SYNTHESIS AND RAFT POLYMERIZATION OF ARGININE CONTAINING MONOMER TO INVESTIGATE THE CELL MEMBRANE TRANSLOCATION

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by
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ABSTRACT

SYNTHESIS AND RAFT POLYMERIZATION OF ARGININE CONTAINING MONOMER TO INVESTIGATE THE CELL MEMBRANE TRANSLOCATION

In this study, a higly cationic biosynthetic polymer, poly(Arginine Methyl Ester Methacrylamide) (p(AMME)) has been designed as a potential component of intracellular delivery systems for biological macromolecular therapeutics such as nucleic acids. Accordingly, an arginine derivative monomer; Arginine Methyl Ester Methacrylamide (AMME) was synthesized by the reaction of an active ester monomer, pentafluorophenylmethacrylate (PFMA) and the L-arginine methyl ester hydrochloride (AME) in the presence of excess triethyl amine. AMME was then polymerized via both conventional free radical polymerization and reversible addition-fragmentation chain transfer (RAFT) polymerization. The polymers p(AMME) were characterized via nuclear magnetic resonance and gel permeation chromatography techniques. The conventional free radical polymerization experiments yielded polymers with uncontrolled molecular weights and wide molecular weight distribution, whereas RAFT polymerizations performed both in aqueous solution and organic solvent yielded polymers with controlled molecular weights and narrow molecular weight distributions. The RAFT polymerization kinetic experiments showed the RAFT-controlled character of AMME polymerization in the presence of 4-cyano-4-(ethylthiocarbonylthioylthio) sulfanylpentanoic acid (ECT) as a RAFT agent. The cytotoxicity of P(AMME) before and after aminolysis was determined via MTT assay using A549 human lung cancer cell line. P(AMME) before aminolysis of the RAFT end-group displayed dose-dependent toxicity after 24 hours incubation with cells. It was highly toxic to cells at 25 μM concentration, killing almost more than 60% of cells after 24 hours incubation. On the other hand, the aminolyzed polymer has no significant toxicity in the concentration range studied (upto 500 μM), which was comparable with octaarginine, a widely used transfection agent. In conclusion, well-defined arginine-polymers synthesized in this study show potential for further investigations as potential components of intracellular delivery systems for therapeutics.

ÖZET

HÜCRE MEMBRANINDAN TRANSLOKASYONU İNCELEMEK İÇİN ARJİNİN İÇEREN MONOMERİN SENTEZİ VE RAFT POLİMERİZASYONU

Bu çalışmada, oldukça katyonik biyosentetik polimer olan poli(Arjinin Metil Ester Metakrilamid) (p(AMME)) , nükleik asit gibi biyolojik makromoleküllerin taşınmasını sağlayan ilaç taşınım sistemlerinin potansiyel bir parçası olarak dizayn edildi. Bu amaçla, arjinin türevi monomer; Arjinin Metakrilmaid Metil Ester (AMME) aktif ester monomer, Pentaflorofenil Metakrilat (PFMA) ve L-Arjinin Metil Ester Hidroklorür (AME)'ün aşırı trietilamin varlığındaki rekasiyonu ile sentezlendi. AMME, sonrasında hem serbest radikal polimerizasyonu ile hem de Tersinir Ekleme-Parçalanma Zincir Transferi (RAFT) polimerizasyonu ile polimerleştirildi. Polimerler p(AMME) Nükleer Manyetik Rezonans Spektrometresi ve Jel Kramatografisi aracılığıyla karakterize edildi. Geleneksel Serbest Radikal Polimerizasyonu deneyleri kontrol edilemeyen moleküler ağırlıkta ve geniş moleküler ağırlık dağılımına sahip polimerlerle sonuçlandı, oysa ki organik çözücülerde ve sulu çözeltilerde gerçekleşen RAFT polimerizasyonları kontrollü moleküler ağırlıkta ve dar moleküler ağırlık dağılımına sahip polimerler sonulandı. RAFT polimerizasyonu kinetik deneyleri RAFT kontrollü karakterini, 4-siyano-4-(etiltiokarboniltioyltio) sülfanil pentanoik asit (ECT)'nin RAFT ajanı olarak kullanıldığı polimerleşmelerde gösterilmiştir. P(AMME), RAFT uç grupları aminoliz olmadan önce 24 saatlik hücre inkubasyonu sonucunda doza bağlı bir toksisite göstermiştir. 25µM hücreler için oldukça toksik olup, %60'dan fazla hücreyi 24 saatlik inkubuasyon sonrasında öldürmüştür. Diğer yandan, aminoliz olan polimer önemli sayılacak bir sitotoksisite göstermemiştir (500µM'a kadar), ki bu oktaarjinin sıkça kullanılan bir transfeksiyon ajanı ile kıyaslanabilir. Sonuç olarak, iyi tanımlı arjinin polimerleri bu çalışmada ileri araştırmalar için potansiyel hücreiçi ilaç taşınım sistemlerinin bir parçası olacak şekilde sentezlenmiştir.

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CHAPTER 1

INTRODUCTION

Intracellular delivery of macromolecular therapeutics is one of the most important concerns in biotechnology. Not every therapeutic can efficiently enter cells, due to the size, solubility and chemical properties. Such therapeutics need viral or non-viral (synthetic) agents for delivery into cells. Since most macromolecular therapeutics and nanosize carrier systems are taken into cells by endocytosis via proteoglaycan receptors, designed carrier systems need to be in accord with and also utilize characteristics of the cellular membranesand intracellular pH changes for an efficient delivery.

Octaarginine (R8) is a peptide derived from viral TAT protein and frequently used in intracellular drug delivery due to its ability to cross cellular membranes, relatively low toxicity and low immune response. Here in this study, we designed a new arginine containing monomer, Arginine Methacrylamide Metyhl Ester (AMME), to obtain well defined arginine polymers via reversible addition-fragmentation chain transfer (RAFT) polymerization as a potential arginine-mimicking system for intracellular drug delivery applications.

In this study P(AMME) is synthesied by RAFT polymerization to control molecular weight of the polymers at different medium. Those polymers are used for polymer-cell interaction study, MTT Assay. Toxicity of RAFT polymers are defined in the wide range of polymer concentration which is compromising for the conjugation with most of the drugs.

CHAPTER 2

LITERATURE REVIEW

2.1. Intracellular Drug Delivery

Phospholipids are lipids composed of the hydrophilic phosphate groups and the hydrophobic alkyl chain, covalently attached to each other. Phospholipids in aqueous solutions self-assemble intobilayer systems by bringing their hydrophobic tails together and exposing hydrophilic head groups to solution. Majority of cell membranes is composed of phospholipids. Phospholipid bilayer covers cell and seperates cytosol and organnels of the cell from the hazardaous extracellular enviroment (Ohki & Aono, 1970).

Certain therapeutics such as nucleic acid-based therapeutics show their activity in the cytosol or nucleus of the cell. Either way therapeutics need to pass through phospolipid barriers. Crossing mammalian cell membrane depends on the physicochemical properties of the therapeutics. Small, non-polar organic molecules can passively diffuse through the phospholipid-based cell membrane, whereas small polar organic or inorganic molecules need enegry (Adenozine Tri Phosphate, ATP) to cross the cell membrane via Active transport through channels or transporters (Chaturvedi & Kurup, 1986; Singer, 1979). Additionally, in the last twenty years, more effective and complex macromolecular arthitectures such as nucleic acid- and protein-based molecules have been designed as better therapeutics.However, cell membrane is impermeable also to those large and polar molecules.Similarly, most of nanoparticulate drug delivery vehicles such as gold nanoparticles, polymeric micelles and nanoparticles, cannot cross cellular membranes due to their large supramolecular structures. Those therapeutics and delivery vehicles which are not able to cross the cell membrane need biocompatible, non-toxic membrane destabilizing agents to pass through cellular membranes and reach into the cytosol of cell. Oligonucleotides (small interfering RNA, plasmids etc.), proteins (hormones, peptides, enzymes etc.), even magnetic beads can be transported through cellular membranes by such agents (Begley, 1994; Boletta et al.,

1996; Federico et al., 1996; Lebolch et al., 1995; Verhoef, Schipper, Romeijn, & Merkus, 1994).

Macromolecules and nanoparticles are generally taken into cells via endocytosis by the proteoglycan receptors (F-actin, caveolin, clathrin) as it is seen in Figure 2.1. Based on the mediating receptor type endocytosis is classified as clathrin dependent, raft/caveolin dependent or macrocytosis (Aplin & Hughes, 1981; Salisbury, Condeelis, & Satir, 1981). Endocytosis is not an internalization or cell uptake mechanism. It is rather a 'digestion' mechanism of cell. When a macromolecule is recognized by the receptors on the cell surface, cell membrane ruffles and forms a vesicle, called endosome by covering macromolecule. The first form of the vesicle is called early endosome with the pH of nearly 6.5. Late endosomes where pH is around5 are then formed (Jimbow et al., 1997; Lemichez et al., 1997; Morre, Peter, Morre, & Van Alstine, 1998). Those changes in the pH can be used to trick theescape mechanism of therapeutics, before combination of endosomes with lysosomes which are full of degradation enzymes such as proteases, nucleases, esterases, etc. Macromolecules can also be directly transported through the cell plasma membrane via energy independent pathways, if the macromolecule or membrane destabilizing agent has the proper helicity, amphilicity and chemical composition (Bell et al., 2003; Funhoff, van Nostrum, Koning, et al., 2004; Lindsay, 2002; Paul et al., 2003).

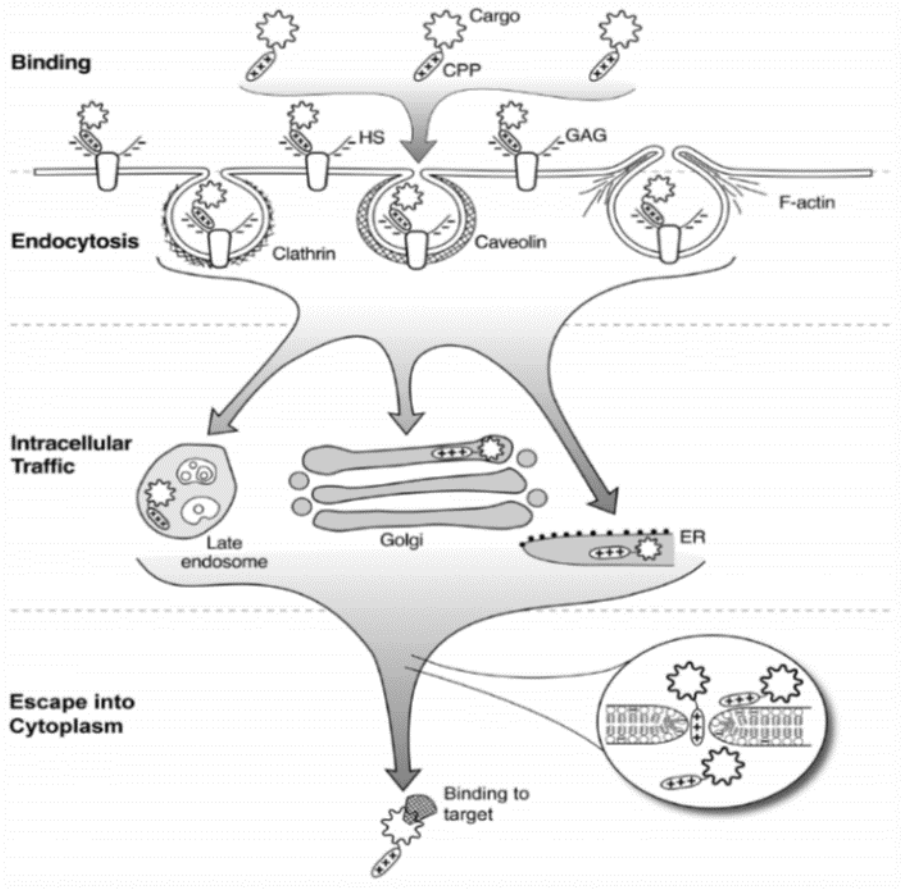

Figure 2.1.Illustration of cargo delivery conjugated to cationic cell penetrating peptides (CPPs) through the endosome via proteoglaycan receptors. (Source:Melikov & Chernomordik, 2005)

2.1.1. Intracelullar Drug Delivery Systems

Endosomal escape is a key tool for intracellular drug delivery that involes destroying the late endosome membrane via different mechanisms before lysosome conjugation to endosomal vesicle (Varkouhi, Scholte, Storm, & Haisma, 2011). Still, some of the systems generated by the researchers based on preventing activity of the lysosomal enzymes, in stead of endosomal escape via lysomotrophic agents (Tietz, Yamazaki, & Larusso, 1990). Andalousi et al. 2006 reported the enhanced internalization and activity of splice correcting peptide nucleic acids (PNAs) conjugated with the cell penetrating peptides (CPPs), Tat, penetrain and transportan. PNAs are synthetic molecules that are not affected by proteases and nucleases and can be complimentary to DNA or RNA sequences selectively due to the lack of electrostatic repulsions created by the phosphate groups. However PNAs's intracellular uptake is

very poor showing low bioavaibility. Thus, they are generally conjugated to CPPs. PNA-CPPs uptake is mediated by the endocytosis. (El-Andaloussi, Johansson, Lundberg, & Langel, 2006).

Viruses and bacteria generally disturb endosomal membrane by viral peptides or exotoxins (for bacteria) (Miller, Griffiths, Lenard, & Firestone, 1983). Viral vectors can disrupt endosom vesicles in different ways depending on the type of the viral vector. Some viruses have fusogenic peptides on their surface. For example, Haemagglutin is a protein found in the coat of the influenza virus. It is found in coil conformation at physiological pH. At endosomal pHs its conformation changes into helical, hydrophobic state that leads to the fusion with the endosome membrane(Wiley & Skehel, 1987).

It is also found that some photosentizers are capable of disrupting endosomal membrane by light activation, called PhotoChemical Internalization (PCI). In 2009 Cabral et al. showed the photochemical releasing of the camptothecin (CPT) inside polymer micelles of poly (ethylene glycol)-*b*-poly (glutamic acid) with dendrimer phthalocyanide (DPc). DPc generally localizes at the surface of the endosomal membrane, and in the presence of irradiant creates singlet oxygen which destroys endosomal membrane and releases the camptotechin. (Cabral et al., 2009)

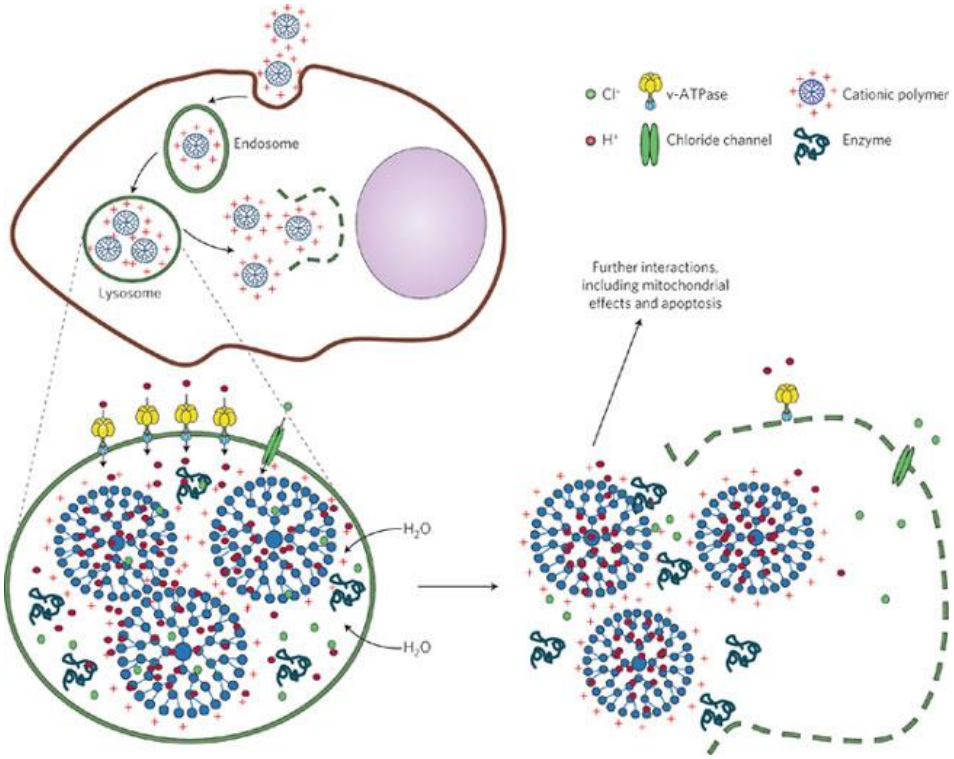

Figure 2.2. Illustration of ‘proton sponge effect’.
(Source:Nel et al., 2009)

Proton sponge effect or pH buffering effect is another endosomal escape pathway encountered, as it can be seen in Figure 2.2. This phenomena can be mediated by viral or non-viral agents only if they have a buffering capacity betweeen pH 5 and 7.4 at cationic stage. After cationic delivery system is taken up by endosome vesicles, it attracts protons like a sponge. Upon this, proton pumps present on the endosome membrane pump protons into the endosome. Chloride (CI^-) ions also enter endosome to balance charge density. Following that, water molecules diffuse into endosome vesicle to balance the osmotic pressure created by the ions. After a point (as the pH of endosome decreases, protonation of the molecule increases, Cl^- pass increases, consequently water passing increases) membrane can not resist against increasing pressure on the membrane and ultimately ruptures, releasing all the materials in the vesicle to cytosol (Pozharskii & Ozeryanskii, 2012). In 2014 Kurtuluş et al. showed the synthesis of a biosynthetic spermine-like polymer, poly(2-(((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl)amino) ethyl methacrylate (p (BocAEAEMA)) synthesized via Reversible Addition Fragmentation Chain Transfer Polymerization (RAFT). P(BocAEAEMA) was deprotected to poly(2-(aminoethyl) amino) ethyl methacrylate P(AEAEMA). P(AEAEMA) showed buffering capacity between pH 5.0 and 7.4 and showed promising cell viability on NIH 3T3 cells compared to widely used poly(ethylene imine) cationic polymer. They also formed polymer-DNA polyplexes at different N/P ratios to show the condensation of DNA with the cationic polymer. (Kurtulus et al., 2014)

2.2. Polyarginine in Intracellular Drug Delivery Systems

Certain peptides have an ability to pass through the membranes without disturbing the cell. Those kind of peptides are called cell penetrating peptides (CPPs) or protein transduction domain peptides (PTD). CPPs have been generally derived from the TAT, penetratin and oligoarginine species (Kardinal et al., 1999; Lindgren, Hallbrink, Elmquist, et al., 2000; Lindgren, Hallbrink, Prochiantz, & Langel, 2000). CPPs have been divided into different groups according to their chemical structure and physico-chemical properties. These are (i) basic aminoacids carrying peptides; HIV-1 Tat protein and oligoarginine residues are examples, (ii) amphiphilic peptides composed of basic or acidic and hydrophobic minoacids; penetratin which is a peptide segment

between 43 and 58 amino acid residue of the *Drosophila* antennapedia protein, is an example in this class, (iii) chimera type is a neuropeptide galanin and bee toxin protein mastoparans related segment transportan are examples for that type of CPP, (iv) the last type of the CPPs is the hydrophobic peptides. eMmbrane translocating segment of the fibroblast growth factor is an example (Futaki, 2006).

