SYNTHESIS AND CHARACTERIZATION OF WELL-DEFINED DIMETHYLAMINOETHYL METHACRYLATE POLYELECTROLYTES FOR NON-VIRAL ANTISENSE OLIGONUCLEOTIDES DELIVERIES

SYNTHESIS AND CHARACTERIZATION OF WELL-DEFINED DIMETHYLAMINOETHYL METHACRYLATE POLYELECTROLYTES FOR NON-VIRAL ANTISENSE OLIGONUCLEOTIDES DELIVERIES

By

XIAOPIN JIN, B. ENG.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

For the Degree of

Master of Applied Science

McMaster University

MASTER OF APPLIED SCIENCE (2003) McMASTER UNIVERSITY

(Chemical Engineering) Hamilton, Ontario, Canada

TITLE: Synthesis and Characterization of Well-defined Dimethylaminoethyl Methacrylate Polyelectrolytes for Non-viral Antisense Oligonucleotides Deliveries

AUTHOR: Xiaopin Jin
B.Eng. (Nanjing University of Chemical Technology, Nanjing, China)

SUPERVISOR: Dr. Shiping Zhu

NUMBER OF PAGES: xvii, 121

ABSTRACT

Cationic polyelectrolytes have attracted growing attention in the field of non-viral oligonucleotides (ONs) deliveries because of their ability to bind ONs by electrostatic interactions for efficient cellular uptake. However the formation of electrostatic polymer/ONs complexes and their biological effects are still poorly understood. The relationships between polymer structure and complexation performance have not been well established. The objectives of this research are to synthesize and characterize well-defined and well-controlled cationic polyelectrolytes and to evaluate the effects of polyelectrolyte chain properties on ONs complexation. Poly(2-(dimethylamino) ethyl methacrylate) (polyDMAEMA) and its derivatives are used as the polymer candidate. A fluorescein-labeled oligonucleotide, 5'-FGCGGAGCGTGGCAGG-3' (F: fluorescein), is used as the oligonucleotide candidate.

Low-molecular-weight cationic polyDMAEMA samples having narrow molecular weight distribution were synthesized by living anionic polymerization (LAP) and atom transfer radical polymerization (ATRP) methods. Fully charged polyDMAEMA quats were prepared by sequential quaternization of polyDMAEMA samples, as well as by direct ATRP of the quaternized DMAEMA monomer. An aqueous GPC calibration method was first developed for the characterization of these cationic polyelectrolytes. It was found that the type of counter-ion has little effect on the hydrodynamic volume of polyDMAEMA quat. Therefore the dimethyl sulfate salt of polyDMAEMA provided a reliable calibration standard for other types of quaternized DMAEMA homopolymers.

Cationic block copolymers of polyDMAEMA with 2-hydroxyethyl methacrylate (HEMA) and polyethylene glycol (PEG) were also prepared by ATRP. It was found that the order of monomer addition, solvent type, temperature, and molecular weight of macroinitiator have significant effects on the living feature of the polymerization. Well-controlled block copolymers were obtained when polyHEMA was used as the macroinitiator.

The complexation capability of the prepared polyelectrolytes with oligonucleotides (15 mer) was evaluated by a fluorescence technique. It was found that the complexation performance depends on polymer molecular weight, charge density, and counter-ion type, as well as polymer concentration and block composition. The polymer sample that has double molecular weight of the ONs gave the optimal complexation performance.

Acknowledgements

I would like to express my sincere gratitude to Prof. Shiping Zhu for his supervision of this research. It has been a real honor and pleasure to work with him. His enthusiasm, insight, energy, and high standard are amazing and truly unforgettable. Not only do I value his academic quality and reputation, but also his personal trait and friendship. He will continue to be a source of motivation for my career.

I would also like to thank Prof. Youqing Shen, a former group member now in University of Wyoming USA, for his collaboration in polymer preparation. Thanks must also go to Prof. Yingfu Li and John C. Achenbach, Department of Biochemistry, McMaster University, for their collaboration in oligonucleotide complexation testing and Prof. Steve P. Armes and Dr. Yuting Li, University of Sussex UK, for sample donation.

I am also grateful to my colleagues: Aileen Wang, Wenjun Wang, Sam Zhen, Leming Gu, Santiago Faucher, Wei Feng, Zhibin Ye and Fahad AlO'baidi for their help and suggestions. I thank the departmental staff: Kathy Goodram, Julie Birch, Lynn Falkiner, Doug Keller, Gord, Slater, and Justyna Derkach, for their kind help and support.

In addition, I acknowledge the Department, the University, and the Ontario Graduate Scholarship program for their financial supports.

"A friend in need is a friend indeed." A very special thank-you must go to my personal friends: Joanne Du and her family, Huining Xiao and his family, Aileen Wang, Lina Liu and her family, Luwei Liu and her family, Ying Lin, Shiyan Li, Lei Guo, Liping Gu, Ting Sheng, Juming Ding, and Redshine Goldberg for their help, friendship and love.

As always, the generosity, patience, support, and love of my family, especially my dear daughter Hannah, have been essential to the successful completion of this study. They make everything worthwhile!

TABLE OF CONTENTS

Abstract
Acknowledgements
Table of Contents
List of Figures
List of Tables
List of Schemes

Chapter 1 Introduction 1
1.1. Polyelectrolytes 1
1.2. Bio-active and bio-compatible polymers 3
1.2.1. Bioactive polymers 3
1.2.2. Biocompatible polymers 5
1.3. Bio-application of polyelectrolytes 7
1.3.1. Gene delivery 7
1.3.2. Antisense oligonucleotides delivery 10
1.4. Atom transfer radical polymerization for polyelectrolytes preparation 13
1.4.1. Background of living polymerization 13
1.4.2. Living radical polymerization 14
1.4.2.1. Stable free radical polymerization (SFRP) 15
1.4.2.2. Reversible addition-fragmentation chain transfer polymerization (RAFT) 17
1.4.3. Atom transfer radical polymerization (ATRP) 19
1.4.3.1. Mechanism of ATRP 20
1.4.3.2. Kinetic of ATRP 21
1.4.3.3. Application of ATRP for polyelectrolytes 22
1.4.3.3.1. Synthesis of well-defined polyelectrolytes 23
1.4.3.3.2. Synthesis of water-soluble block copolymers 25

1.5. Research Objectives..... 27
1.6. Contents of thesis..... 28

Chapter 2 Characterization of well-defined poly(2-dimethylamino) ethyl methacrylate) quaternary salt..... 31
2.1. Introduction..... 31
2.2. Experimental section..... 33
2.2.1. Materials..... 33
2.2.2. Preparation of polymer samples..... 34
2.2.2.1. Synthesis of polyDMAEMA..... 34
2.2.2.2. Preparation of polyDMAEMA quaternary salts..... 34
2.2.3. Measurements..... 35
2.2.3.1. NMR spectroscopy..... 35
2.2.3.2. Gel permeation chromatography..... 35
2.3. Results and discussion..... 36
2.3.1. Quaternization of polyDMAEMA..... 36
2.3.1.1. Effect of solvent..... 38
2.3.1.2. Effect of quaternization agent..... 39
2.3.1.3. Effect of polymer molecular weight..... 40
2.3.2. Characterization of polyDMAEMA quaternary salt by aqueous GPC.... 40
2.3.2.1. Effect of ionic strength of mobile solution on MW determination..... 41
2.3.2.2. Effect of charge density on MW determination..... 44
2.3.2.3. Effect of counter ion type of quaternary salt..... 45
2.3.2.4. Aqueous GPC calibration method of polyDMAEMA quaternary salt..... 46
2.3.3. Application of calibration of polyDMAEMA quaternary salt. 49
2.4. Conclusion..... 51

Chapter 3 Atom transfer radical polymerization of 2-(*N, N*-dimethylamino) ethyl methacrylate and 2-hydroxyethyl methacrylate block copolymer..... 53
3.1. Introduction..... 53
3.2. Experimental section..... 55
3.2.1. Materials..... 55
3.2.2. Synthesis of initiator..... 56
3.2.3. Polymer preparation..... 57
3.2.3.1. Synthesis of homo-polymers..... 57
3.2.3.2. Synthesis of block copolymers..... 58
3.2.4. Measurements..... 59
3.3. Results and discussion..... 60
3.3.1. Homo-polymerization of DMAEMA..... 60
3.3.2. Homo-polymerization of HEMA..... 61
3.3.3. Block ATRP of poly(DMAEMA-b-HEMA)..... 65
3.3.4. Block ATRP of poly(HEMA-b-DMAEMA)..... 66
3.3.5. Effect of water as accelerator..... 73
3.3.6. Preparation of poly(HEMA-b-DMAEMA) with various block molecular weights..... 78
3.4. Conclusion..... 81

Chapter 4 Complexation of well-controlled low-molecular-weight poly-electrolytes with antisense oligonucleotides..... 82
4.1. Introduction..... 82
4.2. Experiment section..... 84
4.2.1. Materials 84
4.2.2. Preparation of cationic polymers 85
4.2.3. Oligonucleotides and buffers..... 87
4.2.4. Preparation of polymer/oligonucleotides complexes..... 88
4.3. Measurements..... 88

4.4. Results and discussion 89
4.4.1. Complexation kinetics 90
4.4.2. Effect of polymer molecular weight 96
4.4.3. Effect of charge density and counter ion type 100
4.4.4. Effect of hydrophilic block 102
4.5. Conclusion 104

Chapter 5 Conclusion and recommendation 106
5.1. Contributions of this thesis work 106
5.2. Recommendations 109

References 112

List of Figures

Figure 1.1. Polymer and polyelectrolyte chain behavior in solution..... 2

Figure 1.2. The electrostatic interaction and condensation of cationic polyDMAEMA and anionic plasmid DNA..... 5

Figure 1.3. Complexation of hydrophilic biocompatible block copolymer with plasmid DNA..... 7

Figure 1.4. Inhibition of mRNA translation by antisense oligonucleotides..... 11

Figure 1.5. Scheme of molecular weight vs conversion for living polymerization..... 13

Figure 1.6. Conversion vs time and semilogarithmic plot of a typical ATRP.... 22

Figure 2.1. ^{1}H NMR spectrum of polyDMAEMA (A, top) and quaternized polyDMAEMA (B, bottom)..... 37

Figure 2.2. Effect of eluent ionic strength on separation of polyDMAEMA-DMSQ in sodium sulfate solution. (a) M_n = 78500; (b) M_n = 7580..... 43

Figure 2.3. Effect of quaternization degree on the hydrodynamic volume of polyDMAEMA quaternary salt..... 44

Figure 2.4. Effect of quaternization degree on the GPC retention time of different molecular weight polyDMAEMA-DMSQ samples..... 45

Figure 2.5. Effect of counter ion type on the hydrodynamic volume of polyDMAEMA quaternary salts..... 46

Figure 2.6. GPC overlay traces of polyDMAEMA-DMSQ standards in 0.3M Na_2SO_4-0.1 (w/v) NaN_3 solution..... 48

Figure 2.7. Calibration curve of polyDMAEMA-DMSQ in 0.3M Na_2SO_4-0.1 (w/v) NaN_3 solution..... 48

Figure 3.1 Kinetic plot and (b) Mn and polydispersity for the ATRP of DMAEMA in 50% w/v MeOH at room temperature and 60 °C..... 62

Figure 3.2 (a) Kinetic plot and (b) Mn and polydispersity for the ATRP of HEMA in 50% w/v MeOH at room temperature and 60 °C. 64

Figure 3.3 GPC chromatograms of polyDMAEMA and poly(DMAEMA-b-HEMA)..... 67

Figure 3.4 (a) Kinetic plot and (b) Mn and polydispersity for the block ATRP of poly (HEMA-b- DMAEMA) in 50% w/v MeOH at room temperature..... 69

Figure 3.5 GPC chromatograms of poly(HEMA-b-DMAEMA). The samples were prepared in methanol at room temperature with different block molecular weights..... 70

Figure 3.6 (a) Kinetic plot and (b) Mn and polydispersity for the block ATRP of poly(HEMA-b- DMAEMA) in 50% w/v MeOH at room temperature and 60 °C..... 74

Figure 3.7 (a) Kinetic plot and (b) Mn and polydispersity for the block ATRP of poly (HEMA-b-DMAEMA) in 50% w/v MeOH at 60 °C..... 75

Figure 3.8 (a) Kinetic plot and (b) Mn and polydispersity for the block ATRP of poly (HEMA-b-DMAEMA) at room temperature with H_2O as accelerator

accelerator. 77

Figure 3.9 ^{1}H NMR spectra of polyHEMA and poly (HEMA-b-DMAEMA) with peaks assigned, in Methanol-d_4..... 80

Figure 4.1 The relative fluorescence intensity as a function of the molar ratio of amino units / nucleotides. The fluorescence of the F-ON solution without polymer addition was set to 100%..... 90

Figure 4.2 Kinetic study of fluorescence intensity as function of time for two polyDMAEMAs complexation with ONs..... 91

Figure 4.3 The dependence of complexation kinetics on the polymer molecular weight..... 93

Figure 4.4 The effects of polymer molecular weight on the complexation of polyDMAEMA with F-ON..... 97

Figure 4.5 The effect of charge density on the complexation of polyDMAEMA quats with F-ON..... 101

Figure 4.6 The effect of counter ion on the complexation of polyDMAEMA quats with F-ON..... 102

Figure 4.7 The effect of polyHEMA block on the complexation of polyDMAEMA with F-ON..... 103

Figure 4.8 The effect of PEG block on the complexation of polyDMAEMA-MCQ with F-ON..... 104

List of Tables

Table 1.1	Examples of biologically active moieties.....	4
Table 2.2.	Table 2.1. Effects of solvent, quaternization agent, agent/polymer ratio, reaction time and polymer molecular weight on quaternization efficiency at room temperature.....	39
Table 2.2.	GPC data of molecular weight and MWD of polyDMAEMA and polyDMAEMA-DMSQ.....	47
Table 2.3.	GPC calibration data of polyDMAEMA-MCQ samples with polyDMAEMA-DMSQ as standard.....	50
Table 3.1	Summary of the synthesis parameters and molecular weight data for ATRP of HEMA at room temperature.....	65
Table 3.2	Summary for the preparation of poly(HEMA-block-DMAEMA) by ATRP under different conditions.....	72
Table 3.3	Summary of the synthesis parameters and molecular weight data for block copolymerization of polyHEMA-DMAEMA via ATRP in MeOH at room temperature.....	79
Table 4.1	Properties of DMAEMA-based polyelectrolytes.....	86
Table 4.2	Summary of DP, number of protonated amino groups, lowest FI and corresponding molar ratio of amino units over ON bases for polyDMAEMA samples.....	99

List of Schemes

Scheme 1.1	Structure of (a) poly (2-dimethylamino) ethyl methacrylate; and (b) poly (2-dimethylamino) ethyl methacrylate quaternary salt.....	3
Scheme 1.2	Scheme 1.2. Mechanism of SFRP.....	15
Scheme 1.3	Structure of Diallyldimethylammonium chloride and Styrenesulfonate sodium salt.....	17
Scheme 1.4	Mechanism of RAFT.....	18
Scheme 1.5	Structure of poly (sodium 4-styrenesulfonate-b-sodium 4-vinylbenzoate) and poly(N,N-dimethylvinylbenzylamine-b-(ar-vinylbenzyl) trimethylammonium chloride) block copolymers.....	19
Scheme 1.6	Mechanism of ATRP.....	20
Scheme 1.7	Structure of a range of non-ionic, anionic and cationic hydrophilic monomers.....	23
Scheme 2.1	Synthesis and subsequent quaternization of polyDMAEMA.....	35
Scheme 2.2	Structure and molecular weight of polyDMAEMA quaternary salt with or without counter-ions.....	49
Scheme 3.1	Synthesis of OEGBr initiator.....	56
Scheme 4.1	Three definable steps in the complexation of cationic polymers with anionic F-ON: the performance of high molecular weight polymer versus low molecular weight polymer.....	95

Scheme 4.2 The diffusion/shielding effect of high-molecular-weight polyDMAEMA on the complexation..... 98

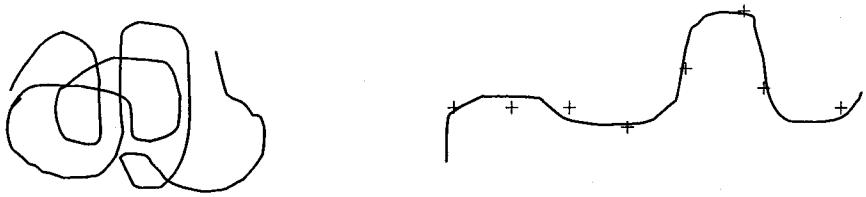
Chapter 1 Introduction

1.1. Polyelectrolytes

Polyelectrolytes are defined as macromolecules carrying a large number of ionizable sites (Armstrong, et. al., 1996). There are two categories of organic polyelectrolytes: one derived from natural sources and the other derived from synthetic sources. Natural polymers from biological sources include proteins, polysaccharides, nucleic acids, and etc. Synthetic polymers may vary from the monomer repeat unit and the nature of their charge including polyacrylamide (PAM), poly(acrylic acid), poly(2-dimethylamino) ethyl methacrylate (polyDMAEMA), and etc.

Polyelectrolytes are mainly classified into two types: anionic and cationic depending upon the nature of residual charge on the polymer in aqueous solution. As the repeat unit may be derived from a single monomer or multiple monomers, polymers can be subdivided into homopolymer and copolymer. The latter includes random, alternating, block and graft polymer.

Because of the charged groups on polymer chain, polyelectrolytes in dilute solution normally expand coil dimension more than nonionic polymers as seen in Figure1.1.



A polymer molecule assumes a random coil

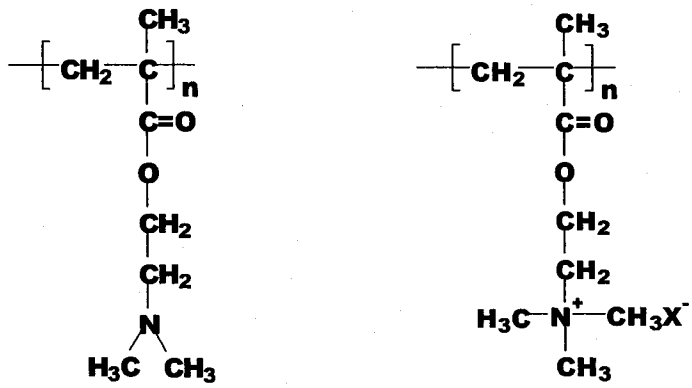
A polyelectrolyte expands because of charge repulsion

Figure 1.1. Polymer and polyelectrolyte chain behavior in solution

Polyelectrolytes have attracted great attention because of their original properties such as solubility in water, ability to interact with charged particles and surface, and chemical and biological stability. They are widely used in many fields, such as agriculture, paper manufacture, water treatment, oil recovery, medicine, biotechnology, and electronics as flocculation and retention agents, thickening and gelling agents, prolongers of drugs, biocatalysts, sensors, gene delivery vectors and so forth (Hoover, et. al., 1970; Creutz, et. al., 1998, 2000; Sassi, et. al., 1996; Rungsardthong, et. al., 2001).

The most versatile and useful type of polyelectrolytes is the cationic polymers, which are mainly comprised three classes: ammonium (amines and quaternaries), sulfonium, and phosphonium quaternaries. The cationic polyelectrolytes studied in this work are 2-(dimethylamino)ethyl methacrylates based (co)polymers and their quaternary salts which are one of the most extensively used types of cationic polymers (Scheme 1.1).

Scheme 1.1.



(a) (b)

X = Cl, I, CH_3SO_4

(a) poly (2-dimethylamino) ethyl methacrylate; (b) poly (2-dimethylamino) ethyl methacrylate quaternary salt.

1.2. Bio-active and biocompatible polymers

1.2.1. Bioactive polymers

Bioactivity can be defined as the interaction of some agent, usually chemical, on a biological system (Gebelein, et. al., 1985).

Biological active polymers include natural polymers and soluble synthetic polymers. Obviously the natural proteins, enzymes, polysaccharides, and nucleic acids are biologically active. Synthetic soluble polymers display biological activity owing to their charge, microstructure, chain conformation, etc.

There are basically three main types of bioactive polymeric systems: (1) polymers with inherent biological activity; (2) polymers entrapped and immobilized bioactive species; and (3) polymers as carriers of bioactive agents.

As the carriers, normally polymers serve as matrices in devices that permit controlled release of active substance over a long period of time. The active substance may be liberated from the intact matrix or through bioerosion or biodegradation of the polymer.

Polymers bounded with bioactive moieties have been widely used in vitro and/or vivo, such as diagnostic assays, biosensors, drug delivery, and toxin removal from blood (Piskin, et. al., 1984; Ratner, et. al., 1976). A variety of biological active species are shown in Table1.1 (Piskin, et. al., 1984).

Table 1.1. Examples of biologically active moieties

Active moieties	**Functions**
Enzymes	Reaction catalysis
Antibodies	Response of immune system to the presence of an antigen
Antigens	A substance that causes a response of the immune system
Antibiotics	Substance that is able to strongly inhibit growth of bacteria or that causes death of the bacteria
Antibacterial	Substance that that causes death of the bacteria
Hormones	Contraceptives
Antisense nucleotides	Blocking of protein expression

Bioactive polycations, such as polyDMAEMA, act mainly through their interaction with negatively charged surfaces such as cell surface, especially those of tumor cells, DNA, antisense oligonucleotide. Molecular weight and charge density play decisive roles in activity. An example of electrostatic interaction and condensation of cationic polymer and anionic plasmid DNA is shown in figure 1.2.

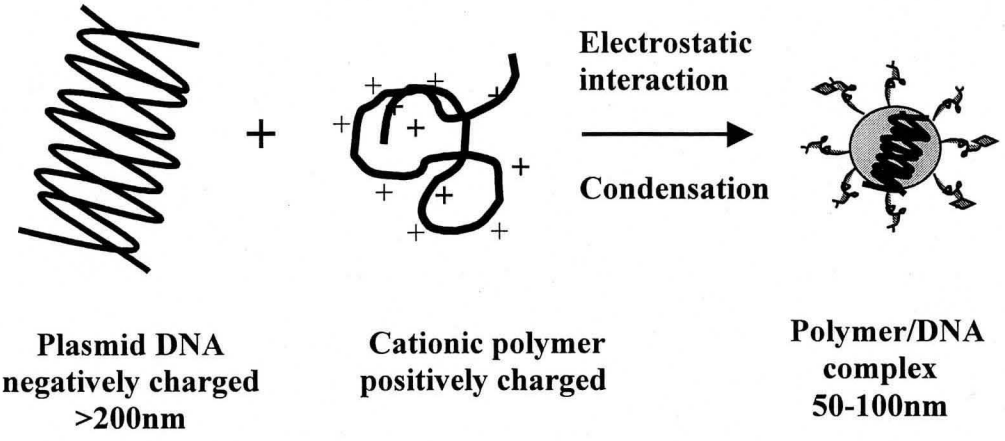


Figure 1.2. The electrostatic interaction and condensation of cationic polyDMAEMA and anionic plasmid DNA

1.2.2. Biocompatible polymers

As bioactive polymeric systems are considered for human or animal use, the most important characteristic required is biocompatibility. There is no absolute definition of biocompatibility because we have to considerate the situation of a given material in a given application for a given duration. Black (1981) defined biocompatibility as "the biological performance of a given polymer in a specific application that is judged suitable to that situation". Williams (1987) defined that biocompatibility is "the ability of a material to perform with an appropriate host response in a specific application". In a simple word, a biocompatible material should disrupt normal body functions as little as possible. It will not cause thrombogenic, toxic, or allergic inflammatory response in

vivo. It must not stimulate changes in plasma proteins and enzymes or cause any adverse effects on the biological system.

The good examples of biocompatible polymers are polyethylene glycol (PEG) and poly(2-hydroxylehtyl) methacrylate (polyHEMA). PEG has been widely used in foods, cosmetics and medications. PEG-conjugated prodrugs have been demonstrated to be an effective means for delivery of the amino-containing anticancer agent as well as a variety of other drugs (Greenwald, et. al., 2000). Recently, PEG was commercially used to conjugate to biologically-active proteins, such as polypeptides and interferons, to improve of the properties of circulating blood, water solubility or antigenicity in vivo (Gillbert, et. al., 1999). PolyHEMA has been used as biocompatibilizers, contact lenses, and hydrogels (Ruckenstein, et. al., 1998; Roovers, et. al., 1985; Mathew, et. al., 1993; Chen, et. al. 1989).

Bioactive polyelectrolytes used as gene delivery carriers have shown high cytotoxicity depending upon the molecular weight and charge density. PEG has been commonly developed to improve the biocompatibility of polymer/DNA complexes by copolymerization or grafting or blocking on polyelectrolyte's backbone (Rungsardthong, et. al., 2001; Andersin, et. al., 2002; Dubruel, et. al., 2000). Figure1.3 shows the complexation of PEG contained hydrophilic biocompatible block copolymer with plasmid DNA.

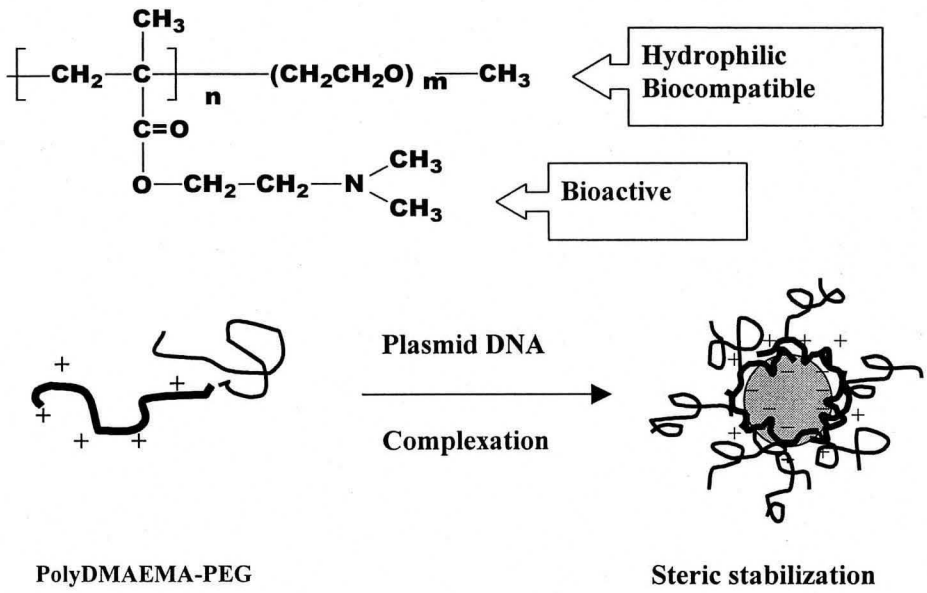


Figure 1.3. Complexation of hydrophilic biocompatible block copolymer with plasmid DNA

1.3. Bio-application of polyelectrolytes

1.3.1. Gene delivery

Gene therapy has attracted tremendous attention for treatments of genetically based diseases, such as cancers, AIDS or cardiovascular diseases (Walsh, et. al., 1993; Huber, et. al., 1994; Herrmann, et. al., 1995). Deficient genes can be replaced, unwanted gene expressions can be modulated by introducing highly selective nucleic acids as inhibitors and/or modulators. In gene therapy, DNA is considered as prodrug for synthesis of therapeutic protein in cells (Middaugh, et. al., 1998; Ledley, et. al., 1996). To act as prodrug, DNA must be introduced into the nucleus of target cells. There are three barriers for cellular uptake DNA: 1) large size; 2) hydrophilicity; and 3) negative

charge. Therefore, in order to efficiently deliver DNA into targeted type of cells, a suitable carrier is required to condense DNA into particles that can be readily endocytosed by cultured cells.

There are two general approaches for the delivery of nucleic acids. One is viral vector, and the other is non-viral delivery system mainly using cationic polymers or liposomes. Viral vectors show excellent transfection efficiencies. However, undesired immune responses, limited carrying size of DNA and potential oncogenicity are fatal.

Liposomes are microscopic closed vesicles consisting unilamellar or multilamellar phospholipid membranes with the size varying from 20nm to 4μm in diameter. Because of their ability of encapsulation or incorporation of nucleic acid they have been widely used for efficient delivery of nucleic acids (Felgner et. al., 1987, 1994). In 1992, Legendre and Szoka (1992) found that the gene expression of cationic liposomes was 100 times higher than that of neutral and anionic liposomes. Since then much effort has been directed to develop cationic liposomes for efficient delivery of nucleic acids in vitro and in vivo (Philip et. al., 1993; Zhu et. al., 1993; Liu et. al., 1995; 1997; Xu and Szoka, 1996). The morphologies of liposomes have significant effect on the complex performance. Templeton et. al. (1997) reported that bilamellar invaginated vesicles gave an optimal DNA delivery compared to small unilamellar vesicles and multilamellar vesicles. A variety of cationic liposomes have been developed to interact with DNA. The best known systems are DOTAP (N-1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniumethyl sulphate) and DOTMA (N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-

trimethylammonium chloride) (Lipofectin®) which are now commercially available (http://web.bham.ac.uk/can4psd4/).

The use of liposomes for gene delivery provides several advantages. A major advantage is the lack of immunogenicity. In addition they can deliver different types and large sizes of nucleic acids including plasmid DNA, RNA, synthetic ribozymes, antisense molecules, and viral nucleic acids. Moreover, functional cationic liposomes, such as ligand- or antibody-bearing liposomes targeted for specific cell-surface receptors, can be designed to enhance the transfection efficiency and targeting ability. Other advantages include low costs and relative ease in large-scale production of liposomes.

However, there are some limitations on liposome gene deliveries. For instance, the transfection efficiency and gene expression are low. The half-life time is short in the circulation system because of the clearance of liposomes by serum proteins and phagocytic cells in reticuloendothelial system (Litzinger, et. al., 1997). In addition the size of the liposomes is too large and their surface charge density is too high. Furthermore, the stabilities of liposomes to mechanical rupture and flow shear are often poor.

