Synthesis and Self-Assembly Behavior of Multi-arm Star Amphiphilic Polyelectrolyte Systems and Their Applications in the Delivery of Biomolecules

He Weiguo Elaine

School of Mechanical & Aerospace Engineering

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Abstract

Amphiphilic polyelectrolytes have attracted increasing attention due to their potential applications in the field of pharmaceutical science and biotechnology. The biocompatible polymers can self-assemble into nano-scale micelles, which provide similar structure and function as natural carriers. Biocompatibility, size and morphologies of the vehicles are important considerations in the design of suitable delivery systems, where they can be accomplished by manipulating block compositions, structure and lengths. Novel multi-arm star shape PEO was grafted with weak polybase or polyacid to produce stimuli-responsive amphiphilic polyelectrolytes. The three-dimensional branched structure offers greater proportion of end functional groups compared to linear structure of identical molecular weights, which induces greater solubility and more attractive aggregation behavior.

The four-arm poly(ethylene oxide)-b-poly(2-(diethylamino)ethyl methacrylate) (PEO-b-PDEAEMA) and poly(ethylene oxide)-b-poly(methacrylic acid) (PEO-b-PMAA) block copolymers with tetrahedral structure were successfully synthesized by the atom transfer radical polymerization technique to yield well-defined amphiphilic block copolymers of narrow polydispersity. The polymerization degree of four-arm PEO-b-PDEAEMA block copolymer was determined from the relative intensities of NMR spectra at 3.66 ppm (-CH₂CH₂O of the PEO block) and 4.02 ppm (-
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OCH₂CH₂N- of the PDEAEMA block), obtained a chemical structure of 4-arm PEO₅₆-b-PDEAEMA₇₄. The polymerization degree of the four-arm PEO-b-PtBMA copolymers was calculated from the peak intensity ratio of 3.58 ppm (-OCH₂CH₂O-) of PEO block and 1.40 ppm (-C(CH₃)₃) of tBMA block, to yield 4-arm PEO₅₆-b-PtBMA₈₈ block copolymer.

The self-assembly behaviors of four-arm PEO-b-DEAEMA in aqueous solution were studied as a function of pH. At low pH, DEAEMA (2-(diethylamino)ethyl methacrylate) segments were protonated, and the polymeric chains exist as fully extended star shape unimers. By increasing the pH of environment, the DEAEMA groups were deprotonated and became hydrophobic resulting in the formation of spherical core-shell micelle comprising of a hydrophobic DEAEMA core surrounded by a folded hydrophilic four-arm PEO corona. The hydrodynamic size of the micelle and conformational transitions was studied in detail at various pH. In addition, the ionic strength in solution also controls the self-assembly behavior of amphiphilic polymeric systems since it mediates the electrostatic interactions between the macroions, counterions, and solvent molecules. The effect of salt on the aggregation behavior of four-arm polyelectrolyte was investigated, where salt concentration alters the electrical potential surrounding the polyions and suppresses electrostatic repulsions of charged polymeric segments.
The star shape four-arm PEO-\textit{b}-PDEAEMA block copolymer was evaluated as a potential vector for gene delivery since it could condense therapeutic DNA forming a core-shell structure that can cross a number of biological barriers. At physiological pH, the star shape four-arm PEO-\textit{b}-PDEAEMA block copolymer possessed positively charged amine groups that interacted with negatively charged plasmid DNA to form polymer/DNA complexes. The mechanism and physicochemical properties of the complex formation were investigated at various molar ratios of amine and DNA segments (N/P). The capacity of the star block polymer to condense DNA was demonstrated through gel electrophoresis and ethidium bromide exclusion assay. The hydrodynamic radius of polyplexes were investigated by dynamic and static light scattering, where approximately 15 polymeric chains were required to condense a plasmid DNA. The addition of monovalent salt into the polymer/DNA mixture significantly alters the size of the complexes, which would have an impact on cell transfection.

The conformation transition of four-arm PEO-\textit{b}-PMAA block copolymer over the course of neutralization was investigated. The multi-arm block copolymer existed as an extended unimer at high pH due to the negatively charged carboxylate and hydrophilic PEO segments. The block copolymers self-assembled into core-shell micelles and large spherical aggregates that flocculated at very low degree of neutralization ($\alpha$). Such behavior was controlled by the fine balance of electrostatic,
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hydrophobic and hydrogen bonding interaction forces. The thermodynamic parameters obtained from the isothermal titration calorimetric technique at different salt concentrations indicated that the energy to extract a proton from a charged polion was reduced with the addition of salt that favors the neutralization process.

The four-arm PEO-b-PMAA block copolymer serves as a reservoir for drug loading through the combination of electrostatic attraction, hydrogen bonding and hydrophobic interactions. With the star shape architecture, the polymer possesses higher densities of terminal functional groups and three-dimensional tetrahedral structure that induces different association properties and interactions with drug compared to linear structured polymer of identical molecular weights. The negatively charged carboxylate on polymer chains interact with cationic drug through electrostatic interaction to form polymer/drug complexes that were stabilized by biocompatible hydrophilic PEO segments. The hydrodynamic radius ($R_h$) of the polymer/drug complexes varied from 32 nm to 55 nm for different amounts of drug in the present of polymer solution, which is a suitable size for drug delivery. Drug selective membrane was prepared and the high efficiency selective electrode system was used to monitor the release kinetics of IPH from multi-arm PEO-b-PMAA star polymer. The release exponent was greater than 0.5 indicating non-Fickian type diffusion mechanism and the release behavior was dominated by the chain relaxation induced by ion exchange with the effect of pH.
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List of Symbols and Abbreviations

ATRP  Atom transfer radical polymerization
ATRA  Atom transfer radical addition
\( \alpha \):  Degree of neutralization
\( pK_{\text{app}} \):  Negative logarithm of the apparent dissociation constant
\( pK_0 \):  Negative logarithm of the intrinsic dissociation constant
G:  Gibbs energy (kJ/mol)
R:  Gas constant (8.314 J/mol\cdot K)
K:  Equilibrium constant
\( N_A \):  Avogadro constant (6.02x10\(^{23}\)/mol)
\( k_B \):  Boltzmann constant (1.38x10\(^{-23}\)J/K)
\( \psi \):  Electrostatic potential
n:  Number of binding sites
\( A_2 \):  Second virial coefficient
CMC:  Critical micellization concentration
DLS:  Dynamic light scattering
SLS:  Static light scattering
\( R_g \):  Gyration radius
\( R_h \):  Hydrodynamic radius
List of Symbols and Abbreviations

$\rho$: Ratio of $R_g$ and $R_h$

$n$: Refractive index of the solvent

$N_{aggregation}$: The aggregation number

GPC: Gel permeation chromatography

HPLC: High performance liquid chromatography

$\bar{M}_n$: Number average molecular weight

$\bar{M}_w$: Weight average molecular weight

$\frac{\bar{M}_w}{\bar{M}_n}$: Molecular weight distribution

PDI: Polymer distribution index

$\bar{DP}$: Degree of polymerization

NMR: Nuclear magnetic resonance

TEM: Transmission electron microscope

ITC: Isothermal titration calorimetry

$\Delta H$: Enthalpy change (kJ/mol)

HLB: Hydrophilic-lipophilic balance

OsO$_4$: Osmium tetroxide

PEO: Poly(ethylene oxide)

PDEAEMA: Poly((diethylamino)-ethyl methacrylate)

PrBMA: Poly(tert-Butyl methacrylate)

PMAA: Poly(methacrylate acid)

EGFP: Enhanced green fluorescence protein
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<th>Symbol</th>
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<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>ZP</td>
<td>Zeta potential</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffers Saline</td>
</tr>
<tr>
<td>IPH</td>
<td>Imipramine hydrochloride</td>
</tr>
<tr>
<td>EMF</td>
<td>Measuring electromotive force</td>
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Chapter One

Introduction

1.1 Background

Water-soluble amphiphilic block copolymers are a class of functional polymers with interesting aggregation behavior that are being utilized in a number of new applications in separation, pharmaceutics, drug delivery and biotechnology [Choucair and Eisenberg, 2003]. The amphiphilic block copolymers can self-assemble in aqueous solution to form micelles with a hydrophobic core and hydrophilic corona. With the polyelectrolyte block, the morphologies of the polymers are pH-responsive and the association behaviors are controlled by hydrophobic interaction and electrostatic force between charged groups [Gaucher et al., 2005]. The ionizable polymeric chains become charged by accepting and donating protons as the environmental pH changes. By adjusting the pH, reversible conformational changes and phase transitions of polymeric chains occur, which is an important property for functional polymer applications. The ionic strength of the solution is another factor that affects the aggregation properties of polymer in solution in addition to block characteristics and compositions, chain length, temperature and pH. Electrostatic repulsions between charged groups are suppressed by ions of small electrolytes in solution, which affect the aggregate morphology and size.
A wide variety of linear amphiphilic block copolymers have been synthesized and their aggregate behaviors have been studied in different types of media. However, relatively limited research has been reported on star-shape polyelectrolytes. Multi-arm star polymers possess three-dimensional macromolecules and compact structure, which has the potential for biomedical applications [Ishizu et al., 2003]. End-functional pendant groups on multi-arm have highly dense functionality that permits further modification. Multi-arm PEOs are water-soluble and biocompatible, which provide the hydrophilic block structure. Weak polybases or polyacids are grafted onto the arms of star PEO to produce pH-responsive polyelectrolytes.

Biocompatible polyelectrolytes composed of ionic and hydrophilic segments that spontaneously associate with polyanionic DNA to form complexes have been reported [Liaw et al., 2001]. The core of the polyion complex comprising of DNA and the polycation is coated by a layer of hydrophilic polymer. The characteristic core-shell structure endows the micellar aggregates with high colloidal stability and reduced interaction with blood components in micellar DNA delivery system.

The cationic drug bound to negative charged polyacid to form a poly/drug complexes in aqueous solution are attractive systems for potential applications in drug delivery. The nanoparticle characteristics and aggregation behaviours have attracted significant interest since they are critical factors in the field of nanomedicine. For drug release
studies, the drug selective electrode system provides a more efficient and low interference technique compared to the widely used dialysis method, where the concentration of drugs are analyzed by UV-vis or high performance liquid chromatography (HPLC).

1.2 Objectives and scope

There are three objectives in this project. First, the star-shape block copolymers containing biocompatible polyethylene-oxide (PEO) block and polyelectrolyte segments, such as poly(2-(diethylamino)ethyl methacrylate) (PDEAEMA) or polymethacrylic acid (PMAA) are synthesized. The di-block copolymers possess three dimensional structures with varying linear structure and they self-assemble to form micelles in aqueous solution. By adjusting the pH environments, the polybase or polyacid accept or donate protons resulting in reversible conformational transitions, which provide potential delivery applications for DNA and drug respectively. Four-arm PEO-\(b\)-PDEAEMA and PEO-\(b\)-PtBMA were synthesized via atom transfer radical polymerization (ATRP). The ATRP mechanism facilitates the control growth of polymeric chains resulting in a well-defined chain length (molecular weight) and polydispersity. Well-defined polymers with narrow molecular weight distributions were characterized by NMR and GPC techniques to investigate the composition,
structure and molecular weight.

Second, the physical properties of the star polymers in aqueous solutions were investigated since the molecular-level association mechanism and the controlling factors are important in biomedical applications. The aggregation characteristics, such as shape, particle size, and aggregation number were studied at various conditions by changing pH and ion strength of the solutions. At various degrees of neutralization, the polybase or polyacid possesses different charge densities. The balance between electrostatic repulsion and hydrophobic interaction controls the self-assembly behavior. By continuously changing pH, the dissociation constant (pK) and Gibbs energy (ΔG) were obtained to confirm the presence of different conformational transitions. The aggregation behaviors were also studied in various salt concentrations since small electrolytes form ion environment that shield polymer chains thereby altering the morphology of the polymer in solution. Static and dynamic light scattering techniques were used to measure the microstructure ($R_h/R_g$) and average molecular weight of particles, which are confirmed by TEM. Tensiometer was used to determine the critical micellization concentration (CMC), which is an important parameter to assess their suitability as delivery vehicles.

Third, some preliminary studies of amphiphilic block copolymers for DNA and drug delivery were carried out. Gene therapy has evolved into a promising therapeutic
modality to treat and manage a diverse array of diseases. The complex polymer/DNA is driven by electrostatic interaction of oppositely charge biomolecules. The mechanism of complex formation was investigated using various techniques, such as agarose gel electrophoresis, Ethidium Bromide displacement assay, zeta potential, light scattering, isothermal titration calorimetry and transmission electron microscopy. The polymer micelles encapsulated drugs demonstrated various attractive properties, such as suitable size for enhanced permeability and retention effect, high stability in aqueous medium and solubilization of water insoluble drugs. Through various chemical, physical, or electrostatic interactions the hydrophobic regions served as reservoirs for drugs loading to fulfill various designed specific functionalities. The size of polymer/drug complexes, binding mechanism and release behaviors were studied by various technologies.

1.3 Survey of the thesis

The report consists of nine chapters. Chapter 1 presents a brief introduction to this research work. Chapter 2 reviews the literatures on star polymers, atom transfer radical polymerization (ATRP), amphiphilic block copolymers and micelles formation. Details of the synthesis techniques of four-arm PEO-\(b\)-PDEAEMA and four-arm PEO-\(b\)-PMAA block copolymers, characterization and materials are described in
Chapter 3. Chapter 4 describes the experimental procedures and theory used in this research. Chapter 5 investigates the association and conformational transition of the star polybase in aqueous solutions by considering factors, such as pH and small electrolytes concentrations. In Chapter 6, the conformation transition of four-arm PEO-\(b\)-PMAA over the course of neutralization was investigated in detail. Chapter 7 reports on the interactions between four-arm PEO-\(b\)-PDEAEMA and plasmid DNA. Chapter 8 describes the binding mechanism and drug release behaviors of IPH from the four-arm PEO-\(b\)-PMAA block copolymer. Chapter 9 includes the conclusions, major findings of this research and recommendations for future works.
Chapter 2
Literature Review

2.1 Star-block copolymers

The design of polymer architectures is an effective way to achieve specific functionalities since it greatly affects the properties and functions of polymeric materials. Generally, there are three major types of macromolecules, linear, branched and cross-linked polymers [Qiu and Bae, 2006]. The class of branched polymers includes graft copolymer, star-shape, hyperbranched and dendritic polymers. Star polymers were classified into branch polymers consisting of more than three linear polymer chains as arm segments linked together at one end of each polymer chain by a common junction [Hirao et al., 2005]. We synthesized four-arm star polymers, which are block copolymers that mimic the so-called star-block copolymer systems [Hadjichristidis et al., 2001]. Each arm in the star-block copolymer is a di-block copolymer that possesses physical properties that are dramatically different from those of the corresponding homopolymers [Berlinova et al., 1997].

The star-shaped polymers are more compact than linear block copolymer or conjugated polymer of similar molecular weight and composition due to their higher
segment densities. They exhibit a smaller hydrodynamic radius and lower solution viscosity since they contain different number of homopolymeric arms or heteroarms of various compositions. Interesting properties concerning both micellization and microphase separation were observed in star-block copolymers as compared to corresponding diblock copolymers. Since the properties of star-branched polymers may be quite different in bulk, melt, and solution from those of linear polymers of similar molecular weights, they have been widely investigated from both synthetic and theoretical points of view. In using a star polymer for any drug vectors application, the smaller hydrodynamic radius is important for complete renal excretion. The structure of the star polymer makes it feasible to use higher molecular weight PEGs; which improves the hydrophilic/hydrophobic balance and protection of the hydrophobic core. The unimolecular micelles derived from star polymers possess higher stability compared to micelles formed from amphiphilic polymers because these unimolecular micelles contain covalently fixed branching points.

There are several types of star-block copolymers, such as simple AB graft, A\textsubscript{n}B\textsubscript{n}, (AB)\textsubscript{n} and ABC star polymer. (AB)\textsubscript{n} star-block copolymer consists of n-arm star diblock; A\textsubscript{n}B\textsubscript{n} star-block copolymers have equal numbers n of A and B arm. If a star copolymer consists of three types of polymeric chains, it is called an ABC star-shaped copolymers. The schematic representations of the four types of star polymers are shown in Figure 2.1 [Ishizu et al., 1999].
2.1.1 (AB)_n star-block copolymers

Well-defined star polymers can be synthesized by either “arm-first” or “core-first” methods. For the “arm-first” synthesis method, a reactive linear mono-functional polymer was first synthesized by living polymerization. Thereafter a multifunctional cross-linking agent was initiated to form the star polymers, from which a number of arms were anchored. The star polymer will be well-defined since the star polymer has only one junction and the number of branches emanating from the junction corresponds to the functionality of the coupling reagent [Hadjichristidis et al., 1978].

In the “core-first” methods, multifunctional initiators are used to grow arms by sequential living polymerization, which is suitable for the preparation of multi-armed (AB)_n star-block copolymers [Young and Fetters, 1978].

For living anionic polymerizations, a typical example is the star-block copolymer...
polystyrene-block-polysoprene (PS-b-PI)$_n$ that was synthesized by cross-linking
diblock-carbanionic chains with divinyl benzene (DVB), which produced a core
junction acting as a small nodule [Alward et al., 1986]. The length of branch segments
was controllable since they were prepared by the living process. In the case of living
cationic polymerizations, amphiphilic (AB)$_n$ star-block copolymers were synthesized
by Higashimura and co-workers [Higashamura et al., 1988]. The pendant ester group
leads to water-soluble polymers.

2.1.2 A$_n$B$_n$ star-shaped copolymers

A$_n$B$_n$ star-block copolymers have been called “heteroarm” or “miktoarm” (mixed arm)
star polymers, whose arm segments differ in molecular weight and chemical
composition [Hadjichristidis et al., 1999]. Such star-branched polymers revealed
interesting and unique properties in solution as well as in the solid state that originated
from possible heterophase structures. These heterophase dissimilar structures are
usually phase-separated at the molecular level to promote self-assembly. The
structures facilitate the fabrication of many new nanoscopic suprastructures and
nanomaterials, which offer the possibility for the development of nano-devices
[Avgeropoulos et al., 2002; Sotiriou et al., 2002].

Using the living and controlled polymerization techniques, two or more reactions are
required for introducing different arms and the intermediate polymers may need to be isolated in each reaction step. Several research groups have reported the syntheses of asymmetric star-branched polymers by living radical polymerization systems using multi-functionalized initiators [Erdogan et al., 2004; He et al., 2004]. Tsitsilianis et al. prepared A_nB_n star copolymers by using a three-step anionic process [Tsitsilianis et al., 1990; 1991]. Initially, the star molecules were made through the polymerization of a small amount of bis-unsaturated monomers using a living precursor polymer. The precursor chains were linked with the resulting cores which were subsequently used to initiate the polymerization of another monomer. The new branches were grown from the core. Amphiphilic star-shaped polymers with heteroarms of vinyl ethers have been prepared using the living cationic polymerization technique. The living polyvinyl ether chains undergo linkage reactions through a bifunctional vinyl ether into a star-shaped polymer [Kanaoka et al., 1991].

2.1.3 **ABC star-shaped copolymers**

ABC star-shaped polymers have three different arms, which present new microdomain morphologies. The asymmetric star-branched polymers were synthesized mainly using two methods via living anionic polymers. One of the methods is based on the continuous reaction of living anionic polymers with multifunctional chlorosilanes, which takes advantage of the fact that the Si-C bond
present different reactivities to the living polymers. [Iatrou et al., 1995; Pispas et al., 2003]. The ABC star polymers comprising of PS, PI, and poly(methyl methacrylate) (PMMA) with three different arms have been synthesized by Sioula et al. [Sioula et al., 1997]. Another method is based on the chemistry of functionalized DPE (Diphenylethylene) derivatives [Hirao et al., 2006]. DPE derivatives were applied to synthesize the asymmetric star-branched polymers by Fujimoto et al. [Fujimoto et al., 1992]. The non-polymerizable DPE-functionalized poly(dimethylsiloxane) macromonomer was synthesized with PSLi using the living linkage reaction. Subsequently, the ABC asymmetric star was synthesized through the anionic polymerization of tert-butyl methacrylate. The advantage of DPE derivatives is the capability of participating in both living linkage and polymerization reactions to link two different arms.

2.1.4 Star polymers containing PEO

The ethylene oxide monomer consists of an epoxide ring, with two corners of the molecule consisting of -CH₂- linkages and the third corner containing an oxygen atom, -O-. In the presence of metallic catalyst systems, the monomer forms a chain having the repeating unit -CH₂-CH₂-O- to form poly(ethylene oxide) (PEO). PEOs are nonionic, water-soluble, and generally classified as hydrophilic polymers. The PEO was classified into different grades based on their molecular weights which range
from 200 to $7 \times 10^6$ Da. Products with molecular weights below 25,000 Da are viscous liquids or waxy solids and are commonly referred to as poly(ethylene glycols) (PEGs). PEO has found numerous applications in coatings, and foaming, solubilizing, thickening and emulsifying agents. The PEO is also widely used in industries, such as papermaking, printing, detergent, paints and personal care. It is one of the very few synthetic polymeric materials that are approved by the U. S. Food and Drug Administration for use as food additives and pharmaceutical ingredients. PEO can be safely used in many specialized applications, based on studies done on dogs, mice and rabbits [Liaw et al., 2001] and marine organisms [Jonkers et al., 2005]. These researchs showed that the toxicity of high molecular weight PEO is very low in oral applications. Because of its large molecular weight, it is not easily absorbed by intestinal tissues. The well-known properties of PEO and its regulatory acceptability have helped to extend its applications in biomedical and pharmaceutical preparations and drug delivery systems.

Poly(ethylene oxide) (PEO) star polymers are regarded as a particularly promising class of materials since they represent variable building blocks for structured polymeric networks, such as hydrogels or amphiphilic network systems. PEO blocks consisting of a hydrophilic component together with hydrophobic arms can respond to external stimuli such as changes in the pH, temperature, type of solvent etc. The amphiphilic copolymers containing PEO blocks as hydrophilic segments are of
significant value and find broad applications as catalysis, surface-active, and ion-conducting materials. The combination of hydrophilic and hydrophobic moieties on the same macromolecule yields materials with many interesting features. The compositions, molecular weight and the topology of amphiphilic copolymers influence the phase-transition, crystallization, ion-conductivity, and self-aggregation behavior of these polymers. Dendritic macromolecule with generations of PEO chains is another type of star-shaped PEO which is obtained by consecutive branching at each arm and subsequent anionic polymerization of ethylene oxide.

Star-shaped PEO can be prepared by anionic ring-opening polymerization of ethylene epoxide with multi-alcohol or hydroxyl-functionalized dendrimer as the initiators [Comanita et al., 1999; Knischka et al., 2000]. ATRP is a useful method in the preparation of star copolymers which use the transition metal compound as the carrier of a halogen atom in a reversible redox process [Du and Chen, 2004; Chen et al., 2006]. In the ATRP polymerization process, monomethoxy PEO 5000 was used as the precursor macroinitiator to react with 2-bromoisobutyryl bromide to form an active species. The divinyl coupling reagents were added to the PEO macroinitiator chain ends to form short block copolymers. Subsequently, the block copolymers containing the divinyl units react with each other to form cross-linked centers which are the formation of PEO star polymers. The high molecular weight star-PEO systems were formed via star-star coupling.
Star-shaped copolymers consisting of poly(ethylene oxide) (PEO) and polystyrene (PS) were synthesized by sequential anionic polymerization of ethylene-oxide and atom transfer radical polymerization (ATRP) [Angot et al., 2000]. The aggregation behavior and surface morphology of the three arm PEO\textsubscript{3}-b-PS\textsubscript{3} star polymer have been studied by Francis and co-workers [Francis et al., 2002]. Tri- and tetra-functional initiators were used to anionically polymerize ethylene-oxide to produce tri- and tetra-armed PEO stars. The -OH end groups of PEO star branches were converted to 2-bromopropionate groups. The end-functionalized tri- and tetra-bromoester PEO stars were macroinitiators for the ATRP of styrene which produce the amphiphilic star block copolymers PEO\textsubscript{n}-b-PS\textsubscript{n} (n=3 or 4).

Star-block copolymers (PEO-b-PAA)\textsubscript{3} consisting of three poly(ethylene oxide) (PEO) arms was derived by a ‘core first’ approach [Hou et al., 2003]. Using an approach that was similar to the synthesis of PEO\textsubscript{3}-b-PS\textsubscript{3}, the -OH end groups of three-arm PEO stars were converted to three bromo-ester functionalized groups. The PrBuA blocks were linked with the bromo-ester segments by atom transfer radical polymerization. For the ‘arm first’ methodology, a divinyl monomer was applied as the linking agent to access star-block copolymers incorporating an inner PAA segment and a peripheral PEO layer. Preformed PEO-b-PrBuA diblock copolymers were reacted with divinylbenzene in the anisole media in the presence of CuBr/PMDETA. Hydrolysis was applied to generate the double-hydrophilic star-block copolymers (PAA-b-PEO)\textsubscript{n}. 
2.2 Atom transfer radical polymerization (ATRP)

In 1994, Wayland et al. reported that organometallic derivatives of cobalt tetramesitylporphyrin [(TMP)Co-R] could initiate and control the polymerizations of acrylates to produce well controlled block copolymers. The number average molecular weight increased linearly with monomer conversion and low polydispersities (Mₚ/Mₚ ranging from 1.1 to 1.3) was obtained [Wayland et al., 1994]. In 1995, Matyjaszewski and Wang studied the controlled polymer systems using 1-phenylethyl chloride as initiator and CuCl, complexed by 2,2'-bipyridine, as catalyst. The group synthesized styrene homopolymers which have predetermined molecular weight, narrow molecular weight distribution and high conversion rate. With the same technique, block copolymers of styrene and methacrylates were synthesized with a negligible amount of irreversible transfer and termination [Wang and Matyjaszewski, 1995]. These polymer synthesis methods demonstrated the “living character” of the resulting polymers, the low polydispersity index and the controlled nature of the process which the molecular weights increased with conversion increase. Because the additions of haloalkanes to alkenes involved atom transfer steps, Matyjaszewski and Wang termed the new method as “atom transfer radical polymerization” (ATRP).
2.2.1 ATRP: General Introduction

In the early twentieth century, conventional radical polymerization was widely used to produce polymeric materials, such as plastics, rubbers and fibers. Some advantages of radical polymerization are significant, such as a large variety of vinyl monomers, well tolerance of impurities, mild reaction conditions and convenient temperature range (typically from 0 to 100°C). However a major drawback exists for the conventional radical polymerization which is the lack of control over the polymer structure. Polymers with high molecular weights and large polydispersities generally happened due to slow initiation, fast propagation and subsequent transfer or termination. These features are reflected in the physical and mechanical properties of the resultant polymers. In order to alter and improve these properties, living polymerizations were developed to produce well designed and controlled polymers in 1950s.

Living polymerizations include anionic, cationic, and ring-opening polymerizations, which have few distinctive characters comparing with the traditional radical polymerization. The initiating step is faster than chain increment which prevents the chain transfer and termination and therefore the resultant polymers have narrow molecular weight distributions. The molecular weight of product is controlled by the amount of monomers and initiators which is linearly proportional to the conversion rate. After the first polymerization step, copolymer with predetermined structural and
functional groups can be produced with adding another type of monomer. Living polymerization method is suitable for the preparation of well-defined polymers with controlled chain end functionalities and the synthesis of well-designed copolymers with special structures. However, these polymerizations need to be carried out in the conditions without moisture and at very low temperatures. Only a limited number of monomers can be used, and the presence of functionalities in the monomers can cause some side reactions. Living polymerization techniques are limited to employe for the polymerizations process in the absence of irreversible chain transfer and chain termination. The complex reaction process and high cost in industry obstruct the applications of this technique in the field of polymer science.

