a controlled/living process:\textsuperscript{169}:

- Polymerization continues until all monomer is consumed.
- $M_n$ vs. conversion is a linear function.
- The number of polymer chains is constant.
- Degree of polymerization can be controlled by reagent stoichiometry.
- Narrowly-dispersed polymers are produced.
- Telechelic polymers are produced.

Because the rate of termination has a square dependence on the active radical concentration, the number of termination events in CRP can be limited by introducing an agent that restricts the concentration of active radicals. For successful control over molecular weight and to maintain livingness of a radical polymerization, the number of active chains must be much less than the number of dormant chains. $R_p$ is decreased due to a reduction in active radicals; as well, the number of termination events is greatly suppressed, allowing less than 10\% chains to terminate during the course of the reaction. By comparison, over 99\% of chains will terminate by either combination or disproportionation in conventional FRP. Because termination events are limited in CRP, a number of architectures have been synthesized (Figure I-6) including block, star and graft copolymers.\textsuperscript{170, 171}
Several CRP techniques exist, including iniferter-mediated polymerization (IMP), stable free radical polymerization (SFRP),\textsuperscript{172, 173} atom transfer radical polymerization (ATRP),\textsuperscript{174, 175} and RAFT polymerization.\textsuperscript{170, 176} While IMP and SFRP suffer from many drawbacks including application to a limited number of monomer families, ATRP and RAFT polymerization have not only successfully controlled the polymerization of a wide variety of monomer families, but reactions can be conducted under ambient conditions (aqueous solution, room temperature). RAFT polymerization was reported concurrently by the CSIRO\textsuperscript{177, 178} and Michelet\textsuperscript{179, 180} groups in 1998 and has since become the most versatile of the CRP techniques. As illustrated in Figure I-7, the number of papers with the keyword RAFT or MADIX (macromolecular design via
the interchange of xanthates) has increased significantly since its first report in 1998.\textsuperscript{181} The utility of RAFT polymerization, along with the demand for well-defined polymers with specific functionality, has prompted significant research in this area. The mechanism, chain transfer agent (CTA), kinetics, and polymerization conditions described below provide a fundamental knowledge-base of RAFT polymerization and reasons why this method has unparalleled versatility.

![Figure 1-7. Number of articles appearing in peer-reviewed journals with the keyword RAFT or MADIX. SciFinder Scholar accessed on November 1, 2009.](image)

The RAFT Mechanism

Initiation. As in conventional free radical polymerization (FRP), a primary radical source is required to initiate polymerization. In Step Ia of Scheme I-7, an initiating species (1) decomposes to an active radical. The active radical then adds to monomer
(M), producing a polymeric radical. A variety of initiators have been employed in RAFT polymerization, including photolytic, thermolytic, and redox sensitive species.\textsuperscript{182}

\textit{Pre-equilibrium.} Once a polymeric radical species (2) is formed, it quickly adds across the double bond of the CTA (3) resulting in an intermediate radical (4). The rate constant for the addition of (2) across the thiocarbonylthio double bond is denoted by $k_{\text{add}}$. The intermediate radical (4) may fragment to release either a radical R-group (5) or a polymeric radical (2). As shown in Scheme I-7 (IIB-C), the radical species (5 or 2) may then add across the double bond of a monomer to chain extend the polymer. The pre-equilibrium stage is completed once all R-groups have fragmented and reinitiated polymerization to form (6 or 9). The length of the pre-equilibrium stage, and the success of RAFT polymerization, depends on the relative magnitudes of $k_{\text{add}}$, $k_{\beta}$, $k_{\alpha}$, and $k_{\beta}$. In order to achieve narrowly dispersed polymers, all chains should exit the pre-equilibrium at approximately the same time.
Scheme I-7. Proposed mechanism of RAFT polymerization. (I) Initiation provides the source of free radicals necessary for polymerization. (II) The pre-equilibrium occurs until all R-groups (5) have been expelled from the intermediate radical and have reinitiated polymerization to form new propagating chains (7). (III) During the main equilibrium growing polymeric radicals reversibly add across the macroCTA (6 or 9) to form an intermediate radical species (8). (IV) Termination generally occurs through the combination of two radicals.

Main equilibrium. The main equilibrium proceeds via a degenerative chain transfer mechanism in which polymeric radical species (2 or 7) add to the macroCTA (6) to form an intermediate radical containing polymeric end groups (8). Given the identical
nature of the polymeric end-groups on the intermediate radical (8), each side has an equal probability of being expelled as a polymeric radical (2 or 7) capable of propagating with monomer. The rapid equilibrium between dormant and active species provides each chain with equal opportunity to grow, thus resulting in narrowly dispersed polymers. While the number of monomer units which are added between transfer reactions is affected by several factors including the monomer and CTA concentration, in favorable conditions less than one monomer is added between transfer reactions.\textsuperscript{183}

*Chain termination.* Coupling or disproportionation of radicals can occur within any system containing active propagating radicals. When combination is the predominant mode of termination, the number of dead chains equals half the number of initiator-derived chains; if disproportionation is the primary mode of termination then the number of dead chains equals the number of initiator-derived chains. In order to limit termination events in RAFT polymerization, a high ratio of \([\text{CTA}]_0/\text{[I]}_0\) is utilized to limit the number of active chains capable of terminating. If appropriate reaction conditions are selected, the number of dead chains should be < 5%.

*Key Aspects of RAFT Polymerization*

As with conventional free radical polymerization, RAFT polymerization may be applied to a wide number of functional vinyl-based monomers. However, key aspects of RAFT polymerization such as CTA selection must be considered in order to maintain a well-controlled system. In the subsequent section, consequences of the RAFT mechanism will be described, including kinetic features which allow the rational design of block copolymers.
Chain transfer agent selection. For a given polymerization, certain CTAs will be more effective in controlling molecular weight and maintaining narrow polydispersity due to the effects of the Z and R-group. As seen in Figure I-8, the CTA has four key features which determine its effectiveness: the activating “Z” group, a reactive double bond, a weak single bond, and the “R” leaving group.

![Key components of a thiocarbonylthio chain transfer agent.](image)

The Z-group serves as a stabilizing or destabilizing agent for the radical adduct as well as dictates the reactivity of the thiocarbonylthio group towards radical addition. The Z-group maintains its position at the active chain end throughout the polymerization and thus affects polymerization kinetics at all conversions. The Z-group must activate the double bond towards radical addition while balancing the stability of the radical adduct. If the Z-group provides too much stabilization, retardation can occur due to the slow fragmentation of the polymer chain from the adduct. While bond dissociation energies have been utilized to predict the stability of a variety of CTA adducts, the zwitterionic canonical forms must also be considered.
As will be discussed below, the R-group must be carefully chosen in order to minimize an initialization period. The stability of the R-group must be balanced so that it successfully fragments from the radical adduct and reinitiates polymerization before adding back to the CTA. While steric bulk, electron withdrawing groups, and radical stabilizing groups enhance fragmentation from the radical adduct, these factors can decrease the capacity of the R-group for reinitiation.

Initialization period. The initialization period, also termed the pre-equilibrium, encompasses the time period during which the R-group of the CTA undergoes fragmentation and subsequently initiates growth of a new polymer chain. Once all R-groups have completed step 2b of Scheme I-7, the initialization period is complete. Klumperman et al. measured initialization times in the polymerization of N-vinyl pyrrolidone (NVP) via in situ nuclear magnetic resonance (NMR) spectroscopy. As shown in Figure I-9, a linear decrease in CTA and monomer occurs until 275 minutes. Correspondingly, a linear increase in single monomer adduct is observed for the same time period. After 275 minutes, all CTA is consumed and a change in slope of monomer consumption is observed.
**Figure I-9.** Concentration profiles of the species involved in the initialization of N-vinylpyrrolidone polymerized at 70 °C in deuterated benzene. 191

*Pseudo first order kinetics.* Assuming a constant number of radical species, the kinetics of RAFT polymerization may be described by pseudo first order kinetics. In Equation 1, the rate of polymerization, $R_p$, is determined by the product of the concentration of free radicals in solution ([M]), the concentration of monomer ([M]), and the rate constant of propagation ($k_p$)

$$\frac{-d[M]}{dt} = R_p = k_p[M][M] = k_p[M] \left( \frac{f k_d [I]}{k_t} \right)^{1/2} \quad (1)$$

Often, the concentration of free radicals is described by a relationship between the concentration of initiator ([I]), initiator efficiency ($f$), the rate constant of initiation ($k_d$), and the rate constant of termination ($k_t$). As seen in Equation 2, by integrating Equation 1 with respect to $t$, $[M]_0$, and $[M]_t$, the monomer concentration at a predetermined time, yields Equation 3.
\[-J_{[M]_0}^{[M]_t} \frac{d[M]}{[M]} = \int_{t=0}^{t=t} k_p [M \cdot] \, dt\]  

\[\ln \frac{[M]_t}{[M]} = k_{app} t \quad \text{where} \quad k_{app} = k_p [M \cdot]\]

The slope of the pseudo first order kinetic plot, \(k_{app}\), should remain constant throughout the polymerization. A negative deviation in slope from the theoretical value indicates chain termination events thus reducing the number of radicals while a positive deviation indicates an increase in the number of radicals. Induction periods result in a positive x-intercept.

Deviations in the kinetic plot may often be corrected by adjusting the \([\text{CTA}]_0:[I]_0\) ratio. The concentration of initiator and the rate of initiator decomposition must be balanced to provide an acceptable rate of polymerization while limiting the number of dead chains. Well-designed experiments typically have a high ratio of \([\text{CTA}]:[I]\) though polymerizations conducted at ambient temperatures may utilize ratios of 1:1 due to the low number of radicals generated. The temperature dependent nature of rate constants, including \(k_d\) can be expressed empirically by the Arrhenius equation (4)

\[k = Ae^{\frac{-E_a}{RT}}\]

where \(A\) is the pre-exponential constant, \(R\) is the gas constant, \(E_a\) is the activation energy and \(T\) is the absolute temperature. As seen from Equation 4, the rate constant will significantly decrease at low temperatures.
Control of molecular weight. By utilizing a set ratio of [M]:[CTA] and controlling conversion (\(\rho\)) of the polymerization, a predetermined molecular weight may be targeted. The theoretical molecular weight may be calculated according to Equation 5, where \(M_n\) is the number average molecular weight, \([M]_0\) is the initial concentration of monomer, \([CTA]_0\) is the initial concentration of CTA, \(M_{MW}\) is the molecular weight of the monomer, and \(M_{CTA}\) is the molecular weight of the CTA. Often, \(M_{CTA}\) is considered negligible and not included in Equation 5.

\[
M_{n,\text{Theor}} = \frac{[M]_0 \times M_{MW} \times \rho}{[CTA]_0} + CTA_{MW}
\]

A plot of \(M_n\) versus conversion can provide insight to the control of a given RAFT polymerization. A positive y-intercept and linear slope indicates uncontrolled polymerization in the early stages of the reaction and can be minimized by choosing appropriate CTA/monomer pairs and decreasing the concentration of initiator. Molecular weight overshoot indicates a loss of CTA. A positive deviation in slope, often observed at high conversions for dithioester mediated polymerizations, is attributed to loss of CTA.

Incorporation of functionality. The facile incorporation of \(\alpha, \omega\) and side-chain functionality on RAFT-synthesized polymers is perhaps the most significant advantage of the process. CTAs have been utilized to directly and indirectly introduce chain-end functionality respectively through the R- and Z-groups.\(^1, 171, 176, 192\) Commonly utilized pre- and post-polymerization chemistries are shown in Scheme I-8. Carbodiimide chemistry, one of the most frequently used methods to introduce chain-end functionality,\(^104, 125, 192-198\) was utilized by D’Agosto and coworkers to conjugate \(N-\)
hydroxysuccinimide (NHS) to the carboxylic acid R-group of 2-[[2-phenyl-1-thioxo]thio]-propanoic acid. Bulmus and coworkers recently reported the synthesis of a novel trithiocarbonate containing an activated disulfide, allowing facile functionalization with thiol containing compounds such as bovine serum albumin (BSA). A variety of “click” chemistries (maleimide, azido and thiol-ene), which are defined by highly efficient and reagent-specific reactions, have also been utilized to conjugate biomolecules to RAFT-synthesized polymers.

Scheme I-8. Functionalization of the α, ω chain ends of RAFT-synthesized polymers.

Furthermore, RAFT polymerization is highly tolerant to a range of vinyl-based monomers, allowing the synthesis of an impressive set of side-chain functional materials often directly in water without requiring protecting group chemistry. As seen in Figure I-2 and I-3, styrenics, (meth)acrylates and (meth)acrylamides containing neutral,
anionic,\textsuperscript{4, 5, 209} cationic, zwitterionic,\textsuperscript{210, 211} and reactive functionality have been successfully homo- or copolymerized utilizing RAFT. Of particular significance to the biomaterial field is the ability to (co)polymerize monomers containing activated esters, primary amines, and protected thiols. These functionalities allow the facile post-polymerization conjugation of bioactive species to the polymer side chains. Scheme I-9 depicts the simplicity with which a reactive polymer scaffold can be converted to a polyvalent biomaterial. Activated esters, such as $N$-acryloxy succinimide (NAS, \textit{M}\textsuperscript{9}) and $N$-methacryloxy succinimide (NMS, \textit{M}\textsuperscript{10}), have been statistically incorporated into a number of RAFT polymers, providing a nucleophilic reactive site.$^{212, 213}$ Amine-containing small molecules are frequently conjugated to backbones using this chemistry (Scheme I-9) resulting in a polyvalent conjugate. Conversely, a primary amine containing monomer (i.e. $N$-3-aminopropyl methacrylamide, APMA, \textit{M}\textsuperscript{14}) may be incorporated into the polymer via the polymerization of the acidified monomer; subsequent deprotonation of the pendent amine groups allows nucleophilic attack on an activated ester small molecule. By converting the primary amine to its protonated form during polymerization, CTA aminolysis and hence loss of control is circumvented.$^{214-216}$ Another facile method to incorporate bioactive compounds is the (co)polymerization of activated thiols. Bulmus and coworkers reported the first successful polymerization of an activated thiol, pyridyldisulfide ethyl methacrylate (PDSM, \textit{M}\textsuperscript{12}), and subsequently demonstrated the ability to conjugate various small-molecule thiols, including the tripeptide glutathione.$^{217}$
Scheme I-9. Synthesis of polyconjugates via the reaction of activated side chains with reactive molecules.

The various routes to functionalize end-chain and side-chain groups on RAFT polymers in addition to the ability to control the molecular weight provide an unprecedented opportunity for the rational bottom-up design of biorelevant materials. With the rapidly growing areas of gene delivery, tissue engineering, and other biomedical fields, there is an emerging need for polymers which can be tailored for high performance applications. As detailed in the previous sections, recent advancements in RAFT polymerization provide an unprecedented opportunity to design polymers with specific compositions and architectures, allowing the delineation of structure-property relationships. In the ensuing work, RAFT-synthesized polymers will be utilized to rationally design materials of biomedical relevance.
CHAPTER II

OBJECTIVES OF RESEARCH

Recent advances in the controlled polymerization of a variety of monomers have allowed the rational design of functional polymers for biomedical applications. The McCormick Research Group has been at the forefront of the field, determining polymerization conditions for a number of monomers classes, including styrenics, acrylamides, methacrylamides, and acrylates, thus allowing the design of functional block copolymers with potential applications in therapeutic delivery and diagnostic testing. In particular, the development of conditions for the controlled synthesis of the temperature-responsive P(NIPAM) (M\textsubscript{5}), non-immunogenic, hydrophilic P(HPMA) (M\textsubscript{3}), and tertiary-amine containing P(DMAPMA) (M\textsubscript{13}) has provided an opportunity to design polymeric platforms for temperature-responsive gels and theranostic delivery vehicles.