Recent studies generally focused on the arginine-rich peptides due to their high transfection and low toxicity properties. A computational investigation through Swissprot Databaseon proteins which carry more than seven arginine residues in their primary sequence revealed the existence of 591 proteins. Interestingly, it was found that 295 of these proteins were identified as viral coat of the viruses and others were generally RNA-DNA binding proteins, strongly suggesting a relation between oligoarginine molecules and cell transfection process (Vives, Macian, Seguer, Infante, & Vinardell, 1997). In the same context, HIV carries an arginine rich peptide, i.e. TAT peptide, on its coat. Since arginine rich viral proteins do not create any strong immune response and show low cytotoxity at infected cells, overcoming HIV and curing AIDS have been a big challenge. These drawbacks for HIV and other viral infections can be translated to an advantage for delivery systems by mimicking those proteins in structure and property. However there still is a big question need to be answered; How can arginine-rich peptides, which are basic and hydrophilic molecules, easily pass through the hydrophobic biological membranes? So far this has been explained based on different evidences, however, the nature of arginine molecule itself is the answer to the question.

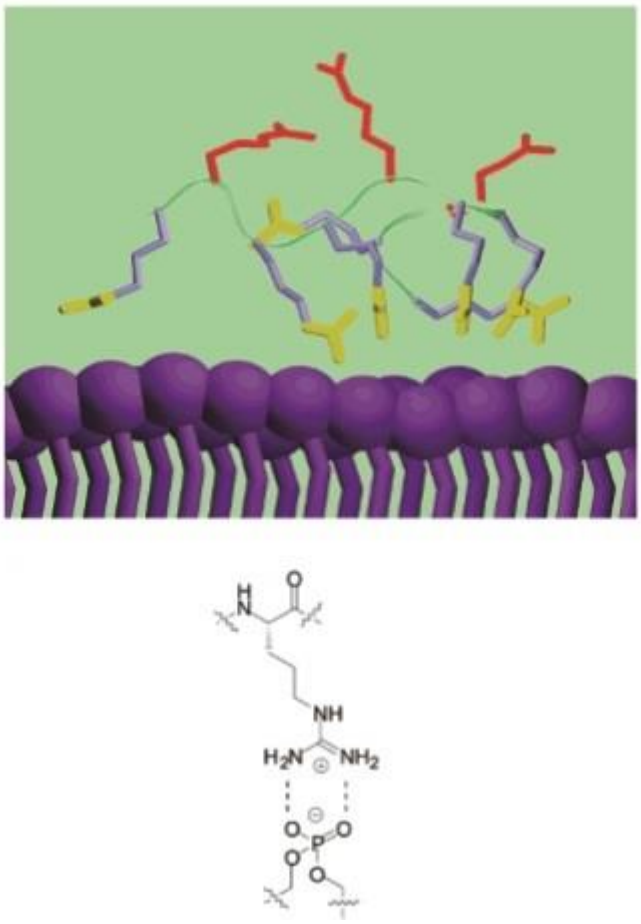

Figure 2.3. Illustrated electrostatic interaction and hydrogen bond formation of arginine's guanidine group and the phospholipid bilayer of the cellular membrane. (Source:Rothbard et al., 2002)

Figure 2.3 describes the interaction of arginine and membrane lipid bilayer. The group on the arginine structure which creates a bidentate hydrogen bonding with the lipid molecus in Figure 2.3 is called 'guanidine'. Actually guanidin is the key of the arginine. Guanidine group makes the residue hydrophilic and provides solubility to the arginine containing systems in the plasma. Again this group carries primary and secondary amines with delocalized resonance structure making polyarginine residues always cationic at the body pH. In 2003 Luedtke et al. carried out very valuable studies by comparing amine, guanidine and arginine, in order to understand which structure has the key role for cellular uptake of the drugs. They labelled an antibiotic, Tobra with a flourophore BODIPY and added amine, guanidine or octaarginine to the same group on the drug structure. Tobra-BODIPY-guanidine conjugate was found to have the highest cellular uptake. The uptake of Tobra-BODIPY-octaarginine conjugate was close to that

of guanidine conjugate according to both flourescence activated cell sorting (FACS) and flourescence microscopy results. It is found that amine conjugates are not taken up into cells whereas guanidine and octaarginine conjugates show similar high cellular uptake. These results suggest that guanidine group enables the cellular uptake of the arginine-containing peptides (Luedtke, Carmichael, & Tor, 2003). Ryser et al. showed for the first time that polycations of amino acids deliver the radio-labelled albumin to the tumor cell lines more effectively than the neutral or anionic polymers of amino acids do (Ryser, 1971). In 2000, it was found that homopolymers carrying more than six L-arginine residues show more cell internalization than other cationic homopolymers of the same length including lysine, ornithine and histidine oligomers (Wender et al., 2000).

Studies showed that arginine rich CPPs do not harm the plasma membrane and show low toxicity (Futaki, 2005). Using arginine rich CPPs, proteins, oligonucleotides; small DNA or RNA fragments, magnetic beads, liposomes have been delivered into cells. (Fawell et al., 1994) Nakase et alshowed that as the number of arginine residues in the peptide structure increases, internalization of the peptide into cell also increases. However when the peptide contains more than 8 arginine residues, cytotoxity of the peptides on cells increases. The internalization of an arginine containing peptide with increasing arginine residues by cells can be seen in Figure 2.4. In the same study the authors also showed the effect of chirality of the peptides on internalization and cytotoxity. L-octaarginine (L-R8) and D-octaarginie (D-R8) did not show remarkable differences for internalization aspect, however D-R8 was found to be slightly more toxic than the L-R8 peptide.

Endocytosis is still the most possible pathway for internalization of the arginine-rich CPPs. Cytochalasin D is the inhibitor of the F-actin polymerization on the cell membrane which plays role as a receptor in endocytosis. Ethylisopropylamiloride (EIPA) is also inhibitor of macropinocytosis. In the presence of those inhibitors separately, the internalization of arginine rich CPPs into cells decreases. These results suggest that arginine rich CPPs are taken by cells via energy or endocytosis dependent pathway. However, experiments repeated at 4°C also showed arginine-rich CPP internalization by cells, indicating that arginine-rich peptides use both energy dependent and independent pathways while crossing the cellular membrane (Nakase et al., 2004).

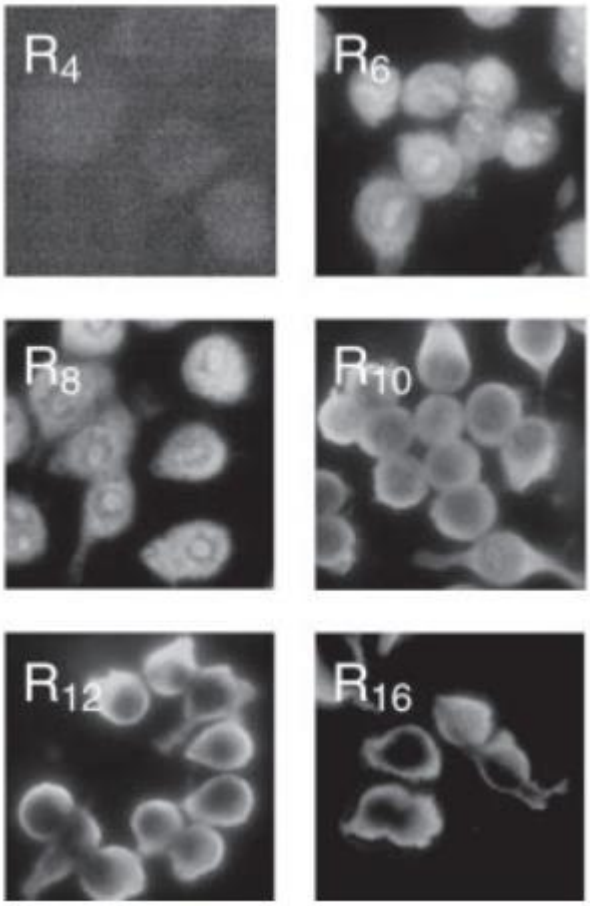

Figure 2.4. Flourescence microscope images of RAW264.7 cells treated with flourescent labelled polyarginine peptides (1 μM) with increasing arginine content (R_4, R_6, R_8, R_{10}, R_{12}, R_{16}) for 3 hours (Source:Futaki et al., 2001).

In another study, branched and linear structures of arginine-rich CPPs with varying number of arginine residues were compared. Only the number of arginine residues in the chain was found to be important for internalization of the peptides by cells. Both linear and branched peptides showed similar cellular uptake profiles when they carry the same number of arginie residues in their structure. Cell viability experiments showed that arginine-rich peptides having branches structure were more cytotoxic and branched or linear peptide chain with arginine residues more than 8, showed higher cytoxicity (Figure 2.5) (Suzuki et al., 2002).

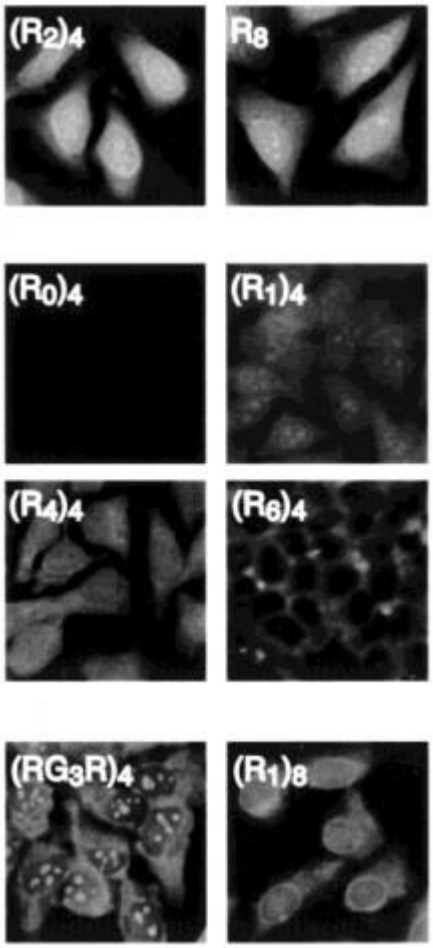

Figure 2.5.Celullar internalization of the branched and linear flourescent labelled polyarginine peptides by *HeLa* cells. (Source:Futaki, Nakase, Suzuki, Zhang, & Sugiura, 2002)

All these studies create a strong reason for us to synthesize well-defined poly(arginine) mimicking biosynthetic polymers and compare the cytotoxicity and cellular uptake profiles of these polymers with octaarginine peptide as a reference CPP.

2.2.1. Biosynthetic Poly(arginine) Mimicking Polymeric Systems

After revealing the potential of TAT protein and its derivatives in intracellular drug delivery, many research groups have focused on developing guanidine containing, cationic, biosynthetic, poly(arginine) mimic polymers. Different routes that have been followed to obtain poly(arginine) mimic polymers include the synthesis and

polymerization ofguanidine-containing monomers, post-polymerization modification of known functional polymers (e.g. PEG) with guanidine or arginine residues, or modification of peptides. In 2004 Funhoff et al. synthesized 2-methacrylic acid 3-guanidino propyl ester (Figure 2.6) and performed its free radical polymerization in aqeous media at pH 5. The authors used polymers with varying compositions to prepare polyplexes with DNA at varying nitrogen: phosphate (N/P) ratios. Zeta potential and dynamic light scattering measurements and cell infection studies showed that as the cationic charge on the polyplex surface inceresed (high N/P ratio) internalization of those polyplexes by COS cells also increased. It was also stated that the polyplexes entered cells via endoctic pathways, as there was no internalization of polyplexes at 4 °C (Funhoff, van Nostrum, Lok, et al., 2004). In 2011, Treat et al. used the monomer 3-guadinopropyl methacrylamide (GPMA) to obtain cell infecting polymers via Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization. Both GPMA homopolymers and HPMA-*b*-GPMA block copolymers were synthesized. 4-cyano-4-(eyhylsulfanylthiocarbonylsulfanyl) pentanoic acid (CEP) was used as a chain transfer agent and 4,4′-Azobis(4-cyanovaleric acid) as an initiator in sodium acetate-acetic acid buffer at pH 5.2. RAFT polymerization kinetics showed linear increase in molecular weight with monomer conversion and a good control over both the number average molecular weight and molecular weight distribution (M_w/M_n). Cell internalization of FITC labelled polymers was investigated. Homopolymer of HPMA was not internalized by KB cells either at 4 °C or at 37 °C. Homopolymer of GPMA was internalized by KB cells at both temperatures. HPMA-*b*-GPMA block copolymers were internalized by KB cells more efficiently than GPMA homopolymer at both temperatures. Although the density of the internalized polymers in cells at 4 °C was low, there was still internalization. Thus, it can be said that both endocytic and non endocytic pathways are used by those polymeric systems to penetrate the cell membrane (Treat et al., 2012).

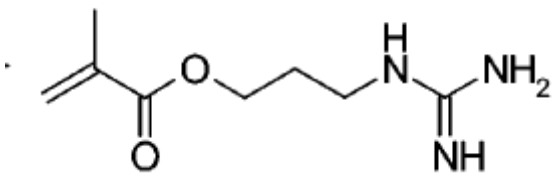

Figure 2.6. Structure of 2-methylacrylic acid 3-guanidinopropyl ester.

Penetrating peptide sequence is important for cellular uptake. However now it is understood that helicity and amphilicity of the polymer or peptid are also important for membrane intreaction. Amphilicity can be achieved by addition of hydrophobic blocks to the polymer. Helical peptide structure inreases the cellular uptake. This may not be a problem for natural CPPs such as TAT and penetrain but problem may occur for synthetic peptides (such as octaarginine) and polymers. In 2013, Tang et al. hypothesized that aif polyarginine mimic cancontain hydrophobic parts and helical secondary structure, it would yield a better system for intracellular drug delivery. Polyarginine itself forms expanded random coils at physiological pH due to the repulsion betweenguanidin side chains. To form a helical structure, polyarginine needs an environment with a pH valuehigher than pH 12.5 where it is completely deprotaned. Poly(γ-(5-aminohexyl)-L-glutamate) ($PAHG_{57}$) was designed for this purpose by placing amine groups 11 sigma bonds away from backbone to obtain helical structure and this long alkyl chain makes molecule amphiphilic at the same time. Cell uptake studies showed that helical, amphiphilic and polyarginine mimic molecule showed 2-4 fold greater cellular uptake compared to Arg_9 and Tat-1 peptides.

Polymeric drug delivery systems in micellar forms have been used to protect cargo molecules in their hydrophobic cores and supply favourable pharmacokinetics and biodistrubution of therapeutic molecules throughout body. Studies showed that cationic micelle delivery systems were more cytotoxic compared to zwitterionic ones (Kim, Binauld, & Stenzel, 2012). To achieve this barrier zwitterionic arginine mimic monomer synthesis was performed by treating arginine's alpha amine with the methacrylic anhydride to obtain Arginine Methacrylate (M-Arg). Triblock copolymers were prepared by RAFT polymerization. Poly(methyl methacrylate)-*block*-poly(ethylene glycol methyl ether methacrylate)-*block*-poly(M-Arg) was formed to generate zwitterionic micelles. Methylation of M-Arg to Methyl Arginine Methacrylate (Me-M-Arg) by converting carboxyl group of arginine to methyl ester resulted in the formation of micelles having cationic effect due to the absence of the carboxyl group of arginine. Cell viability results showed that zwitterionic micelles had nearly 100 % cell viability wheras cationic micelles caused significant cell death. Another micelle based delivery system was studied by Qi et al., (2012) for delivery of siRNA. Poly (ethylene glycol)-*block*-poly(ε-caprolactone)-*block*-poly(L-lysine) (mPEG-*b*-PCL-*b*-PLL, AG_0) triblock copolymer was synthesized and the amine groups of lysine was changed with guanidine group by reaction of polymer with 1-guanyl-3,5-dimethylpyrazole

hydrochloride yielding AG_S polymers. Viability and cell uptake comparison of the polymer systems on HeLa human cervix cancer cells showed that guadinylated lysine carrying AG_S copolymers were less cytotoxic and showed efficient gene silencing functionality. Figure 2.7 shows the micelle structure and internalization of those polymeric systems into cells. As it is seen AG_0 Polymer block cannot escape from the endosomes whereas AG_S polymer block escape properly from the endosomal membrane. This study showed that the guadinylated lysine polymer conjugated siRNA gene knockdown was more efficient than the octaarginine conjugated siRNA systems. The only structural difference of the poly(arginine) and the guadinylated poly(lysine) is the distance of the guanidine group from the backbone. For guadinylated poly(lysine) guadine group is one carbon farther away from the polymeric backbone than that in poly(arginine), supplying reasonable amphilicity to the polymer and making it more efficient than the octaarginine system. (Qi et al., 2012).