In the past decade, much effort has been focused on developing systems where milder conditions can be used. Living radical polymerizations (LRP) including few radical polymerization techniques provide simple and robust routes in the synthesis of well-defined, low-polydispersity polymers and the fabrication of novel functional materials. Several polymerization systems have been applied to control molecular weights and end functionalities, such as atom transfer radical polymerization (ATRP), nitrooxide mediated polymerization (NMP) [Benoit et al., 2000], stable free radical polymerization (SFRP) [Gaynor et al., 1995; Goto et al., 1998] and reversible additional fragmentation chain transfer polymerizations (RAFT) [Chiefari et al., 1998]. The rapid dynamic equilibrations between a minute amount of growing free radicals
and an abundance of dormant species were established in all of these methods.

Atom transfer radical polymerization (ATRP) is a controlled/"living’ radical polymerization based on a copper halide/nitrogen based ligand catalyst [Wang and Matyjaszewski, 1995]. Using the significant method, molecular weights of polymers were determined by the ratio of consumed monomers to introduce initiators and the polydispersities are generally low (M_w/M_n < 1.3). A wide range of monomers such as styrenes, acrylates and methacrylates including a variety of functional groups can be used by this technique. ATRP allows for the preparation of more precisely controlled polymers and many new materials. The polymers have different topologies (linear, branched, hyperbranched, stars, etc.) and the composition of the polymeric chains varies (statistical/gradient copolymers, block copolymers, grafts, etc.) [Patten and Matyjaszewski, 1998]. The end groups of polymers are well-defined and end functionalities can be easily achieved since they are derived from the variable initiators containing functional groups.

### 2.2.2 Principles of ATRP

ATRP originates from atom transfer radical addition (ATRA) (also called Kharasch Addition Reaction), which is an efficient method for carbon-carbon bond formation in organic synthesis [Curran, 1988]. A transition-metal catalyst acts as a carrier of the
halogen atom in a reversible redox process as shown in Scheme 2.1.

\[
\begin{align*}
R-X + M_t^n & \rightarrow R^\cdot + M_t^{n+1} - X \\
 & \downarrow \\
R\text{-CH}_2\text{C} & \rightarrow (\text{Alkene}) \\
 & \downarrow \\
Y_1 & \text{CH}_2 \text{C} \quad (\text{Alkene}) \\
 & \downarrow \\
Y_2 & \\
\end{align*}
\]

(Target product) (Intermediate radical species)

Scheme 2.1 Metal-Catalyzed Radical Addition Reaction (Kharasch Addition Reaction).

Initially, the transition-metal species ($M_t^n$) abstracts halogen atom X from the organic halide ($RX$) to form the oxidized species ($M_t^{n+1}X$) and the carbon-centered radical ($R^\cdot$). Thereafter the radical ($R^\cdot$) participates in intermolecular radical addition to alkenes with the formation of the intermediate radical species ($RY^\cdot$). The reaction between $M_t^{n+1}X$ and $RY^\cdot$ results in a target product ($RYX$), and regenerates the reduced transition-metal species ($M_t^n$), which further promotes a new redox process. The fast reaction between $RY^\cdot$ and $M_t^{n+1}X$ suppresses bimolecular termination between alkyl radicals and introduces a halogen functional group X into the final product. It is possible to have a continuous atom transfer additional reaction in this
process if the macromolecule RYX has strong reaction activity for $M_t^n$ and with sufficient monomers.

ATRP is a radical process using a transition metal combined with a suitable ligand as catalyst. The catalyst complex establishes a reversible equilibrium between growing radicals and dormant species. A general mechanism for ATRP is shown in Scheme 2.2.

\[
\begin{align*}
R-X + M_t^n/Ligand & \xrightleftharpoons[k_d]{k_a} \text{R·} + X-M_t^{n+1}/Ligand \\
\text{monomer} & \xrightarrow{k_t} \text{termination}
\end{align*}
\]

Scheme 2.2 Transition-Metal-Catalyzed ATRP.

The active species is generated through a reversible redox process catalyzed by a transition metal complex ($M_t^n$/Ligand). A halogen atom (X) was abstracted from a dormant species (R-X). This process occurs with a rate constant of activation ($k_{act}$) and deactivation ($k_{deact}$). Polymer chains grow by the addition of intermediate radicals to monomers in a manner similar to a conventional radical polymerization. Because a dynamic equilibrium between dormant species and growing radicals is established, the majority of these growing polymer chains are dormant species that still preserve their abilities to grow. Termination reactions ($k_t$) through radical coupling and
disproportionation also occur in ATRP. However only a few percent of the polymer chains undergo termination in a well-controlled ATRP. Typically, less than 5% of the total growing polymer chains terminate during the initial stage of the polymerization. A successful ATRP will have not only a small contribution of terminated chains, but also a uniform growth of all the chains. The fast initiation and rapid reversible deactivation in ATRP contribute to chains growth. The ATRP fulfills the principles of the controlled radical polymerization, which the initiation is fast and provides a constant concentration of growing polymer chains. The termination is suppressed by propagating radicals throughout the polymerization and keep the concentration of active species.

For the ATRP, there are few factors in influencing the reaction, such as temperature, transition-metal, ligands, structures of organic halide and monomers. From theory and experience, the conjugative and induced effect can weaken the strength of C-X bond. Such findings guide the selection of initiators and affect the applicable monomers.

### 2.2.3 Monomers of ATRP

Various monomers have been successfully polymerized using ATRP. Typical monomers can be classified into four types, namely styrenes, acrylates and methacrylates, methacrylamides, and acrylonitrile [Matyjaszewski and Xia, 2001].
2.2.3.1 Substituted styrenes

Styrene and its derivatives (Figure 2.2) were reported as the monomers in copper, iron, rhenium, and ruthenium catalytic ATRP systems; and the majority of polymerizations were conducted using the copper-based systems.

![Substituted styrenes](image)

Figure 2.2. Various styrenes polymerized by ATRP.

2.2.3.2 Acrylates and Methacrylates

The robustness of the ATRP technique is well demonstrated by the polymerization of several functional methacrylates.

Figure 2.3 shows the structure of 2-(dimethylamino)ethyl methacrylate (DMAEMA), methyl methacrylate (MMA), glycidyl acrylate, and 2-hydroxyethyl acrylate.

![Selected functional acrylates and methacrylates](image)

Figure 2.3. Selected functional acrylates and methacrylates monomers of ATRP.
Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) is water-soluble polymer that find applications in the fields of environmental protection, drug delivery and sensors [Zhang et al., 1998]. Amphiphilic block copolymers of DMAEMA form micelles which can be used as stabilizers in dispersion polymerizations and carriers in drug delivery. PDMAEMA was polymerized by ATRP with CuBr/1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) as catalyst and methyl 2-bromopropionate (MBP) as initiator. The reactions were carried out at 50°C in a relatively polar solvent, such as dichlorobenzene. Polymers with molecular weights up to $M_n=20,000$ Da and with $M_w/M_n=1.25$ were reported. The group found that successful polymerization of DMAEMA requires polydentate ligands, preferably tetradentate, to avoid the displacement of the ligand on the copper complex by polymer chains [Cho et al., 1997].

Well-defined AB block copolymers of DMAEMA with methyl methacrylate (MMA), methyl acrylate (MA) were successfully synthesized by ATRP with polydispersity about 1.2 [Zhang and Matyjaszewski, 1999]. Starting from difunctional polyacrylates, ABA triblock copolymers were prepared using well-defined PMMA or PMA as macroinitiators. PMMA with chlorine end groups was used as the macroinitiator to maximize chain extension. The block copolymerization with DMAEMA was carried out with CuCl/HMTETA as the catalyst system in dichlorobenzene at 90°C. For the PMA macroinitiator, bromine end groups were preferred in the present of
CuCl/HMTETA as the catalyst complex. The halogen exchange process increased the blocking efficiency, since PMA was a less efficient macrorinitiator.

Moreover, poly(glycidyl acrylate) is an interesting functional acrylate since its pendant oxirane rings can be opened and functional modifications are possible [Matyjaszewski et al., 1997]. Poly(2-hydroxyethyl acrylate) (poly(HEA)) is a water-soluble polymer with applications in the fields of coatings and biomaterials [Coca et al., 1998].

### 2.2.3.3 Methacrylic acid

Polymers based on acrylic acid have become increasingly important in applications for pharmaceutical and biomaterials. However, acrylic acid or methacrylic acid is difficult to be directly polymerized using ATRP because of interactions between carboxylic acid functionalities and the copper catalyst. In reaction, carboxylic acids react with Cu\textsuperscript{II} species by displacing the halogen atom from metal carboxylates which inhibit deactivation. Additionally, protonations of nitrogen based ligand systems in ATRP may occur, which disrupt its coordination to the Cu center. Therefore, precursors of poly(acrylic acid) were synthesized by ATRP, such as PrBA, after which the carboxylic acids were deprotected yielding well-defined poly(acrylic acid) [Davis and Matyjaszewski, 2000]. The polymerization condition of tBA was in bulk
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at 90°C, using [MBP]/[CuBr]/[dNbpy]=1/1/2. Well-defined polymers with molecular weights up to $M_n=50,000$ Da and polydispersity as low as $M_w/M_n < 1.2$ were obtained. Low molecular weight $PtBA$ (6000 Da) was also synthesized using CuBr/N,N,N′,N″,N″-pentamethyldiethylenetriamine (PMDETA) catalytic system at 60 °C. Polymerization control was optimized by the addition of a small amount of CuBr2/PMDETA (5% relative to CuI) and 25 vol% of N,N-dimethylformamide (DMF) to homogenize the catalyst. After the polymerization reactions, the tert-butyl groups were hydrolyzed by refluxing the polymer in 1,4-dioxane in the presence of hydrochloric acid. Complete hydrolysis of the ester groups was characterised using $^1$H NMR and FTIR. Other acid protecting groups have been used as long as they remain stable under the applied polymerization conditions as shown in Figure 2.4. For the synthesis of well-defined poly(benzyl methacrylate), the benzyl group was removed under mild conditions by hydrogenolysis.

![Figure 2.4. Structures of protected methacrylic acids polymerized by ATRP.](image-url)
2.2.3.4 Methacrylamides and other Monomers

Methacrylamides and other functional monomers were polymerized using ATRP, such as \(N\)-(2-hydroxypropyl)methacrylamide (HPMA), \(N, N\)-dimethylacrylamide (DMAA), \(N, N\)-diethylacrylamide (DEAA) and \(t\)-butylacrylamide (tBAA). Acrylonitrile and pyridine-containing polymers were also polymerized under ATRP conditions.

2.2.4 Initiators of ATRP

In the presence of metal catalysts, the initiators generate radical species that determine the number of growing polymer chains. The suitable initiators require an apparent initiation rate constant greater than the apparent propagation rate constant. The initiation should occur fast and quantitatively and the dormant polymer chain end should be stable during the polymerization. If the initiations are fast the number of growing chains is constant, which results in negligible transfers, terminations and side reactions. The structure and reactivity of monomers and metal complexes were main considerations in the selection of initiators. The organic halides with an active carbon-halogen bond and polyhalogenated compounds were used as initiators in ATRP. Compounds with a weak \(R-X\) bond can also be used as ATRP initiators, such as \(N-X\), \(S-X\), and \(O-X\). The structures of the initiator are analogous to the structures of the
polymer end groups in most cases. Polymeric chains with halogen end groups in an ATRP catalyst system can be used as macroinitiators to synthesize block and graft copolymers. Few types of macroinitiators have been used including polymers prepared by ATRP, commercially available polymers and polymers obtained by other polymerization techniques.

The reactivity of initiator is one of the main considerations for the choice of initiators. Halogens (X) in the initiators (R-X) include chlorine, bromine, and iodine. The general order of bond strength in the alkyl halides is R-Cl > R-Br > R-I. Thus the reactivity of the C-X bond increases in the order Cl < Br < I, but the stability of the C-X bond decreases vice versa. Alkyl chlorides should be the least efficient initiators and alkyl iodides should be the most efficient. Iodine works well for acrylate polymerizations in copper-mediated ATRP and has been found to controlled polymerization of styrene in ruthenium- and rhenium-based ATRP. Fluorine is not used because the C-F bond is too strong to undergo homolytic cleavage. However, alkyl iodides are light sensitive which can form metal iodide complexes with an unusual reactivity and the R-I bond may possibly be cleaved heterolytically. Bromine and chlorine are the most frequently used halogens [Matyjaszewski et al., 1998]. Multiple functional groups may increase the activity of alkyl halides. Tertiary alkyl halides are better initiators than secondary ones, where both are better than primary alkyl halides. Successful initiation in ATRP also depends on the catalyst and the
method or order of reagent addition.

### 2.2.5 Catalysts of ATRP

A variety of transition metal complexes have been studied as ATRP catalysts including copper, iron, molybdenum, chromium, ruthenium, rhenium, nickel and palladium complexes. The position of the atom transfer equilibrium and the dynamics of exchange between the dormant and active species depend on the nature of the catalyst. The metal center attacks the halogen at the chain end and is oxidized via a single electron transfer followed by halogen abstraction. The growing radical species are generated. Thereafter, the oxidized metal center donates the halogen back to the radical growing species, along with reduction of the metal center. For example, the lower oxidation state of the metal center (Ru(II)) should be more stable than its higher counterpart (Ru(III)) which establish an extremely low concentration of the radical species and a fast reversible reaction with the halogen. It is suggested that the catalytic activity increases with increasing electron density of the metal center or with decreasing redox potential of the complex. The catalyst should give one electron to the halide terminal upon the onset of radical generation. There are several prerequisites for an efficient transition metal catalyst. The metal center should have at least two readily accessible oxidation states separated by one electron. The metal center should have reasonable affinity toward a halogen. The coordination sphere
around metal should be expandable upon oxidation to selectively accommodate a (pseudo)-halogen. The ligand should complex relatively strongly with the metal. The position and dynamics of the ATRP equilibrium should be appropriate for the particular system.

Copper catalysts are superior in ATRP due to the versatility and cost. Cuprous halides complexed by two molecules of bpy were used as catalysts. The polymerization was tolerant to a variety of functional groups, such as -OH and -NH₂, and insensitive to additives, such as H₂O, CH₃OH, and CH₃CN. Multidentate aliphatic amines as the ligands greatly reduced the cost of the catalyst and increased the rate of polymerization. Copper(I) catalyst is effective in a tetrahedral or square planar configuration, which can be achieved in the cationic complexes with tetradeinate ligands or two bidentate ligands. Copper(II) forms cationic trigonal bipyramidal structures with tetradeinate ligands, which apparently forms square pyramidal neutral complexes with the longer Cu-X bond in the apical position. Better solubility of the transition-metal complex is achieved by adding long alkyl substituents to the ligand. Figure 2.5 [Matyjaszewski and Xia, 2001] shows few copper complexes used as ATRP Catalysts.
2.2.6 Ligands for ATRP

Ligands solubilize the transition-metal in organic media to control solubilities of catalyst in the reaction mixture and to ensure stability of the complexes in different monomers, solvents, and temperatures. Ligands adjust the redox potential of the metal center for appropriate reactivity and dynamics for the atom transfer reaction. This is important in the polymerization of acidic monomers and the monomers which can strongly complex transition metals such as pyridine-, amide-, or amine-containing monomers. Proper design of ligands is important in the polymerization under heterogeneous conditions, such as in water or ionic liquids. The efficiency of the catalyst for ATRP depends on the partition coefficients and the temperature. Furthermore, ligands may also facilitate the removal and recycling of the catalysts.

Nitrogen based ligands worked well in copper- and iron-mediated ATRP since the
coordination chemistry of transition-metal complex greatly affects the catalyst activity. The electronic and steric effects of ligands are important. Catalytic activity or efficiency is reduced when there is excessive steric hindrance around the metal center or the ligand has strong electron-withdrawing substituents. Although monodentate ligands are suitable for the most of transition metals employed in atom transfer radical addition (ATRA), they do not promote controlled copper-mediated ATRP. For ATRP a variety of multidentate nitrogen ligands have been successfully developed. The activity of ligand is usually higher for bridged and cyclic systems than for linear analogues. Figure 2.6 [Matyjaszewski and Xia, 2001] lists examples of bi-, tri- and tetradentate nitrogen-based systems used in copper-mediated ATRP.

Figure 2.6. Examples bi-, tri- and tetradentate nitrogen-based ligands used in copper-mediated ATRP [Matyjaszewski and Xia, 2001].
2.2.7 Examples of block copolymers synthesized by ATRP

The presence of an activated alkyl halide on a polymer chain end enables the synthesis of di-, tri-, or multiblock copolymers using ATRP. The growth of subsequent blocks can be achieved using an isolated macroinitiator or in-situ addition of a second monomer to a reaction.

The simplest polymerization is block copolymerization within the same class of monomers such as methacrylates, acrylates, or styrenes. Two early examples were the syntheses of poly(butyl methacrylate)-b-poly(methyl methacrylate) [Granel et al., 1996] and poly(methyl methacrylate)-b-poly(butyl methacrylate)-b-poly(methyl methacrylate) [Kotani et al., 1996] copolymers prepared by sequential monomer addition. For the comonomers belonging to different classes, poly(methyl methacrylate)-b-poly(n-butyl acrylate)-b-poly(methyl methacrylate) ABA triblock copolymer was synthesized by polymerization of the MMA segments onto a difunctional poly(n-butylacrylate) macroinitiator [Shipp et al., 1998].

2.3 Amphiphilic block copolymers

Amphiphilic block copolymers have affinity for two different types of environment, which are hydrophilic and hydrophobic environments. These molecules typically
consist of an aliphatic hydrocarbon chain connected with a hydrophilic group (often charged), which are similar to smaller systems, such as surfactants and lipids. Typical amphiphilic molecules with molecular weight of the order of 500 are referred to as “small” molecules. Amphiphilic polymers generally possess larger molecular weights, which are 10~1000 times larger than the typical “small” amphiphilic molecules. These large amphiphilic polymers are block copolymers, where a block of one type of homopolymer is sequentially attached to a block of another type of homopolymer.

Systems containing amphiphilic structure achieve self-assembled morphologies at the nanometer scale dimensions. Tuning the components and appropriate conditions leads to the desired microstructures that have potential applications in diverse fields, such as coatings, paints, personal care products, pharmaceuticals and biomaterial delivery systems.

### 2.3.1 Formation of micelles

Amphiphilic moleculars spontaneously self-assemble in aqueous solutions to form associating colloids or complex fluids. Depending on their mean aggregation number, molecular volume and critical hydrocarbon chain length, they can self-assemble into spherical or cylindrical micelles [Soo and Eisenberg, 2004]. The surfaces of micelles are formed by the hydrophilic heads of the monomeric molecules, where hydrophobic
tails lie inside the aggregate. The shapes of micelles are dependent on the polarity of solvents. Inverse micelles can be formed if the hydrophilic head is not compatible with the solvent, such as in oil. Equilibrium thermodynamics shows that cylindrical aggregates have a polydisperse distribution of sizes, whereas the sizes of spherical aggregates grow definitely.

The range of amphiphilic molecular concentrations to form micelles is a relatively narrow. Below the concentration limit virtually no micelles are detected and above the limit virtually all additional amphiphilic molecules form micelles [Horbaschek et al., 1998]. Many properties of amphiphilic molecular solutions plotted against the concentration appear to change at a different rate above and below this range of concentration. By extrapolating the loci of such a property above and below this regime until they intersect, a critical value may be obtained which is commonly referred to the critical micelle concentration (CMC). Micellization occurs in dilute solutions of block copolymers in selective solvent at a certain temperature. At higher concentrations, the micelles can form into a lattice above a critical gel concentration (CGC) [Luo et al., 1992]. When the concentration is fixed, micellization can occur on changing the temperature above or below the critical micellar temperature (CMT), depending on whether the self-assembly process is endothermic or exothermic. In most case, the micellization of block copolymer occurs via a closed association process. The dynamic equilibrium between micelles can be reached with a narrow
molar mass and size distribution. The micelle structure depends on the length of the polymer block forming the micellar core and the length of the corona.

2.3.2 Models of micellar association

There are two physical models that can be used to describe the association of molecules into micelles [Elias, 1973]. Firstly, in an open association model, there is a continuous distribution of micelles containing 1, 2, 3,..., n molecules, with an associated series of equilibrium constants. However, the open association model does not predict the existence of CMC since CMC is observed for block copolymer micelles. The closed association model is more applicable and better to describe the behavior of surfactants. The CMC does not correspond to a thermodynamic property of the system. It can be simply defined phenomenologically as the concentration at which a sufficient number of micelles is formed that can be detected by various physical techniques. Thermodynamically, closed association corresponds to an equilibrium between molecules (unimers), $A$, and micelles, $A_p$, containing $p$ molecules:

$$ A \leftrightarrow (1/p) A_p $$

(2.1)

with an associated equilibrium constant,

$$ K = [A_p]^{1/p} / [A] $$

(2.2)

For an advancement of the equilibrium from left to right by a fractional extent $\alpha$, $K$ is
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given by;

\[ K = \frac{\beta}{(1-\alpha)} \left( \frac{\alpha}{p\beta} \right)^{1/p} [A]^{-1+1/p} \]  \hspace{1cm} (2.3)

Where \( \alpha \) is the advancement of the equilibrium at which the CMC is detected and \( \beta = 1-\alpha + \alpha/p \). If the association number is large \((1/p \to 0)\) and independent of temperature then \( K \approx [A]^{-1} \) and the standard Gibbs energy of association is given by;

\[ \Delta G^0_{mic} = -RT \ln[K] \approx RT \ln[A] \]  \hspace{1cm} (2.4)

Under this condition, for molecules and micelles in equilibrium at a concentration just above the critical micelle concentration, the following equation is applied;

\[ \Delta G^0_{mic} = RT \ln[cmc] \]  \hspace{1cm} (2.5)

Otherwise, Equation 2.3 must be used to obtain \( K \). For a small association number, say \( p=10 \), the error in approximating \( K \) by \( 1/\text{cmc} \) is large; calculation of \( \Delta G^0_{mic} \) via Equation 2.5 requires \( p \geq 50 \). For a large association number, the standard enthalpy of micelle formation is then given by;

\[ \Delta H^0_{mic} = RT \left( \frac{d \ln(cmc)}{d(1/T)} \right) \]  \hspace{1cm} (2.6)

However, \( \Delta H^0_{mic} \) and \( T\Delta S^0 \), determined from the temperature dependence of the Gibbs energy, are less sensitive to the association number than \( \Delta G^0_{mic} \) itself [Yang et al., 1995]. Assuming that \( \Delta H^0_{mic} \) is approximately constant within a certain temperature range, Equation 2.6 can be integrated to yield;

\[ \ln(cmc) = \frac{\Delta H^0_{mic}}{RT} + \text{const} \]  \hspace{1cm} (2.7)

Thus the logarithm of CMC can be plotted against the inverse temperature to extract
information on the micellization enthalpy. Equivalently, the logarithmic concentration can be plotted against the inverse critical micelle temperature.

In this study, the CMC was determined from plots of surface tension versus logarithmic concentration. The surface tension decreases rapidly with increase concentration up to the CMC, beyond which the trend approaches a plateau.

2.3.3 Polyacids and anionic block copolymers

The most representative weak polyacids are poly(acrylic acid) (PAA) [Philippova et al., 1997] and poly(methacrylic acid) (PMAA) [Torres-Lugo and Peppas, 1999], which possess carboxylic group with $pK_a$ of around 5~6. By adjusting the pH, the stimuli-responsive polymers undergo ionization/deionization transitions. Their carboxylic pendant groups accept protons at low pH, and releasing protons at high pH. Therefore, they are transformed into polyelectrolytes at high pH which electrostatic repulsion forces between the molecular chains. The hydrophobic interaction controls several processes, such as the precipitation/solubilization of molecular chains, deswelling/swelling of hydrogels, and hydrophobic/hydrophilic characteristics of surfactants. PMAA exhibits an abrupt phase transition that compared to the continuous phase transition of PAA. PMAA has a compact morphology before a critical charge density is reached because the methyl groups in PMAA induce a
stronger hydrophobic interaction force. The compact conformation of PMAA in the uncharged state is called a ‘hypercoiled’ conformation [Tonge and Tighe, 2001]. Introducing a more hydrophobic moiety can offer a more compact conformation at the uncharged state and a more dramatic discontinuous phase. For examples, poly(2-ethyl acrylic acid) (PEAA) and poly(2-propyl acrylic acid) (PPAA) containing more hydrophobic characteristics were studied, and they provided a more compact conformational structure at low pH [Murthy et al., 1999]. Figure 2.7 gives the structures of the four polyacids.

![Figure 2.7 Representative pH-responsive polyacids: PAAc, PMAAc, PEAAc and PPAAc.](image)

Typical classes of anionic polyelectrolytes are polyacrylates and polymethacrylates in the neutralized form. Various morphologies of PS-\(b\)-PANa aggregates have been extensively studied by Eisenberg and co-workers [Zhang and Eisenberg, 1995]. The characteristics of the vesicles can be controlled by varying the copolymer compositions, relative block lengths, copolymer concentration, nature of solvent, polydispersity of the hydrophilic block and additives (ions, homopolymers, and surfactants). The unilamellar bilayer vesicles were prepared by PS-\(b\)-PAA and other
amphiphilic block copolymer as shown in Figure 2.8 [Burke et al., 2001].

Figure 2.8 Representative micrographs of various types of vesicles: (A) small uniform vesicles (PS\textsubscript{410}-b-PAA\textsubscript{13}), (B) large polydisperse vesicles (PS\textsubscript{100}-b-PEO\textsubscript{30}), (C) entrapped vesicles (PS\textsubscript{200}-b-PAA\textsubscript{20}), (D) hollow concentric vesicles (PS\textsubscript{132}-b-PAA\textsubscript{20}), (E) onions (PS\textsubscript{260}-b-P4VPDecI\textsubscript{70}), and (F) vesicles with tubes in the wall (PS\textsubscript{100}-b-PEO\textsubscript{30}) [Burke et al., 2001].

Diblock and triblock copolymers with a PMAA hydrophilic block and hydrophobic blocks 2-\((n\text{-methylperfluorobutanesulfonamido})\) ethyl methacrylate (FMA) were synthesized by sequential anionic polymerization [Busse et al., 2002]. Despite the expected higher hydrophobicity, the copolymers are not very surface active. PS-\(b\)-PMA micelles in aqueous solution were studied by potentiometric titration, light scattering and fluorometry [Stepanek et al., 2000]. The conformational changes of the shell-forming blocks and the exposure of accessible carboxylic groups for neutralization proceeded rather slowly.

### 2.3.4 Polybases and cationic block copolymers

The polybases are pH-responsive polymers where their aggregation behaviors are affected by pH. The representative weak polybases are poly(\(N,N'\text{-dimethyl}
aminoethyl methacrylate) (PDMAEMA) and poly(N,N′-diethyl aminoethyl methacrylate) (PDEAEMA) with amine groups on the side chains. The amine groups gain protons in acidic condition and release protons in basic condition. PDEAEMA has longer hydrophobic groups at the ends of the amine groups than PDMAEMA, which produces stronger hydrophobic interactions at high pH. PDEAEMA homopolymer undergoes an abrupt precipitation above pH 7.5 due to the deprotonation of amino groups and hydrophobic molecular interactions [Lee et al., 2002]. PDMAEMA was reported to show a temperature sensitivity similar to PNIPAAm [Okubo et al., 1998].

The block copolymers comprising of tertiary amine and methacrylic acid form micelles in solution, where the size and aggregation number of micelles were controlled by the changes of pH, ionic strength and temperature of the solution. It is interesting that diblock poly(2-(dimethylamino)ethyl methacrylate-\(b\)-poly(2-(diethylamino)ethyl methacrylate (PDMA-\(b\)-PDEA) polymer is double hydrophilic at low pH. However the PDMA-\(b\)-PDEA form micelles at high pH due to the deprotonated hydrophobic PDEA blocks [Butun et al., 1997; Lee et al., 1999]. When the hydrophilic block length keep constant, micelle size and association number decreased with the increase of the length of hydrophobic block. The concept of ‘schizophrenic’ AB diblock copolymers represents self-assembly systems that can form both micelles with A blocks in the micellar core and also reverse micelles with B
blocks in the micellar core as shown in Figure 2.9 [Butun et al., 1998].