Building on the concept of rationally-designed polymers for biomedical applications, the overall goals of this research are to: (1) design temperature-responsive BAB and ABA triblock copolymers for potential applications as scaffolds for cell growth (2) synthesize polymer-decorated AuNPs capable of binding, protecting, and delivering siRNA.

Realization of the first goal is contingent upon attainment of objectives 1-6, which focus on elucidating polymer architecture, block length, and composition on material properties critical for cell growth applications. The second goal, design of polymer-decorated AuNPs capable of delivering siRNA, is based on specific objectives.
7-9 involving the synthesis of polymer-stabilized AuNPs and subsequent characterization of their ability to serve as gene delivery vehicles.

Specific research objectives are to:

1. Design well-defined, stimuli-responsive BAB triblock copolymers \([A=P(AM)\text{ or }P(DMA), B=P(NIPAM)]\) via RAFT polymerization capable of temperature-induced gelation.

2. Investigate the effects of polymer concentration and block length on the BAB sol-gel properties including the storage modulus \((G')\), loss modulus \((G'')\) and gelation temperature \((T_{gel})\).

3. Investigate the effect of salt on the BAB gel mechanical properties, \(T_{gel}\), and CGC.

4. Design well-defined, stimuli-responsive ABA triblock copolymers \([A=P(DMA), B=P(NIPAM)]\) via RAFT polymerization capable of temperature-induced gelation.

5. Determine the mechanism of gel formation via small angle x-ray scattering.

6. Investigate the effect of A and B block lengths on \(G'\) and \(G''\).

7. Develop a synthetic route for the formation of polymer-stabilized AuNPs with high siRNA loading capacity.

8. Characterize polymer-stabilized AuNPs via dynamic light scattering, small angle x-ray scattering, UV-Vis spectroscopy, zeta potential, and transmission electron microscopy.

9. Investigate the effect of Au reduction conditions on the nanoparticle stability and oligonucleotide binding capacity.
(10) Characterize polymer-stabilized AuNP/siRNA complexes by agarose gel electrophoresis, enzymatic degradation assays, and cell cytotoxicity assays.

(11) Evaluate the therapeutic efficacy of siRNA loaded polymer-stabilized AuNPs through gene knockdown studies.

The research discussed herein has been divided into three sections which are unified by a central theme of tailoring block copolymers for biotechnological applications. In the first section, BAB triblock copolymers [A=P(AM) (M1) or P(DMA) (M2), B=P(NIPAM) (M5)] capable of thermoreversible gelation are synthesized by RAFT polymerization. The polymers are characterized with respect to their biorelevant properties including moduli and gelation temperature. In the second section, temperature-responsive triblock copolymers of an ABA architecture (A=P(DMA), B=P(NIPAM) are synthesized and characterized. The gelation mechanism is investigated by temperature-controlled SAXS studies while oscillatory rheometry is utilized to characterize the mechanical properties. In the third section, a series of P(HPMA-b-DMAPMA) (M3-b-M13) diblock copolymers synthesized by RAFT is utilized for the in situ formation of polymer-stabilized Au nanoparticles (AuNPs) capable of forming neutral, sterically stable complexes with small interfering RNA. Enzymatic degradation assays indicate Au carriers provide protection against nucleases while in vitro cell tests confirm intracellular delivery of siRNA.
CHAPTER III
EXPERIMENTAL

This chapter describes materials and experimental procedure utilized in research detailed in this dissertation. The latter has been divided into three sections. In the first section, the synthesis and characterization of BAB triblock copolymers are described. The second section provides experimental details on the synthesis and characterization of ABA triblock copolymers. The in situ formation of polymer-stabilized Au nanoparticles and subsequent stabilization of siRNA is described in the final section.

Materials

All chemicals were purchased from Fischer or Aldrich at the highest available purity and used as received unless otherwise noted. N-isopropylacrylamide (NIPAM) (M5) was recrystallized from hexane. Acrylamide (AM) (M1) was recrystallized three times from acetone. N,N-dimethylacrylamide (DMA) (M2) was vacuum distilled. 2,2’-azobis[2-(2-imidozolin-2-yl)propane] dihydrochloride (VA-044) and 4,4’-azobis(4-cyanopentanoic acid) (V-501) were recrystallized from methanol. N-[3-(dimethylamino)propyl] methacrylamide (DMAPMA) (M13) was purified by vacuum distillation. N-(2-hydroxypropyl)methacrylamide (HPMA) (M3) was synthesized according to literature procedure. The difunctional chain transfer agent (CTA) 2-(1-carboxy-1-methylethylsulfanylthiocarbonylsulfanyl)-2-methylpropionic acid (CMP) was used without further purification (gift from Noveon, Inc.). 4-Cyanopentanoic acid dithiobenzoate (CTP) was synthesized according to literature procedure. Structures for the aforementioned compounds are shown seen in Figure III-1. Type I Collagen from rat tails (high concentration, 8.14 mg/mL) was purchased from BD Biosciences. Gaussia
Luciferase (Gluc) was synthesized according to previous literature reports. The sense and antisense sequences of siRNA against Gluc are respectively

AGATGTGCAACTTTTGCTACCGCATCT and antisense

AGGAGATGCCTAGGAAAAGTTGCACATCT. Two strands of a 59-nucleotide (nt) model small interfering ribonucleic acid (siRNA) against human survivin were chemically synthesized and labeled with cyanine-3 (Cy3) separately. Unlabeled sense strand, 5′-AGCCCUUUCUCAAGGACCACCGCAUCU-3′, was purchased from Integrated DNA Technologies. Cy3-labeled antisense strand, 3′-

UUUCGGGAAAGAGUUCUGUGGGCGGUAGAGGA-5′ was purchased from Biosynthesis Inc. A negative siRNA control was purchased from Ambion. A549 cells (human lung cancer cells), SKOV3 (human ovarian cancer cells), HeLa (human cervical cancer cells) and KB cells (human nasopharyngeal cancer cells) were purchased from American type Culture Collection (Manassas, VA). Riboshredder™ RNase Blend was purchased from Epicenter Biotechnologies. Plasmid pCMV-Gluc and Gaussia luciferase assay kit were purchased from New England biolab. Celltiter 96 Aqueous one solution cell proliferation assay kit was purchased from Promega.
Figure III-1. Compounds used in the synthesis of temperature-responsive triblock copolymers and diblock copolymers with requisite properties for the \textit{in situ} formation and stabilization of Au nanoparticles.
Synthesis and Characterization of BAB Triblock Copolymers

General Procedure for the Synthesis of a Difunctional P(NIPAM) macroChain Transfer Agent

Based on previous kinetic studies, polymerization conditions were chosen to produce a DP=900 or DP=675 NIPAM macroCTA at 90% conversion. The polymerization was conducted for 10 h at 30 °C in aqueous media at pH 5.0 with an initial monomer concentration ([M]₀) of 0.5 M (Scheme III-1). To synthesize the P(NIPAM₉₀₀) macroCTA, the following components were added to a round-bottomed flask: VA-044 (I₁) (10.2 mg, 31.5 μmol), NIPAM (M₅) (9.97 g, 88.1 mmol), CMP (CTA₁) (25.1 mg, 88.9 μmol), and deionized water (177 mL). The round-bottomed flask was sealed with a septum and purged with nitrogen for 30 minutes. After 10 h in a 30 °C water bath the polymerization was terminated by exposure to air. The P(NIPAM₉₁₀) macroCTA (P₃) (Mₙ=103,200, PDI=1.07) was purified via dialysis against an acidic solution at 4 °C followed by lyophilization. A similar procedure was utilized for the synthesis of P(NIPAM₆₇₅) (P₁) (Mₙ=76400, PDI=1.06). Polymer structures are shown in Figure III-2.
Scheme III-1. Preparation of BAB triblock copolymers via aqueous RAFT polymerization.

Figure III-2. P(NIPAM) macroCTAs (P1, P3) and chain-extended BAB block copolymers (P2, P4, P5).
General Procedure for the Chain Extension of a Difunctional P(NIPAM) macroChain Transfer Agent with AM

The chain extension of P(NIPAM$_{675}$) macroCTA (P1) with AM (M1) was conducted at 27 °C in aqueous media with an initial monomer concentration ([M]$_0$) of 0.5 M (Scheme III-1). Briefly, the following components were added to a round-bottomed flask: VA-044 (I1) (4.24 mg, 13.1 μmol), P(NIPAM$_{675}$) macroCTA (1.0 g, 13.1 μmol), AM (0.74 g, 10 mmol), and deionized water (21 mL). The round-bottomed flask was septum sealed and purged with nitrogen for 30 minutes. After 1 h in a 27 °C water bath the polymerization was terminated by exposure to air. P(NIPAM$_{337}$-$b$-AM$_{705}$-$b$-NIPAM$_{337}$) (P2) (as determined by $^1$H NMR) was purified via dialysis against an acidic solution at 4 °C followed by lyophilization. The polymer structure may be seen in Figure III-2.

General Procedure for the Chain Extension of a Difunctional P(NIPAM) macroChain Transfer Agent with DMA

The chain extension of P(NIPAM$_{910}$) macroCTA with DMA (M2) was conducted at 27 °C in aqueous media with an initial monomer concentration ([M]$_0$) of 0.5 M (Scheme III-1). To chain extend the macroCTA, the following components were added to a round-bottomed flask: VA-044 (I1) (3.16 mg, 9.78 μmol), P(NIPAM$_{910}$) (P3) macroCTA (1.01 g, 9.78 μmol), DMA (0.88 g, 8.9 mmol or 0.44 g, 4.5 mmol), and deionized water (17.8 mL or 8.9 mL). The round-bottomed flasks were sealed with septa and purged with nitrogen for 30 minutes. After 1 h in a 27 °C water bath the polymerization was terminated by exposure to air. The polymers were purified via dialysis against an acidic solution at 4 °C followed by lyophilization P(NIPAM$_{455}$-$b$-
DMA_{210-b-NIPAM_{455}} (P4) (M_n=124,100, PDI=1.06) and P(NIPAM_{455}-b-DMA_{277-b-NIPAM_{455}}) (P5) (M_n=130,700, PDI=1.08). Polymer structures may be seen in Figure III-2.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) was used to determine $M_n$, $M_w$, and PDIs for (co)polymers. Polymers were analyzed on a Viscotek-TDA (0.02 M LiBr DMF eluent, 1.0 mL/min, 60 °C, Viscotek I-Series Mixed Bed low MW and mid MW columns, 302 nm RI, viscosity, 7 mW 90° and 7° true low angle light scattering detectors). The $dn/dc$ of each polymer was determined at 632.8 nm in DMF at 60 °C using a Viscotek refractometer and Omnisec software. The $M_n$ of the inner blocks was determined by taking the difference between the $M_n$ of the triblock copolymer and homopolymer.

Dynamic Light Scattering of BAB Triblock Copolymers

Dynamic light scattering studies of the block copolymers in aqueous solution were conducted using a Malvern Instruments Zetasizer Nano Series instrument equipped with a 22 mW He-Ne laser operating at $\lambda=632.8$ nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 tau digital correlator electronics system. Samples (1 mg/ml) were allowed to dissolve in deionized water overnight. The sample temperature was increased from 25 °C to 50 ºC in increments of 1 °C. Samples were allowed to equilibrate for 10 min at each temperature increment.

Rheometric Studies of BAB Triblock Copolymers

Rheological measurements of the BAB polymer solutions were performed with a Rheometrics SR-5000 controlled stress rheometer to determine the gel points, storage moduli ($G'$), and loss moduli ($G''$) of the temperature-responsive polymers. All
measurements were performed using a 25 mm cone and plate geometry with an angle of 0.1 radians. An insulated ring was placed around the geometry to prevent water evaporation. Before performing measurements, the polymers were allowed to dissolve overnight in either deionized water or 140 mM NaCl/20 mM phosphate buffer. Collagen gels were prepared according to the alternate gelation procedure provided on the product specification sheet. Copolymer solutions were presheared for 2 min at a constant applied shear stress of 5 Pa before dynamic temperature ramp measurements were taken. A frequency of 1 rad/s was used. Materials were heated with a Peltier plate at a rate of 0.5 °C min⁻¹.

**Pulsed Field Gradient Nuclear Magnetic Resonance (PFG NMR) Studies of BAB Triblock Copolymer Gels**

The time-dependent diffusion coefficient of water (D_{app}) was determined using PFG NMR methods. Spectral data were obtained with a Varian UNITY Inova 500 MHz spectrometer using a standard 5mm 2 channel probe equipped with gradients. Self-diffusion coefficients were determined from the negative slope of a log-attenuation plot (log ψ versus γ²g²δ²(Δ- δ/3)), where ψ is the echo attenuation, γ is the proton gyromagnetic ratio, δ is the width of the gradient pulse, g is the magnitude of the applied field gradient, and Δ is the total diffusion time. The standard Stejskal-Tanner sequence, illustrated in Figure III-3, was utilized with an acquisition time of 0.5 s, a recycle delay of 5 s, and gradient pulses of 0.8-1.0 ms. The total diffusion time was varied from 20 to 900 ms, and gradient amplitude ranged from 20 to 80 G/cm to ensure the signal was attenuated ~80%. The spectral width was 50 kHz and the number of scans for each spectrum ranged from 8-16. Exponential line broadening was applied prior to
Fourier transformation of the FIDs. Gradient calibration was performed using a deionized water standard prior to data collection. Samples consisted of 10 wt% polymer dissolved in 140 mM NaCl/20 mM phosphate buffer pH 7.4.