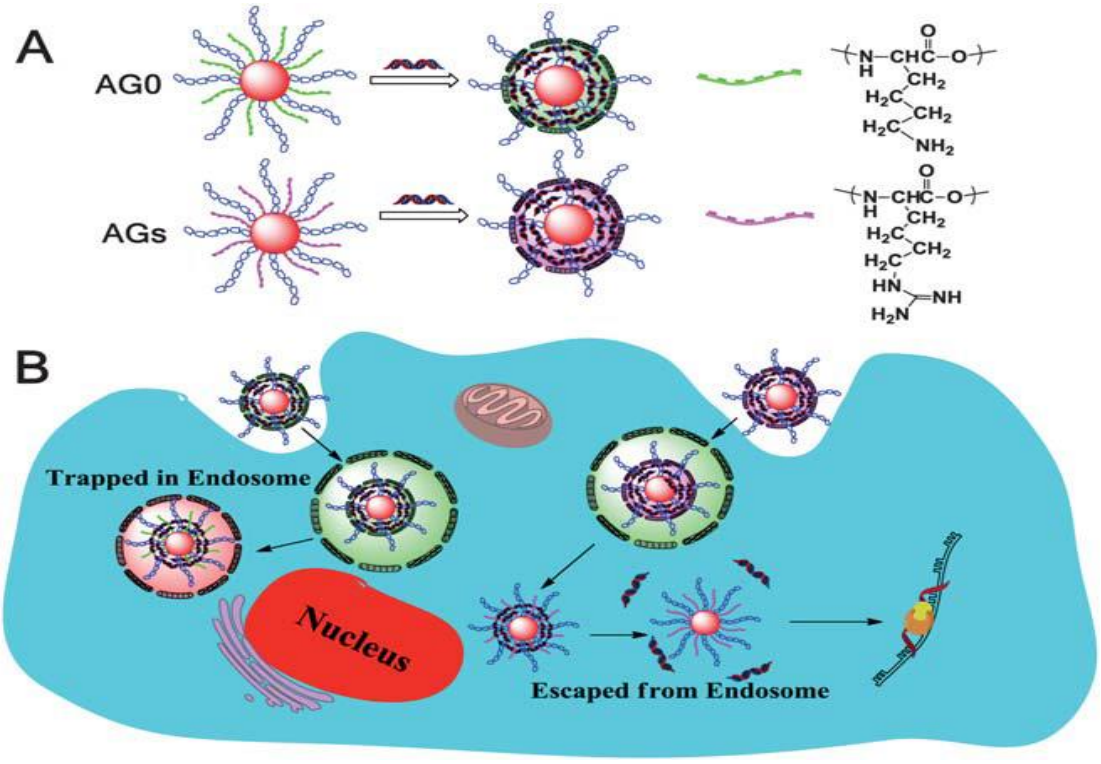

Figure 2.7. Formation of biodegradable cationic micelles self-assembled from siRNA and mPEG-*b*-PCL-*b*-PLL (AG_0) and from siRNA and guanidinated mPEG-*b*-PCL-*b*-PLL (AG_s) triblock copolymers (A). Pathways of these two kinds of micellar nanoparticle in a cancer cell (B). (Source:Qi et. al, 2012)

2.3. A Controlled Polymerization Technique: RAFT Polymerization

Controlled/Living radical polymerization techniques allowcontrol over molecular weight of polymers and are theoretically carried out in the absence of termination reactions (living property). These techniques generally yield well-defined polymers. Nitroxide mediated polymerization (NMP), reversible addition-fragmentation chain transfer (RAFT) polymerization and atom transfer radical polymerizaton (ATRP) are widely used controlled radical polymerizations. In nitroxide mediated polymerization technique, polymers are obtained without any purification. However, this technique offers limited monomer structure choice due to the high temperature reaction medium (Konkolewicz, Thorn-Seshold, & Gray-Weale, 2008). Atom transfer radical polymerization requires substituted monomers in order to protect propogating radicals. This technique also requires organometallic catalyst that contains transition metals. In biotechnology and biomedical applicaitons this generally constitutes a drawback of this technique (Trapa, Huang, Won, Sadoway, & Mayes, 2002). Variety of monomer types that can be used, mild reaction condtions, relatively low toxic reagents required and versatility makes RAFT polymerization more promising technique among the other types of controlled radical polymerization for biomedical applications (Rizzardo, Chiefari, Mayadunne, Moad, & Thang, 1999; Rizzardo, Chong, Evans, Moad, & Thang, 1996).

In 1998 CSIRO was the first group to report RAFT polymerization. Up to day RAFT polymerization has become one of the most important polymerization techniques as ito allows the synthesis of complex polymer arthitectures (star, block and hyper branched), and polymers with narrow molecular weight distribution and definedend-group functionality (comes from RAFT agent) that enables the bio-conjugation of polymers.yes. These properties of RAFT polymerization makes it suitable technique for biotechnology applications (Boyer et al., 2009; Li, He, & Yang, 2000).

RAFT polymerization can be applied to various type of monomers in the presence of a suitable chain transfer agent (RAFT agent) and a radical initiator. RAFT agents are organic compounds having commonly a thiocarbonylthio group. The general and accepted mechanism of RAFT polymerization is given in Figure 2.8. At the propogation step, R group in its structure initiates the growth of polymeric chains and the Z group activates the C = S bonds to radical addition and helps to protect the adduct

radical. Thus, R group should be better leaving group than propogating radical and it should reinitiate monomer (Chong et al., 2003). For this reason, although various types of monomers can be polymerized with this technique, still selection of a RAFT agent according to structure of monomer and solubility is very important to prevent unwanted side reactions. (Levesque, Arsene, Fanneau-Bellenger, & Pham, 2000). Mechanism of RAFT and kinetic studies gives theoritical equation on contorlling molecular weight at Equation 2.1.

$$M_{n(theo)}=\frac{[M_0]}{[RAFT_0]}\ x\ M_{w(monomer)}\ x\ Conv.\% + M_{w(RAFT)} \quad (2.1)$$

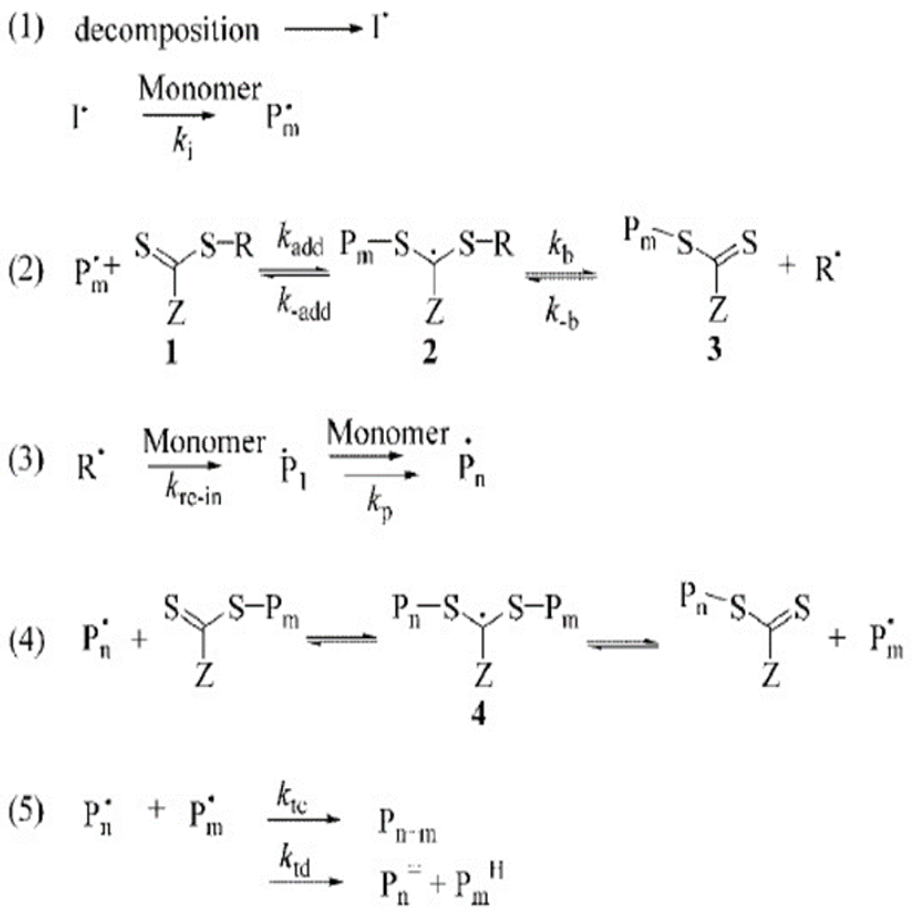

Figure 2.8.Generally accepted mechanism of RAFT.
(Source: Boyer et al., 2009)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Pentaflourophenol (99% purity) and methacryloyl chloride (97% purity) were purchased from Sigma-Aldrich to use for the synthesis of the pentaflourophenyl methacrylate according to the procedure given by Barz et al. (Barz et al., 2008). L-arginine methyl ester dihydrochloride (98% purity), triethylamine (99.5%) and N,N-dimethylformamide (99.5%) were purchased from Sigma-Aldrich for the synthesis of Arginine Methacrylate Methyl Ester (AMME) monomer. 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (97%, granular) and 4,4'-Azobis(4-cyanovaleric acid) were purchased from Sigma Aldrich and used as initiators for RAFT and free radical polymerizations. 4-Cyano-4-(phenyl-carbonothioylthio) pentanoic acid (CPADB) was purchased from Sigma-Aldrich and used as a chain transfer agent (CTA) for RAFT polymerizations. Ethanethiol, carbondisulfide, sodium hydride (57-63 % dispersed in mineral oil), iodine, sodium sulphate were purchased from Sigma-Aldrich for synthesis of the chain tranfer agent (CTA), 4-Cyano-4 (ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) according to the adapted procedure from Moad et al. (Moad, Chong, Postma, Rizzardo, & Thang, 2005). 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.

Ethyl acetate, hexane, dichloromethane (DCM), diethylether, methanol and N'N-dimethylacetamide (DMAc, HPCL grade $\geq$ 99.9%) were purchased from Sigma. Dialysis membrane (MWCO=500-1000 Da) was purchased from Spectrum® Laboratories. Deuterium oxide (D_2O), deuterium chloroform ($CDCl_3$), deuterium

dimethylsulfoxide (DMSO-d), deuterium methanol were purchased from Merck for Nuclear Magnetic Resonance (NMR) Spectroscopy analysis.

DMEM (Dulbecco's Modified Eagle's Medium) medium, L-glutamine, trypsin 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. A-549 cell line was donated by Bioengineering Research and Application Center, İzmir Institute of Technology, İzmir, Turkey.

3.1.2. Instruments

3.1.2.1. Nuclear Magnetic Resonance (NMR)

In this study, ^{1}H NMR and^{19}F NMR spectroscopies (Varian, VNMRJ 400 spectrometer) were used to determine the chemical structure of compounds synthesized and the conversion of the monomers during polymerizations. For analysis, samples were dissolved at 10 mg/ml in deuterated solvents depending on their solubilities.

3.1.2.2. Gel Permation 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) were used. The mobile phase was N, N dimethylacetamide (DMAc) containing 0,05 % w/v LiBr.

Polymers were synthesized in a aqeous buffer were analyzed at Yıldız Technical University, Istanbul. The chromatogram was taken using purified polymer. A GPC system (Viscotek) equipped with a light scattering detector was used in this measurement. The mobile phase for GPC was 0.1 M acetic acid with 0.15 M NaCl. An Eprogen CATSEC300 (25cmx0,46cm) column was used. Flow rate was 0.5 mL/min.

3.1.2.3. UV-Visible Spectrophotometry

UV-visible spectrophotometry was used to investigate hydrolysis and aminolysis kinetics of the RAFT agents, 4-cyano-4-(phenyl-carbonothioylthio) pentanoic acid (CPADB) and 4-cyano-4 (ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) . UV-visible light absorbance of the solution measured by a Thermo Scientific evolution 201 UV-visible spectrophotometer in the range between 200 nm and 600 nm using quartz cuvettes.

3.1.2.4. Microplate Reader

In cytotoxicity analysis, a Thermo Electron Corporation Varioskan microplate reader was used to measure absorbance at 540 nm. 96 well-plates were used. ass Spectrometer

3.1.2.5. Mass Spectroscopy

Mass spectrometry analysis was performed on an autoflex III MALDI TOF/TOF MS system (Bruker Daltonics, Bremen, Germany). The instrument was operated in positive ion reflectron mode with a reflectron acceleration voltage of 21 keV. The ions were generated using 337-nm nitrogen laser and the spectra were acquired over a 150 – 800 m/z mass range. For each spectrum, at least 2000 laser shots were averaged at a laser frequency of 100 Hz and the system was externally calibrated using matrix mixture. All data were processed with Igor Pro Software package (WaveMetrics, Lake Oswego, OR).

3.2. Methods

3.2.1. Synthesis of L-Arginine Methyl Ester

L-Arginine (30mmol) was suspended in methyl alcohol (15 ml). Separetely in another container, concentrated sulphuric acid was added to solid sodium chloride salt to produce hydrogen chloride (HCl) gas. HCl (g) was connected to L-arginine mixture and bubbled for 15 minutes. After this period of time, HCl (g) was removed and the reaction flask was put into the oil bath at 100°C and stirred for 15 minutes. Solvent was removed and residue was again dissolved in 15 ml methanol. This procedure was repeated twice. Resulting foam was redissolved in methyl alcohol. Diethyl ether was added to the mixture until it became turbid. Solution was kept at room temperature for 5 hours. The mixture was then placed in a refrigerator to complete crystallization. As the crystallization was completed solvent was removed using a rotary evaporator.

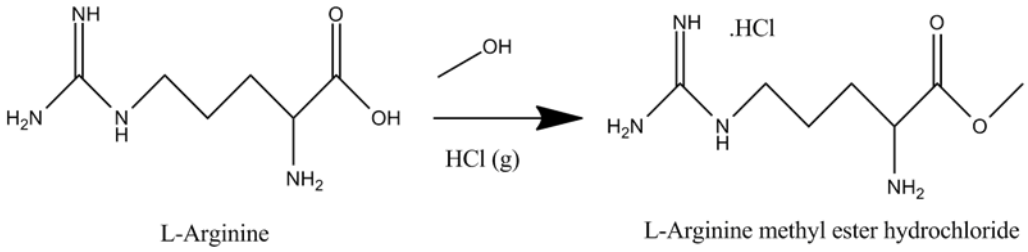

Figure 3.1. Synthesis of L-Arginine methyl ester hydrochloride

3.2.2. Synthesis of Pentaflourophenyl Methacrylate

Active ester monomer, pentaflourophenyl methacrylate (PFMA) was synthesized according to the procedure given by Eberhardt et al. (Eberhardt, Mruk, Zentel, & Theato, 2005). Figure 3.2 explains the experimental scheme of the synthesis. Pentaflourophenol (5.4 g, 29.3 mmol) and 2.6-lutidine (3.5 ml, 30.0 mmol) were dissolved in dichloromethane (50 ml). Under cooling, methacryloyl chloride (3.0 ml, 32.7 mmol) was added and the whole mixture was stirred for 3 h at 0 °C. After removing the ice bath, the reaction mixture was kept at room temperature overnight.

After filtration of the solution to remove the precipitated 2.6-lutidine hydrochloride, the filtrate was washed twice with equal volume of water (approximately 30 ml) and dried over MgSO4. The solvent was removed and the remaining liquid was distilled under reduced pressure yielding a colorless product (4.9 g, 70%) by vacuum distillation. The final product, PFMA was analysed by NMR using $CDCl_3$ as solvent. .

^{1}H NMR ($CDCl_3$, δ in ppm): 6.43 (s, H, CH_3-$\underline{CH_2}$-COO-), 5.89 (s, H, CH_3-$\underline{CH_2}$-COO-), 2.06 (t, 3H, $\underline{CH_3}$-CH_2-)

^{19}F NMR ($CDCl_3$,δ in ppm): -162.90 (2F), -158.63 (F,para), -153.17 (2F).

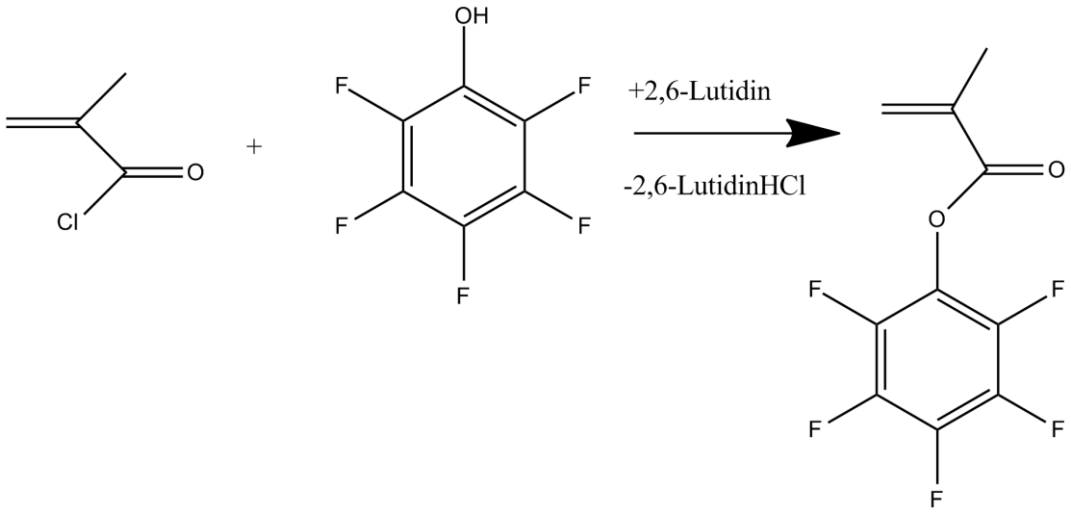

Figure 3.2. Reaction scheme of pentaflourophenyl methacrylate (PFMA).