![Figure 2.9 Schematic representation of the formation of micelles and reverse micelles for an AB diblock copolymer [Butun et al., 1998].](image)

2.3.5 Star shape amphiphilic block copolymers

The micellization behaviors of linear diblock and triblock copolymers were extensively studied. However, the different morphologies of polymer aggregates are controlled by three different factors, including the interfacial tension between micellar core and solvent outside the core, the degree of stretching of core-forming blocks and repulsive interactions between corona forming chains. The architectures of block copolymer affect the formation of aggregates. Miktoarm polystyrene-\(b\)-polysisoprene (PS-\(b\)-PI) star copolymers form spherical micelles in n-decane [Allgaier et al., 1996; Pispas et al., 2000; Sotiriou et al., 2002]. The aggregation number and the size of these micelles were smaller than the corresponding linear diblock copolymers. The advantages of amphiphilic block copolymers are that they are suitable as delivery vehicles for biomaterials or drugs, where aqueous solution is required as the media for such delivery applications.
Although a large number of star copolymers were synthesized, only a few of them were studied with respect to their micellization behavior. In this project, the four-arm star amphiphilic block copolymers were synthesized by ATRP, which produced polymers with a narrow molecular weight distribution. The micelles formations of these star polymers were studied as a function of pH which is an effective stimuli changes in aqueous media. The delivery of biomolecules using these polymers was also investigated.

### 2.3.6 Polymers for gene delivery application

Much attentions have been focused on gene therapy for the treatment of human diseases arising from defective genes in the field of medicine, pharmaceutical sciences and biotechnology [Pack et al., 2005]. An efficient delivery system is required to transfer genetic materials encoded within plasmid DNA or RNA to targeted tissues and organs [Seeman, 2005; Bazan-Peregrino et al., 2007]. Non-viral vectors have several advantages over viral vectors because of their lower immunogenicity, absence of endogenous virus recombination and their ability to package DNAs in wide size range [Crystal, 1995; Pfeifer and Verma, 2001; Haider et al., 2005; Trentin et al., 2005]. Polymeric delivery systems can be designed to optimize their physicochemical and biological properties by molecular diversity, chain density and chemical structures [Dang and Leong, 2006]. Currently, cationic
polymers spontaneously condense polyanionic DNA, which is a prerequisite for
gene transfer in most types of cells [Haag and Kratz, 2006]. Self-assembled block
copolymers consisting of a cationic chain and a neutral hydrophilic segment will form
polyion complex with DNA [Bromberg et al., 2006]. The core-shell polyplex
structures surrounded by a hydrophilic polymer have a high colloidal stability which
reduces the interaction with blood components, thereby protecting the therapeutic
genes from premature degradation in systemic transfer medium.

High cation density of polyethylenimine (PEI) [Boussif et al., 1995],
poly(dimethylaminoethyl methacrylate) (PDMAEMA) [Cherng et al., 1996] and their
derivatives [Verbaan et al., 2005; Adams et al., 2006; Patnaik et al., 2006] contributed
to consistent transfection efficiency in the field of gene delivery. Diethylaminoethyl-
dextran was studied in non-viral gene delivery systems with relatively low
transfection efficiency [Mack et al., 1998; Gavalas and Chaniotakis, 2000; Ahn et al.,
2004; Benham and Mielke, 2005; Dubruel and Schacht, 2006]. Various polyamines
gene carriers have been widely studied as potential gene carriers since the protonated
polyelectrolyte with high molecular weight formed nanoparticles with DNA [Wu and
Wu, 1987; Park and Healy, 2004; Bromberg et al., 2006; Yamagata et al., 2007].
However, the Poly(Lysine)/DNA complexes showed a relatively high cytotoxicity and
a tendency to aggregate and precipitate, depending on the ionic strength [Choi et al.,
1998; Liu et al., 2001; Duncan and Izzo, 2005]. Polysaccharides, such as
cyclodextrins and chitosan, are relatively non-toxic and non-immunogenic [Xu et al., 2006; Chan et al., 2007]. DNA was conjugated to the polymers to prevent inter-particulate aggregation of the complexes, to increase complex solubility and stability, and to eliminate problems associated with cytotoxicity.

2.3.7 Controlled drug release using amphiphilic block copolymers

Self-assembled block copolymers exhibiting stimuli-responsive behaviors have been used extensively as versatile nanomedicine delivery platforms in biomedical applications [Brannon-Peppas and Blanchette, 2004; Betancourt and Brannon-Peppas, 2006; Farokhzad and Langer, 2006; Tong and Cheng, 2007]. The synthetic biomimetic polymers contain hydrophilic and hydrophobic block domains, which could form micelles with core/shell structures at the suitable environmental conditions [Soo and Eisenberg, 2004; Vriezema et al., 2005; Yow and Routh, 2006; Dai et al., 2008]. The hydrophilic segments located between the core and external aqueous medium provide stability of the micellar system. By manipulating the chemical, physical or electrostatic interaction, the hydrophobic regions can be tailored to serve as reservoirs for drug loading and encapsulation [Magid, 1998; Nakamura and Shikata, 2007]. An external stimulus (pH, ionic strength, temperature, light, electric field or magnetic field) can induce a change in the characteristics of the polymer/drug complex, such as the conformation, solubility, association and the release behavior of
drug molecules under specific conditions [Checot et al., 2007; Yang et al., 2007]. The polymer micelles with encapsulated drugs possess various attractive properties, such as optimal size for enhanced permeability and retention effect, high stability in aqueous medium and solubilization of hydrophobic drugs [Allen and Cullis, 2004; Napier and Desimone, 2007; Xu et al., 2007].

A variety of polymeric nanoparticles have been developed and evaluated as delivery vehicles for drug molecules, therapeutic proteins and genes. Temperature responsive poly(N-isopropylacrylamide) (PNIPAm) undergoes a sharp coil to globule transition, where the polymeric chains are transformed from hydrophilic to a hydrophobic character with increasing temperature [Zhang et al., 2007; Jin et al., 2008]. Polyethylene-oxide (PEO) and polypropylene-oxide (PPO) block co-polymers known as Pluronics, Poloxamers and Tetronics are commercially available and widely used in the pharmaceutical industry due to the biocompatible and stealth properties of polyoxyethylenes [Moghimi and Hunter, 2000; Xiong et al., 2006; Chiappetta and Sosnik, 2007]. Poly(ethylene oxide)-block-poly(L-amino acid)s is useful for the chemical conjugation of drugs as they can be chemically modified to yield functional groups enhancing the loading of therapeutic compounds [Lavasanifar et al., 2002; Nishiyama and Kataoka, 2006].
3 Chapter Three
Synthesis of Four-arm PEO Star Block Copolymers by ATRP

3.1 Synthesis of four-arm PEO macroinitiator

3.1.1 Materials

The four-arm hydroxy-end-capped PEOs [degree of polymerization (DP) = 56 per arm; $M_w/M_n=1.08$] were purchased from NOF (Tokyo, Japan). 2-bromoisobutyryl bromide and Triethylamine were purchased from Aldrich to be used without further purification. AR grade Toluene was dried using sodium metal under argon environment at 110°C for three days.

3.1.2 Procedure for the synthesis of four-arm PEO macroinitiator

Four-arm PEO was added into a three-neck round-bottom flask and dissolved in 200 ml of dry toluene. To remove the trace amount of water in PEO, 50 ml of toluene was distilled from the reaction mixture via the azeotropic technique. The reaction mixture was cooled to 0°C in an ice bath and triethylamine (1.5 molar equiv to PEO end) was added. Under continuous stirring, 2-bromoisobutyryl bromide (1.5 molar equiv to PEO
end) was added dropwise over one hour period using a pressure-equalizing funnel. After stirring the reaction mixture for 24 h at room temperature, triethylamine hydrobromide was removed by filtration. The reaction solution was concentrated by rotary evaporation prior to precipitation in ten fold excess of n-hexane. The polymer was then filtered, and dried under vacuum. The above procedure was repeated twice to ensure complete coupling of the end groups.

### 3.1.3 Purification of four-arm PEO macroinitiator

The four-arm PEO macroinitiator was dissolved in water at pH 9 and extracted with dichloromethane, where the organic layer was recovered, and dried over magnesium chloride and subsequently filtered. The solvent was then removed under vacuum to produce the purified macroinitiator, which was stored in a desiccator to prevent contact with moisture. The four-arm block copolymer, PEO-\textit{b}-PDEAEMA was synthesized by ATRP as described in Scheme 3.1. Four arms hydroxy-end-capped PEO was coupled with the initiator, 2-bromoisobutyryl bromide to produced Br-terminated four-arm PEO macroinitiator with $M_n=10750$ Da, $M_w/M_n =1.14$ and 96% yield.

$$\text{Scheme 3.1 Synthentic scheme for producing the four-arm PEO macroinitiator.}$$
3.2 Synthesis of four-arm PEO-\textit{b}-PDEAEMA block copolymer by ATRP

3.2.1 Materials

(Diethylamino)ethyl methacrylate (DEAEMA, Aldrich, 99\%) was purified by passing through a basic alumina column, stirred over CaH\textsubscript{2} and distilled under vacuum. CuCl (99.99\%), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA, 97\%), and anisole (anhydrous, 99\%) were purchased from Aldrich and used without further purification. The synthesized four-arm PEO macroinitiator was stored in the desiccator prior to use.

3.2.2 Procedure of synthesis of four-arm PEO-\textit{b}-PDEAEMA block copolymer

The Schlenk flask was connected to the vacuum line and moisture was removed by repeated application of vacuum with purging with argon. Under an argon environment, Br-terminated four-arm PEO macroinitiator and CuCl were charged into the Schlenk flask that was tightly sealed with a rubber septum. Using an argon washed syringe, deoxygenated anisole and the monomer (volume ratio of anisole:DEAEMA = 2:1) were introduced into the flask and the mixture was stirred at room temperature for 10 min until it became homogeneous. The reaction mixture was degassed using three
freeze-thaw cycles. Once the mixture approached the room temperature, degassed ligand (HMTETA) was introduced using an Ar-purged syringe. The flask was placed in a thermostated oil bath kept at 90 °C. The reaction mixture became dark green and turned progressively more viscous, indicating the progress of polymerization. The reaction extent was monitored by sampling small amounts of the aliquots in 20 mins interval for GPC analyses. The polymerization was terminated after 1.5 h by the addition of THF. The catalyst was removed by passing the reaction mixture through a basic alumina column and flush by THF. The polymer solution was concentrated by rotary evaporation and subsequently recovered by dropwise addition to excess amounts of n-hexane. The unreacted DEAEMA monomer and other contaminants were dissolved in n-hexane. The n-hexane solvent was subsequently removed in a vacuum oven at room temperature to yield a light yellow powder (yield=86%).

Scheme 3.2 shows the synthesis of four-arm PEO-\(b\)-PDEAEMA block copolymer.

![Scheme 3.2 Synthetic scheme for producing the four-arm PEO-\(b\)-PDEAEMA block copolymer.](image)
3.2.3 Characterization of four-arm PEO-b-PDEAEMA block copolymer

The composition, structure and molecular weight of four-arm PEO-b-PDEAEMA block copolymer were characterized by GPC and NMR techniques.

3.2.3.1 Gel permeation chromatography

Gel Permeation Chromatography (GPC) of the copolymers was performed on an Agilent 1100 apparatus equipped with a differential refractometer as the detector. Tetrahydrofuran (THF) was used as the mobile phase with a flow rate of 1.0 ml/min. Polystyrene (PS) is used as the standard calibration polymer. The samples were dissolved in THF first and then filtered through a filter (0.45 μm) to remove impurities. The sample concentration was around 10 mg/ml.

GPC traces of four-arm PEO-b-PDEAEMA block copolymer and Br-terminated four-arm PEO macroinitiator are shown in Figure 3.1. The DEAEMA was copolymerized with the macroinitiator in the presence of CuCl catalyst and HMTETA ligand in anisole at 90 °C. The four-arm PEO-b-PDEAEMA copolymer possessed a $M_n=64690$ Da and a molecular weight distributions $\overline{M}_w/\overline{M}_n=1.29$. As the molecular weight is in good correlation with the theoretical molecular weight which was calculated based
on the amount of DEAEMA monomer added during synthesis and the narrow polydispersity, we confirmed that the copolymerization was well-controlled.

![Figure 3.1 GPC traces of four-arm PEO-b-PDEAEMA block copolymer and Br-terminated four-arm PEO macroinitiator.](image)

**Figure 3.1** GPC traces of four-arm PEO-b-PDEAEMA block copolymer and Br-terminated four-arm PEO macroinitiator.

### 3.2.3.2 Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) spectra were recorded at room temperature using a Bruker DRX400 (400 MHz) Nuclear Magnetic Resonance Spectrometer. Chemical shifts ($\delta$) were given in ppm using tetramethylsilane (TMS) as an internal reference. The polymer sample was dissolved in deuterochloroform (CDCl$_3$) for measurement and the concentration used was about 10 mg/ml.
The NMR results of four-arm PEO-\(b\)-PDEAEMA block copolymer are as follows:

\(^1\)H NMR (400 MHz, CDCl\(_3\), TMS), \(\delta\) (ppm): 4.02 (-OCH\(_2\)CH\(_2\)-), 3.66 (-OCH\(_2\)CH\(_2\)O-), 2.57 (-CH\(_2\)CH\(_3\)-N-), 2.49 (-N(CH\(_2\)CH\(_3\))\(_2\)), 1.74 (-CCH\(_2\)C-), 0.98 (-N(CH\(_2\)CH\(_3\))\(_2\)), 0.83 (-CCH\(_3\)).

Figure 3.2 \(^1\)H NMR spectrum of four-arm PEO-\(b\)-PDEAEMA block copolymer (CDCl\(_3\))

Figure 3.2 shows a \(^1\)H NMR spectrum of a typical four-arm PEO-\(b\)-PDEAEMA block copolymer in CDCl\(_3\). The small peak at \(\delta\) of 1.74 ppm (-CCH\(_2\)C-) indicating the successful graft of DEAEMA onto the four-arm PEO segments. The molar composition of the four-arm star copolymer was determined from the relative
intensities at 3.66 ppm (-CH$_2$CH$_2$O of the PEO block) and 4.02 ppm (-OCH$_2$CH$_2$N- of the PDEAEMA block). Since the four arms chain propagation experienced similar conditions in the polymerization process, the degree of polymerization and the number-average molecular weight ($M_n$) were determined to produce a block copolymer with a chemical structure of 4PO$_{56}$-b-PDEAEMA$_{74}$. Yield was calculated according monomer conversion. The characteristics of four-arm block PEO-\textit{b}-PDEAEMA were summarized in Table 3.1

<table>
<thead>
<tr>
<th>Polymer Structure</th>
<th>$\bar{M}_n$ (Da) (GPC)</th>
<th>$\bar{M}_n/\bar{M}_n$ (GPC)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-arm PEO$<em>{56}$-b-PDEAEMA$</em>{74}$</td>
<td>64690</td>
<td>1.29</td>
<td>86%</td>
</tr>
</tbody>
</table>

3.3 Synthesis of four-arm PEO-\textit{b}-PrBMA block copolymer.

3.3.1 Materials

Tert-Butyl methacrylate (tBMA, Aldrich, 98%) was purified by passing through a neutral alumina column, stirred over CaH$_2$, and distilled under vacuum. CuCl (99.99%), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA, 97%), and anisole (anhydrous, 99%) were purchased from Aldrich and used without further purification. Previously prepared Br-terminated four-arm PEO macroinitiator was taken from desiccator before use.
3.3.2 Procedure of synthesis of four-arm PEO-b-PrBMA block copolymer

The required glassware was dried in oven overnight prior to use. Br-terminated four-arm PEO macroinitiator and CuCl were added to a Schlenk flask. Using an argon washed syringe, deoxygenated anisole and the monomer (volume ratio of anisole: tBMA = 1.5:1) were introduced into the flask and the mixture was stirred at room temperature for 10 min until it became homogeneous. The reaction mixture was degassed three times using freeze-pump-thaw cycles. Once the mixture reached the room temperature, degassed ligand (HMTETA) was introduced using an Ar-purged syringe. The flask was placed in an oil bath with thermostat at 90 °C. The reaction mixture became dark green and turned progressively more viscous, indicating the progress of polymerization. The reaction extent was monitored by sampling small amounts of the aliquots in 20 mins interval for GPC analyses. The polymerization was terminated after 2.5 h by the addition of THF. After the reaction was completed, the catalyst was removed by passing through a neutral alumina column and flush with THF. The polymer solution was concentrated by rotary evaporation and subsequently recovered by dropwise addition into excess amounts of n-hexane. The solvent n-hexane was subsequently removed in a vacuum oven at room temperature to yield a white color powder. Scheme 3.3 shows the synthetic scheme of four-arm block copolymer PEO-b-PrBMA.
Scheme 3.3 Synthetic scheme of the four-arm PEO-b-PrBMA block copolymer.

### 3.3.3 Characterization of four-arm PEO-b-PrBMA block copolymer

#### 3.3.3.1 Gel permeation chromatography

An Agilent 1100 series GPC system equipped with a liquid chromatography pump, photoluminescence gel 5 μm MIXED-C column, and refractive detector was used to determinate the molecular weight and molecular weight distribution. The column was calibrated with the narrow molecular weight polystyrene standards. The GPC eluent was HPLC grade Tetrahydrofuran (THF) stabilized with butylated hydroxytoluene at a flow rate of 1.0 ml/min. The samples were dissolved in THF first and the concentration was around 10 mg/ml.

Figure 3.3 shows the GPC traces of four-arm PEO-b-PrBMA block copolymer and four-arm PEO macroinitiator. If there is any significant amount of macroinitiator present, it can be easily detected from the GPC traces of the final polymer mixture i.e., four-arm PEO-b-PrBMA block copolymer. From the narrow and symmetric single
peak, we confirmed that the copolymerization was well-controlled. The number average molecular weight ($M_n$) of the four-arm PEO-$b$-PtBMA block copolymer obtained from GPC was 60294 Da, which was consistent with the theoretical molecular mass (initiator to monomer molar ratio) calculated based on the amount of monomer added during synthesis. The relative molecular mass distributions ($\bar{M}_w / \bar{M}_n$) is narrow, PDI (polydispersity index) =1.23.

![Figure 3.3 GPC traces of four-arm PEO-$b$-PtBMA block copolymer and Br-terminated four-arm PEO macroinitiator.](image)

### 3.3.3.2 Nuclear magnetic resonance

The $^1$H NMR spectrum for the block copolymer in CDCl3 was recorded using a Bruker DRX400 instrument. Chemical shifts ($\delta$) were given in ppm using tetramethylsilane (TMS) as an internal reference. The polymer samples were
dissolved in deuterochloroform (CDCl₃) for measurement and the concentration used was about 10 mg/ml.

The NMR results of four-arm PEO-b-PrBMA block copolymer are as follows:

\(^1\)H NMR (400 MHz, CDCl₃, TMS), \(\delta\) (ppm): 3.58 (-OCH₂CH₂O-), 1.75 (-CCH₃C-), 1.58 (H₂O), 1.40 (-C(CH₃)₃), 1.02 (-CCH₃).

![Diagram of four-arm PEO-b-PrBMA block copolymer](image)

Figure 3.4 \(^1\)H NMR spectrum of four-arm PEO-b-PrBMA block copolymer in CDCl₃.
Figure 3.4 shows $^1$H NMR spectrum of four-arm PEO-$b$-PtBMA block copolymer in CDCl$_3$. The polymerization degree of four-arm PEO-$b$-PtBMA block copolymer copolymers was calculated from the peak intensity ratio of 3.58 (-OCH$_2$CH$_2$O-) of PEO and 1.40 (-C(CH$_3$)$_3$) of tBMA. The block lengths of the copolymer calculated from $^1$H NMR spectrum were 56 and 88 for PEO and tBMA, respectively. The characteristics of four-arm block PEO-$b$-PtBMA were summarized in Table 3.2

Table 3.2 Characteristics of four-arm block PEO-$b$-PtBMA

<table>
<thead>
<tr>
<th>Polymer Structure</th>
<th>$\bar{M}_w$ (Da)</th>
<th>$\bar{M}_w / \bar{M}_n$</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-arm PEO$<em>{56}$-$b$-PtBMA$</em>{88}$</td>
<td>60294</td>
<td>1.23</td>
<td>89%</td>
</tr>
</tbody>
</table>

3.4 Preparation of four-arm PEO-$b$-PMAA block copolymer

3.4.1 Procedure of hydrolysis of four-arm PEO-$b$-PtBMA block copolymer

The tert-butyl protecting groups of P(tBMA) blocks of four-arm PEO-$b$-PtBMA block copolymer were hydrolyzed under acid environment. The four-arm PEO-$b$-PtBMA block copolymer was dissolved by dichloromethane (DMC) in a round bottom flask and the concentration of polymer was 5 wt%. Trifluoroacetic acid (TFA) was added to the flask as five times than the total molar of tBMA on four-arm PEO-$b$-PtBMA polymer chains. The mixture solution was stirred for 24 hours at room temperature.
The tertiary butyl groups were hydrolyzed with concentrated trifluoroacetic acid, which the extent of hydrolysis was confirmed by FTIR and $^1$H NMR. Thereafter, the polymer solution was concentrated by rotary evaporation and subsequently recovered by dropwise addition into excess amounts of $n$-hexane. The solvent was subsequently removed in a vacuum oven at room temperature. The four-arm PEO-$b$-PMAA was produced from the hydrolysis of 4-arm PEO-$b$-PtBMA.

### 3.4.2 Procedure of dialysis of four-arm PEO-$b$-PMAA block copolymer

The four-arm PEO-$b$-PMAA block copolymer was dissolved in deionized water. The polymer solution was filled into semi-permeable membrane and dialyzed in a container using deionized water, where the water was changed in six hours interval. Until the conductivity of water remained constant, the polymer dialysis was deemed to be completed. The solution was concentrated by rotary evaporation prior to freeze drying to yield a white colour four-arm PEO-$b$-PMAA block copolymer.

### 3.4.3 Characterization of four-arm PEO-$b$-PMAA block copolymer

After hydrolysis and purification, the four-arm PEO-$b$-PMAA block copolymer was characterized by $^1$H NMR, where the 1.40 ppm (-C(CH$_3$)$_3$) of PtBMA segment
disappeared due to the deprotection of tert-butyl groups on the P(\(i\)BMA) blocks. FT-IR (KBr-pellet) showed the broad peak at 3500 cm\(^{-1}\), which is the characteristic absorption for carboxylic acid. The amount of the carboxylic acid on polymer chains was quantified by potentiometric titration as described in Chapter 6, which confirmed that the 4-arm PEO-\(b\)-PrBMA copolymer was fully hydrolyzed to 4-arm PEO-\(b\)-PMAA.

### 3.5 Summary

The weak polybase, four-arm PEO-\(b\)-PDEAEMA, and tertiary butyl group protected weak polyacid, four-arm PEO-\(b\)-PrBMA, were synthesized via the well-controlled ATRP applied with CuCl/HMTETA catalyst system in anisole at 90 °C. Thereafter, the four-arm PEO-\(b\)-PMAA block copolymer was produced by hydrolysis of four-arm PEO-\(b\)-PrBMA. Block copolymers with very narrow PDIs were obtained by two steps process. Few catalysts were tried to synthesize the polymers and copper chloride catalyst is the most effective for polarization of DEAEMA or \(i\)BMA. After hydrolysis, dialysis, and freeze drying, the yellow color four-arm PEO-\(b\)-PDEAEMA and white color four-arm PEO-\(b\)-PMAA block copolymer were obtained. The compositions, structures and molecular weights of these block copolymers were characterized by GPC and NMR techniques, which yielded the four-arm PEO\(_{56}\)-\(b\)-PDEAEMA\(_{74}\) and the four-arm PEO\(_{56}\)-\(b\)-PMAA\(_{88}\) block copolymers.
4 Chapter Four
Analytical Methods and Principles

4.1 Potentiometric titration

4.1.1 Automatic potentiometric titrator

A Radiometer ABU93 Triburette titration system was used to determine the pH and conductivity of solutions, where the base or acid was titrated automatically into the polymer solutions. The system was equipped with a Radiometer pHG201 pH glass electrodes and Radiometer REF201 reference electrodes. The external conductivity meter (model CDM83) was used to measure the changes in conductivity during the titration experiment. The titration instrument is integrated with a standard RS232C interface which was fully controlled by a computer using the ALIQUOT titration software. The electrodes are not affected by the polymer solution as the electrodes yielded identical readings prior to and after the titration experiments. The titration conditions and settings were entered into the software prior to the experiment. Sufficient lag time of about 1 minute was allowed between each dosage to ensure that the reaction has reached equilibrium. The water jacketed titration vessel was maintained at constant temperature 25 °C using a circulating water bath.
An elementary and commonly adopted experimental approach for characterizing polyacid and polybase is to perform titration experiments. In these experiments, a strong base (NaOH) or strong acid (HCl) was added to a solution of weakly charged polyelectrolytes. The pH of solution and the equilibrium dissociation depend not only on the amount of added base but also on the polyelectrolyte concentration and the presence of salt. To prevent the noticeable volume change of titrated solution, 1 M standard NaOH or HCl solution was used as titrants.

### 4.1.2 Potentiometric titration of polyelectrolyte

Potentiometric titration has been applied to characterize natural and synthetic polymeric acids, bases and ampholytes for many decades. The attempt to apply the mass-action law to the electrolytic dissociation of these substances leads to variable “dissociation constants,” changing markedly with the degree of polymer ionization, concentration of polymer, and concentration of added salts.

Weak acid monomers (HA) can undergo dissociation given below:

\[
HA \leftrightarrow H^+ + A^- \tag{4.1}
\]

where \(A^-\) is the charged monomer attached to the backbone, while the dissociated \(H^+\) ions are dispersed in the bulk solution. The dissociation/association is an equilibrium process satisfying the reaction defined by Eq. (4.1). The ionization degree determines
the amount of effective charge on the polyelectrolyte chain, and it also depends on the pH of the solution. At low pH, the polymer is weakly charged, whereas at high pH a larger fraction of monomers are dissociated and the polymer charge saturates to its maximum value. The most visible consequence is the solubility in water. Hydrophobic polyacids become water-soluble at sufficiently high pH, where the polymer charges are strong enough to overcome the hydrophobic character of the polymeric chains. In contrast to low-molecular-weight acids, the charged groups of polyacids are correlated since they are distributed along the polymer backbone. Indeed, the dissociation of one acid group is correlated in a complex manner to the position and number of other charged groups on the polymeric chain. As a result, when the amount of charge varies, the chain conformation is affected and in turn influences the dissociation of other groups.

The acid dissociation constant, $K_a$ is defined by:

$$K_a = \frac{[H^+][A^-]}{[HA]} \quad (4.2)$$

where $[H^+]$, $[A^-]$ and $[HA]$ are the molarities of free hydrogen ion, counterion, and undissociated acid respectively. The ionization degree of acid is given by the expression:

$$\sigma_{HA} = \frac{[A^-]}{[A^-] + [HA]} \quad (4.3)$$

Therefore, the dissociation constant can be obtained by combining Eqs. (4.1) and (4.2):
Chapter 4 Analytical Methods and Principles

\[ K_a = \frac{\sigma [H^+]}{1 - \sigma} \]  \hspace{1cm} (4.4)

For the neutralization reaction, the degree of ionization (\( \sigma \)) is equal to the degree of neutralization (\( \alpha \)), hence the negative-logarithm apparent dissociation constant (pK_{app}) can be expressed by the equation below:

\[ pK_{app} = pH + \log \frac{1 - \alpha}{\alpha} \]  \hspace{1cm} (4.5)

The detail and direct information on the conformational transition can be determined by the analysis of pK_{app} versus neutralization degree (\( \alpha \)) curve.

