In Vitro EVALUATION OF POLY(2-((2-AMINOETHYL)AMINO)ETHYL METHACRYLATE) AS A POTENTIAL siRNA DELIVERY AGENT

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// ABSTRACT

In Vitro EVALUATION OF POLY(2-((2-AMINOETHYL)AMINO)ETHYL METHACRYLATE) AS A POTENTIAL siRNA DELIVERY AGENT

The aim of this thesis is to investigate poly(2-((2-aminoethyl)amino)ethyl methacrylate) (P(AEAEMA)) as a potential siRNA carrier.

For this purpose, an amine containing monomer 2-((tert-butoxycarbonyl) (2-((tert-butoxy carbonyl) amino) ethyl) amino) ethyl methacrylate (BocAEAEMA) was synthesized. Reversible addition-fragmentation chain transfer (RAFT) polymerization was performed to prepare homo- and block co-polymers of BocAEAEMA. The synthesized polymers -$P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$- were characterized via NMR and GPC.

The cytotoxicity of the polymers was investigated *in vitro* using ovarian cancer cell line (Skov-3-luc) via MTT assay. The polymers did not show any toxic effect on cells in 24 h.

The ability of the BocAEAEMA polymers to form polyplexes with siRNA was investigated via gel electrophoresis. $P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ could efficiently form complexes with siRNA at an N/P ratio of 5, 2, and 2 respectively.

Gel electrophoresis analysis revealed that $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ could protect siRNA against serum components at least for 6 h. Block copolymer, when complexed with siRNA at an N/P ratio of 10, could protect siRNA longer (24 h) when compared with the homopolymer.

The size and surface charge of the polyplexes were investigated by DLS. The diameter of the $P(AEAEMA)_{41}$-siRNA complexes was found to be lower than 110 nm at all N/P ratios tested. In contrast, $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA complexes (except the complex prepared at the N/P ratio of 2), displayed aggregation tendency. All polyplexes displayed positive zeta potential. The zeta potential of the homopolymer was found to be higher than the copolymer at the N/P ratio of 2.

Finally, in order to determine siRNA transfection ability of the polymers, luciferase assay was optimized using a commercial transfection reagent lipofectamine RNAimax. The optimized assay conditions will be used in future studies to determine the transfection efficiency of the polymers.

ÖZET

POTANSİYEL BİR siRNA TAŞIYICI AJAN OLARAK POLİ(2-((2-AMINOETİL)AMINO)ETİL METAKRILAT)'IN *In Vitro* DEĞERLENDİRİLMESİ

Bu tezin amacı poli(2-((2-aminoethil)amino)ethil metakrilat) (P(AEAEMA)) 'ın siRNA taşıma potansiyelinin belirlenmesidir.

Bu amaçla, amin içeren monomer 2-((tert-butoksikarbonil) (2-((tert-butoksikarbonil) amino) etil) amino) etil metakrilat (BocAEAEMA) sentezlendi. Daha sonra tersinir katılma ayrışma zincir transfer (RAFT) polimerizasyonu ile BocAEAEMA'nın homo ve blok kopolimer yapıları hazırlandı. Elde edilen polimerler $P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ ve $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$- GPC ve NMR ile karakterize edildi.

Polimerlerin sitotoksik etkileri Skov-3-luc yumurtalık kanseri hücre hattı kullanılarak MTT deneyi ile araştırıldı. MTT sonuçları polimerlerin 24 saat boyunca hücrelere toksik etkisinin olmadığını gösterdi.

Polimerlerin siRNA ile kompleks oluşturabilme yetenekleri jel elektroforezi ile araştırıldı. $P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ ve $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$'ın siRNA ile sırasıyla 5, 2 ve 2 N/P oranlarında etkin kompleksler oluşturabildiği anlaşıldı.

Jel elektroforezi analizleri $P(AEAEMA)_{41}$ ve $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$'ın siRNA'yı en az 6 saate kadar serum bileşenlerinden koruyabildiğini gösterdi. Blok kopolimerin homopolimere kıyasla siRNA'yı daha uzun sürelerde (24 saat) koruyabildiği tespit edildi.

Polipleksler boyutu ve yüzey yükleri DLS ile araştırıldı. Denenen bütün N/P oranlarında $P(AEAEMA)_{41}$-siRNA komplekslerinin 110 nm den küçük olduğu belirlendi. Buna karşın $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA kompleksleri (N/P oranı 2 olan kompleks hariç) agregasyon eğilimi gösterdi. Bütün polipleksler pozitif zeta potansiyele sahip olduğu tespit edildi.