Synthetic cationic polymer carriers are an alternative class of candidates. Cationic polymers show similar advantages as cationic liposomes such as safety (lack of immune), lower cost, and ease of manufacture on a large scale, as well as capability of carrying large DNA molecules (Lehn, et. al., 1998; Lasic, et. al., 1996; Smith, et. al., 1997). The major disadvantages are their low transfection efficiency and cytotoxicity.

Great efforts have also been undertaken to improve the transfection efficiency for polymeric no-viral gene delivery. Several cationic polymers have been currently developed for this purpose, such as polyethylenimine (PEI) (Fischer, et. al., 1999), poly(L-lysine) (Wolfert, et. al., 1999), poly(2-(N,N-dimethylamino)ethyl methacrylate) (polyDMAEMA)) (Cherng, et. al., 1996;. Van de Wetering , et. al., 1997, 1998, 1999 and 2000), and etc. Many factors play crucial roles in transfection/cytotoxicity efficiency of polymer/DNA complexes, for example polymer molecular weight, concentration of polymer and DNA, ionic strength of solvent, order of polymer and DNA additions, and mixing speed. Comparative studies show that polyDMAEMA/plasmid complexes have more efficient transfection than those complexes with well-known carriers such as poly (L-lysine) and DEAE-dextran (DEAE:diethylaminoethyl) (Van de Wetering , et. al., 1998). The stable nanoparticles of polyDMAEMA/DNA plasmids formed at a polymer/plasmid ratio above 2 (w/w). In general, complex sizes of 50-300 nm diameters can be achieved. In order to further improve transfection performance, random, block and graft copolymer of DMAEMA with ethoxytriethylene glycol methacrylate (triEGMA), N-vinylpyrrolidone (NVP), methyl methacrylate (MMA), and poly(ethlylene glycol)(PEG) were designed (Rungsardthong, et. al., 2001; Van de Wetering, et. al.,1998 and 2000).

1.3.2. Antisense oligonucleotides delivery

Since the first use for antigene therapy in 1978 by Zamecnik and Stephenson (Zamecnik, et. al., 1978) synthetic antisense oligonucleotides (AS ONs) have become

very attractive as highly selective nucleic acid drugs for inhibiting disease-causing gene expression.

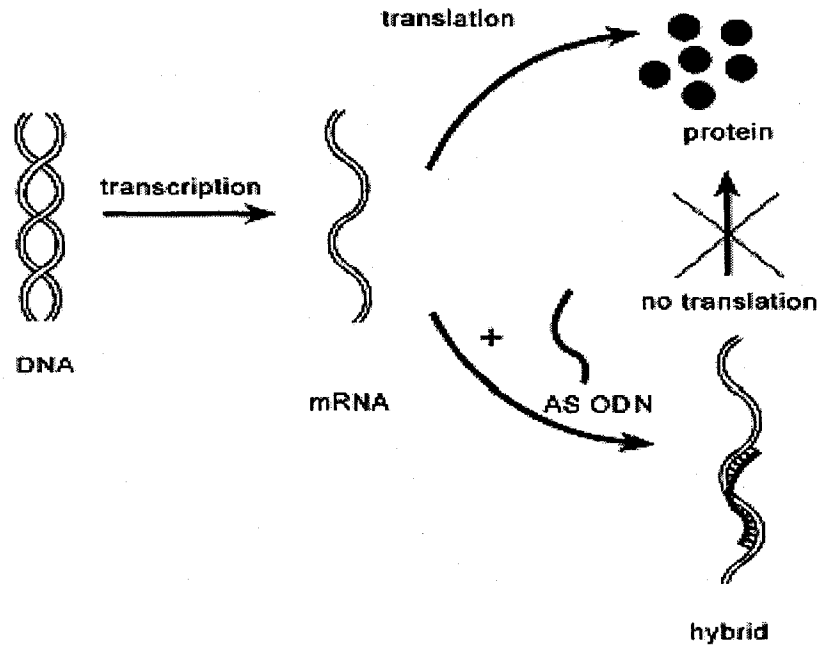


Figure 1.4. Inhibition of mRNA translation by antisense oligonucleotide (Chirila, et.al., 2002).

Differing from traditional drug therapy and gene therapy, antisense therapy use antisense oligonucleotides which display a complementary base sequence to the target messenger ribonucleic acid (mRNA) to interact with the mRNA transcripts, hence to prevent their translation into disease-related proteins (Figure 1.4). It has higher specificity and selectivity than other drug or gene strategies.

Antisense therapy has great potential to treat cancers, AIDS, inflammatory disorders and etc. The first commercial antisense therapeutic product was approved in

1998 (Chirila, et. al., 2002). However, there are several barriers for ONs to reach the cytoplasm or nucleus of target cells to perform the inhibitory functions, including (1) low cellular uptake due to relative large size, negative charge, and hydrophilicity; (2) short lifetime due to rapid degradation and nuclease; and (3) lack of targeting ability. Suitable vector/carrier systems are essential for an efficient delivery of ONs into target cells. Cationic polymers, normally used for gene delivery, have been explored for this purpose because their ability of binding ONs by electrostatic interaction and their multi-functionality.

Several cationic polymers have been developed as carriers for antisense ONs deliveries to improve cellular uptake, degradation resistance, and specificity. They include poly(L-lysine) (Wu, et. al., 1992; Leonetti, et. al., 1988), polyethylenimine (PEI) (Boussif, et. al., 1995; Dheur, et. al., 1999), and poly[2-(dimethylamino)ethyl methacrylate] (polyDMAEMA) (Van Rompaey, et. al., 2000, 2001 a and 2001 b). The use of cationic polymers as carrier results in high affinity to negatively charged membranes and high uptake rates. Unfortunately, increasing positive surface charge also yields high toxicity at both cellular and systemic levels. To overcome these drawbacks, cationic-hydrophilic copolymers have currently been developed to improve polymer biocompatibility and to prolong blood circulation time. Such polymer systems include polyspermine-poly(ethylene oxide) (PS-PEO) (Kabanov et al., 1995), poly(L-lysine)-PEO (Kataoka et. al., 1996), poly(trimethylamino) ethyl methacrylate chloride)-poly[N-(2-hydroxypropyl) methacrylamide] (poly(TMAEMA-b-HPMA)) (Read et. al., 2000).

1.4. Atom Transfer Radical Polymerization for polyelectrolytes preparation

1.4.1. Background of living polymerization

Living polymerization is defined as a polymerization process that undergoes neither chain termination nor chain transfer reaction (Zhu, 1999). The propagating centers of polymer chains exist even at complete monomer conversion for further reaction and therefore enable to synthesize polymers with controlled molecular weights, functionized polymers, and block, star, comb, dendritic copolymers. A typical molecular weight and polydispersity vs conversion plot of living polymerization is described in figure 1.5. The polymer chains grow all the time and the polydispersity decreases with the conversion increase.

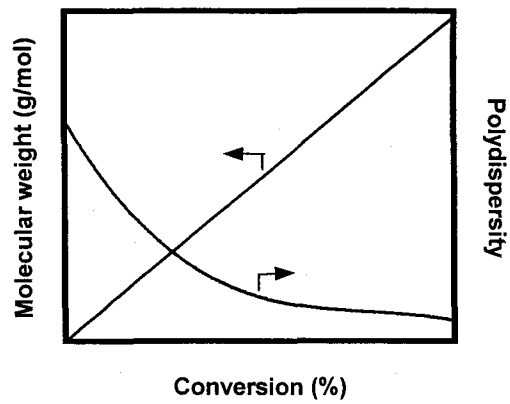


Figure 1.5. Scheme of molecular weight vs conversion for living polymerization.

Living polymerizations that have been used in synthesis of polyelectrolytes include living ionic polymerization (Antoun et. al., 1997; Creutz et. al., 1997; Shen et. al., 2001), group transfer polymerization (GTP) (Beadle, 1993), and living radical polymerization (Hawker, et. al., 1996; Lokaj, et. al., 1997; Zhang et. al., 1998). Under suitable conditions, both living ionic and group transfer polymerization can produce nearly mono-dispersed polymers. However, living ionic polymerization is very sensitive to moisture and traces of impurities, which make the reaction conditions very restrictive. GTP is successful only in a limited number of monomers, such as acrylate and methacrylate and it is required water free conditions. Moreover, GTP is more expensive than radical polymerization. (http://macro.lsu.edu/corecourses/MSweb7/PDF/handouts%20 GTP.pdf)

Comparatively, living radical polymerization is more attractive because it works for most vinyl monomers under mild conditions and it is more tolerant to water, air and impurities. The most currently used living radical polymerizations include stable free radical polymerization (SFRP), reversible addition-fragmentation chain transfer polymerization (RAFT) and atom transfer radical polymerization (ATRP).

1.4.2. Living radical polymerization

The mechanism of living radical polymerization is to equilibrate the active species and various types of dormant species. By converting the reactive centers to dormant species, the concentration of free radicals decreases and hence the transfer and termination reactions were suppressed. The reaction system remains living.

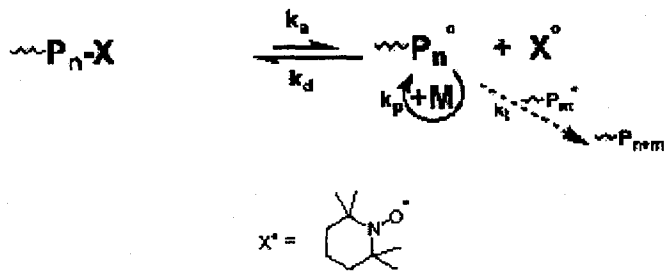
1.4.2.1. Stable free radical polymerization (SFRP)

1.4.2.1.1. Mechanism of SFRP

Since narrow polydisperse polystyrene was first polymerized by nitroxide-mediated stable free radical polymerization (Georges, et. al., 1993), SFRP techniques have been developed to synthesize a variety of monomers (Hawker, et. al., 1996; Keoshkerian, et. al., 1998; Fukuda, et. al., 1996; Benoit, et. al., 2000).

The mechanism of SFRP is illustrated in Scheme 1.2 (Matyjaszewski, 2000a).

Scheme 1.2. Mechanism of SFRP



In this type of process, the propagating species (Pn^{o}) reacts with a stable radical (X^{o}). The resulting dormant species (Pn-X) is not stable and undergoes thermal fragmentation to regenerate stable nitroxide free radicals X^{o} and polymeric radicals Pn^{o}. The nitroxide free radical X^{o} does not initiate any polymer chain, but it can react with carbon-centered free radical at near diffusion-controlled rate. The reversible equilibrium reaction is much in favor of left and most of time the polymeric radical is in its dormant state. Consequently, the concentration of the polymeric free radical is very low, which

can suppress side reactions such as termination by disproportionation and /or combination and keep the reaction in "living" manner.

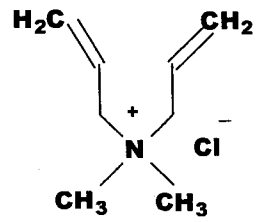
The most commonly used stable radicals are nitroxides, especially 2,2,6,6-tetramethylpiperidinoxy (TEMPO).

1.4.2.1.2. SFRP application for polyelectrolytes

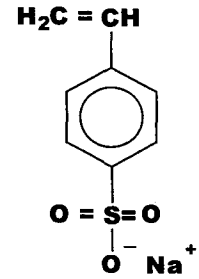
SFRP is attractive for preparation of novel water-soluble polymers, since narrow polydisperse water-soluble polymers cannot be prepared directly by conventional living ionic polymerization systems. Functional groups of hydrophilic monomers have to be protected prior to polymerization and de-protected thereafter. Keoshkerian et al. (1995) developed an SFRP process for narrow-polydispersity water-soluble poly(styrenesulfonate, sodium salt) (Scheme 1.3). A linear increase of molar mass with conversion can be achieved and polymers with narrow polydispersity were obtained. Recently, Burkhart (1996) successfully prepared polydiallyldimethyl- ammonium chloride with 3-carboxy-proxyl nitroxide radical (Scheme 1.3).

Although SFRP is one of the simplest methods of living free radical polymerization, it is applicable to only a few monomers. Neither the polymerizations of DMAEMA nor HEMA by SFRP was successful. Furthermore, the polymerization is slow and alkoxyamine end groups are difficult to transform.

Scheme 1.3. Structure of Diallyldimethylammonium chloride and Styrenesulfonate sodium salt



Diallyldimethylammonium chloride



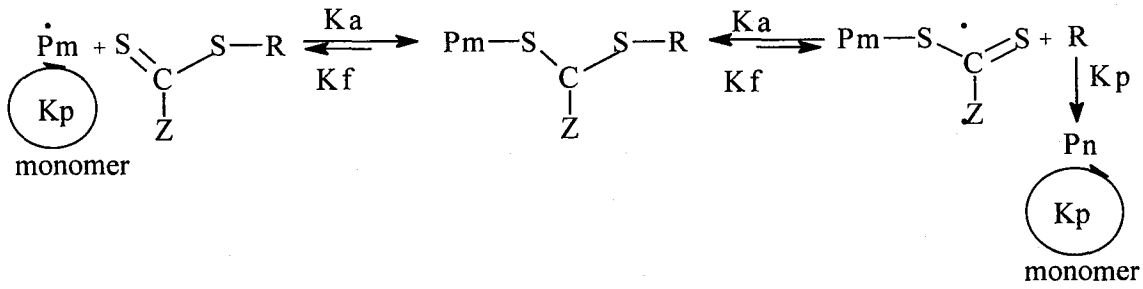
Styrenesulfonate sodium salt

1.4.2.2. Reversible addition-fragmentation chain transfer polymerization (RAFT)

1.4.2.2.1. Mechanism of RAFT

Reversible addition-fragmentation chain transfer polymerization (RAFT) was developed by Thang and his coworkers in 1998 (Chiefari, et. al., 1998). The mechanism of RAFT is illustrated in Scheme 1.4. The chain transfer agent (CTA) reacts with either the primary radical or a propagating polymer chain to form a new CTA and to eliminate R$^{\bullet}$. The reversible addition–fragmentation reactions between active and dormant polymer chains and the S=C(Z)S-mioety allow an equal probability of growth for all chains, leading to a narrow molecular weight distribution.

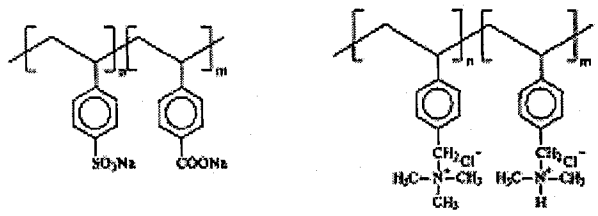
Scheme 1.4. Mechanism of RAFT



1.4.2.2.2. RAFT application for polyelectrolytes

The major advantage of the RAFT technique over other methods for living radical polymerization is its compatibility with a wide range of functional monomers and no particular limitations of reaction conditions. From the economic and environmental view, the preparation of water-soluble (co)polymers via RAFT is most attractive. Rizzardo and his coworkers successfully controlled the polymerization of N-isopropylacrylamide (NIPAM) by RAFT in solution. The polydispersity was as low as 1.03 (Chiefari et. al., 1998). Recently McCormick and his coworkers reported the use of RAFT technique for synthesis of homopolymers and block copolymerms based on the water-soluble styrenic monomers, sodium 4-styrenesulfonate, sodium 4-vinylbenzoate, N,N-dimethylvinylbenzylamine, and (ar-vinylbenzyl) trimethylammonium chloride (Scheme 1.5) (Mitsukami, 2001).

Scheme 1.5. Structure of poly(sodium 4-styrenesulfonate-b-sodium 4-vinylbenzoate) and poly(N,N-dimethylvinylbenzylamine-b-(ar-vinylbenzyl) trimethylammonium chloride) block copolymers



More recently, a thermoresponsible water-soluble block copolymers were prepared from the nonionic monomer NIPAM and the zwitterionic monomer 3-[N-(3-methacrylamidopropyl)-N,N-dimethyl] ammoniumpropane sulfonate (SPP) via RAFT techniques (Heise, et. al., 2001). The result block copolymers with two hydrophilic blocks exhibit double thermoresponsibility in water at high temperature, the poly-NIPAM block forms the hydrophobic "inside" colloidal aggregates that are kept in solution by the "outside" poly-SPP block, and vice versa at low temperature.

However, to date, RAFT has many drawbacks. The RAFT agents are expensive and not commercially available, and the dithio end groups left on the polymer are toxic, colored and odorant that are hard to remove.

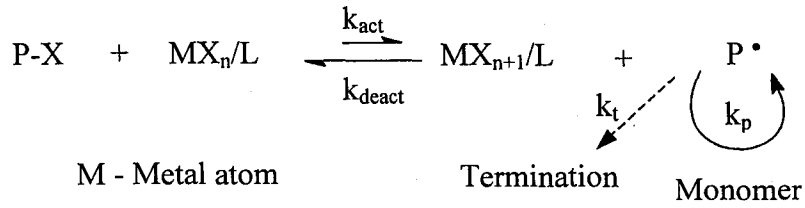
1.4.3. Atom transfer radical polymerization (ATRP)

Since Matyjaszewski's group and that of Sawamoto's developed atom transfer radical polymerization (or metal-catalyzed living radical polymerization) in 1995 independently (Wang et. al., 1995; Sawamoto, et. al., 1996), atom transfer radical

polymerization has been rapidly developed to be the most versatile living radical polymerization technique to precisely and inexpensively control chain-end functionalities. It has been used to prepare a wide range of well-defined polymers including macromonomers, blocks, stars, grafts, statistical copolymers, and functionalized polymers under mild conditions even in the presence of protonic compounds such as water.

1.4.3.1. Mechanism of ATRP

Scheme 1.6. Mechanism of ATRP



A general mechanism for ATRP, see in Scheme 1.6, involves a reversible activation and deactivation equilibrium between the propagating and dormant species with metal complexes acting as a halogen atom transfer reagent (Matyjaszewski, 2001). Metal complex MX_n /L undergoes a one-electron oxidation with simultaneous abstraction of a halogen atom from the carbon-halide bond to generate a radical $P^{\bullet}$ with the rate

constant k_a. The radical has three possible reactions: (1) react with monomer to propagate with rate constant k_p; (2) react with another radical to terminate by coupling or disproportionation with rate constant k_t; or (3) react with the higher oxidation state transition metal complex MX_{n+1}/L and resume to the dormant state P-X with rate constant k_d.

In order to obtain good control over the molecular weight and molecular weight distribution in ATRP, fast initiation, low growing radical concentration and low equilibrium rate constant $K_{eq}(=k_{act}/k_{deact})$ must be fulfilled. To a successful ATRP, the molecular weight of the resulting polymers can be predicted by the theoretical molecular weight as seen in Eq.1.1.

$$M_{th} = (\Delta[M]/[I]_0) \times mw \qquad \text{(Eq.1.1)}$$

where $\Delta[M]$ is the change in monomer concentration, $[I]_0$ is the initial concentration of the initiator, and mw is the molecular weight of the monomer.

1.4.3.2. Kinetics of ATRP

Take a copper-mediated ATRP as an example. Eq.1.2 expresses the rate law of ATRP, assuming 1) fast and equilibrium approximation, and 2) negligible termination.

$$R_p = k_p [M] [P^\bullet] = k_p K_{eq}[M][I]_0 \times [Cu^I] / [Cu^{II}] \qquad \text{(Eq.1.2)}$$

where $[P^\bullet]$ is the concentration of propagation radicals; $[Cu^I]$ is the concentration of low-state oxidation transition metal Cu^I; $[Cu^{II}]$ is the concentration of high-state oxidation transition metal Cu^{II}; and

$$K_{eq} = ([P^\bullet][Cu^{II}])/([I]_0[Cu^I]) \qquad \text{(Eq.1.3)}$$

$$[P^{\bullet}] = K_{eq}[I]_0 \times [Cu^{I}]/[Cu^{II}] \qquad \text{(Eq.1.4)}$$

After integration, we get

$$Ln\frac{[M]_0}{[M]} = \left(k_p K_{eq}[I]_0 \times [Cu^{I}]/[Cu^{II}] \right) \times t \qquad \text{(Eq.1.5))}$$

Eq.1.5 indicates a linear relationship between $\ln([M]_0/[M])$ and t, which means that during polymerization the concentration of active species is constant. The kinetics of ATRP is first-order with respect to monomer. A typical linear semilogarithmic plot of ATRP is shown in Figure 1.6.

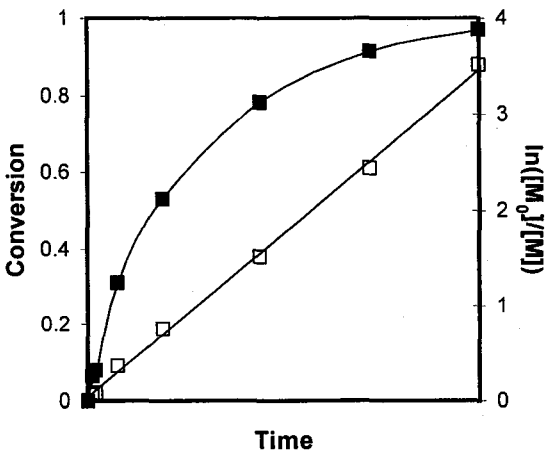


Figure 1.6. Conversion vs time and semilogarithmic plot of a typical ATRP

1.4.3.3. ATRP application for polyelectrolytes

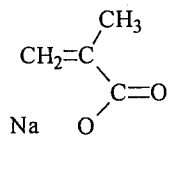
One of the most distinguishable advantages of ATRP is that it has great tolerance to various functional groups on monomers, initiators and in solvent. This in turn leads to direct polymerization of functionalized monomers without protection and

deprotection processes. The functional groups include not only hydroxyl, amino, and amido groups but also ionic and other special groups. Recent advances have been directed toward the extension of ATRP to the preparation of polyelectrolytes or water-soluble polymers with well-defined structures, which has been attracted great attention both in academic and industrial fields, especially for bio-applications.

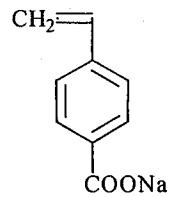
1.4.3.3.1. Synthesis of well-defined polyelectrolytes

Scheme 1.7 is an inventory of a wide range of non-ionic, anionic and cationic hydrophilic monomers for which ATRP has been reported.

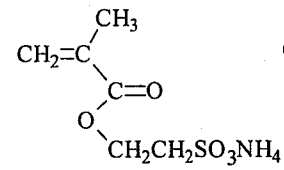
Scheme 1.7. Structure of a range of non-ionic, anionic and cationic hydrophilic monomers



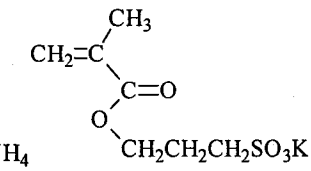
NaMAA



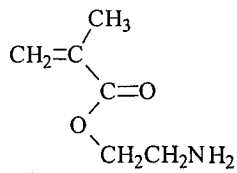
NaVBA



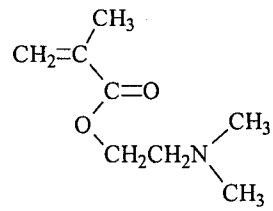
SEM



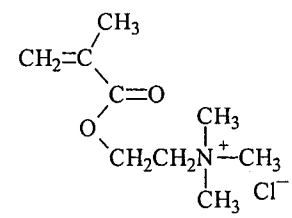
KSPM



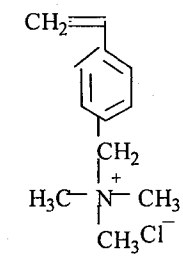
AEMA



DMAEMA



DMAEMA-MCQ



VBTMA

The ATRP of two carboxylic acid-based anionic monomers were first investigated by Armes group. The controlled polymerization of sodium methacrylate (NaMAA) in water was obtained, although the reaction rate was slow even at 90 °C (monomer conversions of 70-80% in 10 h) (Ashford et. al., 1999). In contrast, the ATRP of sodium 4-vinylbenzoate (NaVBA) is rapid at 20 °C, with 95% yield obtained within 25 minutes (Wang et. al., 2000a). The polydispersities were around 1.3 in both cases. Another anionic monomer, ammonium 2-sulfatoethyl methacrylate (SEM) was rapidly polymerized via ATRP in aqueous media at 20 °C (Wang et. al., 2000b) 87% conversion was reached in 3 h. However, the ATRP of potassium 3-sulfopropyl methacrylate (KSPMA) was not successful. The conversion ceased at 40 %.

Several cationic monomers have also been evaluated. PolyDMAEMA has been readily polymerized by ATRP in either organic solvents or aqueous media (Zhang et. al., 1998; Shen et. al., 2000; Zeng et. al., 2000a). The cationic polyelectrolytes can be obtained by quaternizing polyDMAEMA (co)polymers. Moreover, allyl-functionalized macroinitiators of DMAEMA have been prepared via ATRP followed by quaternization with methyl iodide and copolymerized with acrylamide under conventional free radical conditions to yield comb-branched polyelectrolytes (Zeng et. al., 2000b).

Direct ATRP of quaternized monomers has been much more challenging. Vinylbenzyltrimethylammonium chloride (VBTMA) was polymerized by aqueous ATRP to high conversion 95 %. However, the polydispersity was as high as 2.2. Hydrochloride salt of 2-aminoethyl methacrylate (AEMA) can only be polymerized to 75 % conversion via aqueous ATRP (Wang et. al., 2000b).

Much more efforts have been made on quaternary salts of DMAEMA. The first try was made by Guerrini et al (2000). 2-(Methacryloyloxy)ethyl trimethyl- ammonium chloride (MAETACl) was polymerized from the surface of a cross-linked polystyrene latex with CuBr/bipyridine in water at 80 °C to generate hydrophilic shells. However, there were no molecular weight data reported. Very recently, Armes group reported the direct ATRP of poly(trimethylamino)ehtyl methacrylate chloride (polyDMAEMA-MCQ) in protic media (Li et. al., 2003). High conversion can be reached and self-blocking efficiencies were demonstrated in chain extension experiments. Final polydispersities were in the range of 1.19-1.27.

1.4.3.3.2. Synthesis of water-soluble block copolymers

Various block copolymers of hydrophilic monomers have been synthesized by ATRP. In general, there are two basic approaches for synthesizing well-defined block copolymers through ATRP. One is Macroinitiator method, in which the first monomer is polymerized by an initiator with proper R-X. After isolation and purification, macroinitiators with high active carbon-halide terminals (P-X) are yielded. The P-X then initiates the second monomer to produce AB block copolymers. If a bifunctional initiator is used, an ABA triblock copolymer can be prepared. The other is "one-pot" Sequential addition method that polymerizes one monomer first followed by addition of a second monomer to the same pot. If both two monomers can be well-controlled by ATRP, then diblock copolymers can be prepared by this method. It is important to add the second monomer after only 90% conversion of the first monomer to avoid side reactions such as

termination of halide-capped chain ends at high conversion. The Sequential addition method is apparently simpler than the Macroinitiator method, however contamination of the first monomer in the second block is a problem.

The key of synthesizing well-defined block copolymers is to match the reactivity of initiation and propagation. One strategy is to use a proper order of monomer additions. Shipp et al (1998) reported that PMMA-macroinitiator can initiate Methacrylate (MA) or Styrene polymerization and yields well-defined block copolymers, while the reverse order, that is to use PMA or PS macroinitiator to initiate MMA polymerization, yields highly polydispersed block copolymers contaminated with unreacted macroinitiators. Another strategy is to use Cl-ended macroinitiator or mixed halide initiation system R-Br/CuCl to improve the ATRP livingness of methacrylic monomers. For example, low polydispersity PMA-PMMA block copolymers can be prepared using mixed halide initiation system (Matyjaszewski, 1998 b). Poly(MMA-b-DMAEMA) block copolymer can be prepared using PMMA-Cl as macroinitiator and CuCl as catalyst (Zhang et. al., 1999).

Poly(ethylene oxide) is one of most popular water-soluble macroinitiator, which has been used to polymerize various hydrophilic monomers, such as poly(ethylene oxide-b-SEM), poly(ethylene oxide-b-HEMA), poly(ethylene oxide-b-AEMA) (Wang et. al., 2000b) and poly(ethylene oxide-b-2-(dimethylamino)ethyl methacrylate-b-2-(diethylamino)methacrylate) (PEO-DMAEMA-DEMA) triblock copolymers (Liu et. al., 2002).

1.5. Research Objectives

Although polyDMAEMA can efficiently condense DNA or complex with ONs, its transfection efficiency to the target cell is lower compared to the viral carriers and cytotoxicity is slightly higher when polyDMAEMA/DNA ratio or polymer molecular weight is high. Recent studies on polycation gene delivery have revealed that small changes in the molecular structure of polymeric vectors have substantial influences on DNA-binding and condensation, and on toxicity and gene delivery efficiency (Merdan, et. al., 2002). The polymer molecular weight, charge density, and the physical properties of formed polymer/DNA complexes, such as size, play an important role in the transfection/cytotoxicity efficiency. In order to generate optimal gene deliveries, a clear understanding of the mechanisms and capabilities of the polymer/DNA system is essential.

However, well-defined cationic polymer complexes with DNA and/or ONs used as gene delivery, are not well developed. The effects of polymer related properties, such as molecular weight, charge density, on physicochemical and biological properties of polymer/DNA or polymer/ONs complexes are not clear. To date the progress is mainly hampered by how to quantitatively ascertain what molecular weights are suitable for high ratio of transfection/cytotoxicity, and how much cationic charge is enough to neutralize the DNA or ONs and also to provide sufficient residual charge for interactions with cell membrane without causing high cytotoxicity. Therefore, it is essential to be able to

synthesize well-defined polymer/ONs complexes and characterize their physicochemical and biological properties in order to develop an efficient gene delivery.

Controlled/living polymerization such as ATRP offers the possibility of synthesizing well-defined cationic polyDMAEMA and its copolymers, which facilitate the investigation of polymer-related factors governing polymer/DNA complex properties.

The objective of this research is to establish the relationship of well-defined and well-controlled polyelectrolyte structure and ONs complexation performance for non-viral antisense oligonucleotides deliveries. Specific tasks include:

1. To develop a gel permeation chromatography (GPC) calibration method for polyelectrolyte characterization.
2. To synthesize well-defined well-controlled 2-(*N,N*-dimethylamino)ethyl methacrylate (DMAEMA) -based polyelectrolytes and block copolymers by atom transfer radical polymerization technique.
3. To investigate the effects of molecular weight, charge density, and block composition of polyelectrolytes on antisense oligonucleotides complexation performance.