![Figure III-3](image.png)

**Figure III-3.** The standard pulse sequence used in the pulsed field gradient NMR experiments.

**Confocal Microscopy of BAB Triblock Copolymers**

Confocal microscopy samples were comprised of $2.5 \times 10^{-4}$ M 8-anilino-1-naphthalenesulfonic acid (ANS) in a 10 wt% polymer solution (P4 or P5). To prepare the samples, a stock solution of ANS in acetone was utilized. An appropriate volume of the stock solution was transferred to a scintillation vial to give $2.5 \times 10^{-7}$ mol ANS. The acetone was evaporated under a stream of nitrogen. The triblock copolymer (10 mg) was added to the scintillation vial and diluted with 1 mL of 140 mM NaCl/20 mM phosphate buffer pH 7.4. The polymer was allowed to dissolve overnight in the dark at 5 °C. A LSM510 Meta confocal microscope with an excitation wavelength of 488 nm and a LP505 filter was utilized to image the samples. A heating block was used to control the sample temperature.
Small Angle X-ray Scattering of BAB Triblock Copolymers

SAXS profiles were acquired using a seal-tube source, a compact Kratky camera with a micro-process-controlled hot-stage, and a one-dimensional position-sensitive detector. Samples were dissolved in DI water overnight in a scintillation vial. Samples were subsequently loaded into the samples cell and heated to the acquisition temperature for 30 min before a 1 h SAXS profile was acquired. SAXS traces were acquired under a positive pressure of helium. Data reduction was carried out as described in previous reports to desmear absolute intensity.\textsuperscript{223, 224} CuKa x-rays were employed.
Synthesis and Characterization of ABA Triblock Copolymers

General Procedure for the Synthesis of a Difunctional P(DMA) macroChain Transfer Agent

Polymerizations were conducted based on prior literature reports. Briefly, the synthesis of P(DMA) was conducted for 3 h at 25 °C in aqueous media at pH 4.7 with an initial monomer concentration ([M]₀) of 0.5 M (Scheme III-2). A [CTA]₀:[I]₀ ratio of 5:1 was utilized in each polymerization while three ratios of [M]₀:[CTA]₀ were utilized in separate polymerizations, 200:1, 400:1, and 600:1. To synthesize the macroCTA, the following components were added to a 250 mL round-bottomed flask: DMA (M2), CMP (CTA1), VA-044 (I1) and DI water to bring the final volume to 140 mL. The pH was adjusted to a value of 4.7. The flask was sealed with a rubber septum and purged with nitrogen for 30 min at 5 °C. The flask was then immersed in a 25 °C water bath for 3 h before quenching by rapid cooling and exposure to oxygen. The P(DMA) macroCTA was purified by dialysis and lyophilization. P(DMA₁₈₆₆) (P6) (Mₙ=18,400, PDI=1.07), P(DMA₃₉₄₄) (P7) (Mₙ=39,100, PDI=1.06), P(DMA₅₀₀₀) (P8) (Mₙ=49,600, PDI=1.06) may be seen in Figure III-3.
Scheme III-2. Synthetic route for the preparation of ABA triblock copolymers via aqueous RAFT polymerization.
General Procedure for the Chain Extension of a Difunctional \( \text{P(DMA)} \) macroChain Transfer Agent

Polymerizations were conducted based on prior literature reports.\(^{225}\) Briefly, the polymerization was conducted at 25 °C in aqueous media at pH 5.0 with an initial monomer concentration ([M]_0) of 0.5 M. A \([\text{CTA}]_0:[\text{I}]_0\) ratio of 1:1 was utilized in each polymerization. To chain extend the macroCTA, the following components were added
to a round-bottomed flask: NIPAM (M₅), DMA macroCTA, VA-044 (I₁), and DI water to bring the final volume to 200 mL. The pH was adjusted to a value of 5.0. The polymerization solutions were purged with nitrogen for 30 min at 5 °C. The flasks were then immersed in a 25 °C water bath for 1 h before quenching by rapid cooling and exposure to oxygen. The triblock copolymers were purified by dialysis and lyophilization. P(DMA₉₃-b-NIPAM₂₁₃-b-DMA₉₃) (P₉) (Mₙ=42,900, PDI=1.06), P(DMA₉₃-b-NIPAM₃₇₂-b-DMA₉₃) (P₁₀) (Mₙ=55,700, PDI=1.07), P(DMA₁₉₇-b-NIPAM₃₉₂-b-DMA₁₉₇) (P₁₁) (Mₙ=83,700, PDI=1.05), and P(DMA₂₅₀-b-NIPAM₆₀₀-b-DMA₂₅₀) (P₁₂) (Mₙ=117,400, PDI=1.07) can be seen in Figure III-2.

Size Exclusion Chromatography of ABA Triblock Copolymers

SEC was used to determine Mₙ, Mₘ, and PDIs for (co)polymers. Polymers were analyzed on a Viscotek-TDA (0.02 M LiBr DMF eluent, 1.0 mL/min, 60 °C, Viscotek I-Series Mixed Bed low MW and mid MW columns, 302 nm RI, viscosity, 7 mW 90° and 7° true low angle light scattering detectors). The dn/dc of each polymer was determined at 632.8 nm in DMF at 60 °C using a Viscotek refractometer and Omniseck software. The Mₙ of the inner blocks was determined by taking the difference between the Mₙ of the triblock copolymer and homopolymer.

Dynamic Light Scattering of ABA Triblock Copolymers

Dynamic light scattering studies of the block copolymers in aqueous solution were conducted using a Malvern Instruments Zetasizer Nano Series instrument equipped with a 22 mW He-Ne laser operating at λ=632.8 nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 tau digital correlator electronics system. Samples (1 mg/ml) were allowed to dissolve in deionized water overnight.
Temperature was increased from 25 ºC to 50 ºC in increments of 1 ºC. Samples were allowed to equilibrate for 10 min between measurements.

*Rheometric Studies of ABA Triblock Copolymers*

Rheological tests of the ABA polymer solutions were performed with a Rheometrics SR-5000 controlled stress rheometer. A 25 mm cone and plate geometry with an angle of 0.1 radians was utilized for all measurements. An insulated ring was placed around the geometry to prevent water evaporation. Before performing measurements, the polymers were allowed to dissolve overnight in either deionized water or 140 mM NaCl/20 mM phosphate buffer. In order to ensure polymer were tested within the linear viscoelastic regime, dynamic frequency sweep experiments were conducted in triplicate (temperature maintained at 50 ºC with a water bath, 1 Pa). The storage modulus at 50 ºC was determined as the value obtained at 1 rad/s. Samples were allowed to equilibrate for 3 min prior to testing. Dynamic temperature ramp tests were conducted utilizing a Peltier plate to heat the sample at a rate of 1.0 ºC min$^{-1}$. Copolymer solutions were presheared for 2 min at a constant applied shear stress of 1 Pa before dynamic temperature ramp measurements were taken at a frequency of 1 rad/s and stress of 1 Pa. Temperature cycling experiments were conducted using a frequency and stress of 1 rad/s and 1 Pa, respectively. A Peltier plate was utilized to cycle the temperature between 25 and 50 ºC at a ramp rate of 50 ºC min$^{-1}$.

*Small Angle X-ray Scattering of ABA Triblock Copolymers*

SAXS profiles were acquired using a seal-tube source, a compact Kratky camera with a micro-process-controlled hot-stage, and a one-dimensional position-sensitive detector. Samples were allowed to dissolve in DI water overnight in a scintillation vial.
Samples were subsequently loaded into the samples cell and heated to the acquisition temperature for 30 min before a 1 h SAXS profile was acquired. SAXS traces were acquired under a positive pressure of helium. Data reduction was carried per previous reports to desmear absolute intensity.\textsuperscript{223, 224} CuKa x-rays were employed.
Synthesis and Characterization of Polymer-Stabilized Au Nanoparticle/siRNA Complexes

General Procedure for the Synthesis of a P(HPMA) macroChain Transfer Agent

A P(HPMA) macroCTA was prepared according to previous literature reports. Briefly, V-501 (12) (22.5 mg, 0.08 mmol) and CTP (CTA2) (112 mg, 0.4 mmol) were used as the primary radical source and chain transfer agent. Polymerizations were conducted at 70 °C for 3 h in aqueous acetic acid buffer (pH 5.2, 0.27 M acetic acid and 0.73 M sodium acetate) using 1 M HPMA (M3) (11.5 g, 80 mmol). An [M]0/[CTA]0 ratio of 200/1 was utilized while the [CTA]0/I0 was kept at 5/1. The final reaction volume was brought to 80 mL with acetic acid buffer. The round-bottomed flask was septum-sealed and purged with nitrogen for 45 min prior to polymerization. The macroCTA was isolated by dialysis (pH 3-4) at 4 °C followed by lyophilization. P(HPMA70) (P13) (Mn=10,000, PDI=1.05)

**General Procedure for the Chain Extension of a P(HPMA) macroChain Transfer Agent**

Block copolymers were prepared according to previous literature reports. Briefly, a \([M]_0/\text{[CTA]}_0\) ratio of 200/1, \([\text{CTA}]_0/\text{[I]}_0\) of 5/1, and 1 M DMAPMA were utilized in the chain extension of P(HPMA\textsubscript{70}). Block copolymers were prepared by adding V-501 (22.5 mg, 0.08 mmol), P(HPMA\textsubscript{70}) (4.0 g, 0.4 mmol), and 63.7 mL of an HCl-neutralized 1.25 M DMAPMA (M13) stock solution (16.5 g, 80 mmol) to a 100 mL round-bottomed flask. The resulting polymerization solution was divided into four
reaction vessels, septa-sealed, and purged. Flasks were removed from the water bath and quenched at different time intervals to yield copolymers of varying compositions. Polymers were purified by dialysis against DI water and subsequent lyophilization. (See Figure III-5 for polymer structures). P(HPMA$_{70}$-b-DMAPMA$_{24}$) ($\text{P14}$) ($M_n=15,400$, PDI=1.08), P(HPMA$_{70}$-b-DMAPMA$_{49}$) ($\text{P15}$) ($M_n=20,100$, PDI=1.07), P(HPMA$_{70}$-b-DMAPMA$_{84}$) ($\text{P16}$) ($M_n=27,000$, PDI=1.10), P(HPMA$_{70}$-b-DMAPMA$_{104}$) ($\text{P17}$) ($M_n=32,000$, PDI=1.11) can be seen in Figure III-5.

![Polymer Structures](image)

**Figure III-5.** P(HPMA) macroCTA ($\text{P13}$) and chain-extended polymers ($\text{P14-17}$) utilized for the in situ formation of Au nanoparticles.

**Size Exclusion Chromatography of P(HPMA-b-DMAPMA) Copolymers**

Polymers were characterized by aqueous SEC (ASEC) utilizing an eluent of 1 wt % acetic acid/0.10 M Na$_2$SO$_4$ (aq) at a flow rate of 0.25 mL/min at 25 °C, Eprogen, Inc. CATSEC columns (100, 300, and 1000 Å), a Polymer Laboratories LC1200 UV/vis detector, a Wyatt Optilab DSP interferometric refractometer ($\lambda=690$ nm), and a Wyatt
DAWN-DSP multangle laser light scattering (MALLS) detector ($\lambda$=633 nm). Absolute molecular weights and PDIs were calculated using Wyatt OmniSEC software. The $dn/dc$ measurements were performed with a Wyatt Optilab DSP interferometric refractometer ($\lambda$=690 nm) at 35 °C and determined using OmniSEC software.

$^1$H NMR Spectroscopy of P(HPMA-b-DMAPMA) Copolymers

Copolymer compositions were determined with a Varian MercuryPLUS 300 MHz or 500 MHz spectrometer in D$_2$O utilizing solvent suppression with a delay time of 2 s. DMAPMA block composition of diblock copolymers was determined by $^1$H NMR utilizing the relative areas of the methyne proton resonances of HPMA at 3.75 ppm and the dimethyl proton resonances of DMAPMA at 2.72 ppm.

In situ Formation of P(HPMA-b-DMAPMA) stabilized Au Nanoparticles

AuNPs were synthesized via the in situ reduction of NaAuCl$_4$ to Au$^0$. Briefly, P(HPMA$_{70}$-b-DMAPMA$_x$) ($X=24, 49, 84, \text{ or } 104$) and 50 mM phosphate buffer (pH 6.0, 103 mL) were added to a round-bottomed flask equipped with a stir bar to give a final DMAPMA concentration of 1.7 mM. NaAuCl$_4$ was added to the round-bottomed flask to give either a 100:1, 10:1, or 3:1 ratio of [DMAPMA]$_0$:NaAuCl$_4$$_0$. The round-bottomed flask was sealed with a septum and placed in a 50 °C oil bath with stirring for 24 h. The resulting dark red solution was purified by dialysis (50000 mwco tubing) against deionized water at 4 °C to remove unbound polymer. Following dialysis, polymer-AuNPs were lyophilized, analyzed by thermogravimetric analysis for composition, and redispersed in 50 mM pH 6 phosphate buffer at a concentration of 1 mM DMAPMA.

A control reduction reaction was conducted utilizing the P(HPMA$_{70}$) macroCTA as the reducing agent. The reduction contained 70 µM P(HPMA$_{70}$) and 0.17 mM
NaAuCl₄ in 50 mM pH 6.0 phosphate buffer. The solution was heated at 50 °C for 3 d before taking an aliquot and analyzing by UV-Vis spectroscopy.

*Preparation of P(HPMA-b-DMAPMA) stabilized Au Nanoparticle oligonucleotide complexes*

Complexes were prepared at N/P ratios of 0.5-5 at room temperature. An appropriate volume of 1.0 mM DMAPMA HbC-AuNP stock (1-6 µL, 1-6 nmol DMAPMA repeat units) was diluted to 6.6 µL utilizing RNase free water. RNA-polymer-AuNP complexes (Au nanoplexes) were prepared by adding 1.4 µl of a 25 µM survivin siRNA stock (33.9 pmol siRNA, 2 nmol phosphate) to the 6.6 µl solution of HbC-AuNPs. Complexation solutions were immediately vortexed following survivin siRNA addition and allowed to incubate at room temperature for 30 min. Gel-loading buffer (2.5 µL) containing 4 µL of 0.2% bromophenol blue, 8 M urea, and 1X TBE buffer (Trisborate-EDTA) was added to samples prepared for gel electrophoresis. The samples were immediately loaded onto a 1.0% agarose minigel (10 cm x 7 cm) and run for 30 min at 90 V. After staining by ethidium bromide, the agarose gel was imaged by a Bio-RAD Universal Hood II CCD camera.

*UV-Vis Analysis of Au Nanoparticles*

An Agilent 8453 UV-Vis spectrophotometer equipped with a photodiode array was utilized to measure the absorbance of the P(HPMA₇₀-b-DMAPMAₓ) reduction solutions at different time intervals. Samples were prepared by diluting 4 µL of the reaction solution in 400 µL of DI water.
Dynamic Light Scattering and Zeta Potential of Polymer-stabilized Au Nanoparticles

Dynamic light scattering (DLS) and zeta potential measurements of Au nanoparticles under aqueous conditions were performed using a Malvern-Zetasizer Nano Series DLS detector with a 22 mW He-Ne laser operating at $\lambda=632.8$ nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple $\tau$ digital correlator electronics system. Data analysis of DLS measurements was performed using the CONTIN method. Zeta potential measurements were carried out at a theranostic concentration of 1 mg/mL in 0.1 M NaCl/20 mM Phosphate buffer at pH 7.4 in a zeta folded capillary cells purchased from Malvern Instruments. Both DLS and zeta potential measurements were performed in triplicate.

Transmission Electron Microscopy

Transmission electron microscopy measurements were conducted using a JEOL JEM-2100 electron microscope at an accelerating voltage of 200 kV. Samples were prepared by pipetting 2.5-5.0 $\mu$L of a AuNP solution on a carbon-coated copper grid and subsequently drying over night at room temperature. AuNP diameters were determined by averaging the size of 100 particles.

Thermogravimetric Analysis

A TA Instrument Q500 series thermogravimetric analyzer was utilized to determine the composition of polymer-stabilized AuNPs. Lyophilized samples were loaded in a platinum pan and heated to 600 °C at a rate of 10 °C/min under a nitrogen purge (60 ml/min). Sample composition was determined by comparison of polymer and polymer-stabilized AuNP degradation profiles.
Small Angle X-ray Scattering of Polymer-Stabilized Au Nanoparticles

The SAXS profile was acquired using a seal-tube source, a compact Kratky camera with a controlled hot-stage, and a one-dimensional position-sensitive detector. The SAXS profile was acquired on lyophilized HbC-AuNPs. SAXS traces were acquired under vacuum. Data reduction was carried per previous reports to desmear absolute intensity.\(^{223,224}\) CuKa x-rays were employed. The diameter of AuNPs, \(D_{\text{Au}}\), was determined by Equation 6

\[
D_{\text{Au}} = 2\pi q_t^{-1}
\]  

where \(q_t\) is the value of \(q\) where the power-law regions transitions.\(^{226}\) The power-law regime with an exponent of \(-4\) indicates smooth-surfaced primary particles.\(^ {227}\)

Enzymatic Degradation Studies of RNA/Au Nanoparticle Complexes

The kinetics of degradation of free and complexed siRNA with Riboshredder\(^{\text{TM}}\) RNase blend were obtained using a Varian Carey 50 Bio spectrophotometer, monitoring at 260 nm (i.e., the \(\lambda\) max for RNA) in kinetics/time course mode over a time interval of 20 min. P(HPMA\(_{258}\)-b-DMAPMA\(_{24}\))-stabilized AuNPs were complexed with a 59 nucleotide anti-survivin siRNA at an \(N/P\) of 1.85. This stoichiometry produces a near neutral Au nanoplex as indicated by agarose gel electrophoresis experiments (see Chapter IV). Briefly, 10 \(\mu\)L (500 pmol siRNA, 29.5 nmol phosphate) of a 50 \(\mu\)M anti-survivin siRNA stock solution was added to 54.5 \(\mu\)L (54.5 nmol DMAPMA repeat units) of a 1.0 mM DMAPMA AuNP stock solution. The complex solution was diluted to 1.0 mL (10 mM pH 8.0 Tris, 1 mM EDTA, Riboshredder\(^{\text{TM}}\) RNase protocol), vortexed, and placed in
a 1.4 mL quartz cuvette. The spectrophotometer was blanked with a buffer solution containing 54.5 μL of the 1.0 mM DMAPMA AuNP stock solution in order to subtract absorbance due to the AuNP. The initial absorbance at 260 nm (A₂₆₀) of the HbC-AuNP/siRNA complex solution was recorded. After monitoring the baseline for approximately 3 min, 3 μL of 1 unit/μL Riboshredder™ RNase blend was pipetted in the cuvette and inverted to promote mixing. The resulting solution was monitored continuously for 20 min at A₂₆₀ with 536 data points taken per minute.