The thermodynamic parameters such as Gibbs free energy (\( \Delta G \)) were obtained from the titration data. The polymer chains exist as extended chains when charged, but they transformed into a more compact structure when the ionizable groups are not charged. The free energy of polymer chain conformation as a result of the ionization of chargeable groups can be calculated from the equation below:

\[ \Delta G = -RT \int_0^\alpha \alpha d \ln \alpha_{H^+} \]  \hspace{1cm} (4.6)

where \( \alpha_{H^+} \) is the hydrogen ion activity and \( \alpha \) is the degree of neutralization. R is the ideal gas constant and T is absolute temperature. The required energy to extract proton from polyacid (\( \Delta G_{el} \)) is related to the electrostatic potential (\( \Psi \)) on the surface of polyelectrolyte chain if the free energy is only due to the electrostatic interaction.

The \( \Delta G_{el} \) value was obtained from the equation below:

\[ \Delta G_{el} = -N_a e \Psi_a \]  \hspace{1cm} (4.7)
where \( N_A \) is Avogadro's constant and \( e \) is the elementary electric charge. \( \Psi_\alpha \) is the surface electrostatic potential, which was calculated from the solution of Poisson-Boltzmann equation.

Theoretical analysis of the behavior of polyelectrolytes leads to the general potentiometric equation for polymeric acids as the equation below:

\[
pH = pK_o - \log \frac{1 - \alpha}{\alpha} + \frac{0.4343 \left( \frac{\delta F_e}{\delta \nu} \right)}{kT} \kappa
\]  

(4.8)

where \( pK_o \) is an “intrinsic” dissociation constant of the ionizable groups of polymer and independent of ionic strength, \( F_e \) is the electrostatic free energy of the ionized polyelectrolyte molecule carrying \( v \) negatively charged groups, and \( \kappa \) is the inverse Debye radius determined by the concentration of the small ions in solution.

A theoretical reasoning similar to that leading to Eq. (4.8) gives the following expression for the potentiometric behavior of polymeric bases:

\[
pH = pK_o' + \log \frac{1 - \beta}{\beta} + \frac{0.4343 \left( \frac{\delta F_e}{\delta \zeta} \right)}{kT} \kappa
\]  

(4.9)

where \( pK_o' \) is the dissociation constant of the cationic acid conjugate to the single basic group of the polymer, \( \beta \) is the degree of ionization of the polybase, and \( \zeta \) is the number of positive ionized groups carried by each polyelectrolyte molecule.

Thus, theoretically the electrochemical behaviors of polymeric acid or base are
Chapter 4 Analytical Methods and Principles

categorized by a single dissociation constant and also depend on the way in which the electrostatic field energy varies with the number of ionized groups and ionic strength. The factors \( \frac{\delta F}{\delta v} \) and \( \frac{\delta F}{\delta \zeta} \) for polyacids and polybases respectively can be deduced from electrophoresis or evaluated in theory. The intrinsic dissociation constants of some typical acidic and basic polyelectrolytes can be calculated by the method of combined potentiometric titration and electrophoresis [Katchalsky and Miller, 1954].

4.2 Light scattering

4.2.1 Brookhaven BI-200SM laser light scattering system

The Brookhaven BI-200SM Research Goniometer and light scattering system was used for conducting Dynamic Light Scattering (DLS) experiments at angle 90° and Static Light Scattering (SLS) with the change of scattering angles and sample concentrations. BI-9000AT Digital Correlator was equipped for photon counting, which consists of an integrated electronic card controlled by software for mathematical computation. The BI-9000AT produces a contiguous correlation function from 25 ns to 1,310 s in an adjustable delay range. The software programmes include simple particle size analysis by DLS, polymer molecular weight
measurements by SLS (Zimm, Berry and Debye plots) and a programme for alignment and stability monitoring with the BI-200SM. In brief, the light scattering system includes a laser source and rail, beam focusing and steering lens assembly, turntable worm gear and ball bearings, specimen cell assembly, a rigid rotating arm and detector. We used a 200 mW 488 nm Argon-ion laser source. The sample cavity is specially designed to minimize stray light by filling decahydronaphthalene (Decalin) as its refractive index matching with the sample liquid.

The alignment of laser light scattering system was corrected by measuring toluene at measurement angles of between 15 and 155 degree, which were then compared to the measurement at 90 degree. The system was considered to be operating correctly if the deviation of the intensity at each angle was less than 2%. Matching liquid (Decalin) was filtered by pumping the fluid through a filter (200 nm) for about 10 minutes to remove the small particles. In order to maintain consistent results, instrument and laser source need to be warmed up for 30 minutes before the experiment. The intensity of sample measurement was controlled to between 100 to 1000 kilocounts per second (kps) by adjusting the pinhole turret, laser power source or concentration of the sample.

During the light scattering measurement, dust acts as a large scatterer and significantly affects the quality of the correlation function. A large and sudden jump
in the intensity is usually a sign of the presence of dust in the sample. To minimize the presence of dust in sample, the sample should be prepared using a purified solvent, such as Millipore water that has been filtered with a 0.2 \( \mu \text{m} \) filtration membrane. If necessary, glass cell should be soaked in chromic acid to remove stain and subsequently rinsed thoroughly with tap and Millipore water.

4.2.2 Theoretical background of laser light scattering

In 1871, Rayleigh derived an expression of the scattered intensity as a function of angle in a dilute system, where the particles are much smaller than the light wavelength. Debye (1948) extended the light scattering to large particles, which include the effects of intramolecular interference between waves scattering by the different parts of same molecule. Zimm used the Single Contact Approximation (SCA) to derive a formula for the scattered intensity, which represented the results in terms of the reciprocal intensity as a function of the sum of polymer concentration and the square of scattering wave vector. The ‘zimm plot’ provides immediate information on the size and interactions of polymers in terms of the molecular weight \( M \), the radius of gyration \( R_g \), and the second virial coefficient \( A_2 \).

The interaction of light (electromagnetic radiation) with particle can be viewed through the classical mechanism of polarization. The charged particles (electrons and
protons) associated with the atoms and molecules are stretched to form dipoles under the influence of an electromagnetic field. When an electromagnetic wave (light ray) encounters a particle, the wave interacts with the discrete particle. The electron orbits within the constituent molecules of particles are perturbed periodically with the same frequency as the electric field of the incident wave. The oscillation or perturbation of the electron could result in a periodic separation of charge within the molecules, which is called an induced dipole moment. The dipoles were created by electric field and after that absorbed energy from the exciting field to become a source of electromagnetic radiation caused by scattered light. The majority of light scattered by the particles are emitted at an identical frequency as the incident light.

4.2.3 Static light scattering (SLS)

Static light scattering measures the angular dependency of time-averaged intensity of laser light scattered by the particles. The course of the scattered intensity as a function of the detector angle depends on size and structure of the particles. The intensities of light scattered over a period of time are accumulated for a number of sample concentrations. This time averaging removes the inherent fluctuations in the signal, hence the term ‘Static Light Scattering’.

The intensity of the scattered light depends on the molecular weight of the particle
and the size of particles. So that the light scattering is a valuable tool for measuring weight average molecular weight. The application can be extent to polymer solutions in which this dependence on size can be used to measure the radius of gyration of the polymer chain. Light scattering experiments can be used to measure three important physical properties, such as weight average molecular weight ($M_w$), radius of gyration ($R_g$), and the second virial coefficient ($A_2$).

The electromagnetic field is time dependent which can be described by:

$$E_z = E_0 \cos\left(\frac{2\pi ct}{\lambda}\right)$$  \hspace{1cm} (4.10)

where $E_0$ is the amplitude of the electric field, $c$ is the speed of light, and $\lambda$ is the wavelength of light. The subscript $z$ on $E$ represents that the plane polarized light with the light polarized along the $z$ axis. The intensity of scattered light for a small particle can be described by Rayleigh theory:

$$I_z = \alpha p^2 I_{0z} \frac{16\pi^4}{r^2\lambda^4} \sin^2 \theta z$$  \hspace{1cm} (4.11)

where $\alpha$ is the polarizability of the particle and $I_{0z}$ is the intensity of the $z$ polarized incident light. $r$ is the distance to the scattered light detector:

$$I_{0z} = E_0^2$$  \hspace{1cm} (4.12)

For scattering off $n$ moles of particles or $nL$ particles ($L$ is Avagadro’s number) and the dilute solution of volume is $V$, the scattered intensity at $\theta$ is:

$$I_{\theta} = \frac{I_{0} nL 8\pi^4 \alpha_p^2}{V r^2 \lambda^4} (1 + \cos^2 \theta)$$  \hspace{1cm} (4.13)
In ideal polymer solutions with small particles, light scattering only occurs in mediums that have an inhomogeneous index of refraction. Specifically, the polarizability of particles at concentration c is:

\[ \alpha_p = \frac{n_0 c V}{2 m L} \frac{dn_0}{dc} \]

(4.14)

where \( n_0 \) is the refraction index of the solvent and \( (dn_0/dc) \) is the increment of refraction index of the solutions. If \( c \) is \( n M/V \) (g/ml) and substituting into the scattered light intensity:

\[ \frac{i_0^\theta}{I_0} = \frac{2\pi^2 n_0^2}{r^2 \lambda^4 L} \left( \frac{dn_0}{dc} \right)^2 Mc(1 + \cos^2 \theta) \]

(4.15)

In a given scattering experiment, \( I_0 \) and \( r \) will be fixed and \( i_0^\theta \) will be measured. These figures can be combined into one quantity called the Rayleigh ratio:

\[ R_0^\theta = \frac{r^2 i_0^\theta}{I_0} \]

(4.16)

The Rayleigh ratio is independent of the incident light intensity \( (I_0) \) and the distance to the scattered light detector \( (r) \). From the scattering equation, the Rayleigh ratio was given as:

\[ R_0^\theta = KMc \]

(4.17)

where:

\[ K = \frac{2\pi^2 n_0}{\lambda^4 L} \left( \frac{dn_0}{dc} \right)^2 (1 + \cos^2 \theta) \]

(4.18)

The constant \( K \) depends on the solvent properties, \( \lambda \), and \( \theta \), which is a system constant and independent of the concentration of solution and the molecular weight of polymer.
In a diluted polymer solution, the total Rayleigh ratio can be written as a sum of the Rayleigh ratios for scattering of polymers of each possible molecular weight:

\[
\frac{Kc}{R^0_\theta} = \sum_i \frac{c_i}{M_i} = \frac{1}{M_w}
\]  

(4.19)

For the non-ideal solutions virial coefficients and concentration are considered. Thus by expanding gives:

\[
\frac{Kc}{R^0_\theta} = \frac{1}{M_w} + 2A_2c + 3A_3c^2 + \ldots
\]

(4.20)

In general, terms beyond the second virial coefficient can be ignored. The second virial coefficient (slope=2\(A_2\)) was given by the slope of plotting \(Kc / R^0_\theta\) as a function of \(c\) and the intercept will give the molecular weight (intercept = \(1/M_w\)). The effect of large particles was ignored in the extrapolation.

The light can be scattered from different parts of a particle if the particle size is bigger than the wavelength of light. The difference in path lengths can destruct interference resulting in reduced the intensity of scattered light. Considering the correct for large particle size, \(P(\theta)\) is defined as the ratio of the actual and the scattering from small particles:

\[
P(\theta) = \frac{i_\theta}{i_\theta^0} = \frac{R_\theta}{R^0_\theta}
\]

(4.21)

The theoretical result for \(P(\theta)\) is:
\[
\frac{1}{P(\theta)} = 1 + \frac{16\pi^2}{3\lambda^2}\langle s^2 \rangle \sin^2 \frac{\theta}{2} + \ldots
\]  
(4.22)

The higher order terms are normally assumed to be negligible. The scattering intensity as a function of scattering angle for a polydispersed polymer was written as:

\[
\frac{Kc}{R_g} = \frac{1}{M_w} \left( 1 + \frac{16\pi^2}{3\lambda^2} \langle s^2 \rangle \sin^2 \frac{\theta}{2} \right)
\]  
(4.23)

For both non-ideal solutions and large particle effects, non-ideal solution effects analysis are introduced into the large particle analysis:

\[
\frac{Kc}{R_g} = \left( \frac{1}{M_w} + 2A_2c \right) \left( 1 + \frac{16\pi^2}{3\lambda^2} \langle s^2 \rangle \sin^2 \frac{\theta}{2} \right)
\]  
(4.24)

A series of light scattering experiments consist of determining \(Kc/R_g\) at various concentrations and scattering angles. In the Zimm plot technique as shown in Figure 4.1 (the experimental data points are at the grid intersection points except along the \(\theta = 0\) and \(c = 0\) lines.), \(Kc/R_g\) was plotted against \(\sin^2(\theta/2) + kc\) where \(k\) is a constant, and \(k\) is chosen to spread out the data to give equal weights to each variable. By extrapolating the data to zero angles and concentrations, \(R_g\) and \(A_2\) can be obtained from the slopes respectively. A simultaneous extrapolation to zero angle and concentration yields an intercept, which is the inverse of the \(M_w\).
4.2.4 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) explores the relaxation of the fluctuations of polarization when the light hits a moving particle. A monochromatic laser light beam shines onto a solution with particles in Brownian motion resulting in a Doppler Shift, changing the wavelength of the incoming light. This change is related to the particle size. It is possible to compute the spherical size distribution that gives a description of the particle motion in the medium by measuring the diffusion coefficient of particle using the autocorrelation function.

The molecules are constantly moving around and their positions and moment change with time. The average intensities, $<I>$, over long observation time, $T$, can be
expressed by following equation:

\[
\langle I(0) \rangle = \frac{1}{T} \lim_{T \rightarrow 0} \int_0^T I(0,t)dt
\]  

(4.25)

At time, \( t \), and time difference, \( \tau \), the intensity-intensity autocorrelation function is given by the average \( \langle I(t)I(t+\tau) \rangle \), which is a function of \( \tau \). Under the ergodicity assumption of intensity, the autocorrelation function can be expressed by:

\[
\langle I(t)I(t + \tau) \rangle = \lim_{T \rightarrow 0} \frac{1}{T} \int_0^T I(t)I(t + \tau)dt
\]  

(4.26)

In order to study the dynamic properties of scattering system, correlation functions need to be obtained from the time dependent measurements. These functions can then be transformed using Fourier transformation techniques to yield appropriate scattering spectra that contain molecular information on the dynamics of molecules. The autocorrelation function of scattered intensity is described below:

\[
G_2(t) = \langle I(t)I(t + \tau) \rangle
\]  

(4.27)

The normalized form of Eq. (4.27) is shown below:

\[
g_2(t) = \frac{\langle I(t)I(t + \tau) \rangle}{\langle I(t)^2 \rangle}
\]  

(4.28)

where \( I(t) \) is an average value of the product of the scattered intensity at an arbitrary observation time, \( t \), and \( I(t+\tau) \) is the intensity at decay time, \( \tau \). The above expression can be simplified using Siegert relations:

\[
G_2(t) = B \left( 1 + \beta |g_1(t)|^2 \right)
\]  

(4.29)

\[
g_2(t) = 1 + \beta |g_1(t)|^2
\]  

(4.30)
where $B$ is the baseline and $\beta$ is the coherence factor that is normally considered as an adjustable parameter in the data analysis procedure.

The other two correlation functions, field autocorrelation function, $G_1(t)$ and normalized field autocorrelation function, $g_1(t)$, are shown below [Kim et al., 1990]:

\[
G_1(t) = \left( G_2(t) - B \right)^{1/2} \tag{4.31}
\]

\[
g_1(t) = \sqrt{\frac{G_2(t) - B}{B\beta}} \tag{4.32}
\]

The dynamics structure factor, $S(q,c,\tau)$, is defined as follows:

\[
S(q,c,\tau) = \frac{1}{N^2} \sum_{i,j=1}^{N} < \exp\left[ q \cdot \left( r_i(\tau) - r_j(0) \right) \right] > \tag{4.33}
\]

where $r_i(\tau)$ is the position of segment $i$ at time difference, $\tau$. The angular bracket denotes an average over the space-time distance distribution. $g_1(t)$ is related to the relaxation function by:

\[
g_1(t) = \frac{S(q,c,\tau)}{S(q,c,0)} \tag{4.34}
\]

For a system consisting of non-interacting, homogenous, mono-disperse or spherical particles, the normalized field autocorrelation function can be represented by the expression:

\[
g_1(t) = \exp(-\Gamma t) = \exp\left( -\frac{t}{\tau} \right) \tag{4.35}
\]

where $\tau$ is the time difference of the process and $\Gamma = 1/\tau$ is the decay rate.
For polydispersed systems, more than one-decay processes may exist. Thus for multiple decay processes, the following expression will be obtained:

\[ g_f(t) = \int w(\Gamma) \exp(-\Gamma t) d\Gamma \]  \hspace{1cm} (4.36)

where \( w(\Gamma) \) is a continuous distribution function of decay rate.

The decay rate is related to the translational diffusion coefficient, \( D \), by:

\[ \Gamma = Dq^2 \]  \hspace{1cm} (4.37)

The hydrodynamic radius can be determined from the diffusion coefficient through the Stokes-Einstein equation:

\[ D = \frac{kT}{6\pi\eta R_h} \]  \hspace{1cm} (4.38)

where \( k \) is the Boltzmann constant, \( T \) is the absolute temperature, \( \eta \) is the solvent viscosity and \( R_h \) is the polymer particle hydrodynamic radius.

### 4.3 Critical micelle concentration (CMC) measurement

#### 4.3.1 Tensiometer

Dataphysics DCAT 21 tensiometer was used to determine the CMC of polymer solution according to the Wilhelmy plate method. The tensiometer consists of electromagnetically compensated weighing system with an automatic calibrating
function, software-controlled motorized height positioning function for the sample vessel, integrated measuring and control electronics with digital thermometer and liquid temperature control units. The supplementary units include software SCAT and Wilhelmy plates. The measuring range is 1~1000 mN/m and measuring rate is 25 weighing data per second.

### 4.3.2 Wilhelmy plate method to determine CMC

An interface could form due to the interactions between the molecules of liquid and those of any liquid or gaseous substance that is not soluble in the liquid. Energy is needed to alter the formation of this interface or surface. Work is required to change the shape of a given surface known as surface tension.

Wilhelmy Plate method utilizes the interaction between an optimally wettable plate suspended from a precision balance and the surface. The sample holder is height-adjustable to move the liquid container. As the surface is brought to contact with the probe, the sensor will detect changes in the force. It will register the height at which this occurs as the ‘zero depth of immersion’. The plate will then be wetted to a set depth to ensure that there is indeed complete wetting of the plate. The balance record down the maximum tension acts and the sample does not need to be moved during the measurement.
The tension is calculated using the equation:

$$\sigma = \frac{F}{L \cos \theta}$$  \hspace{1cm} (4.39)

where \(\sigma\) is the surface tension, \(F\) is the force acting on the balance, \(L\) is the wetted length and \(\theta\) is the contact angle. The plate should have a high surface energy so that it is made of roughened platinum and wetted. During the measurements, the contact angle is virtually 0° and only the measured force and the length of plate need to be taken into consideration.

The critical micelle concentration (CMC) can be measured by the surface tension measurements as a function of concentration of amphiphilic polymer solutions. Typically, the surface tension is plotted against the logarithm of amphiphilic polymer concentration. With the addition of polymer molecules into solution, the polymer molecules gradually enrich themselves at the water surface. From the determination, the surface tension decreases linearly with the logarithm of the polymer concentration. When the surface is saturated with the polymer molecules, the surface tension no longer has any appreciable influence by adding the polymer into solution. The CMC is obtained from the intersection of concentration and surface tension.
5 Chapter Five
Aggregation Behaviors of Four-arm PEO-\textit{b}-PDEAEMA in Aqueous Solution

5.1 Introduction

Amphiphilic block polymers have attracted increasing attention due to their potential applications in controlled release and biotechnology [Qiu and Bae, 2006]. Polymeric micelles provide similar structures and functions as natural carriers. The nano-scale particles possess properties that facilitate tissue penetration, longer retention time in organism and enhance cellular interactions in specific sites needed for active targeting [Lavasanifar et al., 2002]. The self-assembled aggregate consists of a hydrophobic core and a hydrophilic shell to produce a vehicle suitable for delivering hydrophobic drugs [Glotzer, 2004]. With pH changes, the electrostatic force between ionizable polymeric segments causes a dramatic change in the hydrodynamic volume of polymeric chains [Lukyanov and Torchilin, 2004]. Biocompatibility, size and morphologies of the vehicles are important considerations in the design of suitable delivery systems, which they can be accomplished by manipulating block compositions and lengths [Kumar et al., 2001]. In terms of biocompatibility, poly(ethylene oxide) is one of the more popular synthetic polymeric material as it is
approved by the US Food and Drug Administration.

Water-soluble PEOs grafted with weak polybases that possess hydrophobic characteristic give rise to pH-responsive amphiphilic polyelectrolyte systems. Linear PEO has been widely studied such as in the preparation of biotinylated poly(ethylene oxide)-block-poly(2-diethylamino ethyl methacrylate) (B-PEO-b-PDEA) and PEO-b-PDEA resulting in the formation of spherical core-shell micelle at high pH. Acidic ABC tri-block copolymers consisting of PEO and PDEA were studied, where pH-responsive shell cross-linked micelles were produced. The star-block copolymers offer interesting properties concerning both micellization and microphase separation behavior compared to corresponding linear block copolymers. The greater proportion of end functional groups induces higher solubility in various solvents compared to linear structure polymer in identical molecular weights. Multi-arm PEOs possess three-dimensional branched structures and higher densities of terminal functional groups.

Four-arm PEO was grafted with DEAEMA to yield an amphiphilic and biocompatible polybase. The hydrophilic-lipophobic balance was selected such that spherical core-shell micelle is produced in aqueous solution at high pH. At low pH, the multi-arm block copolymer was fully extended to yield four positively charged segments at the end of PEO chains. The hydrodynamic size of the micelle produced from the
hydrophobic multi polymeric arms can be controlled by varying the pH. In addition, the ionic strength also controls the self-assembly behavior of amphiphilic polymeric systems, since it will mediate the interactions between polymeric chains and solvent molecules at the molecular level. The present study reports the effect of salt on the aggregation behavior of four arm polyelectrolytes dictated by the thermodynamic equilibrium between the various interaction forces that drive the polymer chains to self-assemble.

5.2 Dissociation behaviors of four-arm PEO-\textit{b}-PDEAEMA block copolymer

5.2.1 Potentiometric titration studies

The four-arm PEO-\textit{b}-PDEAEMA block copolymer contained four branches of hydrophilic PEO chains, with pH-responsive DEAEMA segments attached to each end of PEO chains. In acid environment (pH less than 4.5), DEAEMA segments were fully protonated thereby imparting a hydrophilic character to the polymeric chains. With the addition of 1 M NaOH, the DEAEMA segments were gradually deprotonated where the chains became less hydrophilic. This pH-responsive property of PDEAEMA allowed us to control and tune the self-assembly behavior of the block copolymer.
We conducted titration experiments by adding 1 M NaOH into 0.1 wt% of 4PEO_{56-b-PDEAEMA}_{74} block copolymer in 0.001, 0.01, 0.02, 0.05, 0.1 and 0.15 M NaCl solution. Figure 5.1 shows a typical pH and conductivity titration curve as a function of the moles of base for this polymer in 0.05 M NaCl solution. Three regions can be delineated, and two transition points (point A pH ~5.1 and B pH ~9.1) were observed. The polymer solution was prepared at pH of 2.6 by adjusting with HCl. At this pH condition, the solution contained H^+, Na^+, OH^-, Cl^-, and protonated DEAEMA macroions. With the addition of NaOH, the concentration of H^+ and macroion decreased, and the Na^+ ion increased (Region 1). Since the mobility of H^+ ($\lambda_{H^+}^0 = 350$ S cm^2/mol) is much larger than that of Na^+ ($\lambda_{Na^+}^0 = 50.5$ S cm^2/mol), the conductivity of polymer solution decreased to the first transition point A while the pH displayed a sharp increase. Region 2 corresponded to the deprotonation of NH^+(C_2H_5)_2 groups on the PDEAEMA segments with the continual addition of base, as represented by the plateau region on the pH curve. The increase in the concentration of Na^+ ion is responsible for the progressive increase in the conductivity. The second transition point B indicated the end of the deprotonation process, and beyond this point the pH and conductivity increased with the addition of base as described by Region 3.
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Aggregation Behaviors of Four-arm PEO-b-PDEAEMA

Figure 5.1  Potentiometric and conductometric titration curves of 0.1 wt% four-arm PEO-b-PDEAEMA in 0.05 M NaCl solution: (□)pH; (■) conductivity.

The dissociation equilibria of weak basic polyelectrolyte containing amine groups can be expressed by the following equilibrium equation:

\[ [\text{Amine}]^+ \rightleftharpoons [\text{Amine}] + \text{H}^+ \]  \hspace{1cm} (5.1)

To further discuss the dissociation of the polyelectrolyte and the corresponding micelle formation in the presence of salt and with changing pH, the potentiometric data were plotted against the fraction of charged amines ($\alpha$) at various salt concentrations. The magnitude of $\alpha$ was determined by the moles of titrated polyelectrolyte and the titrant added, where $\alpha$ ranged from 0 at un-ionized state of amine groups to 1 at full protonated state. Figure 5.2 showed the pH as a function of the fraction of charged amines ($\alpha$) which was calculated base on the moles of NaOH.
Chapter 5  Aggregation Behaviors of Four-arm PEO-b-PDEAEMA

doses in region 2 of Figure 5.1. At the same $\alpha$ value, the pH was larger for solution containing more sodium chloride. When salt was added into the polyelectrolyte solution, the electrolyte ions formed an ionic atmosphere within the vicinity of the macroions, which shielded the Coulombic interactions between the macroions thus weakened electrostatic interaction. The positively charged amine groups were surrounded by chloride ions, which enhanced the basic property of polybase resulting in an increase of pH.

![Graph showing pH curves of four-arm PEO-b-DEAEMA in various concentrations of NaCl](image)

Figure 5.2  Comparison of pH curves of four-arm PEO-b-DEAEMA in various concentrations of NaCl; (◆) 0.001 M; (■) 0.01 M; (▲) 0.02 M; (◇) 0.05 M; (□) 0.10 M; (Δ) 0.15 M.

The apparent dissociation constant ($K_a$) can be described by Henderson-Hasselbalch equation:
Chapter 5  Aggregation Behaviors of Four-arm PEO-\(b\)-PDEAEMA

\[ pK_{(\alpha)} = pH + \log\left(\frac{\alpha}{1-\alpha}\right) \] \hspace{1cm} (5.2)

and the \(pK_{(\alpha)}\) can be determined from the measured pH and degree of protonation \(\alpha\).

The curves in Figure 5.2 were transformed using Eq. (5.2) to yield Figure 5.3 where the dependence of \(pK_{(\alpha)}\) on \(\alpha\) for the titration of polyelectrolyte solutions in different concentration of sodium chloride was illustrated. \(\Delta G_{el}\) can be derived from graphical integration based on Eq. (5.3):

\[ \Delta G_{el} = 2.30RT \int_{0}^{1} [pK_{(\alpha)} - pK_0] d\alpha \] \hspace{1cm} (5.3)

where \(R\) is the gas constant, \(T\) is the absolute temperature, \(pK_0\) is the negative logarithm of the intrinsic dissociation constant that is independent of \(\alpha\) and was obtained by extrapolating the \(pK_{(\alpha)}\) curves to \(\alpha \approx 0\), \(pK_{(\alpha)}\) is the negative logarithm of dissociation constant at any given \(\alpha\) values and \(\Delta G_{el}\) is the electrostatic Gibbs energy.

