SYNTHESIS OF AMINE CONTAINING WELL - DEFINED POLYMERS VIA REVERSIBLE ADDITION-FRAGMENTATION CHAIN TRANSFER (RAFT) POLYMERIZATION AND THEIR CHARACTERIZATION

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ABSTRACT

SYNTHESIS OF AMINE CONTAINING WELL - DEFINED POLYMERS VIA REVERSIBLE ADDITION-FRAGMENTATION CHAIN TRANSFER (RAFT) POLYMERIZATION AND THEIR CHARACTERIZATION

The aim of the study is to synthesize well-defined, spermine-like, amine containing polymers via reversible addition fragmentation chain transfer (RAFT) polymerization as a potential endosomal escaping agent for intracellular drug delivery applications.

Tert-butyl (2-((tert-butoxycarbonyl) amino) ethyl)(2-hydroxyethyl)carbamate was first synthesized and then methacrylated to yield 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate, (BocAEAEMA). BocAEAEMA was then polymerized via RAFT polymerization. A series of RAFT polymerization kinetics experiments were performed in order to investigate the RAFT-controlled character of polymerizations. The effect of [M]/[R] ratio at constant monomer (0.36 M, 0.72 M and 1.44 M) and initiator concentrations ($3.6x10^{-3}$ M) on polymerization kinetics was first investigated. Linear proportionality between ln $[M]_0/[M]$ and polymerization time, and Mn and conversion, indicated the RAFT-controlled polymerization of BocAEAEMA monomer under the conditions tested.

Boc-AEAEMA polymers were deprotected to yield AEAEMA polymers prior to assays performed to determine cytotoxicity and proton sponge capacity of polymers.

Proton sponge capacity of AEAEMA polymers (5.5 kDa and 8 kDa) and PEI (25 kDa and 60 kDa) was investigated via potentiometric titration using constant polymer ($2.2\ x10^{-5}$ M) or repeating unit ($2.9\ x10^{-5}$ M) concentrations. The proton sponge capacity of p(AEAEMA)was found to be comparable to those of PEIs at the same repeating unit concentration.

AEAEMA polymers did not show cytotoxic effect on NIH 3T3 cells up to 1.6 M concentration, tested via a cell viability assay for 24h and 72 h.

ÖZET

AMİN İÇEREN İYİ TANIMLANMIŞ POLİMERLERİN TERSİNİR KATILMA AYRIŞMA ZİNCİR TRANSFER (RAFT) POLİMERİZASYONU İLE SENTEZİ VE KARAKTERİZASYONU

Bu çalışmanın amacı, potansiyel bir endozomdan kaçış ajanı olarak hücre içi ilaç taşınımı uygulamalarında kullanılmak üzere, iyi tanımlanmış, spermin'e benzeyen, amin içerikli polimerleri tersinir katılma- ayrışma zincir transfer (RAFT) polimerizasyonu ile sentezlemektir.

Öncelikle, tert-butil (2-((tert-butoksikarbonil) amino)etil) (2-hidroksietil) karbamate sentezlendi veamin içeren yeni bir monomer (2-((tert-butoksikarbonil) (2-((tert-butoksikarbonil) amino) etil) amino) etil metakrilat, (BocAEAEMA)) bileşiğinin sentezi içinmetakrilatlandırıldı. BocAEAEMA monomeri RAFT polimerizayonu ile polimerize edildi. Polimerizasyonun RAFT kontrol karakterinin incelenmesi amacıyla bir seri polimerizasyon kinetiği deneyleri yapıldı. [M]/[R] oranının polimerizasyon kinetiği üzerindeki etkileri sabit monomer konsantrasyonunda (0.36 M, 0.72 M and 1.44 M) ve sabit başlatıcı konsantrasyonunda ($3.6x10^{-3}$ M) incelendi. Kinetik grafiklere göre, ln $[M]_0/[M]$ ve polimerizasyon zamanı arasındaki ve Mn ve dönüşüm arasındaki doğrusal orantı, denenen şartlar altında BocAEAEMA polimerlerinin RAFT kontrollü polimerizasyon mekanizmasıyla sentezlendiğigösterdi.

BocAEAEMA polimerlerinin koruma grupları sitotoksite ve proton sünger kapasitesini incelemek amacıyla yapılacak deneyler öncesi uzaklaştırıldı.

Polimerlerin (5.5 kDa and 8 kDa) ve polietileniminin (25 kDa and 60 kDa) proton sünger kapasitesi potansiyometrik titrasyon ile sabit polimer ($2.2\ x10^{-5}$ M) veya tekrarlayan birim ($2.9\ x10^{-5}$ M) konsantrasyonlarında incelendi. AEAEMA polimerlerinin proton sünger kapasitesinin eşit tekrarlayan birim konsantrasyonunda polietileniminlerin kapasitesi ile benzer olduğu bulundu. AEAEMA polimerleri 1.6 M konsantrasyona kadar NIH 3T3 fibroblast hücreleri üzerinde 24 ve 72 saat içerisinde belirgin bir sitotoksik etki göstermedi.

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CHAPTER 1

INTRODUCTION

Intracellular delivery of macromolecular therapeutics is one of the most important concerns in several treatment strategies such as gene therapies and anticancer therapies. When cells encounter a foreign macromolecule, they take up through endocytosis mechanism in which the foreign macromolecules are entrapped inside membrane vesicles called endosomes. In endosomes, pH drops to approximately 5.0. The molecules that cannot escape from endosomes by the help of an endosomal escaping agent are transferred to lysosomal vesicles in which they are degraded by degradative enzymes.

Some synthetic agents that are able to escape from endosomes include certain cationic molecules and poly(cations) such as spermine and poly(ethyleneimine). Spermine is a natural amine that has been used in intracellular drug delivery studies. Spermine containing systems have shown high biocompatibility, nucleic acid binding activity and transfection activity (i.e. transfection is the process of introducing nucleic acids/genes into cells). Importantly, primary and secondary amine groups in spermine provide a proton-sponge effect in acidic environment of endosomes and facilitate endosomal rupturing in intracellular applications (Du et al., 2012). Polyethyleneimine (PEI) is a synthetic poly(amine) that has been widely used in intracellular delivery of genes and nucleic acids. High molecular weight, branched PEI (25 kDa or higher) contains high cationic charge density making it a highly efficient transfection agent (Xu et al., 2011) (Godbey et al., 1999b). The major drawback of PEI is its high dose dependent toxicity (Deng et al., 2009).

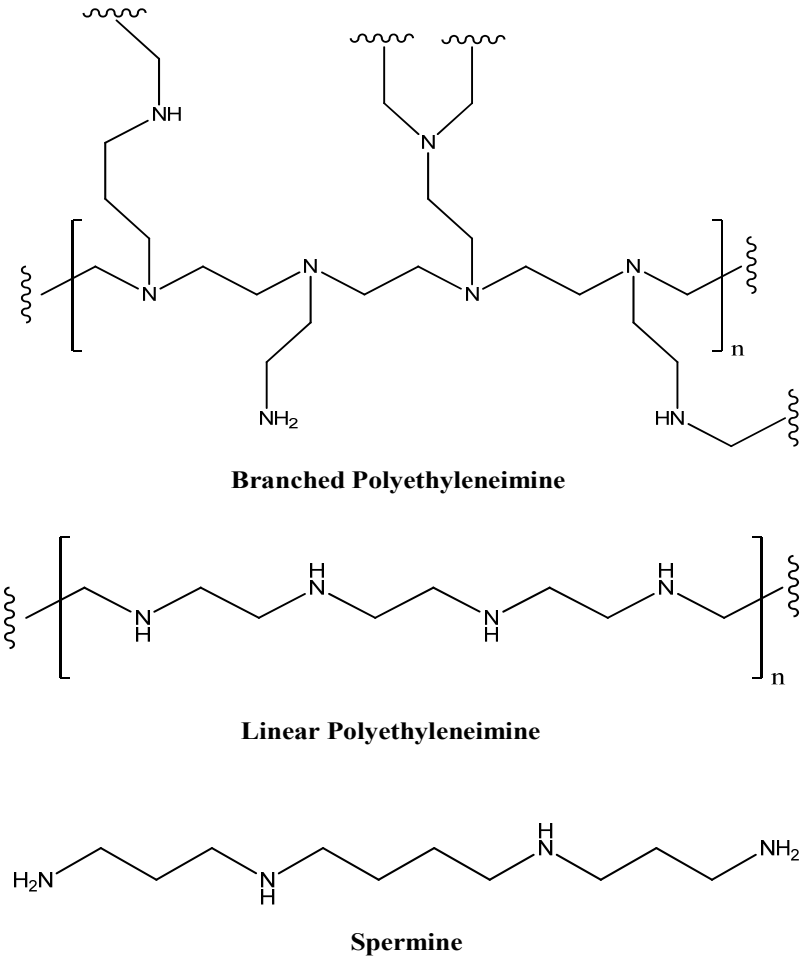


Figure 1.1. Examples of amine containing natural and synthetic endosomal escaping agents using proton sponge effect

All effective endosomal escaping agents showing proton sponge effect have a common property; they contain primary, secondary or tertiary amine groups.

In the literature, various amine containing homo and/ or copolymers such as poly(2-(dimethylamino) ethyl methacrylate) (p(DMAEMA)), poly(aminoethyl methacrylate), poly(*N*-propylethylenimine), poly(1-(2-aminoethyl) piperidine), poly(aniline) and poly(pentaethylenehexaamine), have been prepared via different methods (Figure 1.2) and their DNA binding activity and cytotoxicity were examined and compared with those of polyethyleneimine (Dutta et al., 2011) (Zhu et al., 2010) (Barua S. et al., 2008). Molecular weight, its distribution and chemical structure of amine containing polymers have been found to be important in effective delivery of macromolecules.

Recently, reversible addition fragmentation chain transfer (RAFT) polymerization technique has been used for preparation of amine containing polymers that may have potential for intracellular drug delivery applications (Alidedeoglu et al., 2009) (Treat et al., 2012) (Yanjarappa et al., 2006). The RAFT technique, which is a living/controlled polymerization technique, enables the synthesis of well-defined polymers with controlled molecular weight, narrow molecular weight distribution and controlled structure.

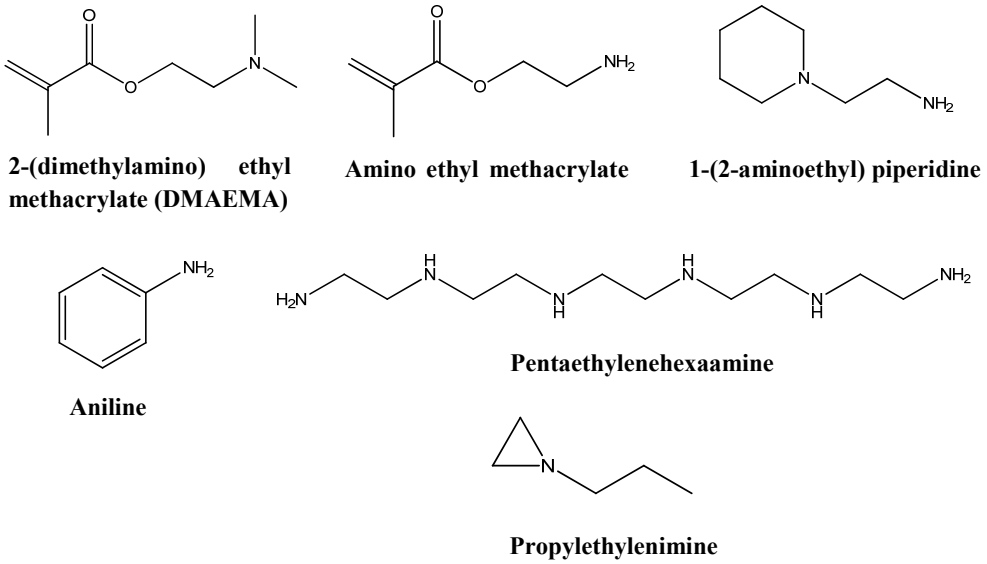


Figure 1.2. The monomers used in preparation of amine containing polymers

The aim of this thesis is to synthesize and characterize a new, spermine-like amine containing polymer, poly(2-((tert-butoxycarbonyl) (2-((tert- butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) via RAFT polymerization as a potential endosomal escaping agent. Firstly, 2-((tert-butoxycarbonyl) (2-((tert- butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate monomer was prepared and its polymers were synthesized via RAFT polymerization. The procedure for monomer and polymer synthesis and characterizations are presented in Chapters 3. Chapter 4 covers the results of new monomer characterization, RAFT polymerization kinetics studies, proton sponge capacity and cytotoxicity assays of polymers.

CHAPTER 2

LITERATURE REVIEW

2.1. Intracellular Drug Delivery

Macromolecular therapeutics such as siRNA/miRNA, peptides, ODN/ribozyme, plasmid DNA have been used increasingly in several therapeutic strategies. In order to show their therapeutic activities, therapeutic macromolecules usually need to localize inside the cells at essential therapeutic amounts. However, these macromolecules encounter many intracellular and extracellular limitations such as *poor transport into cells; non -specific effects on cells; rapid degradation in vitro and in vivo; and rapid elimination from the body* (Kabanov et al., 1995). The extracellular barriers such as liver elimination and kidney excretion can be overcome by increasing hydrodynamic radius of therapeutics and decreasing recognition by reticulo endothelial system (RES).

One of the main limitations of intracellular delivery is the poor penetration of therapeutic macromolecules through the cell membrane. Macromolecules are generally taken up into cell by endocytosis (Figure 2.1) (Nicolazzi et al., 2003). When a molecule approaches to a cell, a vesicle made of plasma membrane, which is called endosome (early and late endosomes) is formed. In early endosome (Figure 2.3. A), pH begins to decrease from neutral to 6 and in late endosome (Figure 2.3. B), pH reaches to acidic pH that is between 5 and 6. The endosome combines with lysosome. In lysosome, the macromolecular therapeutics are degraded by digestive enzymes.

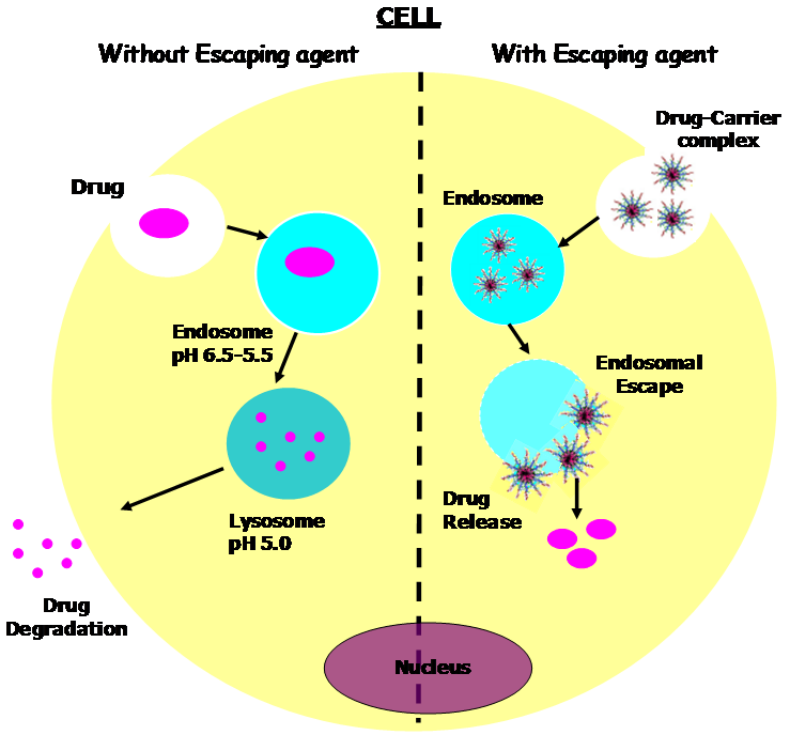


Figure 2.1. Mechanisms of endocytosis with and without endosomal escaping agent

In order to avoid inactivation or digestion of macromolecular therapeutics in lysosomes, endosomal escaping mechanisms which would traffic therapeutics from endosome to cytoplasm are required. In literature, various endosomal escaping mechanisms, such as pH buffering effect (proton sponge effect), pore formation, fusion and photochemical disruption in the endosomal membrane, have been investigated (Varkouhi et al., 2011).

Pore formation mechanism in endosome depends on interaction of membrane tension and line tension which are enlarging pore and closing pore, respectively. In this endosomal escaping mechanism, some agents such as certain peptides have an ability to bind to lipid bilayer that causes an increment internal membrane tension which forms pore. There are some models to explain pore formation in endosomal membrane. In barrel-stave model, escaping agent pushes the membrane to form barrel-like pore (Figure 2.2 (A), (Westphal et al., 2011)). In toroidal model, some α-helical peptides cause membrane curvature via binding to lipid headgroups (Figure 2.2 (B), (Westphal et al., 2011)).

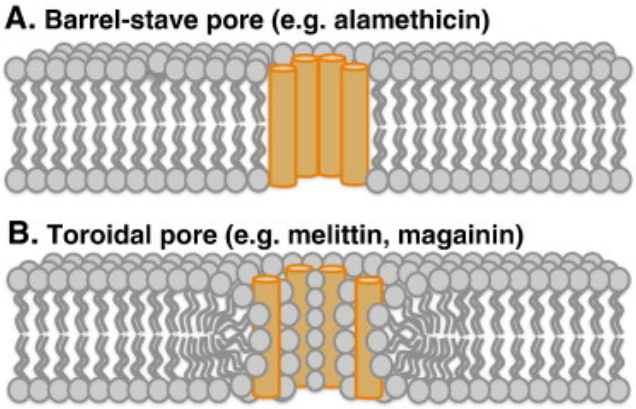


Figure 2.2. Pore formation mechanisms via Barrel-stave and Toroidal models (Source: Westphal et al., 2011)

Most viruses have peptides that fuse through and destabilize the endosomal membrane. In this mechanism, fusogenic peptide undergoes a conformational change in incentive conditions. Haemagglutinin is converted from an anionic, hydrophilic coil at pH 7.4 to a hydrophobic helical conformation at the acidic endosomal pH, 5.0-6.0, which fuses through the endosomal membrane (Plank et al., 1994) (Lear & DeGrado, 1987). In photochemical disruption of endosomal membrane, some photo sensitizers as endosomal escaping agents localize in the endosomal membrane and when they are exposed to light, reactive singlet oxygen is formed and destroys the endosomal membrane. In proton sponge or pH buffering effect, endosomal escaping agents, which have high buffering capacity, are protonated at the acidic endosomal pH. Protonated agent disturbs the acidity of endosome. While ATPase proton pumps transfer protons from cytosol into endosome to conserve acidity, Cl^- ions diffuse into endosome which induces an increment in osmotic pressure. In order to stabilize ionic concentration, water diffuses into endosome which swells and ruptures (Nicolazzi et al., 2003) (Varkouhi et al., 2011) (Liang & Lam, 2012).

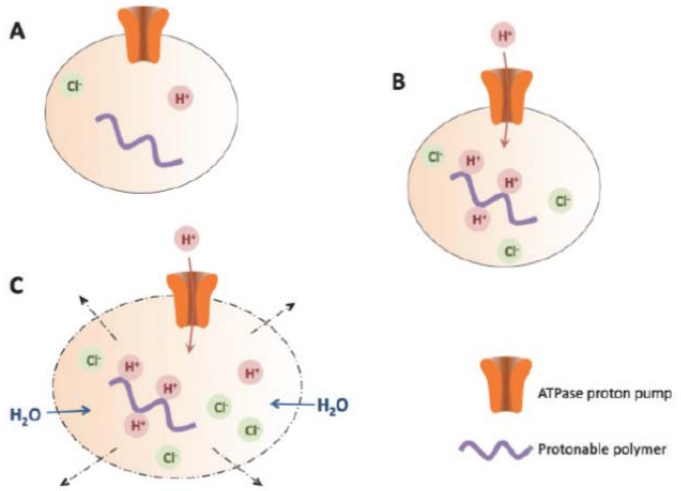


Figure 2.3. The proton sponge hypothesis (pH-buffering effect)
(Source: Liang & Lam, 2012)

Numerous endosomal escaping agents have been derived from viruses and bacteria that have high transfection efficiencies (Rosenfeld M. A. et al., 1991). However virus derived agents are reported as highly immunogenic and potentially mutant. In addition, they may have oncogenic effects (Scales C. W., 2006).

In the past decade, endosomal escaping agents have been developed synthetically based on viruses and bacteria. These synthetic agents are effective, specific, biocompatible and bio- available. In addition, they have lower immunogenicity when compared to virus based agents. There are two major classes of non-viral escaping agents: cationic lipids (Lee & Chu, 1997) (Woodle & Scaria, 2001) and polycations (Thomas & Klibanov, 2003). Positively charged agents could bind to negatively charged macromolecular therapeutics such as DNA and RNA and also cell membrane. In addition, these molecules enhance uptake into cells due to their positive charge and protect therapeutics against degradation (Kabanov et al., 1995).

Studies of non-viral intracellular drug delivery vehicles began with cationic lipids (Cho Y.W. et al., 2003). In a review of Thomas and Klibanov (Thomas & Klibanov, 2003), it is indicated that shape, size and stability of hydrophobic groups of cationic lipids have a key role in transfection of DNA-lipid complexes and interaction with DNA and cell membrane (Zuhorn et al., 2002). In addition, transfection efficiency of cationic lipids can be improved with helper lipids such as dioleoylphosphatidylcholine (DOPC) and phosphatidylethanolamine (dioleoyl PE) (Hui et al., 1996). In a study of Simberg et al. (Simberg et al., 2001), lipoplexes were

prepared using monocationic and polycationic lipid based components ((N-(1-(2,3-dioleoyloxy) propyl) ,N,N,N-trimethylammonium chloride, N-(1-(2,3-dimyristyloxypropyl) -N,N-dimethyl-(2-hydroxyethyl) ammonium bromide) and (2, 3-dioleyloxy-N-[2 (sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanammonium trifluoroacetate)) with or without helper lipids (cholesterol, 1,2-dioleoyl-s*n*-glycero-3-phosphatidylethanolamine and 1,2-dioleoyl-s*n*-glycero-3-phosphatidylcholine). However according to parallel transfection studies, size and phase of prepared lipoplexes were reported as instable.

Nevertheless cationic lipid based systems that are successful in cell culture, have not been useful for *in vivo* delivery because of instability, short serum half-life and toxicity (Gao & Huang, 1995).

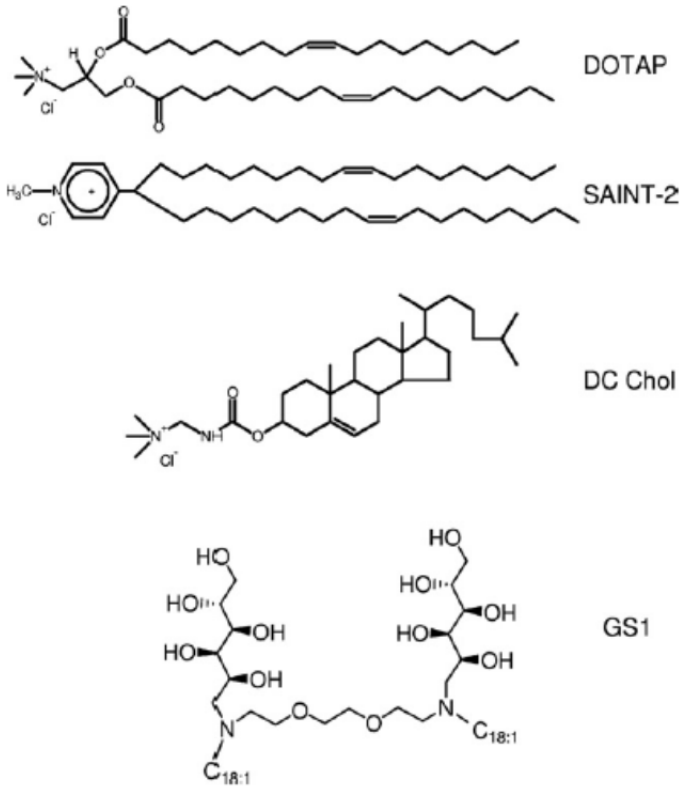


Figure 2.4. Chemical structures of some cationic lipids used in transfection
(Source: Wasungu & Hoekstra, 2006)

2.2. Polycations in Intracellular Drug Delivery

In the recent past, polycations as non-viral intracellular drug delivery agents have been started to be used because of their remarkable and sophisticated properties. Positively charged polymers can interact with negatively charged biological membranes such as endosomal membrane enabling intracellular drug delivery of therapeutics (Gu et al., 2008). Polycations with various molecular weights and molecular architectures can be easily synthesized by using versatile polymer chemistry to improve transfection activity (Cho Y.W. et al., 2003).

There are many naturally occurred (histones, cationized human serum albumin and chitosan) and synthetic polycations (Figure2.5, (Thomas & Klibanov, 2003)). In a study of Balicki et al. (Balicki et al., 2000), plasmid DNA was mixed with histone H2A or Superfect which is a commercial transfection agent, and their activity were compared. According to the results, histone H2A-mediated therapy induced the greatest release of gene and showed a better therapeutic activity in contrast to Superfect. Human serum albumin was cationized by covalent coupling of hexamethylenediamine and then DNA- cationized human serum albumin complex was prepared by Fischer et al. (Fischer et al., 2001). The transfection of DNA into cells was investigated by using NIH 3T3 fibroblasts. According to results, DNA- cationized human serum albumin complex did not show toxic effect. It is concluded that cationized albumin is a promising non-toxic vector for gene delivery (Fischer et al., 2001). In addition to this study, the potential usage of cationic albumin for peptide delivery has been shown by Pardridge et al. (Pardridge et al., 1990).

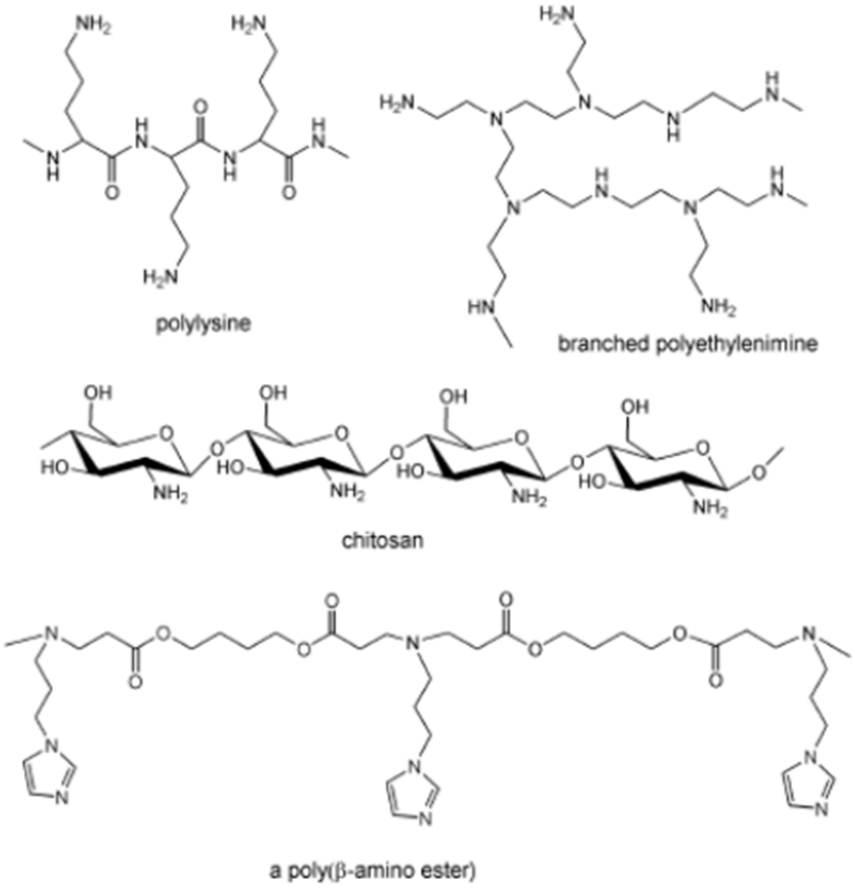


Figure 2.5. Chemical structures of representative polycationic gene delivery agents (Source: Thomas & Klibanov, 2003)

Poly(L-lysine), polyethyleneimine, polypropylenimine and polyamidoamine dendrimers are synthetic polycations whose transfection efficiencies have been investigated in the literature. Recently, new generation polycations such as poly(2-(dimethylamino) ethyl methacrylate (Zhu et al., 2010) and 2-aminoethyl methacrylate (Alidedeoglu et al., 2009) have been also investigated for nucleic acid delivery.

2.2.1. Polyethyleneimine in Intracellular Drug Delivery

Polyethyleneimine (PEI) has been utilized for years in mineral extraction, paper production, shampoo manufacturing, and water purification (Godbey et al., 1999a). It was studied firstly by Boussif et al. as one of the most important cationic intracellular drug delivery vehicles (Sun et al., 2012) (Boussif et al., 1995). PEI, synthesized as

linear or branch type, contains secondary or primary and tertiary amine groups respectively in its backbone and branches (Figure2.6).