For the siRNA control, 10 μL (500 pmol siRNA, 29.5 nmol phosphate) of a 50 μM anti-survivin siRNA stock solution was diluted to 1.0 mL (10 mM pH 8.0 Tris, 1 mM EDTA, Riboshredder™ RNase protocol), vortexed, and placed in a 1.4 mL quartz cuvette. The spectrophotometer was blanked with the 10 mM pH 8.0 Tris, 1 mM EDTA buffer solution. The initial absorbance at 260 nm (A₂₆₀) of the siRNA solution was recorded. After monitoring the baseline for approximately 3 min, 3 μL of 1 unit/μL Riboshredder™ RNase blend was pipetted in the cuvette and inverted to promote mixing. The resulting solution was monitored continuously for 20 min at A₂₆₀ with 536 data points taken per minute.

Cell Culture

All the cancer cells were maintained and proliferated in folate free RPMI 1640 (Gibco, #27016) cell media supplemented with 10% fetal calf serum (FCS), 100 units mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin at 37 °C in 95% air humidified atmosphere and 5% CO₂.
**Toxicity Assay of P(HPMA-b-DMAPMA) stabilized AuNPs**

Cell viability tests were performed utilizing a CellTiter 96 Aqueous one solution cell proliferation assay (Promega). 5000 KB cells were seeded into the 96-well microplate (Nunclon) one day before treatment. Various amounts of the P(HPMA<sub>70</sub>-b-DMAPMA<sub>24</sub>)-AuNP stock solution (1.0 mM DMAPMA) were added to the wells bringing the total volume of medium to 300 μL and the concentration of DMAPMA from 0.5 to 100 μM. Tests were run in triplicate in 3 separate wells. Cells were cultured for 2 days and subsequently 20 μL of CellTiter reagent was added to each well and incubated for 2 hours before measuring the absorbance at 490 nm using a Synergy2 (BioTek) and Gen5 software.

**Construction of Stably Expressing GLuc Cell Line**

KB cells were transfected with pCMV-GLuc (NEB) by DharmaFECT (Dharmacon) according to protocol. 24 h after transfection, 500 μg/mL G418 (Sigma) was added to the culture for 20 d and the medium was changed every other day. The surviving colonies which resisted the selection process were carefully collected and transported into 24-well plates. The positive colonies stably expressing GLuc were verified using a Gaussia Luciferase Assay Kit (New England Biosciences).

Subsequently, the colonies were amplified, stored and used for siRNA evaluation.

**Cell Treatment with AuNP/siRNA complexes for Fluorescence Microscopy**

Prior to treatment, KB cells were seeded on cover glasses in a 12-well plate with 600 μL of folate-free RPMI1640 cell media supplemented with 10% FCS and were cultured for 24 h. Neutral Au nanoplexes (N/P=1.85) were then added to the cell media to give a 100 nM concentration of siRNA. Cells were incubated with Au nanoplexes for
After treatment, the cells were fixed with 4% paraformaldehyde and washed with phosphate buffered saline (PBS) prior to imaging. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) in 12 μL of mounting gel. The cover glasses were then placed on pre-cleaned microscope slides for analysis.

Fluorescence Microscopy

A Nikon fluorescence microscope (Eclipse 80i, Plan Fluor 40X/0.75 DIC M/N2 lens) was utilized to image cells. Image-Pro Plus software was used to process images. Multiple fields were examined for each sample to ensure uniform distribution of AuNP/siRNA and lipofectamine treatments. Cell treatments were repeated three times to ensure reproducibility of the observed results.

Cell Treatment with AuNP/siRNA complexes for Gene Down-Regulation Studies

KB cells cells (3 × 10^4 cells per well) were seeded in 24-well plates (Flow Laboratories, Inc. McLean, VA) in folate free RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% FCS (HyClone, Logan, UT) 24 h before experiments. Cells treatments included either free siRNA, neutral Au nanoplexes, or DharmaFECT/siRNA. In each treatment, cells were incubated with 100 nM GLuc siRNA for 6 h. After this time period the medium was removed, cells were gently rinsed, and fresh medium was added.

Gaussia Luciferase Activity Assay

Expression of GLuc was measured according to the manufacture’s protocol with minor modification. 15 μL of media from stably-expressing KB cell cultures were transported into a white opaque 96-well plate (Coster, Corning, NY) and mixed with 40 μL substrate mixture. Bioluminescence was immediately measured by a Synergy 2
(BioTeK, Winooski, VT). Subsequent analysis was performed based on Gen5 software (BioTeK).
CHAPTER IV

RESULTS AND DISCUSSION

The research discussed herein has been divided into three sections which are unified by a central theme of the bottom up design of biomaterials. In the first two sections, the research is directed towards the rational design of triblock copolymers for physiologically relevant thermoreversible gels. While still focused on the bottom up design of biomaterials, the central theme of the third section involves gene/diagnostic delivery vehicles.

The first section concerns the synthesis and characterization of BAB triblock copolymers containing temperature-responsive outer blocks (NIPAM, $M_5$) and a hydrophilic inner block (AM, $M_1$ or DMA, $M_2$). The effect of inner block length, polymer concentration, and solvent on the gel properties is investigated. By adjusting the polymer concentration, material properties similar to those of collagen are achieved. Temperature-responsive gels are characterized by rheometry, pulsed-field gradient NMR, and SAXS.

In the second section, the focus is shifted from BAB to ABA triblock copolymer gels, in which the hydrophilic outer A blocks are comprised of P(DMA) and the temperature-responsive inner B block is comprised of P(NIPAM). The effects of polymer block length and hydrophilic mass fraction on the gel properties are discussed. Polymer solutions and gels are characterized by rheometry and small angle x-ray scattering (SAXS).

In the third section, the in situ reduction and stabilization of AuNPs utilizing RAFT-synthesized P(HPMA-$b$-DMAPMA) ($M_3$-$b$-$M_{13}$) are described. After
determining the appropriate block length for the formation of stable particles, materials were characterized by UV-Vis, DLS, TEM, TGA, and SAXS. Subsequently, siRNA was complexed to polymer-stabilized AuNPs and characterized by agarose gel electrophoresis and enzymatic degradation studies. Various cancer cell lines were treated with complexes, and monitored for down-regulation of the targeted gene.
Synthesis and Characterization of BAB Triblock (Co)Polymer Gelators

**Overview**

The ability to synthesize narrowly-dispersed polymers with controlled architectures has allowed the design of an increasing number of biorelevant structures. For example, previous work in the McCormick research group focused on the self-assembly of stimuli-responsive di- and triblock copolymers has lead to opportunities in the drug delivery field. Building on this previous work, we report the synthesis and characterization of narrowly-dispersed BAB triblock copolymers capable of self-assembling into physically cross-linked gels. The block lengths can be varied in a facile manner to prepare reversible hydrogel, elastic networks. Poly(N-isopropylacrylamide) (P(NIPAM)) was chosen as the temperature-responsive outer block because of its reversible phase transition temperature (32 °C) near physiological conditions (37 °C)\(^{228}\) and studies indicating its cytocompatibility\(^{229}\). It is well known that the incorporation of hydrophilic monomers increases the lower critical solution temperature (LCST) of P(NIPAM) (M\(_5\)) and, therefore, the hydrophilic inner block length was carefully selected to prevent elevation of the LCST to temperatures above 37 °C.\(^{230, 231}\) Mechanical properties were targeted to that of collagen, a commonly used material for both two and three dimensional cell growth matrices. The effects of inner block length, solvent, and polymer concentration on mechanical properties were investigated. In addition, the apparent pore sizes of the gels were determined through pulsed field gradient NMR.
RAFT Synthesis of P(NIPAM-b-AM-b-NIPAM)

Initial studies were conducted utilizing a triblock copolymer comprised of an P(AM) (M1) inner block and a P(NIPAM) (M5) outer block. In order to obtain a symmetrical triblock copolymer, a P(NIPAM) macroCTA was synthesized with a difunctional trithiocarbonate, CMP (Scheme IV-1). The P(NIPAM\textsubscript{675}) (P1) macroCTA was analyzed by GPC (Figure IV-1, Table IV-1). After dialysis and lyophilization, P1 was chain extended with AM (M1). Utilizing \textsuperscript{1}H NMR (Figure IV-1), the conversion of acrylamide monomer was determined through the ratio of vinyl protons (5.2-6.2 ppm) to the methyne P(NIPAM\textsubscript{674}) protons (3.2-4) at t=0 and t=24 h. Approximately 90\% of the vinyl groups were polymerized at t=24 h, yielding a triblock copolymer with the composition P(NIPAM\textsubscript{337}-b-AM\textsubscript{705}-b-NIPAM\textsubscript{337}) (P2) (Table IV-1). While the M\textsubscript{n} and PDI could not be determined by GPC due to incompatible chromatography conditions, dynamic light scattering and rheometric tests were conducted in order to determine whether the material could form self-assembled structures in response to temperature.
Scheme IV-1. Synthetic route for the preparation of AM (M1) containing BAB triblock copolymers via aqueous RAFT polymerization.

Figure IV-1. SEC trace for P(NIPAM<sub>675</sub>) (P1) macroCTA.
Table IV-1. Reaction Conditions and Gel Permeation Data for the Synthesized Polymers.

<table>
<thead>
<tr>
<th>Experimental Structure, Entry #</th>
<th>Time (h)</th>
<th>Temp (ºC)</th>
<th>M_n (kDa)</th>
<th>M_n inner (kDa)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(NIPAM_{674}), P_1</td>
<td>10</td>
<td>30</td>
<td>76\textsuperscript{A}</td>
<td>-</td>
<td>1.06</td>
</tr>
<tr>
<td>P(NIPAM_{337-705-375}), P_2</td>
<td>24</td>
<td>27</td>
<td>126\textsuperscript{B}</td>
<td>50\textsuperscript{B}</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\textsuperscript{A} As determined by MALLS. \textsuperscript{B} Estimated by the conversion determined from NMR.

Figure IV-2. \textsuperscript{1}H NMR spectrum of a polymerization solution of P(NIPAM\textsubscript{674}) (P_1) difunctional macroCTA in the presence of acrylamide (M_1) monomer at 0 and 24 h. Monomer conversion was determined by normalizing spectra to the methyne proton of P(NIPAM) (3.6 ppm) and subsequently comparing the areas under vinylic protons at t= 0 h and t= 24 h.
Characterization of Temperature-Induced Assemblies of P(NIPAM\textsubscript{337}-b-AM\textsubscript{705}-b-NIPAM\textsubscript{337})

As shown in Figure IV-3, the hydrodynamic diameter (D\textsubscript{h}) of P(NIPAM\textsubscript{337}-b-AM\textsubscript{705}-b-NIPAM\textsubscript{337}) (P\textsubscript{2}) gradually decreases as the temperature is increased from 25 to 38 °C. The decrease in D\textsubscript{h} is due to the collapse of the P(NIPAM) block as the temperature approaches the LCST. As the temperature is increased above 38 °C, the polymer assembles into a single species in the size range of micelles with a constant count rate indicating the material does not precipitate. Because NIPAM homopolymer precipitates at temperatures above the LCST, the DLS results indicate successful chain extension with AM. Additionally, the presence of a single population at elevated temperatures indicates the absence of P(AM) homopolymer impurity. An increase in the phase transition temperature from 32 °C to 38 °C can be attributed to the incorporation of hydrophilic acrylamide.

Figure IV-3. Hydrodynamic diameter as a function of temperature for a 0.1 wt% solution of P(NIPAM\textsubscript{337}-b-AM\textsubscript{705}-b-NIPAM\textsubscript{337}) (P\textsubscript{2}).
Given the evidence of triblock copolymer formation, tube inversion tests were conducted on different wt% polymer solutions heated between 33 °C and 41 °C. As shown in Table IV-2, 2.5-10 wt% solutions were free flowing sols upon inversion within the temperature range tested. However, a 20 wt% solution formed a free-standing gel upon inversion at temperatures ≥ 39 °C. Given these results, rheometric tests were conducted to probe the mechanical properties. As shown in Figure IV-4, the polymer solution exists as a low moduli material below the phase transition temperature. As the temperature is increased, the moduli increase by 5 orders of magnitude which can be attributed to the formation of an interconnected network.

**Table IV-2.** Tube Inversion Test Results for Various Weight % Solutions of P(NIPAM<sub>337</sub>-b-AM<sub>705</sub>-b-NIPAM<sub>337</sub>) (P2). X=Solution. Gel Defined as Free-Standing Material Upon Tube Inversion.

<table>
<thead>
<tr>
<th>Wt% Polymer</th>
<th>Temperature (°C)</th>
<th>2.5</th>
<th>5.0</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>35</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>37</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viscous sol</td>
</tr>
<tr>
<td>39</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Gel</td>
</tr>
<tr>
<td>41</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Gel</td>
</tr>
</tbody>
</table>
Figure IV-4. Dynamic temperature ramp of a 20 wt% gel from P(NIPAM$_{337}$-b-AM$_{705}$-b-NIPAM$_{337}$) (P2) dissolved in deionized water. Storage ($G'$) and loss ($G''$) modulus represented respectively by closed and open symbols.

RAFT synthesis of P(NIPAM-b-DMA-b-NIPAM)

Given the preliminary success of developing a high modulus gel with P(NIPAM$_{337}$-b-AM$_{705}$-b-NIPAM$_{337}$) (P2), research was extended to a second system where the inner A block was comprised of DMA (M2). By using DMA as the inner block, two key advantages over the previous system were expected 1) P(NIPAM-b-DMA-b-NIPAM) can be characterized by GPC due to solubility of both blocks in DMF 2) DMA is less hydrophilic than AM (M1), decreasing the chance of shifting the phase transition above 37 °C. A difunctional P(NIPAM) macroCTA was utilized in the chain extension of DMA to yield a symmetrical triblock copolymer in a two-step synthesis. Scheme IV-2 depicts the synthetic route to achieve a difunctional P(NIPAM) macroCTA which could be subsequently chain extended with DMA. CMP was chosen due to its hydrolytic stability under acidic conditions. After dialysis and lyophilization, the
polymers were characterized by triple detection SEC in dimethylformamide (DMF) (see Table IV-3, Figure IV-5). The $dn/dc$ of each polymer, listed in Table IV-3, was determined using Omnisec software. The triblock copolymers have well-defined molecular architectures with polydispersities below 1.10. A large contribution to mechanical strength of the thermoreversible gels arises from the hydrophobic interactions of P(NIPAM). Hence, a long outer block length was selected to increase the number of hydrophobic associations. Moderate inner block lengths were targeted to facilitate intermicellar bridging at low concentrations while maintaining phase transition temperatures below 37 °C. The P(NIPAM$_{910}$) macroCTA (P3) and triblock copolymer P(NIPAM$_{455}$-$b$-DMA$_{210}$-$b$-NIPAM$_{455}$) (P4) closely approach the respective targeted structures of P(NIPAM$_{900}$) and P(NIPAM$_{455}$-$b$-DMA$_{200}$-$b$-NIPAM$_{455}$).