3.2.3. Synthesis of methyl 5-guanidino-2-methacrylamidopentanoate (Arginine Methacrylamide Methyl Ester, AMME) monomer

Arginine methacrylamide methyl ester was synthesized by reacting active ester PFMA and primer alpha amine of the L-arginine methyl ester dihydrochloride. L-Arginine methyl ester dihdyrochloride (5g, 19.1mmol) was dissolved in dimethylformamide (20 ml). Triethylamine (4.84g, 47.8 mmol) was added to the stirring solution of L-arginine methyl ester. Precipitated triethyl amine hydrochloride salt was removed by simple filtration. Synthesized pentaflourophenyl methacrylate (PFMA) ;(5.12g 19.1mmol) was added dropwise to the strirring solution at room temperature.

The reaction mixture was left at room temperature overnight. Precipitated triethylamine hydrochloride salt was again removed by simple filtration.

^{1}H NMR (D_2O, δ in ppm): 1.13 (q, 9H, $N(CH_2\underline{CH_3})_2$), 3.08 (q, 6H, $N(\underline{CH_2}CH_3)_2$)

Solvent was removed using rotary evaporator andvaccum oven. Product was dissolved in methanol (1 ml).Diethyl ether (100 ml) was then added to precipitate the desired product. The precipitate was collected by centifuragtion. The solvent was removed by rotary evaporator. Finally, a yellow-brown product was collected. The final product was characterized by NMR using deuterium oxide as solvent.

^{1}H NMR (D_2O, δ in ppm): 5.58 (s, H, $\underline{CH_2}CH_3CONH$), 5.36 (s, H, $\underline{CH_2}CH_3CONH$), 4.34 (m,H, $CH_2CH_3CO\underline{NH}$), 3.62 (s, 3H, $COO\underline{CH_3}$), 3.09 (m, 2H, $NH_2NHCNH\underline{CH_2}$), 1.80 (m,2H, $NHCH_2\underline{CH_2}CH_2$), 1.53 (m, 2H, $NHCH_2CH_2\underline{CH_2}$), 1.79 (s, 3H, $CH_2\underline{CH_3}CONH$)

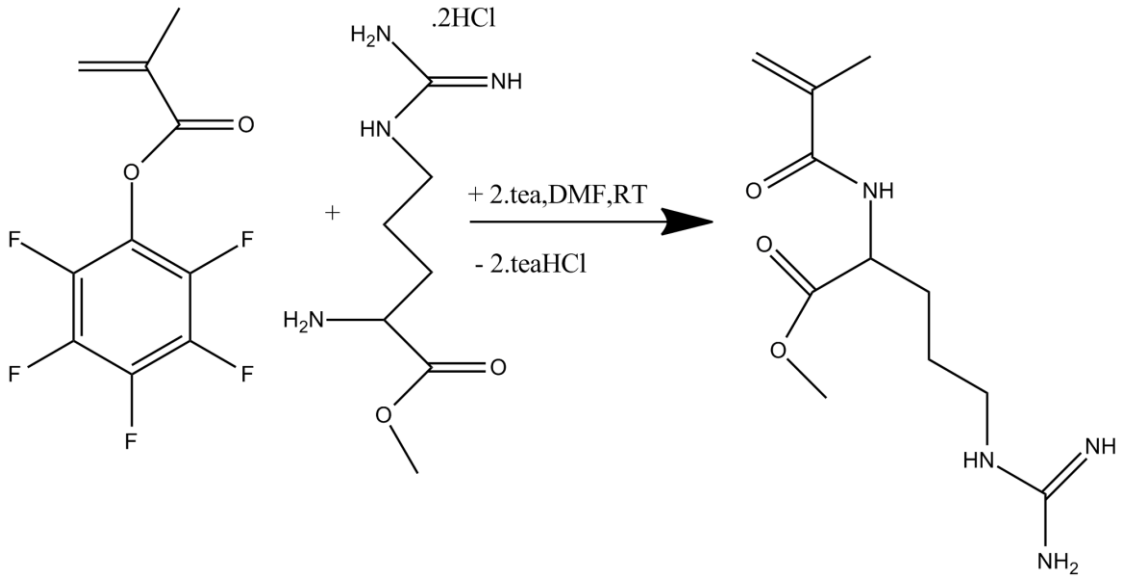

Figure 3.3. Reaction scheme of synthesis of arginine methacrylate methyl ester (AMME).

3.2.4. Synthesis of 4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanyl pentanoic acid

4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) is a chain transfer agent (CTA), suitable to be used in aqeous RAFT polymerizations because of its high solubility in aqueous solutions. Synthetic method was adapted from Moad et al. (Moad et al., 2005). Diethyl ether was dried over sodium wire under nitrogen for one day and dry diethylether (150 ml) was collected to be used as a solvent in the synthesis. Ethanethiol (4.72 g, 76 mmol) was added over 10 min to a stirred suspension of sodium hydride (53-63% in oil) (3.15 g, 79 mmol) in dried diethyl ether (150 ml) at 0 °C. The mixture was allowed to stir for 10 minutes. Carbon disulfide (6.0 g, 79 mmol) was then added to the mixture in an ice bath. Yellow-colored crude ysodium S-ethyl trithiocarbonate (7.85 g, 0.049 mol) was collected by simple filtration. Collected product was suspended in diethyl ether (100 mL) without performing any purification step. The mixture was reacted with iodine (6.3 g, 0.025 mol).A white precipitate of sodium iodine formed.After 1 hour of reaction time, the solution was filtered, washed with aqueous sodium thiosulfate solution with three times, and dried over sodium sulfate.

The crude bis(ethylsulfanylthiocarbonyl) disulfide was isolated from diethyl ether by rotary evaporation. A solution of bis(ethylsulfanylthiocarbonyl) disulfide (1.37 g, 0.005 mol) and 4,4′-azobis(4-cyanopentanoic acid) (2.10 g, 0.0075 mol) in ethyl acetate (50 mL) was refluxed for 18 h. Following rotary evaporation of the solvent, the crude ECT was isolated by column chromatography using silica gel as the stationary phase and 70:30 ethyl acetate:hexane as the eluent.

1H NMR ($CDCl_3$, δ in ppm): 1.36 (t,$SCH_2\underline{CH_3}$), 1.88 (s,$CCN\underline{CH_3}$), 2.3–2.65 (m,$CH_2\underline{CH_2}$), 3.35 q (q,$S\underline{CH_2}CH_3$).

Rf: 0,64 (product)

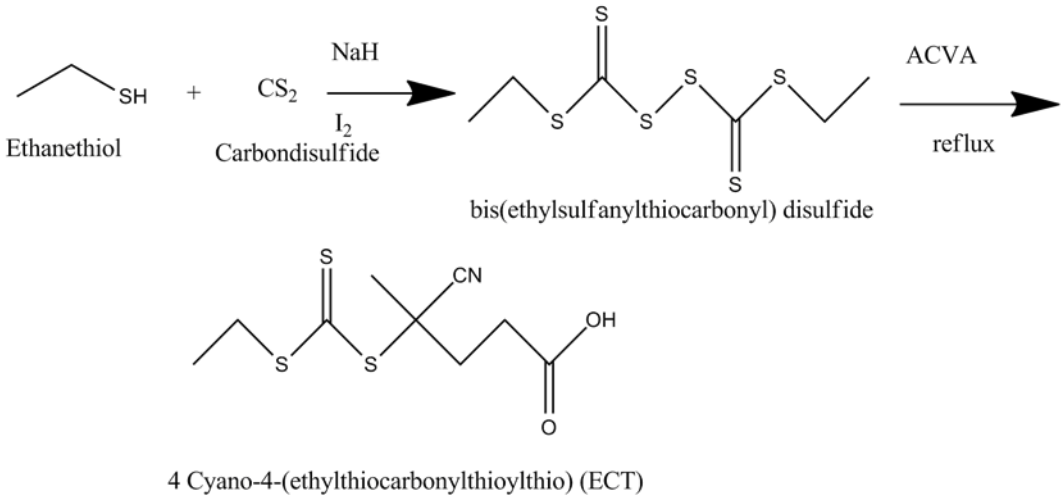

Figure 3.4. Synthesis of ECT as achain transfer agent (CTA)

3.2.5. Free Radical Polymerization of Arginine Methacrylamide Methyl Ester (AMME)

In a typical free radical polymerization, AMME and initiator were dissolved in the solvent. The feed composition and polymerization conditions are given in Table 3.1. The vials containing the polymerization mixtures were sealed with a septum and degassed via nitrogen purging for 15 mins. The vials were then placed in an oil bath at 50°C. As the polymerization time ends, vials were opened to the atmosphere and placed in a refrigerator. The polymerization mixture was characterized by NMR to determine the monomer conversion (Equation 3.1).The mixture was then dialyzed against acidic solution at pH 5.2 using a regenerated cellulose dialysis tubing having 1 kDa molecular weight cutoff for 3 days. The final solution was dried using a lyophilizer. After polymerization and purification, the final polymer was characterized via NMR by dissolving 8 mg sample in 500 µl D_2O or CD_3-OD. ^{1}H NMR (D_2O δ in ppm): 4.30 (m,H), 3.72 (s,3H), 1.90 (s,3H), 3.11 (m,2H), 1.5-2.0 (m,4H)

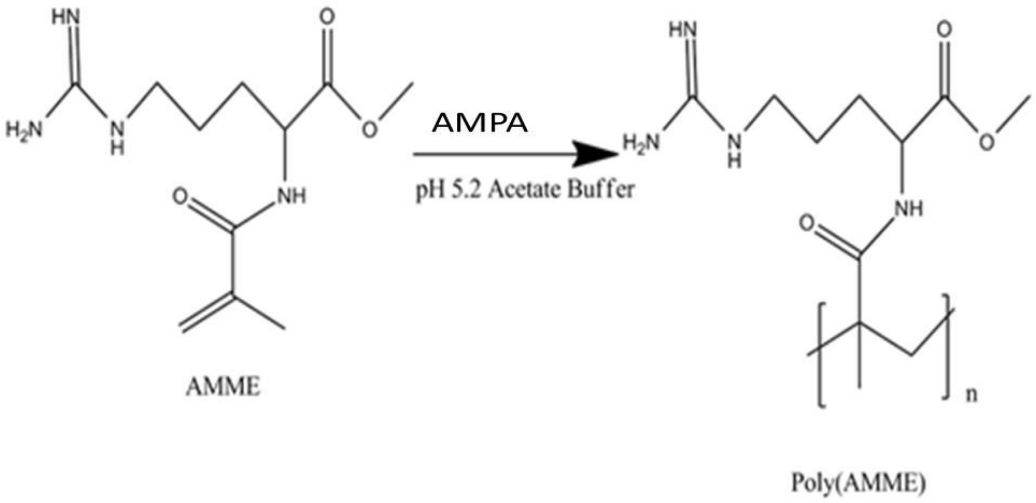

Figure 3.5. Free Radical Polymerization of AMME.

$$Con\% = \frac{(\frac{I_{3H@3.72\,ppm}}{3H} - 1)}{\frac{I_{3H@3.72ppm}}{3H}}\ x\ 100\% \qquad (3.1)$$

Table 3.1. Conventional free radical polymerization conditions.

[AMME] (mol/l)	[Initiator]x10^3 (mol/l)	$[AMME]_0/[Initiator]_0$	Initiator	Solvent	Time (h)
0.2	1	200/1	AMPA	Acetate Buffer	2
0.2	1	200/1	AMPA	Acetate Buffer	4
0.2	1	200/1	AMPA	Acetate Buffer	7

3.2.6. Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization of AMME

RAFT Polymerization of AMME was performed using 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPA) or 2,2′-azobis(2-methylpropionitrile) (AIBN) as an initiator, 4-cyano-4- (ethylthiocarbonylthioylthio) sulfanylpentanoic acid (ECT) as a RAFT agent. As a solvent system, either methanol: acidic acetate buffer (1:1, v:v) mixture or dimethylacetamide was utilized as indicated in Table 3.2. In a typical RAFT polymerization, AMME ($6.49x10^{-3}$mol) was first dissolved in the solvent system (2,5 ml) in a vial. ECT ($5.90x10^{-5}$ mol) was then added into the vial. Finally, initiator ($1.19x10^{-5}$ mol) was added and dissolved in polymerization mixture. The mixture was then placed in an ice-bath and purged with nitrogen for 20 minutes. The vial was sealed and placed into an oil bath at 50^{o}C. Polymerization was performed for a given time as indicated in Table 2. After polymerization was ceased, 10 μl of the polymerization mixture was dried and dissolved in deutareted methanol or water to determine the monomer conversion by ^{1}H NMR analysis. The rest of the polymerization mixture was placed in a dialysis tubing with a MWCO of 1kDa and dialyzed against methanol and aqueous solution mixture where pH was adjusted to pH 4 using HCl. Methanol content of the dialysis system was decreased day by day. In the fourth day, dialysis was performed against neutral ultrapure water. Final polymer was dried in a lyophilizer and characterized by ^{1}H NMR and GPC.

^{1}H NMR (D_2O δ in ppm): 4.30 (m,H), 3.72 (s,3H), 1.90 (s,3H), 3.11 (m,2H), 1.5-2.0 (m,4H)

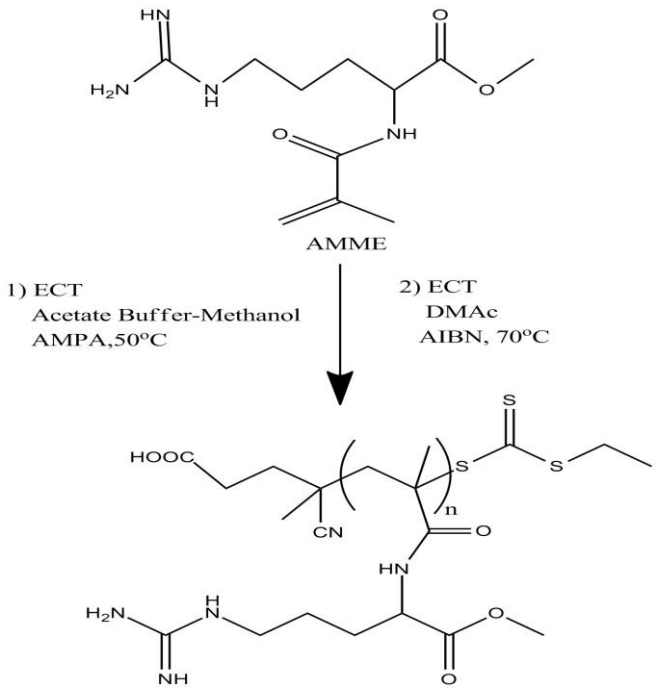

Figure 3.6. RAFT Polymerizations of AMME.

Table 3.2.RAFT Polymerization conditions. Polymerization temperature for experiments performed using AIBN and AMPA was 70 and 50°C, respectively.

[AMME] (mol/l)	[Initiator] x10^3 (mol/l)	$[AMME]_0$/ $[ECT]_0/[Initiator]_0$	Initiator	Solvent	Time (h)
0.4	1	250/3/1	AIBN	DMAc	2
0.4	1	250/3/1	AIBN	DMAc	4
0.4	1	250/3/1	AIBN	DMAc	6
0.4	1	250/3/1	AIBN	DMAc	8
0.4	1	250/3/1	AIBN	DMAc	12
0.4	1	250/3/1	AMPA	Methanol-Buffer	22

3.2.7. Hydrolysis and Aminolysis of 4-Cyano-4-(ethylthiocarbonylthioylthio)sulfanylpentanoic acid (ECT)

Considering its high solubility in aqueous solutions, P(AMME) was intended to be synthesized via aqeous RAFT polymerization. Previous studies have shown that aqeous RAFT polymerization of amine containing hydrophilic monomers provide a straightforward route to well-defined polymers for biomedical applications (Alidedeoglu, York, McCormick, & Morgan, 2009). However, it is also known that aminolysis of RAFT agents occurs in aqueous solutions, which results in the loss of control during aqueous RAFT polymerization. Thus, before the polymerization experiments, degradation of the RAFT agent, ECT in aqueous solution with or without AMME monomer (as an amine-source) was needed to be investigated. Hydrolytic behaviour of the RAFT agent (in the absence of AMME monomer) was investigated as follow: the RAFT agent was dissolved in methanol and mixed with acetic acid buffer pH 5.2 at a v:v ratio of 1:3 (methanol:buffer). The final concentration of the RAFT agent was 0.015M. The solution was immediately placed into an oil bath at 50°C . Every two hours (until 24 hours) 30 µl of the mixture was taken and diluted to 1ml with the blank (3:1, buffer: methanol) solution. The absorbance of the solution was measured at at 295.3 nm. The procedure was repeated in the presence of AMME at a concentration of 0.2 M.

Separately, aminolysis of the RAFT agent was investigated using conditions utilized for RAFT polymerization kinetic experiments. In this experiment, the concentration of AMME was 0.4 M. The aminolysis reaction was followed in DMAc at 70^{o}C.

Degradation of the RAFT agent was calculated according to Equation 3.2 considering the dilution factors. The initial absorbance of the RAFT agent solution was determined using a RAFT agent solution in methanol only.

$$Degradation\ \% = \frac{(A_0 - A_t)}{A_0}\ x\ 100\% \qquad (3.2)$$

3.2.8. In Vitro Cytotoxicity

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a colorimetric assay that is used to determine cell viability via measuring the activity of cellular enzymes that reduce the tetrazolium dye to its insoluble formazan giving a purple color. A549 human lung cancer cells were seeded a day prior to sample exposure at a concentration of 10^4 cells/well in a 96 well- plate and incubated overnight at 37 °C and 5% CO_2 in a humidified atmosphere. Polymer solutions and octaarginine solutions were sterilized by filtrations. Varying concentrations of sterile polymer or octaarginine solutions were transferred to wells. The PBS content of each well was adjusted to be 0.5% (v/v). The final volume of each well was 100 µl. The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 24 or 72 h. After the incubation period, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (5 mg/ml) was added to each well by adjusting the final concentration of dye to be 10% (v/v) and the plates were incubated at 37^{o}C for 4 hours. At the end of the incubation period, plates were centrifuged at 1800 rpm for 10 minutes, supernatants were removed and pellets were dissolved using DMSO. Cell viability was detected by spectrophotometric analysis at 570 and 690 nm (Varioskan Flash, Thermo Electron Corporation, Finland). A calibration curve reported previously (İmran Özer, MSc Thesis, 2014) was used for correlating absorbance measurements with cell viabilities. Percent cell viability was calculated relative to the absorbance of positive control that was cells without any treatment using calibration curve.