Branched and linear types of polyethyleneimine are both synthesized via cationic polymerization from aziridine and 2-substituted 2-oxazoline monomers, respectively. In branched type PEI, branches are formed by specific interactions of growing polymer molecules. In linear type PEI synthesis, firstly poly(N-formalethyleneimine) is formed then hydrolyzed and linear PEI arises (Godbey et al., 1999a).

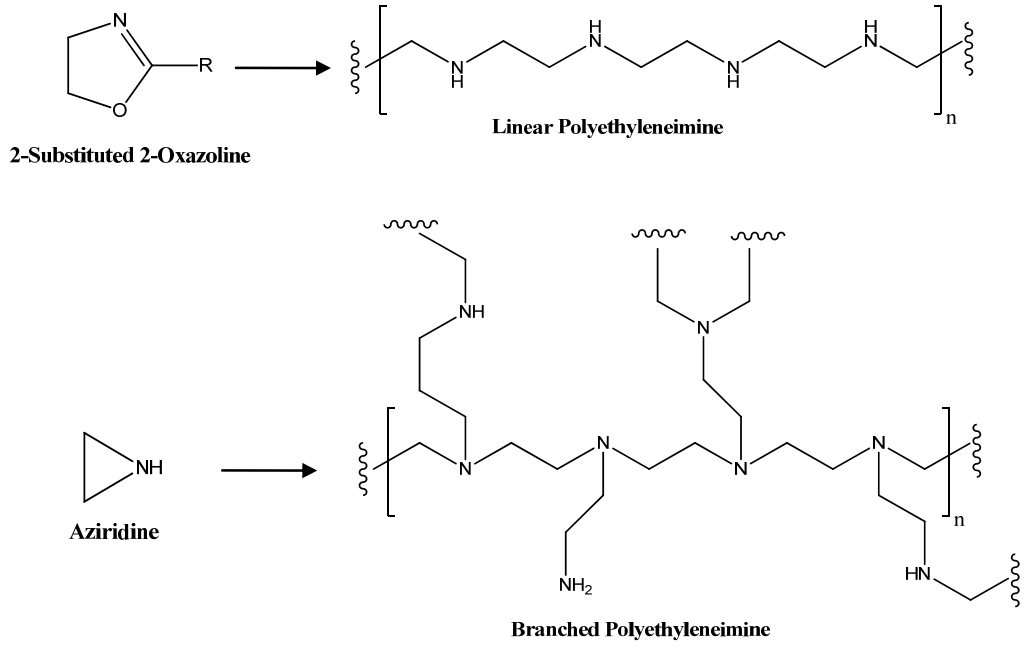


Figure 2.6. Chemical structures of linear and branched polyethyleneimine (Source: Godbey et al., 1999a)

pKa value of primary and secondary amine groups in PEI structure are around 9.5 and 4.5, respectively (Koper et al., 2003). For this reason, primary amines of PEI can be protonated at any cellular pH and PEI behaves as a "proton sponge" (Boussif et al., 1995). Protonation or buffering capacity provides PEI an endosomal escaping capability by "endosomal swelling and rupture" (Figure 2.7) (El-Sayed et al., 2009). Thus, using PEI as endosomal escaping agent together with macromolecular drugs (such as siRNA, ODN, DNA etc.) prevents degradation of drugs by enzymes in lysosome and enhance delivery of them into cell (Boussif et al., 1995; Klemm et al., 1998).

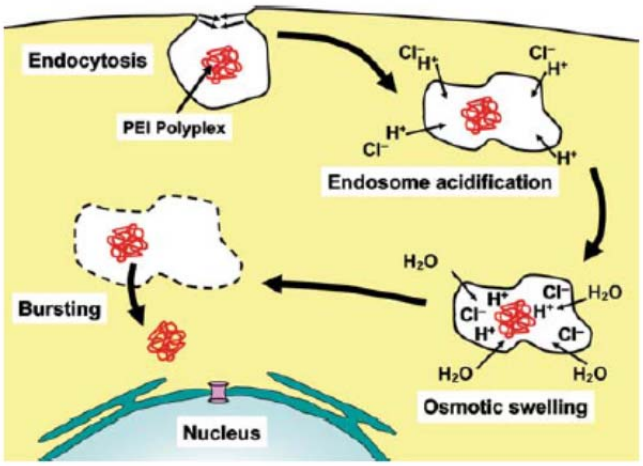


Figure 2.7. Endosomal escaping mechanism of Polyethyleneimine
(Source: El-Sayed et al., 2009)

Molecular weight and architecture (branched or linear) affect buffering capacity (cationic charge density), cytotoxicity and transfection efficiency of PEI. In the literature studies about intracellular delivery of macromolecules, branched polyethyleneimine has been used generally because of its greater transfection activity than linear one (Godbey et al., 1999a).

In a study of Godbey et al. (Godbey et al., 1999b), the effect of molecular weight of PEI on transfection was investigated. 600, 1200, 1800, 10,000 and 70,000 Da PEI's were used in the experiments. According to results, when molecular weight increased, transfection efficiency increased. In addition, 600 1,200 and 1,800 Da PEI's did not show transfection efficiency. It was concluded that lower molecular weight PEI has no transfection efficiency.

In addition, Fischer et al. (Fischer et al., 2003) investigated and compared cytotoxicities of various cationic polymers (high molecular weight PEI (600-1000kDa), Poly(l-lysine) (PLL), diethylaminoethyl-dextran, human serum albumin) using L929 mouse fibroblast and MTT assay and the release of the cytosolic enzyme lactate dehydrogenase as monitoring methods. According to results, high molecular weight PEI and PLL have shown higher cytotoxic effect. It was deduced that molecular weight was a key parameter for charge density and interaction with the cell membrane. When molecular weight increased, cationic charge density increased which caused cell damage (cytotoxicity).

Recently, in order to optimize cytotoxicity and transfection efficiency of polyethyleneimine, copolymers of PEI have been synthesized in literature.

In the study of Nguyen et al. (Nguyen et al., 2000), randomly branched polyethyleneimine (Mole ratios of primary, secondary and tertiary amino groups was 1.3:1.2:1, 2kDa and 25kDa) was grafted with non-ionic polyethers, polyethyleneoxide (PEO) and Pluronic 123 (P123) and their transfection efficiencies were compared to PEIs (2kDa and 25 kDa). P123 grafted PEI (25kDa) has lower transfection activity than non-modified PEI (25kDa). Furthermore, transfection activity of grafted PEI (2kDa) was compared with the non-modified PEI (2 kDa) activity, it was shown that transfection did not improve and remained low. In addition to the study of Nguyen et al., PEI-TEG was synthesized using 600 and 2000Da PEI and triethyleneglycol, then mannose, which was used as a ligand for specific recognition by dendritic cells, was linked to polymers in a study of Sun et al. (Sun et al., 2012). The cytotoxicity and transfection activity of PEI-TEG/DNA and mannose-PEI-TEG/DNA were investigated and compared to 25kDa PEI. *In vitro* cytotoxicity and transfection activity investigations were done using dendritic cell line. N/P ratio (ionisable nitrogen of PEI/ionisable phosphorus of DNA) was used when preparing PEI/DNA complexes and this ratio was increased from 5 to 50. According to results, when ionisable nitrogen increased, cytotoxicity of all PEI/DNA complexes increased. Cell viabilities of PEI-TEG and mannose-PEI-TEG were higher than PEI (25kDa). However for PEI-TEG, when N/P increased to 30, cell viability decreased. In addition, cell viability was nearly 80% for mannose-PEI-TEG at N/P 50. Moreover mannose-PEI-TEG/DNA complex has shown higher transfection efficiency than PEI-TEG/DNA and PEI/DNA. PEI-TEG/DNA complex has shown higher transfection activity than PEI/DNA complex.

In another study, Chen et al. (Chen et al., 2011) developed PEI-antigen (ovalbumin) complex with electrostatic interactions. PLL-antigen complex was also prepared in order to compare delivery of antigen-complex into the cell. According to results, PLL did not improve cross presentation (efficacy of therapeutic vaccines) effect of antigen. PEI improved the cross presentation in appropriate weight ratio. It was concluded that system was stable, flexible, and simple and it had high loading efficiency but PEI was toxic which may have prevented cross presentation.

Moreover, in a study of Xia et al. (Xia et al., 2009), surface of the mesoporous silica nanoparticles (MSNP) was modified with different molecular weights (0.6 kDa, 1.2 kDa, 1.8 kDa, 10 kDa and 25 kDa) of polyethyleneimine in order to enhance cellular

uptake of DNA and siRNA. According to results, PEI modification increased cellular uptake of MSNP-DNA and –siRNA complexes. However, high molecular weight PEI (25kDa) linked MSNP has shown high cytotoxicity. It was concluded that transfection efficiency can be increased when reducing cytotoxicity by selecting appropriate PEI size.

A brush-shaped polymer PHEMA-g-(PEI-b-PEG) with poly(2-hydroxyethyl methacrylate) (PHEMA) backbone and linear poly(ethylenimine)-b-poly(ethylene glycol) (PEI-b-PEG) side chains was prepared and evaluated as a vehicle for potential cancer gene therapy by Lui et al. (Liu et al., 2010). The physicochemical characterizations of brush-shaped polymers were done using ^{1}H NMR and laser light or dynamic light scattering. According to cytotoxicity analysis synthesized PHEMA-g-(PEI-b-PEG) showed lower toxicity than branched poly(ethylenimine) with Mw of 25 kDa. Plasmid DNA was linked to PHEMA-g-(PEI-b-PEG). The internalization of complexes of PHEMA-g-(PEI-b-PEG) and the complexes of PEI (25kDa) with plasmid DNA was investigated and compared using BT474 cells. Results showed that, PHEMA-g-(PEI-b-PEG) complexes were internalized more efficiently, indicating higher gene transfection. Furthermore the complexes of PHEMA-g-(PEI-b-PEG) improved significantly wild-type p53 expression in BT474 cells and they enhanced apoptosis of BT474 cells and sensitivity of BT474 cells to doxorubicin chemotherapy.

Brissault et al. (Brissault et al., 2003) synthesized ethylenimine or *N*-propylethylenimine contained linear polymers via polyethyloxazolines hydrolysis and/or reduction. The polymers' pKa values were defined potentiometrically. The efficiency of DNA complexation was analyzed by gel mobility shift assay that depended on the amount of amino groups. The transfection efficiencies of polymers were investigated for HepG2 cells with respect to cationic charge density and molecular weights. According to the results, transfection efficiency of copolymers depended on the ethyleneimine content. In addition, the capacity of a polymer to capture protons in an acidic environment enhanced transfection efficiency of polymers.

2.2.2. Poly(L-Lysine) in Intracellular Drug Delivery

Poly (L-lysine) (PLL) is a natural cationic homo-polypeptide (Figure2.8). It has been used as food preservatives because of its antimicrobial activity against most gram

positive and gram-negative bacteria, fungi and some kinds of viruses. It has high cationic charge density (pKa: 10.4) (Yu et al., 2011). It is positively charged at neutral pH. It prevents proliferation of microorganisms (Wang et al., 2010). Cationically charged PLL interacts with membrane of microorganisms and causes a fusion with the membrane. PLL, which is water soluble, biodegradable and non toxic, has been used in *non-viral gene delivery,* for the first time by Wu et al. in 1987 (Wu &Wu, 1987).

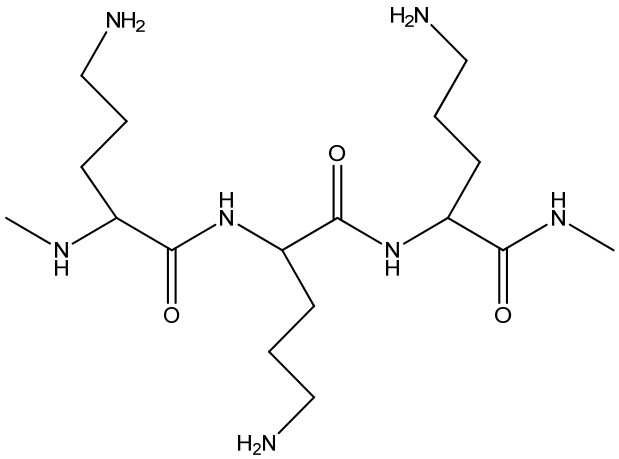


Figure 2.8. Chemical structure of poly(l-lysine)

In the study of Wu et al. (Wu & Wu, 1987), asialoorosomucoid (ASOR)-poly-L-lysine conjugate was prepared and then plasmid, pSV2 CAT was then complexed with the conjugate. In order to test the complex, hepatoma cell lines, Hep G2, asialoglycoprotein receptor (+), and SK-Hep 1, receptor (-) were used. Their hypothesis was that water soluble polycation PLL would form a complex with DNA; hepatocytes would bind to glycoprotein of (ASOR)-poly-l-lysine conjugates with specific receptors for glycoprotein and finally DNA would transfer into cell by receptor-mediated endocytosis. In addition, delivery of DNA-ASOR, DNA-PLL and neat DNA was investigated. According to results, the delivery was not achieved in SK-Hep 1 cells. However (ASOR)-poly-l-lysine-DNA complex was transferred into Hep G2, asialoglycoprotein receptor (+) cells.

In addition, Wagner et al. (Wagner et al., 1992) prepared plasmid DNA-transferrin-poly(l-lysine) and plasmid DNA-poly(l-lysine)-peptide complexes in order to transfer luciferase or galactosidase marker contained genes into K562 cells, HeLa cells, and BNL CL.2 hepatocytes. Peptides were derived from N-terminal sequence of

the influenza virus hemagglutinin subunit HA-2. Poly(l-lysine) and transferrin were used to pack DNA and enable receptor-mediated endocytosis, respectively. Influenza peptides were used to escape from endosome and release into cytosol. The escaping mechanism of hemagglutinin (HA-2)-poly(l-lysine) system was found to be fusion (Varkouhi et al., 2011). The membrane disrupting activities of the peptides were investigated by liposome leakage assay. According to results, influenza peptides showed high leakage activity at lower pH (5-6). The influenza peptides improved the transferrin-poly(l-lysine) mediated gene delivery.

Guo et al. (Guo et al., 2012) was synthesized PEGlated poly(l-lysine)-cholic acid (PLL-CA) amphiphilic cationic polymers to increase siRNA delivey in treatment of Prostate cancer. PEG was attached to PLL-CA polymers via the benzoic imine linker which was cleavable at lower pH. At pH 7.4 PEG-PLLs complexes were stable. Delivery activity and endosomal escape of PEG–PLLs were compared to INTERFRin™ in *in vitro* and *in vivo* assays. According to results, PEGlated copolymers showed high silencing efficiency, low toxicity and immunogenicity.

In addition, pH responsive triblock copolymer systems were designed by inserting poly(l-histidine) between PEG and poly(l-lysine) (PEG-$CH_{12}K_{18}$ and PEG-CK_{30}) to deliver DNA via a nucleolin-independent pathway (Boylan et al., 2012). According to results, poly(l-histidine) improved buffering capacity of the system which was comparable with branched PEI. While PLL does not have buffering capacity, it helps to polymer complexes to attach nucleotids. Human bronchial epithelial cells were used to analyze DNA-triblock polymer complex by a clathrin-dependent endocytic mechanism followed by endo-lysosomal processing. According to results, polymer enhanced 20-fold *in vitro* gene transfer and 3- fold *in vivo* gene transfer to lung airways compared to PEG-CK_{30} DNA nanoparticles while toxicity was constant at an optimized level.

Moreover, in a study of Zhu et al. (Zhu et al., 2012), pH-sensitive polymeric vesicular aggregates composed of cholate grafted poly(l-lysine) (PLL-CA) and an amphiphilic poly(ethylene glycol)−doxorubicin conjugate (PEG-DOX) were prepared. Polymer-drug conjugates had low-pH labile bond. According to results, when PEG-DOX content increased, the permeability of the vesicles reduced. The cell uptake of FITC-dextran loaded PLL-CA25/PEG-DOX vesicles was investigated at pH 7.4 and 6.5. At pH 6.5, strong fluorescence of both the carrier (DOX moieties) and the encapsulated molecules (FITC-dextran) was observed in the cytosol of the cells. The

study was concluded that PLL-CA/PEG-DOX vesicles could escape from endosome/ lysosome and PLL-CA polymers had very effective membrane disruption property at acidic pH close to the extracellular condition of solid tumours.

Furthermore, star block copolymers composed of a hyperbranched polyethyleneimine (PEI) core, a poly(l-lysine) (PLL) inner shell and a poly(ethylene glycol) (PEG) outer shell were designed by Yan et al. (Yan et al., 2012) to deliver protein. The protein insulin was encapsulated by the polymer. According to results, negatively charged protein was linked to positively charged polymers. Furthermore the polymers were found to attach to cell membrane. The fluorescence labelled polymer were observed to localize in cytoplasm after 4h incubation. The insulin release was investigated and according to results, when pH decreased, insulin release increased.

In the study of Hartono et al. (Hartono et al., 2012), mesoporous silica nanoparticles (LP-MSNs) functionalized with poly- L-lysine (PLL) were designed as a new carrier for gene delivery applications. According to results, PLL modified silica nanoparticles enhanced oligo-DNA binding compared to unmodified silica. PLL modified mesoporous nanoparticles showed efficient DNA transfection into cancer cells. It was also observed that nanoparticles exhibited low cytotoxicity and high biocompatibility.

2.2.3. Spermine in Intracellular Drug Delivery

Spermine is a natural polyamine that occurs in all eukaryotes, but is rare in prokaryotes. It is essential for cell growth in both normal and neoplastic tissue. Spermine has strongly basic character and all of its amino groups are positively charged in aqueous solution at physiological pH.

Spermine, which has crystal structure, was separated from human sperm firstly by Leeuwenhoek in 1678. However, in later years it was found that semen pancreas, liver, kidney, ovary, muscle, spleen, lung, brain, testes, thymus, adrenal, and thyroid tissues contain spermine in various amount from 0.26 to 0.003 percent. The chemical structure and formula of Spermine is shown in Figure 2.9 (Cameron, 1927).

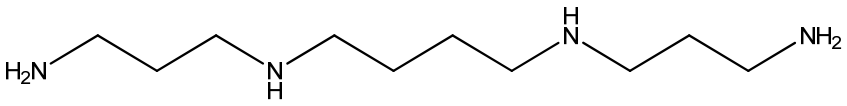


Figure 2.9. Chemical structure of spermine

Pharmacological properties of spermine have been investigated by several researchers. In intracellular drug delivery studies, spermine containing systems have shown higher transfection activity, biocompatibility and nucleic acid binding activity. Importantly, amino groups in spermine provide a proton-sponge effect and facilitate endosomal rupturing in intracellular applications (Du et al., 2012).

In a study of Abdullah et al. (Abdullah et al., 2010), dextran-spermine (D-SPM) cationic polymer was prepared in order to optimize conditions for gene expression of D-SPM/plasmid DNA (D-SPM/pDNA) complexes in cell lines and also in the lungs of BALB/c mice via instillation delivery. According to results of *in vitro* studies, D-SPM protected pDNA partially from nuclease degradation and exhibited optimal gene transfer efficiency at D-SPM to pDNA weight ratio of 12:1.

Galactose-spermine conjugated chitosan was prepared and buffering capacity, DNA binding ability, stability, solubility, transfection efficiency and toxicity of these conjugates were investigated by Alex et al. (Alex et al., 2011) in HepG2 cell lines. According to results, galactosylated chitosan- spermine nanoparticles has good DNA binding ability and high buffering capacity. They were soluble and stable. In addition, according to *in vitro* studies, nanoparticles showed low cytotoxicity and high transfection efficiency. The study was concluded that these chitosan based spermine nanoparticles could be promising in gene delivery applications.

In a study of Du et al. (Du et al., 2012), three different spermine polymers were prepared. Degradability, cytotoxicity, binding ability to DNA and transfection efficiency of polymers were investigated for siRNA and genes to cells of. Half- lives of polyspermine carbamate and polyspermine amide were determined as more than 2 months, whereas the half-life of polyspermine imine half life was 11h. The cytotoxicity of polymers was investigated by using COS-7 and HepG2. While the polyspermine carbamate cytotoxicity was determined the highest, cell viability of polyspermine imine was obtained higher than polyspermine amide. The transfection efficiencies of three polymers were similar for COS-7 cells. However in HepG2 cells, polyspermine carbamate showed higher transfection efficiency than other polymers.

Furthermore, Hong et al. (Hong et al., 2012) synthesized glycerol triacrylate–spermine (GT-SPE) biopolymers via Micheal addition. Plasmid DNA was linked to polymers. Transfection activity and toxicity of polymers were investigated and compared to polyethyleneimine *in vitro* and *in vivo*. The toxicity of GT-SPE was obtained lower than PEI. The GT-SPE-DNA complex displayed high transfection activity and it prevented the degradation of DNA by nucleases.

Amphiphilic cholesterol-spermine polymers were developed by Maslov et al. (Maslov et al., 2012) as non-viral gene delivery agents in order to estimate the structure-activity relationships of novel polycationic lipids and to determine an effective and safe transfectant suitable for the delivery of different nucleic acids such as FITC-labeled oligonucleotide, plasmid DNA and siRNA. According to results, transfection efficiencies of nucleic acids-polymer complexes into HEK293 cells were higher than Lipofectamine 2000.

Moreover, Shen et al. (Shen et al., 2011) designed hydrophobized hyaluronic acid–spermine conjugates (HHSCs) in order to deliver siRNA. The polymers prevented siRNA from nuclease attacks and provided efficient release of siRNA. According to cytotoxicity analysis which was done with MTT assay, siRNA/HHSCs complex showed lower toxicity when compared with siRNA/PEI 25k and Lipofectamine 2000 complexes.

In the study of Wang et al. (Wang et al., 2012), DNA and actinomycin D (ACTD) were binded to spermine to analyze the effects of spermine on the DNA-acting properties of ACTD that were DNA-binding ability and the inhibition of DNA replication and transcription both *in vitro* and *in vivo*. The activity of actinomycin D on DNA was reduced by spermine. Decreasing intracellular amount of polyamines increased the obstruction of actinomycin D transcription, DNA replication and cell viability in cancer cell lines.

Spermine containing polymers polyspermine imidazole-4, 5-amide (PSIA) were synthesized and their nucleic acids delivery activity was investigated by Duan et al. (Duan et al., 2012). Plasmid DNA-polymer complexes were prepared at a weight ratio of 10:1. Plasmid DNA-polymer was found to be stable at pH 7.4. However, plasmid DNA released from the polymer when pH was decreased to 5.8 that is endosomal pH. The release was attributed to the degradation of amide linkages of polymer-DNA complexes. In addition, cytotoxicity and transfection activities of polymer-siRNA complexes were examined using COS-7 cells and HepG2 cells. As a result, PSIA-

siRNA complexes provided efficient cellular uptake and gene silencing with low cytotoxicity. It was suggested that polyspermine imidazole-4, 5-amide would be a promising non-toxic and non-viral nucleic acid carrier with high transfection efficiency.

According to above mentioned studies on polycations, polyethyleneimine is an efficient and commercially available nonviral intracellular drug delivery agent but it is not biodegradable and has toxic effects. Poly(l-lysine) and spermine have high nucleotide binding activity and non-toxic.

2.3. Other Amine-Containing Polymers in Intracellular Drug Delivery

In the literature, amine containing polymers have been synthesized via various methods. In a study of Iwai et al., poly(N,N-dimethylaminoethyl methacrylate) were prepared via photo-induced radical polymerization (Iwai et al., 2013). Transfection efficiency of these cationic polymers was evaluated using tris(hydroxymethyl)-aminomethane (Tris buffer) as a pH adjuster and plasmid DNA (pDNA), HeLa cells, smooth muscle cells, and cardiac fibroblasts. In addition, the effect of pH on transfection was observed. When Tris buffer/ poly(N,N-dimethylaminoethyl methacrylate) ratio was decreased to 2, gene transfection was increased in all the cell lines.

Amphiphilic random copolymers of 2-(dimethylaminoethyl)methacrylate with alkylacrylate (alkyl:hexyl, octyl, dodecyl) was prepared by Dutta et al. (Dutta et al., 2011). Hydrodynamic diameter increased with increasing alkyl chain length at high concentration polymer solutions. The antimicrobial activity of the copolymers was investigated with one Gram-positive bacterium Bacillus subtilis and one Gram-negative bacterium Escherichia coli. According to the results, 2-(dimethylaminoethyl) methacrylate-co-octylacrylate showed more effective antimicrobial activity. DNA was attached to copolymers electrostatically. However, hydrophobic alkyl chains affected the DNA binding process. Cytotoxicity experiments were done at high and low concentrations ($0.1 \leq [P] \leq 1$mg/ml) of copolymers. The copolymers at higher concentrations ([P]>1.2 mg/ml) was toxic to fibroblasts 3T3 cell line while they did not show toxic effect at lower concentrations. It was indicated that copolymers can be used

in pharmaceutical applications. However copolymers were incompatible with red blood cells.

Furthermore, ten different amine containing polymers were synthesized by Barua et al. (Barua S. et al., 2008) via ring opening polymerization of diglycidyl ethers as DNA carriers. As amine containing compound, aniline, butylamine, 1-(2-aminoethyl) piperidine, 4,7,10-trioxa-1,13-tridecanediamine, ethylenediamine, diethylenetriamine, pentaethylenehexaamine, N-2-aminoethyl-1,3-propanediamine and 1,4-bis (3-aminopropyl)piperazine were used. Polymers showed high DNA binding efficiency, significantly higher transfection activity and reduced cytotoxicity when compared to polyethyleneimine.

2.4. Amine Containing Polymers Synthesized via Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization

RAFT polymerization as a living radical polymerization, is one of the most suitable methods for synthesis of amine containing polymers which are used in intracellular drug delivery applications. This polymerization technique allows synthesis of polymers with complex architectures such as block, graft, comb, and star structures, controlled molecular weight, end group functionality and narrow molecular weight distribution (McCormick & Lowe, 2004).

In a study by Paslay et al. (Paslay et al., 2012), statistical copolymers of N-(3-aminopropyl) ethacrylamide (APMA) and N-[3-(dimethylamino) propyl] methacrylamide (DMAPMA) or N-[3-(diethylamino) propyl] methacrylamide (DEAPMA) were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. By changing compositions of monomers, the effect of cationic content (tertiary and primary amine contents) and hydrophobic group (DMAPMA and DEAPMA comonomers) distribution of protonated amines on selective cytotoxicity was investigated. The antimicrobial activity of the polymers was analyzed on E. coli and B. subtilis with respect to time, concentration; amine content buffer type and salt concentration. The antimicrobial activities of polymers were compared to peptides. Cytotoxicity of polymers was also evaluated using MCF-7 cells. Higher number of primary amines contained copolymers prevented bacterial growth completely. However

these copolymers did not affect the growth of eukaryotic cells. This result showed that the polymers were selective.

Low molecular weight (Mn<25kDa) (2-(dimethylamino)thyl methacrylate)-co-aminoethyl methacrylate (DMAEMA-co-AEMA) and (2-(dimethylamino)ethyl methacrylate)-co-aminohexyl methacrylate (DMAEMA-co-AHMA) were synthesized via RAFT polymerization (Zhu et al., 2010). The primary amine groups of aminoethyl methacrylate and aminohexyl methacrylate were protected using di-tert-butyl dicarbonate. After polymerization, polymers were deprotected via acid treatment. Amine content of the polymers was controlled by adjusting comonomer ratios. According to the results of dynamic light scattering and agarose gel electrophoresis experiments, binding activity of DNA to DMAEMA copolymers was higher than the homopolymer of DMAEMA. The amine content enhanced the buffering capacity of copolymers. The cytotoxicity of DMAEMA-co-AHMA-DNA and DMAEMA-co-AEMA-DNA complexes was low when amine/phosphate ratio was 3:1. Transfection efficiencies of copolymer-DNA complexes were determined to be higher than DMAEMA homopolymers. Copolymers revealed transfection efficiency similar to that of PEI (25kDa).

Alidedoglu et al. (Alidedeoglu et al., 2009) synthesized homopolymers of aminoethyl methacrylate (AEMA) and its copolymers with HPMA via aqueous RAFT polymerization by using 4-cyanopentanoic acid dithiobenzoate as a RAFT agent and 2,2'- azobis[2-(2-imidazolin-2-yl)propane dihydrochloride as an initiator. Polymers with controlled molecular weight and narrow molecular weight distribution were obtained.

Copolymers of 2-aminoethyl methacrylamide with N-isopropylacrylamide, 2-aminoethyl methacrylamide with 3-aminopropyl methacrylamide and 3-aminopropyl methacrylamide with methacryloyloxyethyl phosphorylcholine and homopolymers of 3-aminopropyl methacrylamide and 2-aminoethyl methacrylamide were synthesized via aqueous RAFT polymerization using 4-cyanopentanoic acid dithiobenzoate (CTP) as a chain transfer agent and 4,4-azobis(4-cyanovaleric acid) (ACVA) as an initiator at 70°C (Deng et al., 2008). The amino groups were not protected. Instead the hydrochloride salts of monomers were used in the polymerizations. Both homo- and co-polymers with controlled molecular weight and narrow molecular weight distribution were obtained.