1.6. Contents of thesis

This thesis contains five chapters. The first chapter is an introduction. The properties of polyelectrolytes, the bio-applications of polyelectrolytes and the advanced controlled/living polymerization techniques for preparing well-defined polyelectrolytes

are introduced. The mechanism of atom transfer radical polymerization and its application for polyelectrolytes are discussed in detail. The related literature is reviewed.

In Chapter 2, a GPC calibration curve of cationic polyDMAEMA quaternary salt is proposed based on the GPC measurements of quaternized narrow molecular weight distributed polyDMAEMA samples. Detailed information about preparation of cationic polyDMAEMA and characterization is presented. Three major factors of ionic strength of mobile phase, charge density and counter ion of polyelectrolytes to influence the hydrodynamic volume were investigated. Several polyDMAEMA-MCQ samples prepared by direct ATRP were characterized by the newly developed polyelectrolyte GPC calibration method.

In Chapter 3, the ATRP of 2-(*N, N*-dimethylamino) ethyl methacrylate and 2-hydroxyethyl methacrylate block copolymer is presented. The kinetic studies of both homo-polymerization and block co-polymerization are discussed in detail. The effects of the order of monomer addition, reaction temperature and water as an accelerator are evaluated.

In Chapter 4, well-defined DMAEMA based (co)polymers are used as carriers for antisense oligonucleotides delivery. The comlexations of well-defined polymer/ONs complexes were investigated by fluorescence spectrophotometer. The influences of molecular weight, charge density, count-ion and block copolymers on the complexation were systematically evaluated. The kinetic study of complexation of ONs and polyDMAEMA with different molecular weight was also examined in details.

Chapter 5 provides conclusions and engineering significance of the present work. Recommendations for future research in this area are also provided in this chapter.

Chapter 2 Characterization of Well-Defined Poly(2-(dimethylamino) ethyl Methacrylate) Quaternary Salts via Aqueous GPC

2.1. Introduction

Water-soluble poly(2-(dimethylamino)ethyl methacrylate) (polyDMAEMA) and quaternized polyDMAEMA are widely used in many industries, such as wastewater treatment, papermaking, and mineral processing (Cho, et. al., 1997; Chen, et. al., 1997; Zeng, et. al.,2000b). Recently, these polyelectrolytes have attracted considerable interest because of their unique bioactive properties and their important bio-applications as materials for non-biofouling coating for implants, drug delivery, and non-viral gene and antisense oligonucleotides delivery (Vamvakaki et. al., 2001; Van Rompaey, 2000 and 2001 a, b). With these new application developments comes a demand for well-defined polyelectrolytes in order to establish the relationships of material structure and properties.

Many living polymerization techniques for preparing mono-polydispersed polymers are being developed, which provide an opportunity to synthesize well-defined polyelectrolytes. To date polyDMAEMA has been polymerized via living anionic poly-

---

Note: A part of this chapter has been published in Li, Y.; Armes, S. P.; Jin, X. P.; Zhu, S. " Direct Synthesis of Well-defined Quaternized Homopolymers and Diblock Copolymers via ATRP in Protic Media", 2003, 36, 8268. The polymer samples listed in "Table 2.3." were prepared by the Sussex group (Dr. Li and Prof. Armes). My contribution to the paper is the characterization work. We at McMaster developed the aqueous GPC calibration method for the cationic polymers.

merization (Creutz et. al., 1997), group transfer polymerization (Bütün, et. al., 2001), and atom transfer radical polymerization (Zhang, et. al., 1998) in various solvents. Well-defined polyDMAEMA quaternary salts can be prepared by sequential quaternization techniques. Very recently, Armes group successfully synthesized well-defined poly(2-(dimethylamino) ethyl methacrylate methyl chloride) (polyDMAEMA-MCQ) via direct ATRP of DMAEMA-MCQ monomer in protonic media at ambient temperature (Li, et. al., 2003).

However, the characterization of polyDMAEMA and their quaternized derivatives via gel permeation chromatography (GPC) remains to be challenging. Few studies were reported in literatures regarding the GPC analysis of cationic polyDMAEMA. This is because the charged groups on the chains affect the separation via ionic interactions between polymer, solvent, and support of column. For polyDMAEMA, which is soluble in organic solvents such as THF and DMF, this kind of effects in organic GPC is not severe. The adsorption of polymer on column materials can usually be suppressed by adding an ionic salt such as triethylamine, tetrabutylammonium bromide, or LiBr. However, for water-soluble polyDMAEMA quaternary salts, aqueous GPC meets some technical difficulties: 1) lack of available monodisperse water-soluble polymer standards (commercially available standards such as polyethyleneoxide and polyacrylamide are very different in nature from the polyelectrolytes of interest); 2) difficulty in obtaining suitable cationic chromatographic column supports; and 3) inherent difficulty in describing the conformation statistics of polyelectrolyte chains in dilute solution (Rollings, et. al., 1983).

The aim of this study is to develop an aqueous gel permeation chromatography (GPC) calibration method for cationic polyelectrolyte characterization. Our approach is to quaternize the nonionic polyDMAEMA samples that have well-controlled molecular weight and narrow molecular weight distribution prepared by living polymerization and then to generate a GPC calibration curve for the cationic polyelectrolytes. The method will become the first reliable calibration in the field.

In this work, the effects of solvent, quaternization agent, agent/polymer ratio, reaction time, and polymer molecular weight on quaternization efficiency were evaluated in order to optimize the reaction condition. Also studied were the effects of three major factors (ionic strength of mobile phase, charge density, and counter ion of polyelectrolytes) on hydrodynamic volume, and hence the determination of molecular weight. Finally several poly(2-(dimethylamino)ethyl methacrylate, methyl chloride quaternary salt (polyDMAEMA-MCQ) samples prepared by direct ATRP were characterized by the newly developed polyelectrolyte GPC calibration method.

2.2. Experimental section

2.2.1. Materials

Dichloromethane, dimethyl sulfate (Me_2SO_4), methyl iodide (MeI), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), triethylamine, acetone, ethylformamide (DMF), hexane, sodium sulfate (Na_2SO_4) and sodium azide (NaN_3) were purchased from Aldrich and used without further purification.

2.2.2. Preparation of polymer samples

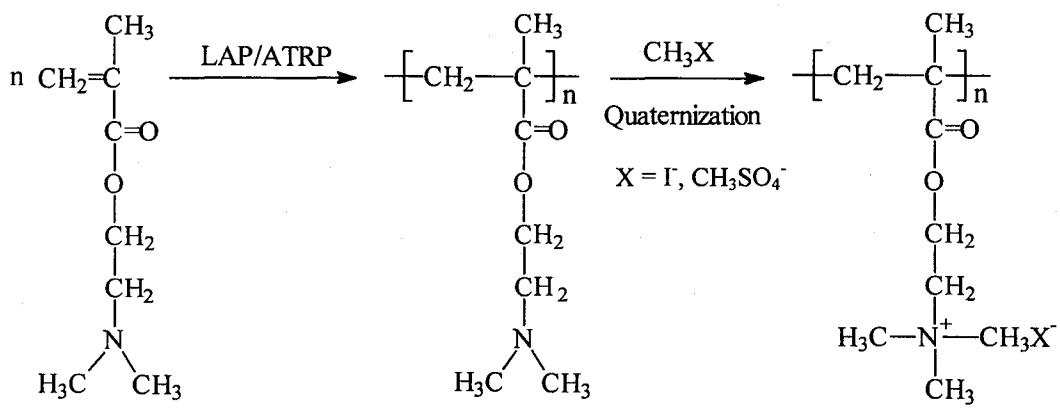
2.2.2.1. Synthesis of polyDMAEMA

The DMAEMA homopolymers were synthesized by both living anionic polymerization (LAP) and atom transfer radical polymerization (ATRP). The well-defined polyDMAEMA samples prepared via living anionic polymerization (Shen, et. al., 2001) were purified by dissolving samples in dichloromethane and re-precipitated in hexane to remove LiCl. The samples were dried under vacuum. The ATRP of polyDMAEMA was carried out in methanol both at room temperature and 60 ^{o}C. The detail procedures were described in Section 3.2.3.1 of Chapter 3.

2.2.2.2. Preparation of polyDMAEMA quaternary salts

Dimethyl sulfate quaternary salt of poly (2-(dimethylamino)ethyl methacrylate) (polyDMAEMA-DMSQ) and methyl iodide quaternary salt of poly (2-(dimethylamino)ethyl methacrylate (polyDMAEMA-MIQ) samples were prepared by quaternizing polyDMAEMA samples with dimethyl sulfate and methyl iodide respectively either in acetone or in DMSO. A typical quaternization procedure shown in Scheme 2.1 was as follows: 1g of polyDMAEMA was dissolved in 10mL of acetone at room temperature. A required molar ratio of dimethyl sulfate was added to the solution dropwisely and was stirred for 4h. The polymer quaternary salt precipitate was isolated and dried in a vacuum oven. PolyDMAEMA-MCQ, donated by Professor S. P. Armes University of Sussex UK, was prepared by a direct atom transfer radical polymerization of DMAEMA-MCQ monomer (Li, et. al., 2003).

Scheme 2.1. Synthesis and subsequent quaternization of polyDMAEMA



2.2.3. Measurements

2.2.3.1. NMR spectroscopy

Monomer conversion, degree of polymer quaternization, and copolymer block composition were determined by ^1H NMR (Bruker ARX-200) using $CDCl_3$, or D_2O as solvents.

2.2.3.2. Gel permeation chromatography

Molecular weights (Mn and Mw respectively) of polyDMAEMA were measured by GPC (Varian MicroPak column G1000, 3000, 7000 HXL) using THF-2%(v/v) triethylamine as eluent at 25°C with RI detector. Narrow polystyrene standards were used for calibration. PolyDMAEMA-DMSQ and polyDMAEMA-MCQ were

characterized by Waters 2690 liquid chromatograph equipped with 4 sets Waters Ultrahydrogel linear 6-13 μm columns and 2410 refractive index detector using 0.1M to 0.35M Na_2SO_4 as eluent containing 0.1% (w/v) of NaN_3. The operating temperature was 30°C and the flow rate was 1.0 ml min^{-1}. All GPC data were recorded and processed using the Windows based Millenium 2.0 software package.

2.3. Results and Discussion

2.3.1. Quaternization of polyDMAEMA

Quaternization of polyDMAEMA with MeI or Me_2SO_4 was carried out at room temperature. Figure 2.1 shows a comparison of ^1H NMR spectrum of polyDMAEMA before and after quaternization. After quaternization, the nitrogen atoms in dimethylamino group ($(CH_3)_2N$) were converted into trimethylammonium ($(CH_3)_3N^+$). The signal of $N(CH_3)_2$ at δ 2.25 disappeared completely while a strong peak for $N^+(CH_3)_3$ appeared at δ 3.2 suggesting a complete quaternization obtained. The quaternization degree (QD) was calculated by the integral of $N(CH_3)_2$ at δ 2.25 respect to that of $N^+(CH_3)_3$ at δ 3.2. The targeted QD were achieved by adjusting the molar ratio of quaternization agents to polyDMAEMA.

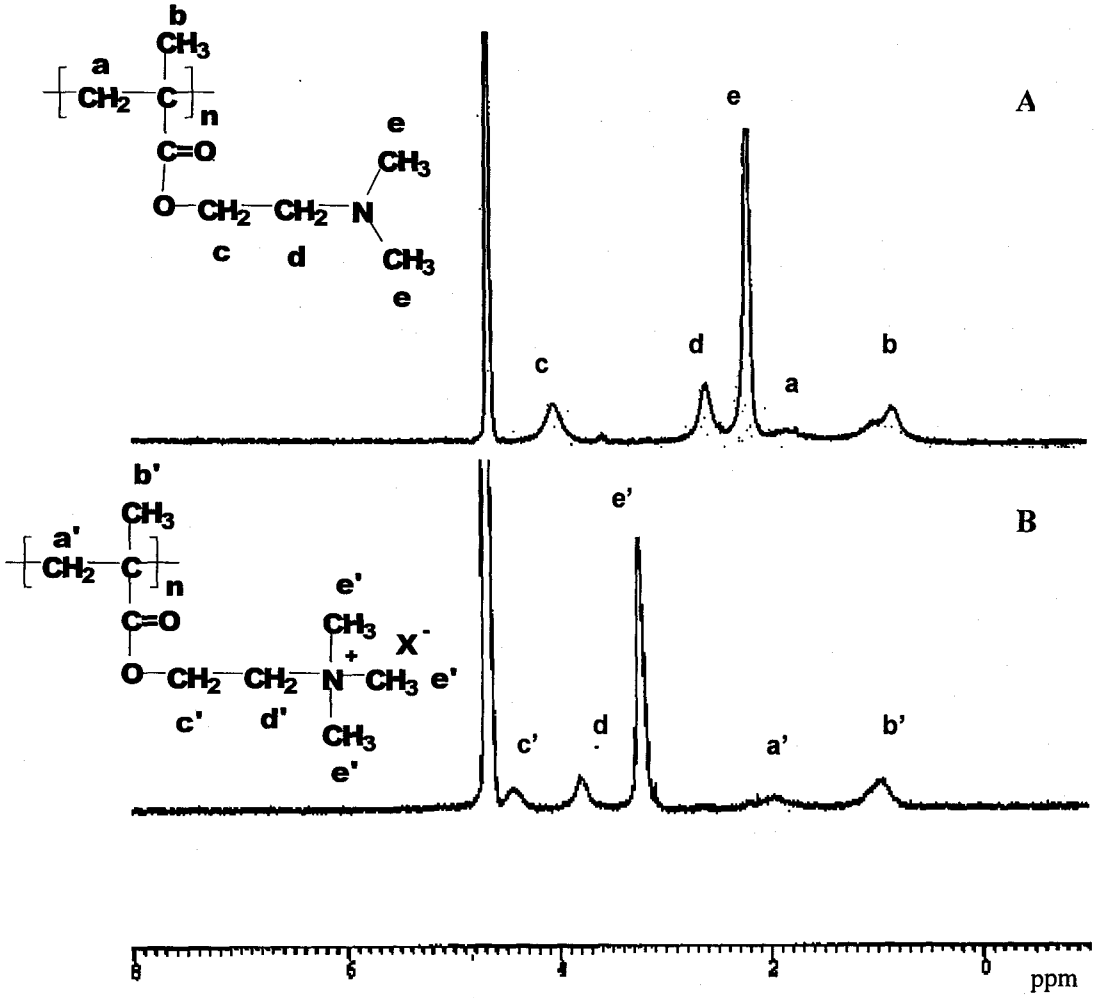


Figure 2.1. ^{1}H NMR spectrum of polyDMAEMA (A, top) and quaternized polyDMAEMA (B, bottom).

The effects of solvent, quaternization agent, agent/polymer ratio, reaction time and polymer molecular weight on quaternization efficiency were evaluated in order to optimize the reaction condition. The result data are summarized in Table 2.1.

Table 2.1. Effects of solvent, quaternization agent, agent/polymer ratio, reaction time and polymer molecular weight on quaternization efficiency at room temperature.

Entry	Mn g/mol	Quaternization agent	Solvent	Quat. agent/polymer molar ratio	time (h)	Quaternization Degree %
5-1	25500	Me_2SO_4	Acetone	1	4.5	64.1
5-2	25500	Me_2SO_4	DMSO	1	4.5	100.0
5-3	25500	Me_2SO_4	Acetone	1	24	84.0
5-4	25500	MeI	Acetone	1	4.5	88.0
5-5	25500	MeI	Acetone	1	4.5	87.3
5-6	25500	MeI	Acetone	2	4.5	96.9
5-7	25500	Me_2SO_4	Acetone	2	4.5	100.0
1-1	4440	Me_2SO_4	Acetone	1	4.5	99.6

2.3.1.1. Effect of solvent

Depending on the solubility of quaternized polyDMAEMA in different solvents, there are two quaternization methods. One is homogeneous method and the other is heterogeneous method. When DMSO was used as solvent, the quaternized polyDMAEMA dissolved in DMSO and the system was homogeneous. When acetone was used as solvent, the polyDMAEMA salt precipitated out very quickly and the system was heterogeneous. A complete quaternization was reached in 4.5 hours using DMSO as

solvent at the stoichiometric molar ratio of Me_2SO_4 and polyDMAEMA (Sample 5-2). However, it was difficult to remove DMSO solvent presumably because of the complexation of DMSO solvent with the polymers. The quaternization efficiency in acetone was lower than in DMSO. A quaternization degree of 64% was reached in 4.5 hours and 84 % in 24 hours using Me_2SO_4 in acetone, as seen Samples 5-1 and 5-3. In the heterogeneous system the quaternization agents experienced more diffusion limitations to reach the amino groups than in the homogeneous system; the mass transfer rates were slow in the precipitated particles. However, increasing the molar ratio of agent/polymer to 2, a full quaternization of polyDMAEMA in acetone was reached in 4.5 h (Sample 5-7).

2.3.1.2. Effect of quaternization agent

MeI is more efficient than Me_2SO_4. MeI at the stoichiometric molar ratio gave an average QD of 87% (Sample 5-5), whereas Me_2SO_4 yielded only 64% in 4.5 h and 84% in 24 h (Sample 5-1 and 5-3). Increasing the molar ratio to 2, a high quaternization degree was reached in 4.5 hours with both Me_2SO_4 and MeI in acetone, as shown in Sample 5-6 and 5-7. However, methyl iodide was readily oxidized. In this work, Me_2SO_4 was selected as the quaternization agent.

2.3.1.3. Effect of polymer molecular weight

The quaternization efficiencies of low molecular weight polymers were higher than those of high molecular weight polymers. For example, Sample 1-1 that has a

number-average molecular weight of 4440 reached a QD of 99.5% when it was quaternized with dimethyl sulfate at the molar ratio 1 for 4.5 hours in acetone. For comparison, Sample 5-1 of molecular weight 25500 had the QD of only 64.1% at the same reaction condition (see Table 2.1). This can be explained in terms of an effect of steric hindrance. Higher molecular weight chains formed larger coils in solution. It is easy for quaternization agent molecules to reach and quaternize the amino groups on the surface of the coils. However, it is difficult to quaternize the amino groups shielded inside the cores of the coils or that of the precipitated particles.

2.3.2. Characterization of polyDMAEMA quaternary salt by aqueous GPC

The principal mechanism of polymer separation in GPC is differential migration of molecules between the flowing solvent and the solvent going through the porous matrix of a GPC column (Yau, et. al., 1979). Smaller molecules access pores more readily and experience longer period of time in column than larger molecules. Larger molecules elute from column earlier. Flory proposed the relationship of molecular weight M and molecular volume as Equation 2.1 (Fox, et. al., 1951).

$$[\eta] = \Phi_0 (\overline{r^2})^{3/2}/M \qquad \text{(Eq. 2.1)}$$

where $[\eta]$ is the intrinsic viscosity, $(\overline{r^2})$ is the mean-square end-to-end distance, and Φ_0 is a constant equal to 3.6×10^{21} dl/cm^3.

Form Eq 2.1 we can see that the size of an uncharged, isolated macromolecule in solution depends on molecular weight, interaction between polymer and solvent, and intramolecular polymer-polymer interactions. However, this is not the case for polyelectrolytes in polar solvents. For polyelectrolytes, the molecular conformation depends on the amount and type of charged species, solvent ionic strength, and molecular weight. It is essential to select chromatographic conditions carefully in order to obtain satisfactory results.

2.3.2.1. Effect of ionic strength of mobile solution on MW determination

Two major factors play important roles in determining the molecular weight and molecular weight distribution of cationic polyelectrolyte via aqueous GPC. One is electrostatic repulsion between charge moieties on chain backbone, which causes an expansion of chain coil and increase local chain stiffness, and thus increases excluded volume (Hunt, et. al., 1989). The other is polymer-support interaction, which is especially problematic for cationic polyelectrolyte. This is because most support matrices bear negatively charged groups (Dubin, et. al., 1988). Therefore the ionic interactions with positive charged groups, such as amino groups of quaternary polyDMAEMA, readily occur. This ionic adsorption causes retardant movement of cationic polymers through porous matrix and lead to low excluded volume (Fukano, et al., 1978). Organic hydrophilic gels are suggested to be used to get efficient and non-adsorption separation for cationic polymers (Strege, et. al., 1989). In this work we selected Waters Ultrahydrogel linear 6-13 μm columns packed with hydroxylated

polymethacrylate-based gel. However it also carries certain amount of residual negative charges originating from carboxyl groups (Hunt et. al., 1989).

A common practice in tackling the problem is the addition of ionic species to suppress electrostatic repulsion and adsorption. Salt solutions, such as 0.05M sodium nitrate or sulfate, are usually sufficient to screening electrostatic repulsion of polyelectrolyte (Hunt et. al., 1989). However, to suppress strong ionic adsorption, a rather high salt concentration is required.

Figure 2.2 shows the overlays of GPC chromatograms for the fully quaternized polyDMAEMA of two different molecular weights in different Na_2SO_4 concentrations. The concentration was varied from 0.1M to 0.35M. At 0.1M, seen in Figure 2.2 (a), the GPC curve was skewed with a sharp leading edge and followed by long tailing, which is a typical sign of adsorption. Increase in salt concentration made GPC curve become symmetric and retention time become short, indicating the suppression of adsorption. With 0.3M Na_2SO_4, the polyelectrolyte effect was eliminated and good separation was obtained.

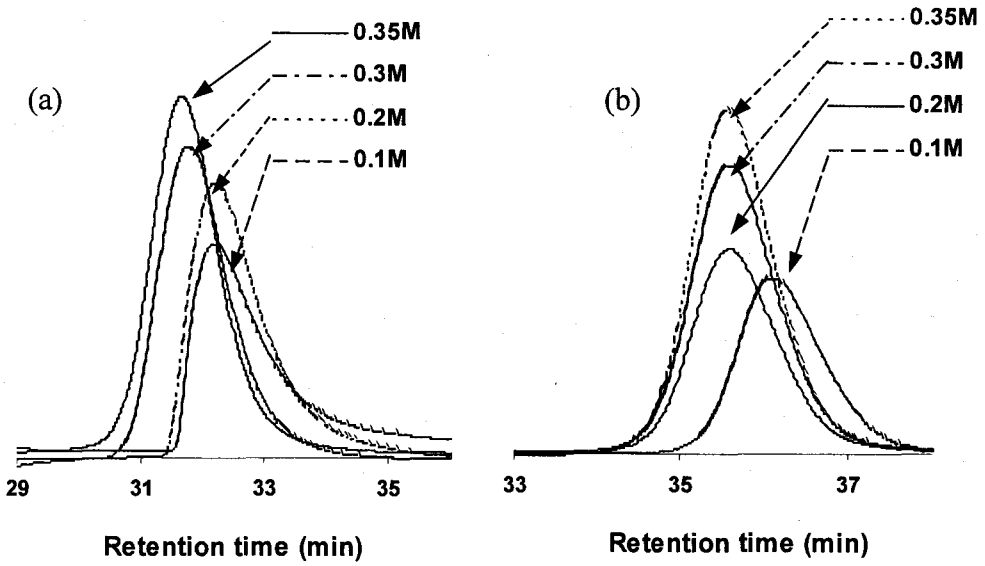


Figure 2.2. Effect of eluent ionic strength on separation of polyDMAEMA-DMSQ in sodium sulfate solution. (a) M_n = 78500; (b) M_n = 7580.

The effects of ionic strength varied with polymer molecular weights. High MW samples (Figure 2.2 (a): M_n=78500) showed the preferential adsorption comparing to low molecular weight samples (Figure 2.2 (b): M_n =7850). This is attributed to the fact that higher molecular weight chains have higher number of charges facilitating multiple contact adsorptions (Howard, et. al., 1980).

The experimental results also revealed that as long as electrostatic repulsion and polymer-support adsorption were suppressed by excess salt, the hydrodynamic volume of polyelectrolyte was confirmed. This means only size exclusion controls the elution, in which state the polyelectrolyte can be fractionated reproducibly.

2.3.2.2. Effect o f charge density on MW determination

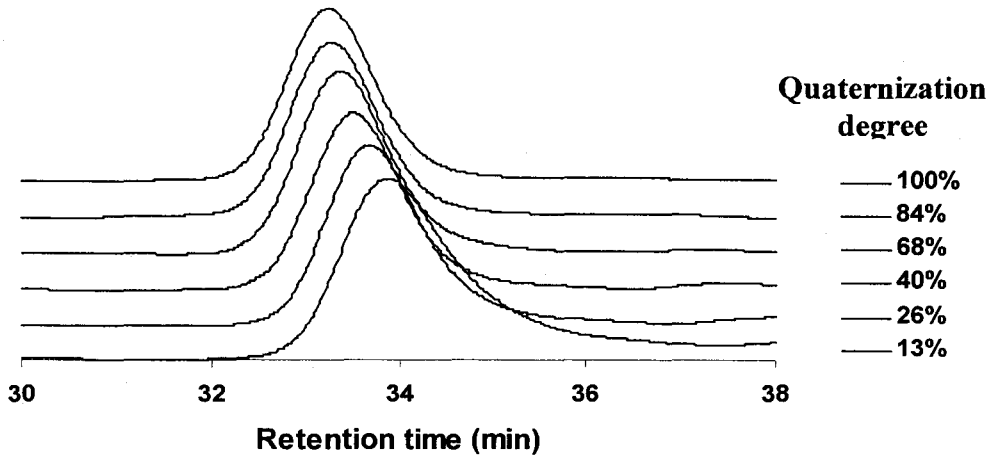


Figure 2.3. Effect of quaternization degree on the hydrodynamic volume of polyDMAEMA quaternary salt.

Charge density is another important factor to influence the determination of polyelectrolyte molecular weight via aqueous GPC. The more extended the conformation of a polymer chain, the more will it be excluded from the pores of column support. Figure 2.3 shows an overlay of GPC traces of polyDMAEMA-DMSQ samples with different degrees of quaternization. Increase of the degree of quaternization of polyDMAEMA increased the amount of quaternized amino groups along polymer chains. Because of intramolecular repulsion, polymer chains expanded and yielded large hydrodynamic volume.

The effects of charge density on the three different molecular weight polyDMAEMA-DMSQ samples were shown in Figure 2.4. In all cases, increasing the degree of quaternization expanded polymer chains and reduced retention time. However,

over 80% QD, the retention time became nearly independent from the degree of quaternization; it depended on the molecular weight only.

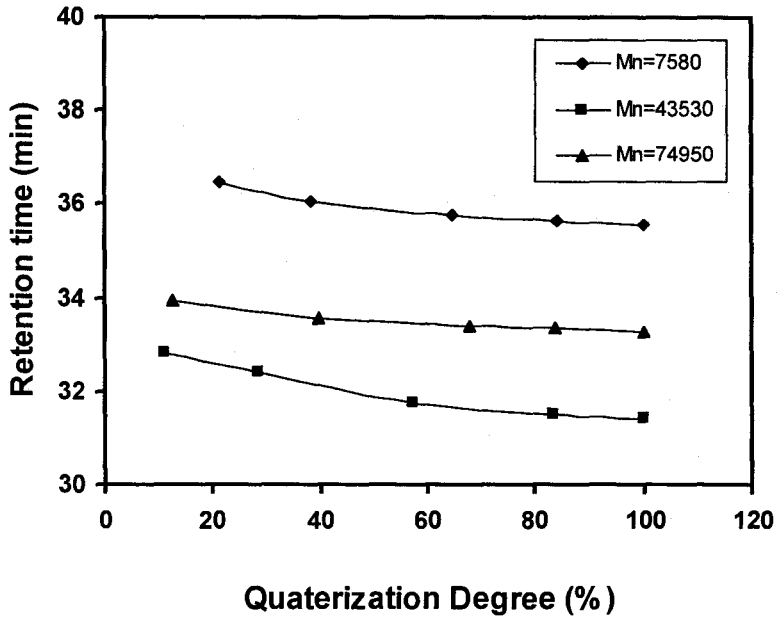


Figure 2.4. Effect of quaternization degree on the GPC retention time of different molecular weight polyDMAEMA-DMSQ samples.

2.3.2.3. Effect of counter ion type of quaternary salt

The effect of counter ion type on retention was tested by fully quaternizing same polyDMAEMA sample with different types of quaternization agents. PolyDMAEMA-DMSQ was quaternized with dimethyl sulfate (counter ion: $CH_3SO_4^-$), while polyDMAEMA-MIQ was quaternized with methyliodine (counter ion: I^-). Figure 2.5 shows the GPC overlay traces of polyDMAEMA-DMSQ and polyDMAEMA-MIQ for

comparison. The GPC result revealed that the counter ion had little effect on the size of polyDMAEMA quaternary salt.

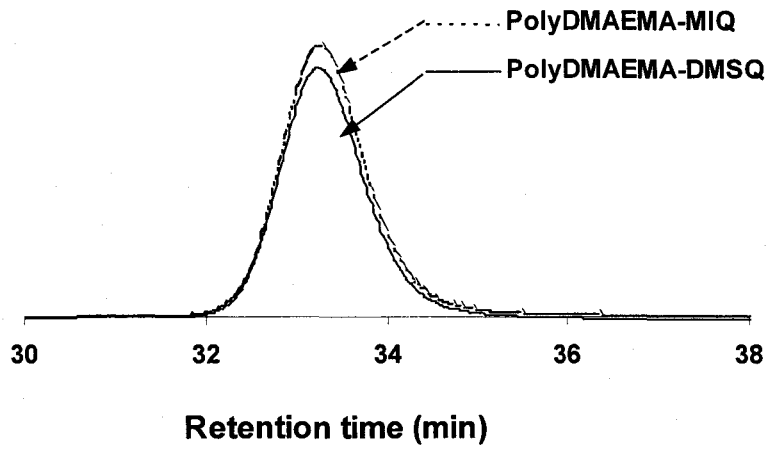


Figure 2.5. Effect of counter ion type on the hydrodynamic volume of polyDMAEMA quaternary salts.