Scheme IV-2. Synthetic route for the preparation of DMA (M2) containing BAB triblock copolymers via aqueous RAFT polymerization.
Table IV-3. Reaction conditions and gel permeation data for the synthesized polymers.

<table>
<thead>
<tr>
<th>Experimental Structure, Entry #</th>
<th>Time (h)</th>
<th>Temp (ºC)</th>
<th>M_n^A (kDa)</th>
<th>M_minner^B (kDa)</th>
<th>PDI^A</th>
<th>dn/dc</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(NIPAM_{910}), P3</td>
<td>10</td>
<td>30</td>
<td>103.2</td>
<td>-</td>
<td>1.07</td>
<td>0.074</td>
</tr>
<tr>
<td>P(NIPAM_{455}-b-DMA_{210}-b-NIPAM_{455}), P4</td>
<td>1</td>
<td>27</td>
<td>124.1</td>
<td>20.9</td>
<td>1.06</td>
<td>0.078</td>
</tr>
<tr>
<td>P(NIPAM_{455}-b-DMA_{277}-b-NIPAM_{455}), P5</td>
<td>1</td>
<td>27</td>
<td>130.7</td>
<td>27.5</td>
<td>1.08</td>
<td>0.084</td>
</tr>
</tbody>
</table>

^A As determined by triple detection GPC conducted in 0.02 M LiBr DMF eluent. ^B As determined by the difference between the triblock and homopolymer molecular weight.

Figure IV-5. SEC traces for the P(NIPAM_{910}) macroCTA (P3) (black), P(NIPAM_{455}-b-DMA_{210}-b-NIPAM_{455}) (P4) (green), and P(NIPAM_{455}-b-DMA_{277}-b-NIPAM_{455}) (P5) (red). A 0.02 M LiBr DMF solution was used as the eluent.

Characterization of Temperature Induced Assemblies of P(NIPAM-b-DMA-b-NIPAM)

The effects of inner block length, polymer concentration, and solvent on the storage modulus (G'), and loss modulus (G''), and gel point (T_gel) of the temperature-responsive polymers were determined through a series of dynamic temperature ramp
The dynamic temperature ramps of P4 dissolved in deionized water and 140 mM NaCl/20 mM phosphate buffer at different weight percents are shown in Figure IV-6. At the specific frequency and stress used in the dynamic temperature ramp experiments, the polymer solutions respond elastically (G’ > G”) at temperatures below the phase transition of the P(NIPAM) blocks. Though G’ is larger than G” at room temperature, the materials are not deemed gels because of their overall low modulus and ability to flow under their own weight. As can be observed in Figure IV-6A, no significant changes in moduli occur for the 2.5 wt% solution dissolved in DI water. The 5.0 wt% solution of P4 exhibits a slight increase in G” but no significant changes in G’.

According to Semenov, the formation of intermicellar bridges does not occur until a critical concentration of flower micelles is reached. In addition, the decrease of G’ to its initial value at 45 °C suggests negligible bridging at these temperatures. At 7.5 wt%, G’ increases as the temperature is raised from 37 to 42 °C but falls back to its initial value at higher temperatures indicating that interconnected flower micelles may form between these intermediate temperatures; however, bridging is not sufficient for the formation of a gel network. Previous reports indicate G’ is related to the lifetime of the junctions and the stability of the micelle core. P(NIPAM) dangling chain ends incur a larger enthalpic penalty at elevated temperatures because of poor outer block solubility. The increased enthalpic penalty leads to a shorter lifetime of the dangling chain ends, an increase in the lifetime of the junctions, and hence an overall higher modulus.

As illustrated in Figure IV-6B, the addition of salt results in a two phase transition (most notably for solutions ≤ 7.5 wt%). Previously, Bergbreiter, Cremer, and coworkers reported the two step phase transition of P(NIPAM) which occurs in the presence of
kosmotropes (water-structure makers) above a certain concentration.\textsuperscript{233, 234} The LCST of P(NIPAM) was reported to have a dependence on both anion concentration and anion type.\textsuperscript{233-235} The two step transition was also affected by the molecular weight and concentration of the polymer.\textsuperscript{234} Hence, the two transitions seen in Figure IV-6B may be explained by the presence of two kinds of anions, Cl\textsuperscript{−} and PO\textsubscript{4}\textsuperscript{−}, as well as the two step phase transition described in the aforementioned literature. Similar trends in the dynamic temperature ramps were seen for P5 (Figure IV-7).
Figure IV-6. Storage ($G'$) and loss ($G''$) moduli plotted against temperature for different concentrations of P(NIPAM$_{455}$-b-DMA$_{210}$-b-NIPAM$_{455}$) (P4) A) in DI water and B) in 140 mM NaCl/20 mM phosphate buffer pH 7.4.
Figure IV-7. Storage (G’) and loss (G”) moduli plotted against temperature for different concentrations of P(NIPAM$_{455}$-b-DMA$_{277}$-b-NIPAM$_{455}$) (P$_5$) A) in DI water and B) in 140 mM NaCl/20 mM phosphate buffer pH 7.4.
The gelation temperature ($T_{gel}$) of these materials was defined as the crossover between $G'$ and $G''$. As shown in Figure IV-8a, $T_{gel}$ decreases as the polymer concentration increases. The concentration dependence of $T_{gel}$ corresponds to the depression in the phase transition temperature of P(NIPAM) with increasing concentration.\textsuperscript{228, 236, 237} Because entries P\textsubscript{4} and P\textsubscript{5} have the same P(NIPAM) block length, the similarity of $T_{gel}$ at various concentrations is not surprising. It should be noted that at 7.5 wt\% P\textsubscript{5} forms a gel whereas P\textsubscript{4} does not. Referring back to Semenov’s theory, the loops on P\textsubscript{5} should extend further into solution than those of P\textsubscript{4}, resulting in overlap and therefore gelation at lower polymer concentrations.\textsuperscript{59} A series of steady stress sweep tests indicate that the triblock copolymers begin overlapping at approximately 7.5 to 10 wt\% polymer. The addition of 140 mM NaCl/ 20 mM phosphate buffer uniformly depresses $T_{gel}$, resulting in systems that gel at temperature below 37 °C. The depression of $T_{gel}$ can be attributed to the disruption of the hydrophobic water shells solvating the isopropyl groups of P(NIPAM).\textsuperscript{238}
Figure IV-8. Gelation temperature ($T_{gel}$) and storage modulus at the gelation temperature ($G'_T_{gel}$) plotted against the solution concentration of the triblock copolymer. Closed and open symbols represent polymers dissolved in DI water and 140 mM NaCl/20 mM phosphate buffer pH 7.4, respectively. $P(\text{NIPAM}_{455}-b-\text{DMA}_{210}-b-\text{NIPAM}_{455})$ (P4) and $P(\text{NIPAM}_{455}-b-\text{DMA}_{277}-b-\text{NIPAM}_{455})$ (P5) represented by squares and triangles respectively.
Figure IV-9. The plateau modulus ($G''_{\text{plateau}}$) as a function of polymer concentration and solvent conditions. Closed and open symbols represent polymers dissolved in DI water and 140 mM NaCl/20 mM phosphate buffer pH 7.4, respectively. P(NIPAM$_{455}$-$b$-DMA$_{210}$-$b$-NIPAM$_{455}$) (P$_4$) and P(NIPAM$_{455}$-$b$-DMA$_{277}$-$b$-NIPAM$_{455}$) (P$_5$) represented by squares and triangles respectively.

Figures IV-8-9 indicate that the storage moduli at the gelation temperature ($G'_{\text{Tgel}}$) and the moduli at the plateau ($G''_{\text{plateau}}$) increase with polymer concentration. These increases can be attributed to the increased number of bridges at higher concentrations as well as an increased number of hydrophobic interactions. When dissolved in DI water, P$_5$ exhibits a slightly larger $G'_{\text{Tgel}}$ than P$_4$ over all tested concentrations, suggesting P$_5$ has more bridges connecting hydrophobic domains. The addition of salts to the solvent results in slight increases in $G'_{\text{Tgel}}$ for P$_4$ and P$_5$. In general, kosmotrope salts disrupt the ordered water shell which solvates the isopropyl group and therefore decrease the solubility of P(NIPAM). As stated previously, a decrease in solvent quality should increase $G'$ by decreasing the lifetime of dangling chain ends.
In order to determine the biological relevance of the material properties, the mechanical properties of collagen, a commonly used *in vitro* cell growth platform, were tested at 37 °C (Figure IV-10, dotted line). Figure IV-10 shows that mechanical properties similar to collagen can be achieved with the synthesized triblock copolymers by adjusting the concentration of polymer in solution. When the polymers are dissolved in DI water, $G''_{37^\circ C}$ increases linearly with the semilog plot above a critical concentration ($C^*$). A dramatic increase in $G''_{37^\circ C}$ occurs above $C^*$ when the polymer is dissolved in 140 mM NaCl/ 20 mM phosphate buffer. One may explain this trend by referring back to Semenov’s theory and basic thermodynamics. Once a critical concentration of micelles is reached, bridging occurs between hydrophobic domains. The lifetime of dangling chain ends is decreased because of the increase in interfacial free energy which occurs as the P(NIPAM) block dangles in the incompatible salt solution. The decreased lifetime of the dangling chain end results in a stronger network.
Figure IV-10. Storage modulus at 37 °C ($G'_{37 \degree C}$) plotted against the solution concentration of the triblock copolymer. Closed and open symbols represent polymers dissolved in DI water and 140 mM NaCl/20 mM phosphate buffer pH 7.4, respectively. P(NIPAM$_{455}$-b-DMA$_{210}$-b-NIPAM$_{455}$) (P4) and P(NIPAM$_{455}$-b-DMA$_{277}$-b-NIPAM$_{455}$) (P5) are represented by squares and triangles respectively. The dotted line is included to show the $G'$ value of a typical collagen gel of 0.4 wt%.

The dependence of $D_{app}$ on total diffusion time $\Delta$ was determined using PFG NMR spectroscopy. For restricted geometries (such as bridged micelles), the diffusion coefficient varies with $\Delta$. At short $\Delta$, $D_{app}$ will be approximately the same as that of bulk water, $D_{free}$. As $\Delta$ increases, H$_2$O molecules will begin to encounter boundaries, thereby making $D_{app} D_{free}$. At a sufficiently long $\Delta$, $D_{app}$ will attain a limiting value. The onset of this limiting time ($t_d$) can be used to describe geometric aspects of the material. For example, the root mean square (RMS) end-to-end distance, $r$, can be determined using equation 7,

$$D_{app} = \frac{r^2}{6t_d} \quad (7)$$
in which $D_{\text{app}}$ is the apparent diffusion coefficient, $t_d$ is the time at which the diffusion coefficient exists, and $r$ is the root mean square end-to-end distance.\textsuperscript{240}

PFG NMR studies were conducted on 10 wt% $P_4$ and $P_5$ in buffer solution. These concentrations are those at which these synthetic gel mechanical properties at 37 °C closely resemble those of collagen. As evidenced in Figure IV-11, the triblock copolymers exhibit similar trends. After 40 ms of diffusion time the diffusion coefficient of water begins to decrease rapidly until a short plateau region is reached at approximately 400-600 ms. The plateau region suggests that all diffusing species are experiencing boundaries on this time scale. Using equation 7, the root mean square end-to-end distance can be calculated to be approximately 75 μm and 68 μm for the initial plateau region of $P_4$ and $P_5$, respectively. To properly support cell growth, the surface features of a scaffold should be smaller than the dimensions of a cell (tens of microns).\textsuperscript{241} The decrease in $D_{\text{app}}$ at $\Delta > 700$ ms may be due to background gradients caused by imperfect shimming and the magnetic susceptibility of the sample. These effects have a more significant impact on measurements taken at long $\Delta$.\textsuperscript{242, 243}
Figure IV-11. The apparent diffusion coefficient of water at 37 °C plotted against the observation time (Δ) for 10 wt% polymer in 140 mM NaCl/20 mM phosphate buffer pH 7.4. Closed and open squares represent P(NIPAM<sub>455</sub>-b-DMA<sub>210</sub>-b-NIPAM<sub>455</sub>) (P<sub>4</sub>) and P(NIPAM<sub>455</sub>-b-DMA<sub>277</sub>-b-NIPAM<sub>455</sub>) (P<sub>5</sub>), respectively.

In order to qualitatively investigate the structure of these gels, confocal microscopy experiments were conducted. The fluorescent probe, ANS, was chosen because of its increased emission intensity in nonpolar environments. Therefore, fluorescence should intensify at elevated temperatures because of the existence of hydrophobic domains. As can be seen in Figure IV-12, the fluorescence intensity increased significantly for P<sub>5</sub> when the temperature was increased to 40 °C. The fluorescent image contains large hydrophobic domains separated by tens of microns in the X-Y plane. The distance between hydrophobic domains observed in these experiments may correlate to the RMS distances calculated in the PFG studies. In addition, a distinct texture can be seen in the differential interference contrast image of P<sub>5</sub> at elevated temperatures indicating morphological changes. Though morphological
changes were observed for P4 (Figure IV-13) these were less significant than those observed for P5.

**Figure IV-12.** Confocal laser scanning microscope images of 10 wt% polymer in 140 mM NaCl/20 mM phosphate buffer pH 7.4 with 2.5 x 10^{-4} M 8-anilino-1-naphthalene sulfonic acid (ANS), a dye with increased emission intensity in nonpolar environments. Image of P(NIPAM$_{455}$-b-DMA$_{277}$-b-NIPAM$_{455}$) (P5) at A) room temperature and B) at 40 °C. Each image contains a differential interference contrast image (upper right), fluorescence image (upper left), and merged image (bottom left).
Figure IV-13. Confocal laser scanning microscope images of 10 wt% polymer in 140 mM NaCl/ 20 mM phosphate buffer pH 7.4 with $2.5 \times 10^{-4}$ M 8-anilino-1-naphthalene sulfonic acid (ANS), a dye with increased emission intensity in nonpolar environments. Image of P(NIPAM$_{455}$-b-DMA$_{210}$-b-NIPAM$_{455}$) (P4) at A) room temperature and B) at 40 °C. Each image contains a differential interference contrast image (upper right), fluorescence image (upper left), and merged image (bottom left).

To determine the morphology of solutions and gels, small angle x-ray measurements were conducted on a 20 wt% polymer solution of P4 at 25 and 50 °C. At room temperature, plots of $qI(q)$ vs $q$, $q^2I(q)$ vs $q$, and $I(q)$ vs $q$ do not show higher order reflections indicating the absence of hexagonally packed cylinders, lamellae, and BCC spheres, respectively. Likewise, increasing the temperature to 50 °C during acquisition of the profile, did not lead to higher order reflections. Thus, the primary mode of gelation appears to be due to the intermicellar bridging of polymer chains.
Figure IV-14. Small angle x-ray scattering on a 20 wt% solution of P(NIPAM$_{455}$-b-DMA$_{210}$-b-NIPAM$_{455}$) (P4) at 25 °C. A) qI(q) versus q plotted to determine the presence of hexagonally packed cylinders B) q$^2$I(q) versus q plotted to determine the presence of lamellae C) I(q) vs q plotted to determine the presence of body center cubic spheres.
Figure IV-15. Small angle x-ray scattering on a 20 wt% gel of P(NIPAM\textsubscript{455}-b-DMA\textsubscript{210}-b-NIPAM\textsubscript{455}) (P4) at 50 °C. A) $qI(q)$ versus $q$ plotted to determine the presence of hexagonally packed cylinders B) $q^2I(q)$ versus $q$ plotted to determine the presence of lamellae C) $I(q)$ vs $q$ plotted to determine the presence of body center cubic spheres.

