

**SYNTHESIS OF WELL-DEFINED FATTY ACID
POLYMERS AS POTENTIAL MEMBRANE
DESTABILIZING AGENTS**

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ABSTRACT

SYNTHESIS OF WELL-DEFINED FATTY ACID POLYMERS AS POTENTIAL MEMBRANE DESTABILIZING AGENTS

The aim of this thesis is to synthesize well-defined, fatty acid polymers via reversible addition fragmentation chain transfer (RAFT) polymerization, as potential membrane destabilizing agents which can be used for intracellular drug delivery applications.

A new methacrylate monomer, derived from an unsaturated fatty acid, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (UDAMA), was synthesized using 10-undecenoic acid as a starting compound. Monomer synthesis was composed of two steps: In the first step, thiol-ene thermal addition of 2-mercaptoethanol to 10-undecenoic acid was performed. The yield of the reaction was 85 %. In the second step, the addition product was reacted with methacryloyl chloride to yield a new monomer, UDAMA. The yield of the second synthetic step was 92%, calculated from ^1H NMR spectroscopy. UDAMA was polymerized via both conventional free radical and RAFT polymerization techniques. Polymers were characterized using ^1H -NMR spectroscopy and gel permeation chromatography (GPC). Linear increase in $\ln [M]_0/[M]$ with polymerization time, and M_n with monomer conversion indicated the RAFT-controlled polymerization of UDAMA monomer under the conditions tested.

The new monomer, UDAMA was also copolymerized with methacrylic acid (MAA) via RAFT polymerization to obtain water-soluble, pH-responsive polymers. Random copolymers of MAA and UDAMA were synthesized using two different polymerization feed composition having 20 mol% or 50 mol% UDAMA content. Copolymerizations were also found to be controlled by RAFT mechanism, as evidenced by measurements via ^1H -NMR spectroscopy and GPC. The pH-responsive behavior of copolymers was demonstrated via UV-visible spectroscopy and dynamic light scattering measurements. Hemolysis assays revealed that the copolymers with 20 mol% UDAMA content demonstrated pH-dependent hemolytic activity.

ÖZET

POTANSİYEL MEMBRAN BOZUCU AJANLAR OLARAK İYİ TANIMLANMIŞ YAĞ ASİDİ POLİMERLERİNİN SENTEZİ

Bu tezin amacı, hücre içi ilaç salımı alanlarında potansiyel membran bozucu ajanlar olarak kullanılmak üzere iyi tanımlanmış, yağ asidi polimerlerinin, tersinir katılma ayrışma zincir transfer (RAFT) polimerizasyonu ile sentezlenmesidir.

Doymamış bir yağ asidi olan 10-undekanoik asitten türevlendirilmiş olan yeni bir metakrilat monomeri, 11-[2-(2-Metil-akriloksil)-etilsülfanil] undekanoik asit (UDAMA) sentezlenmiştir. Monomer sentezi iki basamaktan oluşmaktadır: Birinci basamakta, 2-merkaptetanolün 10-undekanoik asite tiyol-ene termal katılma reaksiyonuyla eklenmesi gerçekleştirilmiştir. Bu basamakta elde edilen ürün verimi % 85'dir. İkinci basamakta ise, katılma ürünü, metakriloil klorür ile reaksiyona sokularak yeni bir metakrilat monomeri (UDAMA) sentezlenmiştir. Ürün verimi, ¹H-NMR spektroskopisi ölçümlerinden %92 olarak hesaplanmıştır. Monomer, serbest radikal ve RAFT polimerizasyonu teknikleri ile polimerleştirilmiştir. Elde edilen polimerler, ¹H-NMR spektroskopisi ve jel geçirgenlik kromatografisi (GPC) ile karakterize edilmiştir. In $[M]_0/[M]$ değerinin zaman, monomer dönüşümün ise M_n değeri ile lineer artması, UDAMA monomerinin RAFT mekanizması kontrollü polimerleştiğini göstermiştir.

Suda çözünebilen ve pH'ya duyarlı polimerler elde edebilmek amacıyla, UDAMA'nın, metakrilik asit (MAA) ile kopolimerleri, RAFT polimerizasyonu ile sentezlenmiştir. UDAMA ve MAA'nın rastgele kopolimerleri, 2 farklı besleme kompozisyonunda (molce % 20 ve % 50 UDAMA içeren) sentezlenmiştir. ¹H-NMR spektroskopisi ve GPC analizlerinin kanıtladığı üzere, kopolimerizasyonların da RAFT mekanizması ile kontrollü gerçekleştiği anlaşılmıştır. Kopolimerlerin pH'ya duyarlı davranışları, UV-görünür spektroskopisi ve dinamik ışık saçılması ölçümleri ile gösterilmiştir. kullanılarak bulanıklık değişimleri ölçülerek değerlendirildi. Hemoliz deneyleri, molce % 20 UDAMA içeren kopolimerlerin pH'ya duyarlı bir şekilde hemolitik aktivite gösterdiğini ortaya çıkarmıştır.

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CHAPTER 1

INTRODUCTION

In certain therapeutic strategies, macromolecular therapeutics need to enter cells at therapeutic concentrations. Macromolecular therapeutics such as DNA, RNA, protein, siRNA and peptides used in certain therapeutic strategies show their therapeutic activity in cell cytosol or nucleus. However, such therapeutics are cell membrane impermeable molecules that cannot be taken up by cells via passive diffusion. Cell membrane is one of the main barriers for these macromolecular therapeutics. Cellular uptake of macromolecules usually occurs by endocytosis mechanism which results in the degradation of the uptaken macromolecules in lysosomal compartments. Endocytosis is the major uptake mechanism of macromolecular therapeutics. Molecules are entrapped in endosome vesicles which have acidic environment and then transferred to lysosomes where reduced pH and degradative enzymes degrade macromolecular therapeutics. In endosome vesicles, the pH is progressively lowered to 5.5 and 6.5, and in lysosome compartments, pH is as low as 4.5.

Efficient delivery of macromolecular therapeutics can be enabled by transfection vehicles called vectors. Vectors can be used to cross cellular membranes. There are two major classes; non-viral and viral vectors. Bacteria and viruses can be used as viral vectors and they are known as efficient delivery agents especially for genes and nucleic acid drugs due to specific proteins present on their outer surface facilitating uptake by cell plasma membrane. Viral vectors can also have capability to destabilize cellular membranes. However viral vectors have potential immunogenic properties and safety concerns. Another problem is that large scale pharmaceutical grade production of viral vectors is very difficult. Non-viral vectors are attractive alternatives over viral vectors because of their minimal toxicity and low immunogenicity. While they have such advantages when comparing to viral vectors, they are less efficient in escaping the endosomal pathway. However by recent technologies non-viral vectors that can mimic endosomal escaping mechanism of viral vectors have been developed. Amphiphilic peptides, cationic lipids, liposomes, amphiphilic polymers are examples of non-viral vectors. Among these vectors, lipid-based systems show high transfection efficiencies.

Lipidic systems enable delivery of highly hydrophobic drugs. However, designing lipidic systems can be problematic due to solubility and stability problems. Polymers are also promising delivery agents that can be tailored and designed using different chemical synthesis methods. pH-sensitive amphiphilic polymers have been designed to act as membrane destabilizing agents to get over the cell membrane barrier (Figure 1.1).

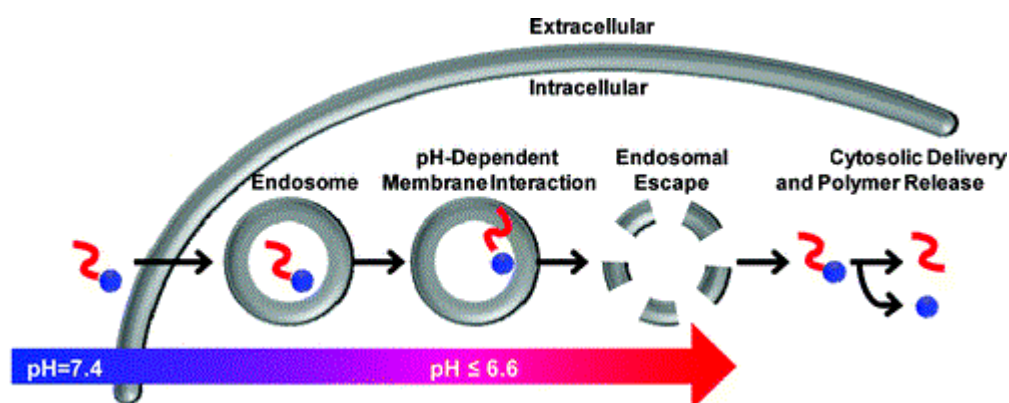


Figure 1.1. A schematic drawing of “smart” pH-sensitive polymers (Source:Duvall et al., 2010)

Membrane-destabilizing amphiphilic polymers can destabilize cell membrane in a pH-responsive manner. Protonable groups of these polymers have ability to sense pH changes in their surroundings and due to amphiphilic character they can undergo from a hydrophilic state at physiologic pH to a hydrophobic, membrane destabilizing state at acidic pH. Such polymeric systems can be tailored to be non-toxic and non-immunogenic. However, polymeric systems that have been developed to date have shown less transfection activity compared to lipid or peptide based systems.

In literature, most of the amphiphilic membrane-destabilizing polymers have been synthesized via conventional polymerization techniques, such as free radical polymerization, leading to polymers with broad molecular weight distributions and uncontrolled molecular weights. Reversible Addition Fragmentation Chain Transfer (RAFT) polymerization is a living polymerization technique that enables the synthesis of well-defined polymers with controlled molecular weight and narrow molecular weight distribution (narrow polydispersity index (PDI)). It also allows the design of complex macromolecular architectures and can be applied to different functional monomers under a wide variety of reaction conditions.

The aim of this thesis is to synthesize well-defined amphiphilic polymers of a fatty acid and characterize pH-responsive membrane-disruptive activity of these polymers. Fatty acids contain both acidic groups and long alkyl chains on the same molecule. While acidic group provides pH-responsive behavior, long alkyl chain may provide membrane-destabilizing activity. The fatty acid used for this thesis is 10-undecenoic acid which is an unsaturated fatty acid that can be modified to compose a suitable monomer for polymerization reactions. The procedures for monomer and polymer synthesis and characterizations are presented in Chapter 3. Chapter 4 covers the results of new monomer characterization, RAFT polymerization kinetics studies, and characterization of polymers.

CHAPTER 2

LITERATURE REVIEW

2.1. Intracellular Drug Delivery

In recent years, macromolecular therapeutics, such as DNA, RNA, protein, siRNA and peptides that have potential to treat various diseases including cancer, viral infections, cardiovascular and neurodegenerative diseases have been developed. However, there are a number of barriers that limit the translation of these therapeutic molecules to clinical use. Cell membrane is one of the major transport barriers that must be overcome. These membrane-impermeable molecules must reach cytosol before showing their biological activity inside cells (Carrasco, 1994). Biological macromolecules, such as proteins and DNA, and synthetic polymeric carriers and nanoparticulate carriers as structures having large hydrodynamic volume, cannot carry out passive diffusion through the plasma membrane of eukaryotic cells. The typical cell uptake pathways for such macromolecules and systems with large hydrodynamic volume are via endocytosis (Figure 2.1). These molecules are entrapped inside the cells by vesicles called endosome. Endosomes have acidic pH environment and most of the entrapped molecules are carried to lysosomal compartments. In the lysosomal compartment carrying the degradation machinery of the cell, pH is as low as 4.5. In addition, there are also a large variety of degradative enzymes in lysosomes. This situation causes the destruction of therapeutic molecules with little or no release to cytosol.(Akinc & Langer, 2002).

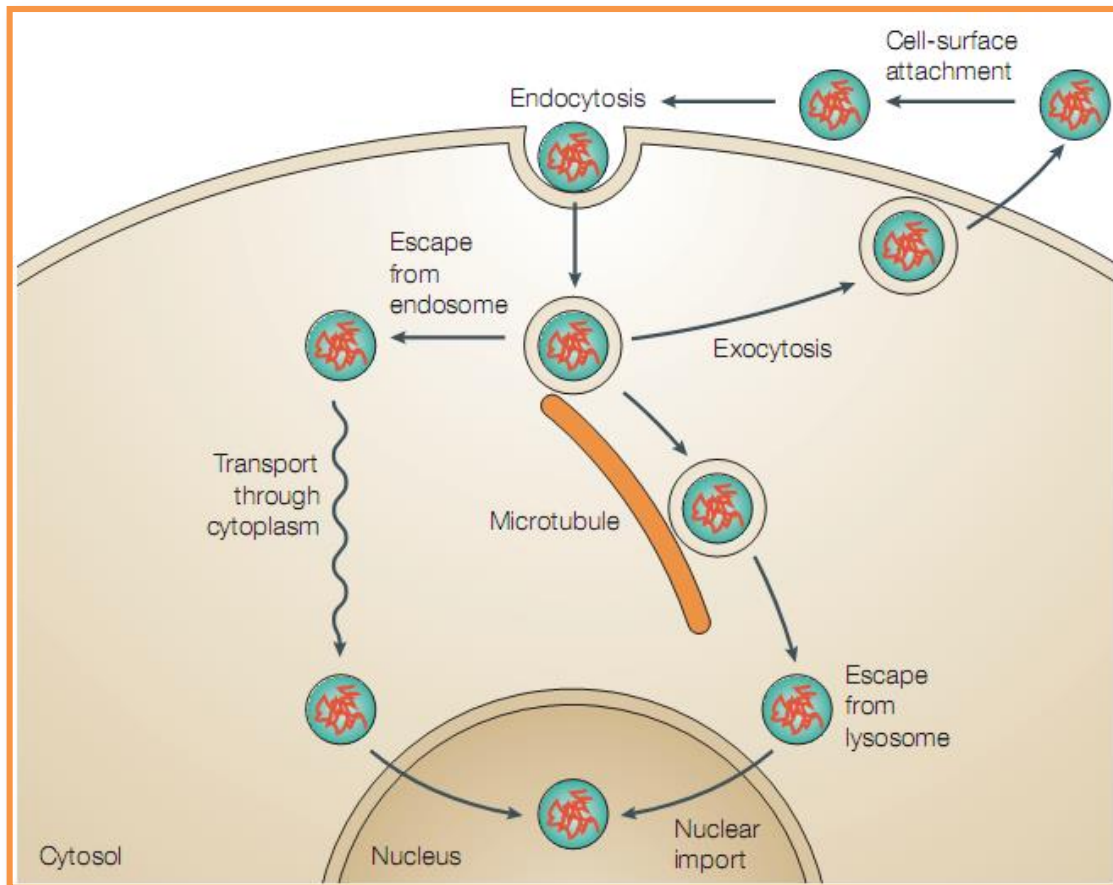


Figure 2.1. Endocytosis of macromolecular therapeutics
(Source: Pack et al.,2005)

Typical endocytosis pathway of a macromolecule can be seen in Figure 2.1. For efficient intracellular delivery of macromolecules, understanding the mechanism of the endocytosis is beneficial. In endocytosis mechanism, most of the entrapped molecules are trafficked to the lysosomes and degraded, which minimizes the therapeutic effect. During intracellular trafficking, rapid recycling of macromolecules out of the cell is about 5 -10 min and lysosomal destruction is about 30 min -1 hr.(Jones, Gumbleton, & Duncan, 2003). In a study by Berg et al., the delivery of protein drugs to the cytoplasm of mouse fibroblast cells, was less than % 5 due to endocytosis mechanism (K. Berg et al., 1999). Nucleic acid drugs are delivered approximately less than % 20 (Bijsterbosch et al., 2002).

Delivery vehicles called vectors enable intracellular delivery of macromolecular therapeutics by overcoming endocytosis pathway. In literature, there are two types of vectors; natural systems called viral vectors including viruses and bacteria, and

synthetic vectors called non-viral vectors including amphiphilic peptides, cationic lipids, liposomes, and polymers. Viral vectors use their own integral surface proteins facilitating uptake through the cell membrane. Viral vectors are highly efficient in transfection of cells. However, there are important safety concerns for *in vivo* applications of viral systems due their potential toxicity, immunogenicity and cancerogenicity. They are difficult to large-scale pharmaceutical grade production.(C. E. Thomas, Ehrhardt, & Kay, 2003).

Non-viral vectors have been designed to serve as simple models of viral counterparts. They offer minimal toxicity and low immunogenicity. Non-viral vectors are promising intracellular drug delivery systems as they can mimic cell penetrating systems of viral vectors and they can be potentially safer than viral counterparts. Desired function of the system can be tailored through versatile synthetic means. The main disadvantage of non-viral vectors is that they are usually less efficient in endosomal escaping when compared with viral vectors. There are a large number of studies in literature to improve the endosome escaping efficiency of non-viral vectors (Akhtar, 2005).

Potential endosomal escaping mechanisms which would facilitate the escape of therapeutics from endosome to cytosol include pore formation in the endosomal membrane, fusion with the membrane and pH buffering of endosomes.

Pore formation generally occurs based on the interplay between a membrane tension causing the enlargement of the pore and a line tension causing the closure of the pore. Peptides which have specific affinity to membrane lead to reduction of line tension. Some models are offered to explain pore formation in the endosomal membrane. In the barrel-stave pore model, escaping agent can destabilize the membrane to form barrel-like pore (Fig. 2.2 (A), (Westphal, Dewson, Czabotar, & Kluck, 2011)). In toroidal model, escaping agents such as peptides aggregate in the membrane by directing perpendicularly, curving the membrane and forming a hole where agents can line on cell membrane. (Fig. 2.2 (B), (Westphal et al., 2011)).

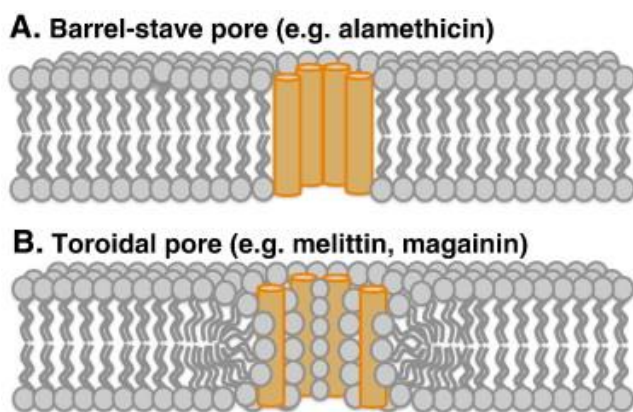


Figure 2.2. Pore formation mechanisms via Barrel-stave and Toroidal models.
(Source: Westphal et al.,2011)

For proton sponge effect which is also known as pH buffering effect, delivery agents should possess high buffering capacity in the endosomal pH range. A well-known polymer displaying proton-sponge effect is polyethylenimine (PEI). PEI contains protonable secondary and tertiary amine groups, and shows high buffering capacity and transfection efficiency. The agents which have high buffering capacity in the endosomal pH range cause inflows of protons and chloride ions into endosomes due to endosomal acidification leading to osmotic pressure difference in endosomal vesicle and swelling of the vesicle, which ultimately results in the rupture of endosome (Figure 2.3) .(Otmane Boussif et al., 1995; Varkouhi, Scholte, Storm, & Haisma, 2011).

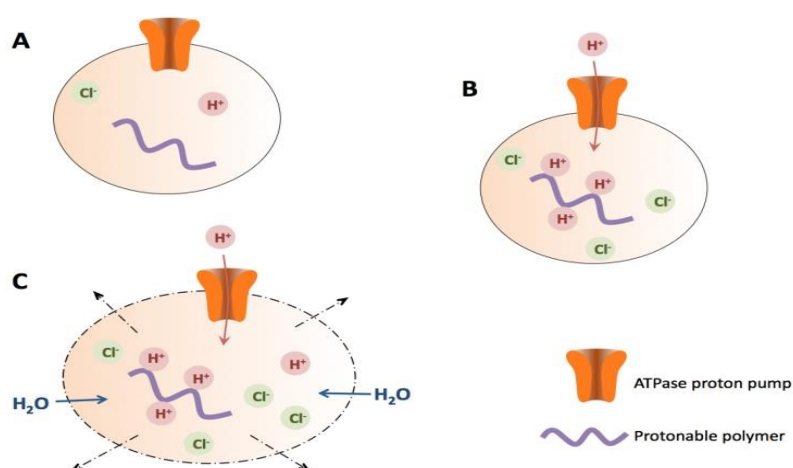


Figure 2.3. The proton sponge hypothesis (pH-buffering effect)
(Source: Liang and Lam, 2012)

Fusion in the endosomal membrane is another proposed theory for endosomal escaping pathways of macromolecules. Fusogenic peptides such as heamagglutinin can be given as an example for this pathway. Such peptides have specific binding affinity to cell membrane. This affinity causes conformational change upon a trigger such as pH change. Anionic fusogenic peptides are converted to adopt a hydrophobic conformation due to the acidic environment of endosomal vesicles, which leads to fusion with lipid bilayer.

2.2. Membrane Destabilizing Systems for Intracellular Drug Delivery

In literature several natural and synthetic membrane destabilizing systems have been developed.

2.2.1. Membrane Destabilizing Proteins and Peptides

In literature, there are several proteins and natural and synthetic peptides which can destabilize endosomal membrane and are used for intracellular drug delivery. These proteins/peptides fuse with endosomal membrane and facilitate the escape of their cargo to the cytosol.