2.3.2.4. Aqueous GPC calibration method of polyDMAEMA quaternary salt

A series of low molecular weight and narrow molecular weight distribution polyDMAEMA-DMSQ samples were prepared by fully quaternizing the well-defined polyDMAEMA samples with dimethyl sulfate $(CH_3)_2SO_4$. The molecular weights of the polyDMAEMA precursors were measured by both organic GPC and ^{1}NMR. The data were summarized in Table 2.2.

Table 2.2. GPC data of molecular weight and MWD of polyDMAEMA and polyDMAEMA-DMSQ.

Poly-DMAEMA	$M_{n,\ GPC}$ [a]	$M_{w\ GPC}$	$M_w/M_{n,\ GPC}$	PolyDMAEMA-DMSQ	$M_{n,\ Cal}$ [b]
1	4440	4620	1.04	Q1	7580
2	6420	6930	1.08	Q2	10960
3	11730	12080	1.08	Q3	20020
4	16400	18370	1.12	Q4	28000
5	25500	26520	1.04	Q5	43530
6	37820	39310	1.04	Q6	64560
7	45950	47790	1.04	Q7	78430

a: measured by GPC using THF as eluent and polystyrene as standard.

b: calculated based on the $M_{n,\ GPC}$ of polyDMAEMA x [mw of (DMAEMA-DMSQ)/mw of DMAEMA]

The narrowly distributed polyDMAEMA-DMSQ samples were then used as standards for the characterization of polyDMAEMA based polyelectrolytes via aqueous GPC using 0.3M of Na_2SO_4 – 0.1% (w/v) NaN_3 as eluent. Figure 2.6 shows the GPC overlay traces of the polyDMAEMA-DMSQ standards and Figure 2.7 shows the calibration curve using polyDMAEMA-DMSQ as standard.

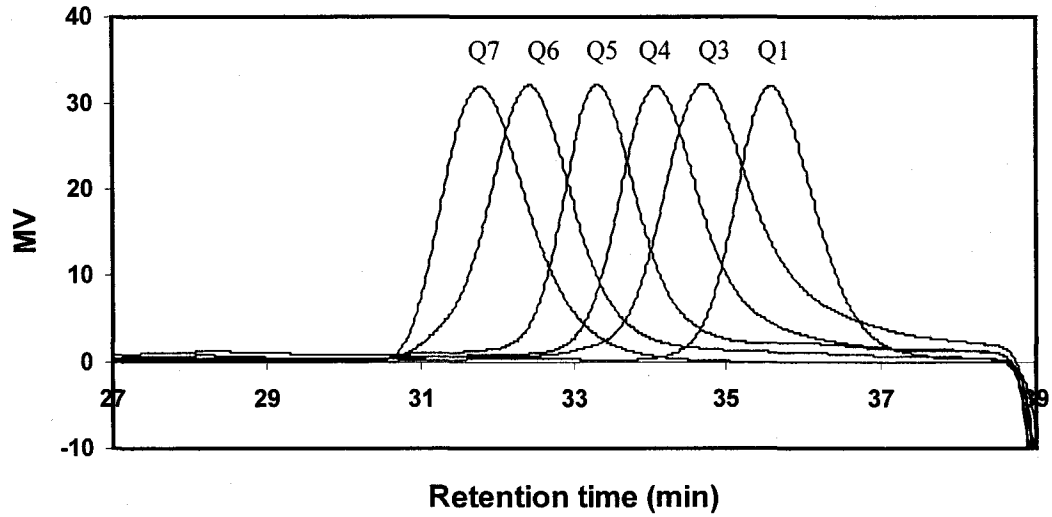


Figure 2.6. GPC overlay traces of polyDMAEMA-DMSQ standards in 0.3M Na_2SO_4-0.1 % (w/v) NaN_3 solution.

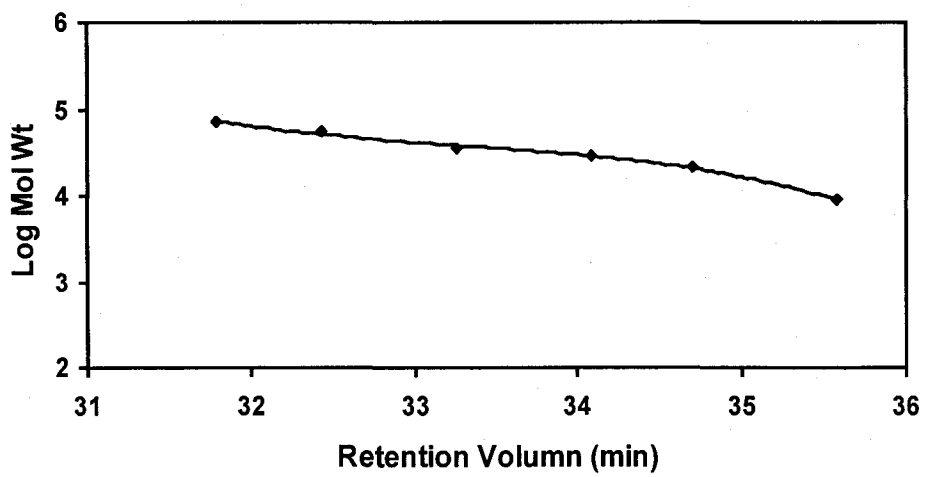


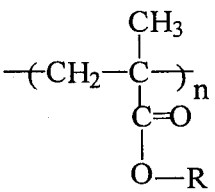
Figure 2.7. Calibration curve of polyDMAEMA-DMSQ in 0.3M Na_2SO_4-0.1% (w/v) NaN_3 solution.

2.3.3. Application of calibration of polyDMAEMA quaternary salt

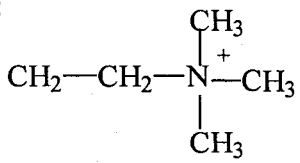
PolyDMAEMA-MCQ and polyDMAEMA-MIQ samples (Scheme 2.2) prepared by direct ATRP techniques were characterized by the developed polyDMAEMA-DMSQ calibration method using aqueous GPC. The molecular weights and molecular weight distributions of the samples are summarized in Table 2.3.

As proven that the counter ion type, such as I^- and $CH_3SO_4^-$ of polyDMAEMA quaternary salts, had little effects on the polymer hydrodynamic volume, we used the molecular weights of polyDMAEMA-DMSQ without including the molecular weight of the counter ion $CH_3SO_4^-$ for generating the GPC calibration curve.

Scheme 2.2. Structure and molecular weight of polyDMAEMA quaternary salt with or without counter-ions

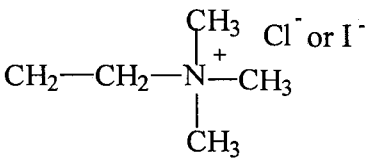


R :



Molecular weight of unit

172 g/mol



207.5 g/mol

(or I^-: 299 g/mol)

Table 2.3. GPC calibration data of polyDMAEMA-MCQ samples with polyDMAEMA-DMSQ as standard

No.	Target Structure	$M_{n,\,GPC}$ Without counter ion	$M_w/M_{n,\,GPC}$	$M_{n,\,th.}$ With counter ion	$M_{n,\,GPC}$ With counter ion
1	PolyDMAEMA-MCQ_{30}	8340	1.18	6225	10060
2	PolyDMAEMA-MCQ_{60}	10600	1.23	12450	12800
3	PolyDMAEMA-MCQ_{100}	16480	1.25	20750	19880
4	PolyDMAEMA-MIQ_{50}*	9030	1.13	14950	15700

* The degree of polymerization is confirmed by NMR measurement.

The molecular weights of polyDMAEMA-MCQ and polyDMAEMA-MIQ obtained from the GPC measurements were corrected with the counter ions Cl^- and I^-, respectively. The calculation equation was described as follows:

$$\mathbf{MW_{polyDMAEMA\text{-}MCQ} = MW_{GPC} \times (207.5\ g/mol\ /\ 172\ g/mol)} \quad \text{(E.q. 2.2)}$$

The molecular weights of polyDMAEMA-MCQ and polyDMAEMA-MIQ estimated by this calibration method were very close to the theoretical values except the low molecular weight sample, as shown in Table 2.3.

2.4. Conclusion

Low polydispersity polyDMAEMA samples were prepared by living anionic polymerization and ATRP of DMAEMA monomer. These polymer samples were fully characterized by an organic GPC and ^1H NMR, before subsequently quaternized to cationic polyDMAEMA salts. The cationic polymers were used as polyelectrolyte standards for aqueous GPC measurements, especially for polyDMAEMA-based polyelectrolytes. The quaternization agent type, agent/polymer ratio, solvent and molecular weight of polyDMAEMA had significant effects on the quaternization efficiency. The ionic strength of solution and the charge density were two major factors to influence the determination of molecular weight. The following conclusions were reached based on the studies:

1. In the aqueous GPC study, it was found that the counter ions of polyDMAEMA quaternary salts had little effect on the hydrodynamic volume of polyelectrolytes. The polyDMAEMA-DMSQ calibration curve was applicable for the characterization of polyDMAEMA quaternary salts.
2. In optimizing chromatographic conditions, it was found that the separation of polyelectrolytes by GPC depended on the ionic strength of solution. High concentration of electrolyte salt was required to suppress adsorption between cationic polyelectrolytes and column support, especially for high molecular weight polymers. 0.3 M Na_2SO_4-0.1%(w/v) NaN_3 solution was selected as an optimal solution for

aqueous GPC measurement within studied molecular weight range. The polymer hydrodynamic volume increased with the increase of degree of quaternization.

3. In quaternization, at the stoichiometric mole ratio of quaternization agent/ polymer, the MeI had higher quaternization efficiency than Me_2SO_4. Homogeneous quaternization in DMSO provided higher quaternization efficiency than heterogeneous quaternization in acetone. Higher molecular weight polymers were more difficult to be quaternized than lower counterparts due to the diffusion effect.

Chapter 3 Atom Transfer Radical Polymerization of 2-(*N*, *N*-Dimethylamino)ethyl Methacrylate and 2-Hydroxyethyl Methacrylate Block Copolymer

3.1. Introduction

Bioactive polyDMAEMA has been currently developed as carriers for gene delivery and antisense oligonucleotides delivery 1) to enhance cell uptake; 2) to enhance system stability against nuclease degradation; and 3) to increase specificity and selectivity. However, there are some disadvantages. First, these carriers have shown high cytotoxicity. Secondly, the complexes of polymer/DNA or polymer/ oligonucleotides are often poorly water-soluble and tend to aggregate in aqueous solution because of charge neutralization (Kabanov, et. al., 1995). An approach to overcome this problem is to develop biocompatible water-soluble block copolymers. The hydrophilic biocompatible blocks of copolymer linked with polyDMAEMA blocks provide an ability to stabilize the complexes and to form micelles in aqueous solutions (Andersin, et. al., 2002; Dubruel, et. al., 2000).

Poly(2-hydroxyethyl methacrylate) (polyHEMA) are very important hydrophilic biocompatible polymers. PolyHEMA and its block copolymers can be used as biocom-

---

Note: This work has been published in Jin, X. P.; Shen, Y.; Zhu, S. "Atom Transfer Radical Polymeriation of 2-(N, N-Dimethylamino) ethyl Methacrylate and 2-Hydroxyethyl Methacrylate Block Copolymer", Macromol. Mater. Eng., 2003, 288, 925.

patibilizers, contact lenses, and hydrogels (Ruckenstein, et. al., 1998; Roovers, 1985; Mathew, et. al., 1993; Chen, et. al., 1989). Besides bioactivity, polyDMAEMA exhibits reversible pH-, salt-, and temperature-induced micellization in aqueous media (Zeng, et. al., 2000a; Vamvakaki, et. al., 1999; Bütün, et. al., 2001a). PolyDMAEMA-based block copolymers can be cross-linked to form nanospheres with cross-linked shell, which can act as nanoreactors and drug delivery vehicles (Zhang, et. al., 2000). Combined the bioactivity and other unique solution properties of polyDMAEMA and the biocompatibility of polyHEMA, well-controlled poly(DMAEMA-block-HEMA) hydrophilic-hydrophilic block copolymers can be very useful for preparing suitable gene delivery and other nanostructural "intelligent" materials and devices for biomedical applications.

Many polyDMAEMA- or polyHEMA-based hydrophilic and amphiphilic diblock copolymers have been prepared using various living polymerization techniques. For examples, DMAEMA was copolymerized with methylmethacrylate (MMA), methyl acrylate (MA), styrene, and ethylene oxide (Wang et. al., 1995; Vamvakaki, et. al., 1999; Zhang, et. al., 1999). HEMA was copolymerized with MMA, styrene, monomethoxy-capped oligo(ethyl glycol) methacrylate (OEGMA) (Ruckenstein, et. al.,1998; Beers, et. al., 1999; Robinson, et. al., 2001). However, there was no much effort made in preparing DMAEMA-HEMA copolymers with well-controlled block molecular weights. Recently, Huang, et al. (2002) reported grafting polymerization of HEMA film on gold surfaces by atom-transfer radical polymerization (ATRP) in aqueous media at room temperature. DMAEMA was added as a second monomer for further polymerization to evaluate the

living character of polyHEMA chains. However, there were no characterization data for the block copolymers and the control block lengths were not known.

Atom transfer radical polymerization (ATRP) is a versatile technique for preparation of well-defined polymers with different compositions, macromolecular architectures, and end-functional groups (Wang et. al., 1995; Sawamoto, et. al., 1996; Zhang, et. al., 1999; Matyjaszewski, 1998, 2001; Beers, et. al., 1999; Kamigaito, et. al., 2001). The process is proven to be effective in synthesizing hydrophilic functional polymers and block copolymers in polar solvent such as methanol and water. This chapter reports the experimental atom transfer radical block copolymerization of DMAEMA and HEMA in methanol via both Macroinitiator and Sequential addition methods. The effects of order of monomer addition, temperature, and molecular weight of macroinitiator on the living feature of homopolymerization and block copolymerization were investigated in a great detail. In the light of high rates of aqueous ATRP systems (Lobb, et. al., 2001; Wang, et. al., 1999a, 2000; Perrier, et. al., 2000; Huang, et. al., 2002), small amount of water is used and evaluated as a possible accelerator to promote the block copolymerization rate.

3.2. Experimental section

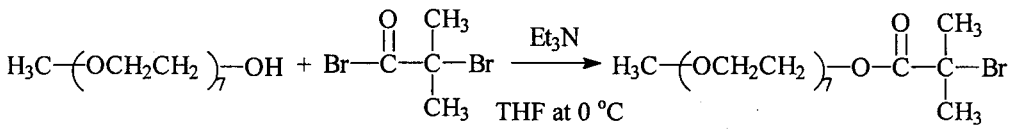
3.2.1. Materials

2-(*N, N*-Dimethylamino)ethyl methacrylate (DMAEMA) (Aldrich) was distilled over CaH_2 prior to use. 2-Hydroxyethyl methacrylate (HEMA) (Aldrich) was dissolved in water (25% vol. of monomer) and washed four times with an equal volume hexane.

NaCl was added to salt out the monomer. The monomer was dried over $MgSO_4$ and distilled under vacuum prior to use. Cu(I)Cl, Cu(II)Cl_2 and 2,2'-bipyridine were obtained from Aldrich and used without further purification. Methoxy oligo(ethylene glycol) (M_n = 350 g/mol), 2-bromoisobutyryl bromide, and triethylamine were used as received from Aldrich. Other reagents, such as tetrahydrofuran (THF), methanol (MeOH), *N, N'*-dimethylformamide (DMF), and hexane were all from Aldrich and used as received.

3.2.2. Synthesis of OEGBr initiator

Scheme 3.1. Synthesis of OEGBr initiator



Oligomeric methoxy polyethylene glycol 2-bromoisobutanate (OEGBr) was prepared following the procedure reported by Wang et. al. (2000c), as described in Scheme 3.1. To a solution of methoxy oligo(ethylene glycol) (20 g, 57.14 mmol) and triethylamine (12.07 ml, 85.71 mmol) in 60 ml of dried THF cooled in an ice-water bath was added dropwisely 2-bromoisobutyryl bromide (20.1 g, 85.71 mmol) diluted in 50 ml THF. A white precipitate, triethylamine–hydrogen bromide salt, was formed immediately. The mixture was magnetically stirred for 1 h at 0 °C and then over night at room temperature for a complete conversion. Triethylamine–hydrogen bromide salt was

filtrated and washed with 10 ml of THF three times. After THF was evaporated, the yellow crude product was washed with 100 ml of 1M $NaHCO_3$ solution for three times, followed with 100 ml of deionized water for three times. The product was extracted with diethyl ether and dried over Mg_2SO_4. The diethyl ether was evaporated under vacuum. Yield: 61.0% (17.4 g) with respect to methoxy oligo(ethylene glycol). ^1H NMR ($CDCl_3$, 200 MHz): 4.3 ppm (t, 2H), 3.74 (t, 2H), 3.63 (m, 24H), 3.54 (t, 2H), 3.35 (s, 3H), 1.91 (s, 6H).

3.2.3. Polymer preparation

3.2.3.1. Synthesis of homo-polymers

ATRPs of DMAEMA and HEMA were carried out in methanol at room temperature and 60 °C using the same procedures. In a typical experiment, 17.54 mg (0.177 mmol) of Cu(I)Cl, 55.29 mg (0.354 mmol) of 2,2'-bipyridine were charged to a 10 ml flask. The flask was sealed with a rubber septum, evacuated and back-filled with ultra-high pure nitrogen for three times. 2 g (17.72 mmol) of DMAEMA, and 2 ml of methanol, pre-degassed with bubbling pure nitrogen for 30 min, were transferred to the reaction flask via a double-tipped needle under vacuum to form a 50% w/v solution. 71.3 μl (0.177 mmol) of OEGBr was also degassed and added to the flask by a micro syringe to initiate the polymerization. The flask was then immersed into a water bath at room temperature. The samples were taken at designed time intervals. Excess methanol containing $CuCl_2$ was added to terminate the reaction. After evaporating the methanol, the mixture was dissolved in THF and the monomer residue was removed by re-

precipitation. The copper catalyst was removed by passing the solution through a column of silica gel. The samples were vacuum dried for characterization. The conversion was determined by ^{1}H NMR. The molecular weights and molecular weight distributions of the polymers were measured by a gel permeation chromatography (GPC) with DMF containing 0.2% w/v of LiBr as eluent, calibrated by polystyrene standards.

3.2.3.2. Synthesis of block copolymers

The block copolymers were prepared by both Macroinitiator and Sequential addition methods. In Macroinitiator method, polyHEMA (or polyDMAEMA), prepared by the above homopolymerization, was used as macroinitiator for the ATRP of the second monomer. For example, 12.59 mg (0.127 mmol) CuCl, 39.69 mg (0.254 mmol) of 2,2'-bipyridine were charged to a 10 ml round-bottom flask. The flask was then sealed with a rubber septum, evacuated and back-filled with ultra-high pure nitrogen for three times. 0.2 ml (2.58 mmol) of pre-degassed DMF was charged by syringe to 2 g (12.72 mmol) of DMAEMA that was pre-degassed with bubbling nitrogen for 30 min. DMAEMA was then transferred to the reaction flask via a double-tipped needle under vacuum and stirred for 10 min. In a 10 ml pear-shaped ampoule, 1.47 g (0.127 mmol) polyHEMA ($M_{n,\,GPC}$ = 11600, M_w/M_n = 1.25) was vacuum degassed and nitrogen back-filled three times. 2 ml of pre-degassed methanol was added. The ampoule was then immersed into an ultrasonic water bath for 20 min until polyHEMA was totally dissolved in MeOH. The prepared polyHEMA solution was then transferred into the flask via double-tipped needle to start the polymerization. The reaction was terminated by adding

excess $CuCl_2$ /methanol solution. The mixture solution was passed through a silica column to remove the copper residues. After evaporating methanol, the concentrated copolymer samples were dissolved in THF and then precipitated by excess hexane. The samples were vacuum dried for characterization. The conversion was measured by ^1H NMR with DMF as an internal standard and methanol-d_4 as solvent. The molecular weights and molecular weight distributions were determined by GPC in DMF containing 0.2 w/v % of LiBr using polystyrene as standard.

In Sequential method, when the homopolymerization of HEMA reached a conversion above 90 %, a degassed DMAEMA/methanol (1/1, w/v) solution was added to the polymerization solution. The kinetic sampling and characterization followed the same procedure as in Macroinitiator method.

3.2.4. Measurements

Nuclear Magnetic Resonance (NMR) Spectroscopy: ^1H NMR spectra were obtained on a Bruker AC-P200 Fourier transform spectrometer (200 MHz) in methanol-d_4 or D_2O solvent.

Gel Permeation Chromatography: The GPC measurements were carried out using Waters 2690 liquid chromatography equipped with a Waters Styragel HR5E 78x300mm linear column connected with two Varian MicroPak columns (G2500 H8, 7000 HXL), and 2410 refractive index detector at 50 °C. *N, N'*-dimethylformamide with 0.2 (w/v)% LiBr was used as solvent. Narrow polystyrene standards (Polyscience Inc)

were used to generate the calibration curve. Data were recorded and processed using Millennium 2.0 software package.

3.3. Results and discussion

3.3.1. Homo-polymerization of DMAEMA

DMAEMA has been polymerized via ATRP in various organic solvents (Zhang, et. al., 1998; Shen, et. al., 2000) and aqueous media (Zeng, et. al., 2000a). The ATRP of DMAEMA in MeOH at 60 °C was first reported by Zeng et al. (2000 b) using allyl-2-bromoisobutyrate as initiator and CuBr complexed with tris(2-di(butyl acrylate)amino ethyl) amine (BA_6-TREN) as catalyst. The monomer conversion could reach 96.3% in 2h. However, the polydispersity was slightly high (M_w/M_n=1.45). The initiator efficiency (f = $M_{n,\ cal}/M_{n,\ GPC}$) was 0.86. In this work, we studied the ATRP of DMAEMA in MeOH using OEGBr as initiator, which effectively initiated the ATRP of HEMA in MeOH (Robinson, et. al., 2001). CuCl was used as catalyst instead of CuBr based on the suggestion that a mixed halide initiation system R-Br/CuCl improves the ATRP livingness of methacrylic monomers (Matyjaszewski, et. al., 1998; Wang, et. al., 1999b).

Figure 3.1(a) shows the polymerization of DMAEMA in MeOH at room temperature and 60 °C respectively. The conversion reached 90 % in 30h at room temperature. Increasing temperature accelerated the polymerization rate. Only 8h was needed at 60 °C to reach the same conversion. In both cases, the plot of $\ln([M_0]/[M])$

versus time exhibited some curvature indicating a radical concentration decrease in the early stage caused by significant radical termination. Before the reaction, RX + Cu(I)X/L $\leftrightarrow$ $R^{\bullet}$ + Cu(II)X_2/L, reached equilibrium, the low Cu(II) concentration favored the forward reaction and resulted in a high radical concentration and subsequent termination. With the accumulation of Cu(II) species and the increase in the reverse reaction rate, the polymerization started to proceed smoothly with a constant radical concentration. Cu(II)Cl_2 was added as a deactivator to suppress the radical termination at the early stage. In the presence of 30% $CuCl_2$, the ln([M_0]/[M]) versus time curve became more linear.

Figure 3.1(b) shows the number-average molecular weights (M_n) and polydispersities (M_w/M_n) of the resulting polyDMAEMA as a function of DMAEMA conversion. The molecular weights of polyDMAEMA increased linearly with the conversion in all cases, demonstrating the living characters of the system. The polydispersities were in the range of 1.2 to 1.3.

3.3.2. Homo-polymerization of HEMA

The ATRP of HEMA in MeOH exhibited a good control over the molecular weights. Figure 3.2 presents the kinetic and molecular weight data for the ATRP of HEMA in MeOH at room temperature and 60 °C. At room temperature, the polymerization of HEMA also experienced an initial curvature in the ln([M_0]/[M]) versus

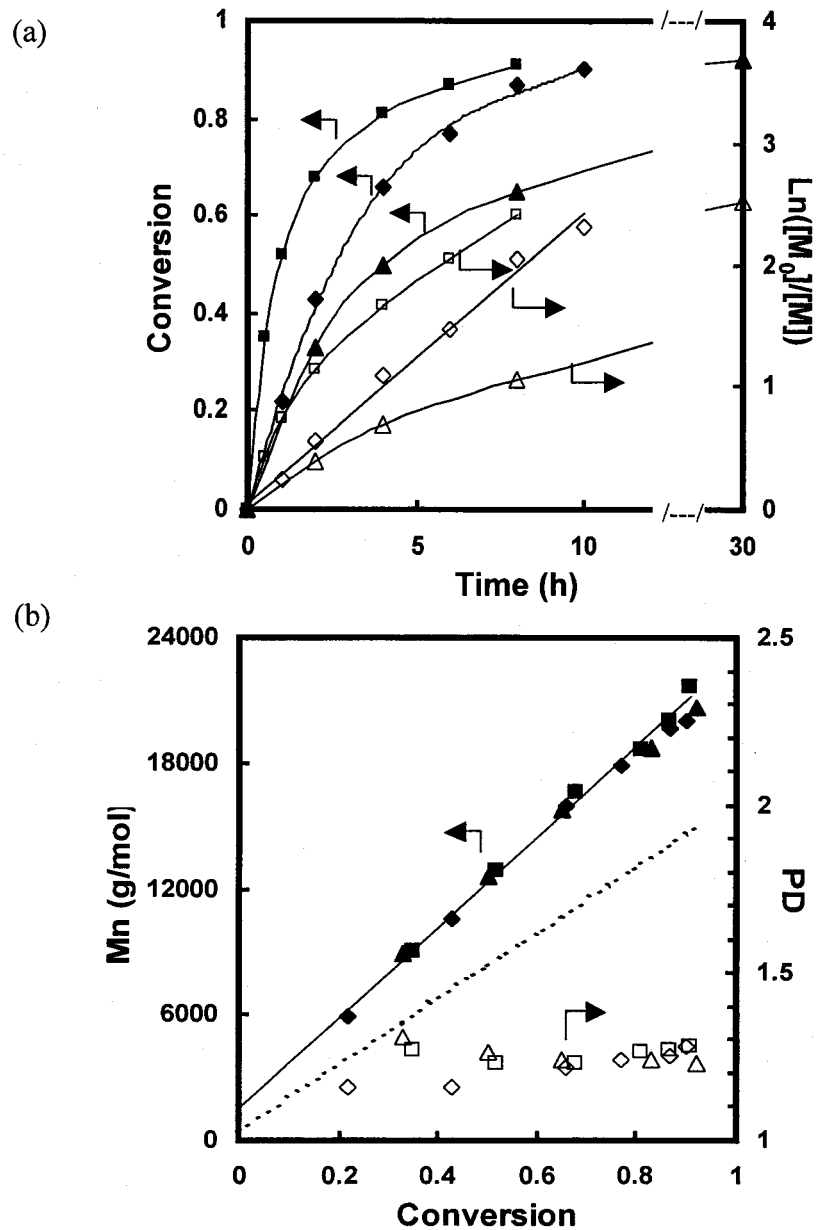


Figure 3.1. (a) Kinetic plot and (b) M_n and polydispersity for the ATRP of DMAEMA in 50% w/v MeOH at room temperature and 60 °C. $[DMAEMA]_0$ = 12.72 mM and $[OEGBr]_0 = [CuCl]_0$ = 0.127 mM. (------) Theoretical line.

(▲, Δ) at room temperature, $[Bpy]_0$ = 0.254 mM;

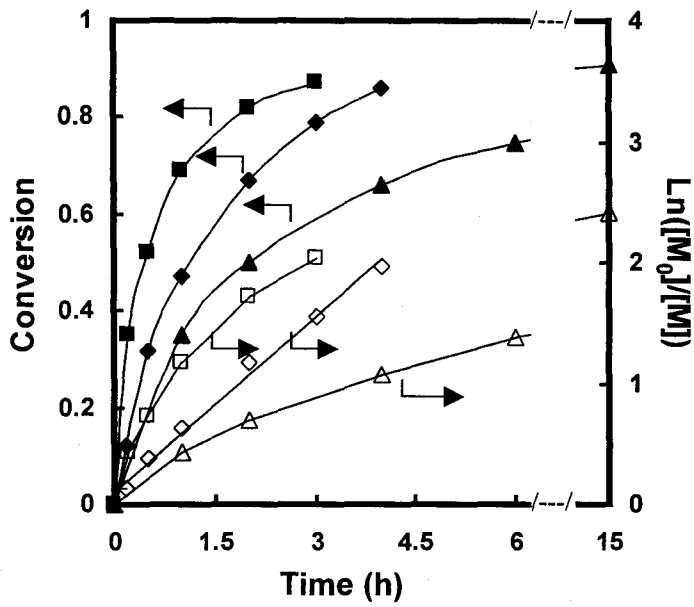
(■, □) at 60 °C, $[Bpy]_0$ = 0.254 mM;

(♦, ◊) at 60 °C, $[Bpy]_0$=0.331 mM and $[CuCl_2]_0$ = 0.038 mM.

time plot. The molecular weight of polyHEMA increased linearly up to 90% conversion and the polydispersity decreased from 1.4 to 1.2. At 60 °C, the polymerization was more rapid. The conversion reached 90% in about 3h. The molecular weight of polyHEMA also increased linearly with conversion. The polydispersities were about 1.3 to 1.4, higher than the values at room temperature. The addition of 30% molar $CuCl_2$ improved the control of the polymerization at 60 °C. The linear increase of $\ln([M_0]/[M])$ versus time indicates the first order with respect to HEMA concentration. The polymer molecular weight was linear with conversion and the polydispersities were slightly lower than those without $Cu(II)Cl_2$.

The molecular weights of polyHEMA measured by GPC in DMF were significantly higher than their theoretical values. The GPC data with a targeted DP of 100 were 3-5 times higher than those measured by NMR (see Table 3.1). Similar results were also reported in literatures. Beers et. al. (1999) reported that the GPC protocol over-estimated true molecular weight of polyHEMA by a factor 2. Armes and coworkers (Robinson, et. al., 2001) found that the GPC gave M_n 5-10 times higher than the NMR data. This discrepancy was caused by the large difference in the hydrodynamic volumes of polyHEMA and polystyrene having the same molecular weights. The molecular weight data of all polyHEMA samples in this work were measured against polystyrene standards in DMF with 0.2 % w/v of LiBr. The hydroxyl groups of polyHEMA complex with DMF molecules through hydrogen bonding and thus extends the chains that have much larger hydrodynamic volumes than polystyrene in DMF.