Scales C.W. et al. (Scales C. W., 2006) synthesized block copolymers of hydroxypropyl) methacrylamide with *N*-[3-(dimethylamino)propyl] methacrylamide

using aqueous RAFT polymerization. According to gel electrophoresis results, copolymers could protect siRNA effectively against degradation and also slowly released siRNA.

Galactosylated N-2-hydroxypropyl methacrylamide block copolymers were prepared with guanidinopropyl methacrylamide used as gene transfection agent targeting hepatocytes (Qin et al., 2011). Block copolymers of N-2-hydroxypropyl methacrylamide with N-3-aminopropyl methacrylamide were synthesized via aqueous RAFT polymerization. Then copolymers were galactosylated and guanidinylated. The cytotoxicity of copolymers was lower than PEI. The copolymer-plasmid DNA complexes which contained 30% primary amine groups and a charge ratio of 12, induced higher transfection efficiency than PEI-pDNA complexes in HepG2 cells. The copolymers combined the advantages of galactose moieties, guanidine groups and HPMA component (Qin et al., 2011).

Well-defined homopolymers and block copolymers of *N*-(2-hydroxypropyl) methacrylamide (HPMA) with 3-guanidinopropyl methacrylamide (GPMA) were prepared via aqueous RAFT polymerization (Treat et al., 2012). 4-Cyano-4-(ethylsulfanylthiocarbonylsulfanyl) pentanoic acid was used as CTA in order to synthesize homopolymers of GPMA in pH 5.5 acetate buffer. According to kinetic analysis, the molecular weight of polymers increased linearly with conversion, indicating RAFT-controlled polymerization mechanism. In order to investigate the living properties of homopolymers of GPMA or macroCTA, block copolymers with HPMA were synthesized. The block copolymers had narrow molecular weight distribution (PDI $\leq$ 1.15). The copolymers showed potential for targeted drug delivery applications.

Copolymers and homopolymers of 3-bis(dimethylamino) propan-2-yl methacrylate, 1-(bis(3-(dimethylamino)propyl)amino) propan-2-yl methacrylate, and 2-((2-(2-(dimethylamino)ethoxy)ethyl)methylamino)ethyl acrylate were synthesized by Yu et al. (Yu & Lowe, 2009) RAFT polymerization in bulk. 1-methyl-1-cyanoethyl dithiobenzoate (CPDB) and AIBN were used as CTA and initiator, respectively. By examining kinetic and molecular weight profiles, controlled manner of polymerizations was demonstrated. According to results, all homopolymers became hydrophobic and phase separated at high pH.

Primary amine containing, well defined block copolymers were synthesized via aqueous RAFT polymerization in acetate buffer at pH 5.2 at 70°C for potential

intracellular siRNA delivery applications. As monomers, *N*-(2-hydroxypropyl) methacrylamide) (HPMA) and *N*-(3-aminopropyl) methacrylamide (APMA) were used. HPMA is a biocompatible and hydrophilic monomer. Amine group of APMA was used to functionalize the copolymers. In order to improve complexation between the copolymer and the negatively charged phosphate backbone of siRNA, poly(*N*-[3-(dimethylamino) propyl]methacrylamide) block polymer was copolymerized with HPMA-co-APMA. Negatively charged siRNA interacted with cationically charged copolymers. The overall charge of complex was found to be neutral. Folic acid was conjugated to copolymers. The delivery of siRNA and gene down-regulation were investigated in KB (folate receptor+) and A549 (folate receptor-) cell lines. Approximately 60% mRNA down-regulation was observed in the KB cell line, while no mRNA down-regulation occured in the A549 cell line. In conclusion, synthesized primary amine-containing copolymers are promising for preparing multivalent polymeric bioconjugates suitable for targeted drug/gene delivery (York et al., 2009).

2.5. Controlled/Living Radical Polymerization Techniques

Controlled/living radical polymerization techniques allow the synthesis of well-defined polymers with living properties. Nitroxide mediated polymerization (NMP), reversible addition-fragmentation chain transfer (RAFT) polymerization and atom transfer radical polymerization (ATRP) are the most recent and widely used controlled/living radical polymerization techniques. All these techniques are effective in the polymerization of a broad range of monomers and each of them gives narrow molecular weight distributions and good control over the molecular weight of the polymers (Konkolewicz D. et al., 2008).

Atom transfer radical polymerization, which was discovered in 1999 by Sawamoto et al., Jin-Shan Wang and Krzysztof Matyjaszewski, requires substituted monomers such as meth(acrylates), styrenes and meth(acrylamides), which are able to stabilize the propagating radicals, appropriate catalysts that contain transition metal compounds and ligands and initiators (Matyjaszewski et al., 2001). In addition, solvent is also very important for this technique because there can be side reactions involving the catalyst system in polar media. The presence of catalyst residues such as copper in

ATRP- synthesized polymers is the main disadvantages of ATRP technique especially for biomedical applications (Trapa P. E et al., 2002).

Nitroxide-mediated polymerization (NMP) is a distinctive technique due to metal free procedure when it is compared to atom transfer radical polymerization. In the NMP technique, bimolecular or unimolecular initiators (nitroxide and/or combination of a traditional free radical initiator with a nitroxide) are required to synthesize colorless and odourless polymers without any purification procedures (Sciannamea V. et al., 2008). However, there are some limitations of NMP technique that are availability and cost of initiators, limited monomer types and the need for high temperature for polymerization.

Although, NMP and ATRP have countless conveniences in polymerizations, it is argued that reversible addition fragmentation chain transfer (RAFT) polymerization is the most versatile technique especially for biomedical applications (Perrier S. et al., 2005; Moad G., 2006).

2.5.1. Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization

As one of the most widely-used controlled/living radical polymerization techniques, RAFT polymerization was first reported by the CSIRO group in 1998. The RAFT polymerization can be used to synthesize a wide range of polymer architectures such as linear, block, star and hyper branched polymers with narrow molecular weight distributions, controlled molecular weights and end-group functionality. RAFT-synthesized well defined, end-group functional polymers are useful building elements in biotechnology and biomedical applications such as intracellular drug delivery applications.

RAFT polymerization of various types of monomers can be performed in the presence of amenable chain transfer agents (RAFT agent) and azo-initiators. Figure 2.10 shows the generally accepted RAFT polymerization mechanism (Semsarilar & Perrier, 2010) . The RAFT agent includes reactive thiocarbonylthio, R and Z groups. The R group is responsible for initiation of the growth of polymeric chains while the Z group activates the thiocarbonyl bond toward radical addition and then stabilizes the resultant adduct radical in the ω-end of the polymer chain. In biomedical applications, living end

group of polymers (thiocarbonylthio) has potential toxicity. In order to overcome this limitation, post polymerization treatments have been easily performed to remove the thiocarbonylthio group from the polymer chain.

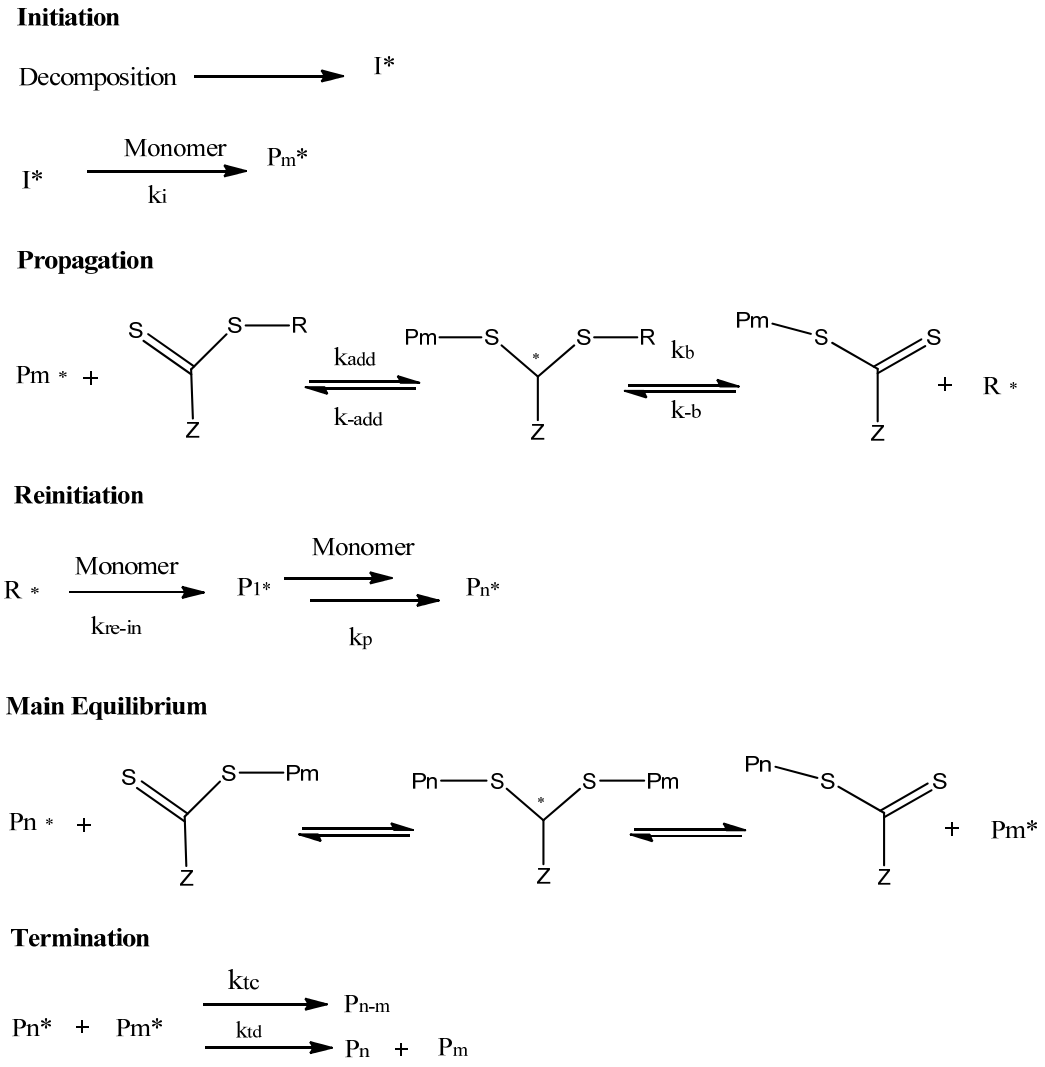


Figure 2.10. Generally accepted RAFT polymerization mechanism
(Source: Semsarilar & Perrier, 2010)

According to kinetics of RAFT polymerization, polymers are synthesized with controlled molecular weight which is determined via Equation 2.1 theoretically.

$$M_{n_{(theo)}} = \frac{[M_0]}{[RAFT_0]} x Mw_{monomer} x \text{ Conv } \% + Mw_{RAFT} \qquad (2.1)$$

2.5.1.1. Hydrolysis and Thioacylation/Aminolysis of RAFT agents

RAFT polymerizations in aqueous media and/or in the presence of amine compounds are relatively difficult to perform since water and amine containing compounds may induce undesirable side reactions with the RAFT agents and the living end-group of the RAFT synthesized polymers (Figure 2.11).

The hydrolysis of thiocarbonyl compounds occurs as these groups are unstable because of energy of the C=S functionality. In thioacylation, which has been used to modify proteins over the 50 years (Levesque et al., 2000), primary and secondary amines can react rapidly with dithioester when they are not protonated.

In the literature hydrolysis and thioacylation/aminolysis reaction of the RAFT agents have been investigated. In a study of Levensque et al. (Levesque et al., 2000), hydrolysis of two different dithioesters was investigated at pH 8.5, 8.0, and 7.5 at room temperature and 35°C. In addition thioacylation/aminolysis reactions of these dithioester were investigated in the presence of lysine. Hydrolysis and thioacylation reaction rates increased with respect to increase in pH and temperature (Bracher et al., 2011).

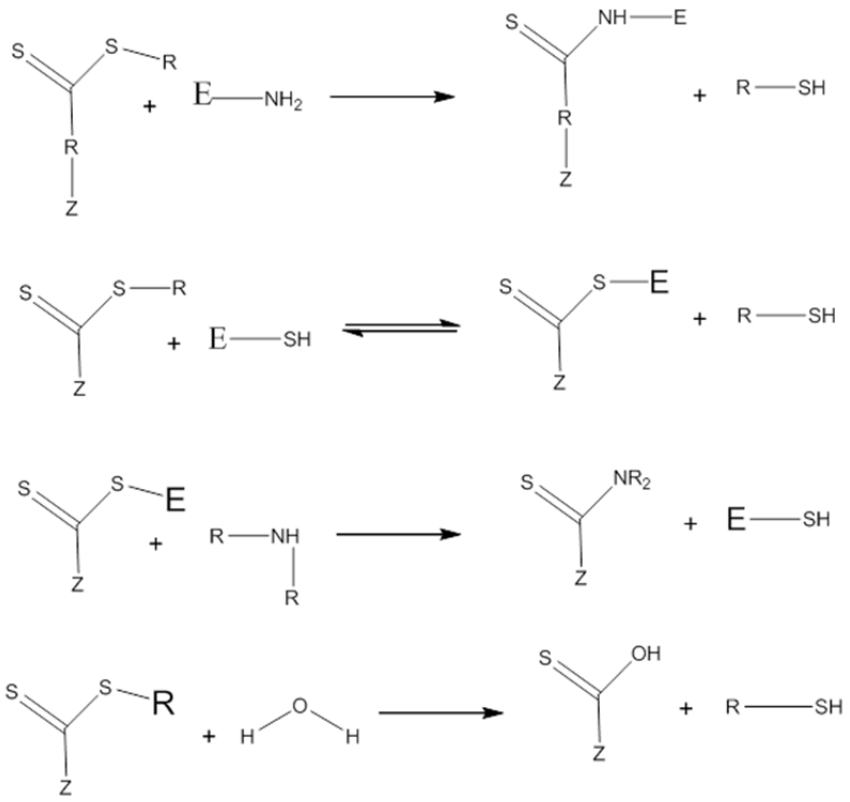


Figure 2.11. Hydrolysis and thioacylation/aminolysis of RAFT agent
(Source: Levesque et al., 2000)

In another study of Thomas et al. (Thomas D. B. et al., 2004), rate constants of hydrolysis and aminolysis reactions for a representative water-soluble chain transfer agent (CTA) cyanopentanoic acid dithiobenzoate (CTP) and the macro-chain-transfer agents (macro-CTAs) of poly(sodium 2-acrylamido-2-methylpropanesulfonate) (AMPSX) and poly(acrylamide) (AMX) were determined at selected pH values (Figure 2.12) (Deletre & Levesque, 1990).

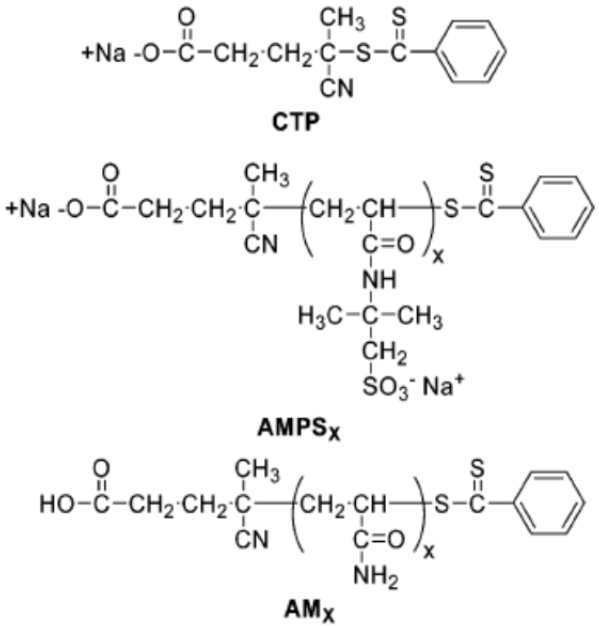


Figure 2.12**.** Dithioester compounds: 4-cyanopentanoic acid dithiobenzoate (CTP) and macro-CTAs of sodium 2-crylamido-2-methylpropanesulfonate (AMPSX) and acrylamide (AMX) (Source: Thomas D. B. et al., 2004)

According to the results, hydrolysis and aminolysis rates both increased with increasing pH and decreased with increasing molecular weight of the dithioester.

Moreover, thioamidation (aminolysis) kinetics were obtained using excess amount of mono- and bi functional amines between 30 and 50°C (Deletre & Levesque, 1990). According to the results, tertiary amine groups did not have an effect on thioamidation reaction rates while primary amines had a significant effect. In addition, reaction rates increased with temperature.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

N-Hydroxyethylethylenediamine (99% purity) and di-*tert*-butyl dicarbonate were purchased from Aldrich to use in the synthesis of *tert*-butyl (2-((tert-butoxycarbonyl) amino)ethyl)(2-hydroxyethyl)carbamate according to the procedure reported by Moura et al. (Moura et al., 2006). Methacryloyl chloride was purchased from Aldrich. Tert-butyl (2-((tert-butoxycarbonyl) amino)ethyl) (2-hydroxyethyl)carbamate was methacrylated according to the procedure reported previously (Burke & Pun, 2010). Chain transfer agent, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB), was purchased from Aldrich. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was used as an initiator after recrystallization twice in methanol. Silica gel (pore size 60 Å, 70-230 mesh) was purchased from Fluka. Acetic acid, sodium acetate, citric acid and mono and dibasic phosphate salts were used to prepare buffer solutions and purchased from Merck. Hydrochloric acid and sodium hydroxide was purchased from Merck and Sigma, respectively. Polyethyleneimine (Mn: 25 and 60 kDa) was purchased from Aldrich and Fluka, respectively.

Toluene, ethyl acetate, hexane, dichloromethane (DCM), trifluoroacetic acid deuterium oxide (D_2O), deuterium chloroform ($CDCl_3$), triethylamine (TEA), hexylamine, diethylether, methanol and N'N-dimethylacetamide (DMAc, HPCL grade ≥ %99.9) were purchased from Sigma. Dialysis membrane (M_{WCO}= 500-1000 Da) was purchased from Spectrum® Laboratories.

DMEM (Dulbecco's Modified Eagle's Medium) medium, L-glutamine, tyripsin and FBS (Foetal Bovine Serum) were obtained from Gibco. PBS (phosphate buffer saline solution, pH 7.1, 0.1 mM) was prepared using relevant mono and dibasic salts and NaCl. Thiazolyl Blue Tetrazolium Blue (MTT) reagent was bought from Sigma-Aldrich. Epitelial (NIH 3T3) cell line were donated by Bioengineering Research and Application Center, İzmir Institute of Technology, İzmir, Turkey.

3.2. Instruments

3.2.1. Nuclear Magnetic Resonance Spectroscopy

In this study, ^{1}H NMR spectroscopy (Varian, VNMRJ 400 spectrometer) was used to determine the chemical structure of compounds synthesized and the conversion of the monomers during polymerizations. For analysis, samples were dissolved at 10mg/ml in deuterium water (D_2O) and chloroform ($CDCl_3$) depending on the solubility of the samples.

3.2.2. Gel Permeation Chromatography

Gel permeation chromatography was used to determine the molecular weight and molecular weight distribution of polymers synthesized throughout the study. A Shimadzu modular system comprising an SIL-10AD auto injector, PSS Gram 30 Å and 100 Å (10 μM, 8x300 mm) columns, an RID-10A refractive-index detector and SPD-20A prominence UV/vis detector calibrated with low polydispersity poly(methyl methacrylate) standards (410-67000g/mol) was used. The mobile phase was N,N-Dimethylacetamide (Houssameddine et al.) containing 0,05 % w/v LiBr.

3.2.3. UV-Visible Spectrophotometry

UV-visible spectrophotometry was used to investigate hydrolysis and thioacylation of RAFT agent 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB). UV-visible light absorbance of the solution measured by a Thermo Scientific evolution 201 UV-visible spectrophotometer in the range between 200nm and 600nm using quartz cuvette. Reference cell was buffer solution at the desired pH (pH 7, pH 5 or pH 3).

3.2.4. Microplate Reader

In cytotoxicity analysis, a Thermo Electron Corporation Varioskan microplate reader was used to measure absorbance at 540 nm. In the study 96 well plate was used.

3.2.5. Column Chromatography Method

Synthesized monomer, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate, was purified by column chromatography method. Silica gel (pore size 60 Å, 70-230 mesh) was used as a stationary phase in preparation of the column. Hexane-ethyl acetate mixtures were used as mobile phase.

3.3. Methods

3.3.1. Synthesis of 2-((Tert-butoxycarbonyl)(2-((tert-butoxycarbonyl) amino)ethyl)amino)ethyl Methacrylate

The amine containing monomer was synthesized according to the procedure shown in Figure 3.1.

The primary and secondary amine groups of N-(2-hydroxyethyl) ethylenediamine was first protected using di-*tert*-butyl dicarbonate according to a study of Moura et al. (Moura et al., 2006). N-(2-Hydroxyethyl)ethylenediamine (0.024mol, 2.43ml, Mw:104.15g/mol) was dissolved in dry DCM (40ml) at -10°C. Di-*tert*-butyl dicarbonate (0.048mol, Mw: 218.25 g/mol) was dissolved in dry DCM (40ml). Di-*tert*-butyl dicarbonate solution was added dropwise into N-(2-hydroxyethyl)ethylenediamine solution at -10°C. The final solution was purged with nitrogen for about 3 hours and stirred for a total of 24h at room temperature. After reaction, tert-butyl hydrogen carbonate formed during the reaction and appeared as a precipitate in DCM was separated by filtration. In order to remove the unreacted N-hydroxyethylethylenediamine, water-DCM extraction was performed 3 times. The organic phases were collected and the solvent was removed using a rotary evaporator.

The final product was dissolved in $CDCl_3$ and then characterized by ^{1}H-NMR. The yield percent was calculated by Equation 3.1 using ^{1}H-NMR spectra of reaction mixture in Appendix A.1.

^{1}H NMR ($CDCl_3$, δ in ppm): 3.71 (t, 2H, -CH_2-**CH_2**-OH), 3.33-3.20 (t, 6H, OH-CH_2-**CH_2**-N($COO(CH_3)_3$-**CH_2**-**CH_2**-NH($COO(CH_3)_3$), 1.49-1.39 (s, 18H -N(COO-**$(CH_3)_3$**)-CH_2-CH_2-NH-(COO**$(CH_3)_3$**), 5.14-4.97 (s, 1H, -CH_2-**NH**-($COO(CH_3)_3$).

$$\text{Yield } \% = \frac{I_{1H\ @3.74ppm}}{I_{1H\ @2.35ppm} + I_{1H\ @3.74}} \tag{3.1}$$

Tert-butyl-2-((tert-butoxycarbonyl) amino) ethyl) (2-hydroxyethyl) carbamate (**1**, BocAEAE) (0.0161mol, Mw: 304.38 g/mol) was dissolved in dry dichloromethane (40ml) at 0°C. Triethylamine (0.0432mol) was dropped into the solution under N_2. The solution was stirred for 30min. Finally, methacryloyl chloride (0.0288mol) was added drop wise to the solution. The final solution, which containing 1/2.7/1.8 molar ratio of [BocAEAE]/[TEA]/[MACl], was stirred at 0°C for 4h under N_2 and for 15h at room temperature.

After reaction, it was observed that HCl salt was formed as a side product. There was no distinct colour change, the solution was light yellow. In order to remove HCl salt, the solution was filtered. Dissolved salt, unreacted methacryloyl chloride and triethylamine were separated by washing reaction solution with brine (three times) and extracting with water three times. Dichloromethane phases were collected and the solvent was evaporated using a rotary evaporator. The final product was further dried in vacuum oven. The product was then purified by column choromatography method using hexane and ethylacetate solutions (Hxn:EA=1:0; 10:1; 8:1; 6:1; 4:1; 2:1; 0:1). High purity monomer was collected using a hexane and ethyl acetate mixture (4:1, v/v).

The product, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino)ethyl) amino)ethyl methacrylate (**2**, Figure 3.1), was obtained as a yellow oil and characterized by ^{1}H NMR spectroscopy using deuterated chloroform ($CDCl_3$) as a solvent. The percent yield of reaction was calculated by Equation 3.2 using ^{1}H-NMR spectra of impure reaction mixture in Appendix A.3.

^{1}H NMR ($CDCl_3$, δ in ppm): 6.11-5.58 (s, 2H, **CH_2**=$C(CH_3)$COO-), 1.94 (s, 3H, CH_2=C-**CH_3**), 4.25-4.23 (t, 2H, -COO-**CH_2**-), 3.50-3.27 (t, 6H, -**CH_2**-N($COO(CH_3)_3$-

$\underline{CH_2}$-$\underline{CH_2}$-NH($COO(CH_3)_3$), 1.45-1.42 (s, 18H -N(COO-**$\underline{(CH_3)_3}$**)-CH_2-CH_2-NH-(COO**$\underline{(CH_3)_3}$**), 4.99-4.79 (s, 1H, -CH_2-**$\underline{NH}$**-($COO(CH_3)_3$).

$$\text{Yield } \% = \frac{\frac{I_{4.22ppm}}{2}}{\frac{I_{3.78ppm} + I_{4.22ppm}}{2}} x100 \qquad (3.2)$$

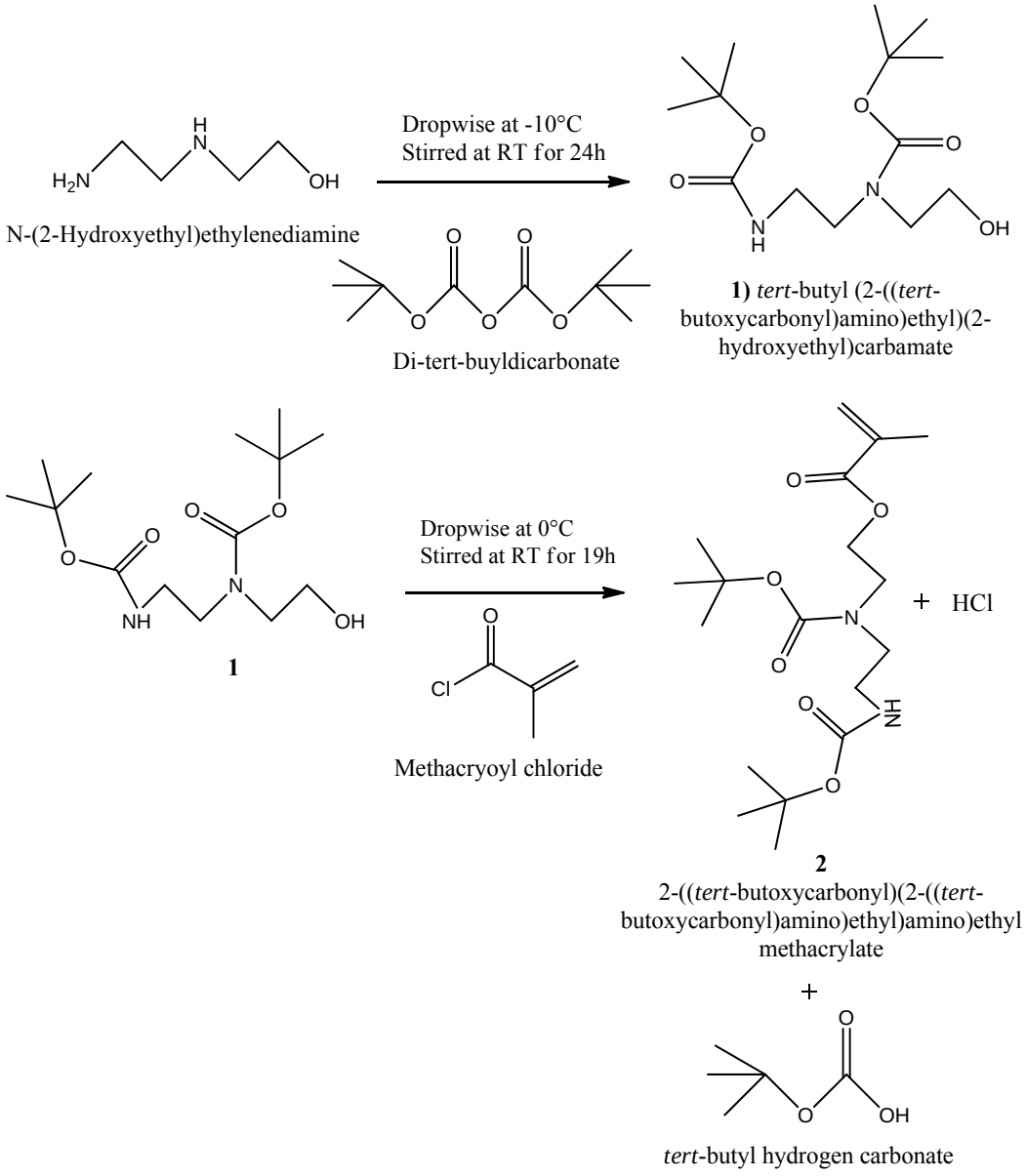


Figure 3.1. Synthesis of 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino)ethyl) amino)ethyl methacrylate

3.3.2. RAFT Polymerization of 2-((Tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl Methacrylate

The polymerization scheme and conditions are given in Figure 3.2 and Table 3.1, respectively. Briefly, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl) amino) ethyl methacrylate (**2**), CPADB and AIBN were dissolved in toluene. The solution was purged with N_2 for 15min. The reaction solution was then immersed in an oil bath at 65°C. At the end of the reaction time, polymerization was stopped by cooling the solution in an ice bath and exposing the solution to air. The solvent was removed in vacuum. Monomer conversions were determined before purification, by analyzing the polymerization mixture via ^{1}H-NMR spectroscopy using $CDCl_3$ as solvent. Monomer conversions were calculated from ^{1}H-NMR spectra using Equation 3.3.