Haemagglutinin (HA) is one of the examples of natural membrane destabilizing proteins. Haemagglutinin (HA) is in the influenza virus coat. The mechanism for the endosomal release of this protein is related to the protonation of carboxylate groups of acidic amino acid residues due to decreased pH in endosomal vesicles. Hydrated coil at pH 7.4 of HA becomes hydrophilic helical conformation at lowered pH and this state can destabilize the endosomal membrane. Schematic representation of how HA can destabilize the endosomal membrane can be seen in Figure 2.4 (Wiley & Skehel, 1987).(Ren, Sharpe, Collier, & London, 1999)

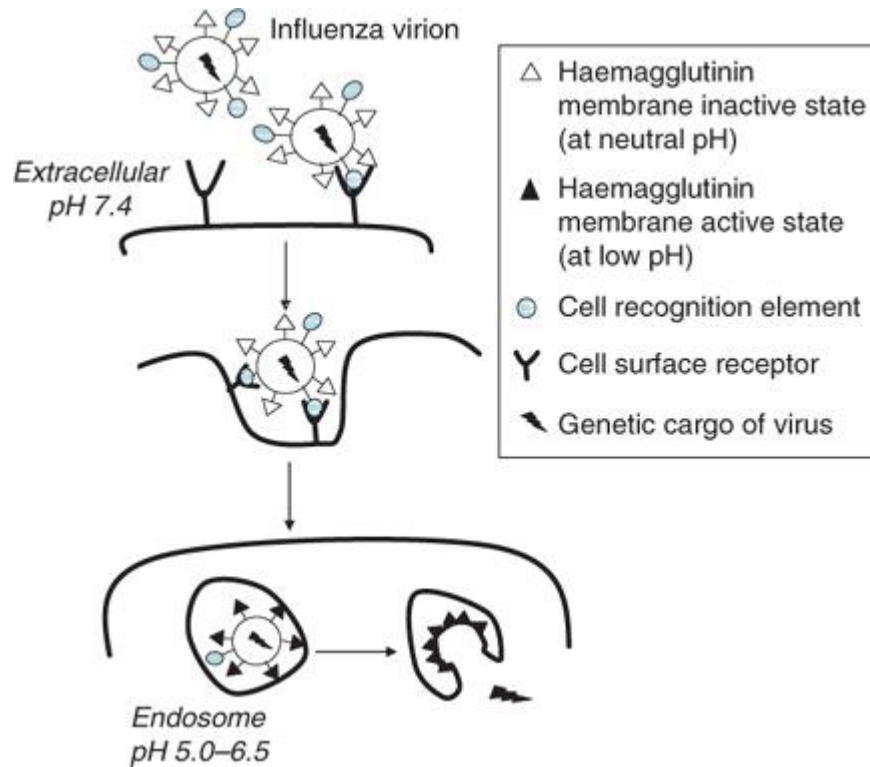


Figure 2.4. Schematic representation of the influenza virus entry to the cell
(Source: Bulmus, 2005)

In a study of Zurbriggen et al. (Zurbriggen, 2003) immunostimulating reconstituted influenza virosomes (IRIVs) that are liposomal carriers having influenza hemagglutinin as a membrane fusogenic agent have been prepared. TAT peptide being the first cell-penetration peptide (CPPs) identified was derived from the transcription activating factor of human immunodeficiency virus 1 (HIV-1). TAT peptide contains mainly arginine and lysine residues. There are a large number of studies with TAT peptide. In the work of Torchilin and co-workers liposome-DNA complexes containing TAT were used to transfect cells *in vitro* and *in vivo*. The efficiency of TAT-liposome associated plasmid DNA transfection was very high. Additionally, when compared to cationic lipid based delivery systems, cytotoxicity of this system was lower (Torchilin et al., 2003).

Membrane-active synthetic peptides were originally designed to mimic membrane-fusion peptide domains of viruses. First studies about the synthetic membrane destabilizing peptides were done by Szoka et al. This group designed and synthesized an acidic amphipathic peptide, GALA. GALA can be described as a repeat

of the sequence glutamic acid–alanine–leucine–alanine. Apolar leucine residues confer a relatively high hydrophobicity on the peptide, whereas polar glutamic acid residues add a pH-sensitive component to the hydrophobicity and the net charge. In the endosomal compartment; glutamic acid residues are protonated at lower pH and adopts the helix state and leucine units are arrayed on one side of protonated glutamic acid to constitute hydrophobic part. GALA was shown to induce membrane fusion (Subbarao, Parente, Szoka Jr, Nadasdi, & Pongracz, 1987), fragmentation, (R. A. Parente, Nir, & Szoka Jr, 1990) and endosomal escaping mechanism by pore formation.(R. Parente, Nir, & Szoka, 1988).

The negatively charged GALA cannot bind with nucleic acid through GALA can only be added as an additional functional component to polyplexes or lipoplexes. KALA is a modified version of GALA by preplacing glutamic acid with lysine partially. KALA is one of the first example of membrane-destabilizing peptides which bind nucleic acids. Although KALA has similar peptide sequence with GALA, membrane destabilization mechanism is different from GALA. When pH is lower, KALA adopts α -helix conformation in a wide pH range and conformational change from amphipathic α -helical to a mixture of α -helix and random coil(Wyman et al., 1997).

By taking sequence of Haemagglutinin subunit HA2 of influenza virus which can mimic endosomal escaping mechanism of viral fusion proteins is another example for membrane destabilizing peptides. The C-terminal end of the peptide is embedded in the viral membrane whereas the N-terminal end contains a fusion peptide with a sequence of hydrophobic amino acids. In the endosomal compartment due to change of pH, the fusion peptide leads to viral genome leakage to cytosol (Stegmann, 2000). In the study of Wagner et al., HA2 was connected to polyplexes containing transferrin/PLL(polylysine)/DNA and this complex structure was found very efficient in respect to intracellular delivery (Wagner, Plank, Zatloukal, Cotten, & Birnstiel, 1992).

Another example for membrane destabilizing peptides is LAH4 peptide and its derivatives that exhibit efficient cell transfection activity. (Kichler, Leborgne, März, Danos, & Bechinger, 2003) Peptides of the LAH4 family which are synthetic cationic amphipathic are mainly composed of histidine residues and hydrophobic amino acids (alanine and leucines). Firstly, these peptides were designed for determination of the interactions of membrane associated proteins.(Bechinger, 1996) When compared to polyethylenimine (PEI) which is a cationic polymer having high buffering capacity,

LAH4 enables higher transfection due to four to five histidine residues present in its central region.. During acidification of endosomes, the imidazole groups of histidine residues become protonated and the peptide changes to hydrophobic state and interacts with the anionic lipids in the endosomal membrane. Membrane destabilization occurs, followed by the release of cargo into the cytosol.(Kichler, Mason, & Bechinger, 2006).

There are many drawbacks of membrane-destabilizing peptides. Synthetic fusogenic peptides have immunogenicity, lack of stability and high cost for large scale applications. Sequence of membrane active peptides approximately varies between 9 and 30 amino acids. Synthesis of large amounts of such peptides is main obstacle for commercialization.(Li, Nicol, & Szoka Jr, 2004) Due to these reasons, non-immunogenic, stable and more cost-feasible systems such as polymers that can mimic action of membrane-destabilizing peptide systems would be very valuable for intracellular drug delivery applications. (Horváth et al., 1998; Kalyan et al., 1994)

2.2.2. Membrane-Destabilizing Polymers

Potential problems about membrane-destabilizing peptides reveal a need for designing alternative systems. Synthetic polymers which contain similar pH-sensitive chemical functionalities and mimic the membrane-active action of synthetic and natural peptides can serve as new vehicles for intracellular drug delivery applications.

2.2.2.1. Membrane Destabilizing Polycations

Certain synthetic polycations have been used as efficient transfection agents for many years. Membrane-Destabilizing cationic polymers are mainly used for intracellular delivery of nucleic acids due to their positive charge. This kind of polymers is made of primary, secondary or tertiary amino groups. They have capability to form electrostatic complexes with DNA. polyethyleneimine (PEI), poly((2-dimethylamino)ethyl methacrylate are examples of cationic polymers.(Eliyahu, Barenholz, & Domb, 2005)

Poly (ethyleneimine) (PEI) was first invented by BEHR in 1995. (Otmame Boussif et al., 1995) PEI is synthesized as linear or branch type, containing secondary or primary and tertiary amine groups, respectively, in its backbone and branches (Figure 2.5)

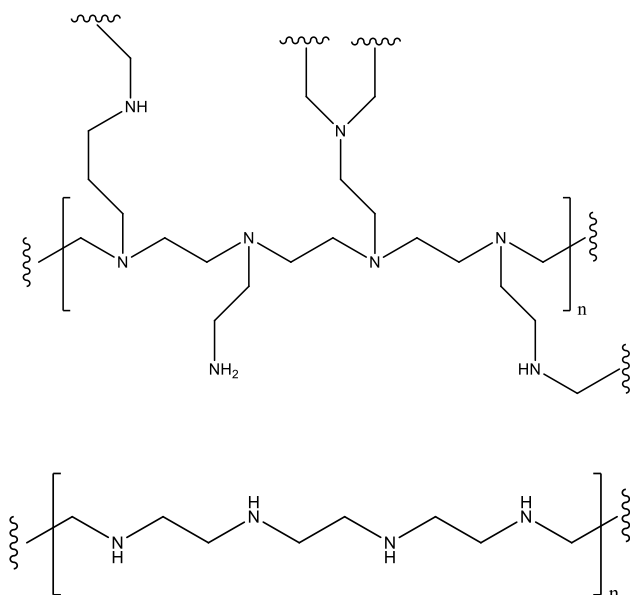


Figure 2.5. Chemical structures of linear and branched polyethyleneimine (Source: Godbey et al.,1999)

Highly branched PEI (e.g 25 kDa) and linear PEI are the mostly used types of polycations for intracellular drug delivery. PEI has significant transfection efficiency when compared to other polycationic systems due to its higher charge density. But this high charge density ends up with high toxicity preventing its *in vivo* use. The pKa value of primary and secondary amine groups in branched PEI structure is around 9.5 and 4.5, respectively (Koper, van Duijvenbode, Stam, Steuerle, & Borkovec, 2003). Primary amines of PEI can be protonated at any cellular pH and PEI behaves as a “proton sponge” (O. Boussif et al., 1995).

Highly positive characteristic of PEI causes several drawbacks. For example; the membrane-disruptive property frequently causes high cytotoxicity. The strong binding with negatively charged proteins deteriorates serum stability of the polyplexes of PEI and nucleic acid drugs.

Modifying PEI with different systems can increase efficiency of PEI. One-step method for preparing a new non-viral gene delivery vector by grafting poly-(2-methacryloyloxyethyl phosphorylcholine) (PMPC) onto the 25 kDa polyethylenimine (PEI 25k) via a TBHP (tert-Butyl hydroperoxide) initiated polymerization was investigated by Sun et al. In this study, different compositions of PMPC grafted PEI system was synthesized. Protein adsorption and cytotoxicity of these polymers were investigated in with PEI 25 kDa. The resulting PEI-PMPC polymers have higher gene delivery efficacy and lower toxicity when compared to 25 kDa PEI. These polymer complexes have gained zwitterionic characteristic. PEI-PMPC6/pEGFP and PEI-PMPC11%/pEGFP complexes work well and exhibit satisfactory gene transfection efficiencies, which are in general higher than that of PEI 25k/pEGFP, especially in the presence of 10% serum. pEGFP used as reporter gene. This work provides an effective way for development of a new biocompatible material based on PEI. (Sun, Zeng, Jian, & Wu, 2013)

In a recent study of Yasayan et al.; synthesized amphiphilic polymers by controlled free radical polymerization techniques and these polymer covered with branched PEI. The behavior of these polymersomes against different pH and buffer strength was investigated. By the covering of branched PEI, these well-defined polymeric vesicles showed enhanced cell uptake. (Yaşayan et al., 2012).

The other example for membrane destabilizing polymers are poly((2-dimethylamino)ethyl methacrylate). In the work of Verbaan et al. (Verbaan et al., 2004), PEG polyplexes were synthesized with poly((2-dimethylamino)ethyl methacrylate) as a graft polymer and AB diblock copolymers. After determination of physicochemical characteristics of polymers, pharmacokinetics and biodistribution of PEG-polyplexes were studied in mice after IV administration. Polymer complexes which prepared via postPEGylation method with PEG₂₀₀₀₀ yielded polyplexes that show minimum interaction with blood components (i.e. albumin and erythrocytes) and also showed substantially prolonged circulation time in mice after IV administration.

In another work, RAFT synthesis and DNA binding of biodegradable, hyperbranched poly(2-(dimethylamino)ethyl methacrylate) were synthesized by Tao and coworkers (Tao, Liu, Tan, & Davis, 2009). The aim of this paper is to target high molecular weights ($M_w \sim 290K$) of the polymer and to ensure effective DNA binding by this way. By the help of suitable disulphide reducing agent, biodegradable hyperbranched PDMAEMA were obtained for potential use as a gene delivery agent.

They synthesized high molecular weight of hyperbranched polymers and analysed their DNA binding ability by agarose gel electrophoresis. Firstly, polymers protonated and mixed with DNA having different positive/negative (P/N) ratio, and the binding was monitored by agarose gel electrophoresis. In the ratio of positive/negative 1:1, the DNA was neutralized and there was no DNA complex observed via the gel. This result was consistent with effective formation of a polymer/DNA complex.

2.2.2.2. Membrane Destabilizing Polyanions

Polyanions that are synthetic non-viral agents are usually composed of both pendant weakly acidic and alkyl groups. Physical characteristic of the polyanions can be affected by the pH of their environment. When pH is above the pKa of the acidic groups of polyanions, ionic repulsion between charged groups can affect the interactions between alkyl groups and this situation causes adoption of an extended coil conformation. When pH is below pKa of the acidic groups, repulsion between acidic group decreases and hydrophobic interactions become dominant. This state can lead polyanion to interact with lipid membranes and destabilize them. (Tonge & Tighe, 2001)

In the study of Thomas et al; pH-responsive phospholipid vesicles mimicking membrane characteristics were synthesized. These phospholipid membranes were used to determine the destabilization effect of amphiphilic polyelectrolytes. It was found that amphiphilic polyelectrolytes can destabilize the membrane vesicles in a pH-dependent manner. The most hydrophobic polymer among the soluble poly(acrylic acid) derivatives, poly(2-ethylacrylic acid) (PEAA), was found not only to adsorb strongly to membranes at low pH but also to permeabilize the cell membrane.(J. L. Thomas & Tirrell, 1992).

Furthermore, hemolytic activity of PEAA rises rapidly when pH is decreased from 6.3 and 5.0 and there is no hemolytic activity at extracellular pH value (i.e. pH 7.4). Red blood cell (RBC) hemolysis by PEAA was consistent with colloid osmotic mechanism(pore formation on the cell membrane due to destabilization) (Stayton et al., 2000)Murthy and his coworkers investigated synthetic polyanionic polymeric systems with increasing pendant alkyl group in their study. Poly (propyl acrylic acid) (PPAA), random 1:1 copolymers of ethyl acrylate (EA) and acrylic acid (AA) and PEAA were

synthesized and their hemolytic activity was investigated. These polymers showed significant hemolytic activity at acidic pH values. Chain length of designed polymers was an important parameter. When alkyl chain is an increased, hemolytic activity of polymers increased proportionally. These results gave an important insight about polymers that can be molecularly engineered to disrupt eukaryotic cell membrane in a pH-sensitive manner within defined and narrow pH ranges.(Murthy, Robichaud, Tirrell, Stayton, & Hoffman, 1999).

In the further studies of this group, a library with poly (acrylic acid) polymers have been characterized. In this library, poly (methacrylic acid) (PMAA), poly (2-ethylacrylic acid) (PEAA), poly (propylacrylic acid) (PPAA) and poly (butyl acrylic acid) (PBAA) were synthesized. These polymers differ in their alkyl side chains by one methylene group. Results of this study showed that the length of the hydrophobic chain is a crucially important parameter. When alkyl chain was increased, hemolytic activity of the polymer increased. However PBAA (polymer with the longest alkyl chain) was hemolytic even at extracellular pH value. PPAA hemolytic activity on red blood cells was one pH unit higher than PEAA. Due to increasing hydrophobic character at acidic pH, the interaction with lipid membrane causes lytic effect. The interaction should be weak enough at neutral pH values (pH 7.4) because of ionization of carboxylic groups. Random 1:1 copolymers of these polymers (acrylic acid and ethacrylic acid) also synthesized. However, hemolytic activity of these polymers were not as effective as PPAA.(Hoffman et al., 2007). (Figure 2.7)

In a study by Bulmus et al.; a glutathione reactive vinyl monomer (i.e. pyridyl disulfide acrylate (PDSA)) was synthesized and copolymerized with butyl acrylate and methacrylic acid to form random copolymers (poly(MAA-co-BA-co-PDSA)) as a membrane-disruptive polymer suitable for preparation of drug conjugates reversible in the presence of glutathione .Membrane-disruptive behavior, cytotoxicity, and intracellular localization of the terpolymers were investigated. Poly (MAA-co-BA-co-PDSA) showed no toxicity on 3T3 fibroblasts and THP-1 macrophage like cells at high concentrations and. The uptake of radiolabeled polymers by macrophage –like cells was also demonstrated.(Bulmus et al., 2003)

Random copolymers of methacrylic acid/N-isopropyl acrylamide/octadecyl acrylate were synthesized by Ladaviere et al. Different copolymers were synthesized via changing ratio of hydrophobic side and several polymers were obtained for determination of effect on the lipid bilayer depended strongly on the chemical structure

and the concentration of polymer. Polymers of high hydrophobicity needed hours to disrupt the membrane. In this study, they found hydrophobicity has had importance for lipid membrane destabilization. But not only hydrophobicity but also charge density of polymer were another important parameter for membrane destabilization. (Ladaviere, Tribet, & Cribier, 2002)

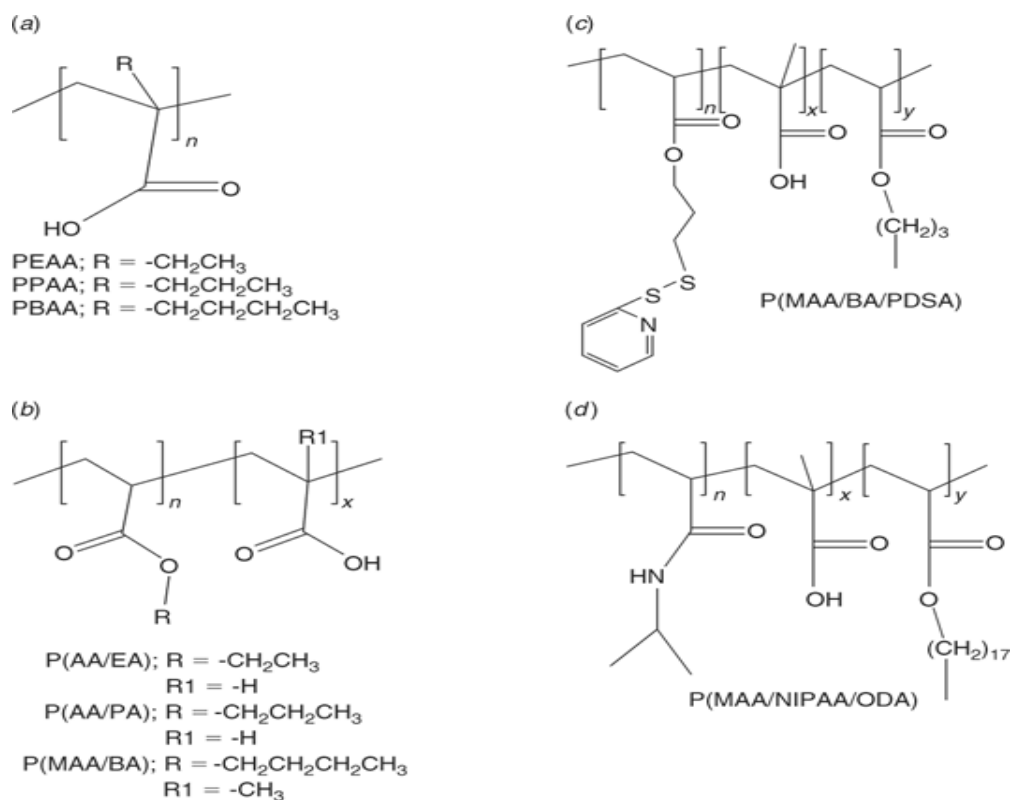


Figure 2.6. a) The chemical structure of the poly(acrylic acid) library, b) Random copolymers of P(AA/EA), P(AA/PA), P(MAA/BA), c) Random copolymers of methacrylic acid/butyl acrylate/pyridyldisulfide acrylate (P(MAA/BA/PDSA)), d) Random copolymers of methacrylic acid/N-isopropyl acrylamide/octadecyl acrylate (P(MAA/NIPAA/ODA)) (Source: Bulmus, 2005)

In a study by Tai et al.; hyperbranched copolymers of propylacrylic acid (PAA) and ethylene glycol diacrylate (PEGDA) were synthesized via one-pot RAFT copolymerization as a pH-responsive polymer. In this study, hyperbranched poly (PAA₉₉-PEGDA₁), poly (PAA₉₀-PEGDA₁₀) and poly (PAA₈₀-PEGDA₂₀) copolymers were synthesized to investigate the pH-dependent membrane destabilizing activity. The results of this work showed that poly (PAA₉₉-PEGDA₁) showed membrane destabilizing activity when compared to other copolymer counterparts as PAA which is

a well-known membrane disruptive polymer presents dominantly in this copolymer composition. When comparing this copolymer with only PAA, since PEGDA is highly hydrophilic, the copolymer displayed slightly reduced hemolytic activity, indicating that non-pH-responsive characteristic may reduce the insertion of copolymer chain into lipid bilayer membranes. (Tai, Duvall, Stayton, Hoffman, & Wang, 2013)

For efficient delivery of small interfering RNA (siRNA), Convertine et al. synthesized diblock copolymers by using reversible addition fragmentation chain transfer polymerization technique. These polymers were composed of a positively-charged block of dimethylaminoethyl methacrylate (DMAEMA) to complex with negatively charged siRNA and an endosomal releasing block composed of propyl acrylic acid (PAA) and butyl methacrylate (BMA) at an equal amount. Several diblock copolymers were analyzed. The terpolymer with composition of 48 % BMA, 29 % PAA and 23 % DMAEMA showed favorable size (25 nm) and charge ratio (4:1) and the greatest hemolytic activity. (Convertine, Benoit, Duvall, Hoffman, & Stayton, 2009)

Yessine *et al.* prepared polyion complex micelles for delivery of nucleic acid based drugs. These complex micelles were formed by a diblock cationic copolymer (poly(ethylene oxide)-*b*-poly(aminomethyl methacrylate), 75/25 mol, Mn 4700), a methacrylic acid copolymer (P(MMA-*co*-EA(ethacrylic acid)-*co*-BMA) butyl methacrylic acid))(buty, MAA 50/40/10 mol %, Mn 19800)). These polymer complexes were combined with an oligonucleotide leading to the formation of ternary polyion complex micelles (30 nm). (Yessine, Dufresne, Meier, Petereit, & Leroux, 2007). Well-defined 30 nm micelles have ability to protect the oligonucleotide against nuclease degradation. In acidic environment, chain clusters can destabilize bilayer membranes and by the help of this behaviour transport and efficient delivery could be achieved.