While these materials exhibited the appropriate mechanical properties for cell growth scaffolds, two major factors limited the \textit{in vitro} application. First, the gels formed from these materials were opaque, thus limiting visibility of cell morphology (Figure IV-16). The ability to visualize the density and shape of cells at different time intervals provides biologists with important qualitative information about the state of the cell. For example, healthy endothelial cells generally have a flat oblong morphology while cells undergoing apoptosis exhibit round, spherical morphologies. Secondly, the materials exhibit a very rapid transition from the liquid to the sol state. Thus, removal of the cell
culture from an incubator for visualization under a microscope is problematic. Because the majority of cells are anchorage dependent, meaning adhesion sites are required for cell growth, the loss of anchorage sites during the dissolution of the gel can adversely affect the cells. Despite these disadvantages, advancements towards the design of physiologically-relevant stimuli-responsive gels were achieved. The mechanical properties could be tuned through both the control over polymer concentration, inner block length, and solvent.

Figure IV-16. Photographs of a 20 wt% solution of P(NIPAM$_{455}$-b-DMA$_{210}$-b-NIPAM$_{455}$) (P4) at 25 ºC and 50 ºC.
Synthesis and Characterization of ABA Triblock (Co)Polymer Gelators

*Overview*

Significant interest in hydrogels has arisen due to their potential applications in drug delivery and tissue engineering.\(^{245-247}\) Over the past decade CRP techniques have facilitated the synthesis of complex architectures capable of forming gels such as ABA and BAB stimuli-responsive block copolymers.\(^{237, 248-254}\) Notably, RAFT polymerization has proven to be one of the more versatile CRP techniques allowing the controlled polymerization of a wide variety of monomers under mild reaction conditions directly in water.\(^9^1\) The synthesis of well-defined amphiphilic polymers is desired because of the ability of these polymers to form an array of morphologies including gels. P(NIPAM)-based assemblies are of particular interest due to the phase transition temperature (32 °C) near physiological conditions.\(^{255}\) Diblock (AB) and triblock copolymers (ABA, BAB, and CBA), in which the A block is permanently hydrophilic and the B and C blocks are either permanently hydrophobic or stimuli-responsive, can form gels through various mechanisms. Significant research indicates that AB and ABA block copolymers form physical gels through the packing of micelles.\(^7^6-8^5\) Building on the previous work in the McCormick research group focused on the self-assembly of stimuli-responsive di- and triblock copolymers as well as the work presented in Section 1 on the assembly of BAB triblock copolymers, we investigated the effects of polymer block lengths and compositions on the gel properties of ABA triblock copolymers.
RAFT synthesis of $P(DMA-b-NIPAM-b-DMA)$

A difunctional chain transfer agent, (CTA) CMP (CTA1) was utilized to synthesize symmetrical triblock copolymers in two steps. In the first step (Scheme IV-3), a hydrophilic monomer (DMA, $M_2$) was synthesized via aqueous RAFT polymerization. Following dialysis to remove excess monomer and lyophilization, the $P(DMA)$ macroCTA was chain extended with NIPAM ($M_5$) to form a hydrophilic-$b$-temperature-responsive-$b$-hydrophilic copolymer.

Scheme IV-3. Synthetic route for the preparation of ABA triblock copolymers via aqueous RAFT polymerization.
The molecular weight and PDI of (co)polymers were determined from SEC (Table IV-4). The low PDIs obtained are indicative of the control exhibited during the polymerization. A range of molecular weights were synthesized with various hydrophilic mass fractions (f) (Table IV-4). By increasing the molecular weight why holding the hydrophilic mass fraction constant, the effect of polymer size versus composition can be distinguished.

**Characterization of Thermo-induced Assemblies**

Dynamic light scattering (DLS) experiments of 0.1 wt% solutions were conducted to verify temperature-induced aggregation in the dilute regime. At 25 ºC, the copolymers exist as unimers while star-like micelles form at 50 ºC with hydrodynamic radii \((R_h)\) between 17 and 40 nm (Table IV-4). Each polymer forms stable aggregates as indicated by a constant count rate. As expected, unimer and aggregate size increases with increasing polymer molecular weight.

Tube inversion tests indicated that 20 wt% polymer solutions formed transparent free-standing physical gels at 50 ºC. In order to demonstrate the reversible nature of the gel, the storage modulus of a 20 wt% sample of P(DMA\(_{93}\)-b-NIPAM\(_{372}\)-b-DMA\(_{93}\)) \((P10)\) was monitored as the temperature was cycled between 25 and 50 ºC. The storage modulus \((G')\) varies by several orders of magnitude as the temperature is cycled above and below the phase transition temperature (Figure IV-17). Additionally, the material responds rapidly and reversibly to changes in temperature. In order to investigate morphological changes which occur in the concentrated regime, SAXS experiments were conducted on 20 wt% polymer solutions at various temperatures. As shown in Figure IV-
18a, at 30 °C no peaks are observed for P(DMA$_{93}$-b-NIPAM$_{372}$-b-DMA$_{93}$) (P10). A primary peak at $q^* = 0.20$ nm$^{-1}$ begins to emerge and intensify as the temperature is increased to 50 °C. Weak higher-order reflections indicative of BCC packing ($q/q^* = 1:√2:√3$) are apparent at 50 °C for P(DMA$_{93}$-b-NIPAM$_{372}$-b-DMA$_{93}$) (P10). The distance between lattice planes, $d_{100}$ and $d_{110}$, at 50 °C are 44 and 31 nm, respectively.
Table IV-4. Physical Properties of Copolymers as Determined by Size Exclusion Chromatography, Dynamic Light Scattering, Small Angle X-Ray Scattering, and Oscillatory Rheometry.

<table>
<thead>
<tr>
<th>Experimental Structure, Entry #</th>
<th>$M_n$&lt;sup&gt;A&lt;/sup&gt; (kDa)</th>
<th>PDI&lt;sup&gt;A&lt;/sup&gt;</th>
<th>$R_g$ at 50ºC (nm)&lt;sup&gt;B&lt;/sup&gt;</th>
<th>$D_{110}$ at 50ºC (nm)&lt;sup&gt;C&lt;/sup&gt;</th>
<th>Lattice Type&lt;sup&gt;D&lt;/sup&gt;</th>
<th>$G'$&lt;sub&gt;50ºC&lt;/sub&gt; of 20 wt% gel (kPa)&lt;sup&gt;E&lt;/sup&gt;</th>
<th>$F$&lt;sup&gt;F&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(DMA&lt;sub&gt;186&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P6</td>
<td>18.4</td>
<td>1.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;394&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P7</td>
<td>39.1</td>
<td>1.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;500&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P8</td>
<td>49.6</td>
<td>1.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;93&lt;/sub&gt;-b-NIPAM&lt;sub&gt;213&lt;/sub&gt;-b-DMA&lt;sub&gt;93&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P9</td>
<td>42.9</td>
<td>1.06</td>
<td>17</td>
<td>26</td>
<td>BCC</td>
<td>3.7</td>
<td>0.43</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;93&lt;/sub&gt;-b-NIPAM&lt;sub&gt;372&lt;/sub&gt;-b-DMA&lt;sub&gt;93&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P10</td>
<td>55.7</td>
<td>1.07</td>
<td>21</td>
<td>31</td>
<td>BCC</td>
<td>3.1</td>
<td>0.33</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;197&lt;/sub&gt;-b-NIPAM&lt;sub&gt;392&lt;/sub&gt;-b-DMA&lt;sub&gt;197&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P11</td>
<td>83.7</td>
<td>1.05</td>
<td>28</td>
<td>38</td>
<td>BCC</td>
<td>2.8</td>
<td>0.47</td>
</tr>
<tr>
<td>P(DMA&lt;sub&gt;250&lt;/sub&gt;-b-NIPAM&lt;sub&gt;600&lt;/sub&gt;-b-DMA&lt;sub&gt;250&lt;/sub&gt;)&lt;sup&gt;1&lt;/sup&gt;, P12</td>
<td>117.4</td>
<td>1.07</td>
<td>40</td>
<td>44&lt;sup&gt;G&lt;/sup&gt;</td>
<td>NA</td>
<td>2.6</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Determined by <sup>A</sup> SEC, <sup>B</sup> DLS of a 0.1 wt% solution, <sup>C</sup> SAXS ($d_{110}=2\pi/q^*$), <sup>D</sup> ratio of $q/q^*$. <sup>E</sup>Value obtained at 1 rad/s from a dynamic frequency sweep (1 Pa). <sup>F</sup>f= permanently hydrophilic mass fraction as determined by SEC. <sup>G</sup>Lattice does not apply due to disordered state.
Figure IV-17. Storage modulus versus time of a 20 wt% sample of P(DMA$_{93}$-NIPAM$_{372}$-DMA$_{93}$) (P10) as temperature is cycled between 25 (■) and 50 °C (▲).

Similar to the molecular weight dependent aggregate size observed in DLS in the dilute regime, $d_{110}$ and $d_{100}$ of polymeric gels increase with increasing polymer molecular weight (Table IV-3, Figure IV-18B). BCC packing is observed in all gels with the exception of P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) (P12). Unlike the lower molecular weight polymers which are able to form ordered arrays, P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) (P12) lacks higher-order reflections indicating a less-defined morphology. However, the appearance of a primary peak indicates correlation hole effects giving density fluctuations on the length scale of 44 nm. The long hydrophilic block may prevent ordered packing through the kinetic trapping of entangled coronas. Given the approximately equal value of f for P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) (P9) and P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) (P12), the differences in ordering may be attributed to block lengths.
Figure IV-18. Small angle x-ray scattering of A) 20 wt% \( P(DMA_{93}-NIPAM_{372}-DMA_{93}) \) \((P10)\) at various temperatures and B) a series of 20 wt% copolymers \((P9-P12)\) at 50 °C. Red arrows indicate primary peaks and higher order reflections. Intensities are scaled by a factor of 10 for clarity.

In order to determine the effect of micellar packing on the mechanical properties of the gel, dynamic frequency sweep tests were conducted at low stress (Figure IV-19). Each polymer gel forms a hard gel \((G' > 1 \text{ kPa})^{256}\) at 50 °C and displays linear behavior.
in the frequency range studied. These mechanical properties are in the range of commonly reported values for hydrophobic-b-hydrophilic polymers. Booth and coworkers reported values of $G'$ near 3 kPa for a 16 wt% sample of poly(oxybutylene$_{20}$-b-oxyethylene$_{610}$) at 50 °C$^{78}$ while Derici et al. observed a storage modulus of 100 kPa for a 30 wt% solution of poly(oxyethylene$_{90}$-b-oxybutylene$_{10}$) at 50 °C$^{77}$.

**Figure IV-19.** Storage modulus data as a function of frequency for 20 wt% P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) (P9) (■), P(DMA$_{93}$-b-NIPAM$_{372}$-b-DMA$_{93}$) (P10) (●), P(DMA$_{197}$-b-NIPAM$_{392}$-b-DMA$_{197}$) (P11) (▲), P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) (P12) (▼) gels at 50 °C.

The storage modulus decreased from 3.7 to 2.6 kPa as the molecular weight increased from 43 to 117 kDa (Table IV-4 and Figure IV-19). The inverse relationship of $G'$ and molecular weight may be related to a second trend in which $d_{110}$ and $d_{100}$ are inversely related to $G'$. The smaller values of $d_{110}$ and $d_{100}$ in the lower molecular weight systems suggest that smaller aggregates comprise the gels, thus leading to higher surface
areas available for entanglements. Conversely, increasing the molecular weight results in larger values of $d_{100}$ and $d_{110}$ thus indicating the gels are comprised of larger aggregates which possess lower surface area available for interactions. In addition, longer outer block lengths may increase steric repulsions resulting in a lower $G'$. Thirdly, ordering appears to slightly increase $G'$ as noted from the low modulus of the disordered P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) ($\text{P12}$). Lastly, $G'$ appears to be independent of the chemical composition of the triblock copolymer. P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) ($\text{P12}$) and P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) ($\text{P9}$) have nearly the same chemical composition as indicated by $f$, but have markedly different mechanical strengths. Thus, lattice size may be adjusted by controlling molecular weight, in turn allowing the tuning of the mechanical properties.

In order to investigate the relevance of these gels in physiological media, dynamic temperature ramps were conducted with 15 and 20 wt% gels of P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) ($\text{P9}$) dissolved in 140 mM NaCl / 20 mM phosphate buffer (pH 7.4). At room temperature, these polymer solutions are viscous with $G''$ two orders of magnitude greater than $G'$. The onset of the sol-gel transition is observed at approximately 31 and 37 ºC for the 20 and 15 wt% gels, respectively. The concentration-dependent onset of aggregation is a commonly observed phenomenon in NIPAM containing polymers.$^{255}$ As expected for packed micellar gels, mechanical properties increase with increasing polymer concentration. The moduli of the 15 and 20 wt% samples are within the same order of magnitude below the phase transition temperature, indicating that differences due to water content are negligible (Figure IV-20). Conversely, above the gelation temperature, the storage modulus of the 20 wt% sample is ~2 orders of magnitude greater.
than that of the 15 wt% sample. Thus the increase in moduli with increasing polymer concentration may be attributed to a higher number of coronal interactions.

Figure IV-20. Storage (closed symbols) and loss modulus (open symbols) data as a function of temperature for 15 (●), and 20 (▲) wt% gels of P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) (P9) dissolved in 140 mM NaCl/20 mM phosphate buffer (pH 7.4).

As with the case of BAB gels investigated, these materials had mechanical properties and gelation temperatures suitable for cell growth applications. Additionally, these materials formed optically transparent gels (Figure IV-21) allowing the potential visualization of cells during culture. However, as demonstrated in Figure IV-21, these materials rapidly transition from a gel to a free-flowing solution. Thus, removal of the culture dish for visualization under a microscope might lead to detrimental effects on the cells.
Figure IV-21. Photographs of a 20 wt% solution of P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) (P9) at 25 °C and 50 °C.
*In Situ* Formation of Polymer-Stabilized Au Nanoparticles and Subsequent Complexation, Stabilization, and Delivery to Cancerous Cells

**Overview**

In this section we have examined the utility of RAFT-synthesized amphiphilic polymers for gene/diagnostic delivery applications. One major challenge for synthetic chemists will be understanding parameters that control self organization of assembled delivery vehicles, for example micelles, vesicles, interpolyelectrolyte complexes, etc. Once such morphologies can be constructed reproducibly, delivery and release under biologically relevant conditions can be pursued in a systematic way.