(a)



(b)

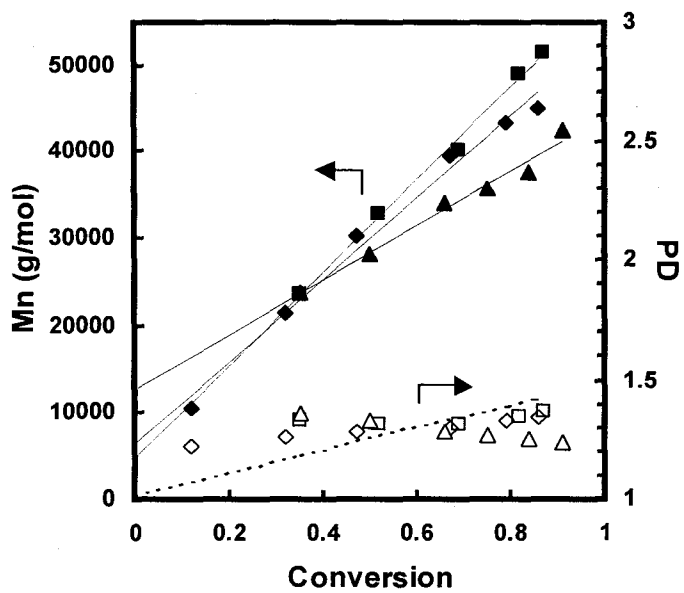


Figure 3.2. (a) Kinetic plot and (b) M_n and polydispersity for the ATRP of HEMA in 50% w/v MeOH at room temperature and 60 °C. $[HEMA]_0$ = 15.37 mM and $[OEGBr]_0$ = $[CuCl]_0$ = 0.154 mM. (------) Theoretical line

(▲, Δ) at room temperature, $[Bpy]_0$ = 0.308 mM;

(■, □) at 60 °C, $[Bpy]_0$= 0.308 mM;

(♦, ◊) at 60 °C, $[CuCl_2]_0$ = 0.046 mM, $[Bpy]_0$ = 0.4 mM.

Table 3. 1. Summary of the synthesis parameters and molecular weight data for ATRP of HEMA at room temperature.

Polymer ID	$M_{n,th}$	DP_{NMR}	$M_{n,NMR}$	$M_{n,GPC}$ [e]	M_w/M_n	$f = M_{n,GPC}/M_{n,NMR}$
H1 [a]	4400	31	4500	21000	1.34	4.7
H2 [b]	7000	52	7260	31200	1.31	4.3
H3 [c]	11560	85	11550	36700	1.25	3.2
H4 [d]	13500	99	13360	45100	1.34	3.4

Reaction conditions: monomer/initiator ratio (HEMA/OEGBr) a =50, b, c =100, d =200; OEGBr/CuCl/Bpy=1/1/2, HEMA/MeOH=1/1.
e: determined by GPC using DMF-0.2% w/v % of LiBr as solvent at 50 ^{o}C, polystyrene as standard.

3.3.3. Block ATRP of poly(DMAEMA-b-HEMA)

Theoretically, a poly(DMAEMA-b-HEMA) sample can be prepared by either DMAEMA or HEMA chain as the first block. However, the attempts to prepare the block copolymer using DMAEMA as the first block were not successful. Figure 3.3 shows the GPC traces of the block copolymers prepared by both methods at room temperature. All the GPC traces of the block copolymers had a shoulder peak at the macroinitiator position, indicating the presence of polyDMAEMA homopolymer. This suggests that a significant fraction of the polyDMAEMA chain ends were dead and could not initiate the HEMA polymerization. At 60 ^{o}C, the loss of the active chain-ends was even more severe. Similar observations were also reported by other groups working on

block ATRP. Liu et al. (2002) recently reported that the PEO-DMAEMA macroinitiator did not initiate the polymerization of 2-(diethylamino) ethyl methacrylate (DEAEMA). The incomplete chain extension was attributed to the loss of terminal halogen atom during the macroinitiator preparation.

3.3.4. Block ATRP of poly(HEMA-b-DMAEMA)

The polyHEMA macroinitiator was prepared at room temperature. The polymerization was terminated at about 85% conversion. After purification, the macroinitiator ($M_{n,\,GPC}$ = 36700, M_w/M_n = 1.25) was used to start the polymerization of DMAEMA in MeOH at room temperature. A conversion of 90% was obtained in 40 h. Compared to the homo-polymerization of DMAEMA under the same condition (92% conversion in 30 h, in Figure 3.1(a)), the block copolymerization was slower. Figure 3.4 shows the kinetics and molecular weight developments of this block copolymerization of DMAEMA.

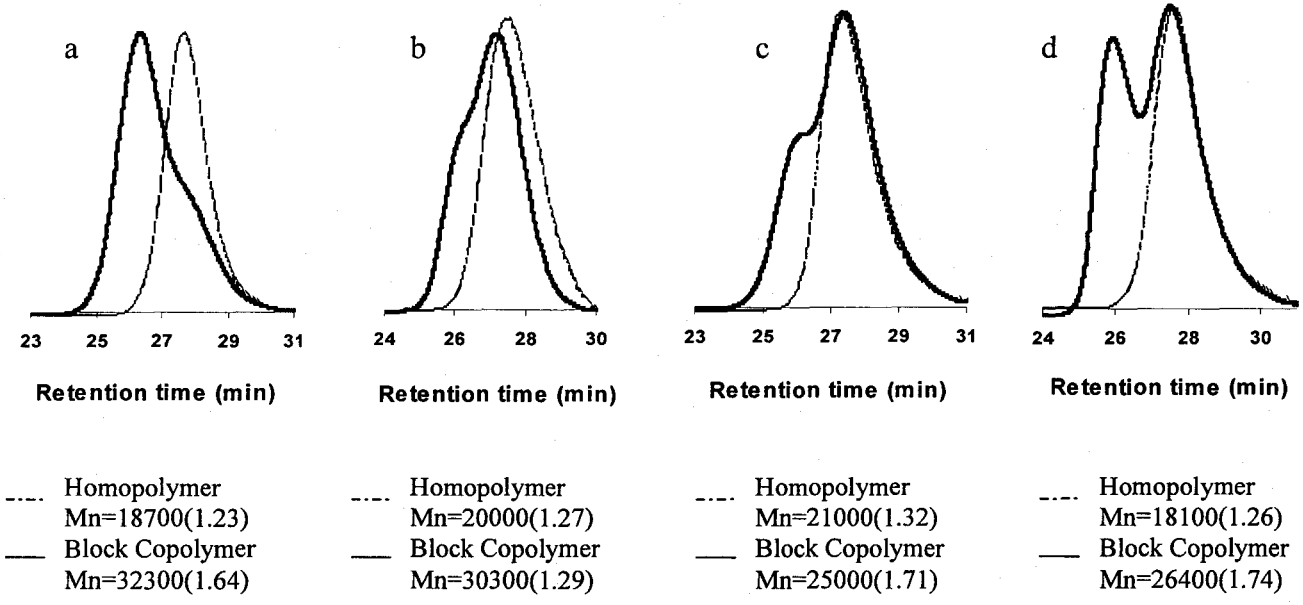
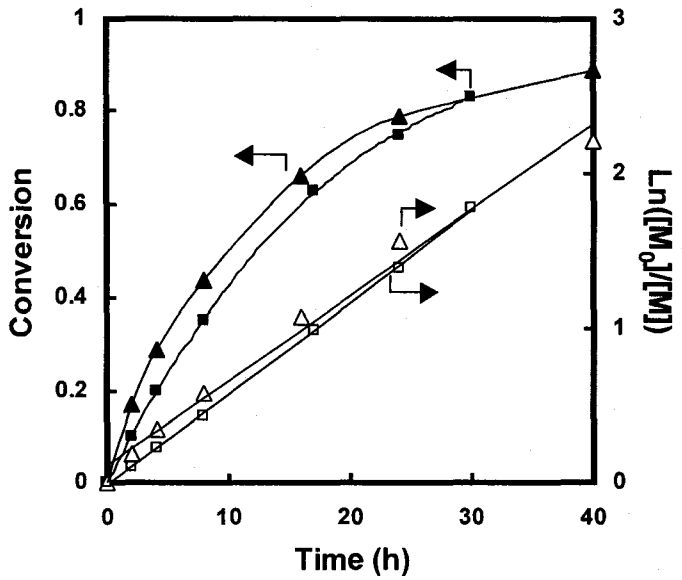


Figure 3.3. GPC chromatograms of polyDMAEMA and poly(DMAEMA-b-HEMA). The samples were prepared in methanol, $[HEMA]_0$ =7.68 mM, and (a) at room temperature via Sequential method, $[HEMA]_0/[DMAEMA]_0/[OEGBr]_0/\ [CuCl]_0/[Bpy]_0$ = 100/100/1/1/2; (b) at room temperature via Macroinitiator method, $[HEMA]_0/[polyDMAEMA]_0/\ [CuCl]_0/[Bpy]_0$ = 100/1/1/2; (c) at 60 °C via Sequential method, $[HEMA]_0/[DMAEMA]_0/[OEGBr]_0/[CuCl]_0/[CuCl_2]_0/\ [Bpy]_0$ = 100/100/1/1/0.3/2.6; (d) at 60 °C via Macroinitiator method, $[HEMA]_0/[polyDMAEMA]_0/[CuCl]_0/[CuI_2]_0/\ [Bpy]_0$ = 100/1/1/0.3/2.6

The copolymerization was first order with respect to DMAEMA monomer up to about 90% conversion, indicating less radical termination at the early stage than in the homopolymerization. This is probably because of the long macroinitiator chains. Due to diffusion limitations, the initiator radicals experience less termination. The polydispersity remained low, <1.3 at 60 % conversion. However, it increased sharply to 2.5 at 90% conversion, indicating side reactions at the high conversion region.

Figure 3.5 shows the GPC traces of poly(HEMA-b-DMAEMA) samples. A complete extension of the macroinitiator chains to block copolymers was evident. This suggests that macroinitiator chain-ends were living and initiated the polymerization of DMAEMA. Since polyHEMA chains are more extended than polyDMAEMA in DMF, the chain length of polyHEMA dominates the hydrodynamic volume of block copolymer. The increase in the hydrodynamic volume, as shown in Figure 3.5 (a), appeared to be moderate because the macroinitiator chains had lengths of about 85 HEMA units and the DMAEMA/macroinitiator ratio was 100. For a comparison, Figure 3.5 (b) shows the chromatograms of poly(HEMA-b-DMAEMA) with 30-unit polyHEMA ($M_{n,\ GPC}$ = 21000) at the DMAEMA/macroinitiator ratio of 200. The hydrodynamic volume increased obviously with the polymerization of DMAEMA. The block copolymer samples were not contaminated by dead polyHEMA chains; the polyHEMA chains were active in initiating DMAEMA polymerization.

(a)



(b)

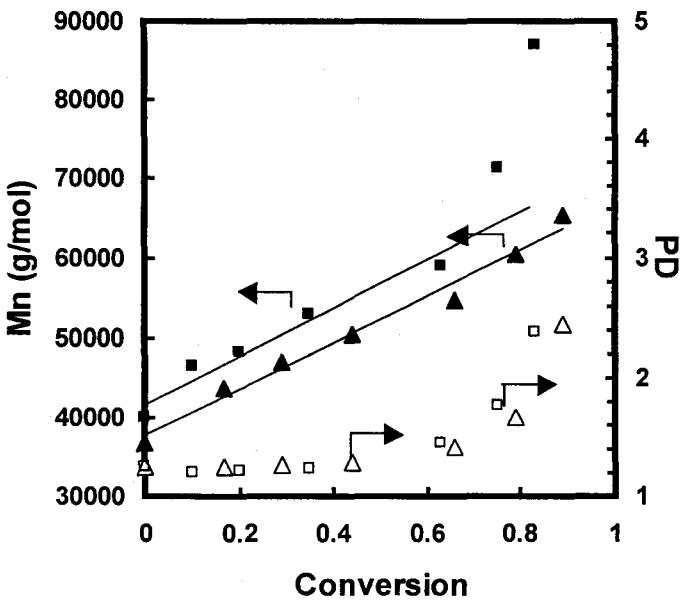


Figure 3.4. (a) Kinetic plot and (b) M_n and polydispersity for the block ATRP of poly(HEMA-b- DMAEMA) in 50% w/v MeOH at room temperature.
(▲, Δ) Macroinitiator method. polyHEMA macroinitiator: $M_{n,\,GPC}$ = 36700, M_w/M_n = 1.25, $[DMAEMA]_0$ = 12.72 mM, $[polyHEMA]_0 = [CuCl]_0$ = 0.127 mM, $[Bpy]_0$ = 0.254 mM; (■,□) Sequential method. $[HEMA]_0 = [DMAEMA]_0$ = 38.42 mM, $[OEGBr]_0 = [CuCl]_0$ = 0.384 mM, $[Bpy]_0$ = 0.768 mM.

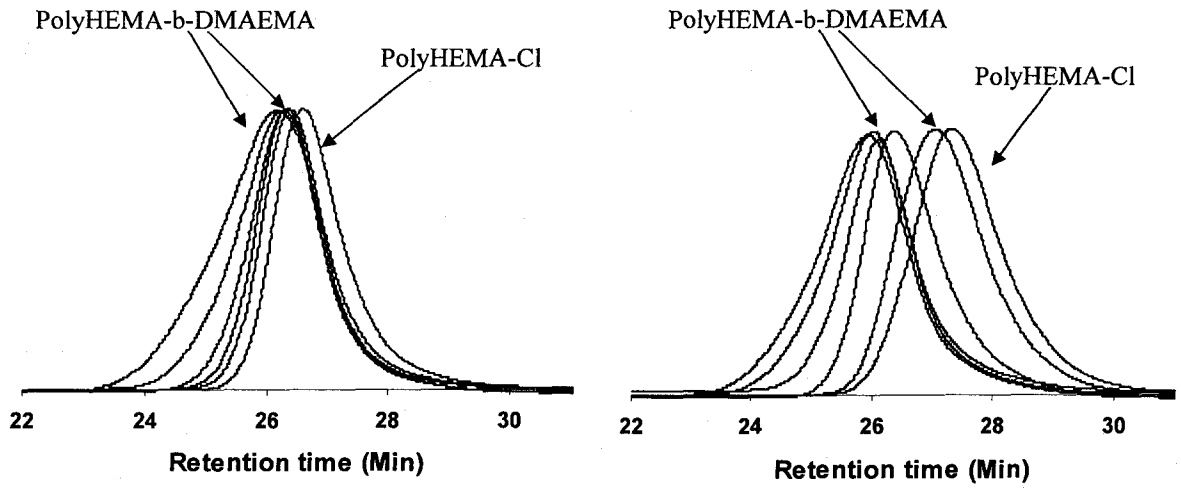


Figure 3.5. GPC chromatograms of poly(HEMA-b-DMAEMA). The samples were prepared in methanol at room temperature with different block molecular weights.

(a) Macroinitiator: $M_{n,\ GPC}$ = 36700, M_w/M_n = 1.25, $[DMAEMA]_0/[polyHEMA]_0/\ [CuCl]_0/[Bpy]_0$ = 100/1/1/2, $[DMAEMA]_0$ = 12.72 mM,

(b) Macroinitiator: $M_{n,\ GPC}$ = 21000, M_w/M_n = 1.31, $[DMAEMA]_0/[polyHEMA]_0/\ [CuCl]_0/[Bpy]_0$ = 200/1/1/2, $[DMAEMA]_0$ = 12.72 mM.

The one-pot synthesis via sequential monomer addition was also evaluated for the preparation of poly(HEMA-b-DMAEMA) samples. After the polymerization of HEMA reached 91% conversion, DMAEMA was added for the block copolymerization. The kinetic data, as presented in Figure 3.4 (a) together with the macroinitiator counterpart for comparison, shows that the polymerization was first order with respect to DMAEMA concentration. The M_n of the block copolymers increased with conversion linearly up to 60% conversion (see Figure 3.4 (b)). After 60% conversion, both M_n and polydispersity increased sharply. The one-pot method, though convenient, has a disadvantage of the contamination of residual HEMA monomers in the DMAEMA block. The second block is virtually a random copolymer of DMAEMA and HEMA. It has been illustrated by Matyjaszewski et al. (2000b) that properties of pure block copolymers prepared by an isolated Macroinitiator method and random/gradient block copolymers by a Sequential addition method can be very different.

Table 3.2 provides a summary for the ATRP preparation of poly(HEMA-b-DMAEMA) under various conditions (temperature, method, and solvent type). The polymerization at 60 ^{o}C was much faster with 67% conversion in 6 hrs compared to 16 hrs at room temperature (B3 versus B1, Macroinitiator method). However, the reaction mixture at 60 ^{0}C became gelled at 8 hrs, in contrast to the homogeneous solution at room temperature throughout the polymerization.

Table 3.2. Summary for the preparation of poly(HEMA-block-DMAEMA) by ATRP under different conditions.

Sample ID	Homopolymer PHEMA					Poly(HEMA-block-DMAEMA)							
	Conv. (%)	$M_{n,\,Cal}$	$M_{n,\,NMR}$	$M_{n,\,GPC}$	PD	Method	T °C	Solvent	Time (h)	Conv. (%)[b]	$M_{n,\,Cal}$[c]	$M_{n,\,GPC}$[d]	PD
B1	85.8	11700	11530	36700	1.25	M	Room	MeOH	16	66	22100	50500	1.28
									24	80	24300	60300	1.66
B2	91.2	12400		39900	1.25	S	Room	MeOH	16	65	22600	58900	1.44
									30	83	25500	87000	2.37
B3 [a]	85.8	11700	11530	36700	1.25	M	60	MeOH	6	67	22200	80200	2.73
									8	Gel			
B4 [a]	90.2	12200		52800	1.39	S	60	MeOH	6	35	17700	74700	2.56
									7	Gel			
B5	85.8	11700	11530	36700	1.25	M	Room	$MeOH/H_2O$ 90/10 v/v	6	61	21300	51300	1.32
									24	87	25400	74300	1.90
B6	85.8	11700	11530	36700	1.25	M	Room	$MeOH/H_2O$ 95/5 v/v	24	86	25200	72500	1.96

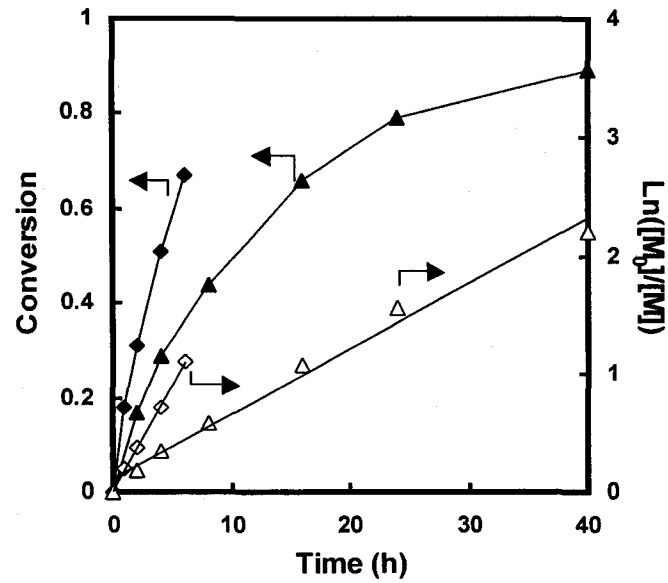
M - macroinitiator method: $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[Bpy]_0=100/1/1/2$;
S - sequential addition method: $[HEMA]_0/[DMAEMA]_0/[OEGBr]_0/[CuCl]_0/[Bpy]_0 =100/100/1/1/2$, except for "a";
a: 30 mol% of $CuCl_2$, B3: $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[CuCl_2]_0/[Bpy]_0=100/1/1/0.3/2.6$; B4: $[HEMA]_0/[DMAEMA]_0/[OEGBr]_0/[CuCl]_0/[CuCl_2]_0/[Bpy]_0 =100/100/1/1/0.3/2.6$;
b: Measured by ^1H NMR with DMF as internal standard;
c: $M_{n,\,Cal} = M_{n,\,Cal}$ (polyHEMA) + ($[DMAEMA]_0/[I]_0$) × mw. DMAEMA × Conv. DMAEMA;
d: Measured in DMF solvent containing 0.2%(w/v) of LiBr with polystyrene as standard.

Figure 3.6. (a) shows that the ln([M_0]/[M]) versus time curve was linear in the pre-gel region. The M_n at 60 ^{0}C increased dramatically at about 40% conversion in contrast to the linear relationship up to 80% at room temperature, as shown in Figure 3.6 (b). The polydispersity increased from 1.3 at the beginning to 2.7 at 67% conversion. The polymerization at 60 ^{0}C with the sequential method had even more severe gel problem, as shown in Figure 3.7 (a) and 3.7 (b).

3.3.5. Effect of water as accelerator

The block ATRP of HEMA and DMAEMA was well-controlled at room temperature, but the polymerization of DMAEMA was very slow. At 60 ^{o}C, the rate was good, but the system became out of control because of gel formation. A system of both good control and high rate is most desired. High rates were often reported with aqueous ATRP systems in literatures (Huang, et. al., 2002; Wang, et. al., 2000a; 2000b; Perrier, et. al., 2002.) The effects of polar solvents on the activation reaction was investigated by Chamhard et al (2000) with butyl acrylate and by Haddleton et al. (2000) with methacrylates. Water acts as a competitive co-ligand for copper species and favors the formation of Cu(II)Cl_2, which moves the equilibrium RX + Cu(I)X/L = R* + Cu(II)X_2/L towards the right side, yielding high radical concentration. In this work, small amount of water (equivalent to 5 and 10% volume of MeOH) was added as accelerator to promote the DMAEMA block co-polymerization. Figure 3.8 (a) shows that the DMAEMA conversion reached 87% in 24h with water compared to over 40 hr without water.

(a)



(b)

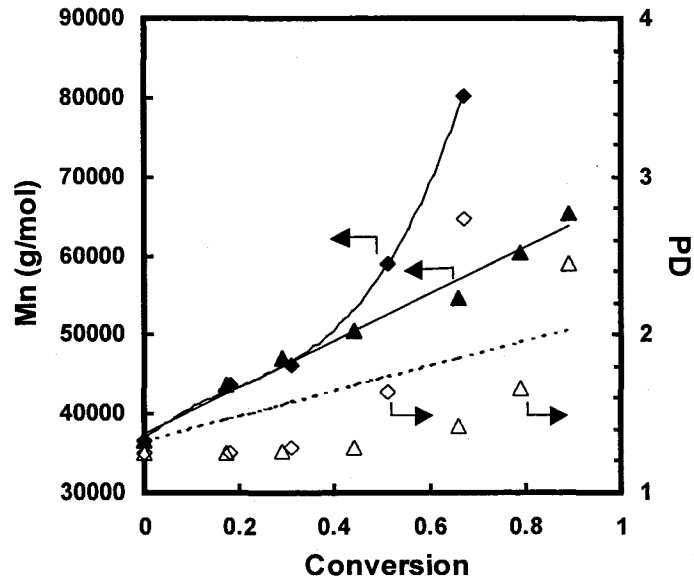
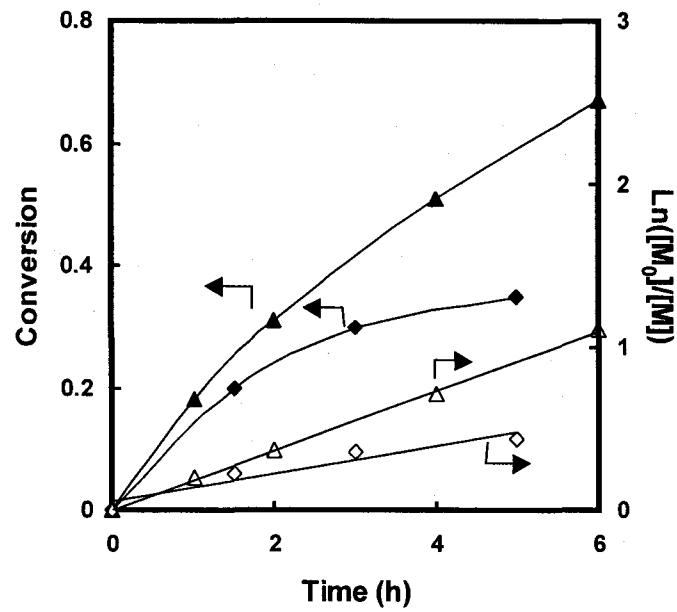


Figure 3.6. (a) Kinetic plot and (b) M_n and polydispersity for the block ATRP of poly(HEMA-b- DMAEMA) in 50% w/v MeOH at room temperature and 60 °C. (▲, Δ) at room temperature, $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[Bpy]_0$ = 100/1/1/2; (♦,◊) at 60 °C, $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[CuCl_2]_0/[Bpy]_0$ = 100/1/1/0.3/2.6, $[DMAEMA]_0$ = 12.72 mM. (------) Theoretical line.

(a)



(b)

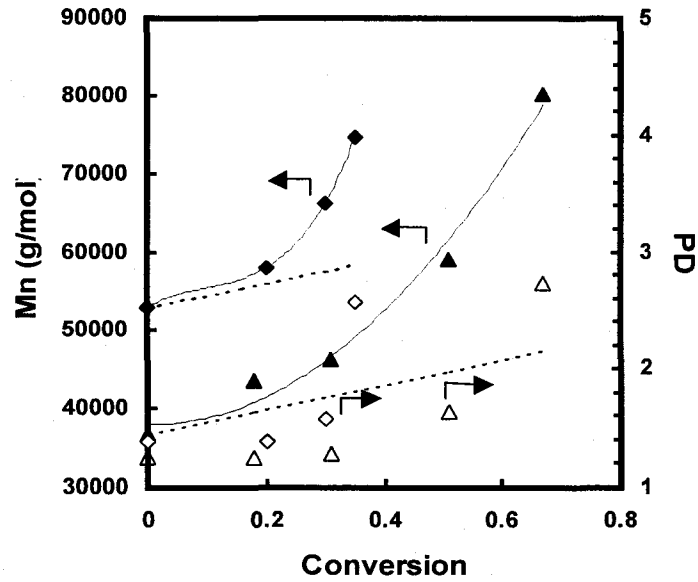
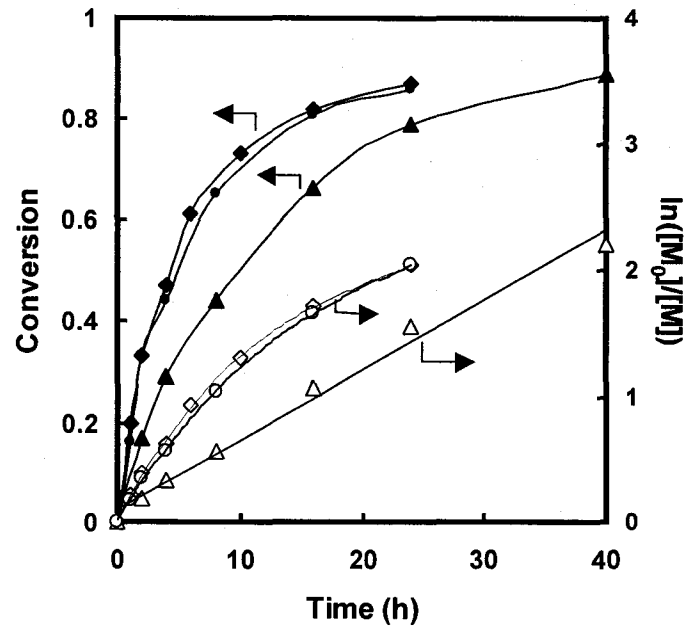


Figure 3.7. (a) Kinetic plot and (b) M_n and polydispersity for the block ATRP of poly(HEMA-b-DMAEMA) in 50% w/v MeOH at 60 °C. (------) Theoretical line. (▲, Δ) Macroinitiator method: $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[Bpy]_0$ = 100/1/1/2, $[DMAEMA]_0$ = 12.72 mM, $M_{n,\ GPC}$ of macroinitiator = 36700 (PD = 1.25). (♦, ◊) Sequential addition method: $[HEMA]_0/[DMAEMA]_0/[CuCl]_0/\ [CuCl_2]_0/[Bpy]_0$ = 100/100/1/0.3/2.6, $[HEMA]_0$ = 7.68 mM, 90% HEMA conversion in polyHEMA preparation.

However, the ln ($[M_0]/[M]$) versus time curve was not linear. The M_n and polydispersity of the copolymers increased linearly up to 65% of DMAEMA conversion, as shown in Figure 3.8 (b). Above this point, both M_n and polydispersity increased sharply, indicating significant side reactions such as gelation.

There are two possibilities for the gel formation in this system. One is caused by hydrogen bonding between the hydroxyl group of polyHEMA and the tertiary amine group of polyDMAEMA. The other is by transesterification through the hydroxyl group of polyHEMA that attacks the ester group of either polyDMAEMA or polyHEMA. The formed gel cannot be dissolved in MeOH and even more polar solvent such as DMF or DMSO. Adding acetic acid (CH_3COOH) to the gel samples or heating the gel samples did not help to dissolve the gels, suggesting that the gels were not formed by hydrogen bonding. We suspected that the gelation was caused by transesterification between HEMA polymer and DMAEMA monomer that yields cross-linkage unit of ethylene glycol dimethacrylate. In order to elucidate the nature of crosslinking, many experiments were carried. However, the results remained inconclusive.