Moreover, Henry et al (Henry, El-Sayed, Pirie, Hoffman, & Stayton, 2006). described a new membrane-destabilizing, pH responsive polymer which was composed of propylamine, butylamine, and pentylamine derivatives of poly(styrene-*alt*-maleic anhydride (PSMA) copolymers. Anhydride group of these copolymers was hydrolyzed and modified using alkylamines to form alkylamine derivatives of PSMA. Pentylamine derivative of PSMA which had lower molecular weight (about 30 kDa) showed greater hemolytic activity.

Membrane destabilizing polyanions can be mimic the endosomolytic properties of fusogenic proteins and enhance the cytoplasmic delivery of therapeutic macromolecules. All of the studies discussed above shows that designing the polymeric

vesicles can be engineered by choosing right parameters such as carboxylic acid side chains or hydrophobic segment for the effective membrane destabilization activity.

2.3. Lipid Based Membrane-Destabilizing Systems

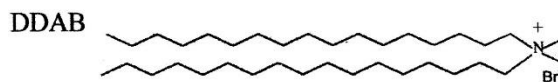
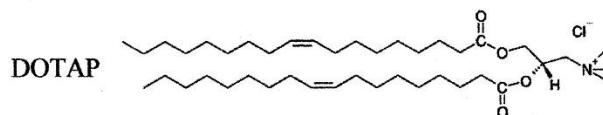
Fatty acids are carboxylic acids containing long aliphatic alkyl chains. This property makes fatty acids amphiphilic. Fatty acids are derived from triglycerides and phospholipids that are essential compounds for the body as a source of ATP. Heart, skeletal and brain tissues prefer mainly fatty acids as a source of energy. Fatty acids which contain 16 to 18 carbon atoms per molecule are the most common in cells. Cell membrane contains fatty acids and uses fatty acids for cell signalling.

There are two types of fatty acids: saturated and unsaturated which have different physical and chemical properties. Unsaturated fatty acids have lower melting points than saturated fatty acids of the same length. For example, the melting point of stearic acid is 69.6°C, but oleic acid which has the same length (18 Carbon-long) contains one cis double bond is 13.4°C. Short chain length in unsaturated fatty acids has more fluidity (J. M. Berg, Tymoczko, & Stryer, 2002).

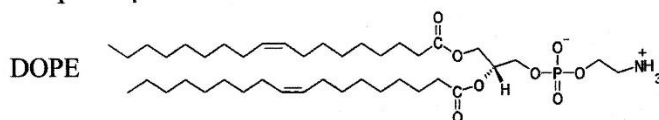
Lipid based systems have crucial importance for the delivery of macromolecular therapeutics. Especially cationic lipids have been studied many years because cationic liposomes were able to complex and condense DNA. Cationic lipid which can be used for transfection, DOTMA, was introduced by Felgner in 1987 was the first example for cationic lipids. The first cationic lipid for transfection, DOTMA, was introduced by Felgner in 1987. DOTMA ether bond was replaced with an ester bond to obtain DOTAP to increase the biodegradability and reduce the toxicity of DOTMA. Cationic amphiphilic lipids should have specific characteristics such as cationic

Head group, hydrophobic anchor, and linker. The positively charged headgroup is necessary for binding and complexation of nucleic acid phosphate groups. (Felgner et al., 1987). (Stamatatos, Leventis, Zuckermann, & Silvius, 1988). The other examples used as lipid based systems; cholesterol, DOPE and DDAB can be seen in Figure 2.7. Such systems are very efficient in cell culture but not efficient for *in vivo* delivery due to lack of instability, short serum half-life and toxicity. (Gao & Huang, 1995).

Cationic Lipids



Helper Lipids



Chol.

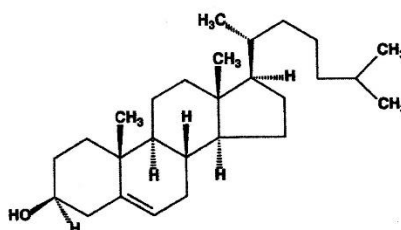


Figure 2.7. The structure of the cationic lipids, DOTAP and DDAB, and helper lipids, cholesterol and DOPE. (Source: Choosakoonkriang et al., 2001)

2.3.1. Fatty Acids as Membrane Destabilizing Systems

Fatty acids can be used in many different applications such as pharmaceuticals, food supplements, coatings and plastics. Especially, unsaturated fatty acids have been modified and used as a renewable material source for green chemistry (Meier, Metzger, & Schubert, 2007). In a work of Turunç et al, methyl 10-undecenoate which was derived from a castor oil was prepared as a set of renewable monomer by thiol-ene addition chemistry. Resulting monomers then polymerized via polycondensation polymerization with the help of a catalysis and hyperbranched polyesters containing thio-ether linkages were obtained. Prepared polyesters derived from fatty acid revealed good thermal properties. These polymers may be an alternative material to petroleum based materials (Türünç & Meier, 2010).

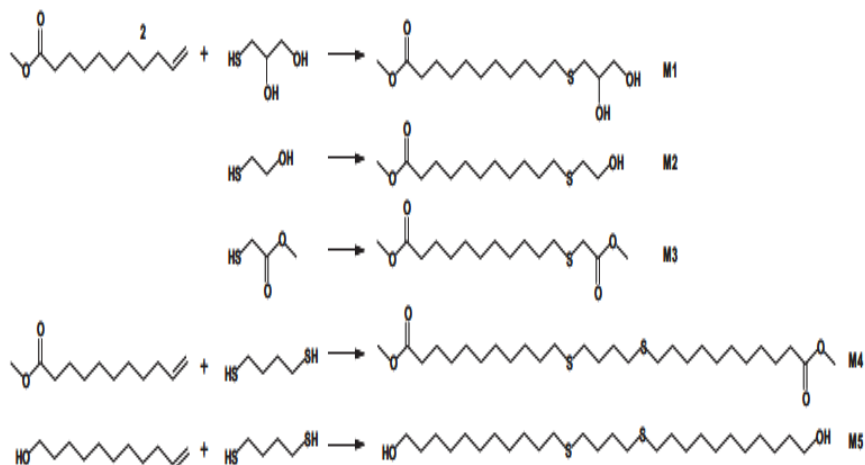


Figure 2.8. Investigated monomers and their preparation from the renewable platform chemical (Source: Türünç and Meier, 2010)

In another study, oleic acid was used as a starting compound and modified with 2-mercaptoethanol for polyurethane synthesis. Modification of oleic was performed via radicalic addition reaction under UV radiation. It was noted that carboxylic acid group of oleic acid generated side reactions with 2-mercaptoethanol. Polyurethane was synthesized via polycondensation. Thermal properties of polyurethanes were found to be similar to commercial polyols (Desmophen 1150). (Desroches, Caillol, Lapinte, Auvergne, & Boutevin, 2011).

Example reports given above generally show applications of fatty acids for materials science. However, fatty acids are essential compounds for drug delivery systems due to their attractive amphiphilic character. In the study of Wolfrum et al., lipophilic siRNA conjugates were synthesized to mediate efficient delivery of siRNA molecules for *in vivo* gene silencing. These lipophilic-siRNA conjugates prepared using cholesterol, oleoyl, docosanyl, myristoyl, lithocolic-oleyl, palmitoyl and lauryl, lipids which had different hydrophobic chain lengths were thought as alternative way to cholesterol based siRNA delivery. Cholesterol based delivery of siRNA was shown to suppress apoB mRNA levels and decrease the levels of plasma apoB and serum cholesterol. This work described several lipophilic conjugates, including several fatty acids and bile acids. Different fatty acid conjugations were performed to compare the efficiency of siRNA delivery. Cholesterol based conjugate was the most effective one. This study showed that lipoprotein particles are efficient delivery vehicles of lipophile-siRNA conjugates *in vivo*. (Wolfrum et al., 2007).

In another study, a lipid library was synthesized and used as drug delivery vehicles. Biomimetic cationic thioether lipids were synthesized via a two-step method based on thiol-yne click chemistry. This library was formed with variable lengths of using 8 different alkyl thiols (alkyl chain length from C6 to C16), 2 alkynyl carboxylic acid linkers bearing a terminal triple bond, and 7 different cationic amines. These systems were determined to mimic glycerol core of phospholipids. In this study, by combination of these groups, more than 100 novel lipids were synthesized. *In vitro* experiments showed that lipid which contains hydrophobic chain with 11 carbon had the greatest transfection efficiency. This work revealed the structure activity relationship in regards to delivery efficiency of macromolecules.

Lipid based systems were also combined with polymers for the delivery of nucleic acid. In the study of Alshamsan et al. oleic and stearic acid modified derivatives of branched polyethylenimine (PEI) were synthesized and used for the delivery of small interfering RNA (siRNA) to B16 melanoma cells *in vitro*. Results of these study revealed that combination of hydrophobic segments with PEI displayed 3-fold higher siRNA protection against degradation in fetal bovine serum when compared with PEI only. siRNAs were effectively delivered approximately to all cells (>90%). The toxic effect of PEI also decreased significantly by the combination with lipid formulation. (Alshamsan et al., 2008)

Yusa et al. synthesized well-defined amphiphilic block copolymers having phospholipid polymer sequences as a novel biocompatible polymeric micelle reagent. Homopolymerization of 2-methacryloyloxyethylphosphorylcholine (MPC) was synthesized with a living polymerization technique, i.e. reversible addition fragmentation chain transfer (RAFT) polymerization. Thioester end-capped polymers were then used as a macro RAFT agent. AB diblock copolymers of MPC and n-butyl methacrylate (BMA) were synthesized and characterized. Polymeric micelles were then determined by static light scattering (SLS), and quasi-elastic light scattering (QELS) data. Increase in poly (BMA) block length increased the size and mass of the micelles. Poorly water-soluble anticancer agent, paclitaxel (PTX) was then loaded into the block copolymer micelles composed of poly (MPC) and poly (BMA) blocks. In this way, the water solubility of PTX was dramatically increased. (Yusa, Fukuda, Yamamoto, Ishihara, & Morishima, 2005)

Synthesis of lipid- α -end-functionalized polymers by RAFT polymerization was described by Bathfield et al. (Bathfield et al., 2008) Phospholipid moiety with dithioester

functionality (Lipid-DT) was synthesized with a high yield (71 %) from a precursor chain transfer agent (CTA). N-Acryloylmorpholine (NAM) was polymerized via RAFT polymerization mediated by lipid-DT CTA and lipid- α -end-functionalized polymers (poly (NAM)) were obtained. These polymeric chains were organized into lipoparticles assemblies composed of a polymer core and a lipid shell. These lipoparticles have shown stability in relatively high ionic strength aqueous solutions.

Poly ethylene glycol (PEG) which is mainly used as a hydrophilic polymer in pharmaceutical industry was conjugated to phospholipids (Inuie et al). Poly (vinyl alcohol) modified with alkyl side chains (fPVA-alkyl) and PEG-phospholipid conjugates (fPEG-lipids) were tested as amphiphilic polymers for cell surface modification. The aim of the study was to understand the interactions between soluble polymers and living cells for cell engineering in various biomedical fields. There was no toxic effect with fPEG-lipids and fPVA-alkyl polymers. The polymers were released from the cell surface without triggering endocytosis. (Inui, Teramura, & Iwata, 2010)

Sevimli et al. (Sevimli, Inci, Zareie, & Bulmus, 2012) synthesized well-defined copolymers of methacrylic acid and cholesteryl methacrylate, poly(methacrylic acid-*co*-cholesteryl methacrylate) P(MAA-*co*-CMA) as pH-responsive non-viral delivery agent. Cholesterol was used as an efficient delivery agent because of its ability to cross cellular membranes. Different statical copolymers with varying cholesterol content (as 2, 4 and 8 mol %) were synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization. Characterization of the polymers revealed that copolymer having 8 mol % CMA formed supramolecular assemblies while the copolymers having 2 and 4 mol % CMA existed as unimers in aqueous solution. Interaction of the polymers with lipid membrane was determined using surface plasmon resonance (SPR). The copolymer containing 2 mol% CMA displayed the greatest polymer–lipid interactions at pH 5.0 and also this polymer showed greatest membrane destabilizing activity.

2.4. Living/ Controlled Radical Polymerization Techniques

Free radical polymerization is a type of chain growth polymerization. Chain transfer may occur for every radical at any and all degrees of polymerization Free radical polymerization is not efficient method for especially biological systems due to polymers are not mono-disperse and molecular weight of polymers were not controlled .

Living/ Controlled radical polymerization techniques allow the synthesis of well-defined polymers having living properties, controlled molecular weight and narrow polydispersity index (PDI). 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. (Braunecker & Matyjaszewski, 2007)

Nitroxide-mediated radical polymerization (NMP) is one of type of controlled radical polymerizations. The main advantage of NMP comes from its simplicity. The polymerization is thermally initiated in the absence of an external radical source or a metal catalyst. In this polymerization technique, alkoxyamine is used as initiator to generate polymers with well-controlled stereochemistry and a very low polydispersity index. The main disadvantage of NMP is availability and cost of initiators, limited monomer types to which the technique is applicable and the need for high temperatures for polymerization. (Sciannamea, Jérôme, & Detrembleur, 2008)

The Atom Transfer Radical Polymerization (ATRP) is another living polymerization technique which was developed by Matyjaszewski in 1994. With the addition of appropriate catalysts that contain transition metal compounds and ligands and initiators, monomers such as meth (acrylates), styrenes and meth (acrylamides) can be polymerized. Solvent choice is very critical due to the catalyst system in polar media. Metal-based catalysts cause disadvantages for generating biomedical polymers using ATRP polymerization technique. (Aamer & Tew, 2007)

NMP and ATRP have many advantages as controlled polymerization techniques, however RAFT polymerization is one of the most versatile and powerful polymerization technique for generating polymers for biomedical applications. (Semsarilar & Perrier, 2010)

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

Reversible addition–fragmentation chain transfer (RAFT) polymerization was firstly reported by CSIRO (Commonwealth Scientific and Industrial Research Organization) group in 1998, providing living characteristics and one of the most efficient polymerization techniques.

RAFT polymerization offers various advantages. By the RAFT polymerization, controlled molecular weight and narrow molecular weight distribution polymers (narrow polydispersity index (PDI)) can be obtained. Also, designing complex architecture polymers such as graft copolymers, and di and triblock copolymers is possible. Many functional monomers can be used with a wide variety of reaction conditions. One of the most important contributions of RAFT polymerization is its suitability for synthesizing polymers for drug delivery. (Boyer et al., 2009)

The RAFT polymerization requires the use of a RAFT agent, i.e. a chain transfer agent. The chain transfer agents used in the RAFT polymerization are organic compounds having a thiocarbonylthio moiety. RAFT agent includes R and Z groups in addition to reactive thiocarbonylthio moiety. Z group activates the thiocarbonyl bond toward radical addition and then stabilizes the resultant adduct radical in the ω -end of the polymer chain. R group is responsible for initiation of the growth of polymeric chains. The living end-group of polymers (thiocarbonylthio) can be modified for further applications. The mechanism of RAFT polymerization can be seen in Figure 2.8. (Bulmus, 2011)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

10-undecenoic acid (99% purity) and 2-mercaptoethanol were purchased from Aldrich. Methacryloyl chloride was purchased from Aldrich. 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) was purchased from Aldrich. 2, 2'-Azobis (2-methylpropionitrile) (AIBN) (Fluka) was used after recrystallization twice in methanol. 4, 4'-Azobis (4-cyanovaleric acid) ($\geq 75\%$) from Sigma Aldrich was used also as initiator. Silica gel (pore size 60 Å, 70-230 mesh) was received from Fluka. Acetic acid, sodium acetate, citric acid and mono and dibasic phosphate salts were purchased from Merck. Hydrochloric acid and sodium hydroxide were purchased from Merck and Sigma, respectively. Poly (ethylene glycol methyl ether methacrylate) and methacrylic acid were purchased from Sigma-Aldrich and de-inhibited by passing through a basic aluminum oxide column before use

Toluene, ethyl acetate, hexane, dichloromethane (DCM), deuterium oxide (D₂O), deuterium chloroform (CDCl₃), triethylamine (TEA), hexylamine, diethylether, methanol and N,N-dimethylacetamide (DMAc, HPCL grade $\geq 99.9\%$) were purchased from Sigma. Dialysis membrane (MWCO= 500-10000 Da) was purchased from Spectrum® Laboratories.

3.2. Instruments

3.2.1. Nuclear Magnetic Resonance Spectroscopy

¹H NMR spectroscopy (Varian, VNMRJ 400 spectrometer) was used to determine the chemical structure of synthesized compounds and the conversion of the monomers to polymers. Deuterium oxide (D₂O) and chloroform (CDCl₃) were used as NMR solvents. For NMR analysis, samples were dissolved at 10 mg/ml concentration

in both deuterium oxide (D₂O) and chloroform (CDCl₃) depending on the solubility of the samples.

3.2.2. Gel Permeation Chromatography

The molecular weight and molecular weight distribution of polymers synthesized throughout the study was determined by gel permeation chromatography. 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 containing 0,05 % w/v LiBr.

3.2.3. UV-Visible Spectrophotometry and DLS Analysis

UV-visible light absorbance of the solutions was measured by a Thermo Scientific Evolution 201 UV-visible spectrophotometer in the range between 200 nm and 600 nm using quartz cuvettes.

For DLS analysis, Malvern NanoZS Particle Analyzer was used to determine hydrodynamic diameter of polymers.

3.2.4. Microplate Reader

A Thermo Electron Corporation Varioskan microplate reader was used to measure absorbance at 540 nm in hemolysis assays. 96-well plates were used for this study.

3.2.5. Silica Gel Column Chromatography

Monomer, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl]-undecanoic was purified via silica gel liquid column chromatography. Silica gel (pore size 60 Å, 70-230 mesh) was used as a stationary phase. Hexane-ethyl acetate mixtures at volume/volume (v/v) ratios of 4/1, 3/1 and 2/1 were used as a mobile phase.

3.3. Methods

3.3.1. Synthesis of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl]undecanoic Acid

11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid was synthesized according to procedure given below. The reaction scheme is shown in Figure 3.1. The first step of monomer synthesis was performed according to a procedure described in literature (Oğuz Türünç, Firdaus, Klein, & Meier, 2012) Briefly, 1 equivalent (equiv.) of 10-undecanoic acid (5 g, 0.027 mole) and 3 equiv. of 2-mercaptoethanol (5 g, 0.081 mole) were dissolved in toluene (25.42 ml) and placed in a round bottom flask set with a magnetic stirrer. The solution was degassed by purging with nitrogen for about 30 min. The reaction mixture was then stirred at 75 °C for 2 days. The reaction solvent was dried via rotary evaporator and collected with dichloromethane (DCM), and then extracted with saturated potassium carbonate (K₂CO₃) solution, brine, and water. The collected organic phase was dried in vacuo. The final product 11-(2-hydroxyethylsulfanyl)-undecanoic acid was obtained as a white precipitate. The product was then dissolved in CDCl₃ and characterized by ¹H-NMR. The yield of the reaction was 85% and calculated from using equation 3.1. The ¹H-NMR spectrum of the reaction mixture is given in Appendix A.1.