Polymers were purified by precipitating the polymerization mixture in hexane (approximately 9 times). The number average molecular weight and molecular weight distribution were determined by GPC using dimethylacetamide DMAc as a mobile phase. Molecular weights were also calculated from ^{1}H-NMR spectrum of purified polymers using Equation 3.4 in which molecular weight of monomer, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl) amino) ethyl methacrylate, is 372.46 g/mol and molecular weight of RAFT agent is 279.38 g/mol.

^{1}H NMR ($CDCl_3$, δ in ppm): 1.76 (s, 2H, -**$\underline{CH_2}$**-$C(CH_3)COO$-), 0.89-0.73 (s, 3H, -CH_2-C**$\underline{(CH_3)}$**COO-)), 4.01 (t, 2H, -COO-**$\underline{CH_2}$**-), 3.50-3.27 (t, 6H, -**$\underline{CH_2}$**-$N(COO(CH_3)_3$-**$\underline{CH_2}$**-**$\underline{CH_2}$**-$NH(COO(CH_3)_3)$, 1.45-1.42 (s, 18H -$N(COO$-**$\underline{(CH_3)_3}$**$)$-CH_2-CH_2-NH-(COO**$\underline{(CH_3)_3}$**), 4.98-4.77 (s, 1H, -CH_2-**$\underline{NH}$**-$(COO(CH_3)_3)$.

$$\text{Monomer Conv \%} = \frac{\frac{\left(I_{1H\text{ at }6.11ppm} + I_{1H\text{ at }5.58ppm}\right)}{2}}{\frac{\left(I_{2H\text{ at }4.24ppm} + I_{2H\text{ at }4.01ppm}\right)}{2}} \text{ x100} \qquad (3.3)$$

$$Mn_{NMR} = \frac{\frac{I_{@4.01ppm}}{2}}{\frac{I_{@7.73ppm} + I_{@7.52ppm} + I_{@7.35ppm}}{5}} \text{ x } Mw_{monomer} + Mw_{RAFT} \qquad (3.4)$$

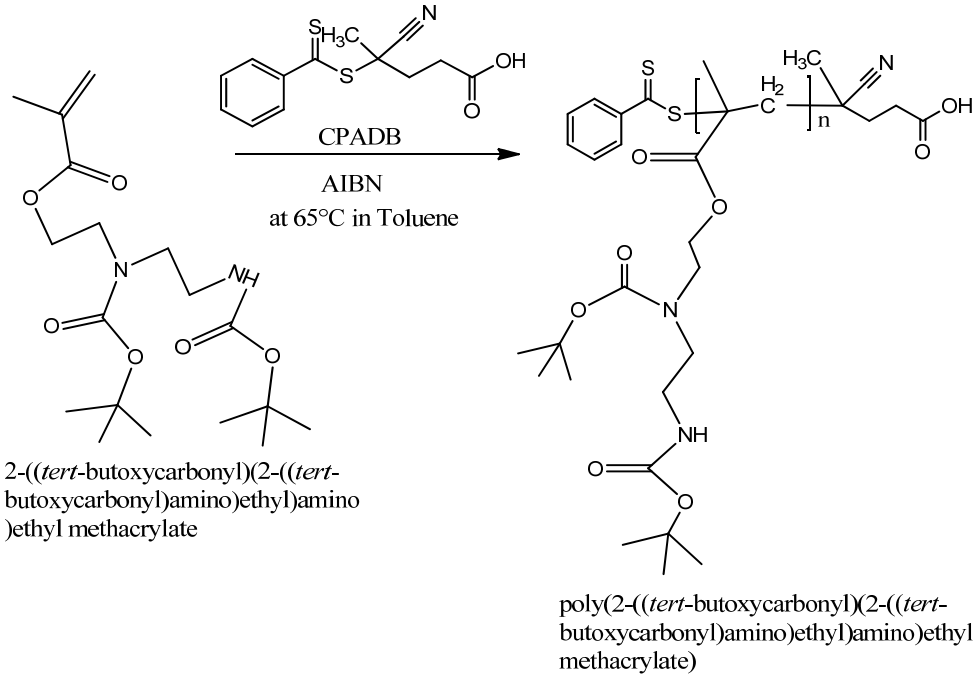


Figure 3.2. Synthesis of poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) (P(BocAEAEMA))

Table 3.1. Polymerization conditions. 2-((tert-butoxycarbonyl)(2-((tert-butoxycarbonyl) amino)ethyl)amino)ethyl methacrylate monomer (BocAEAEMA), initiator (AIBN) and RAFT agent (4-cyano-4-(phenylcarbonothioylthio)pentanoic acid)

Time (h)	[BocAEAEMA] (mol/l)	[CPDBA]x10^2 (mol/l)	[AIBN]x10^3 (mol/l)	$[BocAEAEMA]_0/[CPDBA]_0/[AIBN]_0$
2	0.36	1.44	3.6	25/1/0.25
3	0.36	1.44	3.6	25/1/0.25
4	0.36	1.44	3.6	25/1/0.25
5	0.36	1.44	3.6	25/1/0.25
6	0.36	1.44	3.6	25/1/0.25
8	0.36	1.44	3.6	25/1/0.25
10	0.36	1.44	3.6	25/1/0.25
3	0.36	0.36	0.9	100/1/0.25
4	0.36	0.36	0.9	100/1/0.25
5	0.36	0.36	0.9	100/1/0.25
6	0.36	0.36	0.9	100/1/0.25
8	0.36	0.36	0.9	100/1/0.25
10	0.36	0.36	0.9	100/1/0.25
1	0.72	2.9	7.3	25/1/0.25
2	0.72	2.9	7.3	25/1/0.25
3	0.72	2.9	7.3	25/1/0.25
4	0.72	2.9	7.3	25/1/0.25
5	0.72	2.9	7.3	25/1/0.25
6	0.72	2.9	7.3	25/1/0.25
8	0.72	2.9	7.3	25/1/0.25
10	0.72	2.9	7.3	25/1/0.25
1	0.72	1.44	3.6	50/1/0.25
2	0.72	1.44	3.6	50/1/0.25
3	0.72	1.44	3.6	50/1/0.25
4	0.72	1.44	3.6	50/1/0.25
5	0.72	1.44	3.6	50/1/0.25
1	0.72	0.72	1.8	100/1/0.25
2	0.72	0.72	1.8	100/1/0.25
3	0.72	0.72	1.8	100/1/0.25
4	0.72	0.72	1.8	100/1/0.25
5	0.72	0.72	1.8	100/1/0.25
2	1.44	1.44	3.6	100/1/0.25
3	1.44	1.44	3.6	100/1/0.25
4	1.44	1.44	3.6	100/1/0.25
5	1.44	1.44	3.6	100/1/0.25
6	1.44	1.44	3.6	100/1/0.25
8	1.44	1.44	3.6	100/1/0.25
10	1.44	1.44	3.6	100/1/0.25

3.3.3. Hydrolysis and Aminolysis of 4-Cyano-4-(phenylcarbonothioyl thio) Pentanoic Acid (RAFT agent)

P(AEAEMA) was also intended to be synthesized via aqueous RAFT polymerization of deprotected monomer, 2-((2-aminoethyl)amino)ethyl methacrylate since aqueous RAFT polymerization of amine-containing monomers provides a straightforward route to amine-containing well-defined polymers (Alidedeoglu et al., 2009; McCormick & Lowe, 2004). Before performing aqueous RAFT polymerization of 2-((2-aminoethyl) amino)ethyl methacrylate monomer, a series of experiments was first performed in order to determine the conditions at which the RAFT agent, which is susceptible to aminolysis and hydrolysis, would be stable.

To investigate the hydrolytic susceptibility the RAFT agent, i.e. 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB), was dissolved in dimethylformamide (0.016 M) and phosphate, acetate or citrate buffer solutions at varying pH (pH 7, pH 5 or pH 3) (4.95 ml) was added to the solution. The final solution (3.4 $x10^{-3}$ M) was mixed and put into an oil bath at 65°C. At predetermined time points, an aliquot of 25 µl was taken and diluted to 1000 µl with the relevant buffer solution. The UV-visible light absorbance of the solution was immediately measured in the range between 200 nm and 600 nm. The reduction in the absorbance of the solution at 302-305 nm which is the characteristic wavelength of the RAFT agent was recorded for 24 h.

To examine the thioacylation in the presence of amines, the RAFT agent was incubated with hydroxyethylethylenediamine (the starting compound of monomer synthesis) at pH 5.0 or 3.0. Briefly, hydroxyethylethylenediamine aqueous solution was first neutralized using HCl (5 M) and the solution (3 M) was then added into the buffer solution at the desired pH (pH 5.0 or pH 3.0). The RAFT agent solution in dimethylformamide was added into hydroxyethylethylenediamine solution. In final solution hydroxyethylethylenediamine concentration was 0.17 M and RAFT agent concentration was 3.4 $x10^{-3}$ M. The procedure for hydrolysis reactions was repeated.

The percent degradation of the RAFT agent as a result of hydrolysis or aminolysis was calculated using Equation 3.5 in which A_0 and A_t are the absorbance values at t=0 and t reaction time.

$$\text{Degradation } \% = \frac{(A_0 - A_t)}{A_0} x100 \quad (3.5)$$

3.3.4. Deprotection of Poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) (P(BocAEAEMA))

Three different polymers (p(AEAEMA)), having different molecular weights (Mn_{GPC}: 11.2 kDa, 11.5 kDa and 13.5 kDa) were deprotected in order to investigate their proton sponge and *in vitro* cytotoxicity. For deprotection of amino groups (removal of Boc groups) (Figure 3.3), polymer (4.35 μmol) was dissolved in DCM (1 ml) and trifluoroacetic acid (0.5 ml) was added drop wise into the solution at 0°C. The final solution was stirred for 30 min at room temperature. After reaction, solvent was removed by purging N_2 at room temperature. The reaction mixture was then washed with diethyl ether and chloroform more than three times and finally the sample was dried in a vacuum oven. Finally, the deprotected polymer, poly(2-(amino ethyl) amino) ethyl methacrylate, p(AEAEMA), was characterized by ^{1}H-NMR spectroscopy after dissolving in D_2O as a solvent. Yield percent of the deprotection reaction was calculated using Equation 3.6.

^{1}H NMR (D_2O, δ in ppm): 1.76 (s, 2H, -**CH$_2$**-C(CH_3)COO-), 0.89-0.73 (s, 3H, -CH_2-C**(CH$_3$)**COO-)), 4.15 (t, 2H, -COO-**CH$_2$**-), 3.41-3.3 (t, 6H, -**CH$_2$**-NH-**CH$_2$**-**CH$_2$**-NH_2).

$$\text{Deprotection yield } \% = \frac{\frac{I_{4.15ppm}}{2H}}{\frac{I_{1.48ppm}}{18H} + \frac{I_{4.15ppm}}{2H}} x\ 100 \quad (3.6)$$

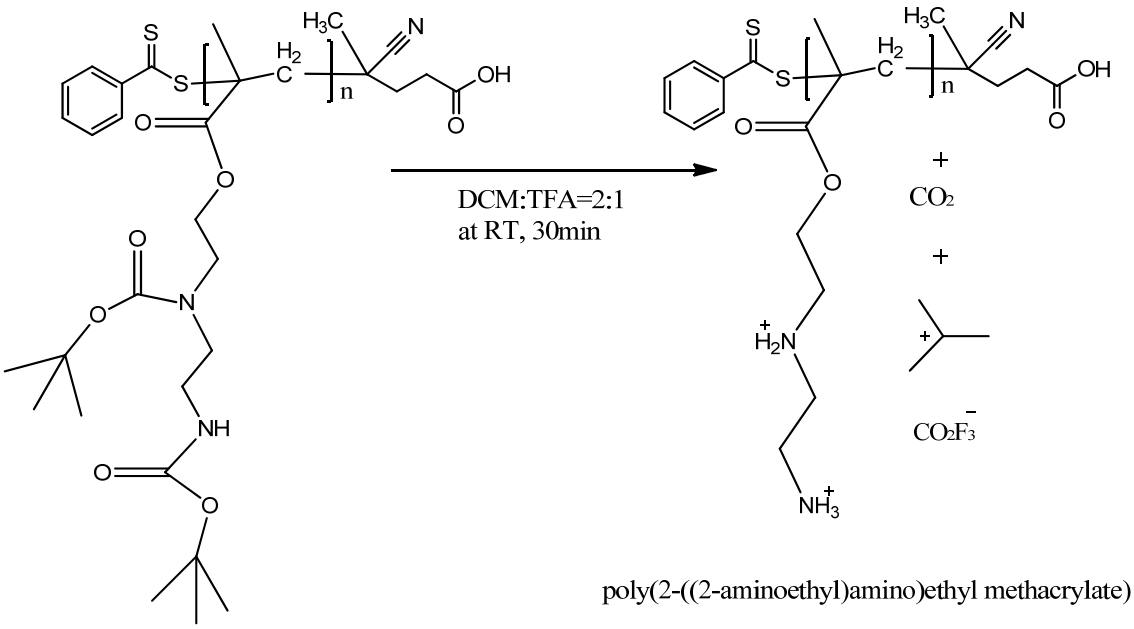


Figure 3.3. Deprotection of poly(2-((tert-butoxycarbonyl)(2-((tert-butoxycarbonyl) amino)ethyl) amino)ethyl methacrylate) to yield poly(2-(amino ethyl) amino) ethyl methacrylate), p(AEAEMA)

3.3.5. Determination of Proton Sponge Capacity of P(2-((2-aminoethyl)amino)ethyl methacrylate) (P(AEAEMA))

Proton sponge capacity of deprotected polymers ($Mn_{theo/NMR}$: 5.5 kDa and 8 kDa, Table B.1) was determined in comparison with a well-known polycation used in intracellular drug delivery, i.e. polyethyleneimine (branched, Mn: 25 kDa and 60 kDa). A protocol reported previously (Benns et al., 2002) was followed for determination of proton sponge capacity of amine-containing polymers. In these experiments, either the molar concentration of polymers or the molar concentration of the repeating units in polymers were kept constant to be able to compare the proton sponge capacity of different types of polymers tested (i.e. p(AEAEMA) having a molecular weight of: 5.5 kDa and 8 kDa based on NMR and PEI having a molecular weight of 25 and 60 kDa). Firstly, aqueous solutions of polymers ([P(AEAEMA)]: $2.2x10^{-5}$ M, $[P(AEAEMA)_{repeating\ unit}]$: $2.9x10^{-5}$ M, in 10 ml) were prepared and the pH of the solutions was adjusted to pH 12 by adding NaOH (40%, w/v). The final solution was

then titrated by addition of HCl solution (0.1 M) until the pH of the solution decreased to 2. The pH of solutions was monitored using a Hanna pH lab meter.

3.3.6. Determination of *In Vitro* Cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay was used to observe the effects of p(AEAEMA) ($Mn_{theo/NMR}$: 5.5 kDa, PDI: 1.35 and $Mn_{theo/NMR}$: 8 kDa, PDI:1.41) on viability of mouse fibroblast NIH 3T3 cell line. The MTT assay measures the ability of the living cells to reduce a tetrazolium dye to its insoluble formazan giving a purple colour (Mosmann, 1983). The cytotoxic effect of PEI (branched, 25 kDa and 60 kDa) was also determined in parallel to p(AEAEMA) for comparison purpose.

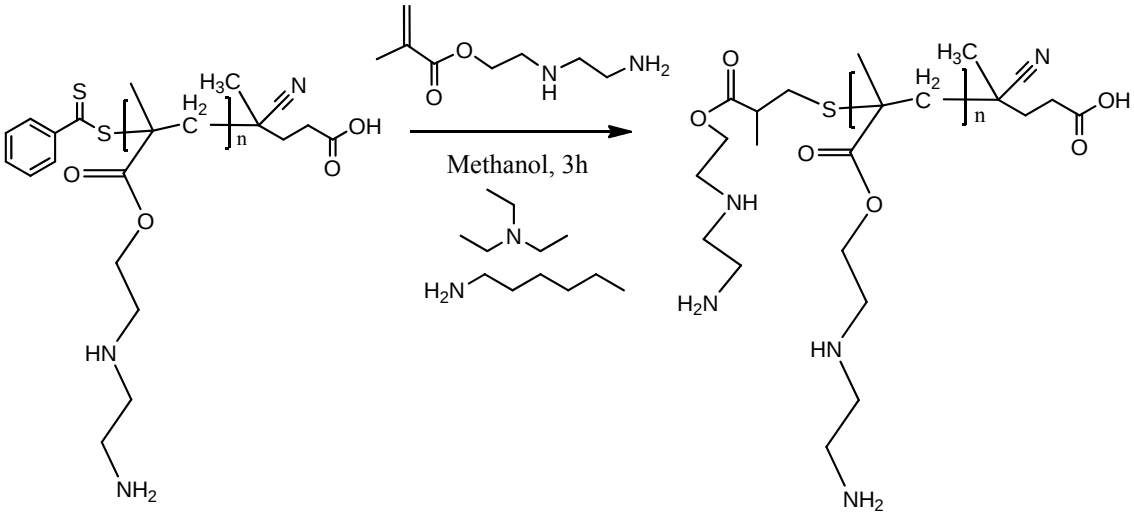
Prior to cytotoxicity assays, the thiocarbonylthio RAFT-end group of p(AEAEMA) was removed from the polymers in order to avoid from possible cytotoxicity of these active groups (Pissuwan et al., 2010). To remove the RAFT-end group, protocols well-known in literature have been performed (Boyer et al., 2009). Briefly, p(AEAEMA) dissolved in methanol was reacted with 2-((2-aminoethyl)amino)ethyl methacrylate monomer (AEAEMA) in the presence of hexylamine (HEA) and triethylamine (TEA) for 3 hours under N_2 atmosphere at room temperature (Figure 3.4). The reaction conditions are given in Table 3.2.

After reaction, the solvent was removed in vacuum. The polymer was then purified by precipitating in diethyl ether. After purification, the polymer was dried under vacuum. The product was characterized by ^{1}H-NMR spectroscopy after dissolving in D_2O. The yield of end-group modification was calculated by Equation 3.7.

$$\text{Yield } \% = 1 - \left(\frac{\left(\frac{I_{7.7ppm-7.32ppm}}{5H}\right)_{\text{after rxn}}}{\left(\frac{I_{7.77ppm-7.32ppm}}{5H}\right)_{\text{before rxn}}} \text{x} \frac{\left(\frac{I_{4.17ppm}}{2H}\right)_{\text{before rxn}}}{\left(\frac{I_{3.95ppm}}{2H}\right)_{\text{after rxn}}} \right) \text{x100} \quad (3.7)$$

Table 3.2. Reaction condition for aminolysis of poly(2-((2-aminoethyl)amino)ethyl methacrylate) before cytotoxicity assay

Polymer	Polymer (mol/l)	HEA (mol/l)	TEA (mol/l)	Monomer (mol/l)	Solvent volume (µl)
5.5 kDa	0.004	0.04	0.04	0.012	663
8 kDa	0.004	0.04	0.04	0.012	988



poly(2-((2-aminoethyl)amino)ethyl methacrylate)

poly(2-((2-aminoethyl)amino)ethyl methacrylate)

Figure 3.4. RAFT end-group aminolysis of poly(2-((2-aminoethyl) amino)ethyl methacrylate)

After removing the thiocarbonylthio RAFT-end group of p(AEAEMA), polymers were purified by dialysis against water using a membrane with a MW cut off 500-1000 kDa for three days. They were then dried using a freeze-dryer. The final product was characterized using ^{1}H-NMR spectroscopy after dissolving in D_2O.

In cytotoxicity analysis, NIH 3T3 cells were seeded a day before to polymer sample exposure at 10,000 cells per well (96 well plate) in culture medium containing 10% FBS/DMEM. Polymer sample stocks were prepared in PBS solution and 5 µl of polymer solution was added at predetermined concentrations to cells. The cells were incubated at 37 °C/5% CO_2 for 24 or 72 h. The culture medium was removed from the

wells after the incubation period. The solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye was prepared with culture medium (10 % v/v). MTT solution (100 µl) was added to wells according to manufacturer's protocol. The plates were incubated at 37 °C for another 4 h and metabolic activity was detected by spectrophotometric analysis. The absorbance of the solutions was recorded at 540 nm. The cell viability (%) was calculated relative to the positive control (cells not treated with polymers) according to Equation 3.8 in which $A_{cell+sample}$ is the absorbance of polymer treated cells and $A_{positive\ control}$ is the absorbance of untreated cells.

$$\text{Cell Viability (\%)} = \frac{A_{cell+sample}}{A_{positive\ control}} x100 \quad (3.8)$$

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Synthesis of 2-((Tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl Methacrylate

In this study, a new, spermine-like amine containing monomer, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate was first synthesized. The synthesis procedure was composed of two steps. The first step was to protect the primary and secondary amine groups of N-hydroxyethylethylenediamine in order to prevent any possible unwanted side reactions such as aminolysis of the RAFT agent during RAFT polymerization of amine containing monomer. The amine groups were protected using di-*tert*-butyl dicarbonate (Boc-) based on a method reported by Moura et al. (Moura et al., 2006). This amine-protection group (Boc-) is widely used in peptide synthesis (Wakselman, 2004). ^{1}H-NMR spectra of N-hydroxyethylethylenediamine before and after protection are shown in Figure 4.1. The characteristic chemical shift of amine groups in N-hydroxyethylethylenediamine was at 2.20 ppm.

After protection of the amine groups with di-*tert*-butyl dicarbonate (Boc anhydride) the amine group chemical shift that appears at 2.2 ppm disappeared almost completely and the characteristic proton signals of Boc group appeared clearly between 1.39 and 1.49 ppm. However, according to the integration of the Boc group protons, there was unreacted excess protection (Boc-) group. From the NMR spectrum of reaction solution before purification in Appendix A.1, the yield of this reaction was determined to be 67% calculated according to Equation 3.1. In order to separate unreacted N-hydroxyethylethylenediamine, water-DCM extraction was done three times (Appendix A.2).

Without any attempt to further purify, this product was reacted with methacryloyl chloride to prepare the final vinyl monomer suitable for free radical polymerization. Tert-butyl (2-((tert-butoxycarbonyl) amino)ethyl) (2-hydroxyethyl) carbamate was methacrylated using a method reported by Burke and Pun (Burke & Pun,

2010). The reaction mixture was characterized by ^{1}H-NMR spectroscopy (Appendix A.3). The yield of reaction was calculated to be 84% by Equation 3.2. The final product (~100% pure) obtained after column chromatography (R_f: 0.19±0.005), 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate was also characterized by ^{1}H-NMR spectroscopy (Figure 4.2). The characteristic signals of vinyl protons, -C**H$_3$** was observed at 1.94 ppm as 3H **(f)** and -C**H$_2$** was obtained at 6.11 ppm (1H) **(d)** and 5.58 ppm (1H) **(e)**. Upon methacrylation the signal of protons of -CH_2- group (-CH_2-**CH_2**-O-COR) which was located at 3.71 ppm (Figure 4.1, **b**) shifted to 4.25-4.23 ppm (Figure 4.2, **b**).

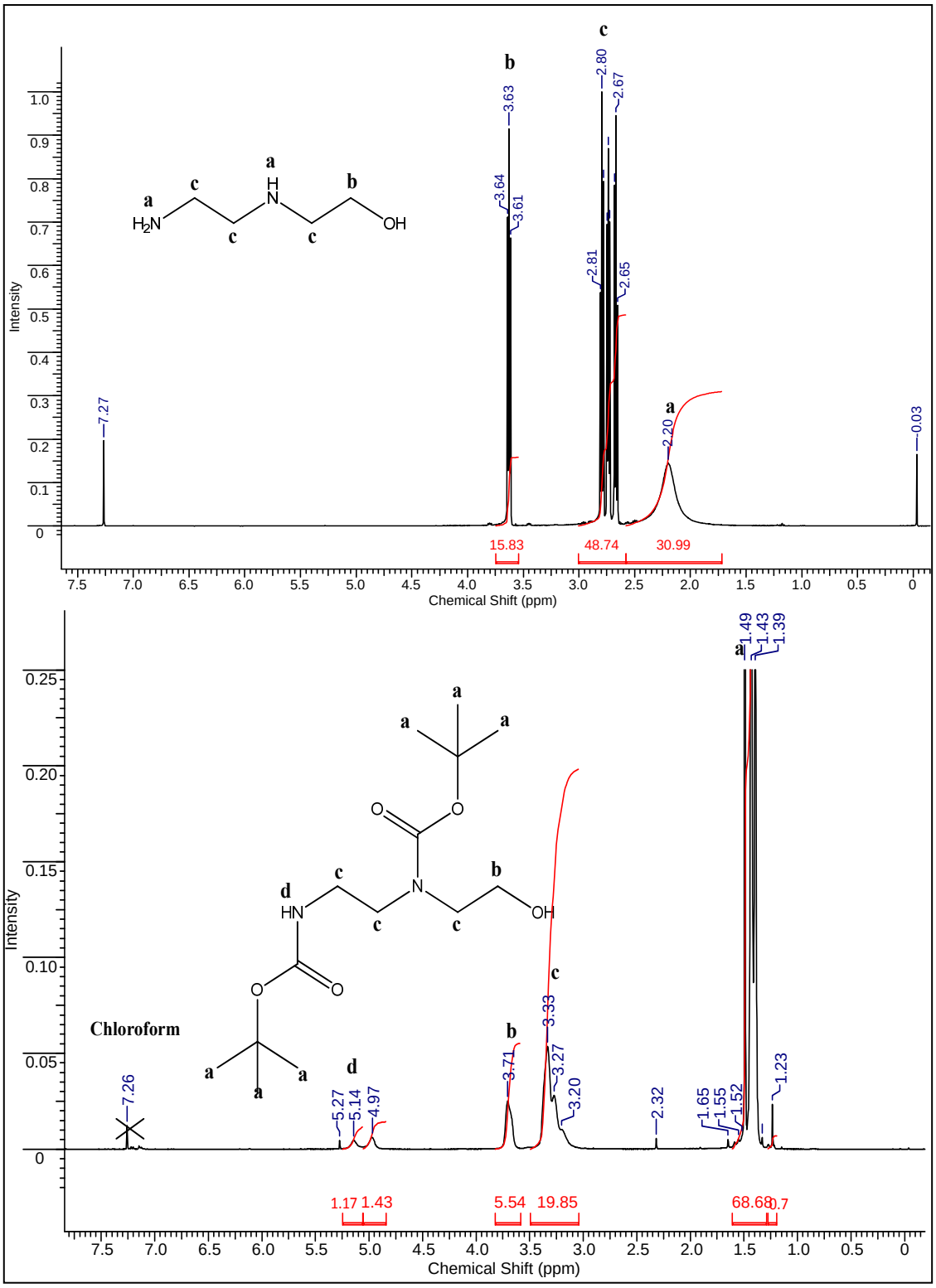


Figure 4.1. [1]H-NMR spectra of N-hydroxyethylethylenediamine before and after protection.