In cytotoxicity experiments, two types of polymers were used. The first type was p(AMME) with RAFT end-group. This polymer has a molecular weight of 17 kDa with a PDI of 1.27, as determined by GPC equipped with light-scattering detector. The second type was p(AMME) without RAFT end-group. This polymer has a molecular weight of 17 kDa with a PDI of 1.27, as determined by GPC equipped with light-scattering detector. The trithiocarbonate RAFT-end group of p(AMME) 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(AMME) dissolved in water was reacted with Arginine Methacrylamide Methyl Ester monomer (AMME) in the presence of ethylenediamine, triethylamine (TEA) and tris (2-

carboxyethyl)phosphine hydrochloride (10/50/110/10 mole ratio respectively, 12mg/ml polymer concentration) overnight at room temperature, as it is schematically given in Figure 3.7.

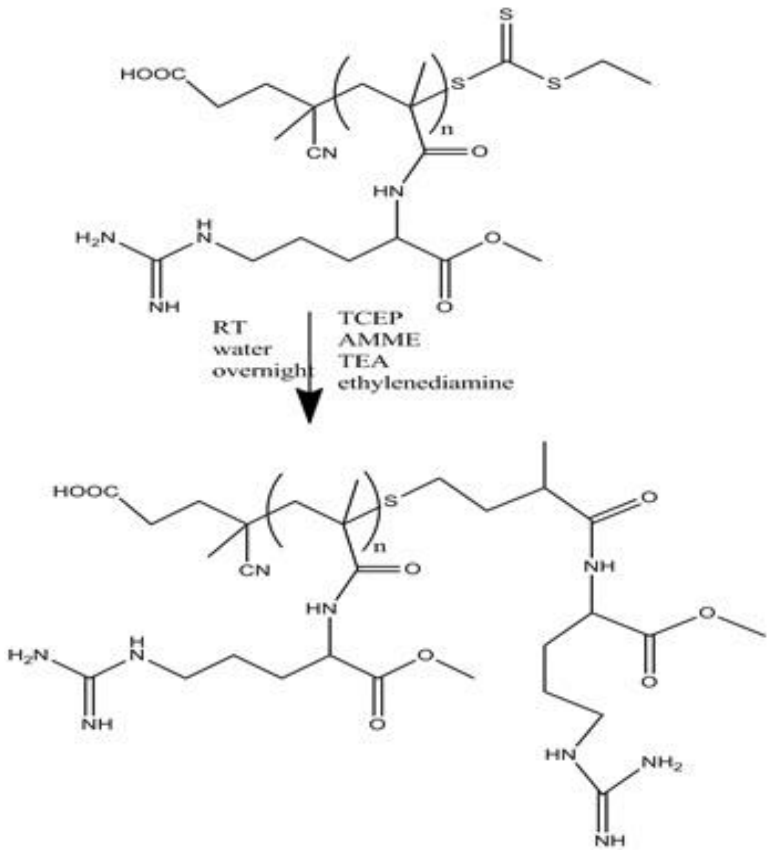

Figure 3.7. Reaction scheme of aminolysis and monomer addition.

After reaction, the reaction mixture was placed into a 3.5 kDa MWCO membrane for dialysis against ultrapure water. After dialysis polymer solution was dried in a lyophilizer. The product was characterized by ^{1}H-NMR spectroscopy after dissolving in D_2O. Also UV-VIS spectrophotometer measurements confirmed the absence of the thiol groups after reaction.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Synthesis of L-Arginine Methyl ester

Methyl ester form of L-Arginine was intended to be used in the synthesis of arginine-containing monomer to avoid any possible side-reactions due to the presence of alpha carboxylic acid group in the structure of L-Arginine. Accordingly, L-arginine methyl ester hydrochloride was synthesized via acid catalyzed esterification reaction. Acid source, hydrochloric acid (g) was obtained by the reaction of the neutral salt, sodium chloride and strong acid sulphuric acid and transferred to the reaction container during esterification. Methanol was used as both solvent to dissolve arginine and a reactant as a carbon source. At the end of the crystallization step, pure product was obtained.Figure 4.1 shows the ^{1}H NMR spectrum of the synthesized L-Arginine methyl ester hydrochloride after crystallization.

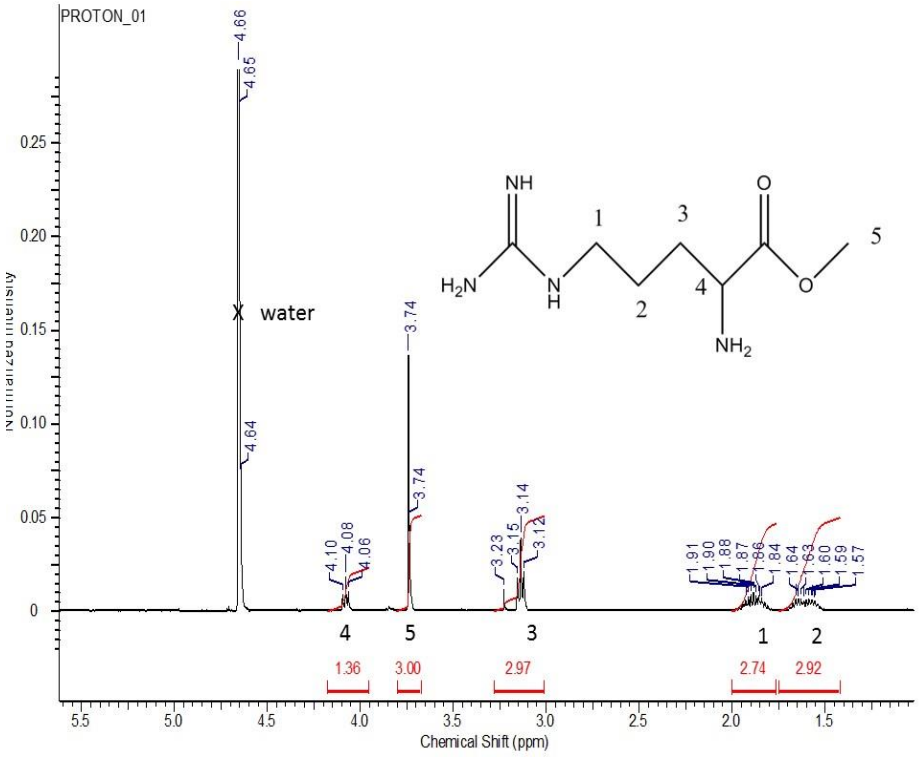

Figure 4.1. ^{1}H NMR spectrum of L-arginine methyl ester after crystallization.

4.2. Synthesis of Pentaflourophenyl Methacrylate

Pentaflourophenyl methacrylate (PFMA) is an active ester compound and selectively reacts with primary amines. It was synthesized by classic methacrylation of pentaflourophenol in a dry solvent under nitrogen to prevent the conversion of methacryloyl chloride which is a methacrylation agent, into methacrylic acid in the presence of water or humidity. Methacryloyl chloride was used to react with the primary alpha amine of the L-arginine methyl ester hydrochloride to form a methacrylate that can be polymerized in the presence of free radicals. After methacrylation, the colorfull reaction mixture was distilled under reduced pressure (approximately 10 mbar) to collect colorless liquid product, PFMA. ^{1}H NMR and ^{19}F NMR spectra of the product was taken by dissolving the sample in deutarated chloroform at Figure 4.2 and Figure 4.3.

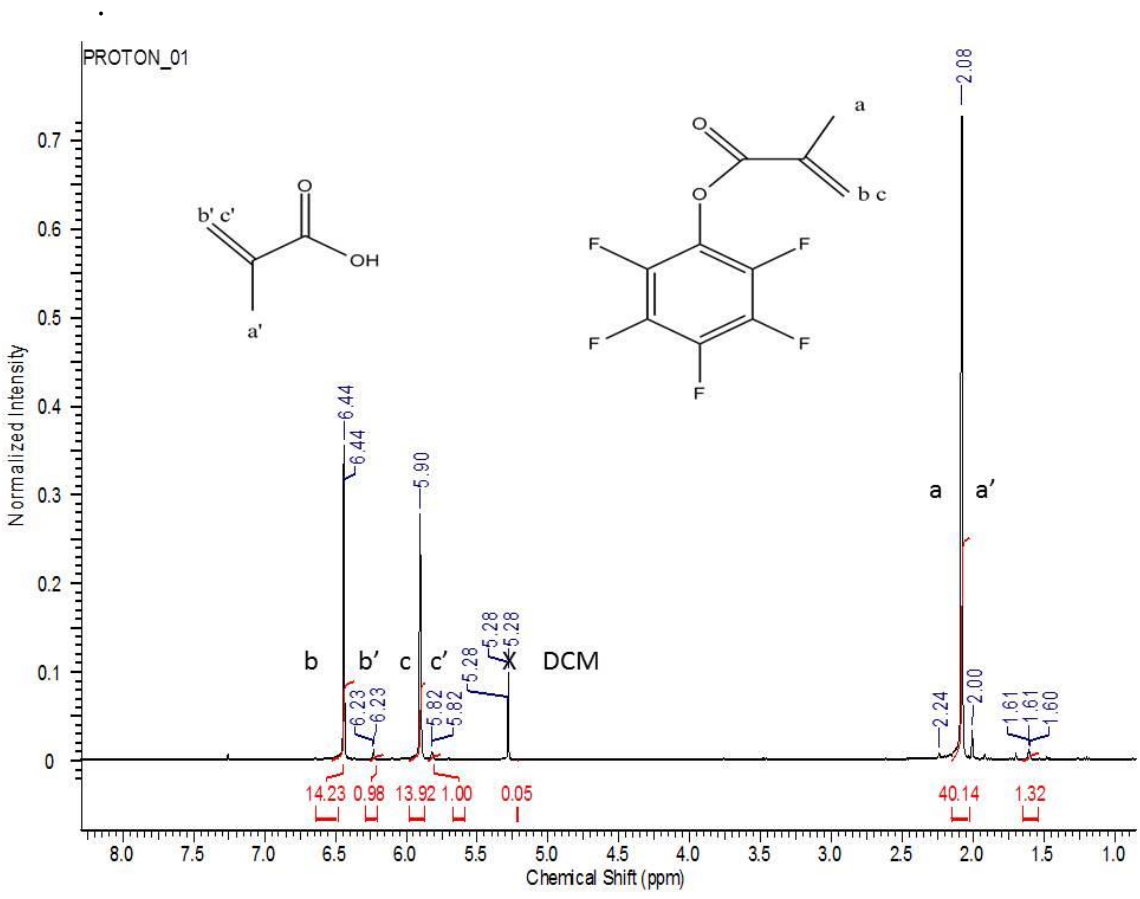

Figure 4.2. ^{1}H NMR spectrum of pentaflourophenyl methacrylate (PFMA) after vacuum distillation.

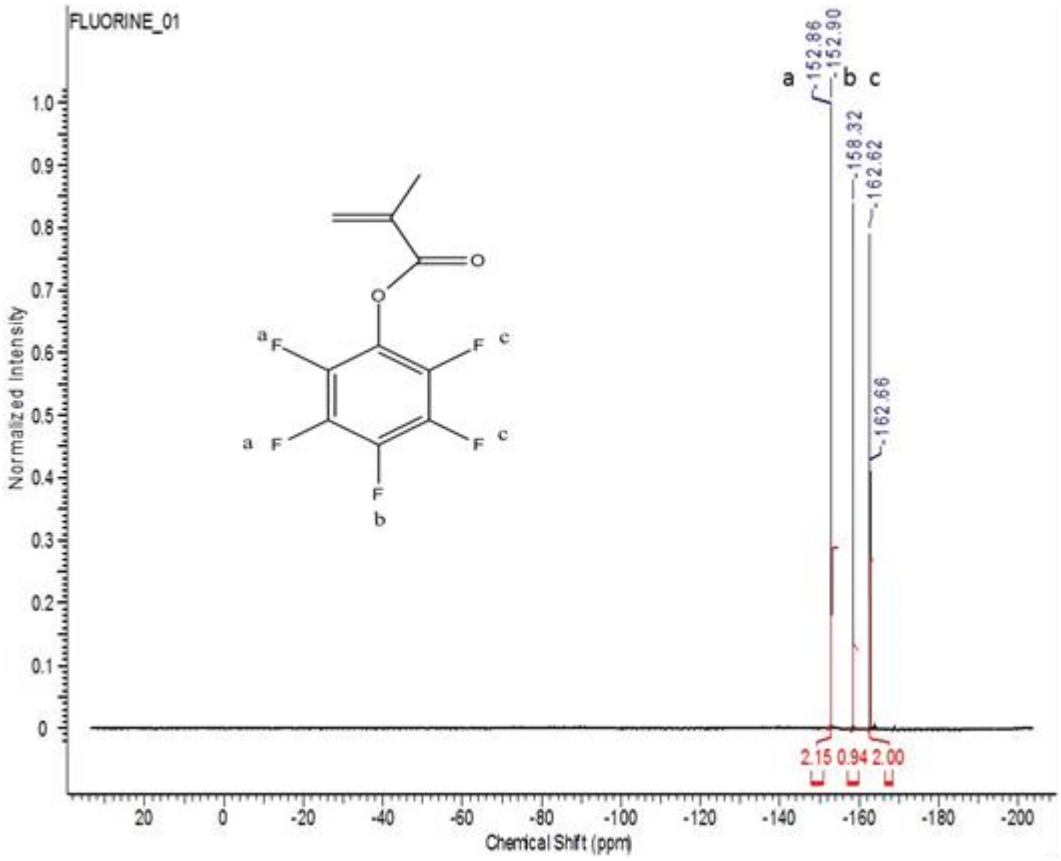

Figure 4.3. ^{19}F NMR of pentaflourophenyl methacrylate (PFMA) after vacuum distillation.

Figure 4.2 shows the presence of the intended compound, PFMA along with the presence of methacrylic acid. 19F NMR spectrum in Figure 4.3 supports the 1H NMR data, evidencing the successful synthesis of PFMA. .The presence of methacrylic acid along with the intended compound did not cause a problem during the monomer synthesis as it could be removed easily by the addition of triethyl amine which is an organic base.

4.3. Synthesis of Methyl 5-guanidino-2-methacrylamidopentanoate (Arginine Methacrylamide Methyl Ester, AMME) monomer

Methyl 5-guanidino-2-methacrylamidopentanoate is a monomeric structure synthesized by the reaction of the active ester PFMA and the L-arginine methyl ester hydrochloride (AME) in the presence of the excess triethyl amine (TEA). In the reaction, TEA forms its hydrochloride salt, also removes methacrylic acid as a salt from reaction medium and supplies high pH to prevent the protonation of the alpha amine of the AME. The reaction occurs through the nucleophilic attack between the amine and pentaflourophenyl groups. Selectivity of the PFMA towards primary amines is strong enough to distinguish the alpha primary amine from the delocalize electron carrying primary amines of the guanidine group of AME. Thus, the guanido amine groups did not need to be protected during the reaction of PFMA and AME. PFMA selectively attacks alpha primary amine of AME when the stoichiometry of the reaction is 1:1 (PFMA : AME). Figure 4.4shows the ^{1}H NMR spectrum of the synthesized arginine methacrylamide methyl ester (AMME) monomer. The spectrum indicates the successful synthesis of the intended structure.

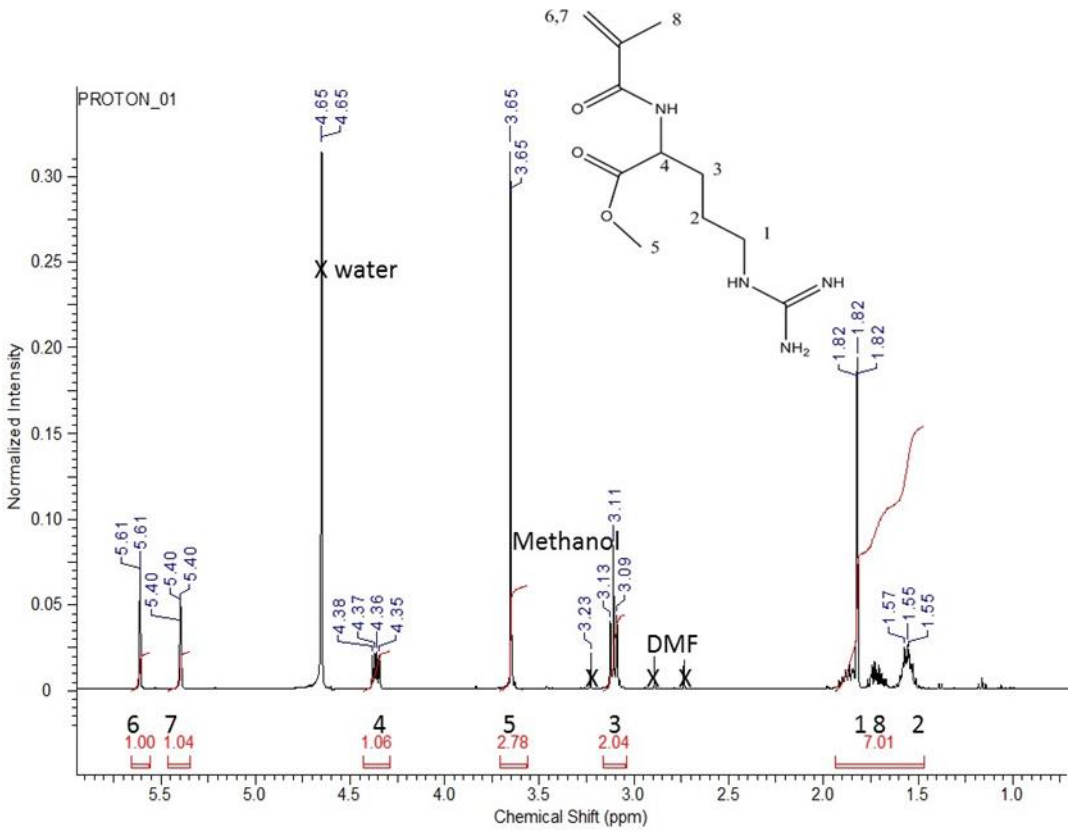

Figure 4.4. ^{1}H NMR spectrum of the synthesized Methyl 5-guanidino-2-methacrylamidopentanoate (AMME).