¹H NMR (CDCl₃, δ in ppm): 3.73 (t, 2H, S-CH₂-CH₂-OH), 2.71 (t, S-CH₂-CH₂-OH), 2.52 (t, CH₂-S-CH₂-CH₂-OH), 2.32 (t, COOH-(CH₂)-(CH₂)₁₄-S-CH₂-CH₂-OH), 1.65-1.27 (14 H, COOH-(CH₂)-(CH₂)₁₄-S-CH₂-CH₂-OH)

$$\text{Yield (\%)} = \frac{I_{2\text{H}} \text{ at } 2.52 \text{ ppm} / 2}{I_{16\text{H}} \text{ at } 1.65 \text{ ppm} - 1.34 \text{ ppm} / 16} \quad (3.1)$$

11-(2-Hydroxy-ethylsulfanyl)-undecanoic acid (Mwt: 246.41 g/mol, 0.322 g, 1.3 mmol) was dissolved in dry tetrahydrofuran (THF) (5 ml) and dry triethylamine (TEA) (0.39 g, 3.95 mmol, 0.53 ml) was added to the mixture and cooled in an ice bath. To this cold mixture, a solution of methacryloyl chloride (3.9 mmol, 0.407 gr, and 0.8 ml) in dry THF (5 ml) was added dropwise under continuous stirring. After the addition was over in 0.5 h, 0.5 gr of butylated hydroxyl toluene (BHT) was added as inhibitor to prevent monomer from unwanted reactions. BHT solution in dry THF (1 ml) was added under N₂ atmosphere. Reaction was continued overnight. The following day, the reaction solution was filtered to remove the solid. THF was evaporated using a rotary evaporator. Product was then dissolved in DCM. The unreacted methacryloyl chloride/methacrylic acid and triethylamine were removed by washing reaction solution with brine (three times) and extracting with water three times. The DCM phases were collected and the solvent was evaporated using a rotary evaporator. The organic layer was collected, dried over anhydrous MgSO₄ and filtrated. Organic layer was then concentrated first by rotary evaporation and then further dried in a vacuum oven (T<30 C°). Finally, a yellowish product was obtained. The product was analyzed via ¹H NMR spectroscopy.

The product was further purified by column chromatography to remove the methacrylic acid. Hexane and ethyl acetate solutions (Hxn: EA= 1:0; 5:1; 4:1; 3:1; 2:1; 1:1; 0:1) were used as a mobile phase. High purity monomer was collected using a hexane and ethyl acetate mixture of 2:1(v/v).

The product, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl]-undecanoic acid (2, Figure 3.1) was obtained as a yellow oil and characterized by ¹H NMR spectroscopy using deuterated chloroform (CDCl₃) as a solvent. ¹H-NMR spectrum of purified monomer is given in results and discussion part. The yield of reaction was 100 % and calculated from ¹H-NMR spectroscopy. Characteristic peak of mercapto group disappeared after reaction.

¹H NMR (CDCl₃, δ in ppm) : 6.11-5.58 (s, 2H, CH₂=C (CH₃) COO-), 1.94 (s, O-3H, CH₂=C-CH₃), 4.27 (t, 2H, -S-CH₂-CH₂-O- CH₂=C (CH₃) COO-), 2.71 (t, S-CH₂-CH₂-OH), 2.52 (t, CH₂-S-CH₂-CH₂-OH), 2.32 (t, COOH-(CH₂)-(CH₂)₁₄-S-CH₂-CH₂-OH), 1.65-1.27 (14 H, COOH-(CH₂)-(CH₂)₁₄-S-CH₂-CH₂-OH))

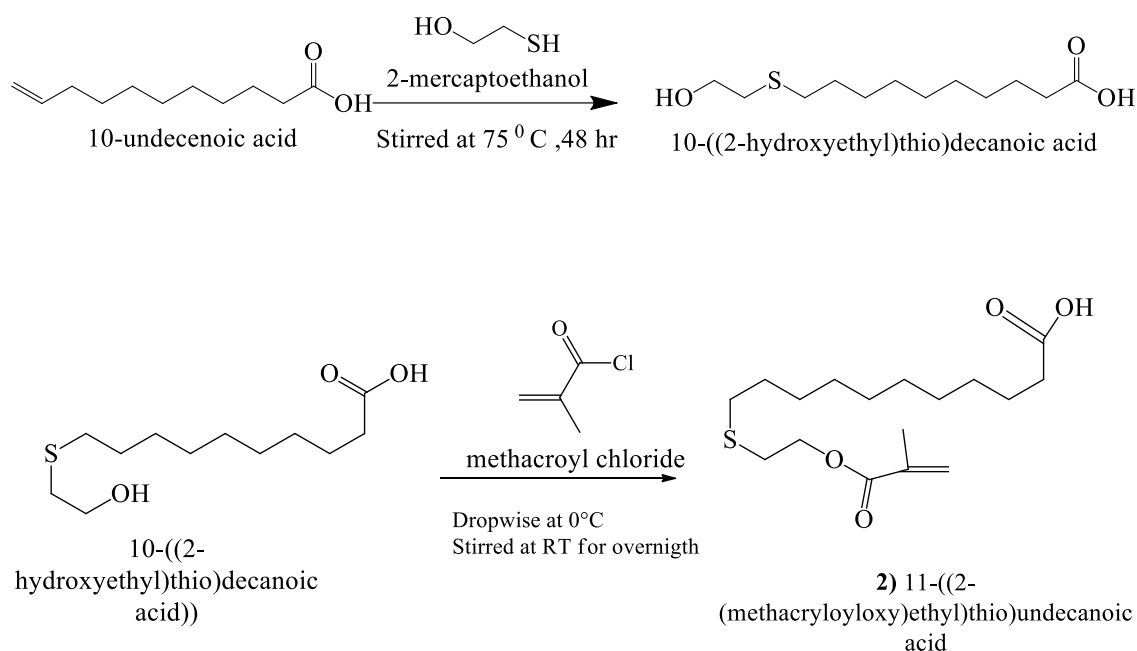


Figure 3.1. Synthesis of 11-((2-(methacryloyloxy)ethyl)thio)undecanoic acid

3.3.2. RAFT Polymerization of 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] Undecanoic Acid

The synthesized fatty acid monomer was polymerized via RAFT polymerization. 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (2), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) and 4, 4'-azobis (4-cyanovaleric acid) (ACVA) were dissolved in an organic solvent. The polymerization scheme and conditions are given in Figure 3.2 and Table 3.1, respectively. The solution was purged with nitrogen for about 20 minutes. 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. Conversion of monomer to polymer was determined before purification by ¹H-NMR spectroscopy using CDCl₃ as a solvent. Conversion was calculated using Equation 3.2.

Polymer purification was performed by precipitating the polymerization mixture in hexane three 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 $^1\text{H-NMR}$ spectrum of purified polymers using Equation 3.3 in which the molecular weight of monomer, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic (2) and the RAFT agent, is 330.48 g/mol and 279.38 g/mol, respectively.

$^1\text{H NMR}$ (CDCl_3 , δ in ppm) : 6.11-5.58 (s, 2H, $\text{CH}_2=\text{C}(\text{CH}_3)\text{COO-}$), 1.94 (s, O-3H, $\text{CH}_2=\text{C}-\text{CH}_3$), 4.27(t, 2H, $-\text{S}-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2=\text{C}(\text{CH}_3)\text{COO-}$), 2.71 (t, $\text{S}-\text{CH}_2-\text{CH}_2-\text{OH}$), 2.52(t, $\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{OH}$), 2.32(t, $\text{COOH}-(\text{CH}_2)-(\text{CH}_2)_{14}-\text{S}-\text{CH}_2-\text{CH}_2-\text{OH}$), 1.65-1.27 (14 H, $\text{COOH}-(\text{CH}_2)-(\text{CH}_2)_{14}-\text{S}-\text{CH}_2-\text{CH}_2-\text{OH}$)

$$\text{Monomer conversion (\%)} = \frac{(2 \text{ H at } 4.24 \text{ ppm} + 1 \text{ H at } 4.01 \text{ ppm} / 2) - (1 \text{ H at } 6.11 \text{ ppm} + 1 \text{ H at } 5.58 \text{ ppm} / 2)}{1 \text{ H at } 4.24 \text{ ppm} + 1 \text{ H at } 4.01 \text{ ppm} / 2} \times 100 \quad (3.2)$$

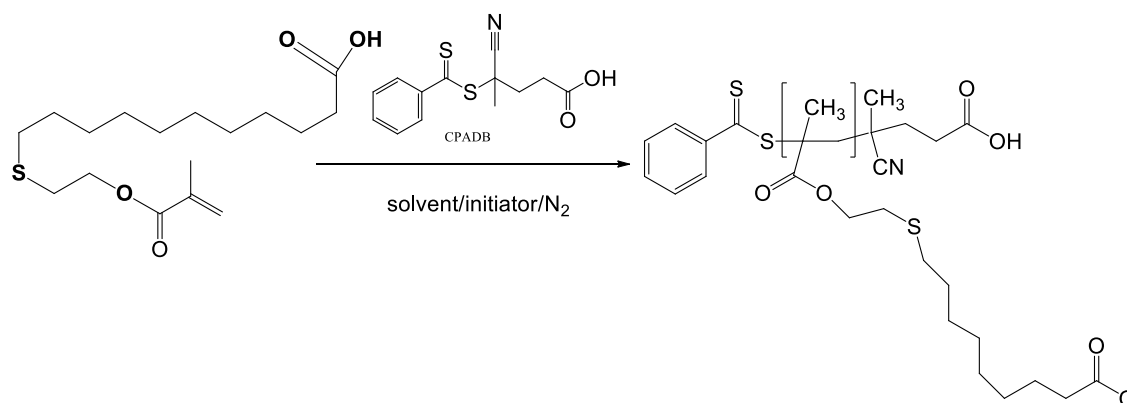


Figure 3.2. Synthesis of poly (11-((2-(methacryloyloxy) ethyl)thio)undecanoic acid), (P(UDAMA)) via RAFT polymerization.

Table 3.1. Polymerization conditions of poly ((11-((2-(methacryloyloxy) ethyl) thio) undecanoic acid), (P(UDAMA). Monomer concentration was 4.2 M.

Polymerization Time (h)	[UDAMA]/[RAFT]/[AIBN] (mol/mol/mol)	Solvent
2	200/2/0.5	Toluene
4	200/2/0.5	Toluene
8	200/2/0.5	Toluene
24	200/2/0.5	Toluene
2	100/2/0.5	Acetonitrile
4	100/2/0.5	Acetonitrile
8	100/2/0.5	Acetonitrile
24	100/2/0.5	Acetonitrile

3.3.3. Synthesis of of 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] Undecanoic Acid and Methacrylic Acid Copolymers

The homopolymer of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid was not soluble in the water. For this reason, random copolymers of fatty acid monomer with methacrylic acid were synthesized. 11-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (2), 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) and 4, 4'-azobis(4-cyanovaleric acid) (ACVA) and methacrylic acid (MAA) were dissolved in an organic solvent. In Table 3.2, copolymerization conditions are given. The solution was purged with nitrogen for about 20 minutes. 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. Resulting polymer mixture was then analyzed via ¹H-NMR spectroscopy.

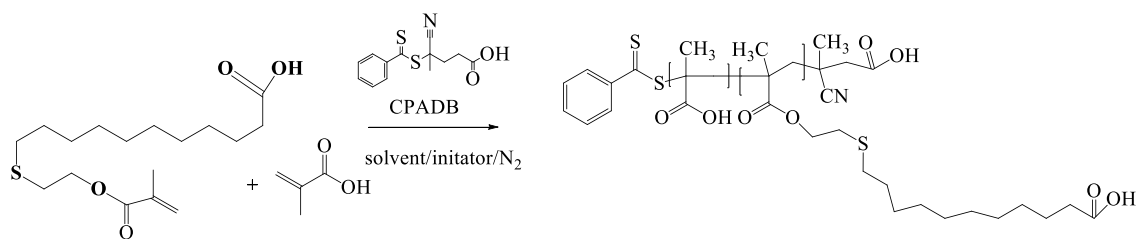


Figure 3.3. Synthesis of poly(11-((2-(methacryloyloxy) ethyl)thio)undecanoic acid) (P(UDAMA))-co-(methacrylic acid) (MAA) via RAFT polymerization

Table 3.2. Copolymerization Conditions. 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] Undecanoic Acid Monomer (UDAMA), Methacrylic Acid (MAA), initiator (ACVA) and RAFT agent (4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid).

Polymerization Time (h)	[UDAMA]/[MAA]/[RAFT]/[ACVA]	Solvent
2	10/40/1/0.25	Methanol
4	10/40/1/0.25	Methanol
6	10/40/1/0.25	Methanol
8	10/40/1/0.25	Methanol
10	10/40/1/0.25	Methanol
2	25/25/1/0.25	Acetonitrile
4	25/25/1/0.25	Acetonitrile
6	25/25/1/0.25	Acetonitrile
8	25/25/1/0.25	Acetonitrile
10	25/25/1/0.25	Acetonitrile

3.3.4. Determination of pH Responsive Behavior and Hydrodynamic Diameters of Polymers

The turbidity change of polymer solutions at varying pH values was investigated via UV-visible spectroscopy to determine pH-responsive behavior of polymers. Phosphate and citrate buffer solutions at two different pH values (5.0 and 7.4) were prepared. Citrate buffer solution at pH of 5.0 was prepared by mixing citric acid (0.1 M) and dibasic sodium phosphate (0.2 M) aqueous solutions. Phosphate buffer solution (0.1 M) at pH 7.4 was prepared by mixing sodium phosphate monobasic (0.1 M) and sodium phosphate dibasic (0.1 M) aqueous solutions. The ionic strengths of the buffer solutions

were adjusted to 0.1 M by the addition of NaCl to yield isotonic solutions. Copolymers which have UDAMA content of 20% (Mn: 5870 g/mol, PDI: 1.21) and 50% (Mn: 10900 and PDI: 1.25) were dissolved in buffer solutions. The final copolymer concentration was 4 mg/ ml. The absorbance of each polymer solution from acidic pH to neutral pH was measured using an UV–visible spectrophotometer (Thermo Fisher) at 500 nm. Quartz cuvettes were used. Measurements were repeated three times.

Dynamic light scattering studies were performed using a Malvern Zetasizer Nano ZS Instrument (Malvern, U.S.A.) The polymer sample solutions at two different pH values were prepared at 2 mg/ml concentration. Measurements were repeated 3 times.

3.3.5. Determination Hemolytic Activity of Polymers

pH-dependent membrane-disruption activity of polymers on red blood cells was tested via hemolysis assay.(Murthy et al., 1999) Briefly, red blood cells were washed with 150 mM saline, and then suspended in 100 mM phosphate buffer solution at pH 5.5 or pH 7.4. The cell suspensions were then diluted to a concentration of 10^8 cell /200 μ l. 200 μ l of cell solution was taken and mixed with 800 μ l of polymer solution dissolved in pH 5.0 or pH 7.4 buffer solution at the desired concentration. The cell and polymer solutions were incubated at 37 °C for one hour. After incubation period, solutions were centrifuged to remove cell debris and supernatants were transferred to a 96-well plate for absorbance measurements at 541 nm. Cell solutions treated with triton X-100 solution and phosphate buffer solution were used as positive and negative controls, respectively. Experiments were performed in triplicate.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Synthesis of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] Undecanoic

In this study, a new monomer, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid derived from an unsaturated fatty acid was first synthesized. The preparation procedure of fatty acid-derived monomer was composed of two steps. Thiol-ene thermal addition of 2-mercaptoethanol to 10-undecenoic acid was the first step of monomer synthesis. The procedure for this reaction was adopted from literature.(Türünç & Meier, 2010). Thiol-ene addition reactions take place by the addition of a S-H bond across a double or triple bond. These reactions are highly efficient and amenable to various solvents and functional groups.(Lowe, 2010) Figure 4.1 shows the result of thiol-ene reaction after purification via extraction with dichloromethane. The disappearance of ene group of 10-undecenoic acid and the presence of characteristic peaks of protons adjacent to sulfur and hydroxyl group at 3.7 ppm and 2.7 ppm, respectively, evidenced the success of addition reaction. The disappearance of proton signals belonging to ene group at 5.8 and 5.0 ppm after addition reaction was clearly seen in the NMR spectrum (Figure 4.1). 10-undecenoic acid has a carboxylic acid group. Esterification of this group can occur upon reaction with 2-mercaptoethanol. However, addition reaction between thiol and ene is more favorable when compared with the esterification reaction between carboxylic acid and hydroxyl groups. Accordingly, esterification was calculated to be only 15%. Since the presence of the ester side product has no effect on the next step of monomer synthesis, i.e. methacryloylation step, it was not attempted to remove this side-product.

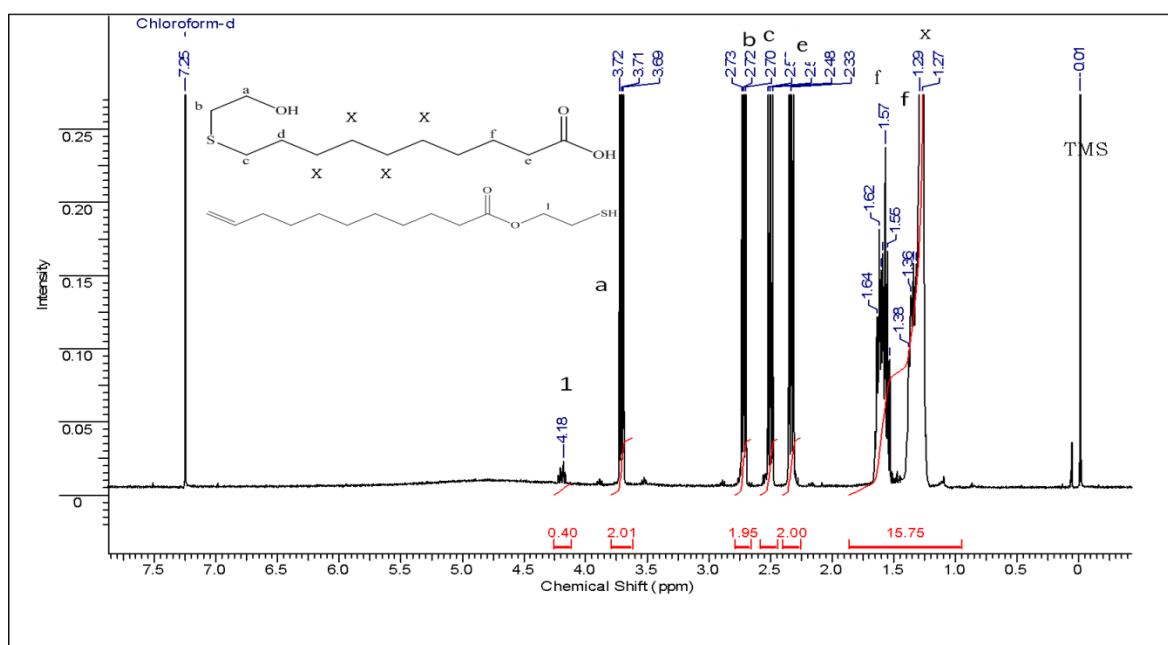
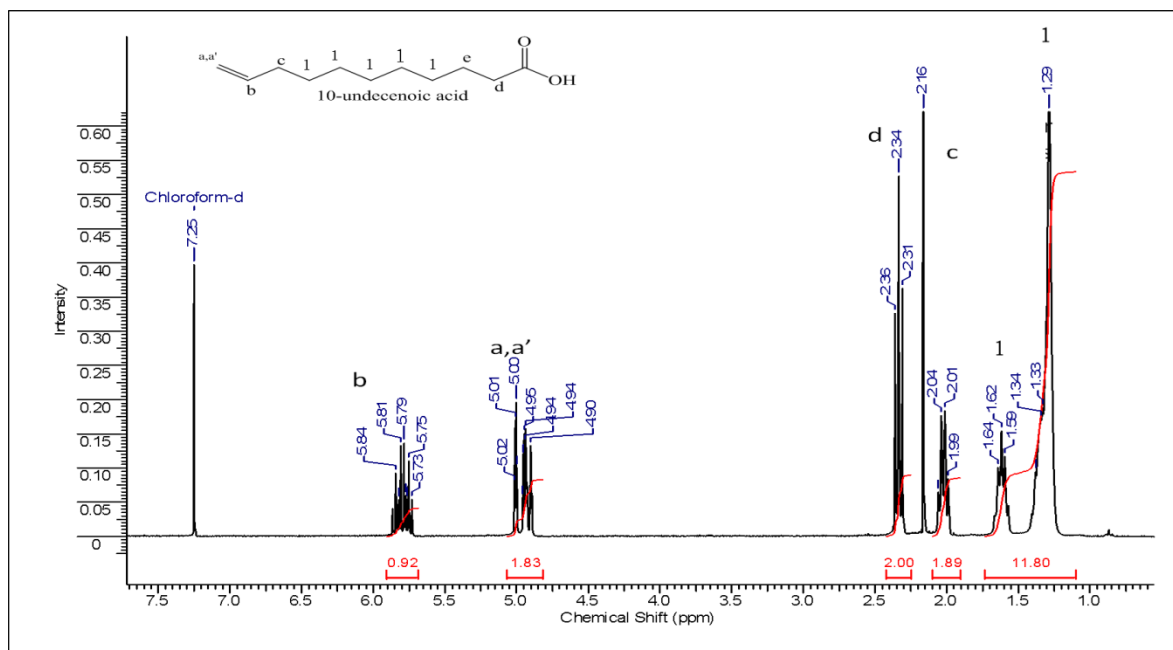


Figure 4.1. ^1H NMR spectra of 10-undecenoic acid (top) and 11-(2-hydroxyethylsulfanyl)undecanoic acid obtained after thiol-ene thermal addition of 2-mercaptoethanol to 10-undecenoic acid (bottom).