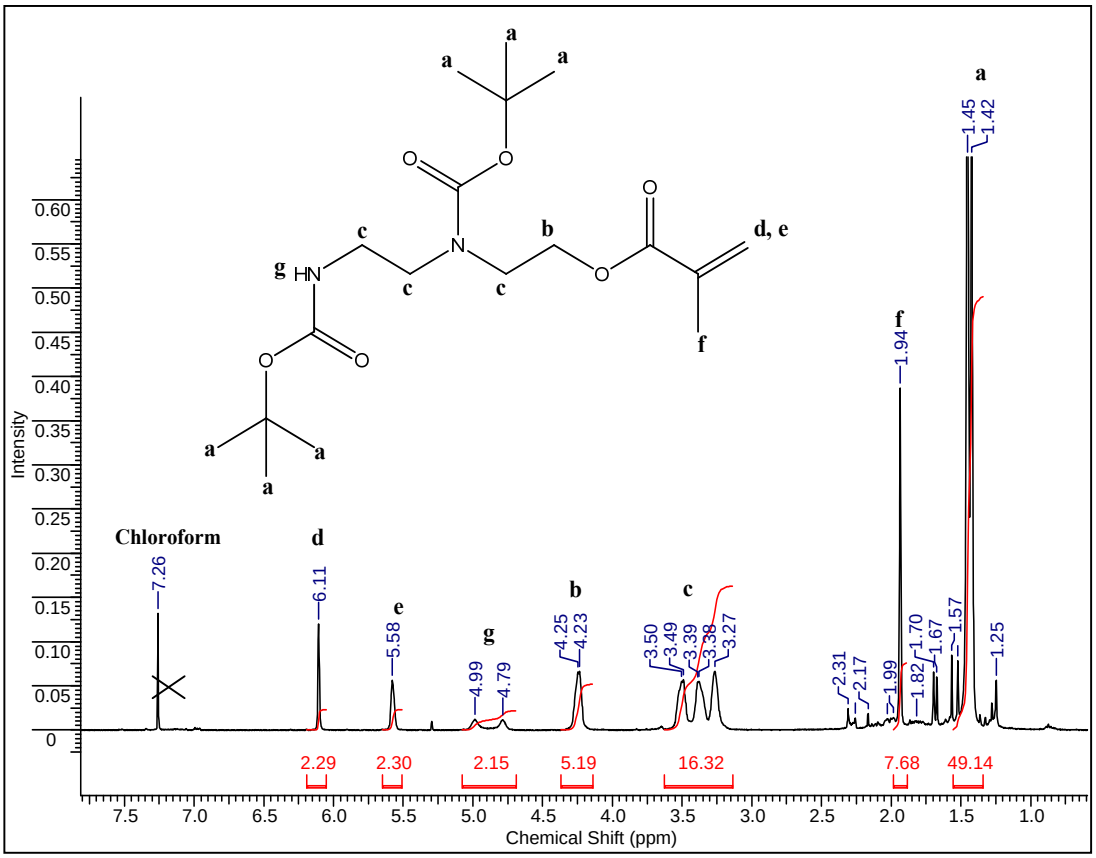


Figure 4.2. [1]H-NMR spectrum of pure 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate obtained after column chromatography

4.2. RAFT Polymerization of 2-((Tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl Methacrylate

2-((Tert-butoxycarbonyl) (2-((tert- butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate was polymerized via RAFT polymerization using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid as a RAFT agent. CPADB has been widely used as a RAFT agent in controlling the polymerization of methacrylates (Barner-Kowollik et al., 2003). The RAFT polymerization of Boc-AEAEMA was performed in toluene as an organic solvent. A well-known azo initiator, AIBN was used as the polymerization initiator. RAFT polymerization kinetics were investigated at three different [Monomer]/[RAFT agent] ratios ([M]/[R]: 25/1, 50/1 and 100/1) and three different monomer concentrations (0.36 M, 0.72 M and 1.44 M). In all polymerizations the ratio of the RAFT agent concentration to the initiator concentration ([R]/[I]) was

kept constant at 1/0.25. Through different sets of polymerizations, two basic effects on the kinetics of RAFT polymerization were investigated: (1) the effects of [M]/[R] ratio and initiator concentration at constant monomer concentration, and (2) the effects of [M]/[R] ratio and monomer concentration at constant initiator concentration. Number average molecular weights and polydispersity indexes of synthesized polymers were determined by gel permeation chromatography (Appendix B.3, B.4 and B.5). Monomer conversion was calculated from ^{1}H-NMR spectra of polymerization mixtures (Appendix B.1) using Equation 3.3. Theoretical molecular weights and number average molecular weights of polymer were also determined using Equation 2.1. and Equation 3.4 in which molecular weight of monomer (Mw) is 372.46 g/mol and molecular weight of RAFT agent is 279.28 g/mol, respectively (Appendix B.2).

Table 4.1 lists the monomer conversions and the number average molecular weights (Mn's) and molecular weight distributions (PDI's) of p(Boc-AEAEMA) synthesized at a fixed monomer concentration of 0.36 M and varying ratios of [M]/[R]/[I] (25/1/0.25 and 100/1/0.25). Here it should be noted that while the monomer concentration was constant, the RAFT agent and also initiator concentrations were accordingly changed in order to vary the [M]/[R] ratio. When the [M]/[R]/[I] ratio was 25/1/0.25 at a monomer concentration of 0.36 M, the RAFT agent and initiator concentrations were 1.44×10^{-2} M and 3.6×10^{-3} M, respectively, while at a 100/1/0.25 ratio, they were 0.36×10^{-2} M and 0.9×10^{-3} M, respectively.

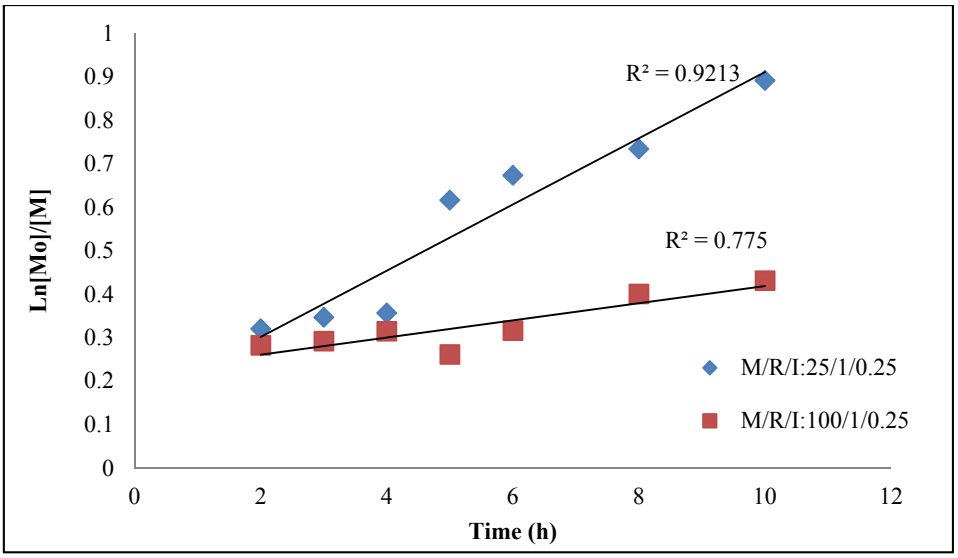
According to the results presented in Table 4.1, the increase in [M]/[R] ratio at a fixed monomer concentration and [R]/[I] ratio, led to an increase in the Mn's and a decrease in monomer conversions, as expected. At a fixed monomer concentration and [R]/[I] ratio (of 4), the increase in [M]/[R] results in a decrease in the amount of initiator used in polymerizations, which leads to the decrease in monomer conversions, in accord with literature (Wang & Zhu, 2003).The increase in Mn with the increase in the ratio of [M]/[R] was a good indication of the RAFT-mechanism controlled polymerization, in accord with Equation 2.1. A gradual increase in monomer conversions with polymerization time was observed at low [M]/[R] ratio, while the monomer conversions at a high [M]/[R] ratio did not appear to depend on polymerization time, which indicated the possible loss of RAFT-controlled mechanism at high [M]/[R] ratio. This was also supported by slightly higher PDI values obtained at high [M]/[R] ratio when compared with those obtained at low [M]/[R] ratio.

Table 4.1. Monomer conversions, number average molecular weights (M_n's) and molecular weigtht distributions (PDI's) of P(Boc-AEAEMA), synthesized at a monomer concentration of 0.36 M and varying ratios of [M]/[R]/[I] and polymerization time.

Time (h)	[M]/[R]/[I]	Conversion %	Mn^{theo} (g/mol)	Mn^{GPC} (g/mol)	PDI
2	25/1/0.25	27	2794	2563	1.14
3	25/1/0.25	29	2980	2839	1.11
4	25/1/0.25	30	3073	3164	1.12
5	25/1/0.25	46	4563	3586	1.14
6	25/1/0.25	49	4842	4253	1.13
8	25/1/0.25	52	5121	4886	1.14
10	25/1/0.25	59	5773	5047	1.15
2	100/1/0.25	25	9591	5936	1.5
3	100/1/0.25	25	9591	3948	1.17
4	100/1/0.25	27	10336	3823	1.16
5	100/1/0.25	23	8846	3796	1.18
6	100/1/0.25	27	10336	5607	1.2
8	100/1/0.25	33	12571	7208	1.21
10	100/1/0.25	35	13316	8276	1.33

Mn^{theo}: theoretical Mn, Mn^{GPC}: Mn obtained by GPC .

Figure 4.3 shows the kinetic plots of polymerizations performed at a fixed monomer concentration of 0.36 M and varying ratios of [M]/[R]/[I] (25/1/0.25 and 100/1/0.25). ln $[M]_0/[M]$ increases linearly with time. As expected, at a lower [M]/[R] ratio, the ln $[M]_0/[M]$ shows a better linearity with all polymerization times, indicating a successful RAFT-controlled polymerization. The linear increase in Mn with monomer conversion can also be clearly seen for polymerizations performed at a [M]/[R] ratio of 25/1, which is also expected for a successful RAFT polymerization based on Equation 2.1.



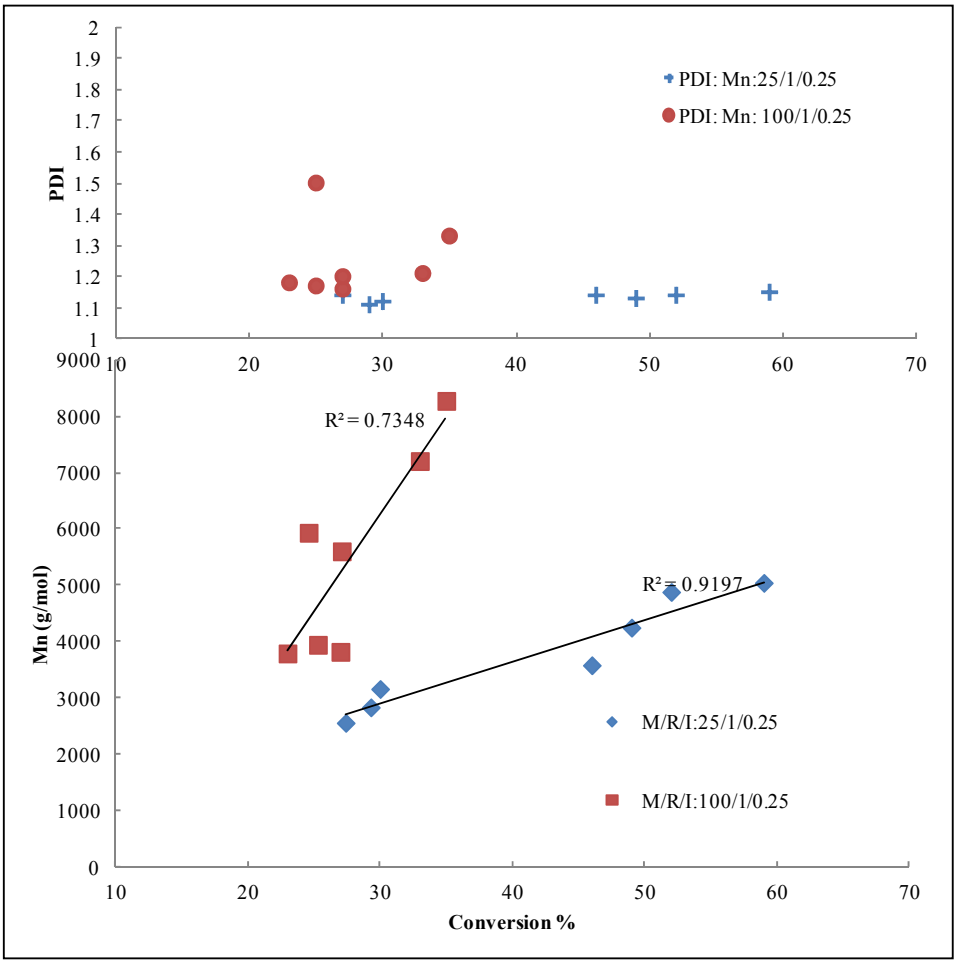


Figure 4.3. Kinetic plots of RAFT polymerization of poly[2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl) amino)ethyl methacrylate]. A) Ln $[M]_0/[M]$ versus time; B) M_n and PDI versus monomer conversion. Mo and M are the monomer concentration in the initial polymerization feed and left after polymerization, respectively.

Similar results were obtained as seen in Tables 4.2 and Figure 4.4 when the polymerizations were performed at a constant monomer concentration of 0.72 M with varying [M]/[R]/[I] ratios (25/1/0.25; 50/1/0.25 and 100/1/0.25). Basically, the increase in [M]/[R] ratio led to an increase in Mn's and a decrease in monomer conversions. The direct proportionality between [M]/[R] ratio and Mn proves that the polymerization of BocAEAEMA was controlled by RAFT mechanism.

In all polymerizations performed at a monomer concentration of 0.72 M, ln $[M]_0/[M]$ increased linearly with time. The linear increase in Mn with monomer conversion was also observed. These are all attributed to the known traits of RAFT-controlled polymerization mechanism (Lowe & McCormick, 2007).

Table 4.3 and Figure 4.5 present the effects of [M]/[R] ratio on the kinetics of RAFT polymerization of BocAEAEMA performed at a constant initiator concentration of $3.6x10^{-3}$ M and [R]/[I] ratio of 4. The [M]/[R] ratio, thus the monomer concentration, was varied to be 25/1 (0.36 M), 50/1 (0.72 M) and 100/1 (1.44 M). At the constant initiator concentration, the monomer conversion per unit time (which can be related to polymerization rate) appeared to increase with increasing [M]/[R] ratio. The increase in [M]/[R] at constant [R]/[I] ratio and initiator concentration results in the increase in monomer concentration used in polymerizations. The increase in monomer concentration leads to an increase in free radical polymerization rate (Odian, 2004; Painter & Coleman, 2009). Increasing [M]/[R] ratio (thus the monomer concentration) at the constant initiator concentration also led to a significant increase in Mn and PDI values. The direct proportionality between [M]/[R] and Mn, which is expected in a RAFT-controlled polymerization (Equation 2.1) indicates the occurrence of a RAFT-controlled polymerization mechanism. However, the control over the polymerization by the RAFT agent gets weaker with the increase in [M]/[R] ratio, which leads to larger PDI values. The kinetic plots (Figure 4.5) show the linear proportionality between ln $[M]_0/[M]$ and polymerization time, and Mn and conversion, indicating the RAFT-controlled mechanism.

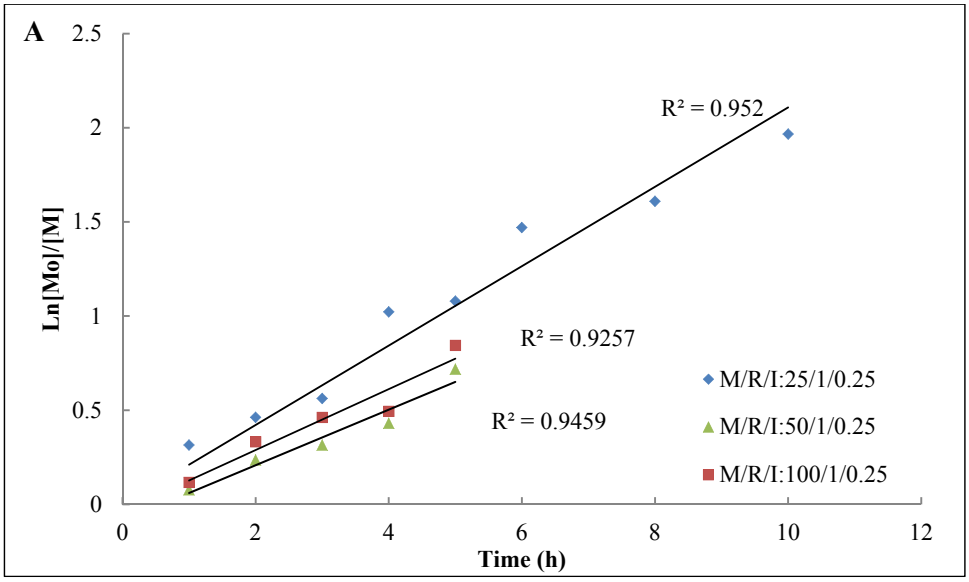
From the kinetic results, it was concluded that the polymerization of new monomer, BocAEAEMA in an organic solvent, toluene using AIBN as an initiator and CPADB as a RAFT agent displayed all characteristics of RAFT-controlled mechanism. The use of a monomer concentration of 0.72 M and a [M]/[R] ratio of 50/1 or 25/1 among the conditions tested provided the best conditions for the RAFT polymerization

of BocAEAEMA, considering the linearity of Mn versus conversion relation and the narrowness of PDI values.

Table 4.2. Monomer conversions, number average molecular weights (M_n's) and molecular weigtht distributions (PDI's) of p(Boc-AEAEMA), synthesized at a monomer concentration of 0.72 M and varying ratios of [M]/[R]/[I] and polymerization time.

Time (h)	M/R/I	Conversion %	Mn^{theo} (g/mol)	Mn^{NMR} (g/mol)	Mn^{GPC} (g/mol)	PDI
1	25/1/0.25	27	2794	1517	4645	1.36
2	25/1/0.25	37	3725	1755	3700	1.3
3	25/1/0.25	55.6	5457	3135	3637	1.14
4	25/1/0.25	64	6239	6343	4982	1.15
5	25/1/0.25	66	6425	4218	5225	1.16
6	25/1/0.25	77	7449	7350	5666	1.16
8	25/1/0.25	80	7729	8629	6060	1.18
10	25/1/0.25	86	8287	undefined	6415	1.18
1	50/1/0.25	8	1769	2212	3035	1.15
2	50/1/0.25	21	4190	3323	4063	1.18
3	50/1/0.25	27	5308	3238	5322	1.22
4	50/1/0.25	35	6797	12172	6956	1.25
5	50/1/0.25	51	9777	undefined	8165	1.28
1	100/1/0.25	11	4376	3243	4781	1.2
2	100/1/0.25	28	10708	8611	5310	1.23
3	100/1/0.25	37	14060	14433	8922	1.34
4	100/1/0.25	39	14805	6509	13564	1.3
5	100/1/0.25	57	21510	11409	14453	1.32

Mn^{theo}: theoretical Mn, Mn^{GPC}: Mn obtained by GPC ; . Mn^{NMR}: Mn obtained by NMR



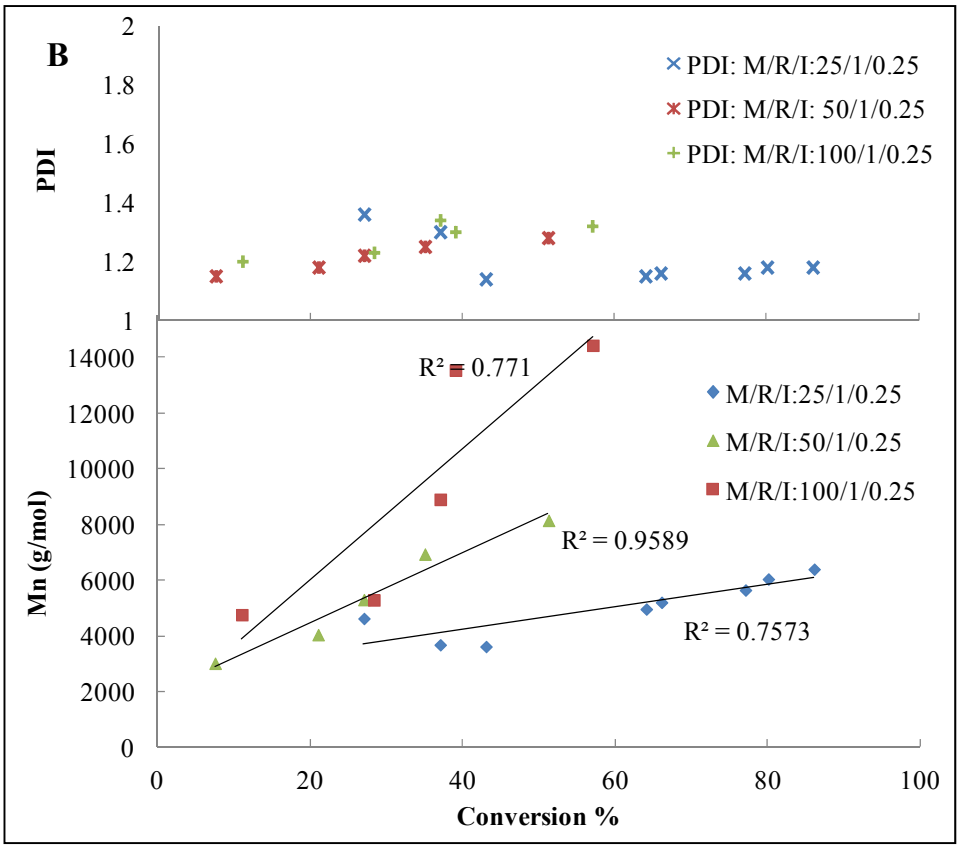
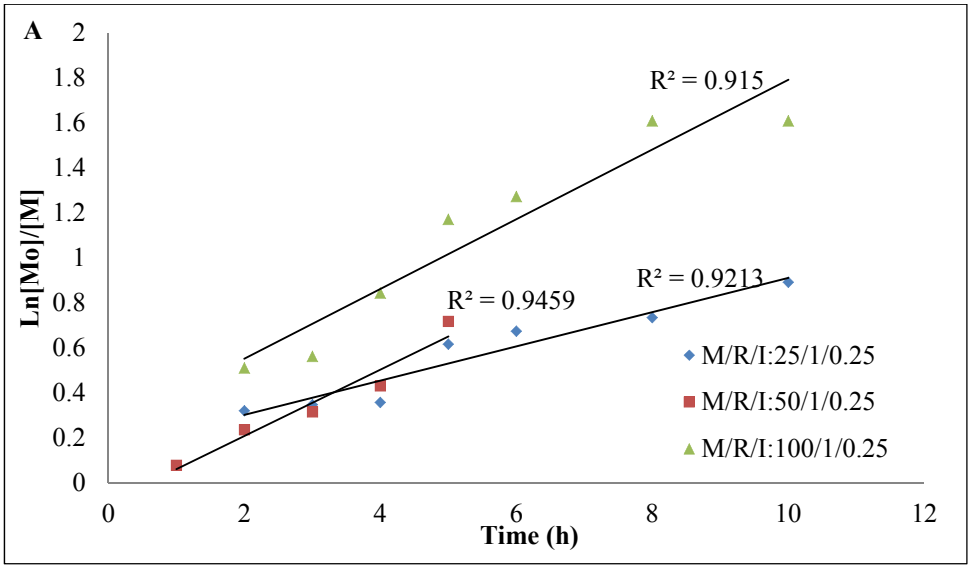


Figure 4.4. Kinetic plots of RAFT polymerization of poly[2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl) amino)ethyl methacrylate]. A) Ln $[M]_0/[M]$ versus time; B) M_n and PDI versus monomer conversion. Mo and M are the monomer concentration in the initial polymerization feed and left after polymerization, respectively.

Table 4.3. Monomer conversions, number average molecular weights (M_n's) and molecular weigtht distributions (PDI's) of p(Boc-AEAEMA), synthesized at a initiator concentration $3.6x10^{-3}$ M and varying ratios of [M]/[R]/[I] and polymerization time.

Time (h)	[M]/[R]/[I]	Conversion %	Mn^{theo} (g/mol)	Mn^{NMR} (g/mol)	Mn^{GPC} (g/mol)	PDI
2	25/1/0.25	27	2795	-------	2563	1.14
3	25/1/0.25	29	2980	-------	2839	1.11
4	25/1/0.25	30	3073	2940	3164	1.12
5	25/1/0.25	46	4563	3902	3586	1.14
6	25/1/0.25	49	4842	------	4253	1.13
8	25/1/0.25	52	5121	5059	4886	1.14
10	25/1/0.25	59	5773	------	5047	1.15
1	50/1/0.25	7.5	1676	2212	3035	1.15
2	50/1/0.25	21	4190	3323	4063	1.18
3	50/1/0.25	27	5308	3238	5322	1.22
4	50/1/0.25	35	6797	12172	6956	1.25
5	50/1/0.25	51	9777	-------	8165	1.28
2	100/1/0.25	40	15178	23057	8473	1.5
3	100/1/0.25	43	16295	-------	8886	1.7
4	100/1/0.25	57	21510	-------	10240	1.5
5	100/1/0.25	69	25979	-------	15192	1.4
6	100/1/0.25	72	27097	43950	14851	1.55
8	100/1/0.25	80	30076	27557	16281	1.41
10	100/1/0.25	80	30076	-------	18901	1.31

Mn^{theo}: theoretical Mn, Mn^{GPC}: Mn obtained by GPC ; . Mn^{NMR}: Mn obtained by NMR



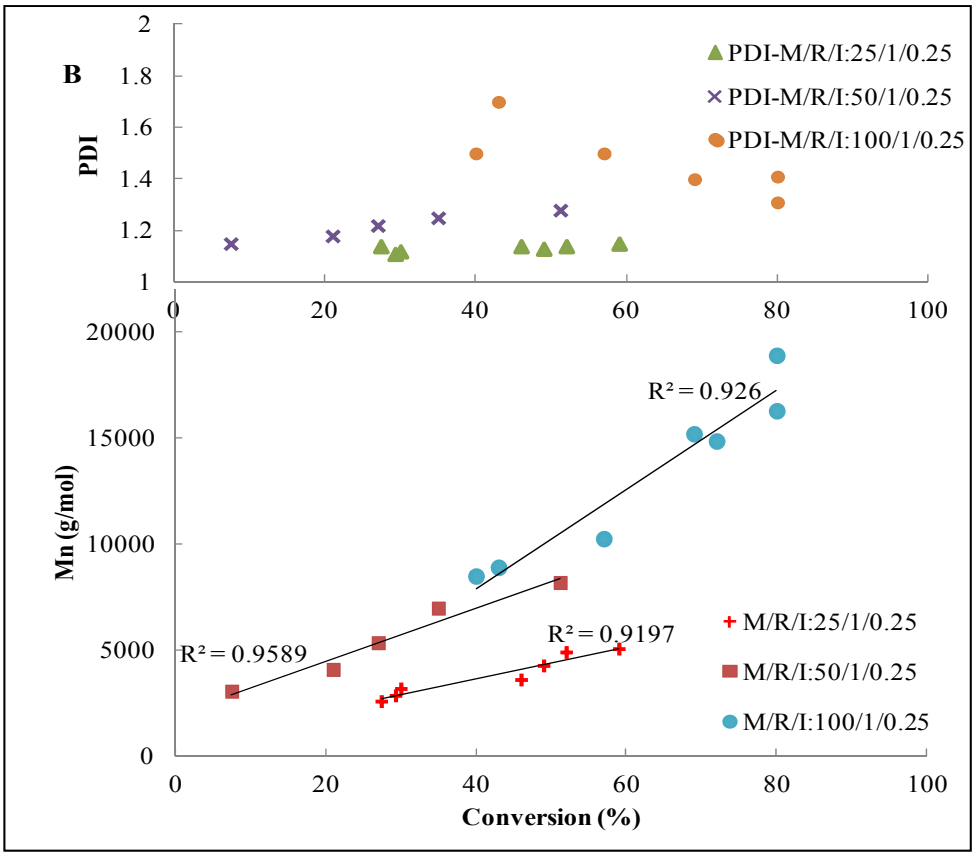


Figure 4.5. Kinetic plots of RAFT polymerization of poly[2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl) amino)ethyl methacrylate]. A) Ln $[M]_0/[M]$ versus time; B) M_n and PDI versus monomer conversion. Mo and M are the monomer concentration in the initial polymerization feed and left after polymerization, respectively.

4.3. Determination of Chain Transfer Constant

The chain transfer constant, C_{tr}, value was determined using a simplified derivation of the Mayo equation (Equation 4.1) (Theis et al., 2005). For this calculation, Mn vs. conversion (Figure 4.3, 4.4 and 4.5) data which had a clear linear relation yielding R^2 value higher than 0.9 were used, since the linear relation between Mn and conversion indicated the occurrence of RAFT-controlled polymerization. According to suggested theory, C_{tr} should be higher than two if there is a reasonable level of control by the chain transfer agent in a polymerisation process (Müller & Litvenko, 1997). In a study of Moad et al. (Moad et al., 2000), C_{tr} values were calculated to be between 26 and 0.03 for various RAFT agents used in polymerizations of styrene and MMA. On the other hand, in the study of Goto et al. (Goto et al., 2001) the C_{tr} values of RAFT polymerization of styrene and MMA were estimated as 6000 and 140, respectively. The variations in C_{tr} values depend on the R and Z groups of the RAFT agents, the monomer and the polymerization temperature.

$$C_{tr} = \frac{[M]_0}{(DP_n^{inst} - 1)x[RAFT]_0} \quad (4.1)$$

In Equation 4.1, DP^{inst}_n is the instantaneous degree of polymerization which is estimated by the extrapolation of experimental molecular weights to zero conversion. $[M]_0$ and $[RAFT]_0$ are initial concentrations of monomer and RAFT agent, respectively.

For all conditions chosen (Figure 4.3, 4.4 and 4.5), DP_n with respect to conversion was drawn (Figure 4.6, 4.7 and 4.8) and DP^{inst}_n was estimated at zero conversion. Chain transfer constant of CPDAB were calculated using Equation 4.1. In Table 4.4, the chain transfer constant values obtained from different sets of polymerizations ($[M]_0/[R]_0$ ratios of 25/1, 50/1 and 100/1 at constant monomer concentration (0.72M and 0.36 M) or at constant initiator concentration ($3.6x10^{-3}$ M) are presented.

Based on these data, the average chain transfer constant was determined to be 25 for BocAEAEMA monomer and CPDAB RAFT agent at 65°C. The calculated chain transfer constant value might indicate a reasonable level of control in the RAFT

polymerization of new amine containing monomer under the polymerization conditions tested.