While the ^{1}H NMR spectrum did not display the protons of the guanidine group on the structure, mass spectrometry (MS) analysis of AMME evidenced the expected structure was successfully synthesized (Figure 4.5). Theoretical mass of AMME monomer is 256.30. The MS analysis indicated mainly the presence of a compound with $[M+H]^{+}$ of 257.205, which confirms well the synthesis of AMME.

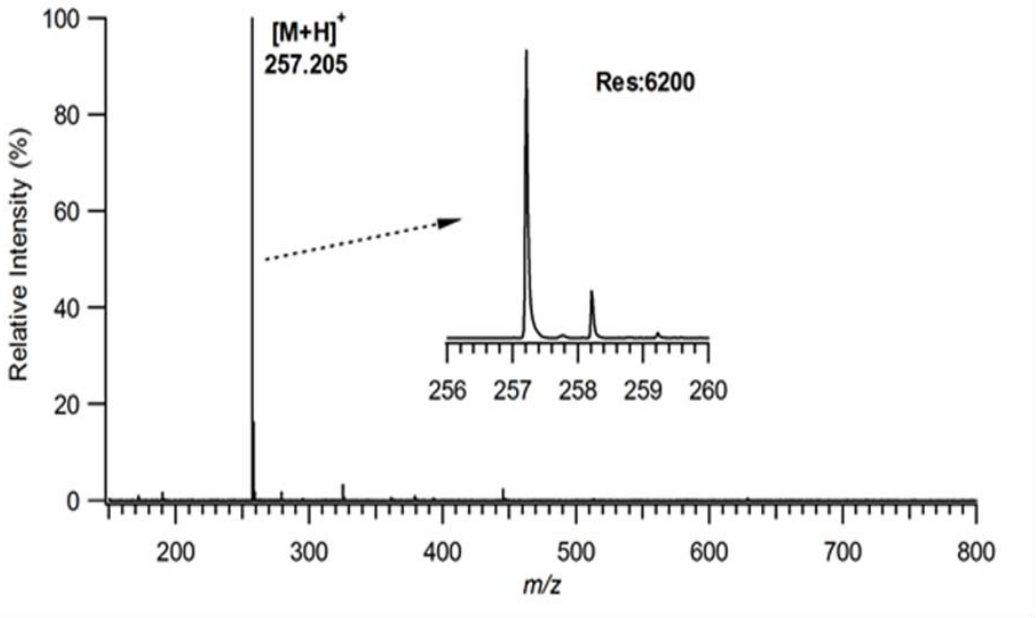

Figure 4.5. MALDI-TOF spectrum of methyl 5-guanidino-2-methacrylamidopentanoate (AMME).

4.4. Synthesis of 4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid

4-Cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) is a RAFT agent which is not commercially avaible and suitable for methacrylamides (Boyer et al., 2009). This RAFT agent is basicly different than the other bulk RAFT agents which have large Z-groups such as dodecyl and phenyl. ECT has ethyl group in its Z-group which increases its solubility in aqeous media and allows aqueous RAFT polymerizations to be performed without solubility issues (Treat et al., 2012). Besides, it is a trithiocarbonate, which means Z group of the agent is connected to a thiol group via another sulphide, making the structure more resistant to hydrolysis and aminolysis. Considering all these advantages, ECT was synthesized adapting a procedure reported previously (Moad et al, 1996). Dry ether was used as reaction solvent to increase the yield of the reaction by preventing loss from the disassociation of the sodium hydride in the presence of humidity. The synthetic route was composed of four steps; the first step was the reaction of ethanethiol with carbon disulphide that gives S-ethyl trithiocarbonate. In the second step, this precursor was reacted with iodine to precipitate

as sodium iodine. Excess iodine was removed by washing the organic phase with sodium thiosulphate, yielding bis-(ethylsulfanylthiocarbonyl) disulphide (bisESTDS) (which is a liquid at room temperature). In the third step, BisESTDS was reacted with 4,4′-azobis(4-cyanovaleric acid). To supply required internal enegy to perform this reaction, reflux was done in ethyl acetate for 18 hours. In the last step, mixture was purified via column chromatography. Figure 4.6 shows the ^{1}H NMR spectrum of the product obtained after column chromatography performed using hexane:ethylacetate (70:30) solvent mixture as eluent and silica as stationary phase.

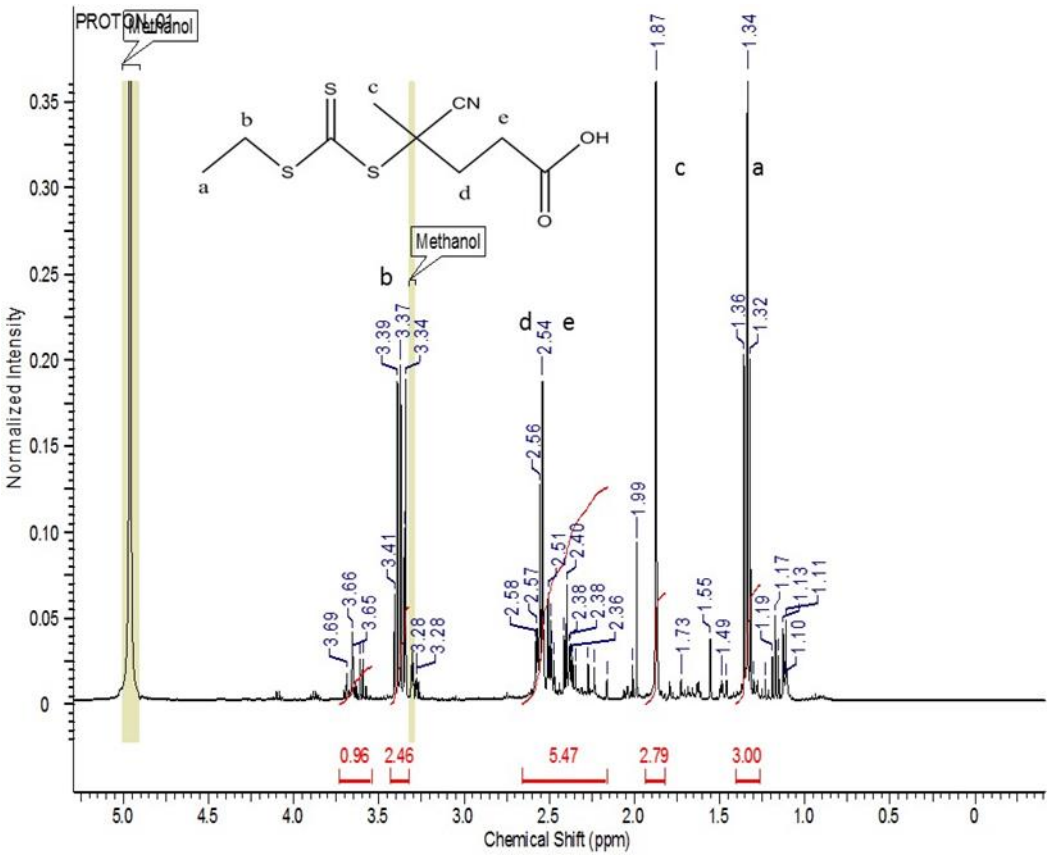

Figure 4.6. ^{1}H NMR spectrum of the synthesized 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) after purification via column chromatography.

4.5. Free Radical Polymerization of Arginine Methacrylamide Methyl Ester

Before attempting to perform RAFT polymerization of AMME monomer, conventional free radical polymerization of AMME was carried out to investigate whether this monomer can be polymerized in the presence of free radicals. AMME was polymerized via free radical polymerization using varying polymerization solvents, monomer/initiator (M/I) ratios and initiator types. In these preliminary experiments, acetonitrile was found to be unsuitable due to the gelation and phase separation during polymerizations, probably due to the insolubility of the polymer formed. AMME monomer was soluble in methanol and also water which allows aqeous polymerization to be performed. For polymerizations performed in water, the pH of the polymerization solution was needed to be controlled due to the strong basic character of AMME monomer because of guanidine groups. Acetic acid (0.73 M) -sodium acetate (0.27 M) buffer at pH 5.2 was therefore used as reaction medium. For polymerizations performed with methanol and water, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPA) was used as an initiator considering the low boiling point of methanol (64.7°C at atmospheric pressure) and also the water-soluble character of AMPA. For polymerizations tested in organic solvents such as acetonitrile as described above, both 4,4′-Azobis(4-cyanovaleric acid) ACVA and 2,2′-Azobisisobutyronitrile (AIBN)were used as radical initiators. AMPA forms radicals at relatively low temperatures (e.g. 50°C) while ACVA and AIBN degrade at higher temperatures (60-70°C).

In all preliminary free radical polymerizations performed in buffer at pH 5.2, methanol or buffer: methanol (35:65) mixture, high conversions of monomer to polymer were obtained. Figure 4.7 shows the polymerization solution after four hours of polymerization at given conditions. The monomer conversion was calculated to be 81% according to Equation 3.1.

Table 3. Conventional Free Radical Polymerization Conditions.

[AMME] (mol/l)	[Initiator]x 10^3 (mol/l)	$[AMME]_0/[Initiator]_0$	Initiator	Solvent	Time (h)	Conv. (%)
0.2	1	200/1	AMPA	Acetate Buffer	2	30
0.2	1	200/1	AMPA	Acetate Buffer	4	81
0.2	1	200/1	AMPA	Acetate Buffer	7	99

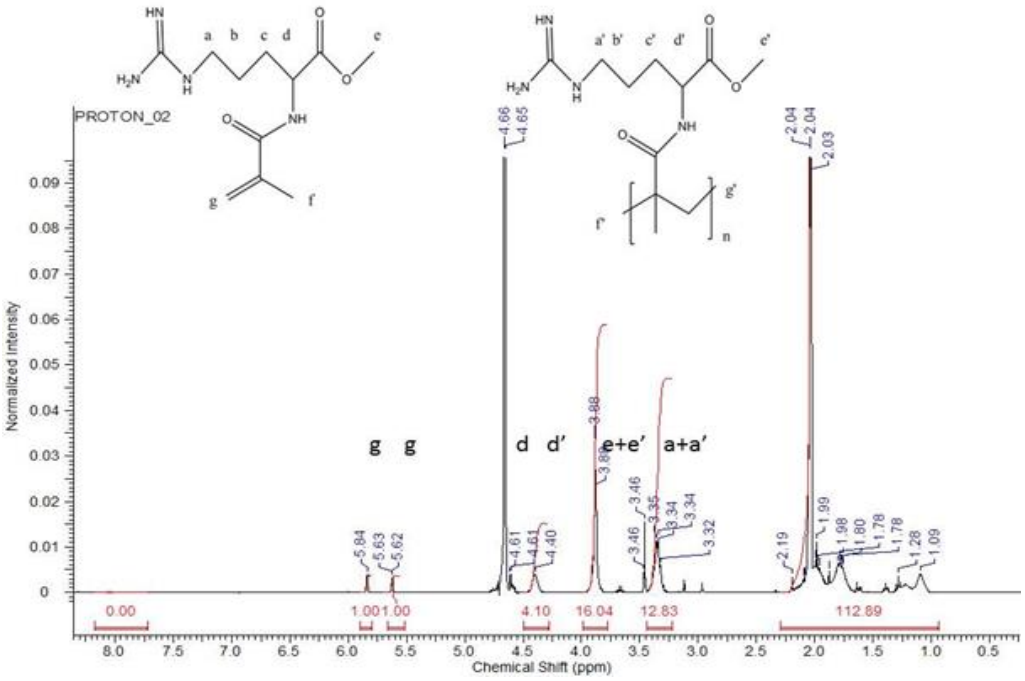

Figure 4.7. ^{1}H NMR spectrum of the conventional free radical polymerization of AMME for 4 hours. Polymerization conditions: [AMME]= 0.2 M, [AMPA]= 10^{-3} M, Solvent; Acetate Buffer, Temperature: 50°C.

Figure 4.8 shows the ^{1}H NMR spectrum of the purified polymers. Purification of the polymers were performed by dialysis against ultrapure water with pH adjusted to pH 3-4 by HCl. The spectrum is a proof that the dialysis method developed for purification of these polymers was effective.

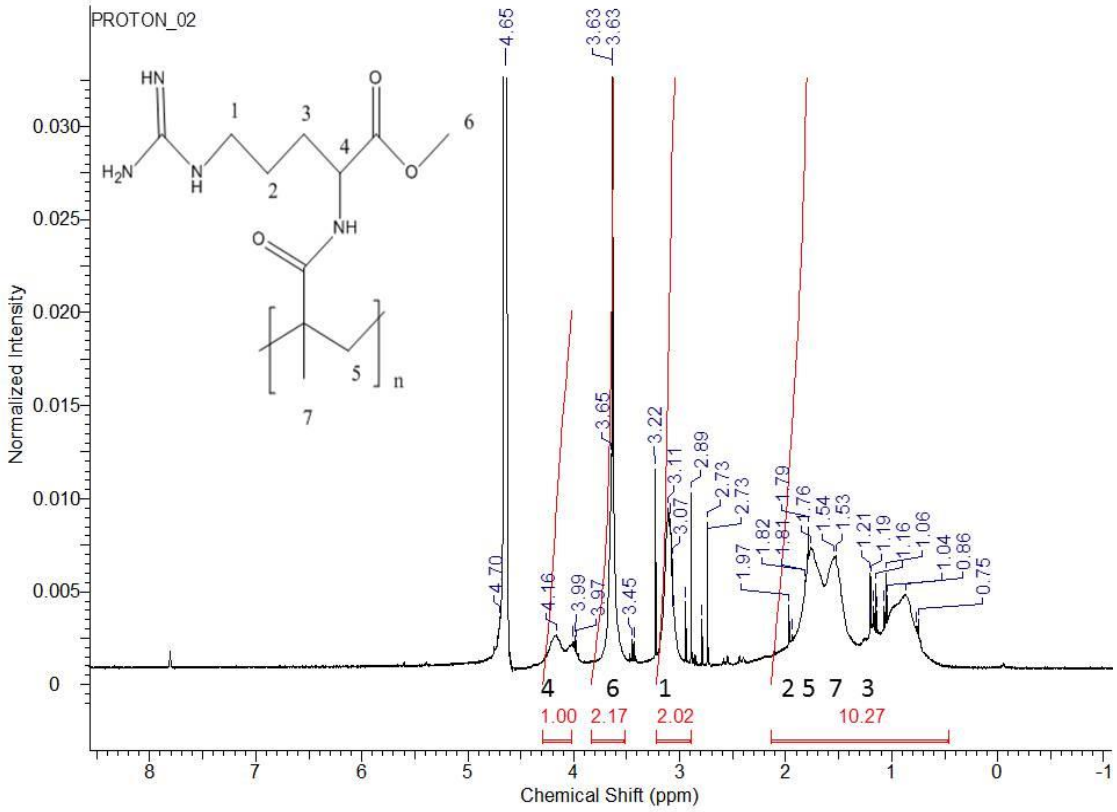

Figure 4.8. ^{1}H NMR spectrum of P(AMME) synthesized via conventional free radical polymerization. The spectrum belongs to the polymer after dialysis.

Figure 4.9 shows the gel permeation chromatogram (GPC) of the purified polymer (P(AMME), obtained at a conversion of 81% using the followingpolymerization conditions: [AMME]= 0.2 M, [AMPA]=10^{-3} M, Solvent; Acetate Buffer, Polymerization Time: 4h,Temperature: 50°C. A GPC system (Viscotek) equipped with a light scattering detector was used in this measurement.

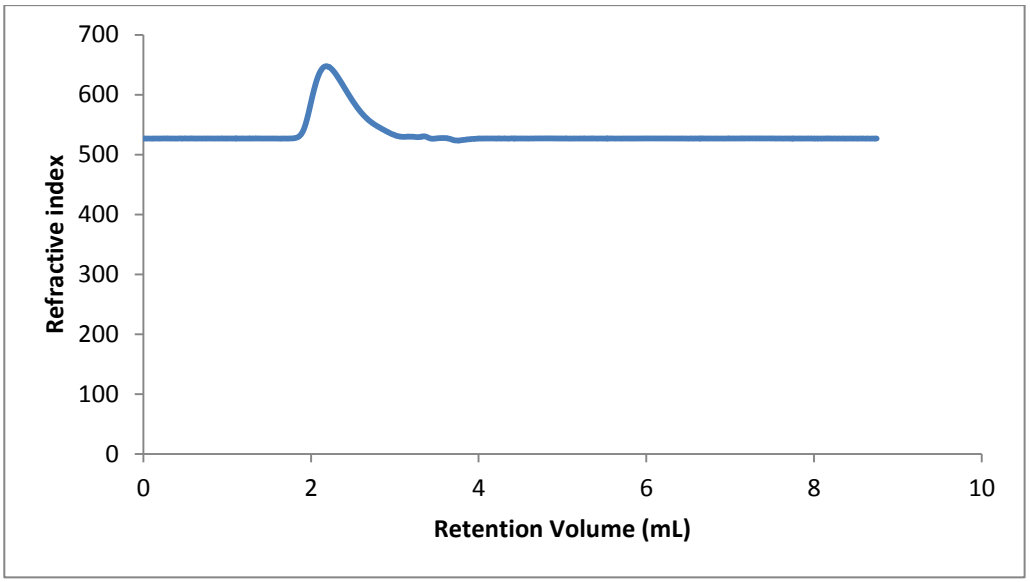

Figure 4.9. GPC chromatogram of P(AMME) synthesized by conventional free radical polymerization (conversion 81%, polymerization conditions: [AMME]= 0.2 M, [AMPA]= 1 mM, Solvent; Acetic Buffer, Polymerization Time: 4h, Temperature: 50°C). The chromatogram was taken using purified polymer. The mobile phase for GPC was 0.1 M acetic acid with 0.15 M NaCl. An Eprogen CATSEC300 (25cmx0,46cm) column was used. Flow rate was 0.5 mL/min

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4.6. Reversible Addition Fragmentation Chain Transfer (RAFT) Polymerization of AMME

RAFT Polymerization of AMME was performed using two different conditions as indicated in Table 4. For polymerization performed in methanol: buffer mixture, p(AMME) was obtained with a monomer conversion of 57% in 22 hours, as calculated from Figure 4.10 using the Equation 3.1. Peaks are assigned for both monomer and polymer at given spectrum.