The specific aim of the research described herein was to develop synthetic conditions for the *in situ* formation of polymer-stabilized AuNPs capable of forming neutral, sterically stable complexes with small interfering RNA. In order to achieve this goal, a hydrophilic-b-cationic polymer was utilized, in which the amine-containing cationic block promoted the *in situ* reduction of Au$^{+3}$ to AuNPs and provided complexation sites for siRNA while the nonimmunogenic, hydrophilic block provided steric stabilization. The reduction conditions, specifically the ratio of amine repeat units to Au$^{+3}$, and the DMAPMA block length were critical to the formation of stable AuNPs. These synthetic conditions also significantly affect the ability of AuNPs to form electrostatic complexes with siRNA. Once the appropriate reduction conditions were determined, the ability of polymer-stabilized AuNPs to protect siRNA from nucleases and deliver RNA was studied.
Synthesis of Polymer Stabilized Au Nanoparticles

A series of P(HPMA-b-DMAPMA)s (M3-b-M13), synthesized via aqueous RAFT polymerization,\textsuperscript{132} (Scheme IV-4) were utilized in the reduction reaction of Au\textsuperscript{+3} to AuNPs due to requisite properties for the stabilization of AuNPs and complexation with siRNA. The HPMA block serves as a nonimmunogenic, neutral, hydrophilic, steric stabilizing block which prevents AuNP aggregation and provides additional steric protection against nucleases which can degrade siRNA. The neutral surface should also reduce opsonization and hence clearance by the MPS. The DMAPMA block serves three roles: 1) promote the \textit{in situ} reduction of Au\textsuperscript{+3} to Au\textsuperscript{0}, 2) coordinate to the AuNP surface via multivalent (N-Au) interactions, and 3) provide cationic sites for the electrostatic complexation of siRNA. As illustrated in Scheme IV-4, the \textit{in situ} formation of hydrophilic-\textit{b}-cationic stabilized AuNPs (HbC-AuNPs) was conducted at 50 °C for 24 h in phosphate buffer (50 mM, pH 6.0) utilizing either a 3/1, 10/1, or 100/1 ratio of [DMAPMA]/[AuCl\textsubscript{4}]-\textsubscript{0} (1.68 mM DMAPMA repeating units). In order to determine the optimal block length required for the formation of stable particles, four polymers with the same HPMA block length (70) but varying DMAPMA block length (24-104) were utilized in the reduction reactions. To aid in the discussion of results, each reduction reaction has been assigned an acronym based on the polymer utilized in the reduction reaction and a subscript referring to the [DMAPMA]/[AuCl\textsubscript{4}]-\textsubscript{0} ratio utilized (Table IV-5). For example, AuNPs synthesized with P(HPMA\textsubscript{70}-b-DMAPMA\textsubscript{24}) (P14) and a 3/1 ratio of [DMAPMA]/[AuCl\textsubscript{4}]-\textsubscript{0} is assigned the entry P14\textsubscript{3}. 
Scheme IV-4. Reaction scheme for the formation of small interfering ribonucleic acid/Au nanoparticle (siRNA/AuNP) complexes (idealized representation). Reduction of AuCl$_4^-$ in the presence of P(HPMA-$_b$-DMAPMA) (M$_3$-b-M$_{13}$) yielding hydrophilic-$_b$-cationic AuNPs (HbC-AuNPs). Complexation of siRNA to HbC-AuNPs yielding an oligonucleotide loaded, polymer-stabilized AuNP (Au nanoplex). Inset illustrates the multivalent coordination of the DMAPMA repeat units with the AuNP surface.
### Table IV-5. Reduction Reaction Conditions for P(HPMA) \((M3)\) and P(HPMA-\(b\)-DMAPMA) \((M3-\textit{b-M13})\) Copolymers and Resulting Physical Properties.

<table>
<thead>
<tr>
<th>Entry(^A)</th>
<th>Polymer</th>
<th>([\text{DMAPMA}]/[\text{AuCl}_4^-]_0)</th>
<th>SPR Band(^B)</th>
<th>Stable AuNPs(^C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13</td>
<td>P(HPMA(_{70}))</td>
<td>see footnote D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P14(_{10})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{24}))</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>P14(_{100})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{24}))</td>
<td>10</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>P15(_{10})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{49}))</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>P15(_{100})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{49}))</td>
<td>10</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>P16(_{10})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{84}))</td>
<td>3</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>P16(_{100})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{84}))</td>
<td>10</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>P17(_{10})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{105}))</td>
<td>3</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>P17(_{100})</td>
<td>P(HPMA(<em>{70-\textit{b-DMAPMA}}</em>{105}))</td>
<td>10</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

\(^A\) Subscript refers to the \([\text{DMAPMA}]/[\text{AuCl}_4^-]_0\) ratio utilized during the reduction. Determined by \(^B\) UV-Vis and \(^C\) physical observation or through monitoring the count rate obtained by DLS. \(^D\) Reductions were conducted utilizing 1.68 mM DMAPMA. For entry P13, which does not contain a DMAPMA block, reduction conditions were chosen to contain the same molarity polymer and AuCl\(_4^-\) utilized during the reduction reaction of P14\(_{10}\).
Successful reduction of Au$^{+3}$ to AuNPs was monitored by the appearance of the surface plasmon resonance (SPR) band at ~520 nm (Table IV-5). For reductions conducted with a [DMAPMA]/[AuCl$_4^-_0$] of 100/1, no SPR band was observed. Based on Turkevich’s description of AuNP formation, a critical mass of Au$^{+3}$ must accumulate before Au nuclei can form.$^{257}$ Thus, the low concentration of Au$^{+3}$ utilized in the reactions P$_{14-17}$ may have prevented Au nanoparticle formation. Increasing the [DMAPMA]/[AuCl$_4^-_0$] to 10/1 and 3/1 lead to the formation of AuNPs as indicated by the SPR band (Figure IV-22), though not all samples were stable (Table IV-5).

**Figure IV-22.** UV-Vis spectra of the in situ reduction of AuCl$_4^-$ in the presence of 1.68 mM DMAPMA repeating units (M$_{13}$) after heating at 50 °C for 24 h. P(HPMA$_{70-b}$-DMAPMA$_{24}$) (P$_{14}$), P(HPMA$_{70-b}$-DMAPMA$_{49}$) (P$_{15}$), P(HPMA$_{70-b}$-DMAPMA$_{64}$) (P$_{16}$). Subscripts refer to the ratio of [DMAPMA]/[AuCl$_4^-_0$] utilized in the reduction reaction.

P$_{16}_3$ and P$_{16}_10$ precipitated several days after the reduction reaction while P$_{17}_3$ and P$_{17}_10$ formed large macroscopic precipitates during the reduction reaction. Since the
concentration of DMAPMA repeating units (1.68 mM) was held constant in each series of reduction reactions rather than the concentration of polymer, the molarity of HPMA repeat units varied from 4.9 mM for P14 to 1.1 mM for P17. Because the P(HPMA) block provides steric stabilization, a reduced concentration could lead to unstable particles. Figure IV-23 illustrates this concept, in which idealized representations of P14 and P17 stabilized AuNPs are shown with approximately the same number of DMAPMA repeat units per particle. The red and blue circles represent, respectively, DMAPMA and HPMA repeat units. Steric stabilization of P17 AuNPs depicted in Figure IV-23 should be significantly decreased as compared to that of P14 AuNPs due to a significantly less dense HPMA shell.

![Figure IV-23. Idealized representation of polymer stabilized Au nanoparticles containing approximately the same number of DMAPMA repeat units per particle.](image-url)
In order to confirm that the DMAPMA units are necessary for AuNP formation, a control reaction was conducted with P(HPMA$_{70}$) ($\text{P13}$). In this reaction, 70 μM of $\text{P13}$ was incubated with AuCl$_4^-$ (0.168 mM) at 50 °C for 72 h. These conditions were chosen to duplicate the molarity of polymer and AuCl$_4^-$ utilized in the reduction reaction of P14$_{10}$. Absence of the SPR band (Figure IV-24) after the $\text{P13}$ reduction indicates AuNPs do not form in the absence of DMAPMA.

![UV-Vis spectra](image)

**Figure IV-24.** UV-Vis spectra of the *in situ* reduction reaction of AuCl$_4^-$ in the presence of 1.68 mM DMAPMA repeating units ($\text{M13}$) of P(HPMA$_{70}$-b-DMAPMA$_{24}$) ($\text{P14}_{10}$) (70 μM), after heating at 50 °C for different time intervals and the *in situ* reduction reaction of AuCl$_4^-$ in the presence of 70 μM P(HPMA$_{70}$) ($\text{P13}$) after heating at 50 °C for 72 h. Reduction reactions were performed in 50 mM pH 6.0 phosphate buffer.

$\text{P14}_{10}$ was chosen for further analysis given its aqueous stability for extended periods of time. Unbound polymer (~14 kDa) was removed from the stabilized AuNPs by dialysis in 50 kDa MWCO tubing. The remaining deep-red solution was lyophilized, analyzed by TGA for polymer concentration (Figure IV-25) and subsequently redispersed
in phosphate buffer (50 mM, pH 6.0) at a DMAPMA concentration of 1.0 mM. The size of redispersed particles was analyzed by TEM (Figure IV-26). Given the high electron density of AuNPs in comparison to polymer chains, only Au cores are visible in TEM. Approximately 100 particles were analyzed utilizing DigitalMicrograph 3.4 Software (Gatan) to determine an average Au core diameter ($D_{Au}$) of 6.5 nm (Table IV-6, Figure 26). In order to determine whether the Au cores seen on the TEM micrograph were comprised of a “cluster” of smaller Au particles or a single large AuNP, small angle x-ray scattering experiments were conducted (Figure IV-27, Table IV-6). The diameter determined by SAXS, 7.8 nm, is in good agreement with the diameter determined by TEM, indicating $\text{P14}_{10}$ AuNPs consist of a single AuNP core.

**Figure IV-25.** Percent weight loss of sample as a function of temperature for $\text{P(HPMA}_{70-b-\text{DMAPMA}}_{24})$ (P14) (---) and $\text{P14}$-stabilized Au nanoparticles ($\text{P14}_{10}$) (---).
Figure IV-26. A) TEM micrograph of P(HPMA$_{70}$-b-DMAPMA$_{24}$)-stabilized AuNPs (P14$_{10}$). B) Distribution and cumulative % of Au core diameter sizes.

Figure IV-27. Small angle x-ray diffraction scattering profile for P(HPMA$_{70}$-b-DMAPMA$_{24}$)-stabilized AuNPs (P14$_{10}$).
Table IV-6. Properties of the P(HPMA$_{70}$-b-DMAPMA$_{24}$) (P14) and (P14$_{10}$)-Stabilized AuNPs P14$_{10}$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>D$_{Au}$ (nm)$^A$</th>
<th>D$_{Au}$ (nm)$^B$</th>
<th>D$_h$ (nm)$^C$</th>
<th>ζ potential (mv)$^D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(HPMA$<em>{70}$-b-DMAPMA$</em>{24}$), P14</td>
<td>-</td>
<td>-</td>
<td>3.8</td>
<td>19</td>
</tr>
<tr>
<td>P14$_{10}$</td>
<td>6.5</td>
<td>7.8</td>
<td>29</td>
<td>-1.1</td>
</tr>
</tbody>
</table>

Determined by $^A$TEM, $^B$SAXS (D$_{Au}$=2π/ν), $^C$DLS, $^D$zeta potential.

Key particle characteristics, including neutral surface charge$^{258}$ and appropriate hydrodynamic diameter (D$_h$)$^{259}$ were characterized by ζ potential and dynamic light scattering. As expected, P14 has a positive ζ potential (19 mV) due to the cationic DMAPMA repeats which may be present at the slipping plane$^{260}$ of the unimers (Table IV-6, Figure IV-28). As illustrated in Scheme IV-4 (inset), coordination of only a few of the 24 DMAPMA repeat units to the AuNP surface should relegate the cationic charge to the inner shell while steric repulsions force the HPMA block to the outer shell resulting in a near neutral ζ potential. As predicted, the ζ potential of P14$_{10}$ (-1.1 mV) is significantly lower than that of unimers and approaches neutrality.$^{260}$ Additionally, the D$_h$ (< 100 nm) has been shown to be critical in the passive delivery of nanoparticles.$^{109}$ $^{259}$ If the unimers were to adopt a random coil confirmation on the surface of the AuNP, the diameter could be predicted by Equation 8

$$2D_{Unimer} + D_{Au} = D_{P14-AuNP}$$  (8)
in which $D_{\text{Unimer}}$ is the hydrodynamic diameter of unimer and $D_{\text{P14-AuNP}}$ is the hydrodynamic diameter of the P14-stabilized AuNP. However, $D_{\text{P14-AuNP}}$ (29 nm, Table IV-6, Figure IV-28) is significantly larger than the value predicted by Equation 8 (14.1 nm), indicating polymer chains adopt an extended conformation once bound to the Au surface, likely due to steric repulsions of neighboring chains. Additionally, unimers are not detected by DLS in the HbC-AuNP sample, indicating dialysis successfully removes unbound polymer.

**Figure IV-28.** A) Zeta potential of P(HPMA\textsubscript{70-}b-DMAPMA\textsubscript{24}) (P14) (▲) and P(HPMA\textsubscript{70-}b-DMAPMA\textsubscript{24})-stabilized AuNPs (P14\textsubscript{10}) (■) B) Hydrodynamic diameters of P(HPMA\textsubscript{70-}b-DMAPMA\textsubscript{24}) (P14) (▲) and P(HPMA\textsubscript{70-}b-DMAPMA\textsubscript{24})-stabilized AuNPs (P14\textsubscript{10}) (■) measured by dynamic light scattering.

**siRNA Complexation and Degradation Studies**

To further explore the potential of the HbC-AuNPs as gene delivery carriers, oligonucleotide complexation studies were conducted with P14\textsubscript{10}. Traditionally, neutral interpolyelectrolyte complexes are achieved by mixing oligonucleotides and polycations...
at an $N/P$ ratio of 1. However, in the case of the HbC-AuNPs, a fraction of the DMAPMA groups interact with the Au surface and thus are not able to bind the oligonucleotide. Thus, the HbC-AuNPs are expected to require an $N/P$ ratio greater than 1 to form neutral Au nanoplexes. Because ζ potential measurements are restricted to determining the surface charge of a species, agarose gel electrophoresis was utilized to determine the $N/P$ ratio yielding a neutral Au nanoplex. As visualized by gel electrophoresis in Figure IV-29A, neutral complexes of RNA and P1410 form when an $N/P$ ratio between 1.5 and 2.0 are utilized. Deviations from this ratio lead to charged nanoplexes as indicated by the migration of the material on the gel. Further gel electrophoresis experiments indicated neutral complexes were formed when an $N/P$ ratio of ~1.85 was utilized, suggesting approximately 54% of the DMAPMA repeat units are available for complexation with siRNA. While the exact mechanism of AuNP formation in the presence of amines is unclear,261-264 that changing the ratio of [DMAPMA]/[AuCl$_4$]$_0$ from 10/1 to 3/1 led to HbC-AuNPs with poor oligonucleotide binding capacities ($N/P > 15$ is required for neutralization, Figure IV-29B). The poor binding capacity may result from a large fraction of the amines coordinating directly with gold, leaving few available sites for electrostatic complexation. Thus, the ratio of [DMAPMA]/[AuCl$_4$]$_0$ appears to be critical in the design of HbC-AuNP oligonucleotide carriers.
Once the neutral point was determined, degradation studies were conducted with Riboshredder™ RNase, a potent mixture of nucleases. In order to demonstrate siRNA protection, 500 nM siRNA (free or complexed) was exposed to a 30 times excess of RNase (3 units/mL, ~ 6 µg/mL) as compared to serum concentrations (0.2 µg/mL). Observation of the hyperchromic effect, an increase in absorbance at 260 nm (Abs$_{260}$) as oligonucleotides are degraded to mononucleotides, can be seen in Figure 5. Because AuNPs absorb at 260 nm, the spectrophotometer was blanked with a buffer solution containing an identical concentration of P$_{14}$$^{10}$ before conducting Au nanoplex degradation studies. Likewise, the spectrophotometer was blanked with a buffer solution before conducting the free siRNA degradation study. As indicated by the 30% increase in Abs$_{260}$ within 3 min (Figure IV-30), free siRNA is rapidly degraded in the presence of 3 units/mL of RiboShredder™ RNase. Conversely, less than a 3% increase in
absorbance occurs for neutral Au nanoplexes exposed to the same amount of RiboShredder™ RNase. The level of siRNA protection, as determined by the relative rates of enzymatic degradation of free and bound siRNA, increases 100 fold when in the Au nanoplex form.