(a)



(b)

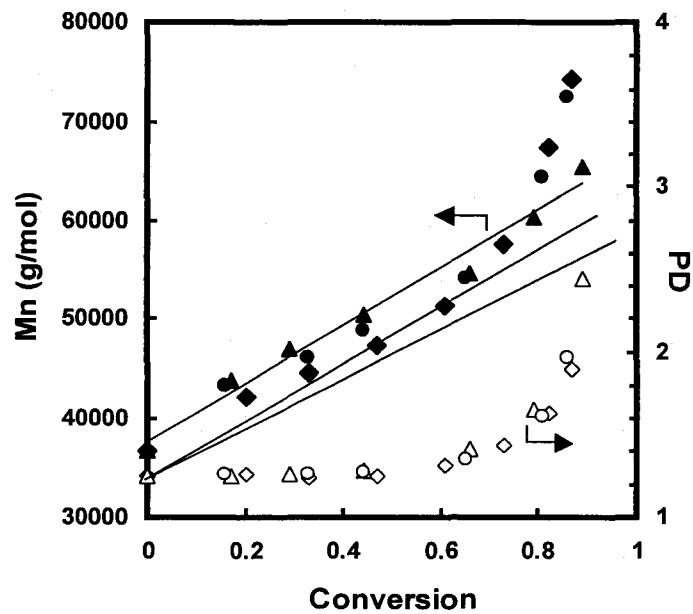


Figure 3.8. (a) Kinetic plot and (b) M_n and polydispersity for the block ATRP of poly(HEMA-b-DMAEMA) at room temperature with H_2O as accelerator. $[DMAEMA]_0/[polyHEMA]_0/[CuCl]_0/[Bpy]_0$ = 100/1/1/2; $[DMAEMA]_0$ = 12.72 mM. $M_{n,\,GPC}$ of macroinitiator = 36700 (PD = 1.25).
(▲, Δ) MeOH; (●, ○) MeOH/H_2O = 95/5 v/v; (♦, ◊) MeOH/H_2O=90/10 v/v

3.3.6. Preparation of poly(HEMA-b-DMAEMA) with various block molecular weights

The block copolymers with different block molecular weights were prepared by varying the monomer/macroinitiator feed ratio. All block copolymerizations were carried out in MeOH at room temperature using polyHEMA as macroinitiator and terminated at about 50% of DMAEMA conversion. The M_n and polydispersity data for the block copolymer samples are summarized in Table 3.3. The M_n data by GPC were not true values due to the difference in the hydrodynamic volumes between the block copolymer and polystyrene standard. However, the relative magnitudes reveal the significant effect of the block composition on the hydrodynamic volume.

^{1}H-NMR was used to determine the block composition. The typical ^{1}H-NMR spectra of polyHEMA and poly(HEMA-b-DMAEMA) are shown in Figure 3.9. The block copolymer structure is clearly confirmed. The block composition was calculated by the NMR signal at δ 2.6-2.8 of the two protons in polyDMAEMA (NCH_2) and the signal at δ 4.0-4.2 of the four protons of OCH_2 in polyDMAEMA and polyHEMA. A good agreement was obtained between the measured compositions and their theoretical values (see Table 3.3). The data in Table 3.3 also show that the block molecular weights were well controlled.

Table 3.3. Summary of the synthesis parameters and molecular weight data for block copolymerization of polyHEMA-DMAEMA via ATRP in MeOH at room temperature.

Sample ID	Macro-initiator	$M_{n,NMR}$ of PHEMA (g/mol)	$M_w/M_{n,GPC}$ of PHEMA [c]	Conv. % of block copolymer	Theoretical DP of block copolymer	DP of block copolymer by NMR	$M_{n,GPC}$ of block copolymer [c] (g/mol)	M_w/M_n of block copolymer [c]
B7 [b]	$PHEMA_{50}$	7260	1.31	54	$PHEMA_{53}$-$PDMAEMA_{54}$	$PHEMA_{52}$-$PDMAEMA_{49}$	53270	1.43
B8 [a]	$PHEMA_{50}$	7260	1.31	50	$PHEMA_{53}$-$PDMAEMA_{100}$	$PHEMA_{52}$-$PDMAEMA_{101}$	60800	137
B9 [a]	$PHEMA_{50}$	7260	1.31	65	$PHEMA_{53}$- $PDMAEMA_{130}$	$PHEMA_{52}$- $PDMAEMA_{140}$	76300	1.42
B10 [b]	$PHEMA_{100}$	13360	1.34	58	$PHEMA_{101}$-$PDMAEMA_{58}$	$PHEMA_{99}$-$PDMAEMA_{60}$	80900	1.47
B11 [a]	$PHEMA_{100}$	13360	1.34	55	$PHEMA_{101}$-$PDMAEMA_{110}$	$PHEMA_{99}$- $PDMAEMA_{110}$	85000	1.47
B12 [a]	$PHEMA_{100}$	13360	1.34	71	$PHEMA_{101}$-$PDMAEMA_{142}$	$PHEMA_{99}$- $PDMAEMA_{137}$	99600	1.55

Reaction conditions: monomer DMAEMA/macroinitiator ratio: a=200, b=100; Macroinitiator/CuCl/Bpy=1/1/2, HEMA/MeOH=1/1 w/v.
c: determined by GPC using DMF-0.2% w/v % of LiBr as solvent, polystyrene as standard.

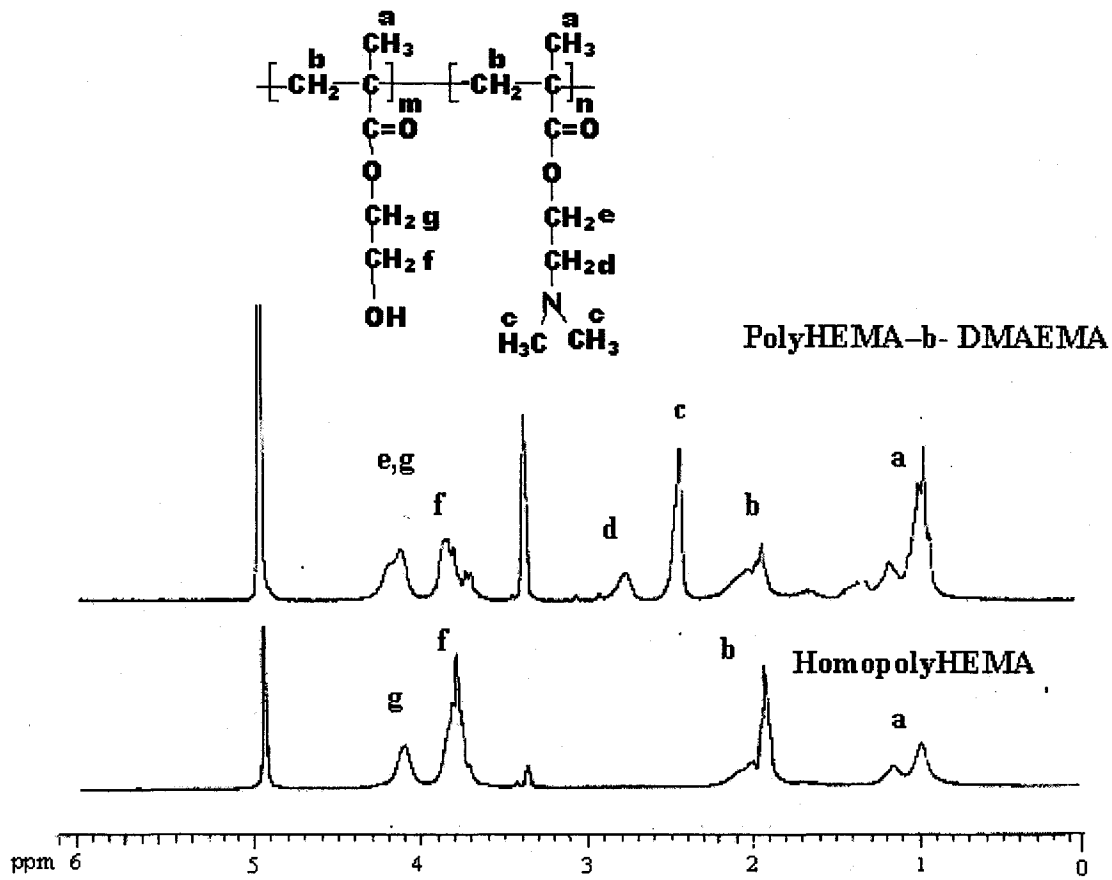


Figure 3.9. ^{1}H NMR spectra of polyHEMA and poly(HEMA-b-DMAEMA) with peaks assigned, in Methanol-d_4.

A recent study by Bories-Azeau and Armes (2002) on the ATRP of DMAEMA in methanolic solvents showed that a small amount of DMAEMA experienced self-catalyzed transesterification to methyl methacrylate. This transesterification appeared to be not significant under the experimental conditions of this work, as evident from the absence of methoxy signal at δ 3.55 in Figure 3.9.

3.4. Conclusion

Based on the studies on the block copolymerization of poly(HEMA-b-DMAEMA) via ATRP, the following conclusions can be reached.

1. The order of polymerization of the two monomers strongly affected the control of the block copolymers. When DMAEMA was used as the first monomer, both Macroinitiator and one-pot Sequential addition method yielded the block copolymer samples that contained significant amount of polyDMAEMA.
2. Well-controlled poly(HEMA-b-DMAEMA) samples with various block molecular weights were obtained through Macroinitiator method at room temperature when polyHEMA was used as macroinitiator to start the polymerization of DMAEMA. The molecular weight increased linearly with conversion up to 90%. Sequential addition method also produced controlled DMAEMA block when the DMAEMA conversion was below 60%, but the polydispersity increased to 2.4 at 90% due to side reactions.
3. Elevating temperature to 60 ^{o}C increased the polymerization rate, but led gel formation. Both the molecular weights and polydispersity increased sharply at 40% conversion.
4. Water accelerated the polymerization rate, but led to the loss of system livingness. Good control was only obtained under 60% of DMAEMA conversion in the presence of 10% water.

Chapter 4 Complexation of Well-Controlled Low-Molecular-Weight Polyelectrolytes with Antisense Oligonucleotides

4.1. Introduction

DMAEMA-based polyelectrolytes are a new class of attractive cationic polymers for delivery applications of gene and antisense oligonucleotides. The reversible pH-, salt- and temperature-sensitivities of the polymers in aqueous media are also important for later release of nucleic acids from the complexes. Recently several research groups have reported the studies of polyDMAEMA as antisense oligonucleotides delivery (Van Rompaey, et. al., 2000, 2001a and 2001b; Read, et. al., 2000; Deshpande, et. al., 2002). A challenge associated with these polymer systems is the control of polymer molecular weight and charge density. ONs are usually 10-30 nucleotides in length and have molar mass in the range of ~3000-9000 g/mol. Most cationic polymers used for antisense delivery, on the other hand, have very high molecular weights and broad molecular weight distributions. The complexation between ONs with cationic (co)polymers can be quite problematic due to the large size differences between the two classes of macromolecules. Another significant drawback is that the resulted polymer/ON complexes show high cytotoxicity, especially for the polymers with high molecular

---

Note: This work has been submitted as a full article, Jin, X. P.; Achenbach, J. C.; Zhu, S.; Li, Y. "Well-defined and Well-controlled Polyelectrolytes for Non-viral Antisense Oligonucleotides Deliveries", to Bioconjugate Chemistry, 2003, July.

weight and/or high charge density.

Therefore there is a significant need for examining the effects of the properties of polyDMAEMA (such as molecular weight, charge density, block composition) on the complexation with antisense ONs and on the delivery of these molecules into cells. For example, it needs to be determined what cationic charge level is adequate to neutralize ONs and also to provide optimal residual charge for interactions with cell membranes without causing much cytotoxicity.

To establish these structure-performance relationships, it is essential to synthesize well-defined and well-controlled polymers and to evaluate their ability to complex with ONs. In previous chapters, we reported the preparations of well-defined and well-controlled DMAEMA-based (co)polymer samples by living polymerization methods, such as living anionic polymerization (LAP) and atom transfer radical polymerization (ATRP). Very recently, Armes group directly synthesized poly(trimethylamino)ethyl methacrylate chloride (polyDMAEMA-MCQ) in protic media by ATRP (Li, et. al., 2003). These advances in polymerization technologies make it possible to prepare well-defined well-controlled polyelectrolytes/ONs complexes for antisense binding studies.

Many methods can be used to study the complexation of cationic polymers and nucleic acids, such as gel electrophoresis, density gradient analysis, fluorescence–based assays. Among these methods, the fluorescence technique is outstanding because it can not only be used to investigate the association of polymer with nucleic acid in vitro but also has a potential of application to study the dissociation of complexes in cytoplasm of cells (Van Rompaey, et. al., 2000; 2001a; 2001b).

In this study, we utilized a series of well-defined low-molecular-weight cationic polyDMAEMA having narrow molecular weight distributions and controlled charge densities to complex with oligonucleotides. Hydrophilic cationic block copolymers of polyDMAEMA-b-HEMA and polyDMAEMA-MCQ-b-PEG prepared by ATRP were also used to evaluate the effects of block-related factors on the polymer/ON complex properties (HEMA: 2-hydroxyethyl methacrylate; MCQ: methyl chloride quat; PEG: poly(ethylene glycol)). The complexation of these polymers with a 5'-fluorescein-labeled oligonucleotide was investigated by fluorescence spectroscopy. The influences of molecular weight, charge density, counter ion type, and block copolymers on the complexation were systematically evaluated. The complexation kinetics of the polymers having different molecular weights was also examined.

4.2. Experimental

4.2.1. Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA) was supplied by Aldrich and distilled over CaH_2 prior to use. 2-Hydroxyethyl methacrylate (HEMA) was supplied by Aldrich and dissolved in water (25% vol. of monomer). The solution was washed four times with an equal volume of hexane. NaCl was added to salt out the monomer. The monomer was dried over $MgSO_4$ and distilled under vacuum prior to use. Methoxy oligo(ethylene glycol), 2-bromoisobutyryl bromide, trichloroacetyl chloride, and triethylamine, as initiator precursors, were used as received from Aldrich. Other reagents, such as Cu(I)Cl, $Cu(II)Cl_2$, 2,2'-bipyridine, dimethly sulfate $(CH_3)_2SO_4$,

tetrahydrofuran (THF), methanol (MeOH), acetone, N, N-dimethylformamide (DMF), hexane were all from Aldrich and used without further purification.

4.2.2. Preparation of cationic polymers

DMAEMA homopolymers:

The DMAEMA homopolymers were synthesized by living anionic polymerization (LAP) as described in Chapter 2, Section 2.2.2.1. The molecular weight and molecular weight distribution of the polymers, summarized in Table 4.1, were characterized by gel permeation chromatography with polystyrene as standard and THF as solvent.

PolyDMAEMA quaternary salts:

PolyDMAEMA-DMSQ samples were prepared by quaternizing the polyDMAEMA samples synthesized by LAP with dimethyl sulfate $(CH_3)_2SO_4$. A typical quaternization procedure was described in Chapter 2, Section 2.2.2.2. The degree of quaternization was measured by ^1H NMR (Bruker ARX-200) using D_2O as solvent. PolyDMAEMA-MCQ, donated by Professor S.P. Armes University of Sussex UK, was prepared by a direct atom transfer radical polymerization of DMAEMA-MCQ monomer. The molecular weights and molecular weight distributions of these polymers are summarized in Table 4.1.

Table 4.1. Properties of DMAEMA-based polyelectrolytes

Polymer	Method	M_n GPC	DP	M_w/M_n GPC
HomopolyDMAEMA [a]				
A1	LAP	4440	28	1.04
A2	LAP	11700	75	1.03
A3	LAP	16400	104	1.12
A4	LAP	25500	160	1.04
A5	LAP	45950	290	1.04
PolyDMAEMA-MSQ				
QA1	LAP + Quaternization	7580	28	1.04
QA2	LAP + Quaternization	19970	75	1.03
QA3	LAP + Quaternization	28000	104	1.12
QA4	LAP + Quaternization	42800	160	1.04
QA5	LAP + Quaternization	78430	290	1.04
PolyDMAEMA-MCQ [b]	Direct ATRP	19880	100	1.25
Block copolymer				
PolyDMAEMA-b-HEMA [c]	ATRP	59700	100/50 [d]	1.39
PolyDMAEMA-MCQ-b-PEO [b]	ATRP	24870	100/50 [d]	1.29

LAP: living anionic polymerization; ATRP: atom transfer radical polymerization
a: Characterized by organic GPC using THF-2%(v/v) triethylamine as eluent at 25°C with polystyrene as standard.
b: Determined by aqueous GPC using 0.3M Na_2SO_4 - 0.1% (w/v) NaN_3 as eluent at 30°C with polyDMAEMA-MSQ as standard.
c: Determined by GPC using DMF - 0.2 w/v % of LiBr as eluent at 50 °C with polystyrene as standard.
d: Measured by ^1H NMR spectroscopy.

Poly(DMAEMA-b-HEMA) block copolymer: The linear DMAEMA-b-HEMA diblock copolymers were synthesized by the ATRP of DMAEMA with polyHEMA as macroinitiator. The detail procedure was described in Chapter 3, Section 3.1.3.2.

PolyDMAEMA-MCQ-b-PEG block copolymer: PolyDMAEMA-MCQ-b-PEG block copolymers, prepared by atom transfer radical polymerization, were donated by Prof. Armes group at Sussex University, UK.

4.2.3. Oligonucleotides and buffers

The fluorescein-labeled oligonucleotide, denoted F-ON and used throughout the study, has the following sequence: 5'-FGCGGAGCGTGGCAGG-3' (F: fluorescein). It was prepared by an automated DNA synthesis via cyanoethylphosphoramidite chemistry and supplied by Keck Biotechnology Resource Laboratory, Yale University. The raw F-ON was purified by reverse-phase high-performance liquid chromatography using a Beckman-Coulter HPLC System Gold equipped with an Agilent Zorbax ODS C_{18} column (4.5x250mm, 5μm) and a 168 Diode Array detector. Solvent A (0.1M triethylammonium acetate pH 6.5) and Solvent B (pure acetonitrile) were used as mobile phase. An optimal separation was achieved by a gradient method (10% B for 10 min, 10-40% B in 65 min) at a flow rate of 0.5 ml/min. The main peak was found to have very strong absorption at both 260 nm (for ON) and 491 nm (for fluorescein). The ONs within central two-thirds of peak width was collected and dried under vacuum. The concentration of purified F-

ON was determined by ultraviolet spectroscopy. The molecular weight of F-ON was 5007 g/mol.

4.2.4. Preparation of polymer/oligonucleotide complexes

Cationic polymer/F-ON complexes with different molar ratios were prepared as follows: A 10μg/ml purified F-ON solution was prepared by diluting the stock solution of purified F-ON with 20 mM HEPES buffer at pH 7.4 with 150 mM of NaCl. The polymer/F-ON dispersions of different molar ratios of amino units and nucleotides were prepared by keeping F-ON concentration at 10μg/ml and adding different volumes of cationic polymer stock solution (1-10μg/μl in HEPES buffer at pH 7.4 with 150 mM of NaCl). The polymer solutions were filtered with 0.45μm pore size filter before added to the F-ON solution to avoid dust particles. The polymer complex dispersions were vortexed for 10s and incubated for 30 min at room temperature before use.

4.3. Measurements

The fluorescence of cationic polymer/F-ON complexes was measured with a Cary Eclipse Fluorescence Spectrophotometer (Varian) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A kinetic study with up to 30 minutes of running time recorded the intensity data at 1-min intervals. The fluorescence measurements of each dispersion were done in duplicate.

4.4. Results and Discussion

At physiological pH 7.4, the average pKa of polyDMAEMA is 7.5 (Van de Wetering, et. al., 1999), indicating that around 50% of tertiary amino groups of polyDMAEMA is protonated. When polyDMAEMA complexs with F-ONs, the positively charged amino groups interact with negatively charged phosphate groups in fluorescein-labeled ONs and cause the reduction in fluorescence intensity. Figure 4.1 shows the relative intensity of fluorescence as a function of the molar ratio of amino units / nucleotides. The fluorescence of the F-ON solution in the absence of cationic polymer was set to 100%. Three definable regions can be identified. At low polymer/F-ON ratios, the fluorescence decreased as polyDMAEMA was added into the F-ON solution. The relative intensity reached the lowest point at an amino/nucleotide ratio 1.3, depending on the chain properties of polyDMAEMA. Further increase in the molar ratio resulted in the polyelectrolyte-assisted dispersion of the F-ON chains with the increase of intensity over 100%. Similar results were observed and explained by Van Rompaey et al. (2001a, 2001b). The decrease in intensity at low amino/nucleotide molar ratios was attributed to self-quenching of the fluorescein-labeled F-ON. The increased fluorescence of the complexes at higher amino/nucleotide ratios was the result of the F-ON redistribution.

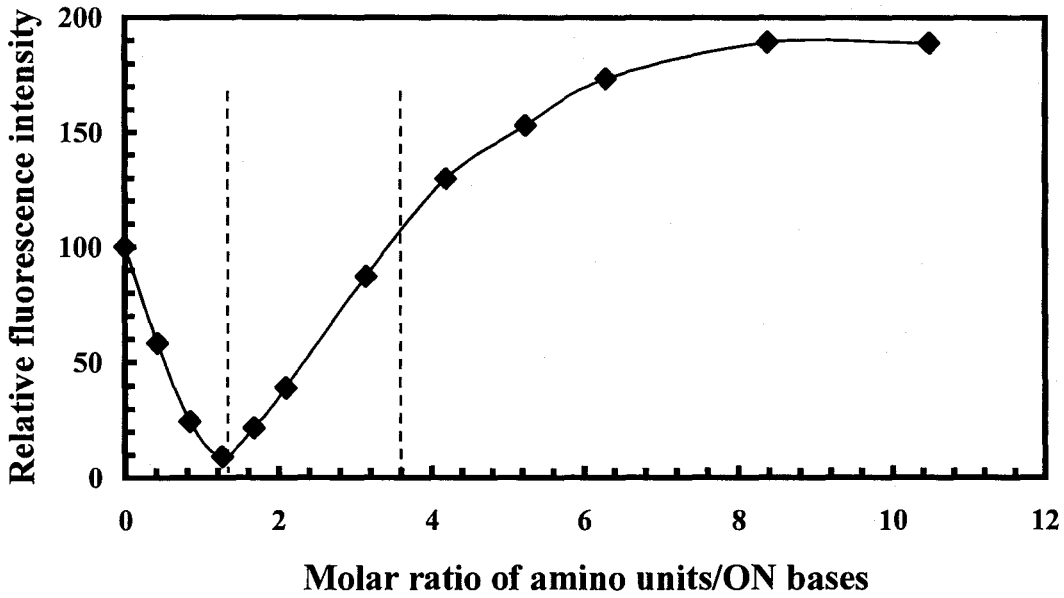


Figure 4.1. The relative fluorescence intensity as a function of the molar ratio of amino units / nucleotides. The fluorescence of the F-ON solution without polymer addition was set to 100%.

Polymer molecular weight, charge density, counter ion type, and hydrophilic block showed significant effects on the formation of the complexes.

4.4.1. Complexation kinetics

To study the kinetics of complexation, polyDMAEMA/F-ON complexes with different molar ratios of amino/nucleotide were prepared with the change in fluorescence intensity followed and recorded every minute for 30 minutes using an auto-kinetic fluorescence spectroscopy.

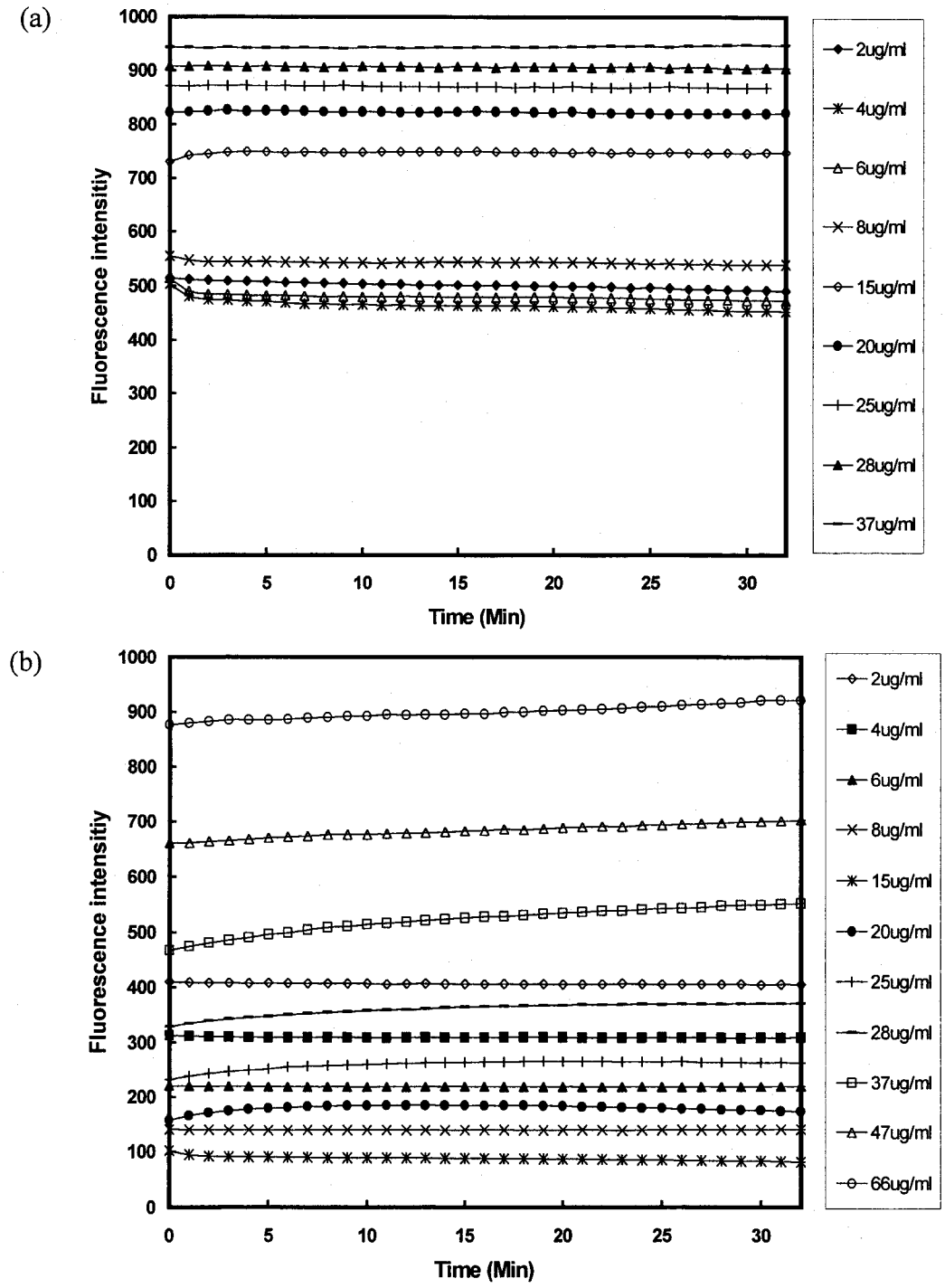


Figure 4.2. Kinetic study of fluorescence intensity as function of time for two polyDMAEMAs complexation with ONs. (a) Sample A1 (DP=28); (b) Sample A5 (DP=290)

Oligonucleotide/polymer complex formation in a buffer solution is a time-dependent process of chain diffusion and charge interaction. Figure 4.2 shows the kinetic data of two different molecular weight polyDMAEMA samples complexed with F-ONs. Before reached to the lowest point of fluorescence intensity, both profiles showed a decrease trend in intensity with the increase of time at low polymer concentration. While an increase trend in intensity with time was observed when adding more polymers after the lowest point. However, the time required to reach constant intensity was different between two regions. An interesting phenomenon was found when comparing the fluorescence intensity versus time at different amino/nucleotide ratios for the two samples, as shown in Figure 4.3. For the low-molecular-weight sample (A1: DP = 28) the fluorescence intensity varied with time at low amino/nucleotide ratios, but not much at high amino/nucleotide ratios. At a ratio of 1, the intensity of the A1/F-ON complex decreased from 92 % in 1 min to 87 % in 30 min. For the high-molecular-weight sample (A5: DP=290), the situation was the opposite: the time affected the performance at high amino/nucleotide ratios, but little at low amino/nucleotide ratios. For example, at a ratio of 8, the fluorescence intensity of Sample A5 increased from 80 % at 1 min to 100 % at 30 min.