Son olarak, polimerlerin siRNA'yı transfekte etme yeteneklerini belirleyebilmek için Lusiferaz Deneyi ticari bir transfeksiyon ajanı -lipofectamine RNAimax- kullanılarak optimize edildi. Bu deneylerden elde edilen bulgular, gelecek çalışmalarda polimerlerin transfeksiyon yeteneklerini belirlemek için kullanılmak üzere kaydedildi.

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CHAPTER 1

INTRODUCTION

The discovery of RNA interference (RNAi) mechanism and the introduction of small interfering RNAs (siRNAs) have led to enourmous progress in the field of gene therapy. The effective and specific gene silencing ability of siRNA molecules has made them valuable tools as potential therapeutic agents (Hannon, 2002; McManus & Sharp, 2002). However, clinical applications of siRNAs have been limited by several structural problems of siRNAs including poor in vivo stability and cellular uptake. (Zhang, Zhao, Jiang, Wang, & Ma, 2007). Various delivery systems have been developed and used to protect siRNA against degradation and carry it into the cell cytosol efficiently. Although some delivery agents have shown successful results at both *in vitro* and *in vivo* applications, still the need for effective and safe delivery systems is frequently reported (R. Kanasty, Dorkin, Vegas, & Anderson, 2013; Whitehead, Langer, & Anderson, 2009).

Most of the gene therapy studies use viral delivery systems due to their high efficiency however, these systems are considered to be toxic and immunogenic especially at repeated administrations (Merdan, Kopeček, & Kissel, 2002; D. Xu et al., 2005). In contrast to viral carriers, non-viral delivery systems draw attention in terms of safer carriers (Merdan et al., 2002).

Among non-viral delivery systems, cationic polymers stand out as carriers for both siRNA and plasmid DNA. Cationic polymers can be produced easily and inexpensively, and modified for various applications. They are capable of forming stable complexes with nucleic acids easily via electrostatic interactions and show higher transfection efficiency when compared with other non-viral delivery systems (Merdan et al., 2002; Wong, Pelet, & Putnam, 2007).

Recently, the importance of cationic diaminoethane motif in nucleic acid carriers has been highlighted in a number of publications (Jin et al., 2011; Miyata et al., 2008; Wagner, 2011). The pH-dependent protonation ability of this motif is known to provide high proton sponge capacity and membrane destabilization capability during endosomal acidification (Miyata et al., 2008; Wagner, 2011). The high transfection efficiency of

the carriers having diaminoethane motif has been attributed to its pH-responsive behaviour (Wagner, 2011).

In this thesis work, poly(2-((2-aminoethyl)amino)ethyl methacrylate) (P(AEAEMA)) a new polymer bearing diaminoethane motifs was evaluated as a potential siRNA carrier. For this aim, the monomer 2-((tert-butoxycarbonyl) (2-((tert-butoxy carbonyl) amino) ethyl) amino) ethyl methacrylate (BocAEAEMA) was synthesized according to the procedure reported by Kurtulus et al. (Kurtulus et al., 2014). Reversible addition-fragmentation chain transfer (RAFT) polymerization was performed to prepare homo- and block co-polymers of BocAEAEMA, namely P(BocAEAEMA) and P(PEGMA)-*b*-P(BocAEAEMA), respectively. The polymers were characterized by Nuclear Magnetic Spectroscopy (NMR) and Gel Permeation Chromatography (GPC). The ability of polymers to form polyplexes (polymer-siRNA complexes) and protect siRNA was determined via gel electrophoresis. The size and surface charge of the polyplexes were investigated by Dynamic Light Scattering (DLS). The effect of polymers on cell viability was determined via MTT assay using Skov-3-luc cell line. In order to determine siRNA transfection ability of the polymers in future, luciferase assay was optimized using a commercial transfection reagent lipofectamine RNAimax.

The literature review, materials and methods, results and discussion of this study are presented in Chapter 2, 3 and 4 respectively.

CHAPTER 2

LITERATURE REVIEW

2.1. Gene Silencing

Gene silencing is a set of techniques that leads to reduction in gene expression level. In order to decrease the gene expression -and as a result prevent the synthesis of protein of interest- in a specific manner, different strategies have been developed by scientists. Although these different strategies have different advantages and drawbacks, commonly their silencing mechanism is based on mRNA hybridization and they are all used to study gene function or to develop therapeutics (Elbashir et al., 2001; Elizabeth R Rayburn & Zhang, 2008).