In the second step of monomer synthesis procedure, 11-(2-hydroxyethylsulfanyl)undecanoic acid was methacrylated using methacryloyl chloride according to a method reported in literature. (Bulmus et al., 2003) The reaction mixture was characterized by ^1H -NMR spectroscopy. The spectrum is presented in Figure 4.2.

The final product, 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid, was purified via silica gel column chromatography and obtained as a yellow oil ($R_f = 0.6$ in Hxn:EA= 2:1; total yield: 92% (Eqn. 4.1)). From the $^1\text{H-NMR}$ spectrum, the yield of the reaction was calculated to be 100 % considering the complete disappearance of the characteristic signals of $-\text{CH}_2$ adjacent to hydroxyl group at 3.7 ppm. The characteristic signals of vinyl protons, $-\text{CH}_3$ was observed at 1.93 ppm (b) and $-\text{CH}_2$ was observed at 6.12 ppm (1H) (a) and 5.58 ppm (1H) (a') (Figure 4.2.). The shift of the characteristic signal of $-\text{CH}_2$ adjacent to hydroxyl group from 3.7 ppm to 4.28 ppm clearly evidenced the occurrence of the intended reaction between methacryoyl chloride and 11-(2-hydroxy-ethylsulfanyl)-undecanoic acid.

$$\text{Yield of reaction \%} = \frac{\text{I @ 4.29 ppm}/2}{\text{I @ (1.65 PPM-1.34 PPM)}/16} \quad (4.1)$$

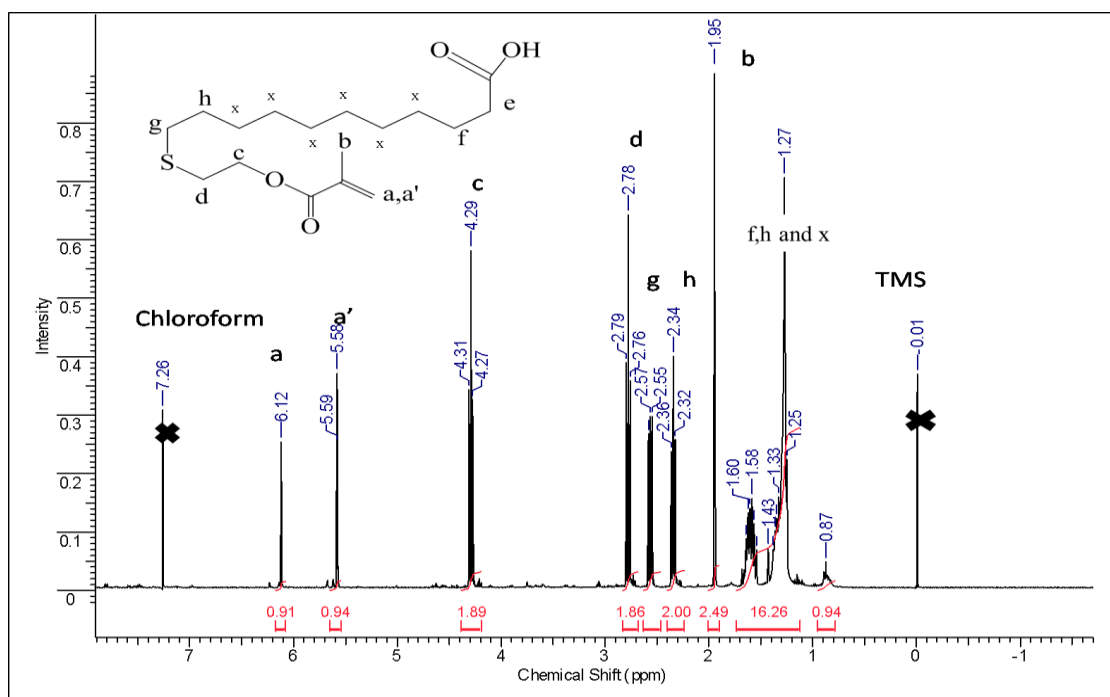
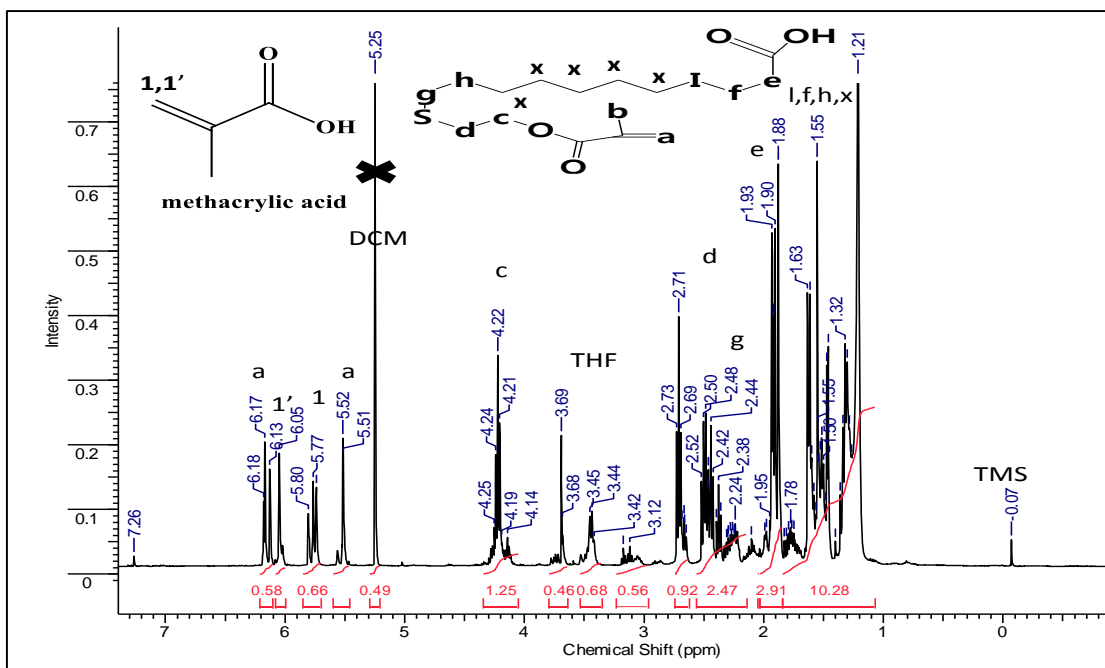


Figure 4.2. ^1H -NMR spectrum of reaction mixture of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid and obtained after column chromatography as pure compound (bottom).

4.2. RAFT Polymerization of 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid

11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (UDAMA) was first polymerized via conventional free radical polymerization to check whether the new monomer can be polymerized via free radical mechanism. For this purpose, UDAMA monomer was polymerized in the presence of AIBN as a polymerization initiator at 65 °C for 12 hours. DMSO-d₆ was used as a solvent. Figure 4.3 shows the ¹H-NMR spectrum of polymerization mixture. The monomer conversion to polymer was calculated, according to Equation 3. 2, to be 77 %. The NMR result proved that the new monomer can be polymerized via free radical polymerization mechanism. The polymerization mixture was further analyzed via gel permeation chromatography (GPC) (Figure 4.3). The GPC analysis showed the formation of a polymer with a number average molecular weight (M_n) of 36277 g/mol and a polydispersity index of (PDI) 1.92. It should be noted that the large PDI value is a characteristic of a non-living free radical polymerization mechanism.

At the next step, the new monomer was attempted to be polymerized via reversible-addition fragmentation chain transfer (RAFT) polymerization using a RAFT agent (4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, CPADB) which has been widely used for controlled polymerization of methacrylates (Chong, Le, Moad, Rizzardo, & Thang, 1999). A well-known azo initiator, AIBN (azobisisobutyronitrile) and ACVA (4, 4'-azobis (4-cyanovaleric acid) was used as a free radical initiator for polymerizations. Toluene or acetonitrile was used as a solvent. Several homopolymerizations of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid were performed. Table 4.1 shows the RAFT polymerization conditions and the results obtained from polymerizations.

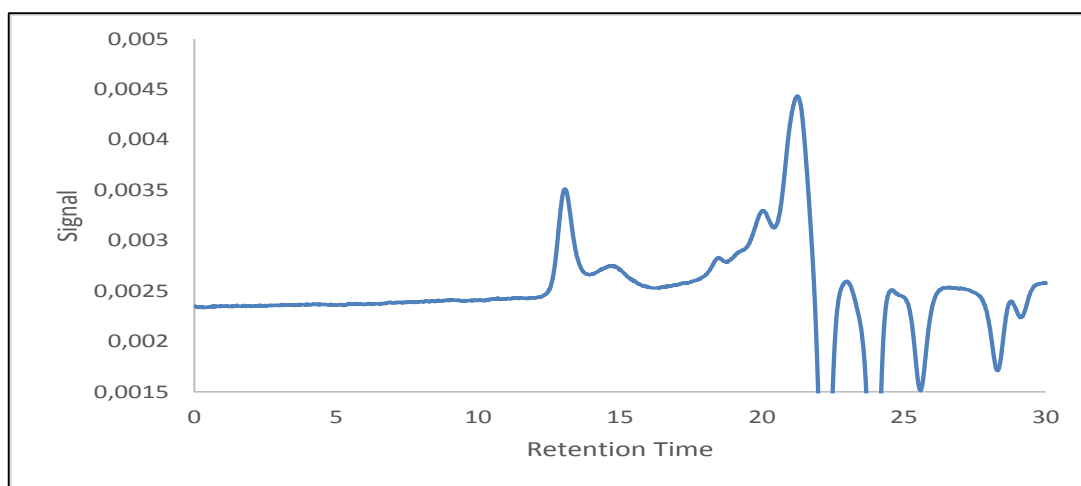
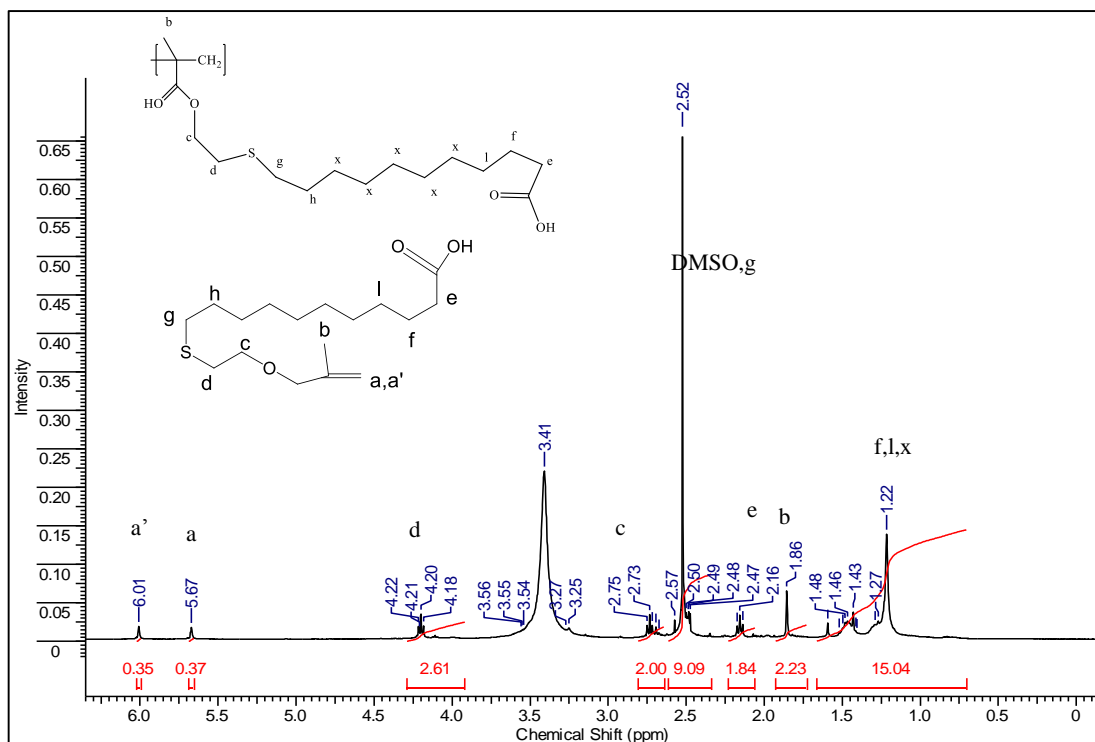


Figure 4.3. $^1\text{H-NMR}$ spectrum of poly 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid synthesized by free radical polymerization. GPC chromatogram of polymerization mixture (bottom). [monomer]= 1M, monomer:initiator (mole:mole)= 200:1

Figure 4.4 shows the $^1\text{H NMR}$ spectrum of RAFT polymerization mixture of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid. The monomer conversion was calculated using Equation 3.2 from the $^1\text{H NMR}$ spectra of polymerization mixtures. According to NMR results presented in Table 4.2, the conversion of the 11-

[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid to polymer increased with time. The number average molecular weights (M_n) of polymers determined by GPC increased with monomer conversion, indicating controlled character of polymerization mechanism. The polydispersity index (PDI) of polymers was below 1.5. Although the molecular weights were well-controlled, relatively high PDI values of polymers were attributed to the occurrence of side reactions such as slight branching. As it can be seen in Table 4.2, the increase in $[\text{Monomer}]_0/[\text{RAFT}]_0$ mole ratio resulted in the increase in molecular weight of polymers. This was attributed to the RAFT-controlled polymerization mechanism as the theoretical molecular weight of RAFT-mechanism controlled polymers is described by Equation 3.3.

The kinetic plots of the polymerizations ($[\text{M}]_0 = 4.2 \text{ M}$, $[\text{Monomer}]_0/[\text{RAFT}]_0/[\text{Initiator}]_0 = 200/2/0.5$ and $100/2/0.5$) are given in Figure 4.5. As it can be seen in the logarithmic monomer conversion ($\ln [\text{M}]_0/[\text{M}]$) versus time graphs, the $\ln[\text{M}]_0/[\text{M}]$ increases linearly with time until a given polymerization time, followed by a slow increase. This may show that after a certain chain length, the polymerizations become very slow possibly due to the increased steric hindrance effects. The linear increase in M_n with monomer conversion was also observed. These are the well-known behavior of RAFT controlled polymerization mechanism. (Barner-Kowollik et al., 2006)

Table 4.1 Results of RAFT polymerization of (11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid. $[\text{Monomer}] = 4,72$

Polymerization Time (h)	M_n (g/mole)	PDI	Conversion (%)
$[\text{UDAMA}]/[\text{RAFT}]/[\text{AIBN}] = 200/2/0.5$			
2	12200	1,4	51
4	17400	1,4	58
8	25600	1,5	68
24	34300	1,4	74
$[\text{UDAMA}]/[\text{RAFT}]/[\text{ACVA}] = 100/2/0.5$			
2	4800	1,1	31
4	7100	1,3	45
8	5700	1,1	40
24	20100	1,4	60

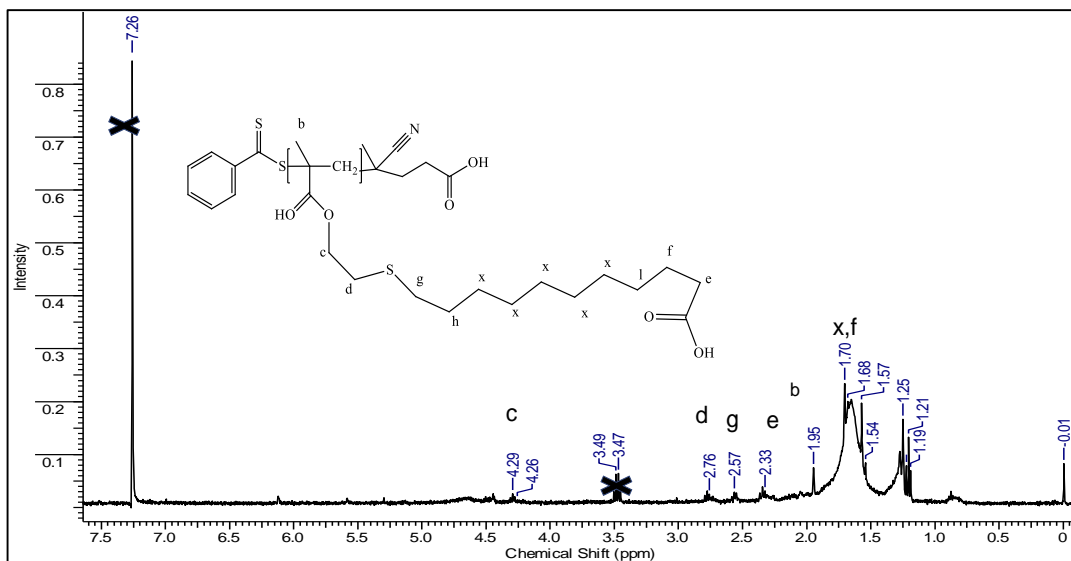
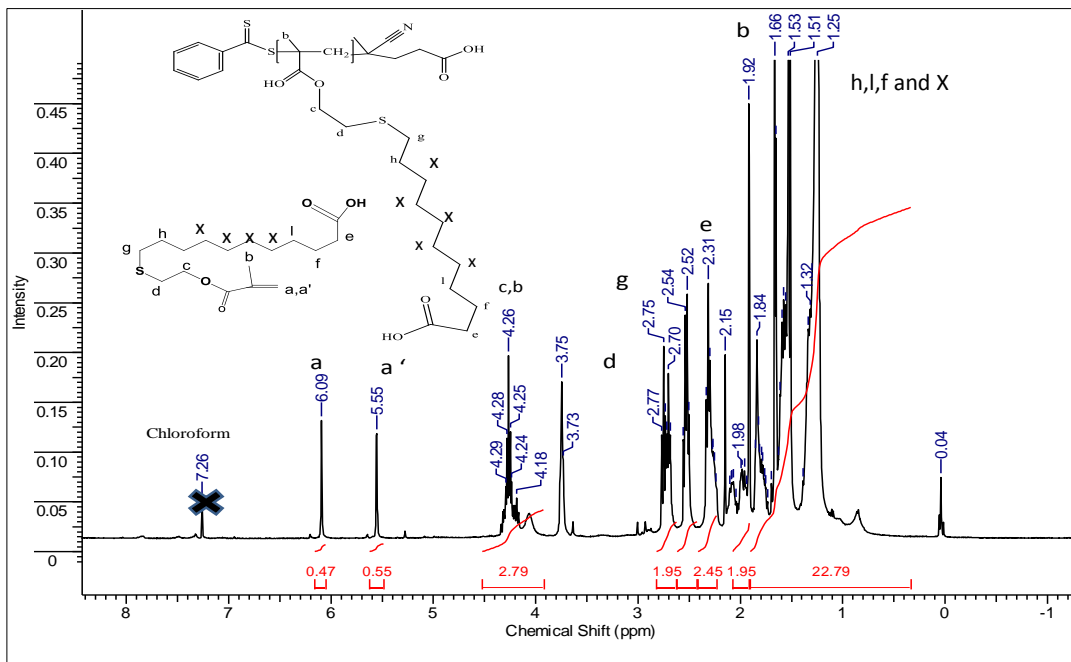


Figure 4.4. $^1\text{H-NMR}$ spectroscopy result of polymerization mixture of UDAMA obtained via RAFT polymerization at 8 hr, and pure P(UDAMA) (bottom), ($[\text{M}]_0 = 4.2\text{M}$; $[\text{M}]_0/[\text{RAFT}]_0/[\text{AIBN}] = 200/2/0.5$).