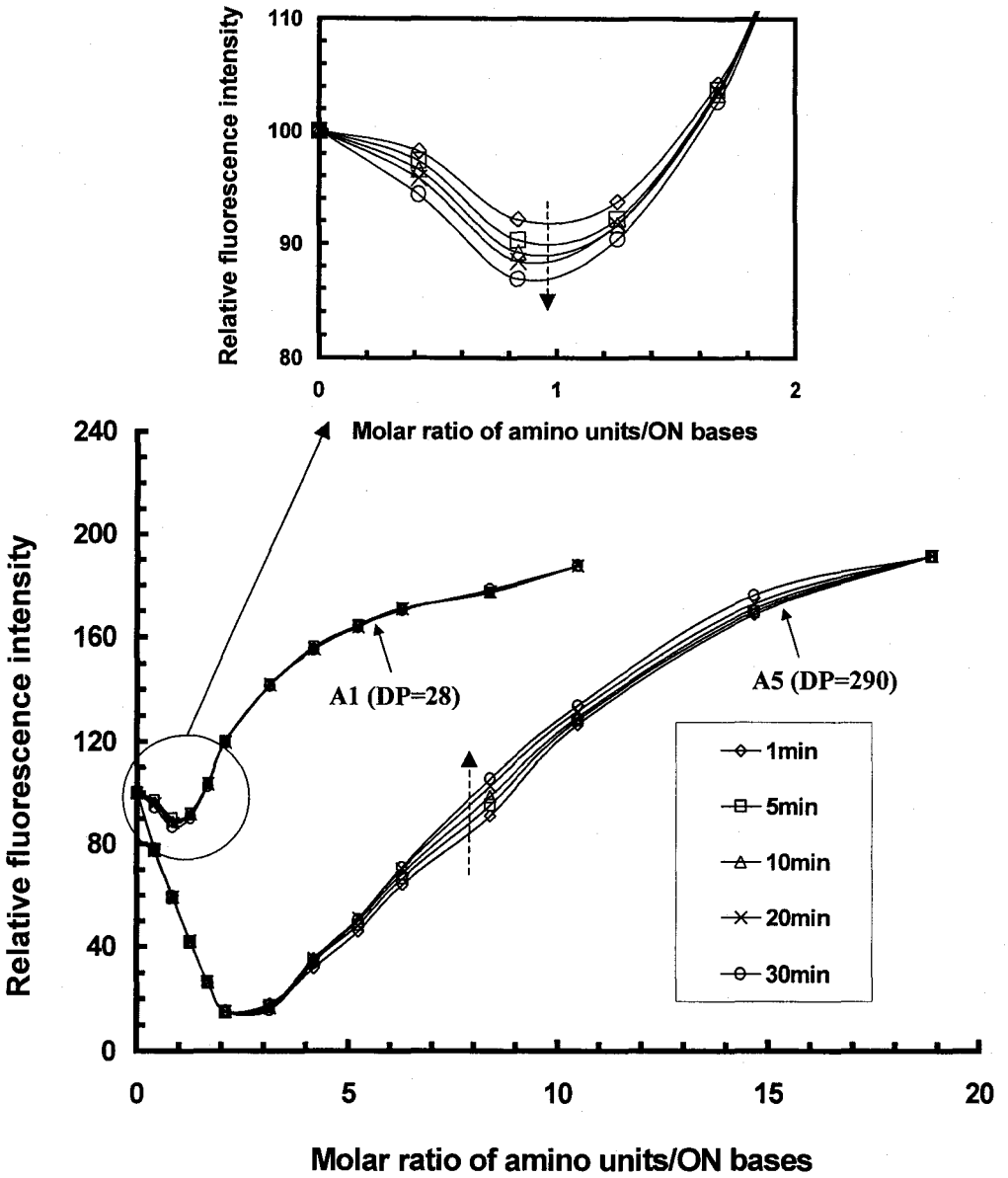
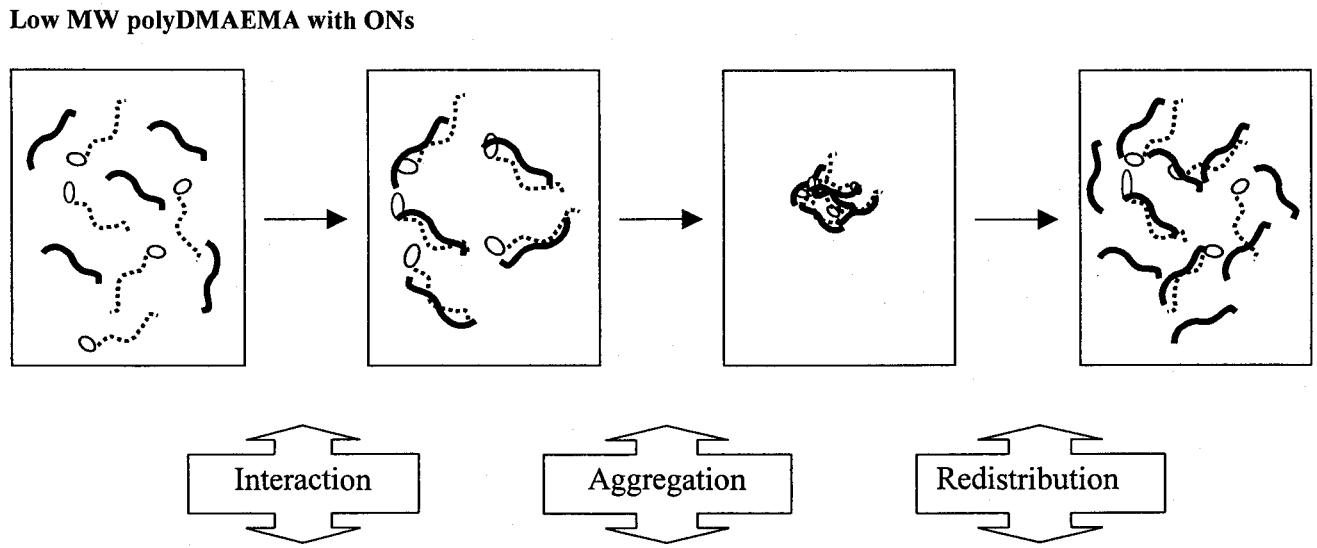


Figure 4.3. The dependence of complexation kinetics on the polymer molecular weight. Samples A1 (DP = 28) versus A5 (DP = 290).

The possible explanation for this peculiar observation is as illustrated in Scheme 4.1. The complexation process can be divided into three definable steps. The first step is the binding between cationic polymer chains and anionic F-ON chains through ionic interactions to form complexes. This step is rapid and does not contribute to the reduction of fluorescence intensity because the complexes are still dispersed in the system. The second step is the aggregation of the complexes to form clusters/particles. The fluorescence intensity decreases as a result of the aggregation. The third step is the redistribution of F-ON into newly added cationic polymer chains and it occurs if an excess amount of polymer is added. This step is accompanied with a recovery of fluorescence intensity. For the low-molecular-weight polyDMAEMA sample, the individual complexes are probably pairs of one F-ON chain with one polyDMAEMA chain. The number of protonated amino groups at pH 7.4 is 14 for sample A1 (DP=28), as seen in Table 4.2. While the F-ONs used here have 15 units. These small complexes aggregate through the translational diffusion of the pairs, which may be the rate-determining step. On the other hand, a high-molecular-weight polyDMAEMA chain can host many F-ON chains. The number of protonated amino groups of sample A5 (DP=290) is around 145 (Table 4.2). An individual F-ON chain is paired with a segment of the polyDMAEMA chain. The aggregation step is thus realized through the diffusion of the paired segments, which is faster than the translational diffusion.

Scheme 4.1. Three definable steps in the complexation of cationic polymers with anionic F-ON: the performance of high molecular weight polymer versus low molecular weight polymer.



When an excess amount of polyDMAEMA is added, F-ONs are redistributed. For a high-molecular-weight polymer sample, this redistribution step is slower because of chain entanglements.

4.4.2. Effect of polymer molecular weight

The effects of polymer molecular weight on the complexation were studied with different molecular weight polyDMAEMA samples (the degree of polymerization or the average number of amino units per polyDMAEMA chain: DP = 28-290). The molecular weight of the cationic polymer clearly influenced the formation of the complexes, as shown in Figure 4.4. Sample A1, which has a molecular weight (Mw = 4440 g/mol; DP = 28) close to that of the F-ON (Mw = 5007 g/mol, DP = 15), clearly lacked complexation ability. The lowest relative intensity of fluorescence was 90 % at the molar ratio of 1. Doubling the polyDMAEMA molecular weight (Sample A2: Mw = 11700, DP = 75) decreased the fluorescence intensity dramatically. The lowest intensity reached 9 % at the molar ratio of 1.3. Further increase in the polyDMAEMA molecular weight (see Sample A4: DP = 160, and Sample A5: DP = 290) did not improve the complexation ability. The lowest intensity of 8 % for A4 and that of 15 % for A5 were observed at the molar ratios of about 2. The high optimal amino/nucleotide ratio indicated that more polymer materials were required to complex the same amount of F-ON with high molecular weight polyDMAEMA. This is attributed to the diffusion/shielding effect. PolyDMAEMA chains were in a form of expanded coils in aqueous media due to the protonated amino groups. F-ON molecules readily complexed with the amino groups at

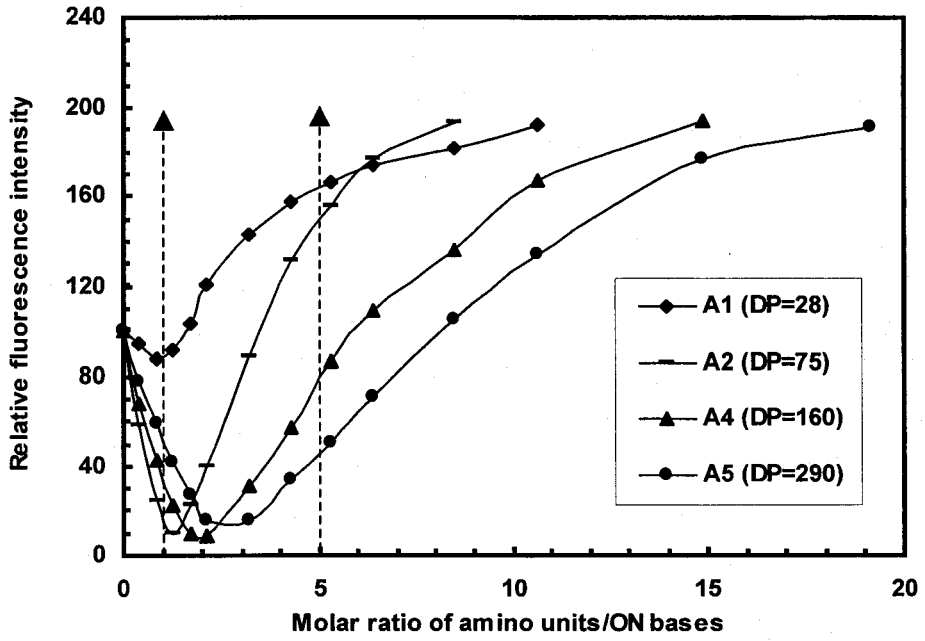


Figure 4.4. The effects of polymer molecular weight on the complexation of polyDMAEMA with F-ON. ♦) Sample A1 (DP = 28); —) Sample A2 (DP = 75); ▲) Sample A4 (DP = 160); ●) Sample A5 (DP = 290).

the coil surface. Upon complexation, the shell of the coil collapsed and formed a shield for the amino groups at the core. It became difficult for F-ON to penetrate the shell due to diffusion limitations, as illustrated in Scheme 4.2.

Scheme 4.2. The diffusion/shielding effect of high-molecular-weight polyDMAEMA on the complexation.

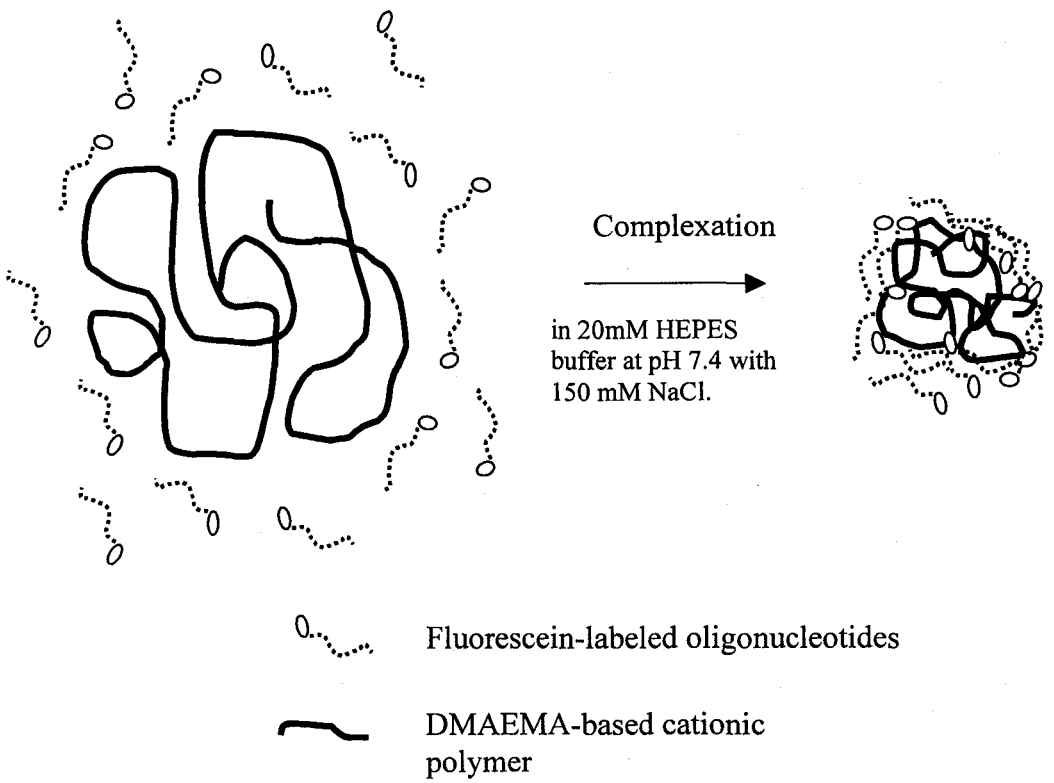


Table 4.2. Summary of DP, number of protonated amino groups, lowest FI and corresponding molar ratio of amino units over ON bases for polyDMAEMA samples.

Sample	DP [a]	No. of $(CH_3)_2N^+H$ at pH 7.4 [b]	Lowest of RFI [c]	Molar ratio of amino unit/ON base at lowest FI
A1	28	14	90%	0.9
A2	75	38	9%	1.3
A4	160	80	8%	2.1
A5	290	145	15%	3.2

a: DP: degree of polymerization

b: Calculated number of protonated amino groups at pH 7.4,

pKa (acidity constant) is 7.5.

Number of $(CH_3)_2N^+H = 1/(1+10^{-pKa}/10^{-pH})$

c: RFI: Relative fluorescence intensity

Table 4.2 summaries the data of the lowest relative fluorescence intensity and corresponding molar ratio of amino units/ON bases for four complexation systems. Sample A2 showed the best complexation capability among four samples with relatively high efficiency (low RFI: 9%) and high effectiveness (low molar ratio: 1.3).

The molecular weight of polyDMAEMA also had significant effects on the redistribution of F-ON chains at high amino/nucleotide ratios. For example, at the ratio

of 5 in Figure 4.4, the relative intensity of Sample A1 was 165%, while those of A2, A4, and A5 were 150%, 80%, and 45%, respectively. This was likely due to different levels of difficulty for F-ON to dissociate from the complexes formed with these polymers. In the high-molecular-weight polyDMAEMA complex systems, the F-ON chains experienced a high level of difficulty to dissociate from original complexes in order to bind newly added polyDMAEMA chains as most F-ON molecules were trapped or partially trapped inside the large complexes. In other words, the high-molecular-weight complexes were more stable than their low-molecular-weight counterparts.

4.4.3. Effect of charge density and counter ion type

The objective of using fully charged polyDMAEMA for a complexation study is to establish the quantitative relationships between charge ratio and complexation performance. A fully charged polyDMAEMA sample is expected to have high complexation ability because of its high charge density. Figure 4.5 shows the comparison for two polyDMAEMA samples of different molecular weights. Samples QA2 and QA4 are the dimethylsulfate quats of A2 and A4. It is of interest to observe that there was no significant improvement, if any, in complexation ability with polymers of higher charge density. The lowest intensities were at the same level, and the optimal amino/nucleotide ratios were also similar. However, the intensity recovery portions were very different between the quats and original samples. The quat complexes, once formed, were not sensitive to the addition of fresh quat polymers, indicating strong binding

between F-ON and quats. From these experiments it appeared difficult for such complexes to release F-ON chains.

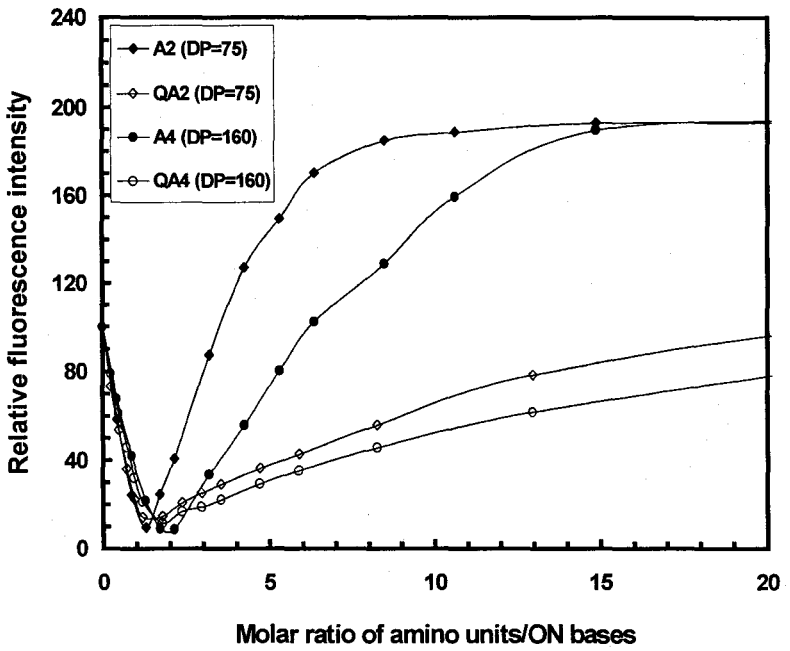


Figure 4.5. The effect of charge density on the complexation of polyDMAEMA quats with F-ON. ♦) polyDMAEMA (A2: DP = 75) versus ◊) polyDMAEMA-DMSQ (QA2: DP = 75); and ●) polyDMAEMA (A4: DP = 160) versus ○) polyDMAEMA-DMSQ (QA4: DP = 160).

Figure 4.6 shows the comparison of polyDMAEMA quats with two different types of counter ion. PolyDMAEMA-DMSQ was quaternized with dimethyl sulfate (counter ion: $CH_3SO_4^-$), while polyDMAEMA-MCQ was quaternized with hydrochloride (counter ion: Cl^-). The former had slightly higher complexation ability than the latter.

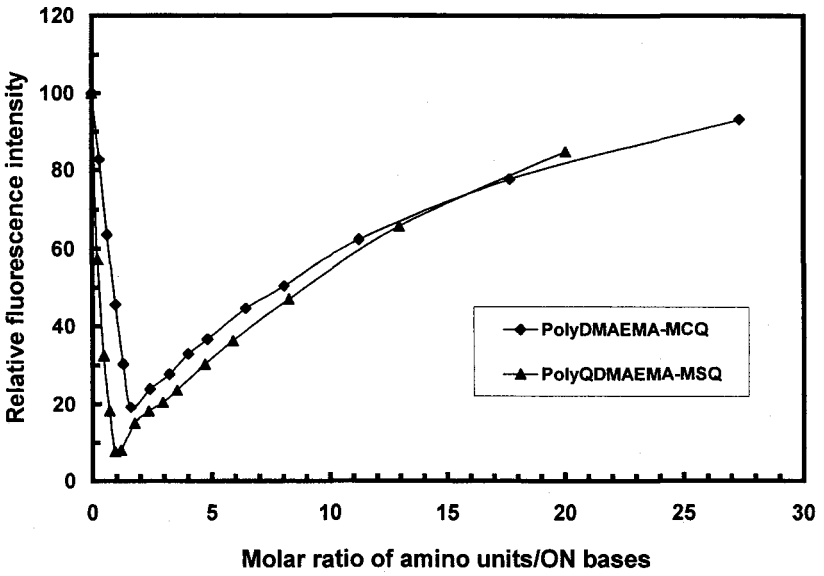


Figure 4.6. The effect of counter ion on the complexation of polyDMAEMA quat with F-ON. ♦) polyDMAEMA-MCQ versus ▲) polyDMAEMA –DMSQ

4.4.4. Effect of hydrophilic block

The use of hydrophilic block, such as PEG, OEGMA, and PHPMA, for improving biocompatibility of cationic polymer/DNA complexes is essential for safe delivery of the nucleic acid. In this work, poly(DMAEMA-b-HEMA) vs. polyDMAEMA as well as polyDMAEMA-MCQ-b-PEG vs. polyDMAEMA-MCQ were compared to assess the effects of the hydrophilic blocks on the complexation performance. Figure 4.7 shows the results of poly(DMAEMA-b-HEMA). The block copolymer appeared to be as good as the homopolymer, if not any better, in terms of the lowest intensity value. The optimal

amino/nucleotide ratio of the copolymer was slightly higher than that of the homopolymer. However, the hydrophilic block significantly affected the intensity recovery and therefore increased the stability of the complexes. The block increased the molecular weight of the total polymer, which may have made it more difficult for F-ON to redistribute. Figure 4.8 shows the comparison between polyDMAEMA-MCQ and its block copolymer with PEG (i.e., polyDMAEMA-MCQ-b-PEG). The PEG block appeared to slightly enhance the complexation and yielded a slightly smaller value of the lowest intensity. The effect of PEG on the recovery portion of the profile was similar to that of polyHEMA. It also increased the complex stability and created an additional barrier for the release of F-ON.

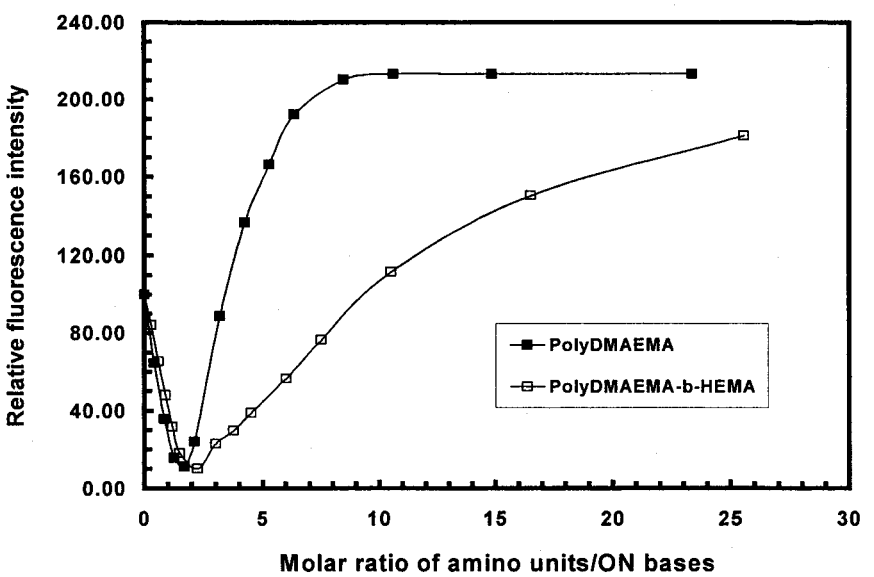


Figure 4.7. The effect of polyHEMA block on the complexation of polyDMAEMA with F-ON. ■) polyDMAEMA versus □) poly(DMAEMA –b-HEMA).

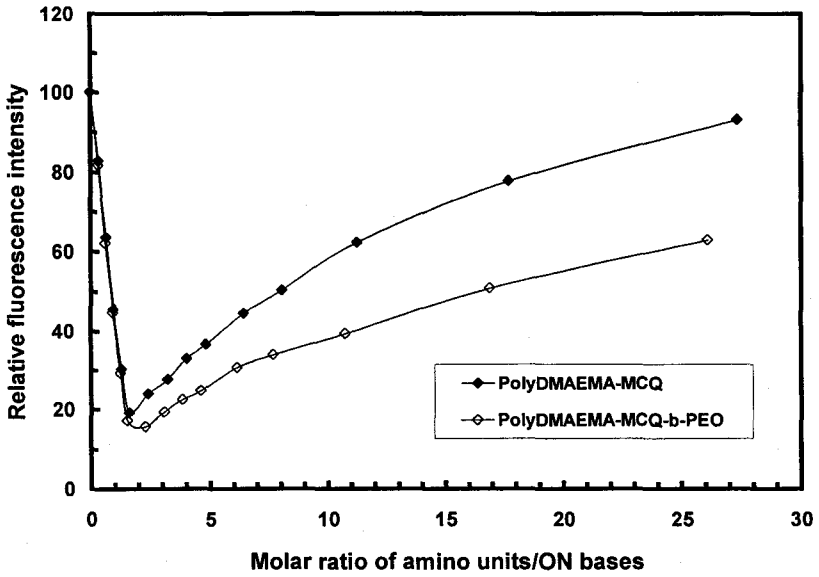


Figure 4.8. The effect of PEG block on the complexation of polyDMAEMA-MCQ with F-ON. ■) polyDMAEMA-MCQ versus □) polyDMAEMA–MCQ-b-PEG.

4.5. Conclusion

Using a 15-unit fluorescein-labeled oligonucleotide F-ON as a model oligonucleotide (ON), we examined the ON complexation behaviors of several well-defined DMAEMA-based cationic polymers of different molecular weights, charge densities, counter ion types, and block compositions. The following conclusions can be drawn from the study.

1. The kinetic study of the complexation of F-ON and polyDMAEMA of varying molecular weights showed that low molecular weight samples experienced diffusion

limitations at low amino/nucleotide ratios, while high molecular weight samples demonstrated diffusion effects at high amino/base ratios.

2. The polymer molecular weight had significant effects on the ON complexation. The polyDMAEMA with a molecular weight doubled that of the selected ON exhibited optimal complexation. However, the polymer sample with a molecular weight similar to the ON was not efficient in complexation. Samples of much higher molecular weights did not improve the efficiency but yielded stable complexes, and imposed diffusion limitations to the redistribution of F-ON.
3. Increasing the polymer charge density did not significantly change the optimal value of amino/nucleotide ratio and that of intensity reduction. The high charge density provided strong interactions with the ON and resulted in stable complexes. The type of counter ions in polyDMAEMA quats played a minor role in complexation.
4. Both poly(DMAEMA-b-HEMA) and poly(DMAEMA-MCQ-b-PEG) block copolymers showed good complexation ability and some steric stability. PEG block slightly improved polyDMAEMA-MCQ interactions with ON, while HEMA block did not show such a change.

Chapter 5 Contributions and Recommendations

5.1. Contributions of This Thesis Work

5.1.1. Aqueous GPC calibration method for polyelectrolyte

There are three significant contributions for the new GPC calibration method reported here to the characterization of polyelectrolytes via aqueous GPC.

The main contribution is that this was the first applicable calibration method for cationic polyelectrolytes in the field. The method was based on the major finding in this study that the difference in hydrodynamic volumes between dimethyl sulfate-quaternized and methyl iodide-quaternized DMA homopolymers was negligible (see Chapter 2 Section 2.3.2.3). Therefore the dimethyl sulfate-quaternized DMAEMA homopolymers can be used as reliable calibration standards for other types of quaternized DMAEMA homopolymers due to the negligible effects of counter ions. The characterization results of methyl chloride- DMAEMA homopolymers using this new calibration method proved its reliability.

The second contribution is that the study provided a practical approach for preparing mono-dispersed polyelectrolyte standards for aqueous GPC measurements via newly developed living polymerization techniques. Water-soluble cationic polymers were prepared either by sequential quaternization of pre-prepared well-defined nonionic polymers or by direct atom transfer radical polymerization of quaternized monomers.

Another contribution is the optimized chromatographic condition reported in this study for the low molecular weight cationic polyelectrolytes ranging from 4,000 to 45,000. The selection of 0.3M Na_2SO_4 - 0.1% (w/v) NaN_3 solution as optimal solution was based on that a high concentration of electrolyte salt was required to suppress adsorption between cationic polyelectrolytes and column support, especially for high molecular weight polymers.

5.1.2. ATRP of poly(DMAEMA-b-HEMA)

Bioactive and biocompatible water soluble poly(DMAEMA-b-HEMA) block copolymers with well-controlled block molecular weights were successfully prepared via ATRP. These block copolymers are very useful for preparing suitable gene delivery vectors because of the improvement of biocompatibility by polyHEMA block. In addition, the block copolymers have pH-, salt-, and temperature sensitivities in aqueous media and can form reversible micelles. PolyDMAEMA-based block copolymers can be cross-linked to form nanospheres with cross-linked shell, and polyHEMA block can form hydrophilic core, which can potentially act as nanoreactors, drug delivery vehicles and other nanostructural "intelligent" devices for biomedical applications.

The atom transfer block copolymerization of 2-(*N,N*-dimethylamino)ethyl methacrylate (DMAEMA) with 2-hydroxyethyl methacrylate (HEMA) reported in this thesis was studied in methanol using Macroinitiator method and "one-pot" Sequential Addition method. The order of polymerization of the two monomers strongly affected the control of the block copolymers. When DMAEMA was used as the first monomer,

both methods produced block copolymer samples containing significant amount of DMAEMA homopolymer chains, because of the elimination of active halogen chain-ends during the preparation of polyDMAEMA. Well-controlled block copolymers with various block lengths were obtained via Macroinitiator method when polyHEMA was used as macroinitiator to initiate the polymerization of DMAEMA. Sequential Addition method, in which HEMA was polymerized first with 90% conversion and DMAEMA was subsequently added, also yielded controlled block copolymers when the polymerization was carried out at room temperature with the DMAEMA conversion below 60%. However, side reactions such as gel formation occurred at high conversion.

Increasing temperature to 60 ^{o}C promoted the copolymerization rate but suffered from early gel formation. The addition of water to the system accelerated the polymerization rate, but led to the loss of the system livingness. Good control was only obtained under 60% of DMAEMA conversion in the presence of 10% water.

5.1.3. Complexation of polyelectrolytes and ONs

The kinetic studies on the effects of polymer-related factors such as molecular weight, charge density, and block composition on physicochemical and biological properties of polymer /ON complexes contribute to a good understanding of the complex system, which is essential for the future design of optimal non-viral vector systems.

Fluorescence measurements revealed that the cationic DMAEMA based polyelectrolytes and their block copolymers have good complexation ability with ONs. The results of this study demonstrated that polymer molecular weight and charge density

had significant effects on the complexation. PolyDMAEMA samples having double molecular weights of the chosen oligonucleotide gave the optimal complexation performance. Kinetic studies showed that high-molecular-weight/high-charge-density polymer samples produced very stable complexes.

The fully charged polyDMAEMA displayed the strongest binding with the ONs. These complexes were therefore less sensitive to changes in environment.

The counter ion of polyelectrolyte also affects the complexation formation. PolyDMAEMA-DMSQ samples had slightly higher complexation ability than polyDMAEMA-MCQ (DMSQ: dimethylsulfate quat; MCQ: methylchloride quat).

Both poly(DMAEMA-b-HEMA) and poly(DMAEMA-MCQ-b-PEG) block copolymers showed good complexation ability and improved steric stability (HEMA: 2-hydroxyethyl methacrylate; PEG: polyethylene glycol). PEG but not HEMA block enhanced the effectiveness of polyDMAEMA-MCQ binding with the ONs.

5.2. Recommendations

The proposed new aqueous GPC calibration method is essential for the characterization of cationic polyelectrolytes. However, to develop a universal calibration method, Mark-Houwink constants k and α of polyDMAEMA and its quaternary salts under various conditions need to be estimated. The estimation requires viscosity intrinsic measurements of the polymers. It has been proven challenging to have accurate measurement of viscosity for polyelectrolytes in dilute aqueous solution using a capillary viscometer. Advanced rheometers can be tried.