The electrostatic Gibbs energy for deprotonating amine groups on PDEAEMA segments can be obtained by integrating the area under each of the \(pK_{(\alpha)}\) curves according to Eq. (5.3). The data of \(pK_{(1/2)}\) that is the apparent dissociation constant at \(\alpha = 0.5\) and \(\Delta G_{el}\) in various salt solutions were summarized in Table 3.1, where \(pK_{(1/2)}\) and \(\Delta G_{el}\) increased with increasing salt concentration. In the presence of large amount of electrolytes, the repulsion between charged amine groups was shielded, which increased the energy required to deprotonate the charged amine groups. The comparisons of \(pK_{(\alpha)}\) curves demonstrated a similar trend for the polymer in low and high salt concentrations respectively. The \(pK_{(\alpha)}\) increased progressively from \(\alpha \approx 0\) to
~0.5 at low \( c_s \) and from \( \alpha \sim 0 \) to ~0.6 at high \( c_s \). At low \( \alpha \), hydrophobic interactions between DEAMEA segments resulted in the formation of micellar aggregates. With increasing charge density on the amine groups, the DEAEMA segments were progressively protonated which induced electrostatic repulsions between charged DEAEMA segments. Hence, micellar aggregates began to swell, which further facilitated the protonation of the amine groups. The microstructure of this system will be discussed in greater details in the light scattering and microscopy sections. At \( \alpha \) greater than 0.5, the \( pK(\alpha) \) exhibited a slightly negative dependence on \( \alpha \) for low salt concentration ranging from 0.001 M~0.05 M. The \( pK(\alpha) \) became independent of \( \alpha \) as the salt concentration increased (e.g. at 0.1 M and 0.15 M) suggesting that further protonation enhanced the polymer charge density resulting in a larger electrostatic repulsive force that hindered the protonation of amine groups.

![Comparison of pK(\(\alpha\)) curves of four-arm PEO-\(b\)-DEAEMA in various concentrations of NaCl (\(\bullet\)) 0.001 M; (\(\blacksquare\)) 0.01 M; (\(\blacktriangle\)) 0.02 M; (\(\diamond\)) 0.05 M; (\(\square\)) 0.10 M; (\(\Delta\)) 0.15 M.](image-url)
Table 5.1 Summary of potentiometric characterization of four-arm PEO-b-PDEAEMA polymer in various salt concentrations.

<table>
<thead>
<tr>
<th>Salt Concentration (M)</th>
<th>p$K_{1/2}$</th>
<th>$\Delta G_{el}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>6.69</td>
<td>5.07</td>
</tr>
<tr>
<td>0.01</td>
<td>6.91</td>
<td>5.13</td>
</tr>
<tr>
<td>0.02</td>
<td>7.13</td>
<td>5.58</td>
</tr>
<tr>
<td>0.05</td>
<td>7.43</td>
<td>6.04</td>
</tr>
<tr>
<td>0.10</td>
<td>7.67</td>
<td>6.55</td>
</tr>
<tr>
<td>0.15</td>
<td>7.71</td>
<td>6.89</td>
</tr>
</tbody>
</table>

5.2.2 Critical micelle concentration

The CMC values of the four-arm PEO-b-PDEAEMA block copolymer were determined by surface tension measurements. Figure 5.4 shows the dependence of surface tension on the polymer concentration at NaCl concentration ranging from 0 to 0.15 M at pH of 10. The intersections of straight lines for the linear concentration-dependent section and the concentration-independent section give the CMC values. As shown in Figure 5.5 the surface tension was plotted against logarithm of polymer concentration.

Since the block copolymer exists as unimers at low pH values, the surface tension measurements were only conducted at high pH. The CMC values were determined to be 13.4, 7.6, 5.4 and 4.5 ppm (w/w) for the four-arm PEO-b-PDEAEMA block copolymer in 0.01 M, 0.05 M, 0.1 M, and 0.15 M NaCl solutions respectively. We
observed that the CMC decreases with salt concentration, which is mainly attributed to the reduction in the solubility of PEO chains in the presence of salt. The low CMC values of the four-arm PEO-\textit{b}-PDEAEMA block copolymer suggest that the micelles at high pH are stable and thus could be potential candidate for applications in enhanced drug delivery.

![Surface tension versus concentration of four-arm PEO-\textit{b}-PDEAEMA polymer at pH 10 in NaCl solutions: (Δ) 0.01 M; (▲) 0.05 M; (◇) 0.10 M; (◆) 0.15 M.](image.png)
Figure 5.5  Surface tension versus logarithm of four-arm PEO-\textit{b}-PDEAEMA polymer concentrations at pH 10 in NaCl solutions: (Δ) 0.01 M; (▲) 0.05 M; (◇) 0.10 M; (◆) 0.15 M.

5.2.3 Dynamic light scattering

The pH-responsive aggregation behavior of four-arm PEO-\textit{b}-PDEAEMA block copolymer was determined by dynamic and static light scattering. The DLS was conducted at a scattering angle of 90° for 0.1 wt% four-arm PEO-\textit{b}-PDEAEMA block copolymer in various NaCl solutions.

Figure 5.6 shows the relaxation time distribution function of 0.1 wt% polymer in 0.01 M NaCl at pH of 9 at different scattering angles. The peak relaxation time shifted towards lower value with increasing angle, confirming that only one type of particle
was present. Figure 5.7 shows the decay rates ($\Gamma$), the reciprocal of peak relaxation time is proportional to the square of scattering vector ($q^2$), which suggest that the distribution function is attributed to the translation diffusion of the scattering objects.

![Diagram showing the angular dependence of decay time distribution functions for 0.1 wt% polymer in 0.01 M NaCl at pH of 9.](image)

**Figure 5.6** The angular dependence of decay time distribution functions for 0.1 wt% polymer in 0.01 M NaCl at pH of 9.

![Diagram showing the relationship between decay rates and $q^2$ for 0.1 wt% polymer in 0.01 M NaCl at pH of 9.](image)

**Figure 5.7** Relationship between decay rates and $q^2$ for 0.1 wt% polymer in 0.01 M NaCl at pH of 9.
Figure 5.8 shows the relaxation time distribution functions (scattering angle of 90°) at different pHs or degree of protonation $\alpha$ for the four-arm PEO-b-PDEAEMA block copolymer in 0.01 M NaCl. At pH values of about 9~10 ($\alpha \approx 0$), the hydrodynamic radius of particles was about 21 nm since DEAEMA was sufficiently deprotonated to acquire a hydrophobic character resulting in the formation of a core-shell micelle surrounded by a hydrophilic 4-arm PEO chains. With the addition of HCl to decrease the pH, the DEAEMA segments became protonated, which induced the swelling of micelles due to enhanced electrostatic repulsion between ionized DEAEMA segments. In 0.01 M NaCl solution, $R_h$ of micelle increased from 21 nm to 56 nm when $\alpha$ was increased from 0 to 0.5. At $\alpha$ ranging from 0.5 to 0.6, the swollen micelles dissociated into unimers where $R_h$ decreased rapidly from 56 nm to 12 nm.

Figure 5.8  Relaxation time distribution functions at scattering angle of 90° for four-arm PEO-b-PDEAEMA in 0.01 M NaCl at different pHs.
The effect of salt on the conformation of four-arm PEO-b-PDEAEMA block copolymer was studied by dynamic light scattering measurements in the course of protonation. The addition of salts suppresses the electrostatic interactions between the charged groups of polymers, which also considerably alter the light scattering behavior of polymer solutions. The apparent hydrodynamic radii in various concentration of salt solution are shown in Figure 5.9. As shown in this figure, the four-arm PEO-b-PDEAEMA block polymer exhibits a swelling-dissociation behavior during the process of protonation in all salt conditions. However, the maximum apparent hydrodynamic radius ($R_h$) of micelle, the extent of swelling, the $\alpha$ value corresponding to the maximum $R_h$ are different according to the amount of NaCl present in the solution. With increasing NaCl concentration, the maximum value of $R_h$ decreases. For example, the peak of $R_h$ is 56 nm in 0.01 M, 48 nm in 0.05 M, 43 nm in 0.1 M and 37 nm in 0.15 M of NaCl solution. The critical onset value of $\alpha$ for the dissociation of micelle to unimers also decreases from 0.5 in 0.01 M to 0.41 in 0.15 M NaCl solution. The differences in the magnitude of $R_h$ and the $\alpha$ value corresponding to the maximum $R_h$ in different salt conditions may be correlated to the diverse conformations at various salt conditions. During the process of swelling, the stiffness of polymer chains caused by the strong electrostatic repulsion of charged amine groups permits the formation of inter-molecular hydrophobic junctions. Therefore, the structure of swollen particle is relatively loose and open, as indicated by the high $R_h$ value. The addition of salt suppresses the electrostatic repulsion of charged amine
groups and increases the flexibility of polymer backbones, which favors the formation of intra-molecular junctions. Hence the structure of swollen particles becomes more compact, yielding a lower $R_h$. The regime representing compact micelle, swollen micelle, micelle to unimer transition and the unimer is also depicted in Figure 5.9.

![Diagram of $R_h$ vs Degree of Dissociation ($\alpha$) for four-arm PEO-b-PEODEAEMA in various NaCl solutions: (■) 0.01 M; (▲) 0.05 M; (□) 0.10 M; (Δ) 0.15 M.](image)

Figure 5.9  $R_h^{\text{app}}$ as a function of $\alpha$ for four-arm PEO-b-PEODEAEMA in various NaCl solutions: (■) 0.01 M; (▲) 0.05 M; (□) 0.10 M; (Δ) 0.15 M.

5.2.4 Static light scattering

SLS was performed to determine the z-average radius of gyration ($R_g$), the weight-average molecular weight ($M_w$), and the second virial coefficient ($A^2$) of particles according to Eq. (5.6):
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Aggregation Behaviors of Four-arm PEO-\(b\)-PDEAEMA

\[
\left( \frac{KC}{R_\theta} \right)^{0.5} = \left( \frac{1}{M_w} \right)^{0.5} \left( 1 + \frac{1}{6} q^2 R_g^2 \right) \left( 1 + A_2 M_w C \right) \tag{5.4}
\]

where \( K \) is an optical constant \((K=4\pi^2 n^2 (dn/dC)^2/N_A \lambda^4)\) with \( n \) the refractive index of the solvent, \( dn/dC \) the refractive index increment of polymer solution, \( N_A \) Avogadro’s constant and \( \lambda \) the wavelength of laser light; \( R_\theta \) is the Rayleigh ratio at scattering angel \( \theta \); \( C \) is the concentration of the polymer solution. The radius of gyration \( R_g \), the second virial coefficient \( A_2 \), and the weight-average molecular weight \( M_w \) were determined by extrapolating the lines to zero concentration and zero angle in the Berry plot.

Several polymer concentrations ranging from 0.04 to 0.1 wt\% in 0.01 M NaCl solution at pH of 6.9 (\( \alpha \sim 0.5 \)) were measured by SLS at scattering angles ranging from 40°-100° at 10° intervals. The resulting Berry plot is shown in Figure 5.10. From the plot, the radius of gyration \( (R_g) \) was found to be 90 nm yielding a \( R_g/R_h \) of about 1.60. The weight-average molecular weight \( (M_w) \) of aggregate is about \( 1.67 \times 10^6 \) g/mol and the second virial coefficient \( (A_2) \) is \( 3.08 \times 10^{-5} \) cm\(^3\) mol/g. Since the \( M_w \) of the single polymer chain determined from GPC is \( 8.37 \times 10^4 \) Da, thus the aggregation number \( N_{agg} \) was calculated to be approximately 20. At pH of 10, \( R_g \) of micelles is about 22 nm and the \( R_g/R_h \) ratio was calculated to be 1.0, which corresponded to the core-shell spherical micellar structure since the aggregation number was low. According to data of the light scattering and evidences of TEM, the DEAEMA segments were
deprotonated and became hydrophobic which induced the formation of core-shell micelle comprising of a hydrophobic DEAEMA core and a hydrophilic four-arm PEO corona. When the pH was decreased to 3 with the addition of HCl, the polymer aggregates dissociates into unimers, yielding a $R_g$ of about 12 nm which corresponded to the molecular contour length of the polymer chain.

![Berry plot for four-arm PEO-b-PDEAEMA in 0.01M salt aqueous at pH of 6.9.](image)

Figure 5.10  Berry plot for four-arm PEO-b-PDEAEMA in 0.01M salt aqueous at pH of 6.9.

5.2.5 Transmission electron microscopy

TEM was conducted to elucidate the morphologies of four-arm PEO-b-PDEAEMA block polymer in 0.01 M NaCl solution at pH of 10 and 6.8. TEM specimens were prepared by dripped polymer solutions onto 200-mesh copper grids precoated with Formvar and stained by osmium tetroxide (OsO4) prior to freeze drying the sample. Figure 5.11 shows the aggregates consisting of compact hydrophobic DEAEMA core
at pH of 10 and a folded four-arm PEO chains as the corona which the diameter of aggregates was about 50 nm. Figure 8b shows the microstructure at the transition point of $\alpha$ about 0.5 for 0.01 wt% four-arm PEO-\textit{b}-PDEAEMA block polymer in 0.01 M salt solution. The micelle became larger with $R_g$ of 90 nm resulting from the contribution of protonated DEAEMA segments at low pH.

![TEM micrographs](image)

**Figure 5.11** TEM micrographs of aggregates form from four-arm PEO-\textit{b}-PDEAEMA block polymer in 0.01 M NaCl solution at pH of (a) 10, (b) 6.8.

The proposed microstucture of the four-arm PEO-\textit{b}-PDEAEMA block polymer at different pH values is shown schematically in Figure 5.12. At pH of 10, compact micelle was formed due to the hydrophobic association of the DEAEMA segments driven by entropic consideration. The entropy was gained when water molecules were released from the disrupted solvent cage surrounding the hydrophobic DEAEMA segments. With increasing $\alpha$, the electrostatic repulsion force between protonated DEAEMA segments and hydrophobic interaction resulted in the stretching of PDEAEMA chains producing a larger micelle. At pH of 3, the polymer existed as
four-arm PEO-\textit{b}-PDEAEMA unimers since the protonated DEAEMA and PEO chain segment possessed hydrophilic characteristics.

![Microstructures](image)

Figure 5.12  The proposed microstructures of the four-arm PEO-\textit{b}-PDEAEMA block polymer in 0.01 M NaCl solution, at pH of (a) 10, (b) 6.8, (c) 3.0.

5.3 Summary

The self-assembly behaviors of a pH-responsive four-arm PEO-\textit{b}-PDEAEMA block copolymer in aqueous solution were examined by potentionmetric titration, surface tensiometry, laser light scattering and transmission electron microscopy. At high pH, the four-arm PEO-\textit{b}-PDEAEMA block copolymer formed spherical core-shell micelle comprising of a hydrophobic DEAEMA core surrounded by a folded four-arm PEO corona. The apparent hydrodynamic radius ($R_h$) of micelle increased from 21 nm to 56 nm when the degree of protonation of the amine groups was increased from 0 to 0.5 in 0.01 M NaCl solution. In higher concentration of NaCl, the micelle shrank due to the electrostatic charge screening of the protonated DEAEMA groups. At low pH, the micelles dissociated into unimers.
6 Chapter Six
Association Behaviors of Four-arm PEO-b-PMAA in Aqueous Solution

6.1 Introduction

Stimuli responsive polymers have attracted increasing attention in biomedical and
drug delivery systems due to their attractive and tunable properties [Alarcon et al.,
2005; Schmaljohann, 2006; Hoffman and Stayton, 2007; Kumar et al., 2007]. Various
chemical or physical stimuli, such as pH, ionic strength, temperature, light, electric
field and magnetic field, can affect the interactions of self-assembly and polymer
chain conformation [Checot et al., 2007; Iatrou et al., 2007]. Under the actions of
external stimuli, the conformation, solubility, degradation or association of the
polymeric chains will change accordingly [Bellomo et al., 2004; Boyer et al., 2007;
Kostina et al., 2007]. The variety of pH in different tissues and cellular compartments
suggests that pH is an important parameter that should be considered in the design of
polymeric system for biomedical applications [Wagner, 2004; Rapoport, 2007].
Poly(methacrylic acid) possesses pH-responsive properties, where the conformation
and solubility of chain segments in aqueous media can be manipulated by adjusting
pH [Bromberg and Ron, 1998; Satturwar et al., 2007]. It adopted extended a random
coil conformation at high pH with a larger hydrodynamic volume induced by
Coulombic repulsive forces between ionized carboxylate groups on polymer chains. In contrast, the hydrophobic interactions between methyl groups result in a lower hydrodynamic volume due to the suppressed hypercoiled morphology at low pH. To stabilize the polymer complexes, biocompatible and hydrophilic poly(ethylene oxide) (PEO) was grafted to the PMAA chain segments making it suitable for drug delivery applications [Soo and Eisenberg, 2004; Nakashima and Bahadur, 2006; Neugebauer, 2007; Schweizer and Taubert, 2007]. In addition, hydrogen bond interactions provide an additional control in defining the conformation of polymer complexes to manipulate the various interactions that control the behaviors of PMAA chains in solution [Kharlampieva and Sukhishvili, 2006; Choi et al., 2007].

Various types of linear pH-responsive poly(ethylene oxide) (PEO)-based copolymers have been reported, such as PEO-\(b\)-PPO [Bromberg et al., 2006; Chiappetta and Sosnik, 2007], PEO-\(b\)-PDMAEMA [Alvarez-Lorenzo et al., 2005], PEO-\(b\)-PEI [Malmsten and Muller, 1999; Nguyen et al., 2000], PEO-\(b\)-PLA [Sanabria-DeLong et al., 2007], and PEO-\(b\)-PCL [Cai et al., 2007]. However, the polymer architecture that defines the shape of a single polymer chain could play an important role since it defines the physicochemical properties of polymer in solution [Heath et al., 2007]. The star shape multi-arm PEOs possess three-dimensional branched structures that may offer interesting properties in self-assembly and association of the polymers in the presence of small molecules [Tezuka and Oike, 2002; Taton et al., 2006]. The higher densities of terminal functional groups on the block copolymer chains induce
stronger interactions compared to linear structure of identical molecular weights. The star polymer undergoes conformation transition with the change of ionization degrees and ionic strengths of the media, both of which have not been extensively studied.

In the previous chapter, the four-arm poly(ethylene oxide)-b-poly(methacrylic acid) (4-arm PEO-b-PMAA) block copolymer was synthesized by atom transfer radical polymerization (ATRP). The dissolution and association mechanism of star polymers in different salt environments were examined from a molecular-level. The dissociation constant and the Gibbs energy ($\Delta G$) were derived from the potentiometric and conductometric titration analysis, and the impact of the ionic environment on the chain conformation and self-assembly were discussed. The critical micelle concentration of the copolymer was investigated to determine a suitable polymer concentration range for potential biological applications. The particle size of polymer cluster during the course of neutralization, the hydrodynamic radius ($R_h$) and the radius of gyration ($R_g$) was determined by light scattering. The interactions between the polymer and alkali were elucidated through thermodynamic quantification using the isothermal titration calorimetric technique. The multi-arm block copolymer was fully extended to yield a three-dimension hydrophilic PEO with negatively charged carboxylate groups at high pH. By adjusting the pH, the micelles experienced changes in their size resulting from the aggregation to produce larger nanostructures caused by hydrophobic interaction of methyl groups and the hydrogen bonding between the methacrylic acid and ethylene oxide segments. The addition of a neutral salt
significantly alters the electrostatic interactions between the macroions, counterions, and solvent molecules resulting in variation of polymer aggregation behaviors.

6.2 Experimental sections

6.2.1 Isothermal titration calorimetry

Microcal ITC system (Northampton, MA) was used to measure the enthalpy changes during the neutralization process of the star block copolymers. The microcalorimeter consists of a reference and a sample cell of 1.35 ml, where both cells are insulated by an adiabatic shield. The sample cell was filled with 0.1wt% of polymer solution, and predetermined volume of titrant (30 mM NaOH solution) was titrated from a 250 μL injection syringe kept at 25.0 ± 0.02 °C. A constant stirring speed (400 rpm) was chosen to ensure an optimum mixing efficiency. The calorimetric data of each injection were recorded by an interactive software automatically and analyzed using the Microcal ORIGIN software. The time interval between each injection was set at 4 mins for all experiments. The blank titration was carried out by adding the titrant to the saline solution, and subsequently subtracted from the base or polymer titration data.
6.3 Results and discussion

6.3.1 Potentiometric and conductometric titrations

The pH-responsive four-arm PEO-b-PMAA block copolymer was studied by potentiometric and conductometric titrations, which provided a fundamental characterization on the conformation and distribution of ions in solution. By adjusting the pH environment, the PMAA chains undergo hyper-coiling, resulting in a change in the conformation of the star polymer block copolymer. The self-assembled nanostructures are stabilized by hydrophilic PEO chains, which impart stealth and biocompatible properties to the system, making them suitable for drug delivery applications.

The dissociation equilibria of weak acidic polyelectrolyte can be expressed by the following equilibrium equation:

\[
HA \leftrightarrow K_a H^+ + A^- \quad (6.1)
\]

where HA, H\(^+\), and A\(^-\) are polyacid, the free hydrogen ion, and corresponding polyanion, respectively. \(K_a\) is the apparent acid dissociation constant and \(pK_a\) is the \(pK_a\) of acidic polyelectrolyte. The equilibria can be quantified by defining the negative logarithm of the apparent dissociation constant, \((pK_a)\), which can be determined from the measured pH and the degree of neutralization \(\alpha\) via the Henderson-Hasselbalch equation:
Chapter 6 Association Behaviors of Four-arm PEO-\(b\)-PMAA

\[ pK_a = pH + \log \frac{1-\alpha}{\alpha} \]  \hspace{1cm} (6.2)

The degree of neutralization, \(\alpha\), of the carboxyl group is defined by:

\[ \alpha = \frac{[BASE] + [H^+] - [OH^-]}{C_{COOH}} \]  \hspace{1cm} (6.3)

Where [BASE], [H\(^+\)], and [OH\(^-\)] are the molarities of added base, free hydrogen ion, and hydroxide ion, respectively, and \(C_{COOH}\) is the total concentration of methacrylic acid groups expressed in moles per liter. The hydrogen and hydroxide ion concentration terms were calculated from the pH, where the activity coefficient is assumed to be unity. The magnitude of \(\alpha\) was determined by the moles of polyelectrolyte and the titrant added, where \(\alpha\) ranged from 0 at un-ionized state of MAA groups to 1 at complete neutralization of MAA groups.

The potentiometric and conductometric titrations were conducted using the ABU93 Triburette Titration System equipped with a Radiometer pHG201 pH glass and REF201 reference electrodes and a CDM83 conductivity electrode system. The water jacketed titration vessel was maintained at a constant temperature of 25 °C using a circulating water bath. In the titration vessel, 50 ml of 0.1 wt % four-arm PEO-\(b\)-PMAA copolymer in various aqueous salt solutions were continuously stirred. The titrant used was 1 M standard NaOH solution (Merck), and sufficient lag time between two dosages was allowed to ensure that the reaction had reached equilibrium.

When NaOH was titrated to the four-arm PEO-\(b\)-PMAA block copolymer solution,
Chapter 6  
Association Behaviors of Four-arm PEO-b-PMAA

both the conductivity and pH were measured simultaneously as shown in Figure 6.1. These two curves revealed the changes in the concentrations of conducting ions, which are $H^+$, $Na^+$, $OH^-$, $Cl^-$ and macroion ($\rho$). The conductivity can be expressed as follows:

$$\Lambda = C_{Na^+}\lambda_{Na^+} + C_{H^+}\lambda_{H^+} + C_{OH^-}\lambda_{OH^-} + C_{Cl^-}\lambda_{Cl^-} + C_{\rho}\lambda_{\rho}$$  \hspace{1cm} (6.4)

where $C_i$ is the concentration of free ion in solution, and $\lambda_i$ is the molar conductivity of the corresponding ion. Since the concentration of $Cl^-$ ion remained constant throughout the titration, the conductivity curve reflects the concentration changes of $H^+$, $Na^+$, $OH^-$ and macro-ions. The figure was delineated into three regions with two transition points (point A $pH\sim4.3$ and B $pH\sim9.7$). In region 1, addition of NaOH to the polymer solution caused the reduction of $H^+$ and increase of $Na^+$ concentrations. Since the mobility of $H^+$ ($\lambda^{0}_{H^+} = 350$ S cm$^2$/mol) is much larger than $Na^+$ ($\lambda^{0}_{Na^+} = 50.5$ S cm$^2$/mol), the conductivity of polymer solution decreased to the first transition point A, where the pH displayed a sharp increase. Since the concentration changes of $H^+$ and $OH^-$ are very small, the conductivity curve increased marginally in region 2 due to the addition of $Na^+$. The increase of pH corresponded to the neutralization of carboxylic groups on the polymeric chains as more NaOH was added. When the titration reached the second transition point B, the four-arm PEO-b-PMAA became fully neutralized which was reflected by the sharp increase pH and conductivity curves. The increase in the conductivity and pH in region 3 was caused by the addition of $Na^+$ and $OH^-$ from the excess NaOH.
The addition of a neutral salt significantly alters the electrostatic interaction between the macroions, counterions, and solvent molecules [Choi et al., 2007]. To achieve a molecular-level understanding on the association mechanism of four-arm PEO-b-PMAA, a series of titration experiments were conducted by adding 1 M NaOH to 0.085 wt% of 4PEO56-b-PDEAEMA74 block copolymer in 0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.15 M and 0.2 M NaCl solutions. Figure 6.2 showed comparison of pH as a function of the degree of neutralization ($\alpha$), which was calculated based on moles of NaOH titrated to the polyelectrolyte solution. At a fixed $\alpha$, the pH decreased with increasing sodium chloride concentrations. The positively charged sodium ions form an ionic atmosphere in the vicinity of negatively charged carboxylate groups that screened the electrostatic interaction between macroions. The polyelectrolyte dissociation was favorable and the acidic property of polyacid is enhanced as indicated by the reduced pH value. Identical pH curves observed for 0.15 M and 0.2
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M NaCl solution suggested that complete shielding of the polyions by small electrolyte ions had been reached.

![Comparison of pH curves of four-arm PEO-b-PMAA in various concentrations of NaCl](image)

Figure 6.2  Comparison of pH curves of four-arm PEO-b-PMAA in various concentrations of NaCl (◆) 0.01 M; (■) 0.02 M; (▲) 0.05 M; (◇) 0.1 M; (□) 0.15 M; (Δ) 0.2 M.

The pK$_a$ was plotted against the degree of neutralization ($\alpha$) for the titration of the four-arm PEO-b-PMAA in different NaCl solutions as shown in Figure 6.3. The pK$_{\alpha}$ decreased at the initial titration stage and reached a minimum at $\alpha$ of ~0.05 for low concentration of salt and $\alpha$ of ~0.1 for high concentration of salt. Thereafter, they increased to $\alpha$ ~ 0.2 and exhibited a plateau between $\alpha$~0.2 to $\alpha$ ~ 0.4, subsequently it increased progressively for $\alpha$ greater than 0.4. At $\alpha$~0, the MAA groups are un-ionized and formed a core surrounded by hydrophilic PEO chains. The MAA groups on polymer chain and the hydrophobic interactions between un-ionized MAA groups produce a compact chain configuration at low ionization degree. With small amount of base addition, the pK$_a$ decreased slightly corresponding to the charge density of
compact structure of the aggregates. As more COOH groups on polymer chain are deprotonated, the electrostatic repulsion between charged groups increased, making the hypercoiled methacrylic acid segments more accessible. At the plateau region ($\alpha$ between 0.2 and 0.4), the charge density was relatively stable resulting from the rearrangement of COO$^-$ groups on the polymer chain, which maintained a constant acidic environment. Further neutralization with the base ($\alpha > 0.4$) enhanced the charge density of polymer chains, and the chain conformation became more extended due to the electrostatic repulsion between charged groups. The hydrophobic forces between the polymer segments could not balance the electrostatic interaction to maintain the structure which destroyed the compact structure.