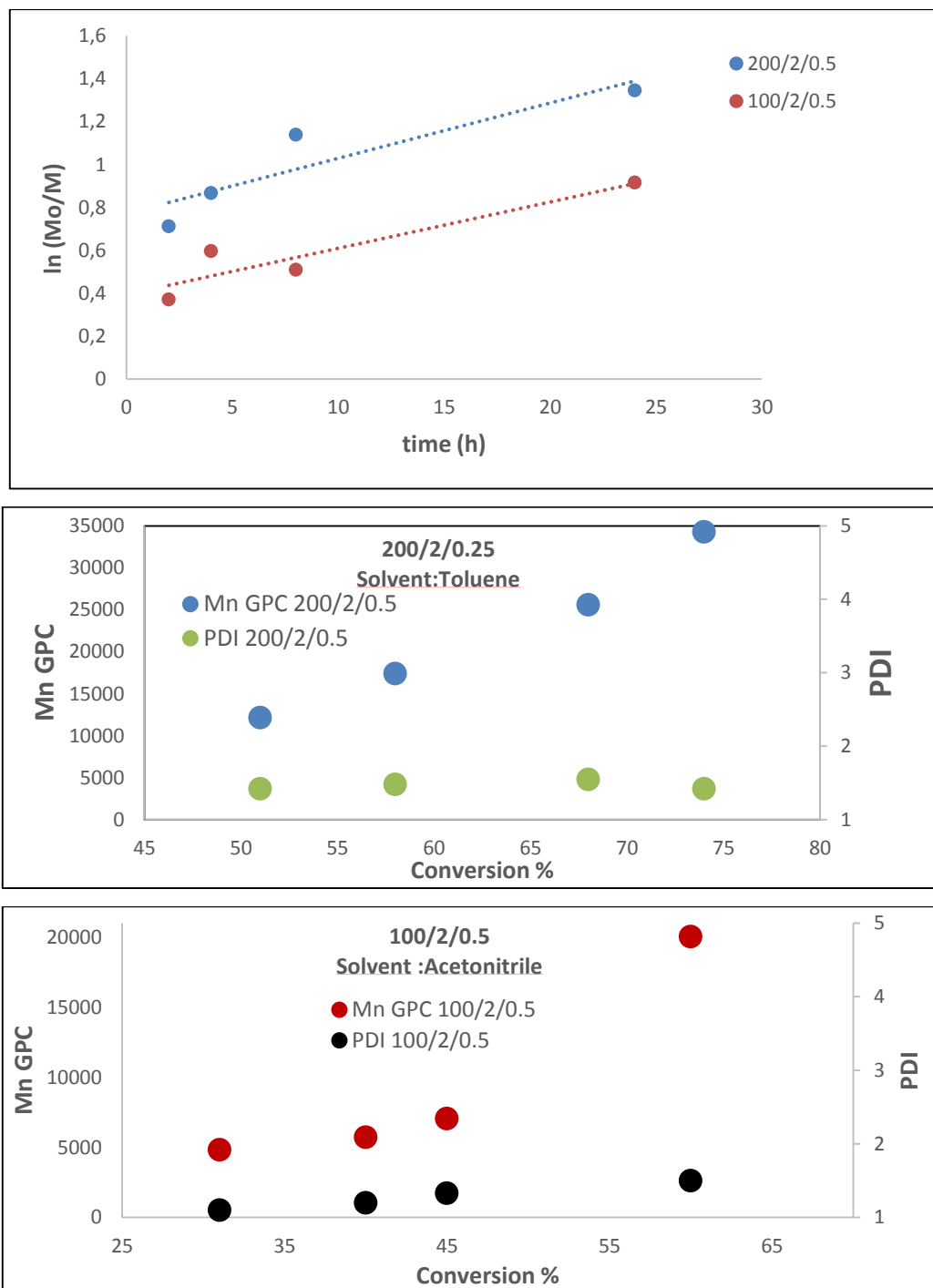


Figure 4.5. Kinetic Plots of RAFT polymerization of poly (11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid) ln (Mo/M) – time, Number average molecular weight (Mn) – conversion and polydispersity index (PDI) – conversion ([M]= 4.2 M; [M]₀/[RAFT]₀/[AIBN]₀= 200/2/0.5 and 100/2/0.5). Solvent: Acetonitrile and toluene.

4.3. Synthesis of of 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] Undecanoic Acid and Methacrylic Acid Copolymers

Following the homopolymerization experiments, random copolymers of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (UDAMA) and methacrylic acid (MAA) were synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization. Since the homopolymers of UDAMA were not water soluble, UDAMA was copolymerized with a highly hydrophilic and acidic monomer, MAA. It was hypothesized that MAA does not only provide water solubility, it also enhances the pH-responsive behavior of UDAMA. RAFT copolymerization of UDAMA and MAA was performed in methanol as an organic solvent. A well-known azo initiator, AIBN was used as the polymerization initiator. 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid was used as the chain transfer (RAFT) agent. Copolymers with varying UDAMA contents (feed UDAMA content: 20 or 50 mole %) were synthesized by changing the [UDAMA]/[MAA]/[RAFT]/[Initiator] mole ratio (10/40/1/0.25 or 25/25/1/0.25) in the polymerization feed at a fixed total monomer concentration (UDAMA and MAA) of 1 M. The monomer conversion was calculated from ¹H-NMR spectra of polymerization mixtures using Equation 4.1 (Figure 4.8).

Monomer conversion % =

$$\left[\frac{1\text{H at } 6.11 \text{ ppm} + 1\text{H at } 5.58 \text{ ppm} / 2}{1\text{H at } 4.24 \text{ ppm} + 1\text{H at } 4.01 \text{ ppm} / 2} (0.2 \text{ for } \% 20 \text{ or } 0.5 \text{ for } \% 50) \text{ UDAMA} + \left(\frac{1\text{H at } 6.23 \text{ ppm} + 1\text{H at } 5.67 \text{ ppm} / 2}{1.86/3} \times 0.8 \text{ for } \% 20 \text{ or } 0.5 \text{ for } \% 50 \text{ MAA} \right) \right] \times 100 \quad (4.1)$$

Table 4.3 lists the monomer conversions, the number average molecular weights (Mn's) and molecular weight distributions (PDI's) of poly (11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid-co- methacrylic acid) p(UDAMA-co-MAA) synthesized throughout the study. According to results presented in Table 4.3 although the monomer conversions were similar, when the UDAMA content increased in the feed, the polymerizations were found to lose RAFT-mechanism mediated control as the PDI and molecular weight values increased significantly. The GPC chromatograms (Figure 4.9) indeed indicated the monomodal molecular weight distribution of polymers obtained at [UDAMA]/[MAA]/[RAFT]/[Initiator] mole ratio of 10/40/1/0.25 while molecular weight distributions showed large dispersity at 25/25/1/0.25, indicating the

loss of RAFT control. This was attributed to the occurrence of side reactions possibly due to the chemical structure of UDAMA. Further optimization experiments on RAFT copolymerization of UDAMA and MAA may be the focus of future studies. GPC chromatograms further showed the increase in molecular weight with time, as expected for a RAFT-controlled polymerization mechanism.

Table 4.2 Monomer conversions, number average molecular weights (M_n 's) and molecular weight distributions (PDI's) of p(11-[2-(2-methyl-acryloyoxy)-ethylsulfanyl] undecanoic acid-co-methacrylic acid) with respect to polymerization time.

Polymerization Time (h)	M_n (g/mol)	PDI	Conversion (%)
[UDAMA]/[MAA]/[RAFT]/[ACVA]= 10/40/1/0.25			
2	4803	1,08	48
4	4366	1,24	65
6	5118	1,28	66
8	5865	1,21	70
10	6141	1,17	71
[UDAMA]/[MAA]/[RAFT]/[ACVA]= 25/25/1/0.25			
2	3802	1,08	48
4	6486	1,08	53
6	10048	1,59	64
8	10940	1,25	62
10	59162	1,74	72

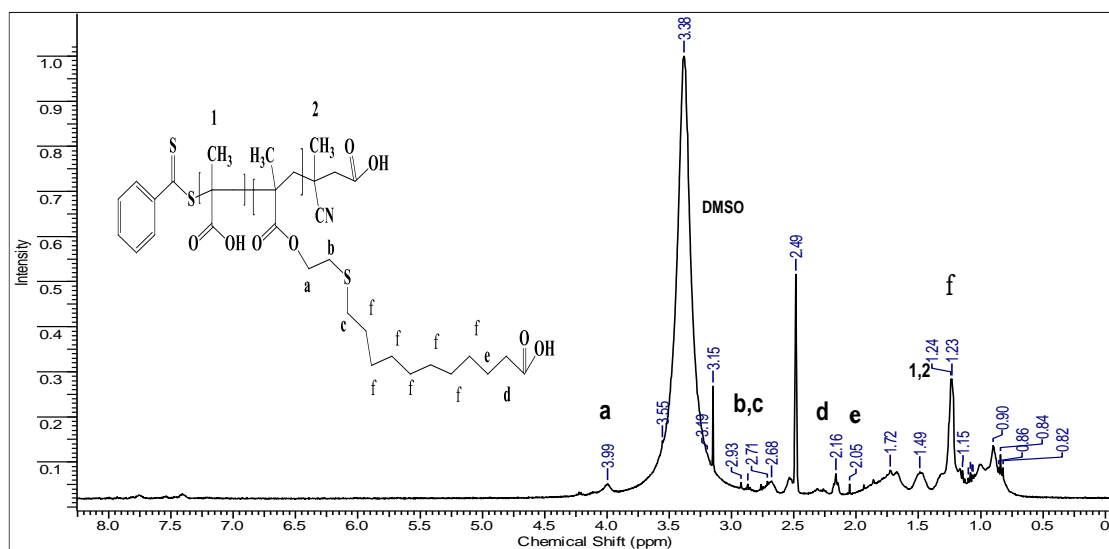
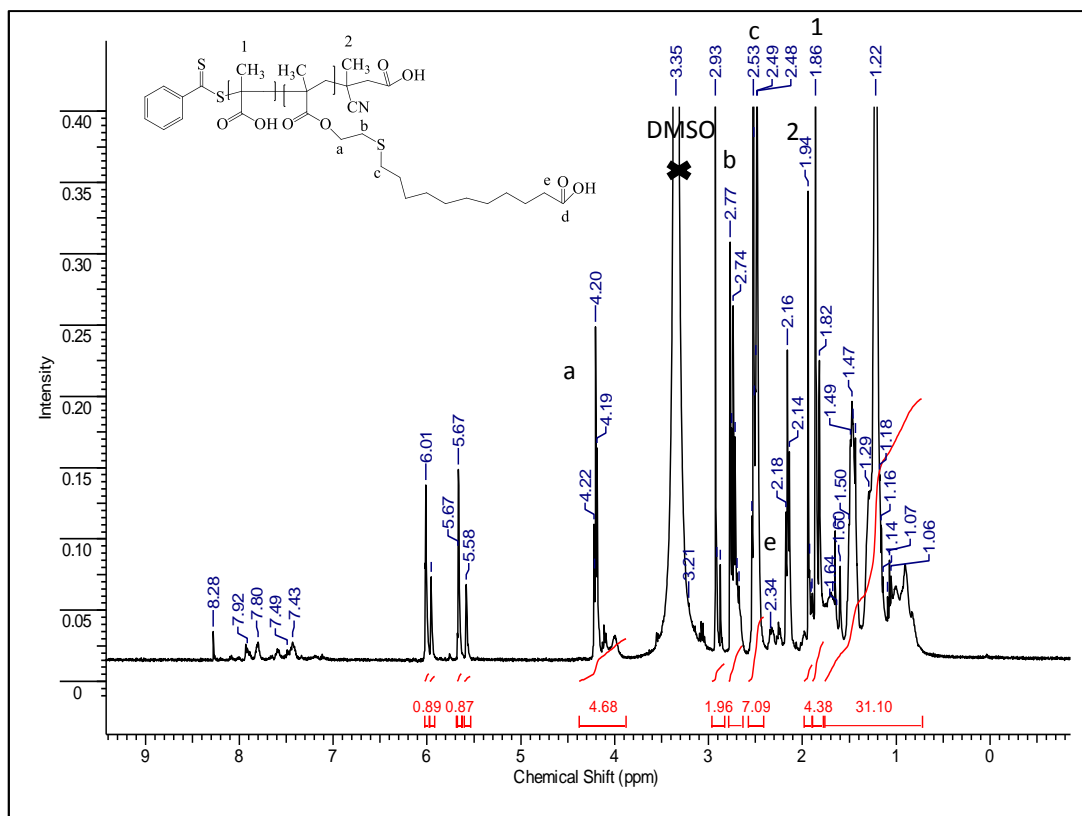


Figure 4.6. A representative $^1\text{H-NMR}$ spectrum of copolymerization mixture of 11-[2-(2-methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid-co-methacrylic acid obtained after 6 hr of polymerization and a representative spectrum of purified p(UDAMA-co-MAA) (bottom). ([Total Monomer] = 1 M; [UDAMA]/[MAA]/[RAFT]/[ACVA]= 10/40/1/0.25)

Figure 4.7 presents the kinetic plots of RAFT copolymerizations of UDAMA and MAA. In all polymerizations, $\ln [M]_0/[M]$ increased linearly with time, indicating pseudo-first order behavior of polymerization. The linear increase in M_n with monomer conversion was also observed. These are all attributed to the known traits of the RAFT-controlled polymerization mechanism.

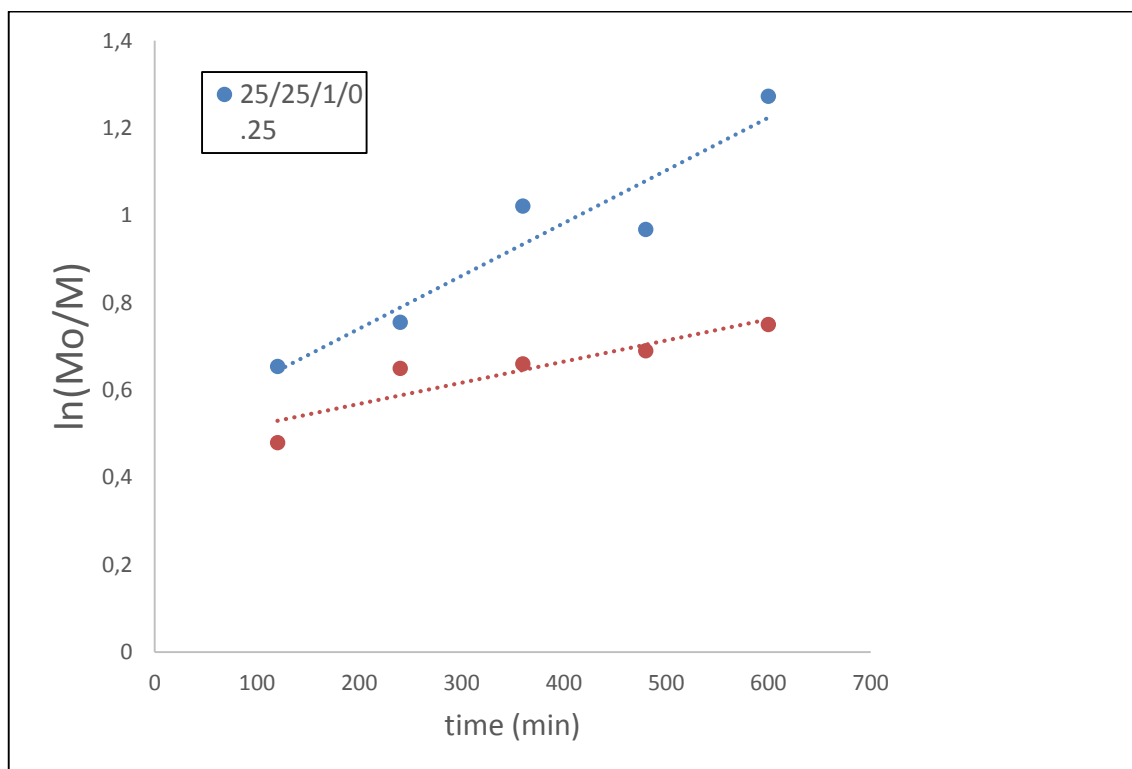


Figure 4.7. Kinetic plots of RAFT copolymerization of poly[11-[2-(2-methylacryloyloxy)-ethylsulfanyl] undecanoic acid-co-methacrylic acid]. A) $\ln [M]_0/[M]$ versus time; B) M_n and PDI versus monomer conversion. M_0 and M are the monomer concentration in the initial polymerization feed and left after polymerization, respectively.

(Cont.on next page)

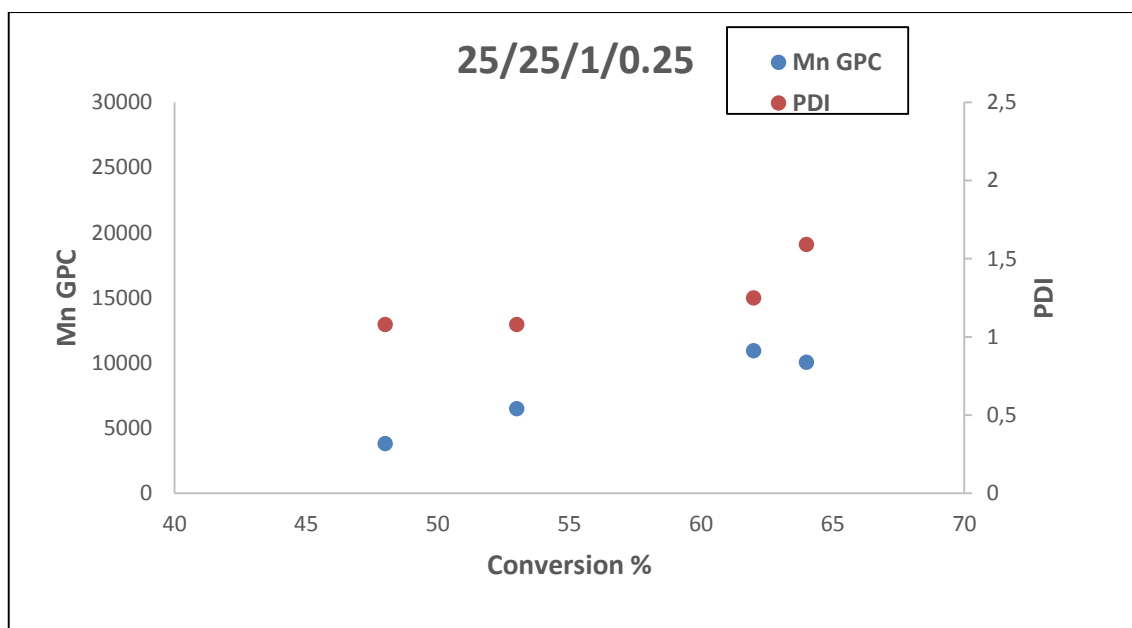
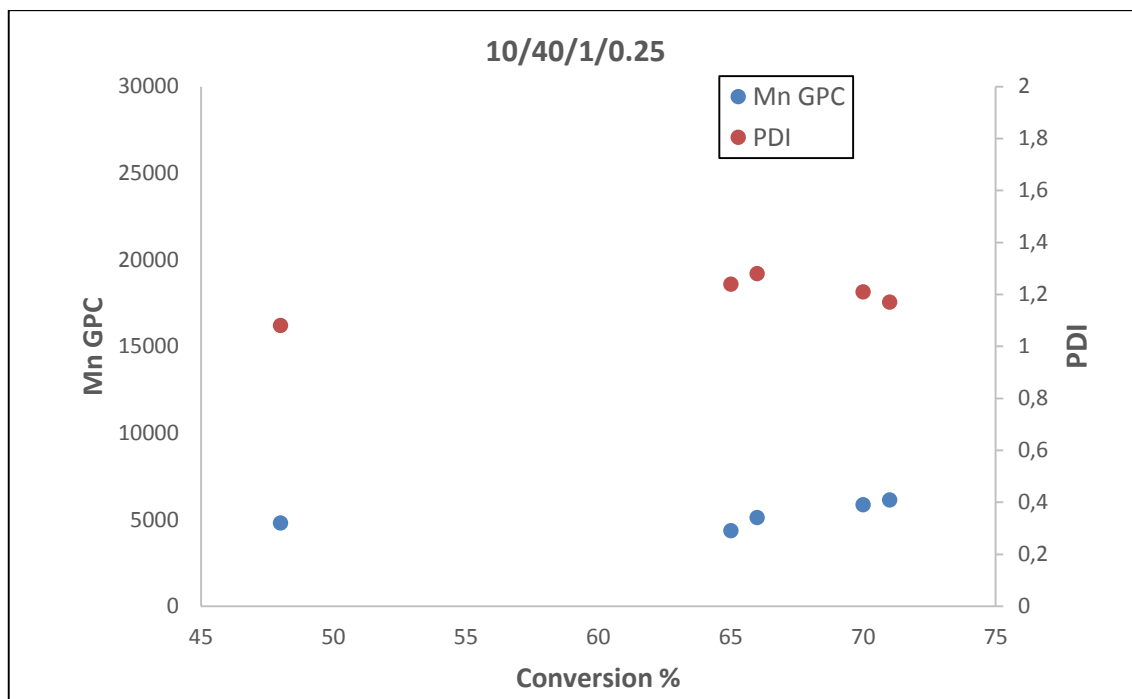


Figure 4.7 (cont.)

4.4. Determination of pH-Responsive Behavior of Copolymers

The pH-responsive phase behaviors of copolymers were studied by measuring the turbidity change of polymer solutions at varying pH values via UV-visible spectroscopy (Figure 4.9).