Table 4. RAFT Polymerization Conditions and Polymer Characterization Results Determined by ^{1}H NMR and GPC Analyses.

[AMME] (mol/l)	$[AMME]_0/[ECT]_0/[Initiator]_0$	Initiator	Solvent	Time (h)	Conv. (%) by NMR	Mn by GPC (g/mol)	PDI by GPC
0.4	250/3/1	AIBN	DMAc	2	19	1800	1.03
0.4	250/3/1	AIBN	DMAc	4	19	1817	1.01
0.4	250/3/1	AIBN	DMAc	6	25	1982	1.00
0.4	250/3/1	AIBN	DMAc	8	29	2362	1.03
0.4	250/3/1	AIBN	DMAc	12	32	2508	1.03
0.2	545/5.5/1	AMPA	Methanol-Acetate Buffer	22	57	17000	1.27

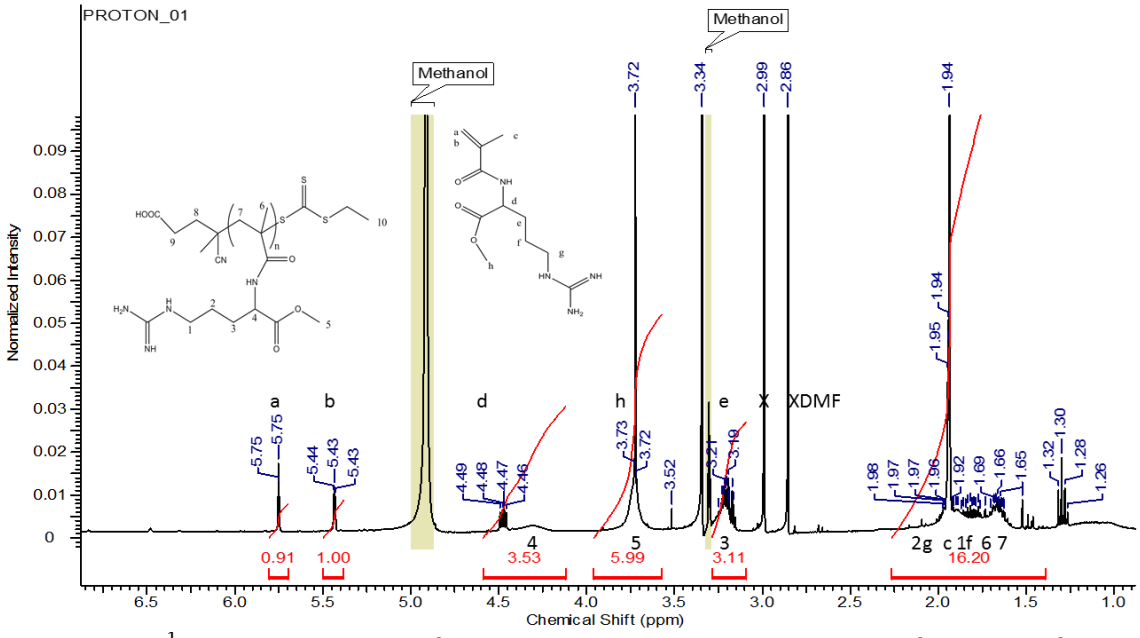

Figure 4.10. ^{1}H NMR spectrum of the RAFT polymerization mixture of AMME after 22 hours. Polymerization solvent was methanol:buffer mixture.

Figure 4.11 indicates the spectrum of the purified polymers obtained after dialysis. The dialysismethod for the RAFT-synthesized polymers was different than that for the polymers synthesized by conventionalfree radical polymerization due to the solubility issues of the excess RAFT agent and low polarity of the polymer end-group.

To overcome the solubility problem faced during dialysis, 50% methanol containing aqueoussolution at pH 4 was used. Content of methanol was gradually decreased as the dialysis solution was changed. The last day of dialysis, ultrapure water was used. Spectrum in Figure 4.11 shows the success of the developed dialysis method. Signals between 2.46 and 2.56 ppm refers to the RAFT end-group of the polymer. From these signals, $M_{n\,(NMR)}$ was calculated as 15641 g/mol by Equation 2.1.

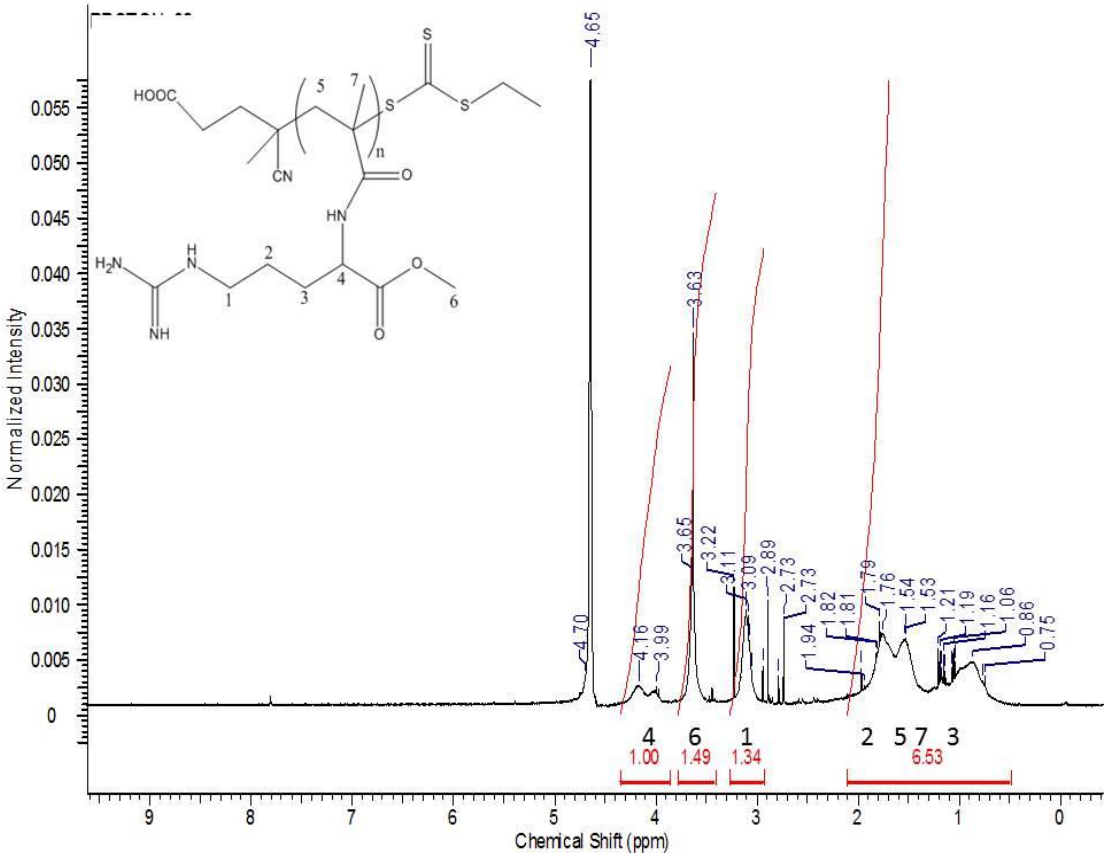

Figure 4.11. ^{1}H NMR spectrum of the RAFT-synthesized p(AMME) after dialysis. P(AMME) was obtained at a $[AMME]_0$/ $[ECT]_0$/$[Initiator]_0$ of 545/5.5/1 in methanol:acetate buffer mixture in 22 h.

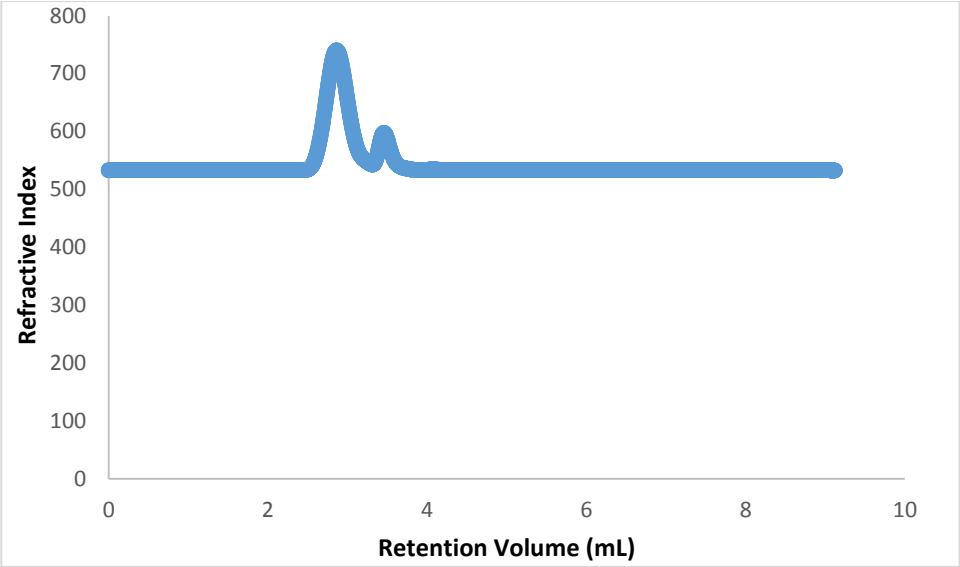

Figure 4.12 . GPC chromatogram of the RAFT-synthesized p(AMME) after dialysis. P(AMME) was obtained at a $[AMME]_0$/ $[ECT]_0$/$[Initiator]_0$ of 545/5.5/1 in methanol:acetate buffer mixture in 22 h.

Control over moleculer weight of p(AMME) synthesized by RAFT polymerization was proven by polymerization kineticstudies performed in dimethylacetamide (DMAc) at 70°C. Figure 4.13 shows the plot of monomer conversion to polymer calculated by ^1H NMR and molecular weight (Mn) of polymers obtained by GPC analysis. As it can be seen, as the conversion of the monomer increases, Mn of the polymers increases linearly. Similarly, ln ($[M_0]$/[M]) (first order) versus conversion plot showed a linear relation (Figure 4.14), which indicates well the RAFT-controlled polymerization mechanism. Figure 4.15 shows narrow molecular weight distrubiton of the polymers against calculated conversion values.

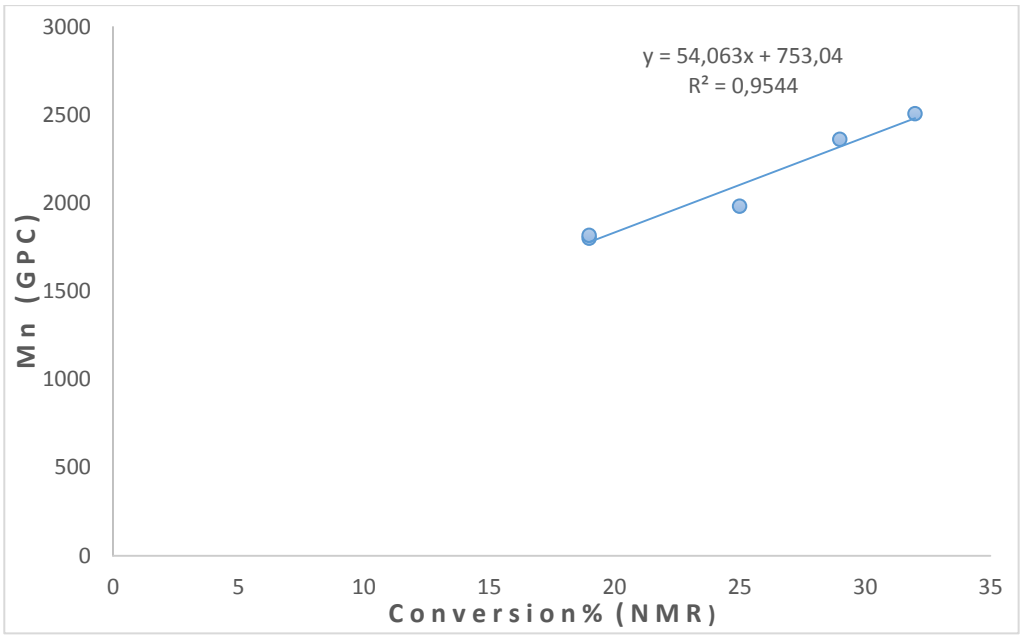

Figure 4.13. Conversion (NMR) versus Mn (GPC) graph.

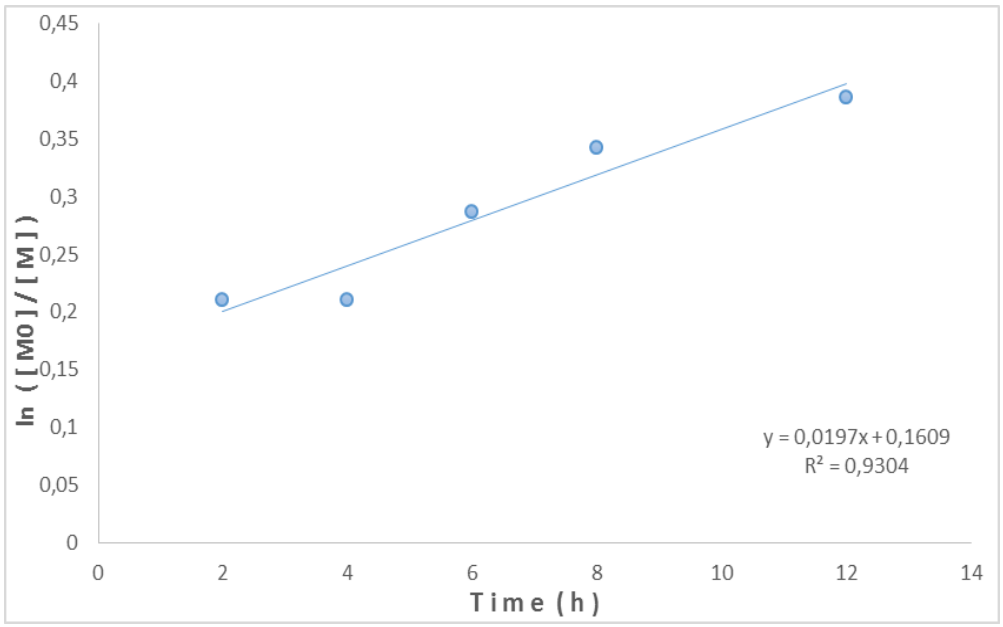

Figure 4.14. Polymerization time (hours) versus ln ($[M_0]/[M]$) graph.

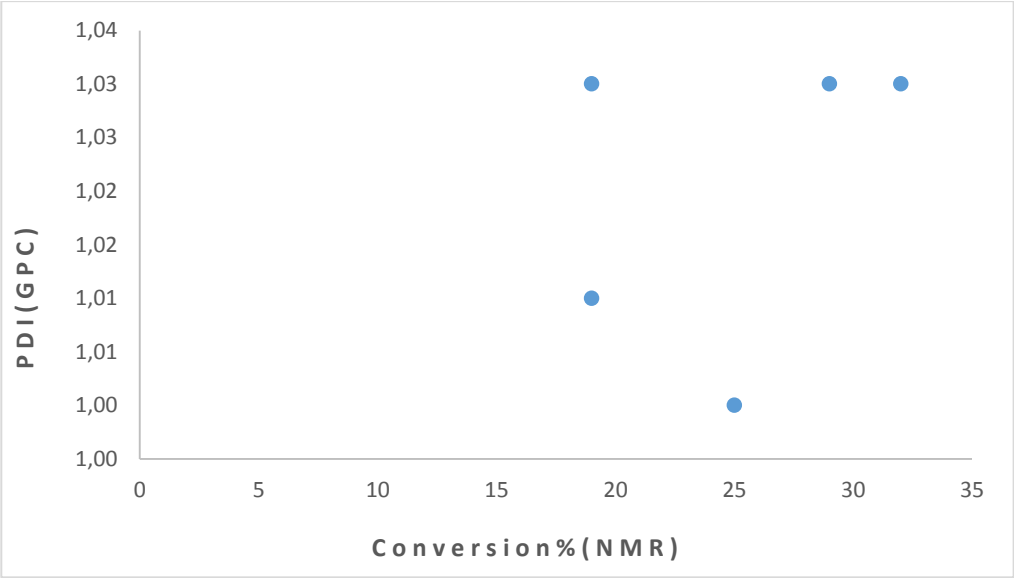

Figure 4.15. Conversion (NMR) versus PDI (GPC) graph.