As depicted by the $pK_a$ curves of the four-arm PEO-$b$-PMAA in various salt solutions, the $pK_a$ was lower at higher salt concentration, and the transition region shifted to higher ionization degree. In the presence of small electrolytes, the electrostatic repulsion was screened by positively charged sodium ions, resulting in a more flexible polymer chain. The degree of swelling became lower with increasing ionization resulting in a more compact structure that caused the transition region to be shifted to a higher ionization degree. The attraction between COO$^-$ and H$^+$ was also screened by salt ions which caused the acidity to increase, resulting in a lower $pK_a$ value in high salt solutions.
The electrostatic Gibbs energy needed to extract a proton from a charged polyion can be obtained by integrating the area under the $pK(\alpha)$ versus $\alpha$ curves. $\Delta G_{el}$ can be derived from graphical integration based on the following equation:

$$\Delta G_{el} = 2.30RT\int_{0}^{1} [pK_{(\alpha)} - pK_{0}] d\alpha \tag{6.5}$$

where R is the gas constant; T is the absolute temperature; $pK_{0}$ is the negative logarithm of the intrinsic dissociation constant that is independent of $\alpha$ and was obtained by linearly extrapolating the $pK_{(\alpha)}$ curve to $\alpha \sim 0$; $pK_{(\alpha)}$ is the negative logarithm of dissociation constant at any given $\alpha$ values and $\Delta G_{el}$ is the electrostatic Gibbs energy. The data of $pK_{(1/2)}$, the apparent dissociation constant at $\alpha \sim 0.5$ and $\Delta G_{el}$ in various salt solutions are summarized in Table 6.1. $pK_{(1/2)}$ and $\Delta G_{el}$ decreased with increasing salt concentrations and $\Delta G_{el}$ approached a constant value at NaCl concentration exceeding 0.2 M. As discussed earlier, the addition of salt favors the
dissociation of carboxylic groups which enhances the dissociation constant $K_a$, resulting in a lower $pK_{(1/2)}$. Since the Coulombic attraction between $H^+$ and $RCOO^-$ is screened by the addition of salt, the energy required to extract protons from the polyacid decreased. With further addition of NaCl, the polymer particles were destabilized and they flocculated due to charged shielding effects.

<table>
<thead>
<tr>
<th>Salt Concentration (M)</th>
<th>$pK_{(1/2)}$</th>
<th>$\Delta G_d$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>6.7</td>
<td>0.37</td>
</tr>
<tr>
<td>0.02</td>
<td>6.5</td>
<td>0.32</td>
</tr>
<tr>
<td>0.05</td>
<td>6.2</td>
<td>0.25</td>
</tr>
<tr>
<td>0.1</td>
<td>6.0</td>
<td>0.20</td>
</tr>
<tr>
<td>0.15</td>
<td>5.8</td>
<td>0.18</td>
</tr>
<tr>
<td>0.2</td>
<td>5.7</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 6.1  Summary of potentiometric characterization of four-arm PEO-$b$-PMAA polymer in various salt concentrations.

6.3.2 Critical micelle concentration

The CMC is an important parameter that describes the self-assembling behavior of block copolymers in solution. Surface tension method was used to determine the CMC values of the four-arm PEO-$b$-PMAA block copolymer, where the surface tension variations with polymer concentrations were monitored. Figure 6.4 shows the CMC of polymer in various NaCl concentrations ranging from 0.01 to 0.15 M at pH of 4. The CMC value was determined to be 65 ppm (w/w) for the four-arm PEO-$b$-PMAA block copolymer in 0.001 M NaCl aqueous solutions. It then decreased to 53, 34 and 28 ppm in 0.002 M, 0.004 M, and 0.01M NaCl respectively. Further increase the salt concentration to 0.05 M did not result in any significant change where the
Chapter 6 Association Behaviors of Four-arm PEO-b-PMAA

CMC remained constant. It is evident that the CMC decreased with increasing salt concentration, which was mainly attributed to the reduction in the solubility of PEO chains in salt solutions. The extremely low CMC values of the four-arm PEO-b-PMAA block copolymer suggested that the micelles were stable and they could be potential candidate for application in enhanced drug delivery. In addition, delivery systems with smaller aggregates may prolong the circulation time in the blood and facilitate their bioavailability [Kuskov et al., 2007].

![Surface tension versus concentration of four-arm PEO-b-PMAA polymer at pH 4 in various salt solutions.](image)

**Figure 6.4** Surface tension versus concentration of four-arm PEO-b-PMAA polymer at pH 4 in (◆) 0.001 M; (■) 0.002 M; (Δ) 0.004 M; (◇) 0.01 M; (□) 0.05 M NaCl solutions.

### 6.3.3 Light scattering

On the basis of previous discussion, the stimuli-responsive behaviors of well-defined water-soluble four-arm PEO-b-PMAA block copolymer in various salt solutions were
demonstrated. Furthermore, the size of aggregates is important for a complete understanding of pH-dependent association behavior with stepwise changes in pH. Dynamic light scattering (DLS) was used to investigate the hydrodynamic radius of 0.1 wt% four-arm PEO-b-PMAA block copolymer in 0.1 M NaCl solution.

Figure 6.5 shows the decay time distributions of four-arm PEO-b-PMAA at different pHs. At high pH environment (α=1), the carboxylic groups on the polymer chains were complete neutralized resulting in a highly charged MAA segments. The repulsive electrostatic interaction between polymer chains yielded unimers in aqueous solution with a hydrodynamic radius of about 12 nm. By adding HCl, the carboxylate groups were transformed to carboxylic acids, and the hydrophobic interaction between the carboxylic acid groups produced micelles at degree of neutralization (α) exceeding 0.3. The apparent hydrodynamic radius ($R_h$) of the aggregates was determined to be about 84 nm. With further addition of HCl, more carboxylate groups were protonated resulting in a reduction of negative charges on the polymer chains. The repulsive electrostatic interactions between ionized carboxylate groups were reduced causing the aggregates to shrink. The relaxation time shifted to a lower value with an increased amplitude, where the $R_h$ was found to be 63 nm and 46 nm at $\alpha$~0.2 and 0.1 respectively. When $\alpha$ reached approximately 0.05, the relaxation time distribution increased and broadened significantly. The hydrogen-bonding between MAA groups induced the formation of larger aggregates with $R_h$ of about 120 nm. The polymer solution was transparent, however it turned opaque and the scattering
intensity increased sharply by 3 orders of magnitude at $\alpha \sim 1$. Even larger particle of 410 nm was detected with the corresponding disappearance of the smaller micelle. The fast and slow modes correspond to the small and large micelles respectively. The graph of degree of neutralization versus $R_h$ and pH was shown in Figure 6.6. The interesting morphological transformation was observed using the TEM, and the trend corresponded well with the trend of the negative logarithm of apparent dissociation constant ($pK_a$) during the course of potentiometric titration.

![Figure 6.5](image)

Figure 6.5  Relaxation time distribution functions at scattering angle of 90° for four-arm PEO-$b$-PMAA in 0.1 M NaCl at different pHs.
Figure 6.6 The graph of degree of neutralization versus R_h (◆) and pH (■).

SLS was used to measure time-average scattered intensities and analyzed microscopic properties of the aggregates, such as the z-average radius of gyration ($R_g$) according to Debye equation:

$$
\frac{KC}{R(q)} = \frac{1}{M_w} \left( 1 + \frac{1}{3} q^2 R_g^2 \right) + 2A_2C
$$

(6.6)

where $K$ an optical constant ($K = 4\pi^2 n^2 (dn/dC)^2 / N_A \lambda^4$) with $n$ the refractive index of the solvent, $dn/dC$ the refractive index increment of polyplexes solution, $N_A$ Avogadro’s constant, $\lambda$ the wavelength of laser light, $R(q)$ the Rayleigh ratio, $q$ the scattering vector and $C$ the concentration of the polymer solution. The absolute excess time-averaged scattered intensity, Rayleigh ratio $R(q)$, is based on the equation:

$$
R(q) = R_{tol,90} \left( \frac{n}{n_{tol}} \right)^2 \frac{I - I_0}{I_{tol} \sin \theta}
$$

(6.7)

where $R_{tol,90}$ is the Rayleigh ratio of toluene at scattering angle 90° with a value of $40 \times 10^{-6}$ cm$^{-1}$, $n$ is the refractive index of the solvent, $I, I_0$, and $I_{tol}$ are the scattered intensities of the solution, solvent, and toluene, respectively, and $\theta$ is the scattering
angle. According the Debye plot, the weighted average molecular weights ($M_w^{app}$) of the four-arm PEO-$b$-PMAA block copolymer was determined to be $7.94 \pm 0.52 \times 10^5$ g/mol in 0.1 M NaCl aqueous solution at $\alpha \sim 0.2$. Since the molar mass of the single star polymer determined from GPC is $4.96 \times 10^4$ g/mol, the aggregation number $N_{agg}$ was calculated to be approximately 16, consisting of 64 MAA chains. The radius of gyration ($R_g$) of the polymeric aggregates in solution was measured to be 68 nm at scattering angles ranging from 50° to 110°. The parameter $\rho (R_g/R_h)$ was calculated to examine the morphology of the aggregates, and found to be about 1.1 suggesting that the particles possessed a spherical structure with a hydrophobic core and hydrophilic corona shell.

6.3.4 Transmission electron microscopic (TEM) studies

A series of TEM images were evaluated to investigate the entanglement effects of MAA segments at low ionization degree. The four-arm PEO-$b$-PMAA block polymer was dissolved in 0.1 M NaCl solution at various degrees of neutralization with 0.1 wt % concentration. Figure 6.7a shows the spherical micelles consisting of compact MAA core and four-arm PEO corona chains at $\alpha \sim 0.1$. The diameter of the aggregates was in good agreement with light scattering data of approximately 46 nm. At $\alpha \sim 0.05$, the hydrogen bond interactions between MAA segments induced the micelles to form larger aggregates. As shown by Figure 6.7b, two different sizes of aggregate were observed, which were in agreement with the results obtained from DLS. With further
reduction in $\alpha$ to 0.02, the large micelles associate to form larger aggregate induced by the association of hydrogen bonding between the palm shape PEO polymer chains which is the outer shell of the particles (Figure 6.7c). Figure 6.7d reveals the presence of large compound aggregates that corresponded to the slow mode as detected by dynamic light scattering.

![Figure 6.7 TEM micrographs of aggregates form from four-arm PEO-$b$-PMAA block polymer at $\alpha$ of (a) 0.1, (b) 0.05, (c) 0.02, and (d) 0.](image)

The association mechanism of four-arm PEO-$b$-PMAA block polymer during the ionization process in aqueous solution is depicted in Figure 6.8. At high ionization
Chapter 6 Association Behaviors of Four-arm PEO-\textit{b}-PMAA

degree, the star polymer exists as negatively charged unimers. When the carboxylic acid groups are partially neutralized at $\alpha \sim 0.3$, the simple core-shell shape micelles are formed driven by hydrophobic MAA segments. The un-ionized PMAA chains are apolar and they tend to form a compact coiled structure to minimize their contact with water molecules due to the hydrophobic effect. When $\alpha$ reaches 0.1, reduced ionization produces weaken electrostatic repulsion, which causes the micelles to shrink into a smaller size while maintaining the core-shell structure. However the micelles tend to aggregate to form larger particle induced by hydrogen bonding between MAA groups as shown in Figure 6.8d at very low degree of neutralization.

![Figure 6.8](image)

Figure 6.8 Proposed microstructure of the four-arm PEO-\textit{b}-PMAA block polymer in 0.1 M NaCl solution at $\alpha$ of (a) 1, (b) 0.3, (c) 0.1, and (d) 0.02.

### 6.3.5 Isothermal titration calorimetric study

The thermodynamics of the dissociation of four-arm PEO-\textit{b}-PMAA block polymer were investigated using ITC by titrating 30 mM NaOH solution into 0.05 wt% of the block polymer in various salt solutions. A thermogram (Figure 6.9) shows the CFB
(cell feedback) heat signal for the step-by-step injections of NaOH to the polymer in 0.15 M NaCl solution. The differential enthalpy curves for polymer solutions at different NaCl concentrations were obtained by integrating the area under the raw signal curve at each injection, and the results are shown in Figure 6.10. The conformational transition of the polymer chains in salt solutions can be deduced from the differential ITC data. At the early stage of neutralization, the most of carboxylic groups are unionized and they are buried inside the large aggregates resulting in low enthalpy. With the addition of NaOH, the OH\(^-\) ions begin to penetrate into the core of micelles and neutralize the carboxylic acid groups. The large aggregates expand to form even larger aggregates, where the enthalpy increases drastically and reaches a maximum at \(\alpha\) of about 0.05. Continuous addition of NaOH results in a drop in the enthalpy at \(\alpha\) of between 0.05 and 0.1, which may indicate that the large aggregates dissociate into micelles. At \(\alpha\) of between 0.1 to 0.3, the enthalpy curves appear to plateau, and this corresponds to the progressive neutralization of the swollen micelles. The enthalpy then decreases from \(\alpha\) of approximately 0.3 in low salt environment (open and filled squares) and \(\alpha\) of approximately 0.9 in high salt environment (open and filled triangles). Since the pronounced exothermic heat was observed, titrations in other salt solutions revealed similar enthalpy curves. However, the shape of the ITC titration curve was affected considerably by the addition of salt. The flat region in the enthalpy curve was observed over a narrower \(\alpha\) range of 0.1 to 0.3 for polymer in low salt solution than that for polymer in high salt solution, where the flat range was from 0.1 to 0.9. The progressively less sensitive titration curves indicated that the
interaction between polymer and sodium hydroxide was more cooperative than in the absence of salt, thus the addition of salt favored the neutralization process of the aggregates.

![Thermogram of CFB for titration of 30 mM NaOH solution into 0.05 wt% of four-arm PEO-b-PMAA block polymer in 0.15 M NaCl solution.](image)

Figure 6.9 Thermogram of CFB for titration of 30 mM NaOH solution into 0.05 wt% of four-arm PEO-b-PMAA block polymer in 0.15 M NaCl solution.

![Differential enthalpy curves for titrating 30 mM NaOH solution into 0.05 wt % four-arm PEO-b-PMAA block polymer in (□) 0.001 M; (■) 0.005 M; (Δ) 0.02 M; (▲) 0.15 M NaCl solution.](image)

Figure 6.10 Differential enthalpy curves for titrating 30 mM NaOH solution into 0.05 wt % four-arm PEO-b-PMAA block polymer in (□) 0.001 M; (■) 0.005 M; (Δ) 0.02 M; (▲) 0.15 M NaCl solution.
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6.4 Conclusions

The novel four-arm PEO-\(b\)-PMAA synthesized by ATRP formed well-defined micelles or large spherical aggregates in aqueous solution depending on the pH. The conformational transitions during the process of neutralization were revealed by the negative logarithm dissociation constant (p\(K_a\)) curves of potentiometric titration, static and dynamic light scattering studies and TEM. The average Gibbs energy to extract a proton from a charged polyion decreased with the increase of salt content in the polymer solution which suggested that the addition of electrolyte favored the neutralization process. The size of spherical core-shell micelles consisting of partially ionized PMAA core surrounded by a hydrophilic PEO corona could be reversibly manipulated by changing the pH. Isothermal titration calorimetric results showed that the neutralization of polyacids with a strong base is an exothermic process dominated by enthalpy. The extremely low CMC made the polymer systems suitable for biological applications.
Chapter Seven
Interactions between Four-arm PEO-b-PDEAEMA and Plasmid DNA

7.1 Introduction

Significant attention has been focused on gene therapy for the treatment of human diseases arising from defective genes in the field of medicine, pharmaceutical sciences and biotechnology [Pack et al., 2005]. An efficient delivery system is required to transfer genetic materials encoded within plasmid DNA or RNA to targeted tissues and organs [Seeman, 2005; Bazan-Peregrino et al., 2007]. Non-viral vectors have several advantages than viral vectors because of their lower immunogenicity, absence of endogenous virus recombination and their ability to package DNAs of wide size ranges [Crystal, 1995; Pfeifer and Verma, 2001; Haider et al., 2005; Trentin et al., 2005]. Polymeric delivery systems can be designed to optimize their physicochemical and biological properties by molecular diversity, chain density and chemical structures [Dang and Leong, 2006]. Currently, cationic polymers spontaneously condense polyanionic DNA, which is a requirement for gene transfer in most cell lines [Haag and Kratz, 2006]. Self-assembled block copolymers consisting of cationic chains and neutral hydrophilic segments form polyion complex with DNA [Bromberg et al., 2006]. The core-shell polycplex structures surrounded by
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a hydrophilic polymer have a high colloidal stability, which reduce the interaction with blood constituents thereby protecting therapeutic genes from premature degradation in systemic transfer medium.

Various cationic lipid/polymer-based transfection reagents have been widely studied as potential gene carriers since the protonated polyelectrolyte with high molecular weight formed nanoparticles with DNA [Wu and Wu, 1987; Park and Healy, 2004; Bromberg et al., 2006; Yamagata et al., 2007]. Polyethylenimine (PEI) [Boussif et al., 1995], poly(dimethylaminoethyl methacrylate) (PDMAEMA) [Cherng et al., 1996] and their derivatives [Verbaan et al., 2005; Adams et al., 2006; Patnaik et al., 2006] contributed to consistent transfection efficiency in gene delivery applications. Diethylaminoethyl-dextran was studied in non-viral gene delivery systems with relatively low transfection efficiency [Mack et al., 1998; Gavalas and Chaniotakis, 2000; Ahn et al., 2004; Benham and Mielke, 2005; Dubruel and Schacht, 2006]. Poly(Lysine)/DNA complexes showed a relatively high cytotoxicity and a tendency to aggregate and precipitate, depending on the ionic strength [Choi et al., 1998; Liu et al., 2001; Duncan and Izzo, 2005]. Polysaccharides, such as cyclodextrins and chitosan, are relatively non-toxic, and non-immunogenic [Xu et al., 2006; Chan et al., 2007]. The DNA was conjugated to these polymers to prevent inter-particulate aggregation of the complex, to increase its solubility and stability, and to eliminate problems associated with cytotoxicity.
Polyethylenimine (PEI) is a commercially available polyamine because it shows high gene transfection efficiency. However, polymer architecture is a critical factor in condensing an extended therapeutic DNA crossing through a number of biological barriers. The star shape multi-arm polymers possess three-dimensional branched structure that offers interesting properties in self-association and condensation ability for plasmid DNA. The higher densities of terminal functional groups on the block copolymer chains induce stronger interactions as compared to linear structure of identical molecular weights.

In this Chapter, the polymer/DNA complexes are preliminary studies to examine the potential for using the 4-arm PEO-b-PDEAEMA block copolymer in gene delivery. The subcellular size of polymer/DNA complexes induced by the stronger interactions could result in higher cellular endocytosis of the entrapped DNA [Prabha et al., 2002]. The polycation with tetrahedral structure condensed DNA into polyplexes with unique morphology that predominantly contributes to successful gene transfection. In this study, the star shape four-arm PEO-b-PDEAEMA block copolymer was evaluated as a potential synthetic vector for gene delivery. By varying the pH and ionic strength, the self-assembled polymers can form size tunable micelles that dissociate into unimers at low pH as reported previously [Alemdaroglu and Herrmann, 2007]. The polymeric chains are positively charged in physiological environment (pH 7.4) due to the partially protonated amine groups. DNA was condensed by the star polymer into smaller structures and the negative DNA charges were masked, which is a necessary
condition for transfection to most types of cells. The ability of star block copolymers to encapsulate DNA was demonstrated as evident from changes in the fluorescence intensity of intercalated ethidium bromide within the DNA. The formation of complexes between the polymers and plasmid DNA was further confirmed by agarose gel electrophoresis assay. The morphology and aggregation behaviors of polyplexes in aqueous medium were studied by light scattering, where the addition of monovalent salt significantly altered the size of complexes in HEPES buffer solution. The mechanism of DNA complexation with the four-arm star block polymer was elucidated, and comparison of polyplex structures was investigated at various N/P ratios and salt concentrations. In-vitro cytotoxicity and transfection efficiency were investigated using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and flow cytometry, where the cell population expressing green fluorescence protein (GFP) and the level of the expressed GFP were quantified.

7.2 Experiments for interactions between four-arm PEO-b-PDEAEMA and plasmid DNA

7.2.1 Preparation of plasmid DNA and polyplexes

The four-arm PEO-b-PDEAEMA star block copolymer was synthesized by atom transfer radical polymerization as described previously and the chemical structure is
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shown in Figure 7.1.

![Chemical structure of four-arm PEO-b-PDEAEMA](image)

**Figure 7.1** Chemical structure of four-arm PEO-b-PDEAEMA.

Plasmid EGFP (pIRES-EGFP-EV71), an expression vector containing the enhanced green fluorescence protein (EGFP), was constructed by grafting picornaviral IRESes from Enterovirus 71 (strain 7423/MS/87) onto a pIRES-EGFP backbone (Clontech, U.S.). The plasmid was amplified in Escherichia coli (DH5α strain) and purified through column chromatography according to the manufacturer’s instructions of EndoFree plasmid mega kits (Qiagen, USA).

Based on the calculated polymer to DNA ratio, the polymers were added to DNA in aqueous solutions followed by vortexing, and incubated for 60 minutes prior to analysis. All complexes of DNA and polymer were freshly prepared prior to use. The N/P ratio was expressed as the molar ratio of nitrogen of DEAEMA units on the
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copolymer chains to phosphorus of nucleotides in plasmid DNA.

7.2.2 Agarose gel electrophoresis assay

The electrophoretic mobility of the polymer/DNA complexes at different polymer/DNA ratios was determined by gel electrophoresis using 1.0 wt\% of agarose gel mixed with 20 µg of ethidium bromide (EtBr) in Tris-acetate EDTA (TAE) buffer. The DNA concentrations are 10 \textmu g/ml for all the samples with various N/P ratios. The polymer concentrations in polymer/DNA complexes solutions were calculated according N/P ratios, which were expressed as the molar ratio of nitrogen of DEAEMA units on the polymer chains to phosphorus of nucleotides in plasmid DNA. The polyplexes were prepared in HEPES buffer at pH 7.4 and HEPES saline buffer containing 150 mM NaCl at same pH with an incubation of 60 mins. The gel was run at 120 V for 45 minutes. The EtBr-stained DNA bands were visualized and photographed on an ultraviolet transilluminator (254 nm).

7.2.3 Ethidium bromide exclusion

Ethidium bromide was added to 200 \textmu L of 20 mM HEPES buffer and 20 mM HEPES saline buffer containing 150 mM NaCl and gently mixed. 0.4 µg of plasmid DNA was added to the mixtures respectively, and the mole ratio of EtBr to nucleotide of plasmid DNA was set to 4/1 and allowed to incubate for 30 min at room temperature.
Fluorescence intensity was measured with three readings at the excitation wavelength ($\lambda_{\text{ex}}$) of 485 nm and emission wavelength ($\lambda_{\text{em}}$) of 590 nm using BMG Laboratories FLUO star optima. The fluorescence of each sample was corrected for the background fluorescence of EtBr in the absence of DNA and the relative fluorescence was calculated as follows:

$$\text{Relative fluorescence } \% = \frac{\text{Fluorescence}_{\text{observed}} - \text{Fluorescence}_{\text{EtBr}}}{\text{Fluorescence}_{\text{pDNA+EtBr}} - \text{Fluorescence}_{\text{EtBr}}}$$

### 7.2.4 Laser light scattering

A series of N/P polymer/DNA complex was measured in 20 mM HEPES buffer solution and in 20 mM HEPES buffer solution with addition of 150 mM NaCl.

### 7.2.5 Zeta potential (ZP) measurement

Utilizing the phase analysis light scattering (PALS) system, the zeta potential of the four-arm PEO-$b$-PDEAEMA/DNA complexes were determined by the Brookhaven zeta potential analyzer equipped with a 671 nm He-Ne laser source. $\zeta$ (mV) was derived from the measured electrophoretic mobilities with five repeated measurements to confirm the repeatability of results. Since the Zeta potential is a function of the surface charge on the particles, it reflects the stability and physicochemical properties of polyplexes, which is a key factor in cell-surface
binding for polyplexes gene delivery.

### 7.2.6 Transmission electron microscopy

The TEM micrographs of polyplexes were observed by a JEOL JEM-2010 electron microscope at an acceleration voltage of 200 kV. Two drops of a selected polyplexes solution were placed onto a carbon coated copper grids and stained by osmium tetroxide (OsO₄). The copper grids were dried in a freeze dryer and then kept in decicator overnight at room temperature prior to measurement.

### 7.2.7 Cell cultures

Neuro-2A cells were obtained from the American Type Culture Collection (CCL-131, ATCC, USA). The cells were incubated in Dulbecco’s Modification of Eagle’s Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37 °C in 5% CO₂ humidified atmosphere.

### 7.2.8 Evaluation of cytotoxicity

The MTS cell proliferation assay is a colorimetric method for cytotoxicity assays. MTS compound was reduced by active reductase enzymes into a colored formazan product, which can be quantified by spectrophotometer. The absorbance of formazan
product is directly proportional to the number of living cells.

MTS assay was applied to evaluate the cytotoxicity of four-arm PEO-\(b\)-PDEAEMA and polyplexes using the CellTiter 96 aqueous one solution cell proliferation assay (Promega). Neuro-2A cells were seeded into 96 well microtitre plates in 200 \(\mu l\) culture medium at a density of \(2 \times 10^4\) cells per well and incubated for 24 h. Thereafter, the culture media were replaced by serum-free Dulbecco's modified Eagle's medium (DMEM) containing a series of polymer solutions or polyplex at different N/P ratios. Cells were incubated for an additional 4 h prior to the replacement of 200\(\mu l\) of fresh culture medium. Each well was added with 20 \(\mu l\) of 5 mg/ml MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution. The cells were incubated for 2 h in an incubator at 37 °C, 5% CO\(_2\), and 95% relative humidity. The optical intensity of each well was measured spectrophotometrically at a wavelength of 490 nm by a microplate reader (Model 680, Bio-Rad). The spectrophotometer baseline was subtracted according to the culture medium without cells.

7.2.9 In vitro transfection

Neuro-2A cells were seeded in a 6-well plate with DMEM solution at a density of 150,000 cells per well and incubated for 24 h at 37 °C under a 5% CO\(_2\) atmosphere prior to transfection. EGFP plasmid containing the enhanced green fluorescence
protein was used to analyze the expression efficiency. Polymer/DNA complexes were prepared in a series of N/P ratio in 20 mM HEPES buffer at pH 7.4, which contain 20 µg/ml of Plasmid DNA and equilibrated for 30 min at 25 °C, thereafter the complexes solution was diluted in tenfold times with serum free DMEM. 2 ml of diluted polyplexes solution was then added to the wells to yield a final concentration of 4 µg of purified plasmid per well and incubated for 6 hours. In addition, naked plasmid DNA and cells treated with only the transfection medium were used as negative controls. The polyplex transfection media were replaced with DMEM solution containing 10% FBS and the cells were further cultured for 48 hours. The culture medium was removed before the cells were rinsed and harvested by centrifugation and re-suspended in 1 ml of 20 mM PBS. All cell samples were sieved through nylon mesh with pore size 40 µm prior to sorting. GFP expression was analyzed using flow cytometers (FACS Canto II, Becton Dickinson). The mean fluorescence intensity of enhanced green fluorescent protein (EGFP) expressing 30,000 individual cells was measured and analyzed using WinMDI version 2.8 (Scripps Research Institute). All transfection experiments were performed in triplicate.