One of the earliest approaches used in the field of gene silencing technology is 'antisense oligonucleotides' (P. C. Zamecnik & Stephenson, 1978). In the late 1970s, the discovery of inhibition of a specific gene product by using short complementary DNA sequences –antisense strategy- has led to intensive research and interest in the corresponding field (Geiser, 1990; Jens, 2003). Researchers have revealed two major mechanisms for the silencing effect of DNA oligos (Jens, 2003; E. R. Rayburn, Wang, & Zhang, 2006). One of the mechanisms is 'physical blockage' that involves the hybridization of single-stranded DNA oligos with complementary mRNA and results in inhibition of translation of the target protein via 'physical arrest'. The other mechanism is 'RNase-H activation' which also involves the hybridization of DNA with complementary mRNA sequences but this mechanism results in RNase-H dependent catalytic cleavage of mRNA. In either way, translation of target protein has been prevented by short oligonucleotide sequences (Jens, 2003; E. R. Rayburn et al., 2006). Although many preclinical and clinical trials have been made and much progress has been achieved with antisense oligonucleotides, there are still some hurdles like efficacy, side effect, off-target that must be overcome in order to develop effective therapeutics (Elizabeth R Rayburn & Zhang, 2008).

Another therapeutic tool that can be examined under the scope of gene silencing is 'ribozymes'. Ribozymes are catalytic RNA molecules that can specifically catalyse

the cleavage of RNAs in a similar way to enzymes (Bruce A. Sullenger & Thomas R. Cech, 1994). Ribozymes can be targeted to prevent various diseases such as cancer and infectious diseases (Bartolome, Castillo, & Carreno, 2004; Grassi, Dawson, Guarnieri, Kandolf, & Grassi, 2004; Bruce A Sullenger & Thomas R Cech, 1994). Because the structure is important for the function of ribozymes, their manipulation is limited (Elizabeth R Rayburn & Zhang, 2008).

RNA interference (RNAi) is a more recently discovered post-transcriptional gene silencing mechanism -transcriptional gene silencing by RNAi machinery has also been reported in the literature (Matzke & Birchler, 2005) (Figure 2.2a)-. It is a natural process which includes the specific inhibition of gene expression by double stranded RNA molecules via sequence-dependent hybridization with interest of mRNA (Hannon, 2002; McManus & Sharp, 2002). Since the discovery of this powerful mechanism, intense research activities have been carried out at the interface of molecular biology and material sciences, revealing that RNAi is a very powerful and promising tool to cure a wide variety of diseases including genetic and neurodegenerative diseases as well as cancer (Aigner, 2006; Elizabeth R Rayburn & Zhang, 2008; Whitehead et al., 2009).

2.1.1. RNAi

In 1998, Fire et al. showed that double stranded RNA (dsRNAs) molecules could cause gene silencing in a nematode called *C. elegans* (Fire et al., 1998). Afterwards, it was understood that the most of the eukaryotic organisms also have this mechanism (Aigner, 2006). This evolutionary conserved process acts against viral infections, transposons and also plays role in gene regulation (Hamilton & Baulcombe, 1999; Hammond, Boettcher, Caudy, Kobayashi, & Hannon, 2001; Tijsterman, Ketting, & Plasterk, 2002).

The mechanism of small interfering RNAs (siRNAs) mediated RNAi includes several biochemical reactions that begin with processing of cytoplasmic dsRNAs by a complex including TAR RNA-binding protein (TRBP), protein activator of protein kinase (PACT) and a ribonuclease called Dicer (Aigner, 2006; D. H. Kim & Rossi, 2007) (Figure 2.1 and Figure 2.2c). The cleavage of dsRNAs leads to the formation of short 21-23 nucleotide length double stranded RNA molecules called siRNA (Hammond, Bernstein, Beach, & Hannon, 2000). These siRNAs are loaded into a

complex called 'RNA-induced silencing complex' (RISC) which contains Argonaute 2 (Ago2) protein, a DEAD-box helicase (Gemin-3), protein of unknown function (Gemin-4) and dicer (Bernstein, Caudy, Hammond, & Hannon, 2001). One of the two strands – sense or passenger strand- of siRNA is cleaved by Ago2 and active RISC is formed (Matranga, Tomari, Shin, Bartel, & Zamore, 2005; Rand, Petersen, Du, & Wang, 2005). Other strand of siRNA –antisense or guide strand- guides the RISC to the target mRNA where the sequence-specific full complementary hybridization occurs between the antisense strand of siRNA and mRNA. The target mRNA is cleaved by the endonucleolytic activity of RISC and this causes the degradation of whole mRNA molecule from unprotected ends by cellular exonucleases (Orban & Izaurralde, 2005). RISC is recovered for other cycles of mRNA cleavage (Hutvágner & Zamore, 2002).