4.7. Hydrolysis and Aminolysis of 4-Cyano-4-(ethylthiocarbonylthioylthio) sulfanylpentanoic acid (RAFT agent)

Before proceeding with RAFT polymerization of AMME, the hydrolysis and aminolysis kinetics of the RAFT agent (ECT) were investigated at varying conditions mimicking possible RAFT polymerization conditions. The thiocarbonylthio group of RAFT agents is susceptible to both hydrolysis and aminolysis, which makes it difficult to perform RAFT polymerizations in aqueous media and/or in the presence of amine-containing monomers (Alidedeoglu et al., 2009). Accordingly, before performing RAFT polymerization of AMME monomer, hydrolysis and aminolysis were performed by incubating ECT in methanol: buffer (pH 5.2) (1:3 v/v) mixture at 50°C in the absence or presence of AMME monomer (0.2 M or 0.4 M), respectively. The final concentration of the RAFT agent was 0.0015 M. Similarly, the aminolysis kinetic was also investigated in dimethylacetamide (DMAc) at 70°C. The absorbance of ECT solution (A_t) was measured at 294nm at given time points. The initial absorbance of ECT solution (A_0) was measured immediately after dissolving ECT in the respective solvent (without adding AMME). The hydrolysis or aminolysis percentage was calculated according to Equation 3.2. Figure 4.16 shows the aminolysis kinetics of ECT in DMAc at 70°C. Figure 4.17 and Figure 4.18show the hydrolysis and aminolysis kinetics of ECT,

respectively, in methanol: buffer mixture at 50°C. As it can be seen in the fFigures, in all cases the degradation of the RAFT agent was less than 10%, making this chain transfer agent ideal for RAFT polymerization of amine-containing monomers such as AMME both in aqueous and organic solvents, even at elevated temperatures.

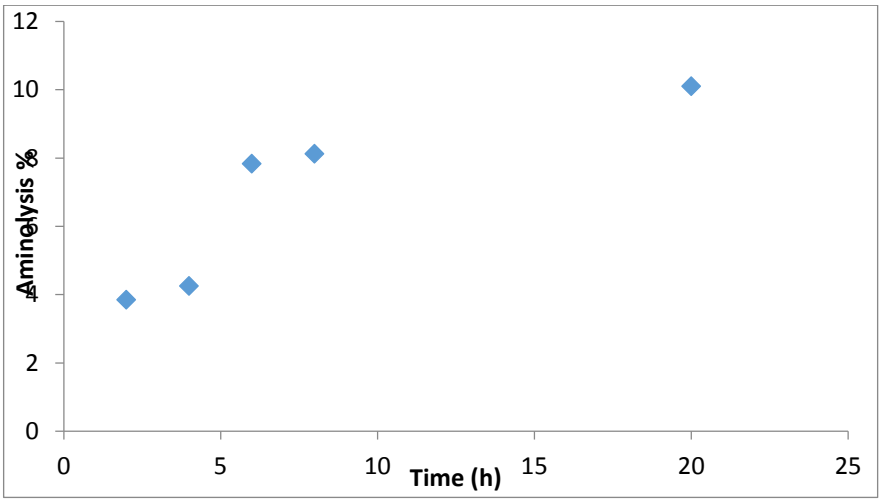

Figure 4.16. Aminolysis degradation percent vs. time graph for 4-cyano-4-(ethylthiocarbonylthioylthio) sulfanylpentanoic acid. Aminolysis was followed via UV-vis spectroscopy at 295nm using AMME of 0.4 M at 70^{o}C in DMAc.

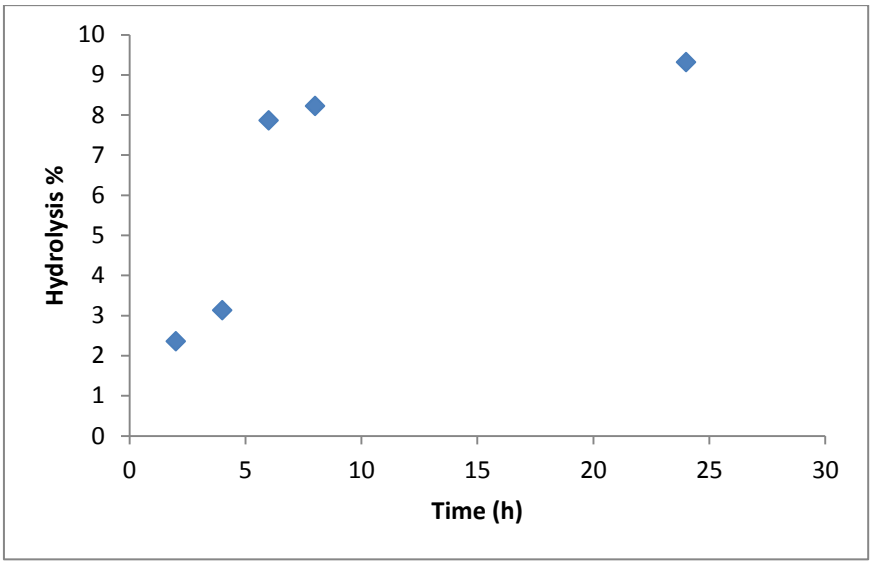

Figure 4.17. Hydrolysis degradation percent vs. time graph for 4-cyano-4-(ethylthiocarbonylthioylthio) sulfanylpentanoic acid. Hydrolysis was followed via UV-vis spectroscopy at 295nm at 50^{o}C in methanol-buffer (1:3 v:v) mixture.

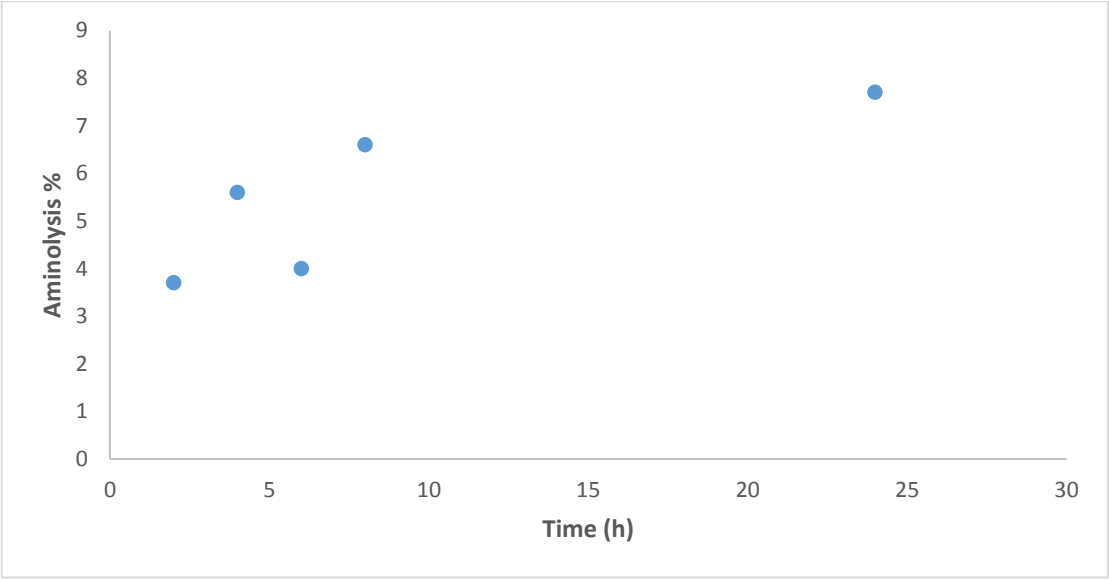

Figure 4.18. Aminolysis degradation percent vs. time graph for 4-cyano-4-(ethylthiocarbonylthioylthio) sulfanylpentanoic acid. Aminolysis was followed via UV-vis spectroscopy at 295nm using AMME of 0.2 M at 50^{o}C in methanol-buffer mixture (1:3 v:v).

4.8. In vitro Cytotoxicity of P(AMME)

A549 (Human lung adenocarcinoma epithelial) cell linewas used for investigating the effect of P(AMME) (Mn=16718 g/mol; PDI 1.22). The cytotoxicity of polymer was compared with that of octaarginine. In order to evaluate the cytotoxicity, a well-known method, MTT was used. Figure 4.19, Figure 4.20 and Figure 4.21 show the effect of octaarginine and P(AMME), respectively, on cell viability. The results shows that both octaarginine and P(AMME)'s cytotoxity is dose- and time-dependent. Both cytotoxic and non-cytotoxic range was defined by MTT assay. Accordingly, toxicity of the P(AMME) shows steep increase above 10 μM concentration for 24 hours incubation. Cells are able to tolerate less than 25 μM P(AMME) which is in the therapeutic range of most drugs.

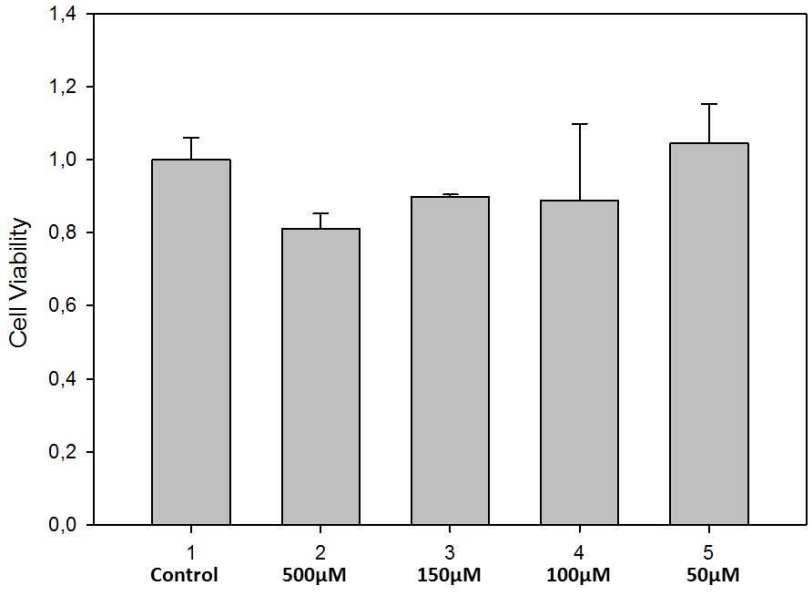

Figure 4.19.Cell viability results of the A549 cell line after incubation with the octaarginine peptide for 24 hours . (1) Control, (2) 500 μM octaarginine (40mM in Arginine Unit), (3) 250 μM octaarginine (2mM AU), (4) 100 μM octaarginine (800 μM AU), (5) 50 μM octaarginine (400μM AU).

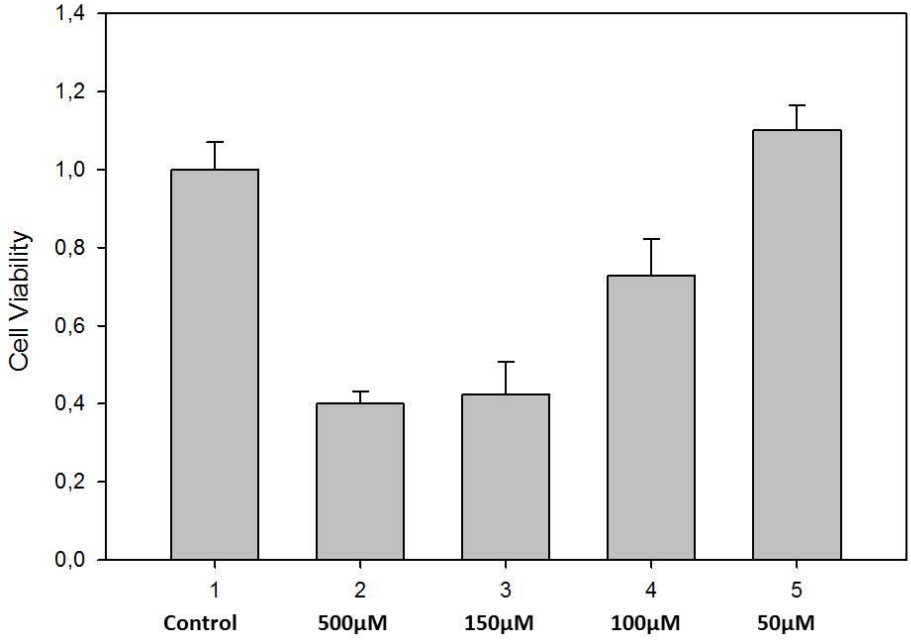

Figure 4.20. Cell viability results of the A549 cell line after incubation with the octaarginine peptide for 72 hours . (1) Control, (2) 500 µM octaarginine, (3) 250 µM octaarginine, (4) 100 µM octaarginine, (5) 50 µM octaarginine.

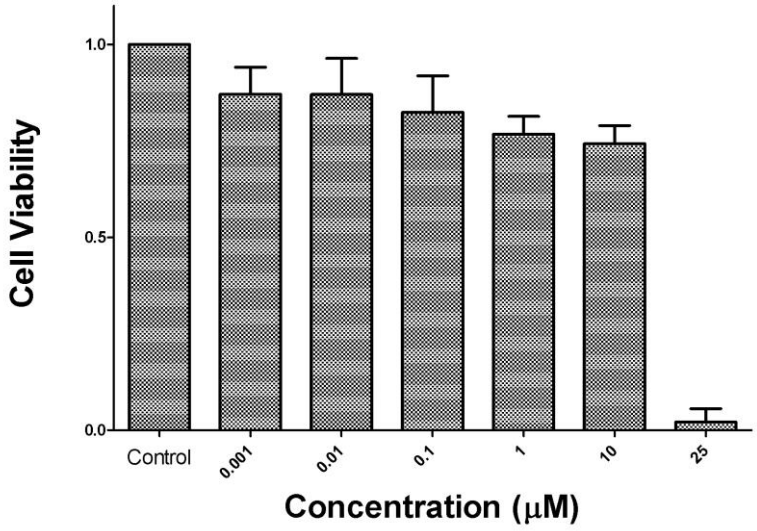

Figure 4.21. Cell viability results of the A549 cell line after incubation with P(AMME) for 24 hours . (1) Control, (2) 0.001 µM P(AMME) (0.06 µM), (3) 0.01 µM P(AMME) (0.66 M), (4) 0.1 µM P(AMME) (6.6µM), (5) 1.0 µM P(AMME) (66 µM), (6) 10 µM P(AMME) (660 µM), (7) 25 µM P(AMME) (1650 µM).

Figure 4.21 shows that p(AMME) is relatively toxic at high concentrations. It is known that for RAFT-polymers, RAFT thiocarbonylthio end-group may be a source of toxicity. Considering this,aminolyzedP(AMME)'s cytotoxicity was also investigated. The aminolyzed polymers contain an additional monomeric unit instead of thiocarbonylthio RAFT end-group. Figure 4.22 gives the cytoxity results of both aminolysed and RAFT end-goup carrying p(AMME)s, showing clearly that removing RAFT end-group eliminates the toxicity of the polymer. P(AMME) synthesized in this study can therefore be considered to have minimal or no cytotoxic effect similar to octaarginine, in the concentration and incubation time ranges tested.

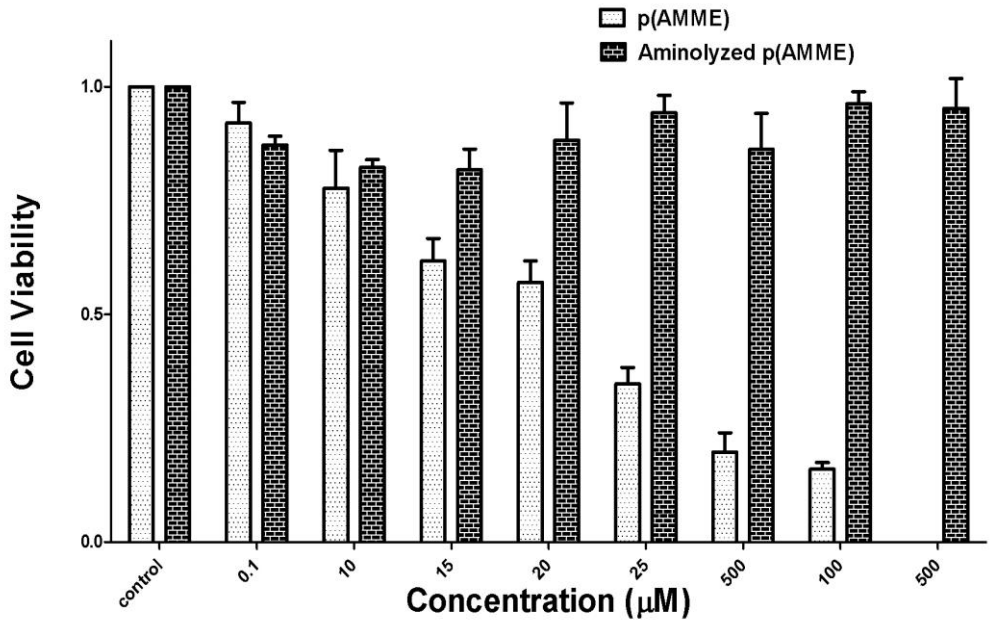

Figure 4.22. Cell viability of A549 cell line after incubation with varying concentrations of both aminolysed P(AMME) and P(AMME) with RAFT thiocarbonyl end-group. The repeating arginine unit concentrations equivalent to polymer concentrations tested are indicated in brackets: 0.1 μM polymer concentration (6.6μM Arginine unit concentration); 10 μM (660μM);15 μM (990μM); 20 μM (1320μM); 25 μM (1650μM); 50 μM (3300 μM); 100 μM (6.6 mM); 500 μM (33mM).

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CHAPTER 5

CONCLUSION

AMME was successfully synthesized via a new synthetic route consisted of three-step reactions without the need for protecting guanidine group of arginine. ECT whichis a previously reported trithiocarbonate RAFT agent was also synthesized due to its less bulky structure, solubulity in polar polvents and water medium and its resistance to aminolysis and hydrolysis.Both conventional free radical and RAFT polymerizations of AMME monomer were performed. Conventional free radical polymerizations yielded polymers with uncontrolled molecular weights and wide molecular weight distributions, whereas in RAFT polymerizations molecular weight of the polymers was controlled by time and monomer conversion. The RAFT-controlled character of polymerization was proven by polymerization kinetic study.Cell viability experiments showed that P(AMME) after aminolysis of the RAFT end-group hasno toxicity upto 500 μM concentration, comparable with octaarginine, a widely used transfection agent

In conclusion, well-defined arginine-polymers as potential components of intracellular delivery systems for therapeutics were sucessfully synthesized by RAFT polymerization. The polymers were found to have no significant toxicity on A549 cell line, comparable with octaarginine, after removal of the RAFT end-group. Further studies may be required to investigate cell internalization and intracellular distribution of arginine polymers synthesized in this study. Synthesis of copolymers with a neutral, biocompatible block such as poly(ethylene glycol) may also be performed in future studies.

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