7.3 Results and discussion

7.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to elucidate the relative amounts of DNA that were free or bound to the polymer as a function of N/P ratio. Two series
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of agarose gel images illustrate the visualization on the interaction of DNA and four-arm PEO-b-PDEAEMA in HEPES buffer and HEPES saline buffer containing 150 mM NaCl as shown in Figure 7.2. Lane 1 is the illumination image of 10 kbase pair ladder and lane 2 shows the image of plasmid DNA intercalating with EtBr. As shown in Figure 7.2a, the illuminating intensity decreased from lane 3 to 11 with increasing N/P molar ratios from 0 to 1.25. The negatively charged DNA neutralized the cationic segments on the polymeric chains, which induced the condensation of DNA resulting in less EtBr being intercalated within the DNA. Beyond N/P of 1.25 (from lane 11 onwards), all available plasmid DNA was complexed with the polymer in HEPES buffer since the illumination had almost disappeared. The agarose gel electrophoresis of polymer/DNA complexes prepared in HEPES saline buffer containing 150 mM NaCl addition at various N/P ratios was reported in Figure 7.2b. A similar trend was observed, where the illuminating intensity decreased with increasing N/P molar ratios (see lane 3 to lane 10). However, the negatively charged DNA was saturated by cationic polymer at N/P = 1, indicating that the binding was reduced compared to polyplexes prepared in HEPES buffer. In the presence of small electrolytes, electrostatic shielding around the polymer segments reduced the binding and subsequent condensation of DNA with the star shape block copolymer. While unbound or partially bound DNA will migrate towards the anode, a reduction was observed with increasing N/P ratio due to the larger polyplexes that possessed lower net negative charges.
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**Figure 7.2** Agarose gel electrophoresis results for four-arm PEO-\textit{b}-PDEAEMA and DNA complexes in Hepes buffer (a) and Hepes buffer containing 150 mM NaCl (b) at different N/P ratios. (1) Ladder, (2) Plasmid DNA, (3) N/P ratio 0.125, (4) N/P ratio 0.25, (5) N/P ratio 0.375, (6) N/P ratio 0.5, (7) N/P ratio 0.625, (8) N/P ratio 0.75, (9) N/P ratio 0.875, (10) N/P ratio 1, (11) N/P ratio 1.25, (12) N/P ratio 1.5, (13) N/P ratio 1.75, (14) N/P ratio 2, (15) N/P ratio 3, (16) N/P ratio 4, (17) N/P ratio 5, (18) N/P ratio 10, (19) N/P ratio 15, (20) N/P ratio 20.

**7.3.2 Ethidium bromide exclusion assay**

Ethidium bromide exclusion assay examines the ability of cationic polymer to condense plasmid DNAs to form polyplexes [Eastman et al., 1997]. Ethidium bromide
binds DNA by intercalating within the DNA base pairs that stretches the double helix of DNA where EtBr fluorescence is enhanced approximately 40-fold upon intercalation with DNA. With more cationic polymer bound to DNA, ethidium bromide cannot access the DNA resulting in a reduction in the fluorescence intensity. Figure 7.3 shows the ethidium bromide exclusion assay conducted for polyplexes prepared in 20 mM HEPES buffer, where the fluorescence intensity decreased dramatically with increasing N/P ratio from 0 to 1.25 confirming that EtBr was displaced by cationic polymer. When more polymers were added, the fluorescence intensity decreased to a very low value due to the compact structure induced by the condensation of plasmid DNA with the block copolymer. The ethidium bromide exclusion assay was also conducted in 20 mM HEPES buffer containing 150 mM NaCl as shown in Figure 7.4. Similar trend was observed where the fluorescence intensity decreased to only approximately 30% of initial intensity with increasing N/P ratio. Sodium ions screened the electrostatic interaction between charged polymer and plasmid DNA, hence the DNA condensation was reduced. The percentage of intercalating ethidium bromide was higher, presumably caused by the formation of less compact polyplexes which was confirmed by light scattering measurements.
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Figure 7.3  Ethidium bromide displacement results for four-arm PEO-\(b\)-PDEAEMA and DNA complexes in 20 mM Hepes buffer (pH 7.4) at different N/P ratios.

Figure 7.4  Ethidium bromide displacement results for four-arm PEO-\(b\)-PDEAEMA and DNA complexes in 20 mM Hepes buffer (pH 7.4) with 0.15 M NaCl addition at different N/P ratios.
7.3.3 Zeta potential measurement

Zeta potentials (ZP) of the DNA/four-arm PEO-b-DEAEMA polyplexes in 20 mM HEPES buffer (pH 7.4) were measured and the results are shown in Figure 7.5. The ZP of plasmid DNA was about -25 mV. The addition of copolymer to DNA induced the condensation of DNA, which resulted in an increase in the zeta potential. The zeta potential of polyplexes became less negative with increasing N/P ratio, approaching the isoelectric point at N/P ratio of about 1. The ZPs became positive at N/P ratio greater than 1.0 and reached an asymptotic value of +15 mV at N/P of about 5. The ZP of homopolymer polyplexes (PDMAEMA/DNA) is about +30 mV at high polymer/DNA ratio [Cherng et al., 1996]. The lower ZP (+15 mV) of four-arm PEO-b-DEAEMA/NDA polyplexes is result by the PEO block shielding the polyplexes.

![Figure 7.5](image_url)  
**Figure 7.5** Zeta potential of the polyplexes as a function of four-arm PEO-b-PDEAEMA/DNA molar ratios.
7.3.4 Dynamic light scattering

In gene delivery system, the size of the polymer/DNA complexes is an important factor for DNA transfection to cell. DLS was used to investigate the hydrodynamic radius of polymer/DNA complexes in various molar ratios and salt concentration in buffer solution.

The four-arm PEO-\textit{b}-PDEAEMA block copolymers spontaneously bind with DNA to form complexes, driven by electrostatic interactions between DNA and oppositely charged polycation. Figure 7.6 shows the decay time distributions at scattering angle of 90° for various ratios of polymer/DNA complexes in 20 mM HEPES buffer solution. At N/P ratio of 0.1, the $R_h$ of the scattering object was 56 nm, which was close to the size of naked 3.7 kbp DNA.[Fishman and Patterson, 1996] When N/P ratio was increased to 0.5, polymer/DNA complexe was formed induced by electrostatic force to produce an aggregate of 74 nm. The size of polyplexes increased with polymer concentrations and reached a maximum of 95 nm at N/P ratio of 1.25, which is in good agreement with the results of agarose gel electrophoresis and ethidium bromide exclusion analysis. The unimodal distribution function shifted to longer relaxation times as N/P ratio was increased from 0.1 to 1.25 as shown in Figure 7.6, suggesting that a single type of particle was present. With further addition of polymer, the relaxation time decreased, and this might be attributed to the rearrangement of DNA structure driven by the minimization of Gibbs free energies.
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from a combination of electrostatic and hydrophobic forces. At N/P of 2 the hydrodynamic radius of polyplexes was 61 nm and it decreased significantly to 37 nm at N/P of 10 and 33 nm at N/P 20, which is suitable for gene transfection application. A small fast decay mode was observed together with slow decay mode in decay time distribution function of polyplexes at N/P ratio greater than 2, suggesting that free unbound polymeric chains co-existed with the complexes.

Figure 7.6 Relaxation time distribution functions at scattering angle of 90° for four- arm PEO-b-PDEAEMA and DNA complexes at different N/P ratios. (◆) N/P ratio 0.1; (◇) N/P ratio 0.5; (▲) N/P ratio 1.25; (△) N/P ratio 2; (■) N/P ratio 10; (□) N/P ratio 20.
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The addition of salt alters the solution properties of polyelectrolytes [Bazan-Peregrino et al., 2007] by destroying the water structure, hydrogen bond and the ion-dipole interaction. The polymer/DNA aggregation behavior was also investigated in 20 mM HEPES buffer solution with the addition of 150 mM NaCl. The DEAEMA segments of polymer chain were partially protonated in buffer solution thereby imparting a hydrophilic character to the polymeric chains. The free negatively charged chloride and hydroxide ions surrounded the positively charged polymer chains. Furthermore, the negatively charged DNA attracted positively charged ions. As shown in Figure 7.7, the hydrodynamic radius of polyplexes increased in the buffer solution with salt addition if the N/P ratio is fixed. At very low N/P ratio of 0.1, the polymer/DNA particles possessed a similar size (about 54 nm) as nake plasmid DNA, however the size of polyplexes increased with polymer concentration increase and reached a maximum of about 106 nm at N/P of 1. As discussed previously, the polyplexes underwent a dramatic compaction in the presence of a condensing agent. However the size of condensed polyplexes in salt solution was larger (about 53 nm) at N/P 20, compared to 34 nm for polyplexes in buffer solution in the absence of salt. In presence of salt the polymer/DNA structure became less compact induced by small ions interfering the electrostatic interaction between DNA and polymer.

The DLS experiment for polyplexes aqueous solution was measure at several scattering angles, and it was found that the decay rates were $q^2$ dependent, which suggested that the decay modes can be attributed to the translational diffusion of
particles in solution. The slopes of $\Gamma$ versus $q^2$ were related to the translational diffusion coefficients $D$ of particles in solution as shown in Figure 7.8.

![Graph showing $R_h, \text{app}$ of four-arm PEO-b-PEODEAEMA polymer/DNA complexes as a function of N/P ratio in buffer solution with and without 0.15 M NaCl addition.]

Figure 7.7 $R_h, \text{app}$ of four-arm PEO-b-PEODEAEMA polymer/DNA complexes as a function of N/P ratio in (■) 20 mM Hepes buffer solution and (▲) the buffer solution with 0.15 M NaCl addition.

![Graph showing relationship between decay rates and $q^2$ for polymer/DNA complexes at N/P ratio 20 in buffer solution with and without 0.15 M NaCl addition.]

Figure 7.8 Relationship between decay rates and $q^2$ for polymer/DNA complexes at N/P ratio 20 in (■) 20 mM Hepes buffer solution and (▲) the buffer solution with 0.15 M NaCl addition.
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7.3.5 Static light scattering

SLS was used to measure time-average scattered intensities, where the microscopic properties of particles such as the z-average radius of gyration ($R_g$) can be determined according to the Debye equation. Two series of radius of gyration ($R_g$) values were obtained for the four-arm PEO-b-PDEAEMA/DNA complexes at various N/P ratios in 20 mM HEPES buffer and 20 mM HEPES buffer containing 150 mM NaCl at scattering angles ranging from 50° to 110° in 10° intervals as shown in Figure 7.9. In the present study, $R_g$ corresponded to the particle size of large particles since the scattering intensity of large particles was much stronger than single polymeric chains. The $R_g$ of plasmid DNA was about 85 nm and 88 nm in 20 mM HEPES buffer and in the buffer containing 150 mM NaCl respectively. With increasing N/P ratio, $R_g$ increased, reaching a maximum of 122 nm at N/P of 1 in 20 mM HEPES buffer solution. The positively charged 4-arm PEO-b-PDEAEMA interacted with negatively charged DNA through electrostatic force resulting in the decoration of polymeric chains on the DNA. However, when N/P ratio exceeded 1, the polyplexes became compact, where $R_g$ decreased dramatically approaching 107 nm, 73 nm and 53 nm at N/P ratio 1.5, 2, 3, respectively. With further increase of N/P ratio, the $R_g$ values remained essentially constant at about 33 nm, suggesting that the DNA was fully condensed into a stable compact structure. In HEPES buffer solution containing 150 mM NaCl, $R_g$ exhibited a similar trend, it increased to a maximum of 138 nm at N/P=1, and it then decreased and approached a stable size of 53 nm (larger than the
33 nm in the absence of salt). Based on the Debye plot, the weighted average molecular weight ($M_w^{\text{app}}$) of the polyplexes in 20 mM HEPES buffer solution was determined to be $4.39 \pm 0.22 \times 10^6$ g/mol at N/P = 3, which remained constant at N/P up to 20. Since the molecular weight of DNA was $3.12 \times 10^6$ g/mol, the average number of aggregated polymeric chains required to condense one DNA molecule was determined to be approximately 15, since the molar mass of one star block copolymer was $8.37 \times 10^4$ g/mol.

![Figure 7.9](image-url)  

**Figure 7.9** $R_g^{\text{app}}$ of polymer/DNA complexes as a function of N/P ratio in (■) 20 mM Hepes buffer solution and (▲) the buffer solution containing with 150 mM NaCl.

The parameter $\rho (R_g/R_h)$ was calculated to examine the morphology of the microstructure of polyplexes, and the trend is shown in Figure 7.10. For plasmid DNA, $\rho$ was approximately 1.5~1.6, which was very close to the theoretical value of a Gaussian chain in a good solvent. With the addition of polymer, $\rho$ decreased and
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reached an asymptotic value of \( \sim 1.0 \). This suggested the polyplexes possessed a spherical structure with a dense core and polymeric corona shell. The positively charged DEAEMA chains interacted with negatively charged DNA to form a compacted core with a shell consisting of the multi-arm PEO chains. Such structure provides stability of complexes and protects the DNA from nuclease degradation.

![Graph](image)

Figure 7.10 \( \rho(R_g/R_h) \) of polymer/DNA complexes versus N/P ratio in (□) 20 mM Hepes buffer solution and (Δ) the buffer solution containing with 150 mM NaCl.

7.3.6 Morphology of the polyplexes

To further elucidate the condensation behavior of four-arm PEO-\textit{b}-PDEAEMA polymer, the morphology of DNA/polymer complexes was examined by transmission electron microscopy (TEM). The images revealed the presence of condensed polymer/DNA complexes in different sizes and demonstrated significantly
morphological differences at varying N/P ratios. At N/P ratio of 1, the polymeric chains (stained with a dye) were trapped within the DNA as shown in Figure 7.11(a). However, the complexes were observed to be weakly associated and loosely packed as suggested by $\rho (R_g/R_h)$ of about 1.3 as discussed previously. Figure 7.11(b) shows the micrograph of polyplexes prepared at N/P ratio of 3 in 20 mM HEPES buffer solution. The DNA was fully condensed by the positively charged polymeric chains to form spherical and discrete nanoparticles, whose size is in good agreement with light scattering results. The micrographs confirmed the structural reorganization of the DNA from Gaussian chain to spherical nanostructures as increasing amounts of block copolymer were introduced. The concentration of polymer in DNA solution is an important factor as the characteristics and stability of polymer/DNA complexes depends on the level of DNA condensation.

Figure 7.11 TEM micrographs of the four-arm PEO-\textit{b}-PDEAEMA block polymer and plasmid DNA complexes prepared in 20mM Hepes buffer at N/P molar ratio of (a) 1 and (b) 3.
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7.3.7 \textbf{In Vitro cell viability assay}

The viabilities of Neuro-2A cell in the four-arm PEO-\textit{b}-PDEAEMA polymer solution and four-arm PEO-\textit{b}-PDEAEMA polymer/DNA complexes at various N/P ratios were investigated by MTS assay. Figure 7.12 shows that more than 90\% of the cells survive at the low N/P ratio, and the cell viability in the polymer media is equivalent to that in polymer/DNA complexes. With increasing N/P ratio, the viable cell count was reduced, where the polymer/DNA complexes were significantly less toxic to cells than free polymers. Unbound polymer in the transfection formulation partially blocked cellular association of DNA complexes and contributed to cellular and systemic toxicity. At N/P greater than 8, the experiments showed the expected low cell viability within 4 h since large amounts of excess polymer were present in the culture media. At an equal molar ratio of DNA in the culture media, the polymers interacted with DNA resulting in a reduction in the positively charged environment that exhibited lower cytotoxicity to cells. The star shape polymer condenses with the plasmid DNA, which undergoes significant structural rearrangement to form compact micellar-like structures surrounded by hydrophilic PEO corona.
7.3.8 Intracellular transfection

EGFP Plasmid is a DNA expression vector containing the enhanced green fluorescence protein (EGFP), which the fluorescence intensity can be quantified by flow cytometers. The cells were seeded in a 6-well plate at a density of 150,000 cells per well. The polymer/plasmid DNA complexes were prepared and then added to the wells to yield a final concentration of 4 µg plasmid DNA per well. After incubation for 6 hours, the cells were harvested and the expression level of the transfected protein can be measured by determining the fluorescent intensity of EGFP in the cells.

The gene transfection efficiency of four-arm PEO-b-PDEAEMA in Neuro-2A cells was evaluated using flow cytometry. The normalized transfection efficiency at
various N/P ratios against the highest efficiency at the N/P 4.5 is shown in Figure 7.13. The transfection levels were very low at N/P ratio of less than 1, which confirms that the plasmid DNA is not fully condensed by the cationic polymer. The percentage of cells expressing EGFP increased with increasing N/P ratio and reached an optimal N/P ratio (from 4 to 4.5). The positively charged polymers possessed strong electrostatic interaction with plasmid DNA, which undergo structural rearrangement and the overall charge of complexes particles became positive. The positively charged polyplexes are attracted to the negatively charged cellular membrane walls resulting in the internalization of DNA into the cells.

Figure 7.13 Transfection efficiency of four-arm PEO-b-PDEAEMA/pDNA polyplexes in Neuro-2A cell line at varying N/P ratios.

The transfection of pEGFP by four-arm PEO-b-PDEAEMA in Neuro-2A cells were also visualized by fluorescence microscopy as shown in Figure 7.14. The number of cells expressing EGFP was related to the N/P ratio, where the efficiency of
transfection was slightly improved with increasing N/P ratio up to the optimal value of 4.5, beyond which cell deaths dominated because of the toxicity of the free polymers.

Figure 7.14 Images of Neuro-2A cells transfected with PEO-b-PDEAEMA/pDNA complex observed under fluorescent microscope at different N/P ratios.

Figure 7.14 Images of Neuro-2A cells transfected with PEO-b-PDEAEMA/pDNA complex observed under fluorescent microscope at different N/P ratios.
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7.4 Summary

A star-shaped multi-arm PEO-\(b\)-PDEAEMA was used to condense extended plasmid DNA in 20 mM HEPES buffer solution at the physiological pH of about 7.4 and in the buffer solution containing 150 mM NaCl. The phosphate anions on DNA interacted with partially protonated amine groups on the polymer backbone to form spherical core-shell structure consisting of a condensed PDEAEMA/DNA core stabilized by PEO corona shell. The size of polymer/DNA complexes increased with increasing N/P, and reached a maximum of about 95 nm and 106 nm at N/P 1.25, where it then decreased to an asymptotic value of about 35 nm in HEPES buffer in 0 mM and 150 mM NaCl solutions respectively. The ratio \(R_g/R_h\) decreased from 1.5 to 1 when N/P ratio was increased, suggesting that the polyplexes underwent a conformation rearrangement and became more compact at high N/P Ratio. The gene transfection efficiency of the multi-arm PEO-\(b\)-PDEAEMA/pDNA complexes in Neuro-2A cells suggested that the three dimensional structure of cationic polymers offers positive characteristics for the delivery of genetic materials.
8 Chapter Eight

Binding Mechanism and Release Studies of Drug/Polymer Complexes

8.1 Introduction

Self-assembled block copolymers exhibiting environmentally responsive behaviors have been used extensively as a versatile nanomedicine platform in biology and medical fields [Brannon-Peppas and Blanchette, 2004; Betancourt and Brannon-Peppas, 2006; Farokhzad and Langer, 2006; Tong and Cheng, 2007]. The synthetic biomimetic polymers contain hydrophilic and hydrophobic block domains, which could form micelles with core/shell structures at the right environmental conditions [Soo and Eisenberg, 2004; Vriezema et al., 2005; Yow and Routh, 2006; Dai et al., 2008]. The hydrophilic segments are located between the core and the external aqueous medium to stabilize the hydrophobic core. Through various chemical, physical, or electrostatic interactions, the hydrophobic regions serve as reservoirs for drugs loading to fulfill designed specific functionalities [Magid, 1998; Nakamura and Shikata, 2007]. An external stimulus, such as pH, ionic strength, temperature, light, electric field and magnetic field, results in a change of the polymer properties, such as the conformation, solubility, association and the release of the drug molecule under
specific conditions [Chan et al., 2007; Checot et al., 2007]. The polymer micelles encapsulated drugs demonstrate various attractive properties, such as suitable size for enhanced permeability and retention effect, high stability in aqueous medium and solubilization of water insoluble drugs [Allen and Cullis, 2004; Napier and Desimone, 2007; Xie et al., 2007].

A variety of polymeric nanoparticles have been developed and evaluated as delivery vehicles for drug molecules, therapeutic proteins and genes. Temperature responsive polymer, such as poly(N-isopropylacrylamide) (PNIPAm) undergoes a sharp coil–globule transition, which the polymeric chains are transformed from hydrophilic to a hydrophobic character with temperature increase [Xie et al., 2007; Jin et al., 2008]. PEO and PPO block co-polymers known as Pluronics, Poloxamers and Tetronics are commercially available and widely used in the pharmaceutical industry due to the highly soluble character of PEO polymers and hydrophobic property of PPO [Moghimi and Hunter, 2000; Xiong et al., 2006; Chiappetta and Sosnik, 2007]. Poly(ethylene oxide)-b-poly(L-amino acid)s is useful for the chemical conjugation of drugs because they can facilitate chemical modification yielding functional groups that are capable of enhancing the loading of therapeutic substances [Lavasanifar et al., 2002; Nishiyama and Kataoka, 2006]. In our studies, poly(methacrylic acid) was selected as the pH-responsive block linked with PEO block, where micelles can form at low pH condition due to the hydrophobicity of MAA segments [Bromberg and Ron,
At high pH, the MAA chain adopted an extended random coil conformation with a larger hydrodynamic volume induced by Coulombic repulsive forces between ionized carboxylate groups on polymer chains. In contrast, the hydrophobic interactions between methyl groups result in a lower hydrodynamic volume due to the suppressed hypercoiled morphology at low pH. Poly(ethylene oxide) (PEO) that is approved by FDA for biomedical applications was grafted to the PMAA chain to reduce the cytotoxicity. In addition, PEO improves the biocompatibility of foreign materials due to its high aqueous solubility and mobility, unique solution properties, minimal interfacial free energy with water and large exclusion volume [Nakashima and Bahadur, 2006; Neugebauer, 2007; Schweizer and Taubert, 2007]. In contrast to the widely reported studies on linear PEO systems, the three-dimensional branched structures possess unique physicochemical properties that could potentially be useful in some applications [Heath et al., 2007]. The star shape multi-arm PEOs possess three-dimensional branched structures combined with other responsive polymeric segments can self-assemble and associate to form nanostructures in the presence of drugs. The higher densities of terminal functional groups on the block copolymer chains induce stronger interactions with drugs compared to linear structure polymer with identical molecular weights.

The application of 4-arm PEO-\textit{b}-PMAA star polymer in drug delivery was studied preliminarily. The pH-responsive PMAA chains were grafted onto the four-arm PEO
using atom transfer radical polymerization (ATRP) to produce a drug delivery vehicle with tetrahedral structure. In various pH environments, the ionization degree of PMAA segments was changed that induces conformation changes in the 4-arm PEO-\(b\)-PMAA star polymer chains. As more COOH groups on the polymeric chains are deprotonated at high pH, the electrostatic repulsion between charged groups increases, making the extended methacrylic acid segments more accessible for the loading of cationic drug molecules. At low pH, the hydrophobic interaction of methyl groups and the hydrogen bonding between the methacrylic acid and ethylene oxide segments causes the PMAA segments to adopt a hyper-coiled conformation that facilitates drug release. A comprehensive understanding on the interaction between drug and delivery vehicle is critical for optimizing drug delivery, since the efficient binding and controlled release triggered by changes in the binding affinity between drug and polymeric matrix are not fully understood. The interactions between the polymer and IPH were elucidated through thermodynamic quantification using the isothermal titration calorimetric technique. The radius of gyration (\(R_g\)) of polymer in aqueous solution and drug/polymer complex was determined by dynamic light scattering. The drug release profiles were monitored by drug selective electrode system, which is a more efficient and practical compared to the widely used dialysis method coupled with UV-vis or high performance liquid chromatography (HPLC).
8.2 Experimental Methods

8.2.1 Materials

The four-arm PEO-\textit{b}-PMAA star block copolymer was synthesized by atom transfer radical polymerization as described previously. The number average molecular weight ($M_n$) of four-arm PEO-\textit{b}-PtBMA block copolymer obtained from GPC was 60294 Da, which was consistent with the theoretical molecular mass (initiator to monomer molar ratio). The relative molecular mass distributions ($M_w / M_n$) is narrow, PDI (polydispersity index)=1.23. The chemical structure of the star block copolymer is 4-arm PEO$_{56}$-\textit{b}-PDEAEMA$_{88}$ as determined by $^1$H NMR and GPC.

Imipramine hydrochloride (IPH), carboxylated poly (vinyl chloride) (PVC) and poly(ethylene-co-vinyl acetate-co-carbon monoxide) (PE-co-PVA-co-CO) were purchased from Aldrich and used as received. Sodium tetraphenylborate (NaTPB) was obtained from Fluka. Deionized water was produced by a Millipore Alpha-Q purification system.

8.2.2 Preparation of drug selective membrane

The polymeric membrane consists of negatively charged carboxylate-modified PVC and IPH drug complex, ion exchanger and suitable plasticizer. Carboxylated PVC (0.5
g) was initially dissolved in 20 ml of tetrahydrofuran (THF). Thereafter imipramine hydrochloride (1.109 g) solution was added dropwise to the THF solution at a volume ratio 1:9 under gentle stirring to form carboxylated PVC/IPH complex. The mixture was precipitated in ten fold excess of de-ionized water, and the complex was filtered and dried at room temperature. The polymeric plasticizer, poly(ethylene-co-vinyl acetate-co-carbon monoxide) (0.18 g) was completely dissolved in dichloromethane under constant stirring at 35°C. Optimum relative amounts of carboxylated PVC/IPH complex (0.114 g) and sodium tetraphenylborate (0.006 g) were dissolved in THF and mixed with plasticizer solution. The mixture was poured into a petri dish of 55 mm in diameter and subsequently the solvent was evaporated at room temperature to yield the drug selective membrane[Tan et al., 2007; Tan and Tam, 2007; Tan et al., 2008; Tan et al., 2008].

8.2.3 Selective electrode system

The drug selective membrane electrode was used to determine the IPH concentrations by measuring electromotive force (EMF) relative to a commercial Ag/AgCl electrode. The measurement electrode was filled with a 1 mM IPH in 10 mM NaBr solution and conditioned for half an hour prior to use. An ABU93 tri-burette titration system with modified Aliquot software was used to record the EMF values. The sample solutions were placed in water jacketed vessel which was maintained at a constant temperature.
of 25 °C using a circulating water bath. The drug selective membrane sensor possesses the high effective response to the primary ion in comparison to the influence of interfering ions owing to the differences in ionic size and consequently their mobilities and permeability [Satturwar et al., 2007]. The electrode was calibrated by measure EMF with various concentrations of IPH range from 0.01 mM to 20 mM in sample vessel. The plot of EMF versus log$(C_{IPH})$ is linear with a slope of 52.1 mV/decade that corresponds to Nernstian behavior (Figure 8.1).

![Figure 8.1 The calibration curve for imipramine hydrochloride selective electrode.](image)

8.2.4 Isothermal titration calorimetry

The calorimetric data were obtained using the Microcal ITC system (Northampton, MA), which consists of a reference cell and a sample cell of 1.35 ml insulated by an
adiabatic shield. The sample cell was filled with 0.05 wt% of polymer solution, which was titrated with 30 mM imipramine hydrochloride solution from a 250 μl injection syringe at 25.0 ± 0.02 °C controlled by a water bath. A constant stirring rate (400 rpm) was applied to ensure an optimum mixing efficiency. The time interval between each injection was set at 6 mins for all the experimental runs. The calorimetric data of each injection were recorded automatically by the software to produce the cell feedback (CFB) output. Microcal ORIGIN was used to integrate each CFB to yield the differential enthalpy curve for each titration.