In the preparation of block copolymers, DMAEMA was not suggested to be used as the first monomer because of the elimination of active halogen chain-ends. Macroinitiator method with polyHEMA as macroinitiator was recommended to prepare well-fined poly(DMAEMA-b-HEMA) block copolymers via ATRP at room temperature. Low conversion less than 60% was also recommended to avoid gel formation and other side reactions when using "one-pot" Sequential addition method or water as accelerator.

As the solubility of polyHEMA in aqueous solution is low, other hydrophilic blocks, such as PEO, GMA (glycerol monomethacrylate), or MPC (2-methacryloyloxyethyl phosphorylcholine), are suggested to be used to improve the solubility and biocompatibility of polymer/ONs complexes. DMAEMA based grafting, brush copolymers and dendrimers can also be used for comparison to obtain optimal non-viral polymeric oligonucleotides delivery.

As the complexation study in this research was based on the selected oligonucleotides with 15 base units, further study is recommended to evaluate the effects of ONs length on the complex formation using large ONs such as 30 or 45 units.

Furthermore, in vitro experiments are required to study cytotoxicity and transfection efficiency of polymer/ONs complexes. Fluorescence technique, which is useful to investigate the association and dissociation of polymers with nucleic acids in vitro, can be used to monitor transfection of complex into cells and to evaluate the effects of molecular weight, charge density, and block type and composition on transfection efficiency and cytotoxicity of complexes.

Reference

Anderson, B. C.; Mallapragada, S. K. Synthesis *and characterization of injectable, water-soluble copolymers of tertiary amine methacrylates and poly(ethylene glycol) containing methacrylates*. Biomaterials, **2002,** 23, 4345.

Antoun, S.; Teyssié, P.; Jéròme, R. Lithium *Diisopropylamide as Initiator for the Anionic Polymerization of Methacrylates.* Macromolecules, **1997**, 30, 1556.

Armstrong, R. W. and Strauss, U. P. *Polyelectrolytes. In Encyclopedia of polymer science and technology*, **1996**,vol. 10, 781.

Arotcarena, M.; Heise, B.; Ishaya, S.; Laschewsky, A. *Switching the Inside and the Outside of Aggregates of Water-Soluble Block Copolymers with Double Thermoresponsivity*. J. Am. Chem. Soc., **2002**, 124, 3787.

Ashford, E. J.; Naldi, V.; O'Dell, R.; Billingham, N. C.; Armes, S. P. *First example of the atom transfer radical polymerization of an acidic monomer: direct synthesis of methacrylic acid copolymers in aqueous media.* Chem. Commun., **1999**, 1285.

Beadle, P. M.; Rowan, L.; Mykytiuk, J.; Billingham, N. C.; Armes, S. P. *Synthesis and characterization of sterically stabilized colloidal dispersions of polypyrrole using novel tailor-made water-soluble block copolymers of narrow molecular weight distribution.* Polymer, **1993**, 34,1561.

Beers, K. L.; Boo, S.; Gaynor, S. G.; Matyjaszewski, K. *Atom Transfer Radical Polymerization of 2-Hydroxyethyl Methacrylate*. Macromolecules, **1999**, 32, 5772.

Benoit, D.; Harth, E.; Fox, P.; Waymouth, R. M.;Hawker, C. J. *Accurate Structural Control and Block Formation in the Living Polymerization of 1,3-Dienes by Nitroxide-Mediated Procedures.* Macromolecules, **2000**, 33, 363.

Black, J. *Biological performance of Materials: Fundamentals of Biocompatibility.* Marcel Dekker, New York, **1981**.

Bories-Azeau, X.; Armes, S. P. *Unexpected Transesterification of Tertiary Amine Methacrylates during Methanolic ATRP at Ambient Temperature: A Cautionary Tale.* Macromolecules, **2002**, 35, 10241.

Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in vivo: Polyethylenimine*. Proc. Natl. Acad. Sci. USA, **1995**; 92: 7297.

Burkhart B.E. http://www.lpsrc.usm.edu/REU/1996/Burkhart/public.html/project/

Bütün, V.; Armes, S. P.; Billingham, N. C. *Selective Quaternization of 2-(Dimethylamino) ethyl Methacrylate Residues in Tertiary Amine Methacrylate Diblock Copolymers.* Macromolecules, **2001a**, 34, 1148.

Bütün, V.; Armes, S. P.; Billingham, N. C. *Synthesis and aqueous solution properties of near-monodisperse tertiary amine methacrylate homopolymers and diblock copolymers.* Polymer, **2001b**, 42, 5993.

Chambard, G.; Klumperman, B.; German, A. L. Effect of Solvent on the Activation Rate Parameters for Polystyrene and Poly(butyl acrylate) Macroinitiators in Atom Transfer Radical Polymerization. Macromolecules, **2000**, 33, 4417.

Chen, D.; Kennedy, J. P.; Kory, M. M.; Ely, D. L. *Amphiphilic Networks: II. Biocompatibility and Controlled Drug Release of Poly[isobutylene-co-2-(dimethylamino)ethyl methacrylate].* J. Biomed. Mater. Res., **1989**, 23, 1327.

Chen, S. J.; Su, F. S.; Wu, Z. Q.; Huang, L.; Li, Z. C.; Li, F. M. *Stimuli-responsive Behavior of N,N-dimethylaminoethyl methacrylate Polymers and Their Hydrogels.* Polym Prepr., **1997**, 38, 534.

Cherng, J. Y.; Van de Wetering, P.; Talsma, H.; Gommelin, D. J. A.; Hennink, W. E., *Effect of Size and Serum Proteins on Transfection Efficiency of Poly([2-dimethylamino]ethyl methacrylate)-plasmid Nanoparticles.* Pharm. Res., **1996**, 13, 1038.

Chiefari, J.; Chong, Y. K.; Ercole, F.; Krstina, J.; Jeffery, J.; Le, T. P. T.; Mayadunne, R. T. A.; Meijs, G. F.; Moad, C. L.; Moad, G.; Rizzardo, E.; Thang, S. H. *Living Free-Radical Polymerization by Reversible Addition-Fragmentation Chain Transfer: The RAFT Process.* Macromolecules, **1998**, 31, 5559.

Chirila, T. V.; Rakoczy, P. E.; Garrett, K. L.; Lou, X.; Constable, I. J. *The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides.* Biomaterials, **2002**, 23, 321.

Cho, S. H.; John, M. S.; Yuk, S. H.; Lee, H. B. *Temperature-induced phase transition of poly(N,N-dimethylaminoethyl methacrylate-co-acrylamide).* J. Polym. Sci. Part B: Polym. Phys., **1997**, 35, 595.

Creutz, S.; Jerome, R. *Effectiveness of Block Copolymers as Stabilizers for Aqueous Titanium Dioxide Dispersions of a high Solid Content.* Prog. Org. Coat, **2000**, *40*, 21.

Creutz, S.; Jerome, R.; Kaptijn, G. M. P.; Van der Werf, A. W.; Akkerman, J. M. *Design of Polymeric Dispersants for Waterborne Coatings.* J. Coat. Technol., **1998**, *70*, 41.

Creutz, S.; Teyssié, P.' Jéròme, R. *Living Anionic Homopolymerization and Block Copolymerization of (Dimethylamino)ethyl Methacrylate.* Macromolecules, **1997**, 30, 6.

Deshpande, M. C., Garnett, M. C., Vamvakaki, M., Bailey, L., Armes, S. P., Stolnik, S. *Influence of polymer architecture on the structure of complexes formed by PEG-tertiary amine methacrylate copolymers and phosphorothioate oligonucleotide.* J. Control. Release, **2002**; 81, 185.

Dheur, S., Dias, N., van Aerschot, A., Herdewijn, P., Bettinger, T., Remy, J. S., Jelene, C., Saison-Behmoaras E. T. *Polyethylenimine but not Cationic Lipid Improves Antisense Activity of 3'-capped Phosphodiester Oligonucleotides.* Antisense Nucleic Acid Drug Dev., **1999**; 9, 515.

Dubin, P. L. Aqueous Size Exclusion Chromatography, **1988**, ch3.

Dubruel, P., Schacht, E. H. *Effect of Polyethylene Oxide Blocks or Grafts on the Physicochemical Properties of Poly(2-(dimethylamino)ethyl Methacrylate) DNA complexes.* J. Bioactive and Compatible polymers, **2000**,15, 279.

Felgner, J. H.; Kumar, R.; Sridhar, C. N.; Wheeler, C. J.; Tsai, Y. J.; Border, R. *Enhanced Gene Delivery and Mechanism Studies With a Novel Series of Cationic Lipid Formulations.* J. Biol. Chem., **1994**. 269, 2550.

Felgner, P. L.; Gadek, T. R., Holm, M.; Roman, R.; Chan, H. W.; Wenz, M. *Lipofection: a Highly Efficient Lipidmediated DNA Transfection Procedure.* Proc. Natl. Acad. Sci. USA , **1987** 84, 7413.

Fischer, D.; Bieber, T.; Li, Y.; Elsasser, H. P.; Kissel, T. *A Novel Non-viral Vector for DNA Delivery Based on Low Molecular Weight, Branched Polyethylenimine: Effect of Molecular Weight on Transfection Efficiency and Cytotoxicity.* Pharm. Res., **1999**, 16, 1273.

Fox, t. G.; Flory, P. J. *Intrinsic Viscosity Relations for Polystyrene.* J. Am. Chem. Soc., 1951, 73, 1904.

Fukano, K.; Komiya, K.; Sasaki, H.; Hashimoto, T. *Evaluation of New Supports for High-pressure Aqueous Gel Permeation Chromatography.* J. Chromatography, **1978**, 166, 47.

Fukuda, T.; Terauchi, T.; Goto, A.; Tsujii, Y.; Miyamoto, T.; Shimizu, Y. *Well-Defined Block Copolymers Comprising Styrene-Acrylonitrile Random Copolymer Sequences Synthesized by "Living" Radical Polymerization.* Macromolecules, **1996**, 29, 3050.

Gebelein, C.G. *Bioactive polymeric systems, An overview.* **1985,** 1.

Georges, M. K.; Veregin, R. P. N.; Kazmaier, P. M.; Hamer, G. K. *Narrow Molecular Weight Resins by a Free-radical Polymerization Process.* Macromolecules, **1993**, 26, 2987.

Gilbert, C. W.; Park-cho, M. *Interferon Polymer Conjugates.* **1999**, U.S. Pat. No. 5,951,974.

Greenwald, R. B.; Choe, Y.H.; Conover, C. D.; Shum, K.; Wu, D.; Royzen, M. *Drug Delivery Systems Based on Trimethyl Lock Lactonization: Poly(ethylene glycol) Prodrugs of Amino-Containing Compounds.* J. Med. Chem., **2000,** 43, 475.

Guerrini, M. M.; Charleux, B.; Jean-Pierre Vairon, J-P. *Functionalized latexes as substrates for atom transfer radical polymerization.* Macromol. Rapid Commun., 2000, 21, 669.

Haddleton, D. M.; Perrier, S.; Bon, S. A. F. *Copper(I)-Mediated Living Radical Polymerization in the Presence of Oxyethylene Groups: Online ^{1}H NMR Spectroscopy To Investigate Solvent Effects.* Macromolecules, **2000**, 33, 8246.

Hawker, C. J.; Barclay, G. C.; Orellana, A.; Dao, J.; Devonport, W. *Initiating Systems for Nitroxide-Mediated "Living" Free Radical Polymerizations: Synthesis and Evaluation.* Macromolecules, **1996**, 29, 5245.

Heise, A.; Trollsas, M.; Magbitang, T.; Hedrick, J. L.; Frank, C. W.; Miller R. D. *Star Polymers with Alternating Arms from Miktofunctional -Initiators Using Consecutive Atom Transfer Radical Polymerization and Ring-Opening Polymerization.* Macromolecules, 2001, 34, 2798.

Herrmann, F., *Cancer Gene Therapy: Principles, Problems, and Perspectives.* J. Mol. Med., **1995**, 73: 157.

Hoover, M. F. *Cationic Quaternary Polyelectrolytes- A Literature Review.* J. Macromol. Sci. Chem., **1970**, A4 (6), 1327,

Howard, G. Barth; J. *Chromatographic Science*, **1980,** 18.

http://macro.lsu.edu/corecourses/MSweb7/PDF/handouts%20GTP.pdf

Huang, W.; Kim, J.; Bruening, M. L.; Baker, G. L. *Functionalization of Surfaces by Water-Accelerated Atom-Transfer Radical Polymerization of Hydroxyethyl Methacrylate and Subsequent Derivatization.* Macromolecules, **2002**, 35, 1175.

Huber, B. E.; Lazo, J. S. Eds. *Gene Therapy for Neoplastic Diseases.* New York Academy of Sciences: New York, NY, **1994.**

Hunt, B. J.; Holding, S. R. *Size exclusion Chromatography*, **1989,** 172.

Kabanov, A. V., Vinogradov, S. V., Suzdaltseva, Y. G., Alakhov, V. Y. *Water-Soluble Block Polycations as Carriers for Oligonucleotide Delivery.* Bioconjugate Chem. **1995**; 6, 639.

Kamigaito, M.; Ando, T.; Sawamoto, M. *Metal-Catalyzed Living Radical Polymerization.* Chem. Rev., **2001**, 101, 3689.

Kataoka, K.; Togawa, H.; Harada, A.; Yasugi, K.; Matsumoto, T.; Katayose, S. *Spontaneous Formation of Polyion Complex Micelles with Narrow Distribution from Antisense Oligonucleotide and Cationic Block Copolymer in Physiological Saline.* Macromolecules, **1996**; 29: 8556

Keoshkerian, B.; Georges, M. K.; Boils-boissier, D. *Living Free-Radical Aqueous Polymerization.* Macromolecules, **1995**, 28, 6381.

Keoshkerian, B.; Georges, M.; Quinlan, M.; Veregin,R.; Goodbrand, B. *Polyacrylates and Polydienes to High Conversion by a Stable Free Radical Polymerization Process: Use of Reducing Agents.* Macromolecules, **1998**, 31, 7559.

Lasic, D. D.; Templeton, N. S., *Liposomes in Gene Therapy.* Adv. Drug Delivery Rev., **1996**, 20, 221.

Ledley, F. D.; *Pharmaceutical Approach to Somatic Gene Therapy.* Pharm. Res., **1996**, 13, 1595.

Legendre, J. Y.; Szoka Jr F. C. *Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes.* Pharm. Res., **1992**, 9, 1235.

Lehn, P.; Fabrega, S.; Oudrhiri, N.; Navarro, J. *Gene Delivery Systems: Bridging the Gap Between Recombinant Viruses and Artificial Vectors.* Adv. Drug. Delivery Rev., **1998**, 30, 5.

Leonetti, J. P., Degols, G., Lebleu, B. *Biological Aactivity of Oligonucleotide-poly(L-lysine) Conjugates: Mechanism of Cell Uptake.* Bioconj. Chem., **1990**; 1, 149.

Leonetti, J. P.; Rayner, B.; Lemaitre, M.; Gagnor, C.; Milhaud, P. G.; Imbach, J. L.; Lebleu, B. *Antiviral Activity of Conjugates Between Poly(L-lysine) and Synthetic Oligodeoxyribonucleotides.* Gene, **1988**; 72, 323.

Li, Y. T.; Armes, S. P.; Jin, X.; Zhu, S. Direct Synthesis of Well-defined Quaternized Homopolymers and Diblock Copolymers via ATRP in Protic Media. Macromolecules, **2003**, 36, 8268.

Litzinger, D. C. Limitations of Cationic Liposomes for Antisense Oligonucleotide Delivery in vivo. J. Liposome Res., **1997**, 7, 51.

Liu, S.; Weaverm, H. V. M.; Tang, Y.; Billingham, N.C.; Armes, S.P. *Synthesis of Shell Cross-Linked Micelles with pH-Responsive Cores Using ABC Triblock Copolymers.* Macromolecules, **2002**, 35, 6121.

Liu, Y.; Liffitt, D.; Zhong, W.; Tu, G.; Gaensler, K.; Debs, R. *Cationic Liposome-Mediated Intravenous Gene Delivery.* J. Biol. Chem., **1995** 270, 24864.

Liu, Y.; Mounkes, L. C.; Liggitt, H. D.; Brown, C. S.; Solodin, I.; Heath, T. D.; Debs, R. J. *Factors Influencing the Efficiency of Cationic-liposome Mediated Intravenous Gene Delivery.* Nature Biotech., **1997** 15, 167.

Lobb, E. J.; Ma, I.; Billingham, N. C; Armes, S. P.; Lewis, A. L. *Facile Synthesis of Well-Defined, Biocompatible Phosphorylcholine-Based Methacrylate Copolymers via Atom Transfer Radical Polymerization at 20 ° C.* J. Am. Chem. Soc., **2001**, 123, 7913.

Lokaj, J.; Vlcek, P.; Kriz, J. *Synthesis of Polystyrene-Poly(2-(dimethylamino)ethyl methacrylate) Block Copolymers by Stable Free-Radical Polymerization.* Macromolecules, **1997**, 30, 7644.

Mathew, J.; Liu, S.Q.; Kodama, M. *Study of Blood Compatible Polymers III: Copolymers of N-benzyl, N-(2-hydroxyethyl) Acrylamide and 2-hydroxyethyl Methacrylate.* Biomaterials, **1993**, 14, 57.

Matyjaszewski, K. *Mechanistic Aspects of Atom Transfer Radical Polymerization.* Journal; General Review, ACS Symp. Ser., **1998a**, 685, 258.

Matyjaszewski, K., *Controlled/Living Radical Polymerization: Progressing ATRP, NMP, and RAFT.* ED.; A.C.S.: Washington, D.C. **2000** (a); Vol. 768.

Matyjaszewski, K.; Shipp, D. A.; Mcmurtry, G. P.; Gaynor, S. G.; Pakula, T. *Simple and Effective One-pot Synthesis of (Meth)acrylic Block Copolymers Through Atom Transfer Radical Polymerization.* J. Polym. Sci. Polym. Chem., **2000** (b), 38, 2023.

Matyjaszewski, K.; Shipp, D. A.; Wang, J. L.; Grimaud, T.; Patten, T. E. *Utilizing Halide Exchange To Improve Control of Atom Transfer Radical Polymerization.* Macromolecules, **1998b**, 31, 6836.

Matyjaszewski, K.; Xia, J. *Atom Transfer Radical Polymerization.* Chem. Rev., **2001**, 101, 2921.

Merdan, T.; Kopecek, J.; Kissel, T. *Prospects for Cationic Polymers in Gene and Oligonucleotide Therapy against Cancer.* Advanced Drug Delivery Reviews, 2002, 54, 715.

Middaugh, C. R.; Evans, R. K.; Montgomery, D. L.; Casimiro, D. R. *Analysis of plasmid DNA from a pharmaceutical perspective.* J. Pharm. Sci., **1998**, 87, 130.

Mitsukami, Y.; Donovan, M. S.; Lowe, A. B.; McCormick, C. L. *Water-Soluble Polymers. 81. Direct Synthesis of Hydrophilic Styrenic-Based Homopolymers and Block Copolymers in Aqueous Solution via RAFT.* Macromolecules, **2001**, 34, 2248.

Perrier, S.; Haddleton, D. M. *Effect of water on copper mediated living radical polymerization.* Macromol. Symp., **2002**, 182, 261.

Philip, R.; Liggitt, D.; Philip, M.; Dazin, P.; Debs, R. *In Vivo* Gene Delivery: Efficient Transfection of T Lymphocytes in Adult Mice. J. Biol. Chem., **1993**, 268, 16087.

Piskin, E., Hoffman, A. S., *Polymeric Biomaterials.* Series E: Applied Sciences, **1984**, 106, 3.

Ratner, B. O.; Hoffman, A. S. In Hydrogels for Medical and Related Applications. J. D. Andrade, ed., ACS Symposium Series 31, Washington D. C., 1976.

Read, M. L.; Dash, P. R.; Clark, A.; Howard, K. A.; Oupicky, D.; Toncheva, V.; Alpar, H. O.; Schacht, E. H.; Ulbrich, K.; Seymour, L. W. *Physicochemical and Biological Characterization of an Antisense oligonucleotide Targeted Against the Bcl-2mRNA Complexed with Cationic-hydrophilic Copolymers.* Euro. J. Pharm. Sci., **2000**; 10, 169.

Rinaudo, M.; Desbrieres, J. *Aqueous gel permeation chromatography of polyelectrolytes and salt exclusion effect.* Europen Polymer J., **1980**, 16, 849.

Robinson, K. L.; Khan, M. A.; de Paz Báñez, M. V.; Wang, X. S.; Armes, S. P. *Controlled Polymerization of 2-Hydroxyethyl Methacrylate by ATRP at Ambient Temperature.* Macromolecules, **2001**, 34, 3155.

Rollings, J. E.; Bose, A.; Caruthers, J. M.; Tsao, G. T.; Okos, M. R. *Aqueous size exclusion chromatography.* American Chemical Society, **1983,** 346.

Roovers, J. in *Encyclopedia of Polymer Science and Engineering.* 2nd ed.; Kroschwitz, J.I., ed.; Wiley; New York, **1985**; 2, 478.

Ruckenstein, E.; Zhang, H. *A novel route to poly(2-hydroxyethyl methacrylate) and its amphiphilic block copolymers.* J. Polym. Sci. Polym. Chem., **1998**, 36, 1865.

Rungsardthong, U.; Deshpande, M.; Bailey, L.; Vamvavaki, M.; Armes, S. P.; Garnett, M. C.; Stolnik, S. *Copolymers of amine methacrylate with poly(ethylene glycol) as vectors for gene therapy.* J. Controlled Release, **2001**, *73*, 359.

Sassi, A. P.; Blanch, H. W.; Prausnitz, J. M. *Characterization of size-exclusion effects in highly swollen hydrogels: Correlation and prediction.* J. Appl. Polym. Sci., **1996**, *59*, 37.

Sawamoto, M.; Kamigaito, M. *Precision Polymer Synthesis by Living Cationic Polymerization.* Trends Polym. Sci., **1996**, 4, 371.

Shen, Y., Zeng, F., Zhu, S., Pelton, R. Versatile Initiators for Macromonomer Syntheses of Acrylates, Methacrylates, and Styrene by Atom Transfer Radical Polymerization. Macromolecules, **2000**; 33, 5399.

Shen, Y.; Zeng, F.; Zhu, S.; Pelton, R. *Novel Cationic Macromonomers by Living Anionic Polymerization of (Dimethylamino)ethyl Methacrylate.* Macromolecules, **2001**, 34, 144.

Smith, J.; Zhang, Y.; Niven, R. *Towards Development of a Non-viral Gene Therapeutic.* Adv. Drug Delivery Rev., **1997**, 26, 135.

Strege, M. A.; Dubin, P. L. *Size-exclusion Chromatography of Cationic Polyelectrolytes on Superose Gel.* Journal of Chromatography, **1989**, 463(1), 165.

Vamvakaki, M.; Billingham, N. C.; Armes, S. P. *Synthesis of Controlled Structure Water-Soluble Diblock Copolymers via Oxyanionic Polymerization.* Macromolecules, **1999**, 32, 2088.

Vamvakaki, M.; Unali, G.-F.; Butun, V.; Boucher, S.; Robinson, K. L.; Billingham, N. C.; Armes, S. P. *Effect of Partial Quaternization on the Aqueous Solution Properties of Tertiary Amine-Based Polymeric Surfactants: Unexpected Separation of Surface Activity and Cloud Point Behavior.* Macromolecules, **2001**, 34, 6839.

Van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin D. J. A.; Hennink, W. E., *2-(dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents.* J. Control. Release, **1998**, 53, 145.

Van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Hennink, W. E. *Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid complexes.* J. Control. Release, **1997**, 49, 59.

Van de Wetering, P.; Moret, E. E.; Nieuwenbrodk, N. M. E.; Van Steenbergen, M. J.; Hennink, W. E. *Structure-activity relationship of water-soluble cationic methacrylate/methacrylamide polymers for non-viral gene delivery.* Bioconjugate Chem., **1999**, 10, 589.

Van de Wetering, P.; Nieuwenbrodk, N. M. E.; Van Steenbergen, M. J.; Hennink, W. E., *Copolymers of 2-(dimethylamino) ethyl methacrylate with ethoxytriethylene glycol methacrylate or N-vinyl-pyrrolidone as gene transfer agents.* J. Control. Release, **2000**, 64, 193.

Van Rompaey, E., Chen, Y., Műller, J. D., Gratton, E., Van Craenenbroeck, E., Engelborghs, Y., De Smedt, S., Demeester, J. *Fluorescence Fluctuation Analysis for the Study of Interactions between Oligonucleotides and polycationic Polymers.* Biol. Chem., **2001a**, 382, 379.

Van Rompaey, E., Engelborghs, Y., Sanders, N., De Smedt, S. C., Demeester, J. *Interactions between Oligonucleotides and Cationic Polymers Investigated by Fluorescence Correlation Spectroscopy.* Pharmaceutical Research, **2001b**, 18, 928.

Van Rompaey, E.; Sanders, N.; De Smedt, S. C.; Demeester, J.; Van Craenenbroeck, E.; Engelborghs, Y. *Complex Formation between Cationic Polymethacrylates and Oligonucleotides.* Macromolecules, **2000**, 33, 8280.

Walsh, C. E.; Liu, J. M.; Miller, J. L.; Nienhuis, A. W.; Samulski, R. J. *Gene Therapy for Human Hemoglobinopathies.* Proc. Soc. Exp. Bio. Med., **1993**, 204(3), 289.

Wang, J. S.; Matyjaszewski, K. *Controlled/"living" Radical Polymerization. Atom Transfer Radical Polymerization In The Presence of Transition-metal Complexes.* J. Am. Chem. Soc., **1995**, 117, 5614.

Wang, X. S.; Lascelles, S. F.; Jackson, R. A.; Armes, S. P. *Facile Synthesis of Well-defined Water-soluble Polymers via Atom Transfer Radical Polymerization in Aqueous Media at Ambient Temperature.* J. Chem. Soc., Chem. Commun., **1999a**, 1817.

Wang, X. S.; Luo, N.; Ying, S. K. *Controlled/living Polymerization of MMA Promoted by Heterogeneous Initiation System (EPN-X-CuX-bpy).* J. Polym. Sci., Polym. Chem., **1999b**, 37, 1255.

Wang X. S.; Jackson, R. A.; Armes, S. P. *Facile Synthesis of Acidic Copolymers via Atom Transfer Radical Polymerization in Aqueous Media at Ambient Temperature.* Macromolecules, **2000 a**, 33, 255.

Wang, X. S.; Armes, S. P. *Scope and Limitations of Atom Transfer Radical Polymerization in aqueous media.* Polymer Prep., **2000 b**, 41(1), 413.

Wang, X. S.; Armes, S. P. *Facile Atom Transfer Radical Polymerization of Methoxy-Capped Oligo(ethylene glycol) Methacrylate in Aqueous Media at Ambient Temperature.* Macromolecules, **2000c**, 33, 6640.

Williams, D. F., ed. *Definitions in Biomaterials. Progress in Biomedical Engineering*, 4. Amsterdam, The Netherlands, Elsevier Publishers, **1987**.

Wolfert, .A.; Dash P. R.; Nazarova, O.; Oupicky, D.; Seymour, L. W.; Smart, S.; Srrohalm, J.; Ulbrich K. *Polyelectrolyte Vectors for Gene Delivery: Influence of Cationic Polymer on Biophysical Properties of Complexes Formed With DNA.* Bioconjugate Chemistry, **1999**, 10, 993.

Wu, G. Y., Wu, C. H. *Specific Inhibition of Hepatitis B Viral Gene Expression in Vitro by Targeted Antisense Oligonucleotides.* J. Biol. Chem., **1992**; 267: 12436

Xu, Y.; Szoka, F. C. *Mechanism of DNA Release Fromcationic Liposome/DNA Complexes Used in Cell Transfection.* Biochemistry, **1996** 35, 5616.

Yau, W. W.; Kirkland, J. J.; Bly, D. D. "*Modern Size Exclusion Liquid Chromatography*"; Wiley: New York, **1979**; 1.

Zamecnik, P. C.; Stephenson, M. L. *Inhibition of Rous Sarcoma Virus Replication and Cell Transformation by A Specific Oligodeoxynucleotide*. Proc Natl Acad Sci U S A, **1978**. 75(1), 280.

Zeng, F.; Shen, Y.; Zhu, S.; Pelton, R. H. *Atom Transfer Radical Polymerization of 2-(dimethylamino)ethyl Methacrylate in Aqueous Media.* J. Polym. Sci. Polym. Chem., **2000a**, 38, 3821.

Zeng, F.; Shen, Y.; Zhu, S.; Pelton, R. H. *Synthesis and Characterization of Comb-Branched Polyelectrolytes. 1. Preparation of Cationic Macromonomer of 2-(Dimethylamino)ethyl Methacrylate by Atom Transfer Radical Polymerization.* Macromolecules, **2000 b**, 33, 1628.

Zhang, X.; Matyjaszewski, K. *Synthesis of Well-Defined Amphiphilic Block Copolymers with 2-(Dimethylamino) ethyl Methacrylate by Controlled Radical Polymerization.* Macromolecules, **1999**, 32, 1763;

Zhang, X.; Xia, J.; Matyjaszewski, K. *Controlled/"Living" Radical Polymerization of 2-(Dimethylamino)ethyl Methacrylate*. Macromolecules, **1998**, 31, 5167.

Zhang, Z.; Liu, G.; Bell, S. *Synthesis of Poly(solketal methacrylate)-block-poly(2-(dimethylamino)ethyl methacrylate) and Preparation of Nanospheres with Cross-Linked Shells.* Macromolecules, **2000**, 33, 7877.

Zhu, N.; Liffitt, D.; Liu, Y.; Debs, R. *Systemic Gene Expression after Intravenous DNA Delivery in Adult Mice.* Science, **1993** 261, 209.

Zhu, S. *Modeling Stable Free-radical Polymerization.* J. Polym. Sci. Part B, Polym. Physics, **1999**, 37, 2692.