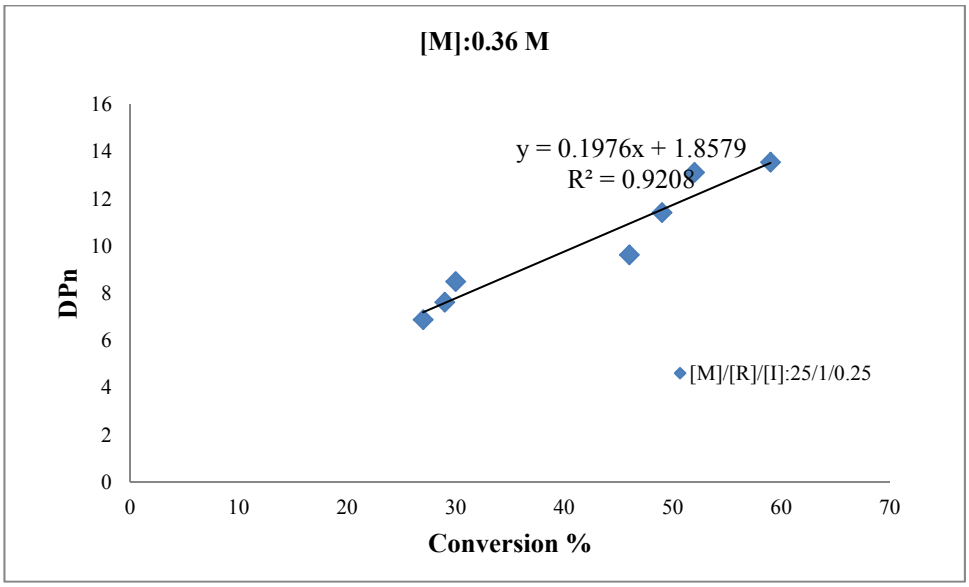


Figure 4.6. DP^{inst}_n estimation for RAFT polymerization of BocAEAEMA at 65 °C in toluene at constant monomer concentration of 0.36 M

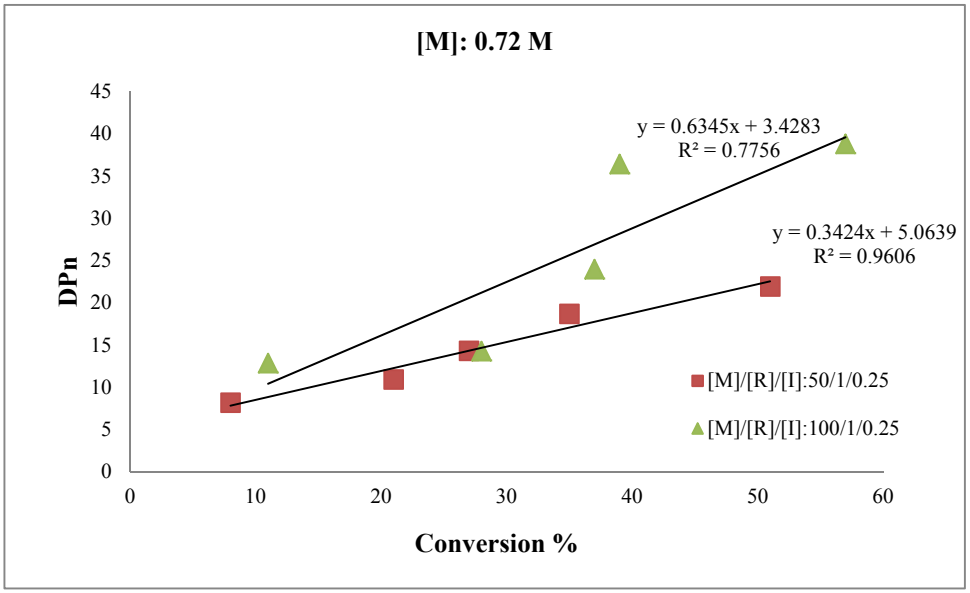


Figure 4.7. DP^{inst}_n estimation for RAFT polymerizations of BocAEAEMA at 65 °C in toluene at constant monomer concentration of 0.72 M

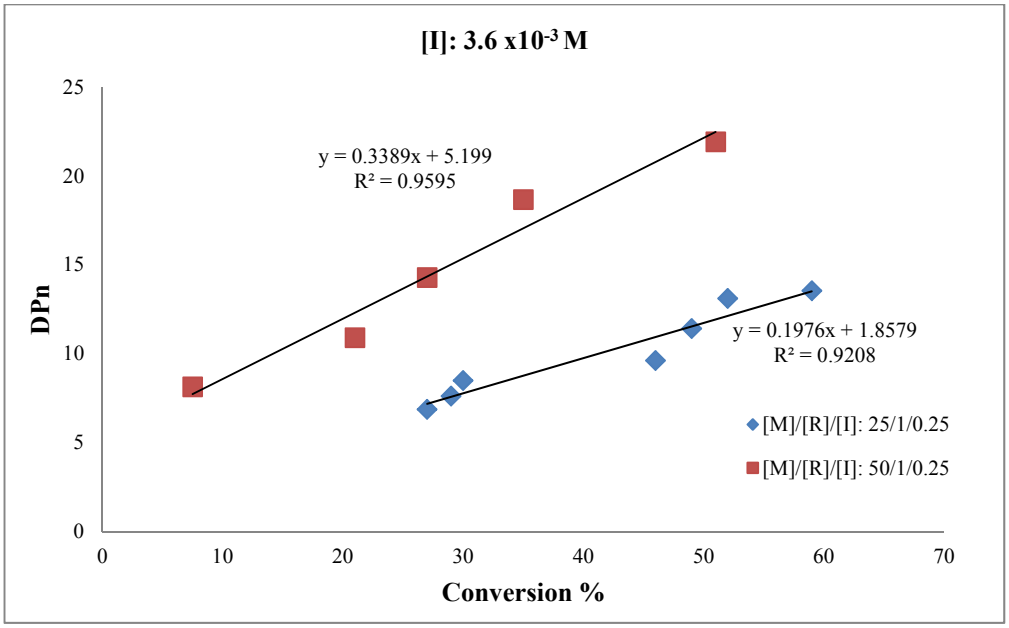


Figure 4.8 DP^{inst}_n estimation for RAFT polymerizations of BocAEAEMA at 65 °C in toluene at constant initiator concentration of 3.6 x 10^{-3} M

Table 4.4. Estimation of the chain transfer constant C_{tr} of the RAFT agent (CPDAB)

[M]	**$[M]_0/[R]_0$**	**DP_n**	**Ctr**
0.36 M	25	1.90	29.1
[M]	**$[M]_0/[R]_0$**	**DPn**	**Ctr**
0.72 M	50	5.10	12.20
	100	3.40	41.67
$[I]x10^2$	**$[M]_0/[R]_0$**	**DPn**	**Ctr**
0.36 M	25	1.9	29.1
	50	5.10	12.20

4.4. Hydrolysis and Aminolysis of 4-Cyano-4(phenylcarbonothioyl thio) Pentanoic Acid (RAFT agent)

After achieving successful results from the RAFT polymerization of the new monomer in toluene, an attempt was made to polymerize directly the unprotected monomer (AEAEMA) via aqueous RAFT polymerization. Aqueous RAFT polymerization of amine containing monomers has been performed by others by applying careful polymerization conditions (McCormick & Lowe, 2004; Paslay et al., 2012; Treat et al., 2012). It is well-known that the RAFT agents are degraded in the presence of water and amine containing compounds and after degradation they lose their livingness character, as already explained in Literature Review section of this thesis. According to the study of Levesque et al. (Levesque et al., 2000), RAFT agent hydrolysis depends on the pH of solution and the temperature. At basic and neutral pHs, hydroxide ions of water react with RAFT agent that leads to a fast degradation on RAFT agent thiocarbonyl thio group in the absence of amine containing compounds (Llauro et al., 2004). In the presence of amine compounds, when pH increases that is higher than the pKa values of amine groups, amine groups deprotonate and react with the RAFT agent thiocarbonyl thio group. When the temperature increases, degradation rate increases that degradation time of RAFT agent decreases.

In this study, the hydrolysis in the presence and absence of N-hydroxyethylethylene diamine (the starting compound for the synthesis of AEAEMA monomer) was investigated at neutral and acidic pHs at 65°C. N-hydroxyethylethylene diamine [HEDA] concentration and [HEDA]/[RAFT agent] molar ratios were used as 0.17 M and 50/1, respectively. The degradation kinetic of the RAFT agent was followed by a UV-vis spectrophotometer. The decrease in the characteristic UV-absorption band of the RAFT agent between 302 and 305 nm was monitored. The UV analysis data are given in Appendix C. The degradation percentage was determined using Equation 3.5.

In Figure 4.9, the hydrolysis percent of the RAFT agent is shown with respect to time at pH 7.4, 5.2 and 3.0. The degradation of the RAFT agent at pH 7.4 was higher when compared with that at acidic pHs and reached about 80% after 24 h. However, the degradation at pH 5.2 and 3.0 was nearly the same, reaching 35% after 24 h. In acidic conditions, hydronium ions ($[H_3O^+]$) occur by interaction of the hydroxide ions with

hydrogen ions of acid. When pH decreases, formation of hydronium ions increases that leads to slow degradation of RAFT agent as expected.

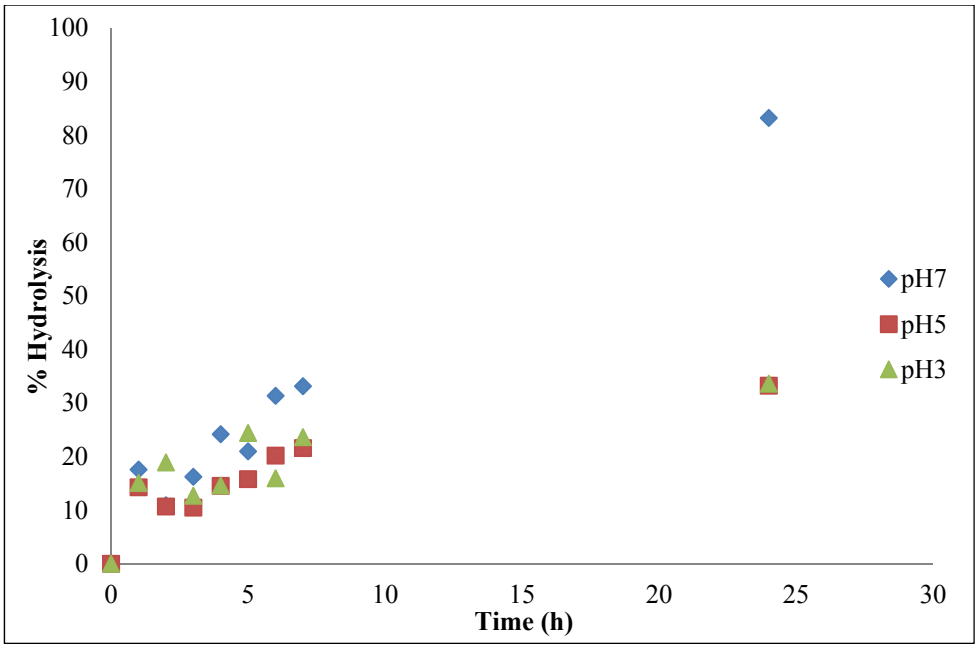


Figure 4.9. Percent hydrolysis of 4-cyano-4-(phenylcarbono thioylthio) pentanoic acid (CPADB) with respect to time at 65°C and pH 7.4, 5.2 or 3.0

The hydrolysis in the presence of the amine compound, HEDA (i.e. thioacylation reaction) was investigated at 65°C and pH 5.2 or 3.0. Figure 4.10 shows the results of this experiment. It can be seen that the RAFT agent aminolyzed much faster at pH 5.2 when compared with the aminolysis at pH 3.0. When the pH of the solution decreases, more amine groups are protonated leads to the slow degradation of the RAFT agent.

The hydrolysis in the presence of amine compound is more significant even at pH 3.0 when compared with the hydrolysis in the absence of amine at the same pH.

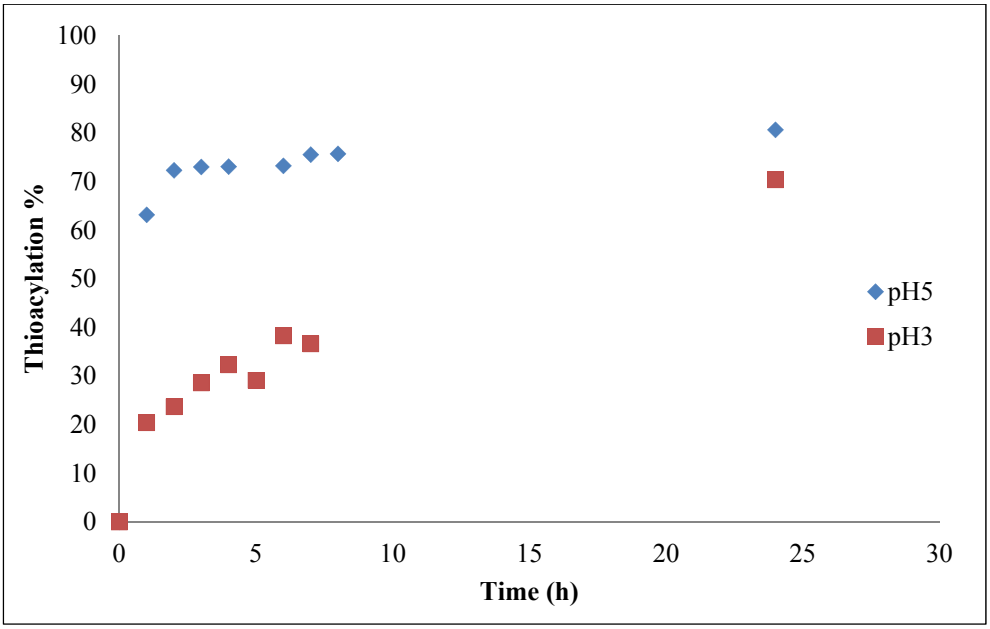


Figure 4.10. Percent aminolysis of 4-cyano-4-(phenylcarbono thioylthio) pentanoic acid (CPADB) with respect to time at 65°C and pH 5.2 or 3.0

Overall the results show that the aqueous RAFT polymerization of deprotected monomer AEAEMA was not possible under the conditions tested due to the fast aminolysis of the RAFT agent. Considering these results, it was not attempted to perform aqueous RAFT polymerization of deprotected AEAEMA monomer.

4.5. Deprotection of Poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) (P(Boc-AEAEMA))

In order to investigate the cytotoxicity and proton sponge capacity, p(BocAEAEMA) was deprotected to yield the final amine-containing, spermidine-like polymer structure. For this aim, 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate polymers were treated with trifluoroacetic acid.

Figure 4.11 shows the NMR spectra of polymers having different molecular weights after deprotection and purification. It should be noted that the NMR spectra were taken in D_2O. The proton signals of the protection groups (Boc-), that make the polymer water insoluble, can be seen at 1.40 and 1.48 ppm in the NMR spectra of deprotected polymers (Figure 4.11). The disappearance of the signals of Boc groups

indicate that the protection groups were cleaved from the polymers successfully to yield 99.4% of p(AEAEMA) that was calculated by Equation 3.6.

Molecular weights of polymers after deprotection were calculated theoretically by Equation 4.2 (Table 4.5). Equation 4.2 is based on molecular weights of the cleaved Boc group (202 g/mol), protected monomer (372.46 g/mol) and molecular weights of protected polymers obtained by NMR and GPC.

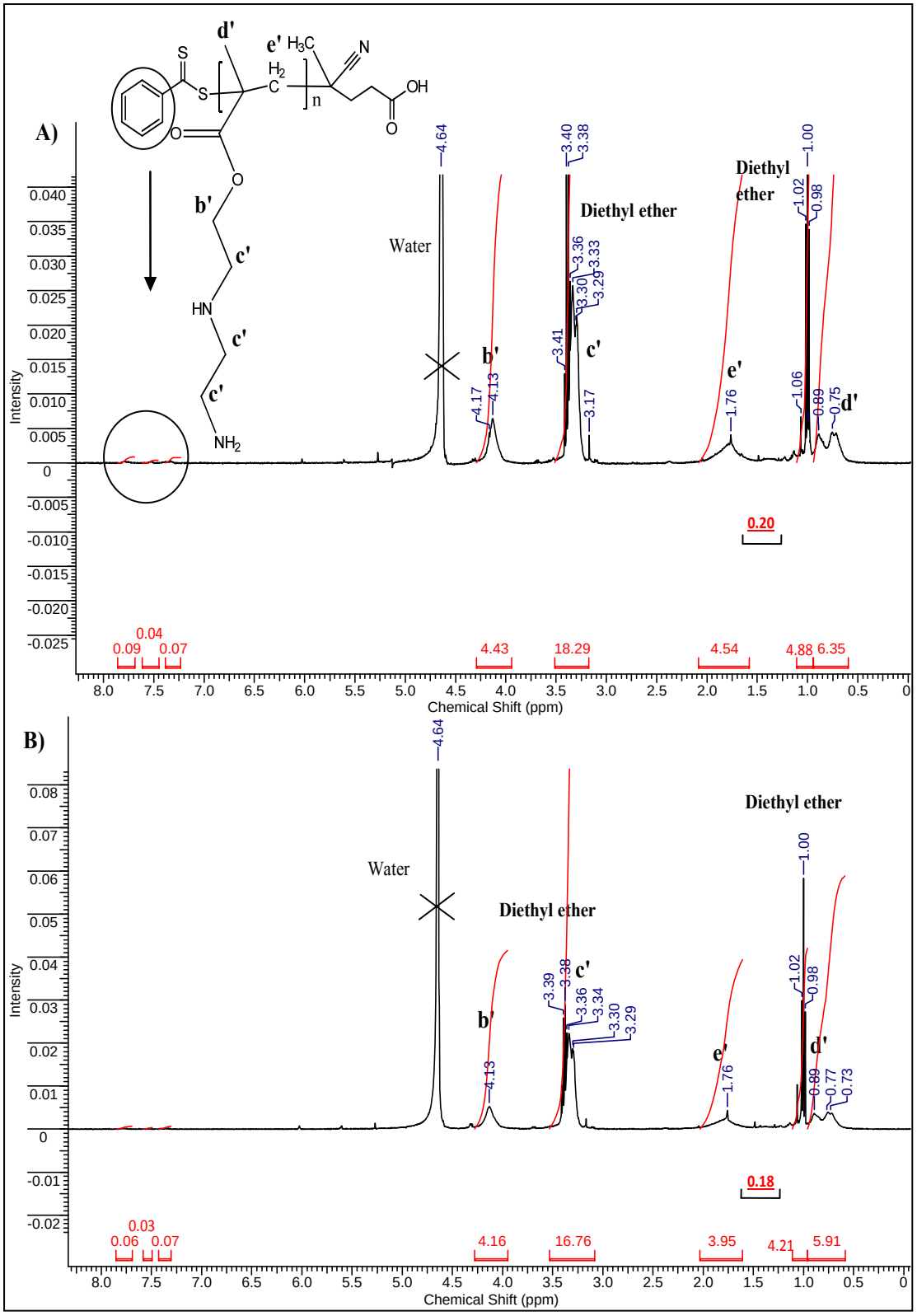


Figure 4.11. ^{1}H-NMR spectra of cleavage of protection (boc-) groups of primary and secondary amine groups of poly[2-((tert-butoxycarbonyl)(2-((tert-butoxycarbonyl) amino) ethyl) amino)ethyl methacrylate]; A) 5.5 kDa and B) 8 kDa

Table 4.5. Molecular weights of polymers before and after deprotection. Molecular weights after deprotection was calculated theoretically based on Equation 4.2

POLYMERS				
BEFORE DEPROTECTION Mn (g/mol)			AFTER DEPROTECTION Mn (g/mol)	
By GPC	PDI	By NMR	By GPC	By NMR
11.5	1.35	12	5.5	5.5
13.5	1.41	16.5	6.5	8

$$M_{n_{theoretical}} = \frac{M_{n_{GPC\ or\ NMR}} - M_{w_{RAFT\ agent}}}{\frac{M_{n_{protectedmonomer}}}{M_{n_{protectedmonomer}} - M_{w_{Boc\ group}}}} + M_{w_{RAFT\ agent}} \quad (4.2)$$

4.6. Determination of Proton Sponge Capacity of Poly(2-((2-aminoethyl)amino)ethyl methacrylate)

Proton sponge capacity of p(AEAEMA) was investigated with respect to polyethyleneimine (PEI), a widely used polymer in intracellular drug delivery due to its proton sponge property. P(AEAEMA) of 5.5 kDa (PDI:1.35) and 8 kDa (PDI:1.41) and PEI of 25 kDa and 60 kDa were titrated with 0.1 M HCl using polymer solutions containing the same polymer (2.2 $x10^{-5}$ M) or repeating unit (2.9 $x10^{-5}$ M) concentration.

According to the titration curve at Figure 4.12 p(AEAEMA) having the molecular weight 5.5 kDa and 8 kDa have a pKa of 6.6 and 7.2, while PEI of 25 kDa and 60 kDa show two pKa values for primary and secondary amine groups that are 9.8 and 5.05 at the same polymer concentration, respectively. The pKa value of p(AEAEMA) was found to be suitable for endosomal pH range.

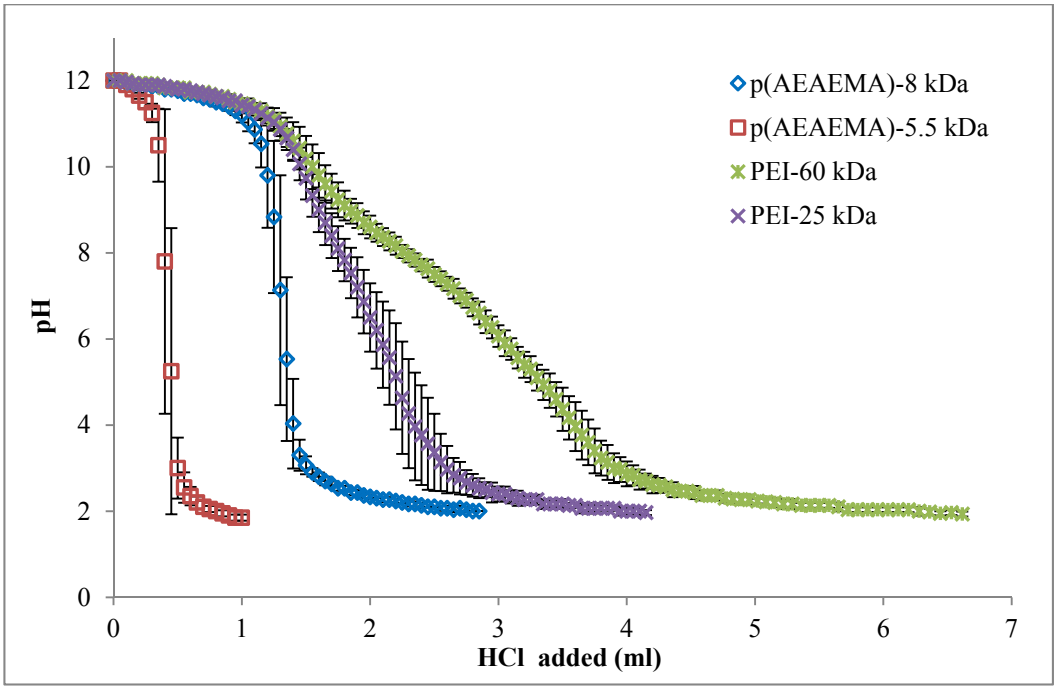


Figure 4.12. Proton sponge capacity of of 2-((2-aminoethyl) amino)ethyl methacrylate polymers (8 kDa and 5.5 kDa) and polyethyleneimine (25 kDa, 60 kDa). The solutions tested contained the same concentration of polymers (2.2 x10^{-5} M).

In addition, proton sponge capacity was obtained at the same repeating unit concentrations. Figure 4.13 shows that p(AEAEMA) has proton sponge capacity comparable to PEI 25 kDa which is one of the most common used polymers in intracellular gene delivery and PEI 60 kDa. At the same repeating unit concentration, the mole number of lower molecular weight AEAEMA polymers present in solution is higher than the mole number of high molecular weight PEI's, which led to high proton sponge capacity.

Table 4.6 shows the mole number of protons required to decrease the pH of different polymer solutions to acidic pH values. The mole number of protons required per mole of repeating unit of AEAEMA polymers was higher than that of PEIs, indicating stronger proton sponge capacity of AEAEMA polymers. To accurately compare the proton sponge capacity, AEAEMA polymers having molecular weights closer to the molecular weight of PEI samples would be required to be tested in future studies.

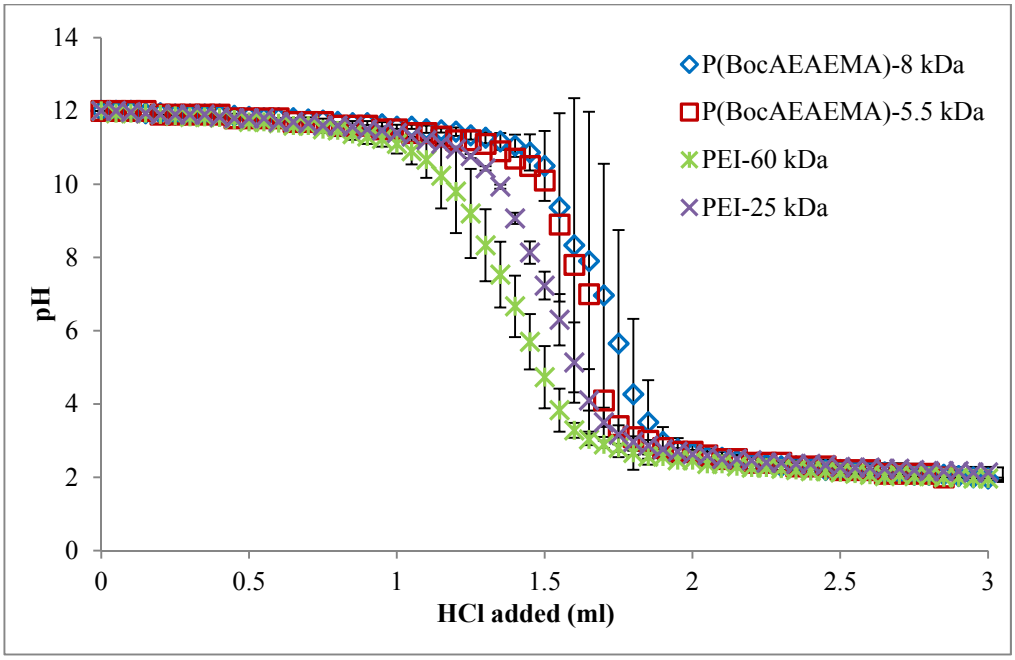


Figure 4.13. Proton sponge capacity of of 2-((2-aminoethyl) amino)ethyl methacrylate polymers (8 kDa and 5.5 kDa) and polyethyleneimine (25 kDa, 60 kDa). The solutions tested contained the same concentration of polymers (2.2 x10^{-5} M).

Table 4.6. Proton sponge capacity results

Polymers	pKa	Mole proton/Mole polymer	Mole proton/Mole repeating unit
PEI (60kDa)	9.8-5.05	1477.3	482.8
	6.9*		
PEI (25kDa)	9.8-5.05	1004.5	517.2
	7.0*		
P(AEAEMA) (8 kDa)	7.2	590.9	620.7
	9.4-5.7*		
P(AEAEMA) (5.5 kDa)	6.6	195.5	551.7
	8.9-5.5*		

*pKA results from same repeating unit concentration data.

4.7. End-group Modification of Poly(2-((2-aminoethyl)amino)ethyl) methacrylate) (P(Boc-AEAEMA))

Before performing the cytotoxicity assays, the thiocarbonylthio RAFT end-group of p(AEAEMA) was removed as this reactive group is potentially toxic. According to the study of Pissuwan et al. (Pissuwan et al., 2010), polymers synthesized via RAFT polymerization using 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid as RAFT agent has no toxicity on NIH 3T3 and CHO-K1 cell lines over 72 h. However, the authors stated that the RAFT end-group cytotoxicity depends on the types of cell line and polymer. Nevertheless in order to avoid from possible toxic effects of the RAFT end-group, the thiocarbonyl thio end group of polymers was cleaved to thiol in the presence of hexylamine, triethylamine and AEAEMA monomer, following a procedure reported widely in literature (Boyer et al., 2009). The ^{1}H-NMR spectra of the polymers after aminolysis and dialysis are shown in Figure 4.14. The spectra show that the RAFT end-group of the polymers removed with a 54.3% reaction yield. Reaction yield was calculated by Equation 3.7.