8.2.5 In vitro release studies

The in-vitro drug release from the copolymer/drug complex was studied by EMF measurements using the drug selective electrode system. A certain amount of IPH was added to 0.1 wt% of four-arm PEO-b-PMAA block copolymer in 10 mM phosphate buffer solution. The mixture was kept in the dark and left to equilibrate in a shaking water bath at 25 °C for 24 h. Ultra-filtration cell with 20 nm filtration membrane was applied to remove the unbounded IPH molecules. The free IPH in the filtrate was determined by the absorbance at 251 nm using UV-vis spectrophotometer. The UV calibration curve was performed in aqueous PBS solution to eliminate the variation caused by solution media.
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The IPH/polymer complex was added to 20 ml of 10 mM PBS at a constant temperature of 37°C with continuously stirring. The amount of released IPH was reflected by the EMF measurements at a regular interval, and the data was recorded automatically by the ABU93 tri-burette titration system.

8.3 Results and discussion

8.3.1 Thermodynamic characterization of drug/polymer binding interactions

Isothermal Titration Calorimetry (ITC) was used to investigate the interactions between the star block polymer and drug. The four-arm PEO-b-PMAA block copolymer consisting of hydrophilic neutral PEO segments and pH-responsive PMAA chains. The dissociation of weak acidic polyelectrolyte was determined by potentiometric titration, where the degree of neutralization, $\alpha$, of the carboxylic group is defined by:

$$\alpha = \frac{[\text{BASE}^+] + [H^+] - [OH^-]}{C_{\text{COOH}}}$$  \hspace{1cm} (8.1)

where $[\text{BASE}]$, $[H^+]$, and $[OH^-]$ are the molarities of added base, free hydrogen ion, and hydroxide ion, respectively, and $C_{\text{COOH}}$ is the total concentration of methacrylic acid groups expressed in moles per liter. The hydrogen and hydroxide ion
concentration terms were calculated from the pH, where the activity coefficient is assumed to be unity. The magnitude of $\alpha$ was determined by the moles of titrated polyelectrolyte and the titrant added, where $\alpha$ ranged from 0 at un-ionized state of MAA groups to 1 at full complete neutralization state.

![Graph](image1)

![Graph](image2)

**Figure 8.2** Calorimetric titration of 30 mM imipramine hydrochloride into 0.05 wt% of four-arm PEO-$b$-PMAA block polymer solution at $\alpha\sim0.3$: (a) thermogram of cell feedback; (b) differential enthalpy curve.
30 mM imipramine hydrochloride solution was injected into the sample cell filled with 0.05 wt % of four-arm PEO-
\textit{b}-PMAA block polymer solution at different degrees of neutralization ranging from 0.05 to 1.0. The thermogram of CFB (cell feedback) heat signal for step-by-step injections of the polymer solution at $\alpha \sim 0.3$ is shown in Figure 8.2a. The differential enthalpy curves were obtained by the integration of the area under the raw signal curves as shown in Figure 8.2b. An exothermic peak over a predefined range of IPH concentrations was observed which corresponds to the interactions between the positively charged drug and anionic macroions of the star polymer.

To further elaborate the interaction for drug/polymer solutions at different pH or degree of neutralization, titration experiments were performed at predetermined $\alpha$. Figure 8.3 shows the enthalpy profiles for the titration of IPH into 0.05 wt % of four-arm PEO-
\textit{b}-PMAA block polymer solution at $\alpha=0.05$, 0.2, 0.3, 0.4, 0.6, 0.8, 1. From $\alpha \sim 0.05$ to 0.2, the increased amplitude of exothermic peak corresponded to the binding of more IPH to the polymeric chains through hydrogen bonding and electrostatic interactions. As $\alpha$ was increased from 0.2 to 0.4, the polymer micelles became less compact due to the deprotonation of carboxylic groups that produced more negative charges on MAA chains resulting in a reduced hydrogen bonding and a lower exothermic process. Further increase of $\alpha$ from 0.4 to 0.8, the exothermic amplitude of the peak increased gradually, where the binding fraction ($\phi$) became
greater. The exothermic peak increased with the degree of neutralization, which corresponded to the enhanced electrostatic binding of imipramine ions to negatively charged carboxylate groups along the PMAA chains. Through ion-exchange interactions, the condensed counterions on the polymeric segments are released where Na$^+$ ions regain their translational entropy. The higher exothermic peak indicated that more imipramine ions participated in the electrostatic interaction with the star polymer with larger number of negatively charged sites. Small endothermic peak was observed for polymer solution at high $\alpha$ value from 0.6 to 1, suggesting that other type interactions between the drug and polymer could be present. The binding may be initiated by the hydrophobic interaction of IPH bound to the polymer, and the neutralization of the negatively charged polymer by IPH. The backbone of PMAA bound with IPH was relatively hydrophobic and the drug induced the aggregation of polymer/drug complexes.
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Figure 8.3  Differential enthalpy curve of titrating 30 mM imipramine hydrochloride solution into 0.05 wt % four-arm PEO-\(b\)-PMAA block polymer at different \(\alpha\): (○) \(\alpha=0.05\); (Δ) \(\alpha=0.2\); (◇) \(\alpha=0.3\); (□) \(\alpha=0.4\); (▲) \(\alpha=0.6\); (♦) \(\alpha=0.8\); (■) \(\alpha=1\).

The concentration of drug for binding with polymer corresponding to the onset of endothermic peak was defined as \(C_1\) and the concentration of drug at binding equilibrium point was named as \(C_2\) corresponding to the saturation of polymer binding with drug. The onset concentration of drug (\(C_1\)) from the enthalpy curves as showed in Figure 8.3 is about 0.04 mM, which is independent of the degree of neutralization. The enthalpy curves became broader with increasing \(\alpha\) resulting in a higher saturation concentration (\(C_2\)). The binding fraction of the IPH in the star polymer solution is given by the following expression:

\[
\phi = \frac{C_2 - C_1}{C_{[COOH]}} \tag{8.2}
\]

where \(C_1\) and \(C_2\) were determined from the differential enthalpy curves (Figure 8.3).
\( C_{[\text{COOH}]} \) is the maximum ion exchange capacity of COOH groups determined from potentiometric titration. The values of \( C_1, C_2, \) binding fraction \( (\phi) \) at different degree of neutralization are summarized in Table 8.1. The binding fractions \( (\phi) \) of IPH increased when more macroions were present in the polymer solution corresponding to the higher degree of neutralization. The enthalpy measured from the ITC experiments is the sum of heat from several contributing factors, such as electrostatic attraction, hydrogen bonding and hydrophobic interactions.

<table>
<thead>
<tr>
<th>Degree of neutralization</th>
<th>( C_1 ) (mM)</th>
<th>( C_2 ) (mM)</th>
<th>Binding fraction ( (\phi) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.04</td>
<td>0.9</td>
<td>0.25</td>
</tr>
<tr>
<td>0.2</td>
<td>0.04</td>
<td>1.22</td>
<td>0.34</td>
</tr>
<tr>
<td>0.3</td>
<td>0.04</td>
<td>1.43</td>
<td>0.40</td>
</tr>
<tr>
<td>0.4</td>
<td>0.04</td>
<td>1.53</td>
<td>0.43</td>
</tr>
<tr>
<td>0.6</td>
<td>0.04</td>
<td>2.28</td>
<td>0.64</td>
</tr>
<tr>
<td>0.8</td>
<td>0.04</td>
<td>2.93</td>
<td>0.83</td>
</tr>
<tr>
<td>1</td>
<td>0.04</td>
<td>3.49</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 8.1 The values of \( C_1, C_2, \) binding fraction \( (\phi) \) of IPH in 0.05 wt% four-arm PEO-\( b \)-PMAA solution at different degree of neutralization.

At low \( \alpha \) range from 0 to 0.2, the carboxylate groups on four-arm PEO-\( b \)-PMAA polymer chains were partially charged and most of them were buried inside the core-shell micelles, induced by hydrophobic interactions between MAA chains. Hydrogen bonds contributed to the formation of drug-polymer complexes since the carboxylic group is a proton donor while IPH is a proton acceptor. Urea was added to the
polymer solution to remove hydrogen-bonding interaction between four-arm PEO-\textit{b}-PMAA and IPH. Urea disrupts existing H-bonding by forming H-bonding with all proton donors and acceptors, which weakens the interactions between drug/polymer as reflected in ITC titration results. Figure 8.4 shows the enthalpy curves for titrating 30 mM IPH into 0.1 wt % of four-arm PEO-\textit{b}-PMAA solution at $\alpha \sim 0.2$ in various concentrations of urea. In the presence of urea, the enthalpy caused by the interactions between IPH and polymer was significantly reduced from -3.57 kJ/mol (without urea) to -2.20 kJ/mol (with 1M urea) and -1.43 kJ/mol (with 2M Urea). This indicates that the extent of H-bonding between carboxylic groups of PMAA and IPH is weakened since most of the proton donating and accepting sites are occupied by urea molecules. This confirms that H-bonding is one of interaction forces during the formation of IPH/four-arm PEO-\textit{b}-PMAA complexes.

Figure 8.4  The enthalpy curves for titrating 30 mM IPH into 0.05 wt% of four-arm PEO-\textit{b}-PMAA solution at $\alpha \sim 0.2$ with various concentrations of urea. (■) 0 M; (♦) 1 M; (▲) 2 M.
The electrostatic interaction for the binding of the polymer and IPH was verified by adding small electrolytes to the solution mixtures. The addition of salt alters the electrostatic interactions between macroions, counterions, and solvent molecules and significantly impacts the binding behavior. The isothermal titration calorimetry curves are shown in Figure 8.5 for 0.05 wt % of four-arm PEO-b-PMAA solution at \( \alpha \sim 1 \) in various NaCl concentrations. The absolute value of differential enthalpy decreased from 6.97 kJ/mol to 5.02 kJ/mol and 2.61 kJ/mol for 0.01M, 0.05 M and 0.15 M NaCl present in the polymer solution, respectively. The small electrolytes present in the solution screen the electrostatic interactions between the macroions and cationic drug via an ionic atmosphere around the charged sites and hence hinder electrostatic attraction between negative carboxylate groups and positive IPH molecules. The free energy of the electrostatic binding decreased in higher salt content, which suggests that the addition of salt reduced the drug binding due to charge-shielding effect. The significant reduction of the enthalpy with the addition of small electrolytes in solution indicates that electrostatic interactions play a major role in the drug loading.
Figure 8.5 The enthalpy curves for titrating 30 mM IPH into 0.05 wt% of four-arm PEO-\textit{b}-PMAA solution at $\alpha \sim 1$ in different NaCl solution: (▲) 0.01 M; (♦) 0.05 M; (■) 0.15 M.

8.3.2 Particles size studies by dynamic light scattering.

The stimuli-responsive association behavior of four-arm PEO-\textit{b}-PMAA block copolymer in solution was studied to gain important insights on the responsive characteristics of the drug delivery vehicle. The greater proportion of end-functional groups induces higher solubility and stabilization in solution compared to linear structure of identical molecular weights [Alemdaroglu and Herrmann, 2007]. Dynamic light scattering (DLS) was used to investigate the hydrodynamic radius of polymer/drug complexes in aqueous solution.
The four-arm PEO-b-PMAA polymer chains were extended due to repulsion interactions between highly charged MAA segments at pH 8. Imipramine hydrochloride protonized at such pH environment provided the possibility for binding onto the polymer through electrostatic interactions. The particles size measurement was conducted for 0.1 wt% of four-arm PEO-b-PMAA polymer in 0.01 M NaCl solution, where the IPH concentration varied from 0 mM to 6 mM covering the entire binding regime. Figure 8.6 shows the relaxation time distribution functions of the polymer/drug complex at scattering angle of 90° in the presence of 0 mM, 3.6 mM, 4.8 mM, and 6 mM IPH. In the absence of IPH, a single mode was observed for the four-arm PEO-b-PMAA polymer at the fully ionization status that corresponded to the star shape unimer of bout 12 nm. As the IPH solution was added into polymer solution at pH 8, the distribution function shifted to a higher relaxation time which indicated the formation of polymer/drug complexes. The apparent hydrodynamic radius ($R_h$) of the particles was determined to be about 55 nm in the polymer solution containing 3.6 mM IPH, where the molar ratio of amine group of IPH and carboxylic groups of polymer is about 0.6. This suggested that the IPH bound to some MAA groups caused the PMAA segments to become more hydrophobic. The hyper coiled polymers together with IPH formed aggregates through hydrophobic interaction between the carboxylic acid groups. With increasing IPH concentration, the distribution peak became more significant and shifted slightly to lower relaxation time, with a corresponding increase in the scattering intensity. This trend is attributed to
more IPH being bound to the polymer chains resulting in the condensation of the polymer/drug aggregates. The $R_h$ of the polymer/drug complex was about 38 nm and 32 nm for 4.8 mM and 6 mM IPH respectively.

![Figure 8.6](image)

Figure 8.6  Relaxation time distribution functions at scattering angle of 90° for four-arm PEO-$b$-PMAA with the presence of different amount of IPH.

The relaxation time distribution functions for the solutions were also measured at different scattering angles in order to determine the dependence of decay rates ($\Gamma$) on square of scattering vector ($q^2$). It is found that $\Gamma$ exhibited a linear relationship with $q^2$, which suggested that the distribution function was attributed to the translation diffusion of the scattering objects. The complex solution remained optically transparent during the entire course of binding brought about by the steric stabilization of the hydrophilic PEO chains on the complexes. However, with further addition of IPH to the complex solution, the ratio of amine group of IPH and carboxylic groups of polymer exceeded 1, and the mixture became turbid and
eventually flocculation occurred. According to the dynamic light scattering analysis of the polymer and drug aggregation, the molar ratio of polymer/drug was optimized for drug loading and release studies.

### 8.3.3 In vitro release studies

The star shape four-arm PEO-\textit{b}-PMAA underwent a conformation transition due to ionization/deionization at varying pH as described in our previous study. PMAA has a compact conformation when a critical charge density is reached. At low degree of neutralization, the block copolymers self-assembled into large spherical aggregates because the methyl groups on PMAA induce a stronger hydrophobic interaction. The drug release studies were conducted in 10 mM phosphate buffer solution at pH 6.1, pH 7.4 and pH 8.3 corresponding to the degree of neutralization of 0.3, 0.7, and 0.9. The p\textit{K}_a of imipramine hydrochloride is 9.5, which means that free imipramine molecules in solution will be protonated in the course of the release process. This suggested that IPH is an ideal model drug for the studies of release mechanisms induced by electrostatic interactions.

Figure 8.7 shows the released behavior of 6 mM IPH loaded four-arm PEO-\textit{b}-PMAA nanoparticles in 10 mM PBS at various pHs at 37°C. At the initial stage, a rapid release of IPH was observed, however the release rate of IPH from the aqueous
dispersion was substantially slower. The release rate of IPH from the star polymer showed significant dependence on the pH of the release media and it decreased with increasing pH of the media. The percentage of IPH released was also affected by pH, which were found to be 94 %, 81 % and 66 % of the loading drug for release media at pH 6.1, pH 7.4 and pH 8.3 respectively.

The semi-empirical equation in the form of power law relationship was applied to describe the drug released from the polymeric systems:

\[
\frac{M_t}{M_\infty} = k t^n
\]  

(8.3)

where \(M_t\) and \(M_\infty\) are the absolute cumulative amounts of drug released at time \(t\) and infinite time, respectively; \(k\) is the release constant; exponent \(n\) describes the kinetic
and the release mechanism, which depends on the geometry of the system. The value of $n$ can be used to determine the type of solute transport in the system, that is $n = 0.5$ indicating diffusion-controlled drug release and $n > 0.5$ suggesting non-Fickian transport. Eq. (8.3) can be modified as the following equation:

$$\log \left( \frac{M_t}{M_\infty} \right) = n \log t + \log k$$  \hspace{1cm} (8.4)

Figure 8.8 shows the linear relation of $\log(M_t/M_\infty)$ as a function of $\log(t)$, where $n$ was derived from the slope of plot. Typically, the data of $M_t/M_\infty$ can be fitted to the linear relationship for drug release up to about 60%. The values of $n$ range from 0.53 to 0.81 for all pHs, which implies that the drug release mechanism represents anomalous transport. The release behavior is dominated by chain relaxation induced by ion exchange. The cationic drug binds to the ionized polymer chains, and the diffusion of drug from the complexes is inhibited by electrostatic attraction. The relatively faster release rate at low pH was due to the partially ionized MAA groups on polymer segments that induced very minimal electrostatic interactions. The release of drug occurs through the diffusion of undissociated drug molecules due to counter-ion condensation. At the initial stage, the burst release was caused by ion exchange between the outer protonated drug on the nanoparticles and cations in media. Thereafter, the polymer/drug complex became less compact due to the smaller interaction of polymer/drug and increasing repulsion between macroions. The relaxed polymer chains are favorite to release agents buried inside the polymer/drug complex
which facilitate the drug release.

![Graph showing the power law model for the release of IPH from four-arm PEO-b-PMAA at various pHs.](image)

Figure 8.8 The calculated experimental data based on the power law model for the release of IPH from four-arm PEO-b-PMAA at various pHs: (■) pH= 6.1; (♦) pH=7.4; (▲) pH=8.3.

### 8.4 Conclusions

At high pH, the star shape four-arm PEO-b-PMAA block polymer chains extended to a three-dimensional tetrahedron unimeric structure of 12 nm in size. As the IPH was added to the polymer, the charged carboxylic groups of polymer were neutralized resulting in the formation of hyper coiled aggregates bound with cationic drug through electrostatic and strong hydrophobic interaction force inducing by methyl groups on PMAA. The particles sizes are 32 nm to 55 nm for different amount of IPH present, which is a suitable size range for drug delivery.
The binding mechanism of polymer/drug comprised of electrostatic attraction, hydrogen bonding and hydrophobic interactions between macroions and drug molecules, which was affected by the properties of media. Isothermal Titration Calorimetry (ITC) was used to measure the enthalpy profiles of drug binding over a predefined range of IPH concentrations. With changes in the media of polymer/drug particles solution, the interaction mechanism was verified to provide important information for drug loading and release.

The distinct drug selective membrane electrode was prepared to monitor the drug release by measuring electromotive force (EMF). The release profiles were determined in different pH environment of the release media, and the release rate decreased with increasing pH of the media. The relatively faster release rate at low pH was due to the partially ionized MAA groups on the polymer segments, which exhibited a relatively small electrostatic interaction forces between the polymer chains and IPH. The values of n determined from the power law model range from 0.53 to 0.81 for all pHs, suggesting that the drug release mechanism is anomalous transport, where the release behavior was dominated by the chain relaxation induced by ion exchange.
9 Chapter Nine
Conclusions and Recommendations

9.1 Conclusions

9.1.1 Synthesis of star shape multi-arm polyelectrolytes

In the materials synthesis part of this research, the polyacid and polybase with three-dimensional tetrahedral structure were successfully synthesized using the ATRP technique. The polymer architecture plays an important role in defining the physicochemical properties of the polymer in solution. The higher densities of terminal functional groups on the block copolymer chains induce stronger interactions compared to linear structure of identical molecular weights.

Well-defined four arm PEO-\textit{b}-PDEAEMA and four arm PEO-\textit{b}-PrBMA block copolymers with narrow PDI were obtained under the reaction condition of CuCl/HMTETA catalyst system in anisole at 90 °C. These polymers were characterized by GPC and NMR to confirm the chemical structures comprising of four arm PEO_{56}[^\textit{b}]-PDEAEMA_{74} and four arm PEO_{56}-PrBMA_{88}. After hydrolysis of four-arm PEO-\textit{b}-PrBMA, the polyacid formed of the block copolymer (four-arm
PEO-b-PMAA) was obtained. The polyelectrolytes were dialyzed and purified prior to their evaluation for gene and drug delivery applications.

9.1.2 Association behaviors of polyelectrolytes in aqueous solutions

The four-arm polyethylene-oxide with polybase and polyacid blocks are stimuli responsive block copolymers that respond to changes in pH and ionic strength. Both polymers could form core/shell micelles structure in prescribed range of pH and the size of micelles could be manipulated by adjusting the environmental conditions. We observed that the four-arm PEO-b-PMAA block copolymers can reorganized from small micelles into large spherical compounds that flocculated at very low degree of neutralization (\(\alpha\)).

A molecular-level understanding on the association mechanism and the microstructure of the aggregate in aqueous medium was examined by potentiometric titration, light scattering, tensiometer, isothermal titration calorimetric and TEM. The effects of salt on the association behaviors of amphiphilic polymer solution were studied by adding small molecular electrolyte (NaCl) into the aqueous polymer solutions.

The four arm PEO-b-PDEAEMA block copolymer was a weak polybase, which exists as unimers at low pH. However, by adjusting the pH, the polymer formed micelles
consisting of a hydrophilic PEO corona and hydrophobic PDEAEMA core. The important findings for multi-arm star copolymers are their compact and stable aggregate morphologies in aqueous solutions, which have potential application as DNA carrier.

The potentiometric titrations provided information on the conformational transition of the four-arm PEO-b-PDEAEMA block copolymer as a function of the degree of protonation. At high pH, DEAEMA groups on the polymer chain were deprotonated, which induced micellization that produced a core-shell micelle consisting of a hydrophobic DEAEMA core and a PEO hydrophilic shell. With the addition of HCl, DEAEMA segments became increasingly charged, and the electrostatic repulsion between the charge segments caused the micelle to swell. When the degree of protonation exceeded 0.5, the micelle started to dissociate due to an overwhelming electrostatic repulsions resulting in the demicellization to unimers. The swelling process of the micelle during the conformational transition was indicated by a plateau range on the curve of pH against degree of protonation. The expansion-dissociation of the polymer was driven by electrostatic repulsion between protonated DEAEMA groups and the balance of forces between hydrophobic interaction of DEAEMA group and electrostatic repulsion controlled the aggregation behaviors of polymer in solution.
With the addition of small electrolyte molecules in the polymer solution, the aggregation behaviors of four-arm PEO-\(b\)-PDEAEMA block copolymer was affected. Ionic species of salt shielded the electrostatic repulsion between protonated DEAEMA groups and stabilized the charged amine groups. The maximum hydrodynamic radius of swollen micelle was reduced with increasing salt concentration. The degree of protonation for the onset of micelle dissociation was reduced and the protonation of four-arm PEO-\(b\)-PDEAEMA block copolymer became more favorable in the presence of salt.

The aggregation behaviors of four-arm PEO-\(b\)-PMAA were studied over the course of neutralization in aqueous solution. At high pH environment (\(\alpha=1\)), the carboxylic groups on the polymer chains were complete neutralized resulting in a highly charged MAA segments. The repulsive electrostatic interaction between polymer chains yielded unimers in aqueous solution. By reducing pH, the carboxylate groups were transformed to carboxylic acids and the hydrophobic interaction between the carboxylic acid groups produced micelles at degree of neutralization (\(\alpha\)) exceeding 0.3. With further addition of HCl, the repulsive electrostatic interactions between ionized carboxylate groups were reduced causing the aggregates to shrink for \(\alpha\) up to 0.1. When \(\alpha\) reached approximately 0.05, the hydrogen-bonding between MAA groups induced the formation of larger aggregates with \(R_h\) of about 120 nm. The polymer solution was transparent, however it turned opaque and even larger particle of 410 nm
was detected at $\alpha \sim 1$.

PMAA possesses a compact conformation with a critical charge density and it exhibit an abrupt phase transition compared to the continuous phase transition of PAA, because the methyl groups in PMAA induce a stronger hydrophobic interaction force. Such behavior was controlled by the fine balance of electrostatic, hydrophobic and hydrogen bonding interaction forces. We observed that the four-arm PEO-$b$-PMAA block copolymers self-assembled into large spherical aggregates that flocculated at very low degree of neutralization ($\alpha$). The thermodynamic parameters obtained by isothermal titration calorimetric technique with different salt concentrations indicated that the energy to extract a proton from a charged polyion was reduced with the addition of salt which favors the neutralization process.

9.1.3 Interactions between polybase and plasmid DNA

The polymeric chains are positively charged in physiological environment (pH of around 7) which could condense an extended therapeutic DNA, which is meaningful for DNA crossing through a number of biological barriers. DNA introduced into cells could unleash many strategies for gene therapy that promise considerable therapeutic possibilities against a wide range of genetic diseases. The four-arm PEO-$b$-PDEAEMA block copolymer bound spontaneously to plasmid DNA to form
complexes, driven by cooperative electrostatic interactions between DNA and oppositely charged amine groups. These processes are thermodynamically and kinetically controlled by the structure of the interacting species and environmental characteristics, including pH and ionic strength. Light scattering studies provided information on the morphology and size as a function of the molar ratio of polymer and DNA. The thermodynamic of binding was determined using the isothermal titration calorimeter. Zeta potential experiment provided information on the surface charges of the complex. The physicochemical properties of the complex were elucidated further by transmission electron microscopy.

The addition of a neutral salt significantly alters the complexes size in Hepes buffer solution, which is an important factor for cell transfection. With salt addition in polymer/DNA complex solution, the complex becomes more loosely packed since the electrostatic interaction is affected by small electrolytes.

**9.1.4 The binding mechanism and release studies for polyacid and drug system**

The polyacid self-assembled into core-shell micelles at low pH and transformed into unimers at high pH. The negatively charged segments on the polymer chains interacted with cationic drug through electrostatic interaction to form polymer/drug
complexes. The four-arm PEO-b-PMAA block copolymer serves as reservoirs for drugs loading to fulfill designed specific functionalities. Meanwhile, the biocompatible hydrophilic PEO segments position between the core and the external aqueous medium to stabilize the hydrophobic core. The drug loading was manipulated by the electrostatic force, hydrogen bonding and hydrophobic interaction corresponding to the molar ratios of polymer over drug.

The hydrodynamic radius ($R_h$) of polymer aggregates and polymer/drug particles were determined by light scattering and range from 46 nm to 84 nm and 32 nm to 55 nm at different pH respectively, which is a suitable size for drug delivery. The thermodynamic parameters and interactions between polymer and drug were studied in detail by isothermal titration calorimetric technique. Release exponent $n$ is greater than 0.5 indicating non-Fickian type diffusion mechanisms. The release behavior was dominated by the chain relaxation induced by ion exchange.

### 9.2 Recommendations for Future Work

#### 9.2.1 Comparison of multi-arm polyelectrolytes

Well-defined four-arm polyelectrolytes were successfully synthesized by ATRP. With
this technique, the star block copolymer with other number of arms can be synthesized for further evaluation. The architecture of polymer significantly affects the morphology, interactions and properties of polymer in solution. It is interesting to compare the properties of polymers comprising various numbers of arms that can be tailored for useful application.

9.2.2 Associated behavior of block copolymer with grafted complementary functional groups

The polybase and polyacid are responsive to changes in the pH environment, which form micelles in a certain pH range. The multi-arm diblock copolymers can be grafted further with appropriate functional groups to form tri-block copolymers. The $pK_a$ of tri-block copolymers are varied to provide important information relevant for the delivery of biomolecules. The aggregation behaviors of polymers in aqueous solution can be studied to elucidate their conformation transition and to verify their application for the delivery of chemical compounds and biomolecules.

9.2.3 Further development for applications in biomolecular systems.

Water soluble amphiphilic block copolymers can spontaneously self-aggregate into micellar aggregates comprising of a hydrophobic core and hydrophilic shell. Polybase
form micelles at high pH ranging from 10 to 100 nm in size and this property is desirable for other biomolecular carriers, such as RNA. With similar interesting microstructure, the polyacid ionized at high pH and the negatively charged segments at neutral pH provide the possibility of binding with proteins.
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