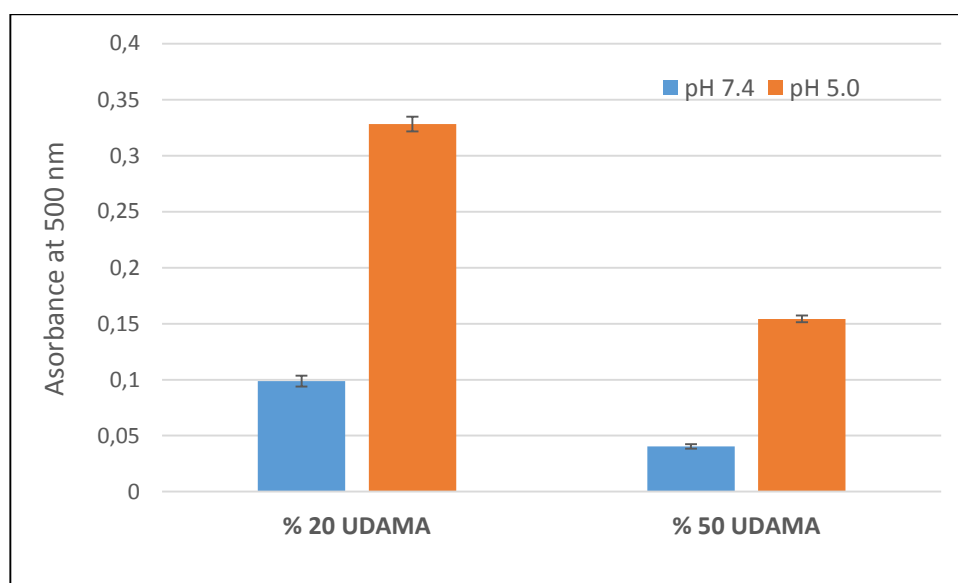


Figure 4.8. Absorbance values of copolymers with varying UDAMA content (20 mol % and 50 mol %) in buffer solutions at pH 5.0 and 7.4. Polymer concentration was 4 mg/ml concentrations

MAA content attributes amphiphilic character to polymers. In acidic pH, carboxylic acid group of polymers are protonated, enhancing the overall hydrophobicity of polymers. Thus, polymers are expected to show pH-responsive phase transition. As the pH of the solution is increased, the COOH/COO^- ratio in the copolymer decreases, making the copolymer chains more hydrophilic. In other words, at higher pH, the copolymer chains are in their most soluble state, causing the solution to appear less turbid. Also, the result of this experiment showed that the copolymer which has 20 % UDAMA content, had higher turbidity at pH 5.0. This was attributed to the molar concentration difference between copolymer solutions used in the measurements as the molecular weight of copolymers was different from each other.

The pH-responsive behavior of copolymers was further analyzed via DLS. The hydrodynamic diameter of copolymers having 20 or 50 % UDAMA content was investigated at pH 5.0 and pH 7.4. The final concentration of polymers in buffer solutions was the same with the concentrations used in turbidity experiment (i.e. 4 mg/ml). The DLS results of copolymer solutions are given in Table 4.3.

Table 4.3. DLS results of the copolymers having 20 and 50 mol % UDAMA content at varying pHs (pH 5.0 and 7.4).

	pH 7.4		pH 5.0	
	20 %	50 %	20 %	50 %
Copolymer UDAMA Content				
Hydrodynamic Diameter (nm)	8,3±1,0	5,9±0,5	10,1±0,0	8,44 ± 0,5

At pH 7.4, because of deprotonation of COOH group, copolymer exists as unimers. At pH 5.0, increasing hydrodynamic diameter suggests self-aggregation and perhaps tendency to aggregate because of protonation of COOH groups. DLS measurements showed slight increase in diameter at high pH, suggesting increase in hydrophobicity and self-organization behavior of copolymers. Moreover, smaller diameter of copolymers with higher UDAMA content was attributed again to the intrachain self-organization of unimers possibly through hydrophobic interactions.

4.5. Hemolytic Activity of Polymers

The hemolytic activity of p(MAA) and copolymers was evaluated at different pH values (pH 5.0 and pH 7.4) using polymer concentrations of 50 or 100 ug/ml. In endocytic pathway, the pH of endosomes gradually decreases. This pH gradient is a key factor in the design of membrane-disruptive polymers which could enhance the endosomal release of drugs. Such polymers are expected to disrupt lipid bilayer membranes at acidic pHs such as 6.0 and lower, but should be non-lytic at pH 7.4. The haemolytic effect of copolymers having a UDAMA content of 20 % and 50 % and homopolymer of MAA was compared with positive control (Triton X-100). Experiments were performed in triplicate. The results are shown in Figures 4.10 and 4.11. Copolymer containing 20 % UDAMA showed the desired haemolytic behaviour

as a potential membrane-destabilizing agent at both polymer concentrations. Hemolytic activity of this copolymer was found to be 100 % at pH 5.0 while it was less than 10 % at pH 7.4.

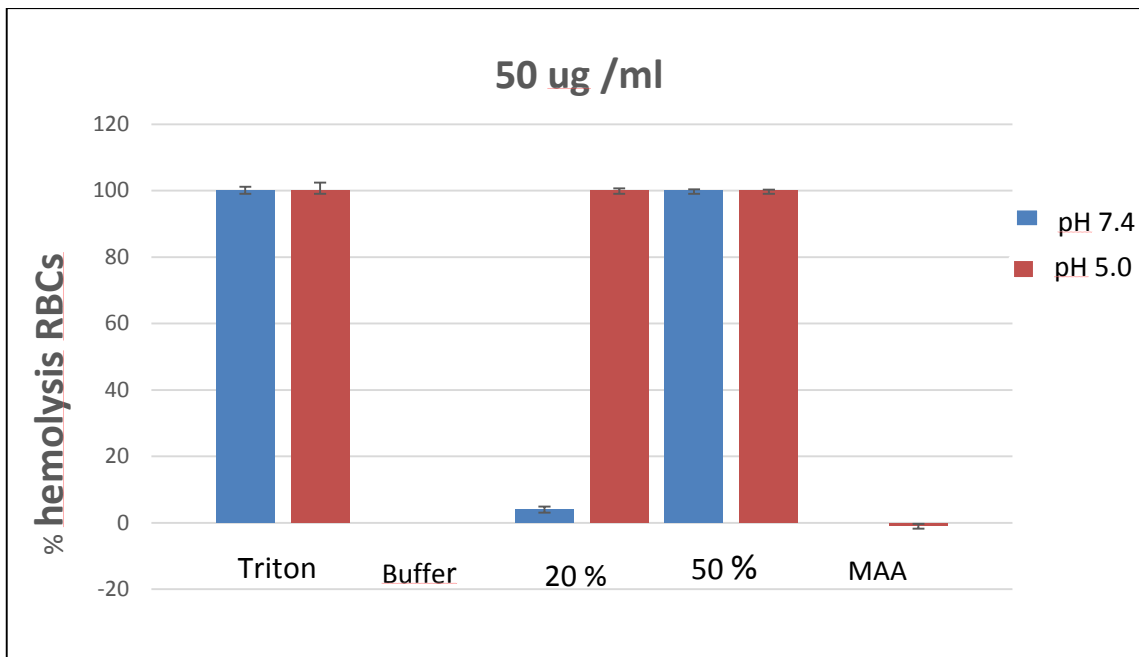


Figure 4.9. The hemolytic activity of polymers (Polymer concentration= 50 ug /ml)

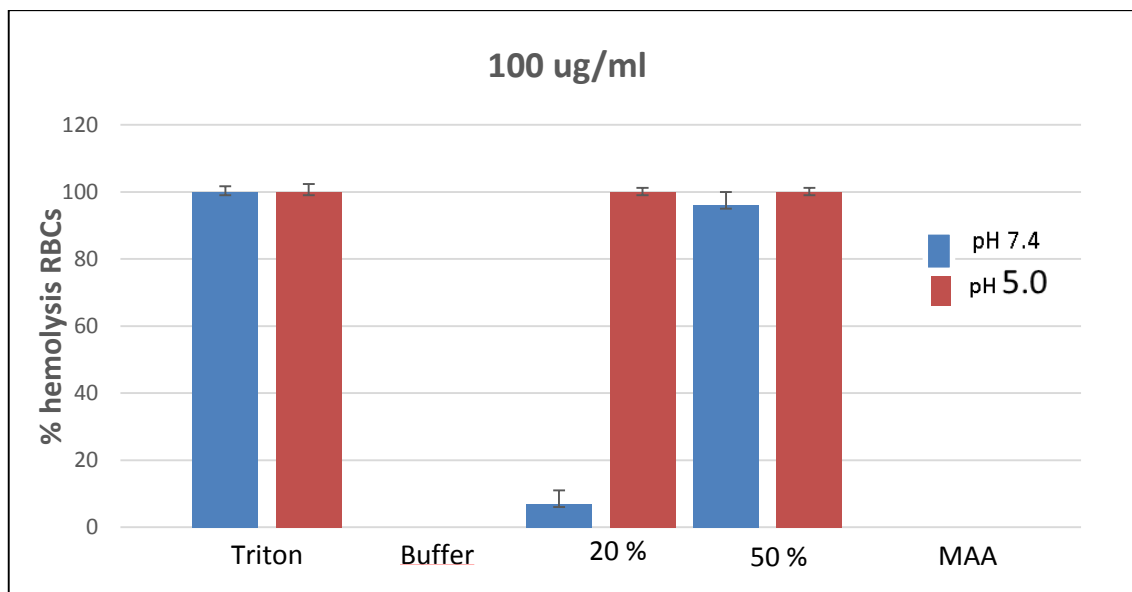


Figure 4.10. The hemolytic activity of polymers (Polymer concentration= 100 ug /ml)

The copolymer structure having 50% UDAMA content was membrane-lytic at both pH values possibly due to the higher hydrophobicity even at neutral pH values when compared with the copolymer having 20% UDAMA. Deprotonation of acidic groups of this copolymer at pH 7.4 apparently does not provide sufficient hydrophilicity to the copolymer due to the higher hydrophobic comonomer (UDAMA) content.

CHAPTER 5

CONCLUSION

The aim of the study was to synthesize and characterize well defined fatty acid polymers as potential membrane destabilizing agents. For this aim, a new monomer derived from unsaturated fatty acid (10-undecenoic acid) was synthesized. 11-[2-(2-Methyl-acryloyloxy)-ethylsulfanyl] undecanoic acid (UDAMA) was then polymerized via RAFT polymerization. Additionally, random copolymers of UDAMA and methacrylic acid (MAA) having two different UDAMA content (20 and 50 mole%) were synthesized via RAFT polymerization.

The monomer was synthesized following a two-step procedure. The first step was converting the unsaturated fatty acid to a saturated fatty acid by thiol-ene thermal addition of 2-mercaptoethanol to 10-undecenoic acid. In the second step, product which was obtained from the first step, was methacrylated according to procedures reported previously. Chemical structures, reaction yields and purity of synthesized compounds were characterized at every step. Pure monomer was obtained with an overall yield of 92 %. The new monomer, UDAMA was then polymerized via RAFT polymerization. This polymerization technique enables the synthesis of well-defined polymers with controlled molecular weights and narrow molecular weight distributions (narrow polydispersity index (PDI)). Monomer conversions, and number average molecular weights and molecular weight distributions were determined using Nuclear Magnetic Resonance (NMR) and Gel Permeation Chromatography (GPC), respectively.

Several monomer concentrations, organic solvents and [monomer]/[RAFT]/[initiator] mole ratios were tested to optimize polymerization reaction conditions. A monomer concentration of 1 or 4.2 M, acetonitrile as a solvent and a [monomer]/[RAFT]/[initiator] mole ratio of 200/2/0.5 were found suitable. From the kinetic plots of RAFT homopolymerization of UDAMA, the logarithmic monomer conversion $\ln[M]_0/[M]$ was found to increase linearly with time. The linear increase in M_n with monomer conversion was also observed. These are attributed to the RAFT controlled polymerization mechanism. Random copolymers of methacrylic acid and UDAMA were also synthesized via RAFT polymerization to overcome water-

insolubility problem of UDAMA homopolymers and also enhance the pH-responsive behavior of p(UDAMA). In copolymerizations, when the UDAMA content increased in the feed, the polymerizations were found to lose RAFT-mechanism mediated control as the PDI and molecular weight values increased significantly. The GPC chromatograms indicated the monomodal molecular weight distribution of polymers obtained at [UDAMA]/[MAA]/[RAFT]/[Initiator] mole ratio of 10/40/1/0.25 while molecular weight distributions showed large dispersity at 25/25/1/0.25, indicating the loss of RAFT control. GPC chromatograms further showed the increase in molecular weight with time, as expected for a RAFT-controlled polymerization mechanism.

pH-responsive behavior of copolymers was investigated via turbidity and DLS experiments. When pH is lowered from neutral pH to acidic pH, turbidity of copolymer solutions increased, indicating the transition of copolymer chains from a hydrophilic state to a hydrophobic state. The DLS measurements showed slight increase in hydrodynamic diameter of copolymers at acidic pH when compared with those at neutral pH, suggesting an increase in hydrophobicity and self-aggregation tendency of copolymers. Moreover, smaller diameter of copolymers with higher UDAMA content indicated the intrachain self-organization of unimers possibly through hydrophobic interactions.

Finally, pH-dependent membrane-disrupting activity of copolymers was investigated via hemolysis assay. The results showed that the polymer having 20 % UDAMA efficiently disrupted red blood cell (RBC) membrane at pH 5.0 (endosome pH) while it did not interact with RBCs at pH 7.4 (extracellular pH).

In summary, new, well-defined, lipid-containing homopolymers and pH-responsive lipid-containing copolymers were synthesized via RAFT polymerization. The copolymer synthesized with a feed composition of 20% mol UDAMA displayed the desired hemolytic activity, envisioning the potential use of this copolymer as an endosome-disrupting agent.

The following investigations can be the focus of future studies:

1. Further optimization experiments on RAFT copolymerization of UDAMA and MAA can be performed to obtain copolymers with better controlled molecular weight characteristics.
2. Copolymers with UDAMA content below and above 20% can be synthesized and characterized to determine the UDAMA content for the best hemolytic activity.

3. Cytotoxicity of copolymers with varying UDAMA content can be performed to determine whether these copolymers possess potential for drug delivery applications.

4. Intracellular distribution of the copolymers with desired hemolytic activity can be investigated using fluorescent labelled polymers and organelle-specific fluorophores.

REFERENCES

- Aamer, K. A., & Tew, G. N. (2007). RAFT polymerization of a novel activated ester monomer and conversion to a terpyridine-containing homopolymer. *Journal of Polymer Science Part A: Polymer Chemistry*, 45(23), 5618-5625.
- Akhtar, S. (2005). Non-viral cancer gene therapy: beyond delivery. *Gene therapy*, 13(9), 739-740.
- Akinc, A., & Langer, R. (2002). Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. *Biotechnology and bioengineering*, 78(5), 503-508.
- Alshamsan, A., Haddadi, A., Incani, V., Samuel, J., Lavasanifar, A., & Uludag, H. (2008). Formulation and delivery of siRNA by oleic acid and stearic acid modified polyethylenimine. *Molecular pharmaceutics*, 6(1), 121-133.
- Bathfield, M., Daviot, D., D'Agosto, F., Spitz, R., Ladavière, C., Charreyre, M.-T. r. s., & Delair, T. (2008). Synthesis of lipid- α -end-functionalized chains by RAFT polymerization. Stabilization of lipid/polymer particle assemblies. *Macromolecules*, 41(22), 8346-8353.
- Bechinger, B. (1996). Towards membrane protein design: pH-sensitive topology of histidine-containing polypeptides. *Journal of molecular biology*, 263(5), 768-775.
- Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). Lipids and cell membranes.
- Berg, K., Selbo, P. K., Prasmickaite, L., Tjelle, T. E., Sandvig, K., Moan, J., . . . Anholt, H. (1999). Photochemical Internalization A Novel Technology for Delivery of Macromolecules into Cytosol. *Cancer research*, 59(6), 1180-1183.
- Bijsterbosch, M. K., Manoharan, M., Dorland, R., van Veghel, R., Biessen, E. A., & van Berkel, T. J. (2002). bis-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides are highly selectively taken up by the liver. *Journal of Pharmacology and Experimental Therapeutics*, 302(2), 619-626.
- Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., & Behr, J.-P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences*, 92(16), 7297-7301.
- Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., & Behr, J. P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A*, 92(16), 7297-7301.

- Boyer, C., Bulmus, V., Davis, T. P., Ladmiral, V., Liu, J., & Perrier, S. b. (2009). Bioapplications of RAFT polymerization. *Chemical reviews*, 109(11), 5402-5436.
- Braunecker, W. A., & Matyjaszewski, K. (2007). Controlled/living radical polymerization: features, developments, and perspectives. *Progress in Polymer Science*, 32(1), 93-146.
- Bulmus, V. (2005). Biomembrane-active molecular switches as tools for intracellular drug delivery. *Australian journal of chemistry*, 58(6), 411-422.
- Bulmus, V. (2011). RAFT polymerization mediated bioconjugation strategies. *Polymer Chemistry*, 2(7), 1463-1472.
- Bulmus, V., Woodward, M., Lin, L., Murthy, N., Stayton, P., & Hoffman, A. (2003). A new pH-responsive and glutathione-reactive, endosomal membrane-disruptive polymeric carrier for intracellular delivery of biomolecular drugs. *Journal of controlled release*, 93(2), 105-120.
- Carrasco, L. (1994). Entry of animal viruses and macromolecules into cells. *FEBS letters*, 350(2), 151-154.
- Convertine, A. J., Benoit, D. S., Duvall, C. L., Hoffman, A. S., & Stayton, P. S. (2009). Development of a novel endosomolytic diblock copolymer for siRNA delivery. *Journal of controlled release*, 133(3), 221-229.
- Desroches, M., Caillol, S., Lapinte, V., Auvergne, R. m., & Boutevin, B. (2011). Synthesis of biobased polyols by thiol-ene coupling from vegetable oils. *Macromolecules*, 44(8), 2489-2500.
- Duvall, C. L., Convertine, A. J., Benoit, D. S., Hoffman, A. S., & Stayton, P. S. (2010). Intracellular delivery of a proapoptotic peptide via conjugation to a RAFT synthesized endosomolytic polymer. *Molecular pharmaceuticals*, 7(2), 468-476.
- Eliyahu, H., Barenholz, Y., & Domb, A. (2005). Polymers for DNA delivery. *Molecules*, 10(1), 34-64.
- Godbey, W. T., Wu, K. K., & Mikos, A. G. (1999). Poly(ethylenimine) and its role in gene delivery. *J Control Release*, 60(2-3), 149-160.
- Henry, S. M., El-Sayed, M. E., Pirie, C. M., Hoffman, A. S., & Stayton, P. S. (2006). pH-responsive poly (styrene-alt-maleic anhydride) alkylamide copolymers for intracellular drug delivery. *Biomacromolecules*, 7(8), 2407-2414.
- Hoffman, A., Stayton, P., El-Sayed, M., Murthy, N., Bulmus, V., Lackey, C., & Cheung, C. (2007). Design of " Smart" Nano-Scale Delivery Systems for Biomolecular Therapeutics. *Journal of Biomedical Nanotechnology*, 3(3), 213-217.

- Horváth, A., Tóth, G. K., Gogolák, P., Nagy, Z., Kurucz, I., Pecht, I., & Rajnavölgyi, É. (1998). A hemagglutinin-based multi-peptide construct elicits enhanced protective immune response in mice against influenza A virus infection. *Immunology letters*, *60*(2), 127-136.
- Inui, O., Teramura, Y., & Iwata, H. (2010). Retention dynamics of amphiphilic polymers PEG-lipids and PVA-alkyl on the cell surface. *ACS Applied Materials & Interfaces*, *2*(5), 1514-1520.
- Jones, A. T., Gumbleton, M., & Duncan, R. (2003). Understanding endocytic pathways and intracellular trafficking: a prerequisite for effective design of advanced drug delivery systems. *Advanced drug delivery reviews*, *55*(11), 1353-1357.
- Kalyan, N. K., Lee, S.-G., Wilhelm, J., Pisano, M. R., Hum, W.-T., Hsiao, C.-L., Hung, P. P. (1994). Immunogenicity of recombinant influenza virus haemagglutinin carrying peptides from the envelope protein of human immunodeficiency virus type 1. *Vaccine*, *12*(8), 753-760.
- Kichler, A., Leborgne, C., März, J., Danos, O., & Bechinger, B. (2003). Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proceedings of the National Academy of Sciences*, *100*(4), 1564-1568.
- Kichler, A., Mason, A. J., & Bechinger, B. (2006). Cationic amphipathic histidine-rich peptides for gene delivery. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1758*(3), 301-307.
- Koper, G. J., van Duijvenbode, R. C., Stam, D. D., Steuerle, U., & Borkovec, M. (2003). Synthesis and protonation behavior of comblike poly (ethyleneimine). *Macromolecules*, *36*(7), 2500-2507.
- Ladaviere, C., Tribet, C., & Cribier, S. (2002). Lateral organization of lipid membranes induced by amphiphilic polymer inclusions. *Langmuir*, *18*(20), 7320-7327.
- Li, W., Nicol, F., & Szoka Jr, F. C. (2004). GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Advanced drug delivery reviews*, *56*(7), 967-985.
- Meier, M. A., Metzger, J. O., & Schubert, U. S. (2007). Plant oil renewable resources as green alternatives in polymer science. *Chemical Society Reviews*, *36*(11), 1788-1802.
- Murthy, N., Robichaud, J. R., Tirrell, D. A., Stayton, P. S., & Hoffman, A. S. (1999). The design and synthesis of polymers for eukaryotic membrane disruption. *Journal of controlled release*, *61*(1), 137-143.
- Pack, D. W., Hoffman, A. S., Pun, S., & Stayton, P. S. (2005). Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery*, *4*(7), 581-593.