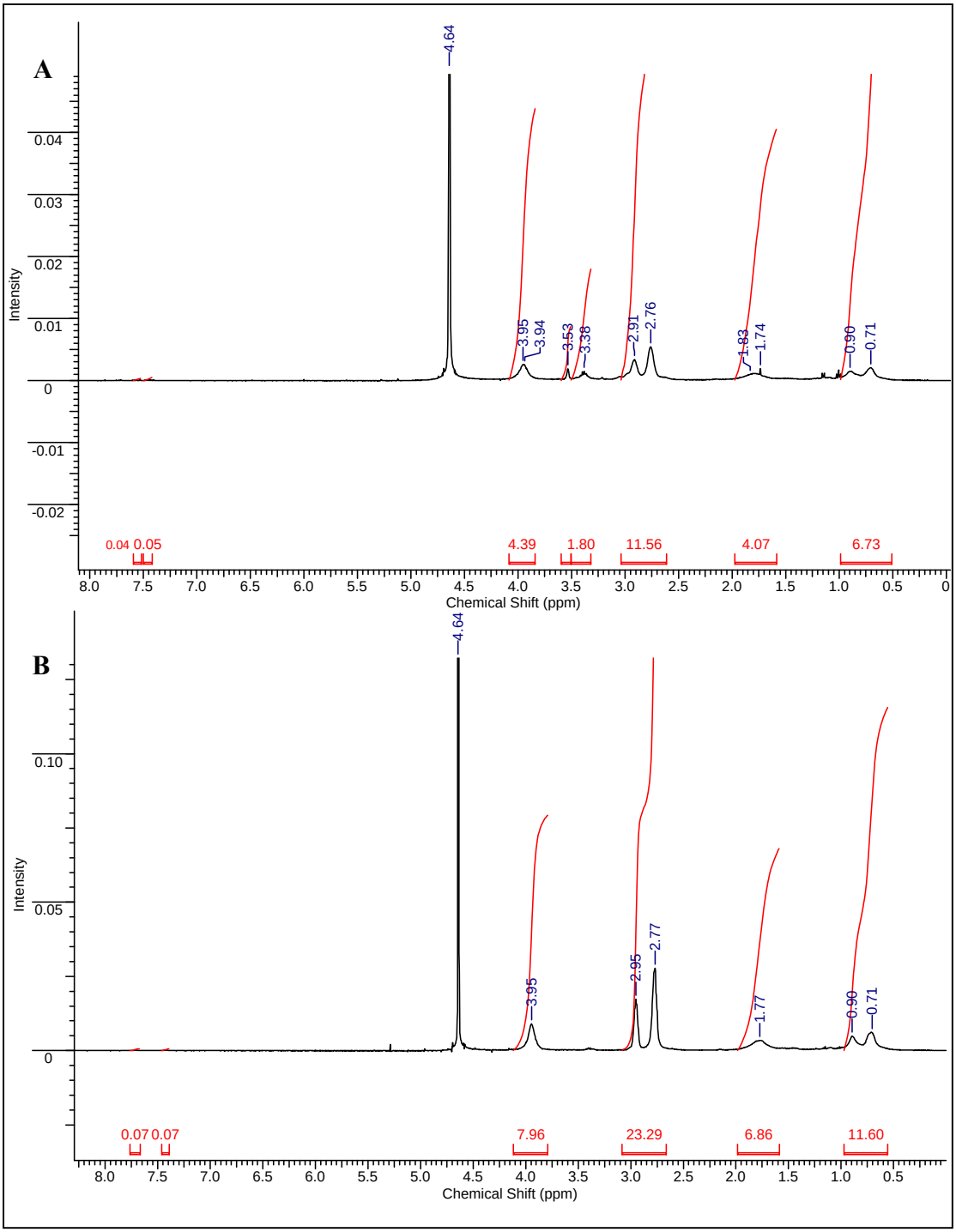


Figure 4.14. [1]H-NMR spectra of aminolysized and dialyzed of poly[2-((2-aminoethyl)amino)ethyl methacrylate]; A) 5.5 kDa and B) 8 kDa

4.8.Determination of *In Vitro* Cytotoxicity

The effects of p(AEAEMA) (Mn_{GPC}:5.5 kDa, Mn_{NMR}:5.5 kDa, PDI: 1.35; Mn_{GPC}:6.5 kDa, Mn_{NMR}:8 kDa kDa, PDI:1.41) on viability of *in vitro* cultured mouse fibroblast NIH 3T3 cell line were investigated and compared with those of PEI (branched, 25 kDa and 60 kDa) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability assay by incubating cells at 37 °C/5% CO_2 for 24 h and 72 h at varying polymer concentrations (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM and 1.6 μM).

In the MTT assay, tetrazole can reduce into purple formazan with the metabolic activity of living cells. The absorbance of purple formazan, which depends on viable cells, was measured using microplate reader at 540 nm. The percentage of cell viability was determined with respect to untreated cells by Equation 3.8.

According to Figure 4.15 and Table 4.7, p(AEAEMA) did not show cytotoxic effect at all polymer concentrations while PEIs (25kDa and 60 kDa) were highly toxic at 1.6 μM, 0.8 μM and 0.4 μM for 24h. PEI (25 kDa and 60 kDa) did not show toxic effect at 0.1 μM and 0.2 μM polymer concentrations.

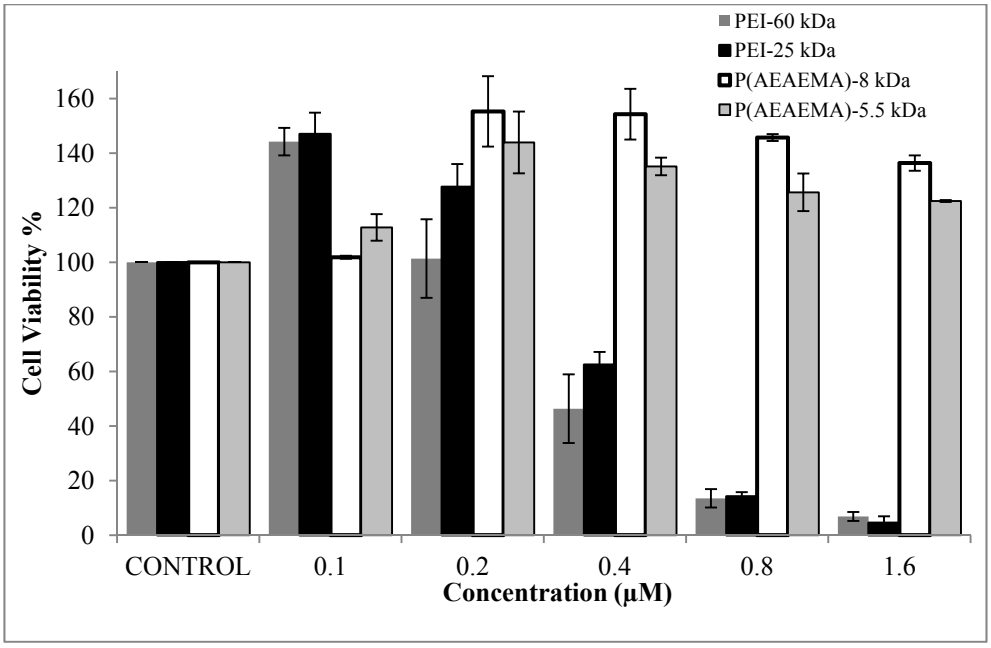


Figure 4.15. Viability of NIH 3T3 cells after incubation with 2-((2-aminoethyl)amino)ethyl methacrylate polymers and PEI (60 kDa and 25 kDa)s for 24 h. Control is the cells with no treatment

Table 4.7. Percent Viability of NIH 3T3 cells after incubation with P(AEAEMA) and PEI (60 kDa and 25 kDa) for 24 h

Concentrations	Polymers			
	PEI (60 kDa)	**PEI (25 kDa)**	**P(AEAEMA) (8 kDa)**	**P(AEAEMA) (5.5 kDa)**
Control	100	100	100	100
0.1 μM	144.2 ±5.1	147 ±7.8	101.9 ±0.6	112.8 ±4.9
0.2 μM	101.4 ±14.4	127.6 ±8.4	155.3 ±12.9	144 ±11.3
0.4 μM	46.4 ±12.6	62.5 ±4.7	154.3 ± 9.3	135.1 ±3.2
0.8 μM	13.5 ±3.4	14.2 ±1.6	145.7 ±1.3	125.7 ±6.9
1.6 μM	6.9 ±1.6	4.5 ±2.4	136.4 ±2.8	122.5 ±0.4

Figure 4.16 and Table 4.8 show the effects of p(AEAEMA) and PEI (25 kDa and 60 kDa) on the cell viability after 72 h incubation time. From the results, AEAEMA polymers did not show significant inhibition on NIH 3T3 cell viability at all concentrations tested for 72 h. However PEIs (25kDa and 60 kDa) showed highly toxic effect at 1.6 μM, 0.8 μM and 0.4 μM polymer concentrations for 72h. The cell viabilities were 97.77 and 84.43 % for PEI 60 kDa at 0.1 μM and 0.2 μM while PEI 25 kDa did not show significant toxicity at these concentrations.

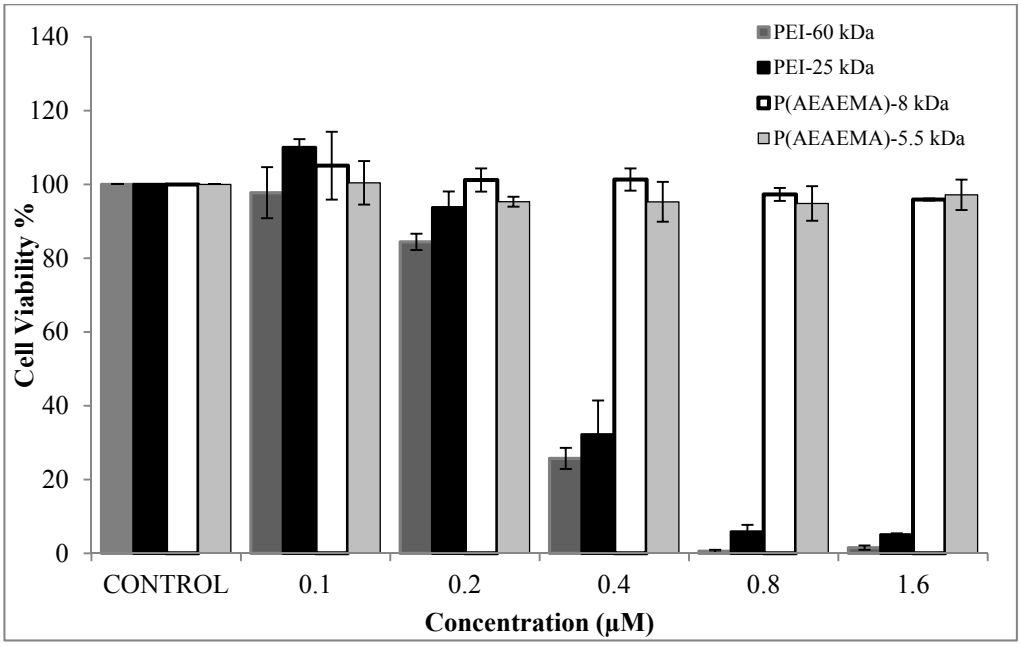


Figure 4.16. Viability of NIH 3T3 cells after incubation with 2-((2-aminoethyl) amino) ethyl methacrylate polymers and PEI (60 kDa and 25 kDa) for 72h. Control is the cells with no treatment

Table 4.8. Percent Viability of NIH 3T3 cells after incubation with P(AEAEMA) and PEI (60 kDa and 25 kDa) for 72 h

Concentrations	Polymers			
	PEI (60 kDa)	**PEI (25 kDa)**	**P(AEAEMA) (8 kDa)**	**P(AEAEMA) (5.5 kDa)**
Control	100	100	100	100
0.1 µM	97.8 ±6.9	110 ±6.9	105.1 ±9.2	100.5 ±5.9
0.2 µM	84.4 ±2.2	93.7 ±4.4	101.2 ± 3.15	95.3 ±1.4
0.4 µM	25.7 ±2.9	32.1 ±9.3	101.3 ± 3	95.3 ±5.4
0.8 µM	0.6 ±0.4	5.78 ±1.9	97.3 ± 1.8	94.8 ± 4.7
1.6 µM	1.5 ±0.6	5 ±0.4	95.9 ± 0.3	97.2 ± 4.1

CHAPTER 5

CONCLUSION

The aim of this study was to synthesize a new, spermine-like, amine containing polymer, poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) via RAFT polymerization as a potential endosomal escaping agent.

The amine groups of N-hydroxyethylethylenediamine were first protected using di-*tert*-butyl dicarbonate followed by methacrylation of tert-butyl (2-((tert-butoxycarbonyl)amino)ethyl)(2-hydroxyethyl)carbamate was methacrylated according to procedures reported previously. Chemical structures, reaction yields and purity of synthesized compounds were characterized at every step. 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate was polymerized via RAFT polymerization. Monomer conversion, number average molecular weight and molecular weight distribution were determined using Nuclear Magnetic Resonance (NMR) and Gel Permeation Chromatography (GPC). In a series of polymerization kinetics experiments, the effects of [M]/[R] ratio at constant monomer or initiator concentrations on RAFT-controlled character of polymerizations were investigated. According to results, at constant monomer concentrations (0.36 M or 0.72 M) and [R]/[I] ratio (1/0.25), the increase in [M]/[R] ratio led to an increase in the Mn's and a decrease in monomer conversions as expected. The linear relation between ln $[M]_0/[M]$ and polymerization time, and Mn and conversion proved the RAFT-controlled polymerization mechanism.

At constant initiator concentration ($3.6x10^{-3}$ M) and [R]/[I] ratio (1/0.25), monomer conversion increased with time when [M]/[R] ratio increased. The increase in [M]/[R] at constant [R]/[I] ratio and initiator concentration induced an increase in free radical polymerization rate, as expected. Increasing [M]/[R] ratio (thus the monomer concentration) at the constant initiator concentration also led to a significant increase in Mn and PDI values. According to results, [M]/[R] ratio and Mn were directly proportional, ln $[M]_0/[M]$ with polymerization time, and Mn with conversion increased linearly. These results indicated that the polymerization of the new monomer was RAFT-controlled under the conditions tested. In this study, best conditions for the

RAFT polymerization of BocAEAEMA was 0.72 M monomer concentration and a [M]/[R] ratio of 50/1 or 25/1 based on the linearity of Mn versus conversion relation and the narrowness of PDI values.

RAFT agent degradation was investigated in the presence or absence of N-hydroxyethylethylenediamine in aqueous solutions at various pHs at 65°C. When pH of the solution increased, RAFT agent degradation increased. Hydrolysis of the RAFT agent (in the absence of amine compound) at pH 7.4 was reached to 80% after 24 h. At acidic pHs (pH 5 and pH 3), RAFT agent hydrolyzed in a similar manner and 35% degradation was obtained after 24 h. According to aminolysis results, in the presence of amine compound RAFT agent degraded much faster at pH 5.2 when compared with the aminolysis at pH 3.0. When the pH of the solution decreases, more amine groups get protonated which decreases the aminolysis of the RAFT agent. Considering all these results, aqueous RAFT polymerization of deprotected monomer AEAEMA was found to be unreasonable due to the fast degradation of the RAFT agent.

Before proton sponge capacity and cytotoxicity analysis of polymers, amine-protection groups of polymers were removed in acidic conditions with a yield of 99.4%. The polymers that were water-insoluble, became water-soluble after deprotection of amine groups.

The proton sponge capacity of AEAEMA polymers was investigated via potentiometric titration and compared with that of polyethyleneimine (25 kDa and 60 kDa). According to results, the pKa values and proton sponge capacities of p(AEAEMA) were comparable with PEI 60 kDa and 25 kDa at the same repeating unit concentration. And at the same polymer concentration, the proton sponge capacities of AEAEMA polymers were lower than PEIs and increased with molecular weight. The pKa values of p(AEAEMA) were perfectly in the range of endosomal pH.

Following the removal of thiocarbonylthio end-group of the polymers (in order to remove any possible toxicity), cytotoxicity analysis of p(AEAEMA) (Mn= 5.5 kDa and Mn= 8 kDa) and PEI (Mn= 25 kDa and Mn= 60 kDa) was performed using NIH 3T3 mouse fibroblast cell line. According to cytotoxicity analysis, AEAEMA polymers did not show cytotoxic effects at all polymer concentrations for 24 h and 72 h while PEIs showed high toxicity at high polymer concentrations.

Future investigations on the topic may include the following suggestions:

1. Higher molecular weight poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) can be synthesized to efficiently compare proton sponge capacity and cytotoxicity of polymers to poly(ethyleneimine).

2. The escape of poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) from endosomes can be investigated in *in vitro* cell culture studies after labeling the polymer with a fluorescent dye.

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APPENDIX A

SYNTHESIS OF 2-((TERT-BUTOXYCARBONYL) (2-((TERT-BUTOXYCARBONYL) AMINO) ETHYL) AMINO) ETHYL METHACRYLATE

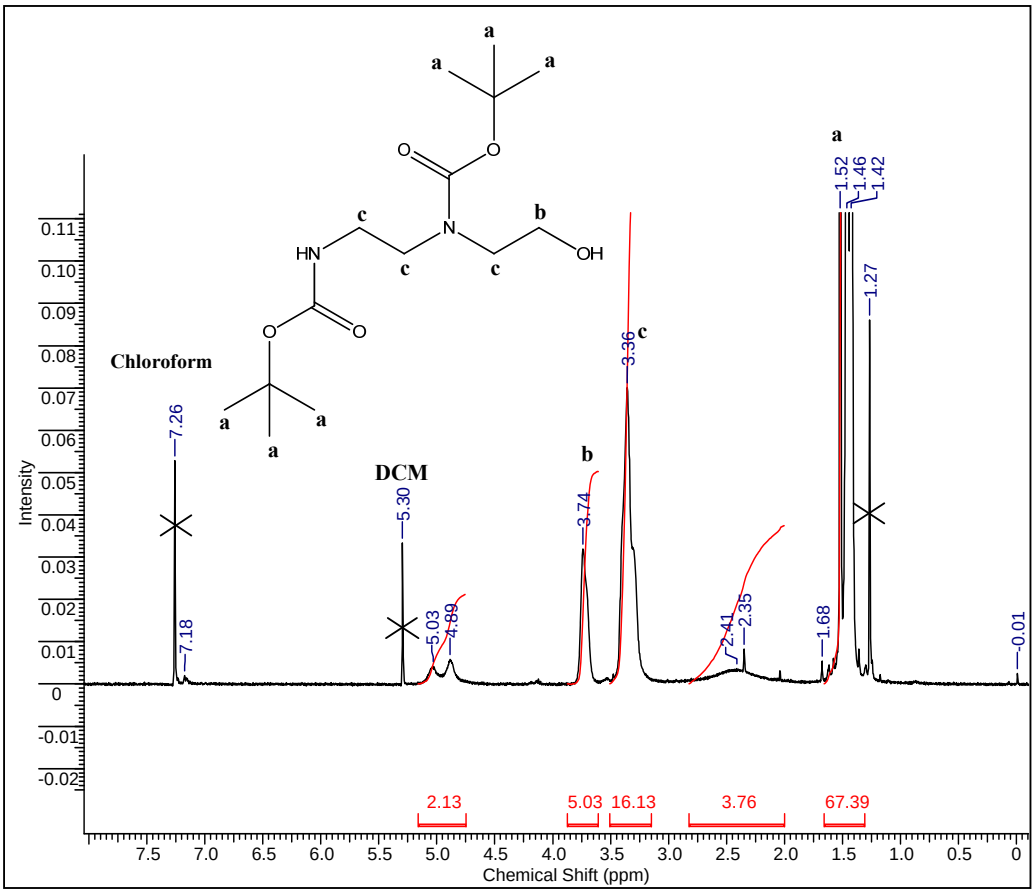

Figure A.1. ^{1}H-NMR spectrum of tert-butyl (2-((tert-butoxycarbonyl) amino) ethyl) (2-hydroxyethyl) carbamate before purification

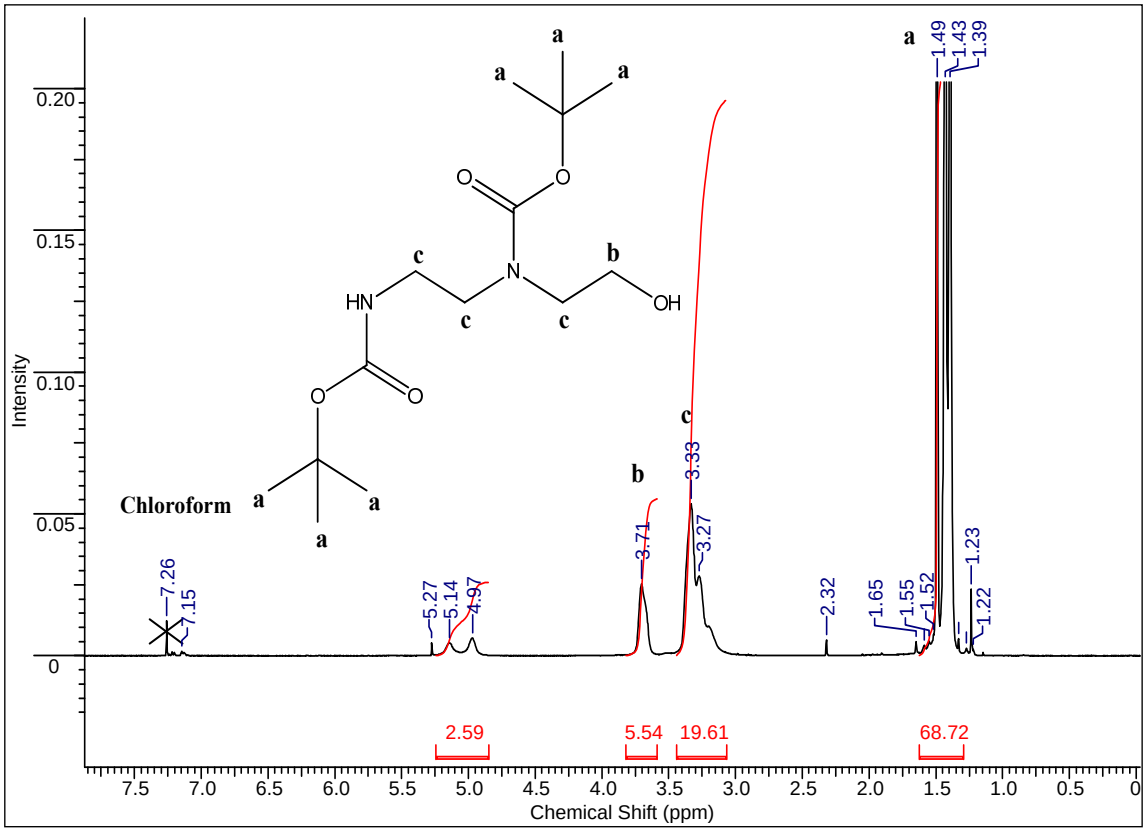

Figure A.2. ^{1}H-NMR spectrum of tert-butyl (2-((tert-butoxycarbonyl) amino) ethyl) (2-hydroxyethyl) carbamate after water-DCM extraction to separate unreacted N-hydroxyethylethylenediamine

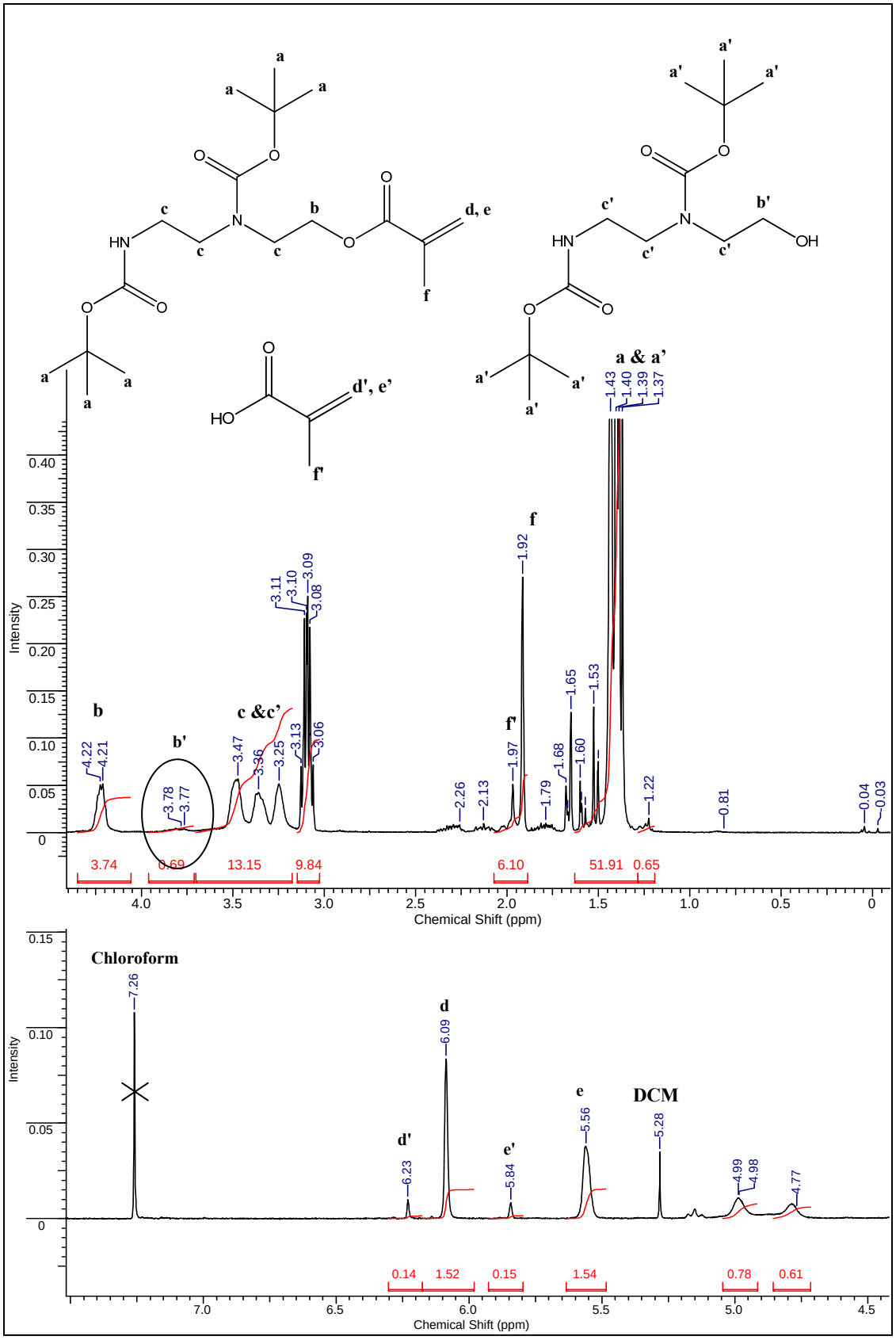

Figure A.3. [1]H-NMR spectrum of Methacrylated tert-butyl (2-((tert-butoxycarbonyl) amino) ethyl) (2-hydroxyethyl)carbamate before purification

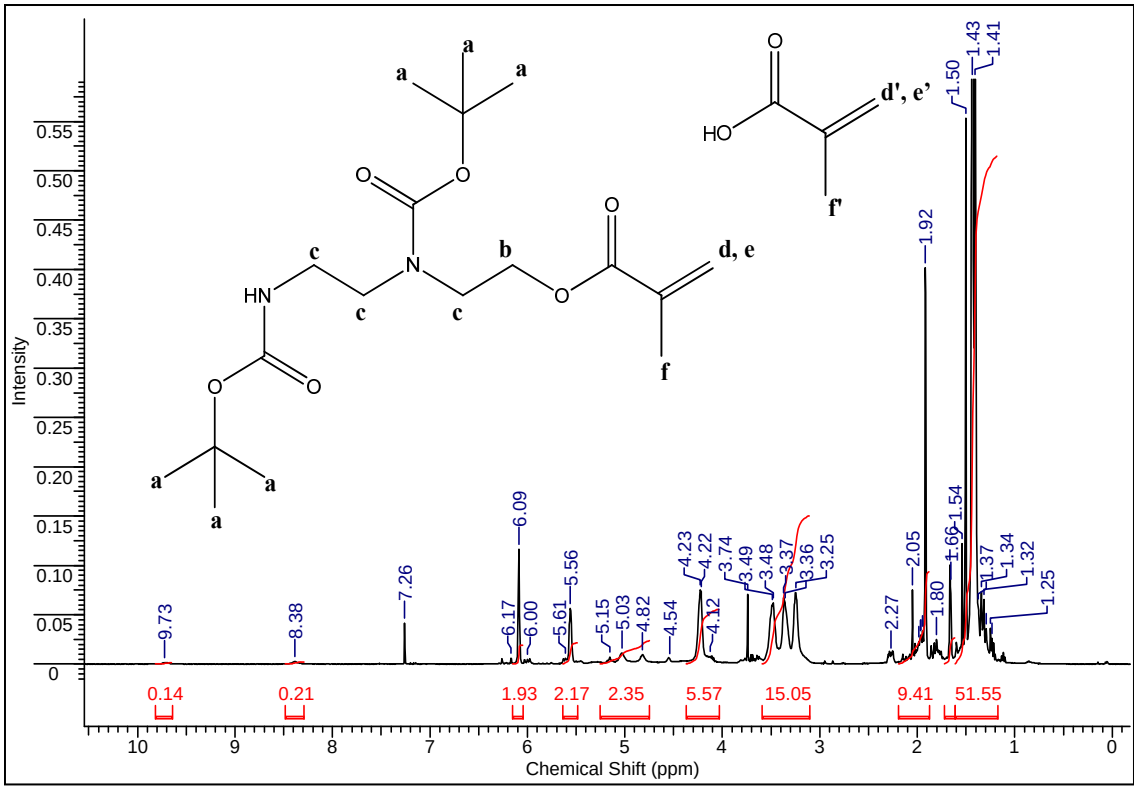

Figure A.4. [1]H-NMR spectrum of Methacrylated tert-butyl (2-((tert-butoxycarbonyl) amino)ethyl) (2-hydroxyethyl)carbamate after extraction

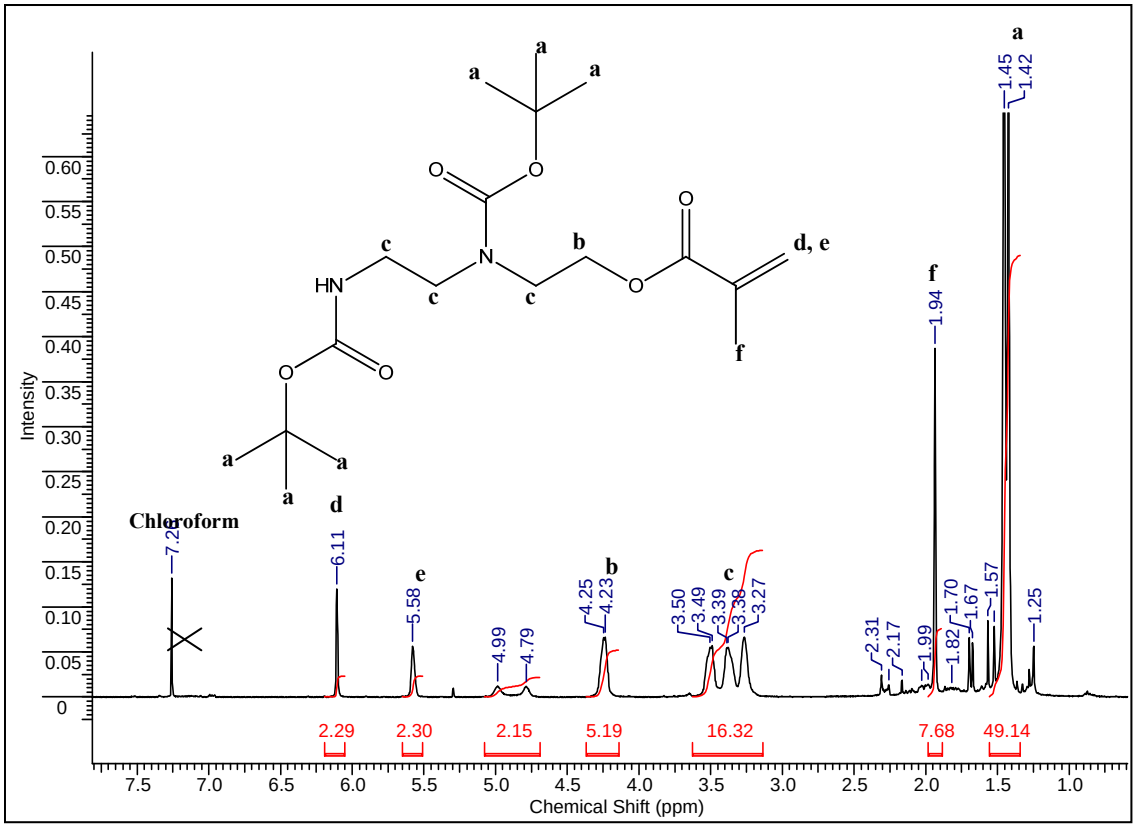

Figure A.5. [1]H-NMR spectrum of purified methacrylated tert-butyl (2-((tert-butoxycarbonyldamino)ethyl)(2-hydroxyethyl)carbamate by column chromatography method.