**Figure IV-30.** Enzymatic degradation of naked and complexed small interfering ribonucleic acid (siRNA) (500 nM) with Riboshredder™ RNase (3 units/mL) as monitored by the percent increase in absorbance at 260 nm. Naked siRNA (---) and neutral P1410 Au nanoplexes (---).

*Delivery of siRNA from polymer-stabilized Au Nanoparticles*

Before cell delivery studies were conducted, the cytotoxicity of P1410 was measured using a CellTitr 96 Aqueous one solution cell proliferation assay (Promega) (Figure IV-31). Cytotoxicity of P1410 AuNPs was found to be negligible in the concentration range studied. After determining the biocompatibility of AuNP carriers, several lines of cancer cells (KB, HeLa, SKOV3, A549) were incubated with P1410 Au nanoplexes (50 nM 59-nucleotide cyanine-3 survivin siRNA134 (Cy3-siRNA)) for 40 min.
Following incubation, cells were rinsed thoroughly with phosphate buffered saline to remove free Au nanoplexes and the cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). As demonstrated in Figure IV-32, all cell lines treated with P14\textsubscript{10} Au nanoplexes exhibited red fluorescence, indicating internalization of Cy3-siRNA. KB cells were also treated with uncomplexed Cy3-siRNA (Figure IV-32) as a negative control. Negligible fluorescence was observed, indicating the polymer-stabilized AuNP carrier was required for delivery.

\textbf{Figure IV-31.} Cytotoxicity of P(HPMA\textsubscript{70}-b-DMAPMA\textsubscript{24}) (P14\textsubscript{10}) stabilized Au nanoparticles (AuNPs) Arrow points to concentration utilized in gene delivery studies.
Figure IV-32. Fluorescent microscope images of A) KB cells B) HeLa cells C) SKOV3 cells and D) A549 cells treated with 100 nM cyanine-3 labeled small interfering RNA (Cy3-siRNA) complexed with P14_{10}. E) As a control, KB cells were incubated with uncomplexed Cy3-siRNA. Cell nuclei were stained with 4’-6-diamidino-2-phenylindole (DAPI, blue). Scale bars= 20 μm.

To determine whether siRNA could be released from the AuNP carrier once internalized, gene down-regulation of a target protein was monitored. KB cell lines stably expressing *Gaussia* Luciferase (GLuc) were treated with 100 nM 59-nucleotide GLuc siRNA (free siRNA and P14_{10} Au nanoplexes) for 6 h. In addition, cells were treated with complexes in the presence and absence of serum to ensure opsonization was
not responsible for the uptake of Au nanoplexes. After incubation, cells were rinsed to remove free Au nanoplexes and fresh medium was added to the well. Figure IV-33 shows the down-regulation of the target gene when incubated with Au nanoplexes in comparison to positive and negative controls. Controls included no treatment, treatment with uncomplexed GLuc siRNA, treatment with nontargeting siRNA control P14\textsubscript{10} complexed with DharmaFECT (commercially available transfection agent) and GLuc siRNA complexed with DharmaFECT. Protein expression levels are down-regulated by approximately 50% in KB cells treated with P14\textsubscript{10} AuNP carriers, indicating internalized carriers were able to release siRNA. As shown in Figure IV-33, relative luciferase expression was similar for cells treated with P14\textsubscript{10} in the presence and absence of serum. This indicates protein absorption to AuNP surfaces does not dictate the successful internalization of the carrier as is the case with SH-siRNA coated AuNPs.\textsuperscript{143-145}

In conclusion, a rationally designed oligonucleotide delivery vehicle was developed from narrowly-dispersed, RAFT-synthesized polymers. A series of P(HPMA-\textit{b}-DMAPMA)s provided requisite functionality for the \textit{in situ} formation of AuNPs. The stability of the AuNPs depended on the DMAPMA block length and the [DMAPMA]/[Au]\textsubscript{0} ratio utilized during the reduction reaction. The hydrodynamic diameter of P14\textsubscript{10} stabilized AuNPs (29 nm) and ζ potential (-1.1 mV) values indicate cationic DMAPMA units are located near the Au surface while HPMA is presented at the surface of the P14\textsubscript{10} AuNP. Complexation studies indicate neutral Au nanoplexes can be formed with over half of the P14\textsubscript{10} DMAPMA repeat units available for siRNA binding. Neutral Au nanoplexes protected siRNA against enzymatic degradation and were successfully delivered to a variety of cancerous cell lines. Compared to other AuNP
systems which require opsonization-based uptake, the current platform can be efficiently delivered in the absence of serum proteins.

**Figure IV-33.** Relative luciferase expression in KB cells 24 h after treatment with 100 nM siRNA. siRNA control (DharmaFECT and a nontargeting siRNA) serves as a negative control for the specificity of *Gaussia* Luciferase siRNA (GLuc). GLuc siRNA (uncomplexed) serves as a negative control for delivery. Dharma/GLuc siRNA (GLuc siRNA complexed with DharmaFECT) serves as a positive control for delivery. GLuc/AuNP, no serum and GLuc/AuNP, serum were neutral complexes of P14 and GLuc siRNA treated in the presence and absence of serum, respectively. Error bars represent the standard deviation from 5 independent tests run in triplicate.
CHAPTER V

CONCLUSIONS

Synthesis and Characterization of BAB Triblock (Co)Polymer Gelators

Temperature-responsive BAB block copolymers capable of forming physical gels under physiological conditions were synthesized via aqueous RAFT polymerization. The use of a difunctional trithiocarbonate CTA facilitated the two-step synthesis of well-defined BAB copolymers with symmetrical outer blocks. The outer B blocks of the triblock copolymers consisted of P(NIPAM) (M5) and the inner A block consisted of either P(AM) (M1) or P(DMA) (M2). While BAB triblock copolymers containing P(AM) inner blocks were capable of forming gels, the material could not be fully characterized with respect to molecular weight and PDI due to incompatible chromatography conditions. To address this issue P(DMA) was utilized in place of P(AM) as a hydrophilic block.

Copolymers comprised of P(NIPAM-b-DMA-b-NIPAM) formed physical gels above the phase transition temperature of P(NIPAM) at concentrations as low as 7.5 wt% copolymer. Mechanical properties similar to those of collagen (G’ = ~200 Pa), a naturally occurring polypeptide used as a three dimensional in vitro cell growth scaffold, have been achieved utilizing reversibly associating polymers. The mechanical properties of the gels as a function of solvent, polymer concentration, and inner block length were investigated. The addition of salt to the aqueous solvent resulted in a depression of T_{gel} and an increase in G’_{37 °C}. Mechanical properties (G’_{T_{gel}}, G’_{37 °C}) increased linearly on a semi-log plot as a function of polymer concentration. While inner block length affected the minimum polymer concentration required for gelation, mechanical properties only differed slightly
when dissolved in DI water whereas negligible differences were noted when polymers were dissolved in pH 7.4 buffer.

Synthesis and Characterization of ABA Triblock (Co)Polymer Gelators

Narrowly-dispersed, temperature-responsive acrylamido-based triblock copolymers capable of self-assembling into star-like micelles and ordered gels have been synthesized via aqueous RAFT polymerization. In dilute solution, star-like micelles formed with $R_h$ values between 17 and 40 nm depending on the polymer molecular weight. Hard gels formed when solutions containing 20 wt% polymer were heated to 50 °C. SAXS experiments indicated that BCC ordering occurs in gels containing lower molecular weight polymers. As $d_{100}$ and $d_{110}$ decreased, the mechanical properties increased due to an increase in micellar surface area available for entanglement.

P(DMA$_{250}$-b-NIPAM$_{600}$-b-DMA$_{250}$) (P12), the largest polymer, exhibited no higher order reflections in the gel state which may be attributed to the kinetic trapping of entangled micelles. Dynamic temperature ramp studies of P(DMA$_{93}$-b-NIPAM$_{213}$-b-DMA$_{93}$) (P9) dissolved in physiologically relevant media indicated that hard gels form just prior to reaching 37 °C at both 15 and 20 wt%. In conclusion, this research demonstrated the ability to tune mechanical properties and ordering of thermoreversible ABA gels through the variation of polymer hydrophilic mass fraction and polymer molecular weight.

In situ Formation of Polymer-Stabilized Au Nanoparticles and Subsequent Complexation, Stabilization, and Delivery to Cancerous Cells

A facile procedure for the synthesis of polymer-stabilized AuNPs which are capable of forming neutral, sterically stable complexes with siRNA was developed. The amine-containing cationic block of P(HPMA-b-DMAPMA) (M3-b-M13) was utilized to
promote the *in situ* formation of AuNPs and subsequently bind small interfering RNA while the nonimmunogenic, hydrophilic block provided steric stabilization. The DMAPMA block length utilized in the reduction reaction was found to be critical for the production of stable AuNPs. Within the series studied, polymers containing shorter DMAPMA block lengths (24 and 49) provided better particle stabilization than polymers containing longer DMAPMA block lengths (84 vs. 105).

In addition to the DMAPMA block length, the ratio of [DMAPMA]/[Au\(^{+3}\)]\(_0\) was critical to the successful development of a AuNP siRNA carrier. As indicated by negligible absorbance at 520 nm, reduction reactions utilizing a 100/1 ratio of [DMAPMA]/[Au\(^{+3}\)]\(_0\) did not produce AuNPs. Reduction reactions which utilized either a 3/1 or 10/1 ratio of [DMAPMA]/[Au\(^{+3}\)]\(_0\) led to the formation of AuNPs, while the particles formed with a 10/1 ratio had a much higher siRNA binding capacity than those formed with a 3/1 ratio.

P(HPMA\(_{70}\)-b-DMAPMA\(_{24}\)) (P\(_{14}\)\(_{10}\)) was analyzed for its ability to protect and deliver siRNA given its solution stability and high oligonucleotide binding capacity. Kinetic degradation profiles were measured for siRNA in the presence and absence of P\(_{14}\)\(_{10}\). The neutral P\(_{14}\)\(_{10}\) siRNA complex exhibited ~100 fold protection as compared to free siRNA. Delivery of Cy3 labeled siRNA from P\(_{14}\)\(_{10}\) to KB, Hela, SKOV3, and A549 cells was confirmed by fluorescence microscopy. Delivery of GLuc siRNA complexed to P\(_{14}\)\(_{10}\) was further confirmed by monitoring relative luciferase expression of treated and untreated cells. Protein expression levels were down-regulated by approximately 50% in KB cells treated with P\(_{14}\)\(_{10}\) AuNP carriers, indicating internalized carriers were
able to release siRNA. The presence of serum did not dictate delivery efficiency, indicating opsonization was not the cause of cellular uptake.
CHAPTER VI

RECOMMENDATIONS FOR FUTURE RESEARCH

In the years immediately following the discovery of RAFT polymerization, the majority of research was focused on mechanistic studies and development of appropriate polymerization conditions. Since then, a wide range of monomers, CTAs, and solvents have been utilized in the controlled synthesis of polymers.\textsuperscript{14, 266, 267} With the ability to control the polymerization of a diverse set of monomers, unprecedented opportunities have opened. For example, room temperature polymerizations conducted in aqueous media\textsuperscript{15} provide a facile route for the modification of proteins while still retaining tertiary structures.\textsuperscript{192} Novel functional monomers, such as glyco-\textsuperscript{192, 268, 269} boronic acid,\textsuperscript{270-272} and amino-based vinyl monomers,\textsuperscript{273, 274} provide opportunities for the synthesis of biorecognizable polymers. For example, Fukuda and coworkers synthesized galactose and mannose-modified RAFT copolymers capable of stabilizing AuNPs and inducing protein specific aggregation.\textsuperscript{268}

Regarding the research presented in this dissertation, several nano- and biotechnology opportunities exist. For example, self-assembled structures can be used as templates for patterned nanomaterials. As shown in Scheme VI-1, a polymer comprised of a stimuli-responsive block and a hydrophilic-\textit{statistical}-reactive block can be assembled into a nanoporous template. After cross-linking reactive functionality in the shell of the micelles, core removal is facilitated by the cleavable moiety incorporated along the polymeric backbone.
Scheme VI-1. The assembly of a [(hydrophilic-statistical-functional)-b-stimuli responsive]) polymer with a cleavable core into a face centered cubic nanoporous template. Following shell cross-linking and core cleavage, a nanoporous material is obtained. Blue area represents a cross-linked network while black circles represent pores formed through the removal of stimuli-responsive cores.

AuNPs can also be employed as templates for complex architectures.\textsuperscript{275,276} For example, the Au cores of poly(hydrophilic-b-cationic)-stabilized-AuNP/RNA complexes can be removed by adding aqua regia or cyanide, leaving a polyelectrolyte complex.\textsuperscript{276} The size of polyelectrolyte complexes formed with H\textsubscript{b}C polymers and RNA is dictated by the N/P ratio and polymer block lengths (Scheme VI-2).\textsuperscript{126,127,132} Conversely, utilizing H\textsubscript{b}C-stabilized AuNPs as a template for polyelectrolyte complexation allows an alternate method to control the size of the complex. Additionally, this route would provide a means to synthesize shell or core cross-linked structures which lack responsive groups and thus otherwise might not assemble.
Scheme VI-2. A) Traditional polyelectrolyte complex formed between a hydrophilic-\textit{b}-cationic polymer and siRNA. B) A templated siRNA complex formed by complexation of siRNA to a hydrophilic-\textit{b}-cationic polymer-stabilized AuNP followed by Au core dissolution.

While the ability of amine-containing polymers to reduce Au\textsuperscript{+3} to Au has been established, several parameters involving this process remain unexplored. The research presented in this dissertation touched on the effect of the N/Au ratio on stable AuNP formation. However, further studies need to be conducted to obtain a fundamental understanding of the processes occurring during AuNP formation. Key parameters which should be further studied include the effects of hydrophilic block length, amine-pendent block length, molarity of amine, and molarity of Au\textsuperscript{+3}, and temperature on stable AuNP
formation. Gaining insight into these areas should facilitate the synthesis of AuNPs with predetermined sizes and binding sites based on reaction conditions.
APPENDIX

*Lower Critical Solution Behavior of P(NIPAM).* Unlike traditional materials which have increased solubility at high temperatures, the solubility of P(NIPAM) decreases as the temperature is raised until it precipitates from solution at the point known as the lower critical solution temperature. The solubility of the polymer can be expressed by Equation 1, where $\Delta G$ is the change in Gibb’s free energy, $\Delta H$ is the change in enthalpy, $T$ is the temperature, and $\Delta S$ is the change in entropy.

\[
\Delta G = \Delta H - T\Delta S \tag{9}
\]

For spontaneous mixing, $\Delta G$ must be negative. When P(NIPAM) is added to water at low temperatures, the enthalpic and entropic terms are negative, resulting in spontaneous mixing.\textsuperscript{277} The formation of hydrogen bonds between water molecules and amide functionalities results in the negative $\Delta H$ while an increase in order resulting from the bound water molecules participating in hydrogen bonding results in the negative $\Delta S$.\textsuperscript{277} At higher temperatures the hydrogen bonding between water molecules and the acrylamide groups is disrupted, leading to a positive $\Delta S$ and $\Delta H$. At a specific temperature, known as the LCST, the polymer will phase separate as $\Delta G$ changes from negative to positive.
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(260) *As a charged particle moves through solution, counter- and coions will diffuse with it. The slippling plane is defined as the boundary where ions no longer move with the particle.*