- Parente, R., Nir, S., & Szoka, F. (1988). pH-dependent fusion of phosphatidylcholine small vesicles. Induction by a synthetic amphipathic peptide. *Journal of Biological Chemistry*, 263(10), 4724-4730.
- Parente, R. A., Nir, S., & Szoka Jr, F. C. (1990). Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry*, 29(37), 8720-8728.
- Ren, J., Sharpe, J. C., Collier, R. J., & London, E. (1999). Membrane translocation of charged residues at the tips of hydrophobic helices in the T domain of diphtheria toxin. *Biochemistry*, 38(3), 976-984.
- Sciannamea, V., Jérôme, R., & Detrembleur, C. (2008). In-situ nitroxide-mediated radical polymerization (NMP) processes: their understanding and optimization. *Chemical reviews*, 108(3), 1104-1126.
- Semsarilar, M., & Perrier, S. (2010). 'Green'reversible addition-fragmentation chain-transfer (RAFT) polymerization. *Nature Chemistry*, 2(10), 811-820.
- Sevimli, S., Inci, F., Zareie, H. M., & Bulmus, V. (2012). Well-Defined Cholesterol Polymers with pH-Controlled Membrane Switching Activity. *Biomacromolecules*, 13(10), 3064-3075.
- Stayton, P. S., Hoffman, A. S., Murthy, N., Lackey, C., Cheung, C., Tan, P., . . . Press, O. W. (2000). Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. *Journal of controlled release*, 65(1), 203-220.
- Stegmann, T. (2000). Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion. *Traffic*, 1(8), 598-604.
- Subbarao, N. K., Parente, R. A., Szoka Jr, F. C., Nadasdi, L., & Pongracz, K. (1987). The pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry*, 26(11), 2964-2972.
- Sun, J., Zeng, F., Jian, H., & Wu, S. (2013). Grafting zwitterionic polymer chains on PEI as a convenient strategy to enhance gene delivery performance. *Polym. Chem.*
- Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics*, 4(5), 346-358.
- Thomas, J. L., & Tirrell, D. A. (1992). Polyelectrolyte-sensitized phospholipid vesicles. *Accounts of Chemical Research*, 25(8), 336-342.
- Tonge, S., & Tighe, B. (2001). Responsive hydrophobically associating polymers: a review of structure and properties. *Advanced drug delivery reviews*, 53(1), 109-122.

- Torchilin, V. P., Levchenko, T. S., Rammohan, R., Volodina, N., Papahadjopoulos-Sternberg, B., & D'Souza, G. G. (2003). Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome–DNA complexes. *Proceedings of the National Academy of Sciences*, *100*(4), 1972-1977.
- Türünç, O., Firdaus, M., Klein, G., & Meier, M. A. (2012). Fatty acid derived renewable polyamides via thiol–ene additions. *Green chemistry*, *14*(9), 2577-2583.
- Türünç, O., & Meier, M. A. (2010). Fatty Acid Derived Monomers and Related Polymers Via Thiol-ene (Click) Additions. *Macromolecular rapid communications*, *31*(20), 1822-1826.
- Varkouhi, A. K., Scholte, M., Storm, G., & Haisma, H. J. (2011). Endosomal escape pathways for delivery of biologicals. *Journal of Controlled Release*, *151*(3), 220-228.
- Wagner, E., Plank, C., Zatloukal, K., Cotten, M., & Birnstiel, M. L. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proceedings of the National Academy of Sciences*, *89*(17), 7934-7938.
- Westphal, D., Dewson, G., Czabotar, P. E., & Kluck, R. M. (2011). Molecular biology of Bax and Bak activation and action. *Biochim Biophys Acta*, *1813*(4), 521-531. doi: 10.1016/j.bbamcr.2010.12.019
- Wiley, D. C., & Skehel, J. J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annual review of biochemistry*, *56*(1), 365-394.
- Wolfrum, C., Shi, S., Jayaprakash, K. N., Jayaraman, M., Wang, G., Pandey, R. K., . . . Ndungo, E. M. (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nature biotechnology*, *25*(10), 1149-1157.
- Wyman, T. B., Nicol, F., Zelphati, O., Scaria, P., Plank, C., & Szoka, F. C. (1997). Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry*, *36*(10), 3008-3017.
- Yaşayan, G., Redhead, M., Magnusson, J. P., Spain, S. G., Allen, S., Davies, M., Fernández-Trillo, F. (2012). Well-defined polymeric vesicles with high stability and modulation of cell uptake by a simple coating protocol. *Polymer Chemistry*, *3*(9), 2596-2604.
- Yessine, M.-A., Dufresne, M.-H., Meier, C., Peterleit, H.-U., & Leroux, J.-C. (2007). Proton-actuated membrane-destabilizing polyion complex micelles. *Bioconjugate chemistry*, *18*(3), 1010-1014.

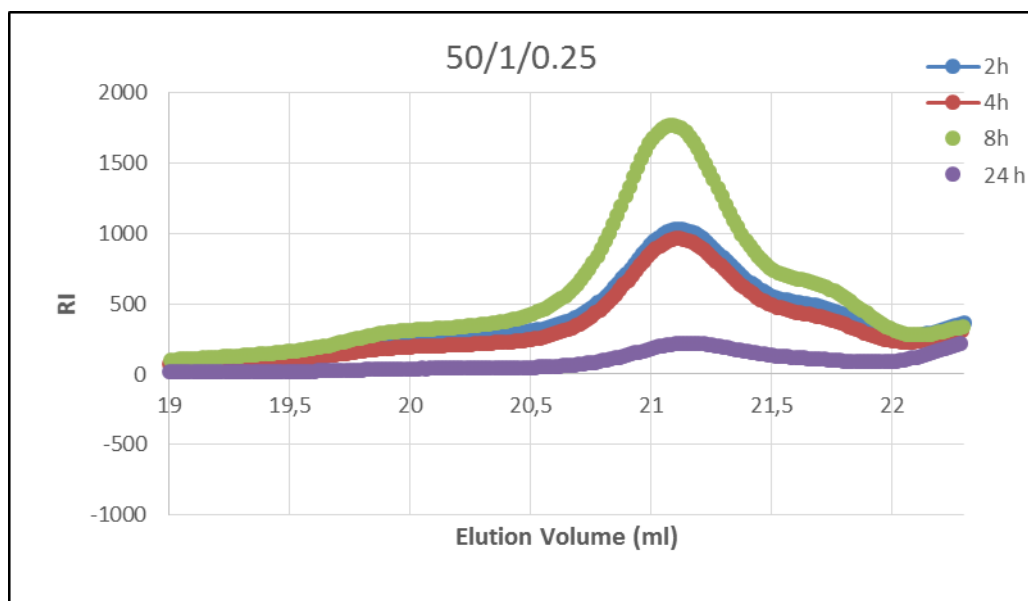
Yusa, S.-i., Fukuda, K., Yamamoto, T., Ishihara, K., & Morishima, Y. (2005). Synthesis of well-defined amphiphilic block copolymers having phospholipid polymer sequences as a novel biocompatible polymer micelle reagent. *Biomacromolecules*, 6(2), 663-670.

Zurbriggen, R. (2003). Immunostimulating reconstituted influenza virosomes. *Vaccine*, 21(9), 921-924.

APPENDIX A

RAFT POLYMERIZATION OF 11-[2-(2-METHYLACRYLOYLOXY)-ETHYLSULFANYL] UNDECANOIC ACID

A)



B)

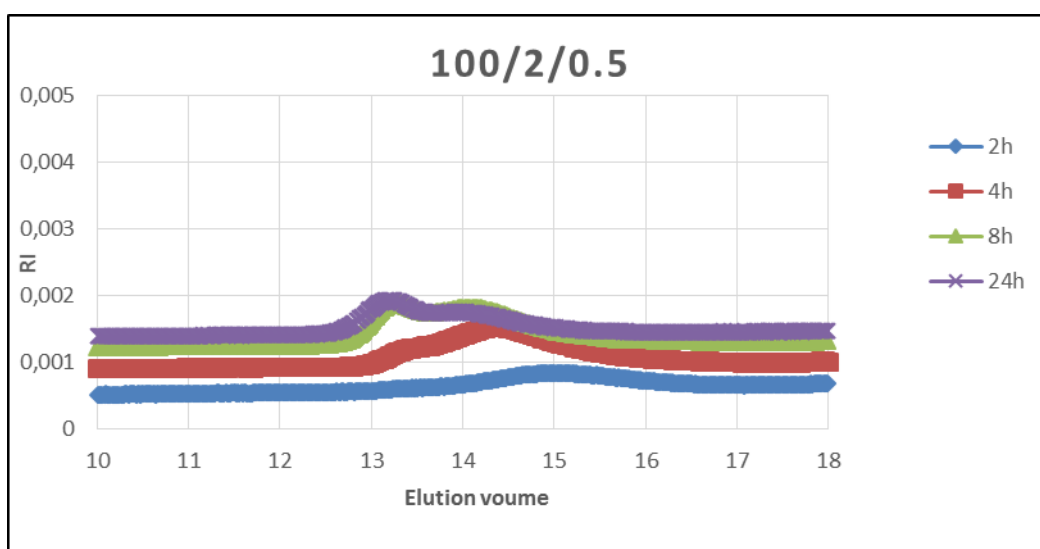


Figure A.1. GPC chromatograms of polymerization mixture when monomer concentration was 4.72 M and $[M]/[R]/[I]$ ratios were A) 50/2/0.25, B) 100/1/0.25

APPENDIX B

SYNTHESIS OF 11-[2-(2-METHYL-ACRYLOYLOXY)-ETHYLSULFANYL] UNDECANOIC ACID AND METHACRYLIC ACID COPOLYMERS

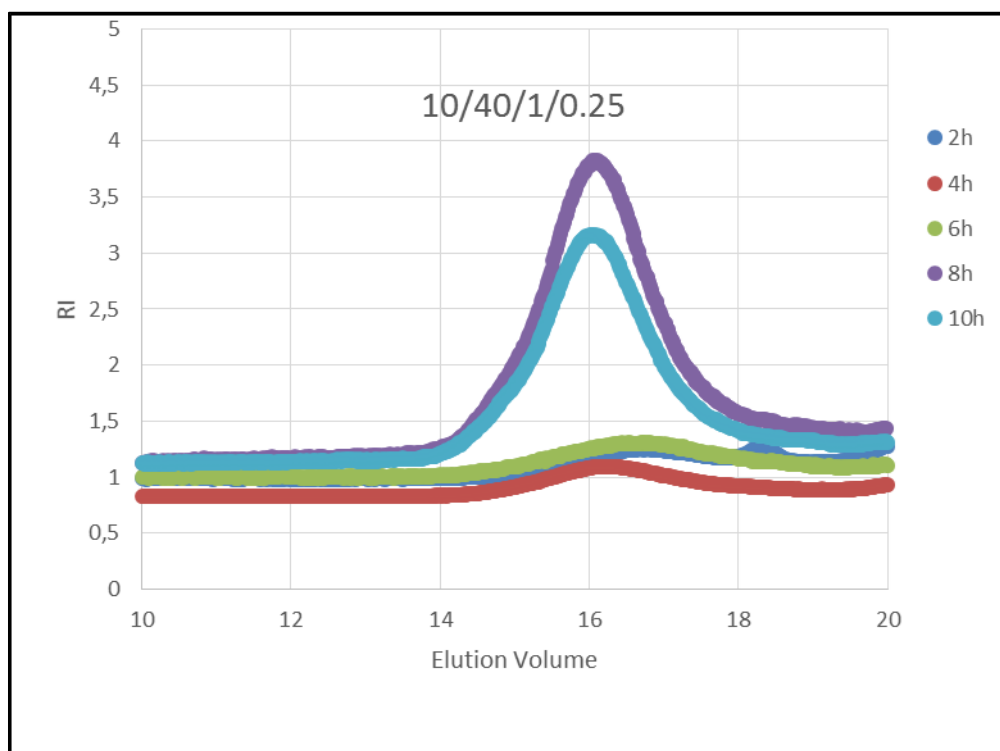


Figure B.1. GPC chromatograms of copolymerization mixture when monomer concentration was 1 M and $[M]/[R]/[I]$ ratios were 10/40/2/0.25.

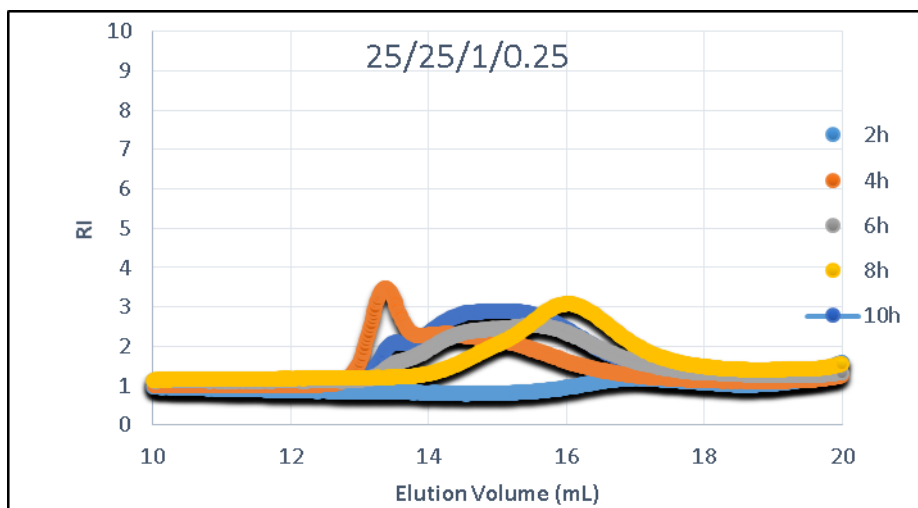
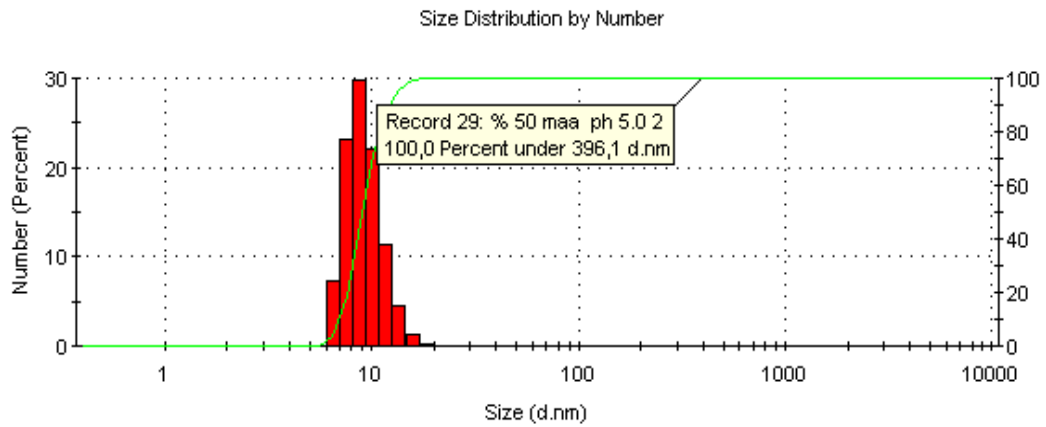


Figure B.2. GPC chromatograms of copolymerization mixture when monomer concentration was 1 M and $[M]/[R]/[I]$ ratios were 25/25/1/0.25

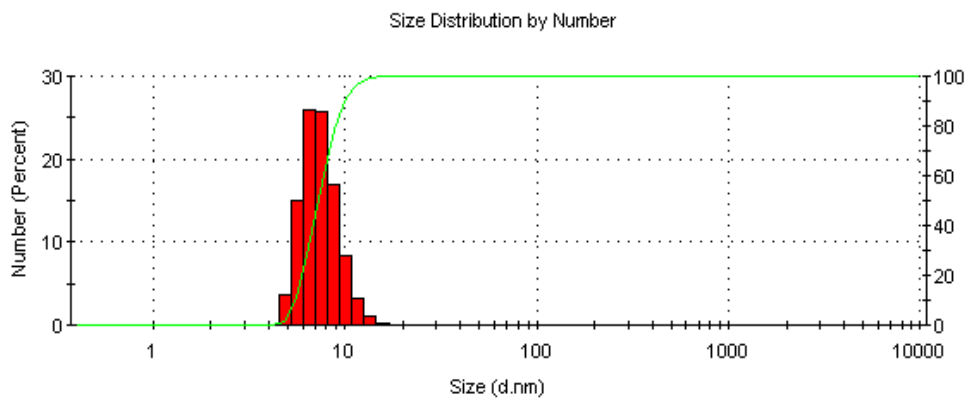
APPENDIX C

DYNAMIC LIGHT SCATTERING (DLS) MEASUREMENTS AT VARYING TEMPERATURE AND pH VALUES

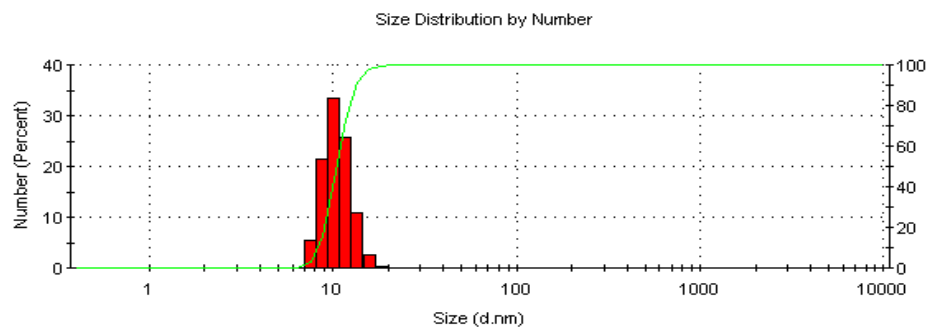
A)



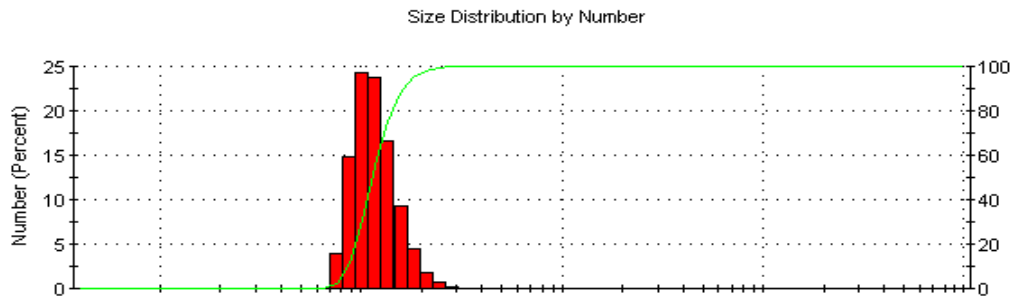
B)



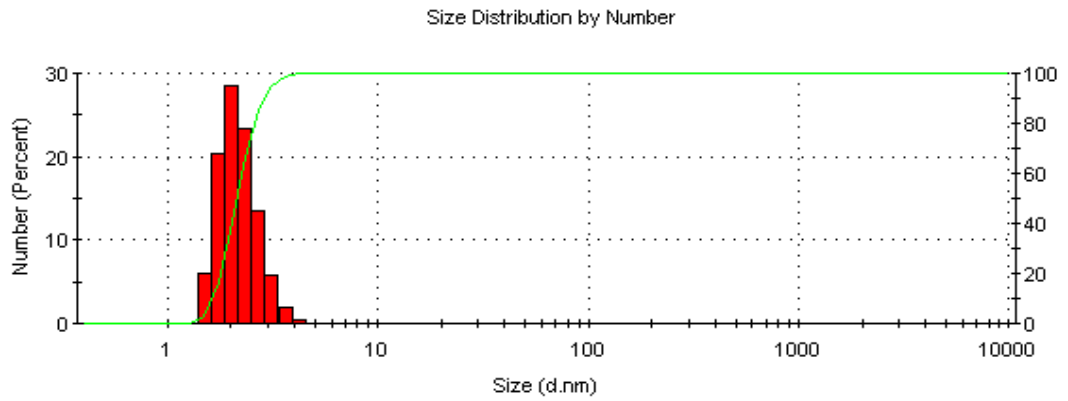
C)



D)



E)



F)

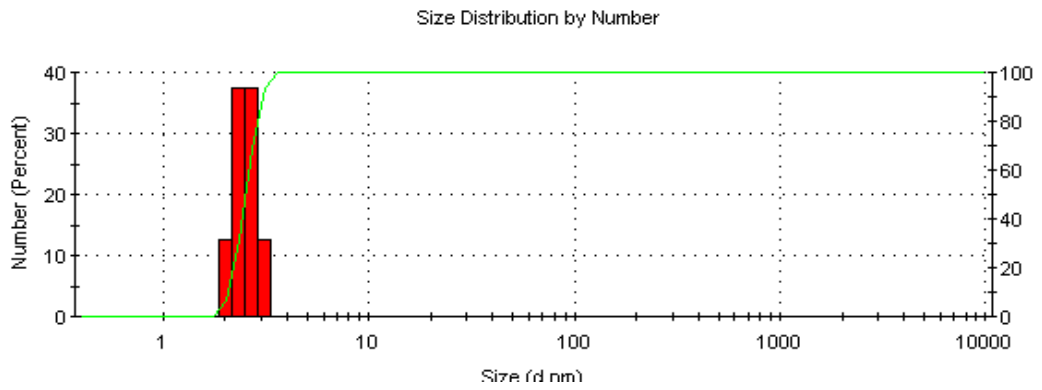


Figure C. 1. Representative Size distribution plots of copolymers analyzed by dynamic light scattering (DLS). (A) 50 mol% UDAMA, (B) 20 mol% UDAMA, and (C) MAA in pH 5.0(upper) and pH 7.4(bottom)