APPENDIX B

RAFT POLYMERIZATION OF 2-((TERT-BUTOXYCARBONYL) (2-((TERT-BUTOXYCARBONYL) AMINO) ETHYL) AMINO) ETHYL METHACRYLATE

Calculation of Monomer Conversion and Molecular Weight by Theoretical and NMR:

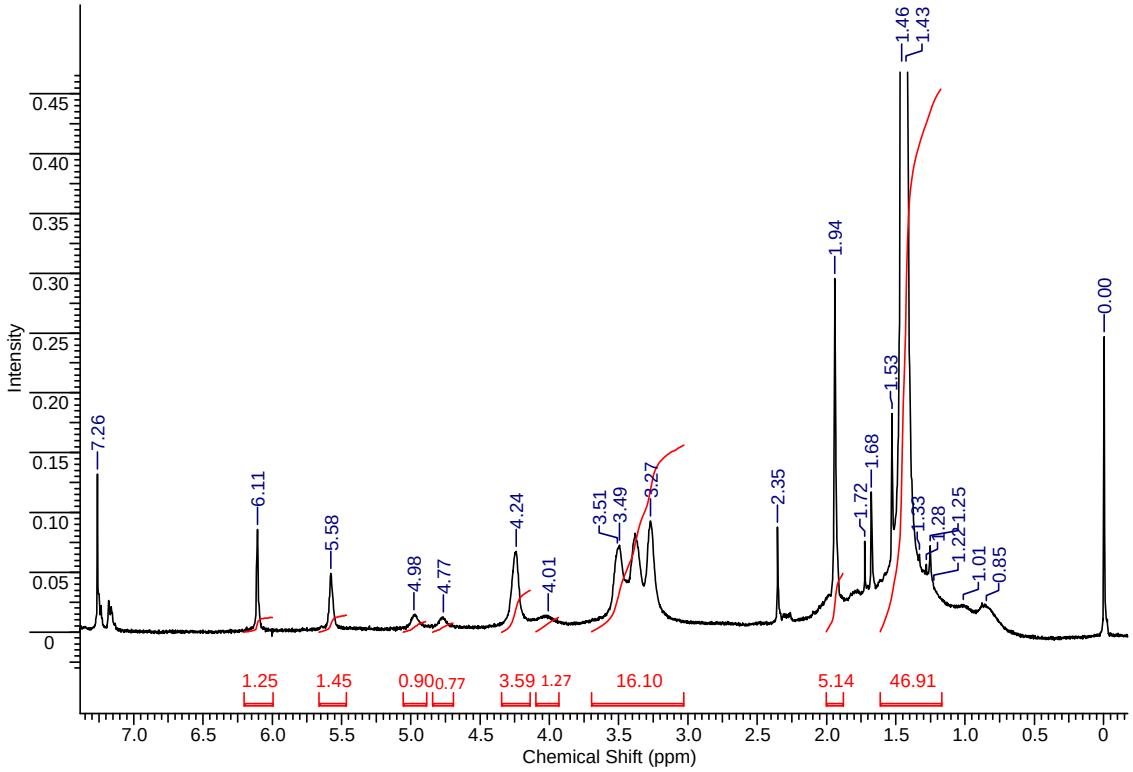

Figure B. 1. ^{1}H-NMR spectrum of polymerization mixture of p(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate), initial monomer concentration was 0.72 M and [M]/[R]/[I]:100/1/0.25. Reaction occurred at 65°C for 3h.

$$\text{Monomer Conv \%} = \frac{\frac{\left(I_{1H\text{ at }6.11ppm} + I_{1H\text{ at }5.58ppm}\right)}{2}}{\frac{\left(I_{2H\text{ at }4.24ppm} + I_{2H\text{ at }4.01ppm}\right)}{2}} \text{ x100} \qquad (3.3)$$

$$\text{Monomer Conv \%} = \frac{\frac{(1.25 + 1.45)}{2}}{\frac{(3.59 + 1.27)}{2}} \text{ x100} = 55.6\%$$

$$M_{n_{(theo)}} = \frac{[M_0]}{[RAFT_0]} x Mw_{monomer} x \text{ Conv \%} + Mw_{RAFT} \qquad (2.1)$$

$$M_{n_{(theo)}} = \frac{0.72 \text{ M}}{0.0288 \text{ M}} x 372.46 \frac{g}{mol} x\ 0.556 + 279.38 \frac{g}{mol} = 5457 \text{ g/mol}$$

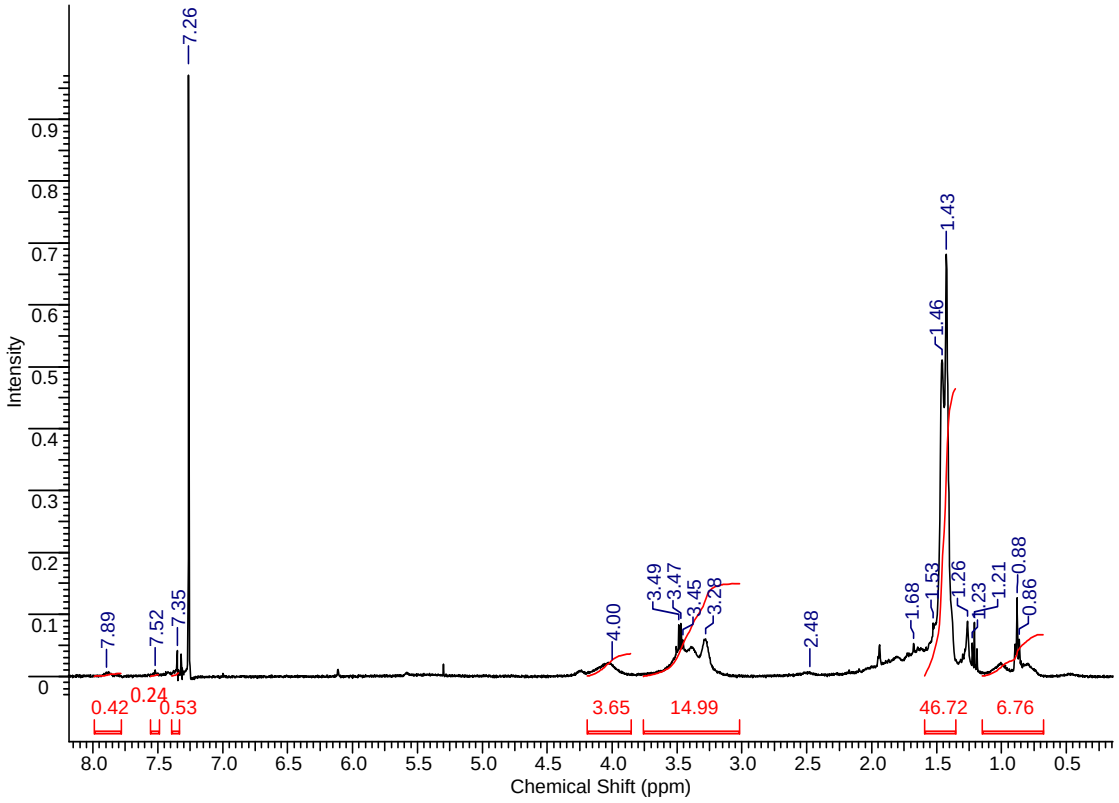

Figure B. 2. [1]H-NMR spectrum of purified P(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate). Initial monomer concentration was 0.72 M and [M]/[R]/[I] was 100/1/0.25.

$$Mn_{NMR} = \frac{\frac{I_{@4.01ppm}}{2}}{\frac{I_{@7.73ppm} + I_{@7.52ppm} + I_{@7.35ppm}}{5}} \text{x } Mw_{monomer} + Mw_{RAFT} \qquad (3.4)$$

$$Mn_{NMR} = \frac{\frac{3.65}{2}}{\frac{0.42 + 0.24 + 0.53}{5}} \text{x } 372.46 \frac{g}{mol} + 279.38 = 3135 \text{ g/mol}$$

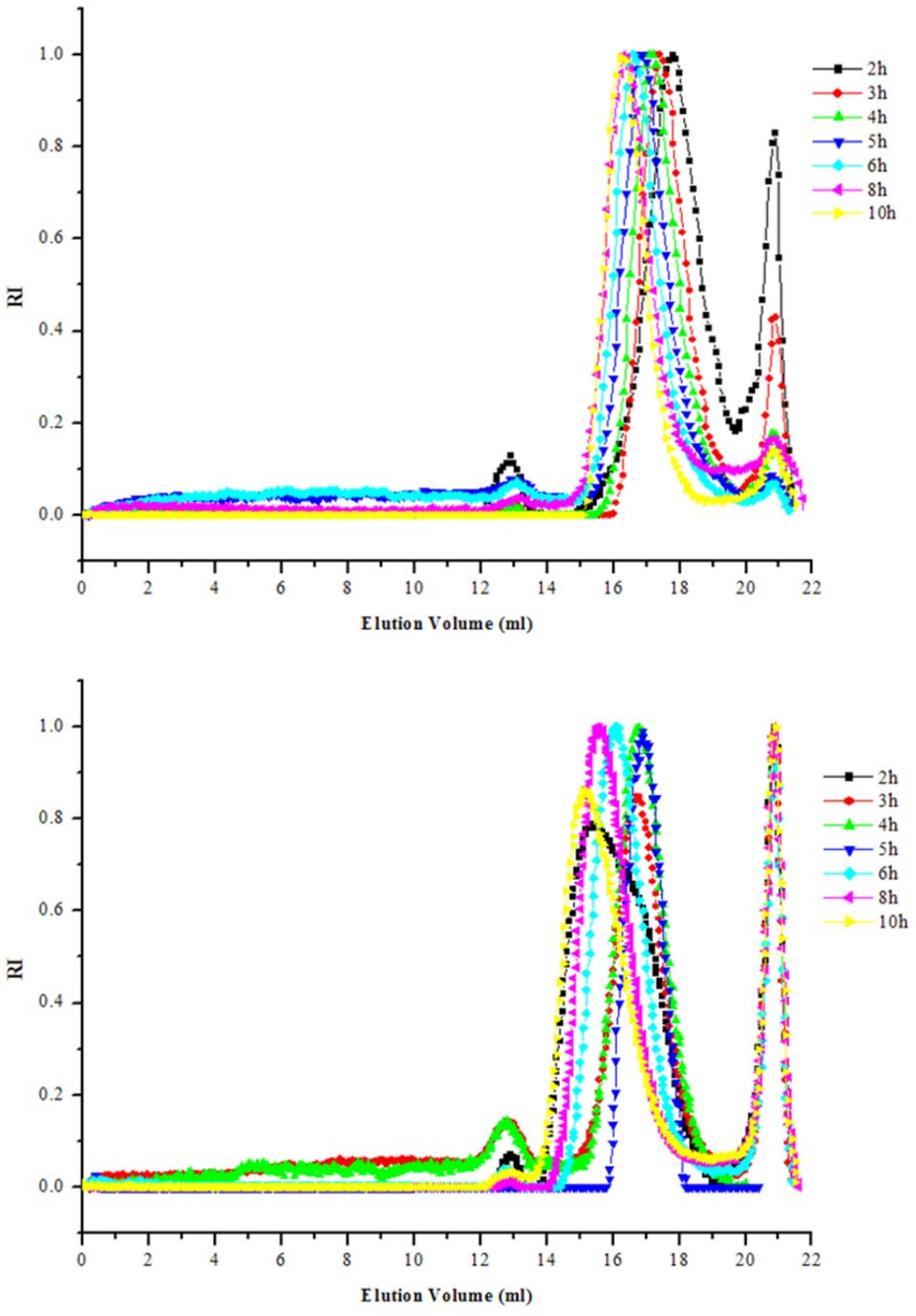

Figure B. 3. GPC chromatograms of polymerization mixture when monomer concentration was 0.36M and [M]/[R]/[I] ratios were A) 25/1/0.25 and B) 100/1/0.25.

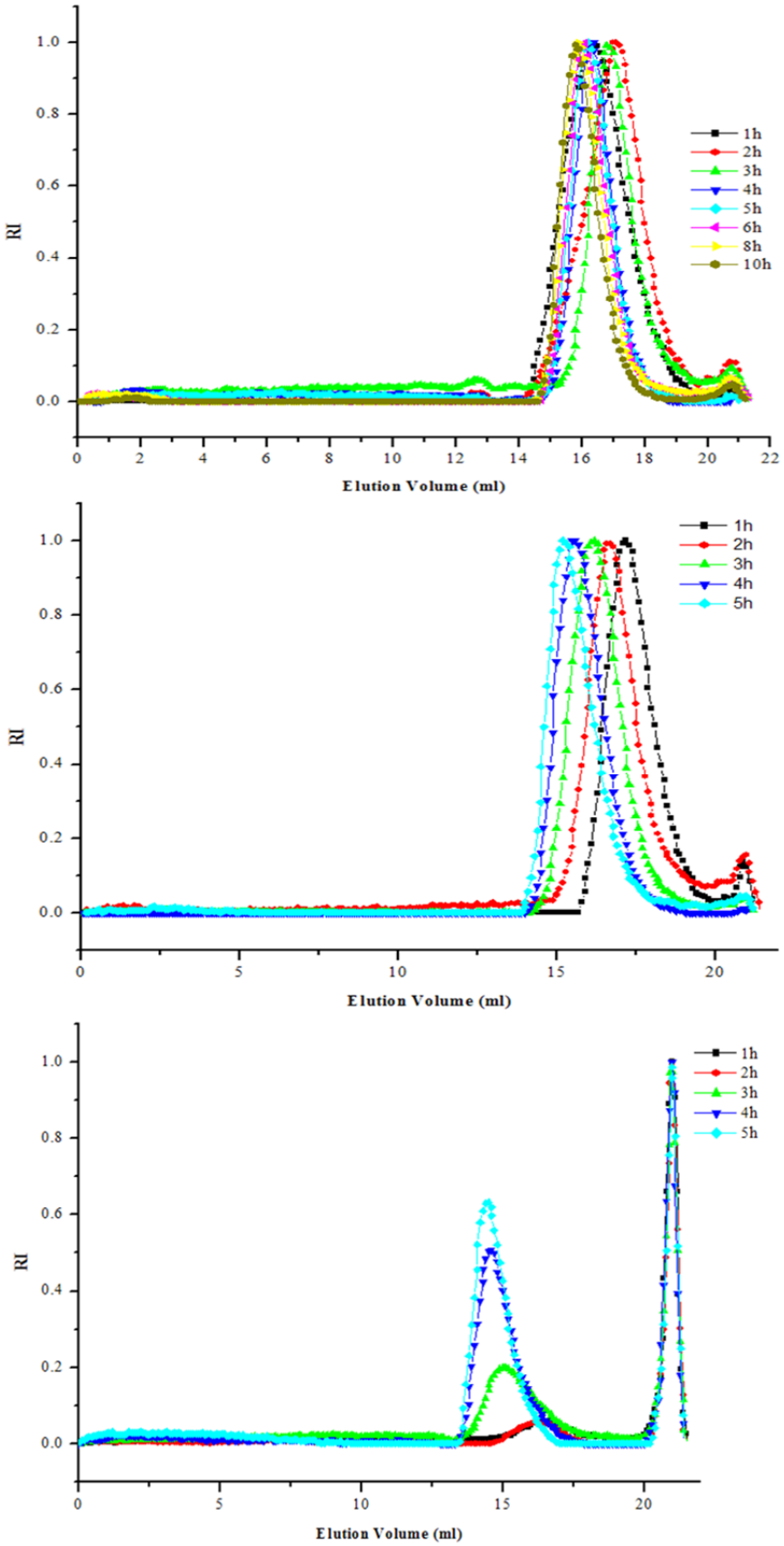

Figure B. 4. GPC chromatograms of polymerization mixture when monomer concentration was 0.72M and [M]/[R]/[I] ratios were A) 25/1/0.25, B) 50/1/0.25 and C) 100/1/0.25.

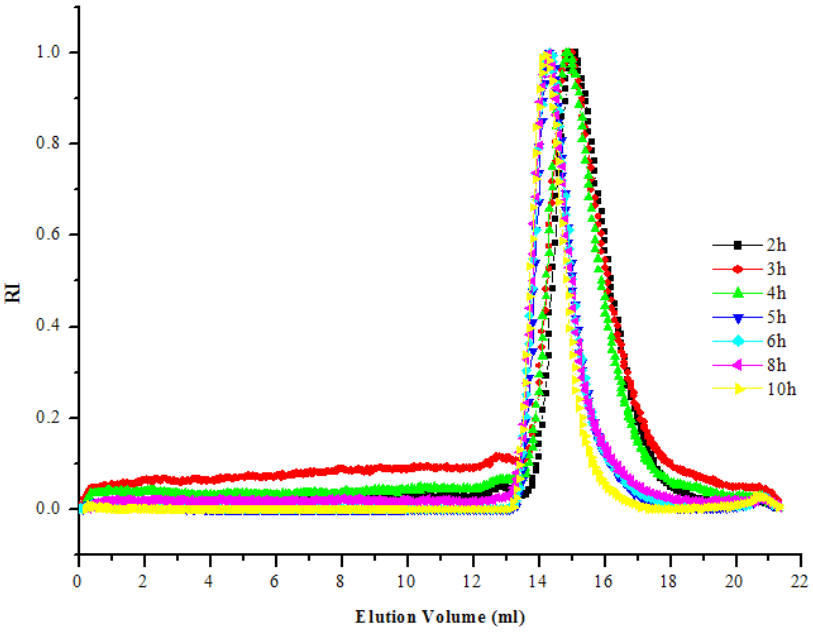

Figure B. 5. GPC chromatograms of polymerization mixture when monomer concentration was 1.44M and [M]/[R]/[I] ratios was 100/1/0.25.

APPENDIX C

HYDROLYSIS AND AMINOLYSIS OF 4-CYANO-4-(PHENYLCARBONOTHIOYLTHIO) PENTANOIC ACID (RAFT AGENT)

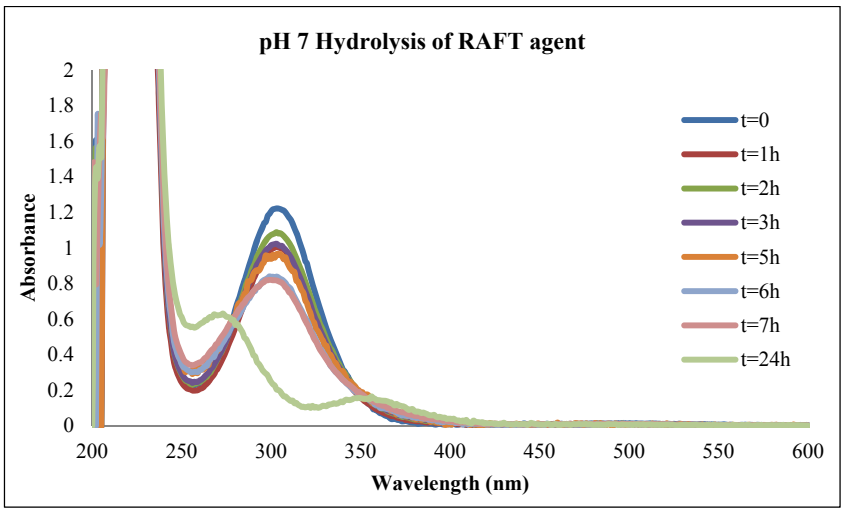

Figure C.1. UV spectra of hydrolysis of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) with respect to time at pH 7.4 and 65°C

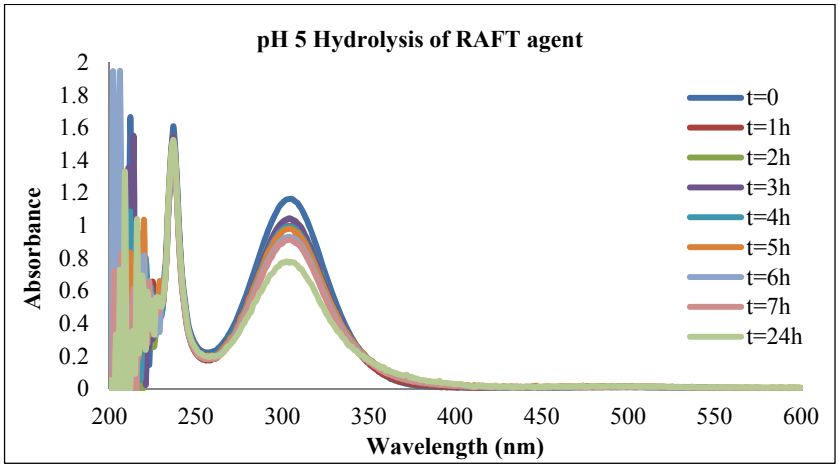

Figure C.2. UV spectra of hydrolysis of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) with respect to time at pH 5.2 and 65°C.

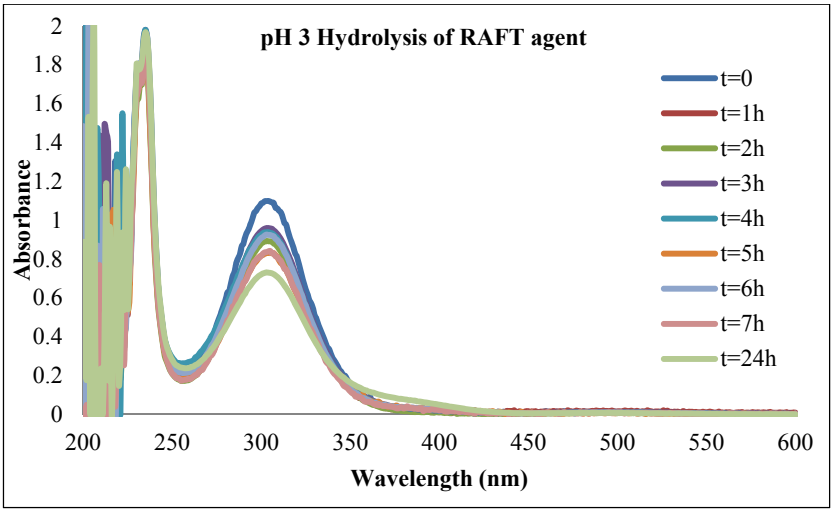

Figure C.3. UV spectra of hydrolysis of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) with respect to time at pH 3 and 65°C.

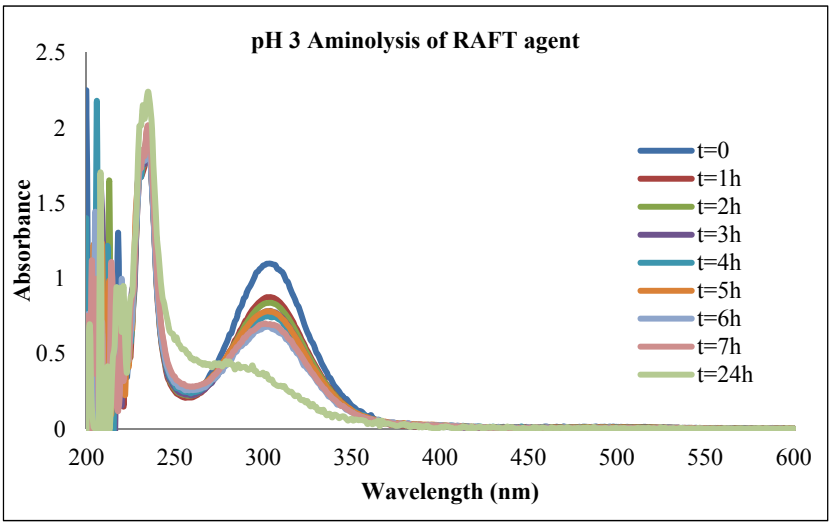

Figure C.4. UV spectra of aminolysis of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) with respect to time in presence of N-hydroxyethylethylenediamine at pH 3 and 65°C.

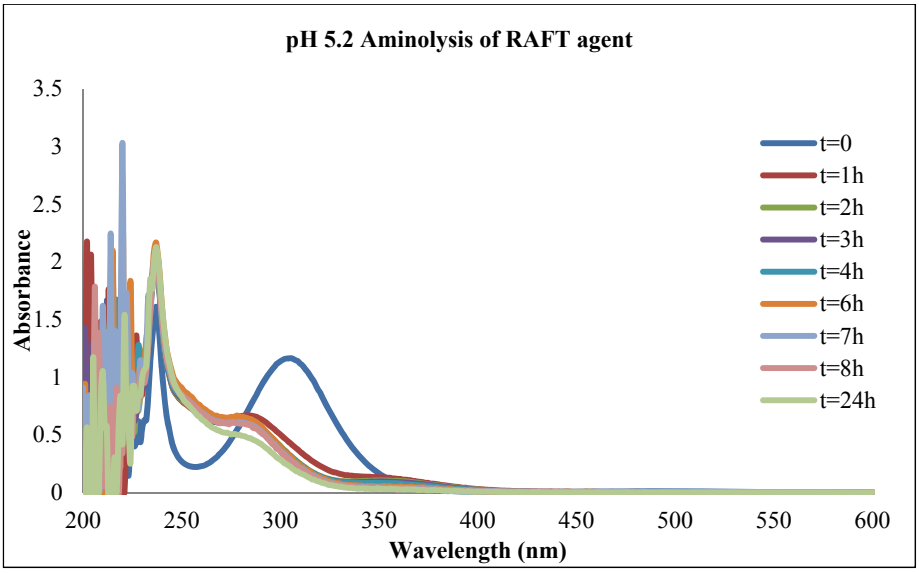

Figure C.5. UV spectra of aminolysis of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) with respect to time in presence of N-hydroxyethylethylenediamine at pH 5.2 and 65°C.