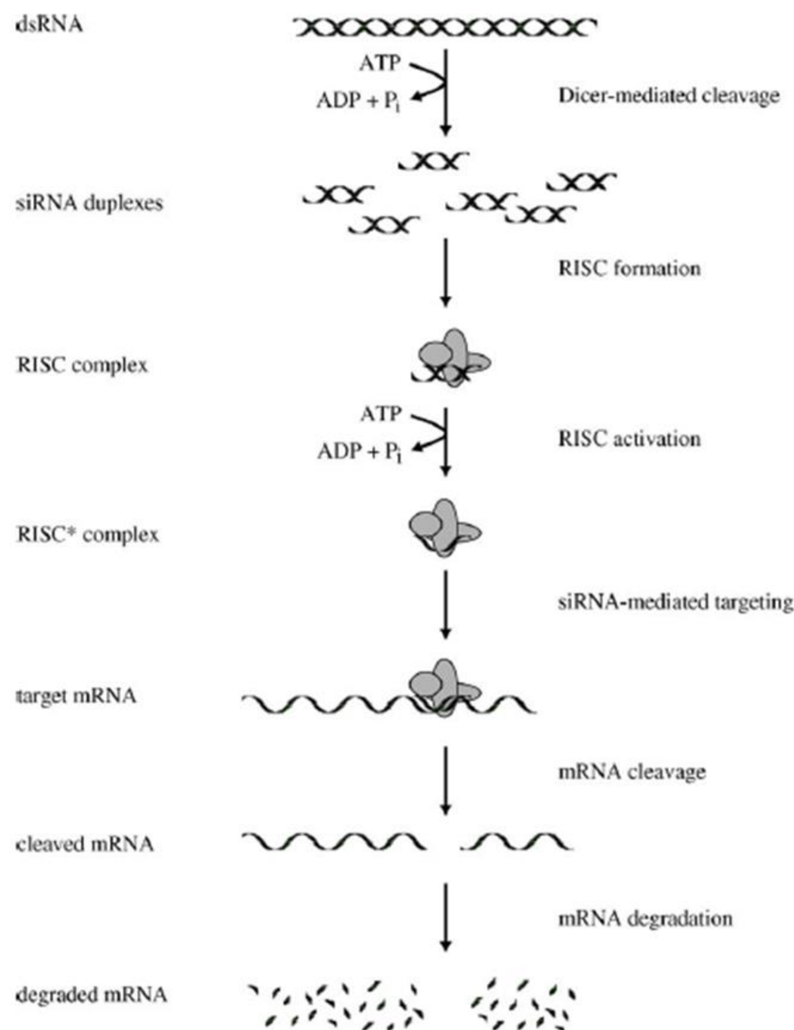

Figure 2.1. siRNA mediated RNAi mechanism in mammalian systems
(Source: Aigner, 2006)

Another member of RNAi is microRNAs (miRNAs). These are non-coding RNA molecules and they are responsible for the regulation of gene expression during development and differentiation (Bartel & Chen, 2004). Formation and action of miRNAs includes several steps (Figure 2.2b). First, primary microRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II (Pol II) from introns and exons (Y. Lee et al., 2004). Second, Drosha –an endonuclease- processes pri-miRNAs and shorter 'stem-loop structures' –precursor miRNAs (pre-miRNAs)- form (Y. Lee et al., 2003). These two steps take place in nucleus. After the second step, pre-miRNAs are transferred into the cytoplasm by exportin 5 (Lund, Güttinger, Calado, Dahlberg, & Kutay, 2004). The complex including Dicer–TRBP–PACT processes pre-miRNAs, leading to the formation of ~22 nt mature miRNAs (Cullen, 2004). These mature miRNAs are loaded into the RISC (Preall & Sontheimer, 2005). 3' untranslated region of target mRNA is recognized by the mature miRNA and the translation is inhibited via partial sequence complementarity (R. C. Lee, Feinbaum, & Ambros, 1993). Afterwards, target mRNA is degraded in P-bodies (Liu, Valencia-Sanchez, Hannon, & Parker, 2005). Unlike the siRNA loading pathway into RISC, the loading pathway of miRNAs into RISC does not include passenger strand cleavage (Preall & Sontheimer, 2005). Instead of cleavage, a helicase activity is used to unwind and remove the passenger strand (Preall & Sontheimer, 2005).

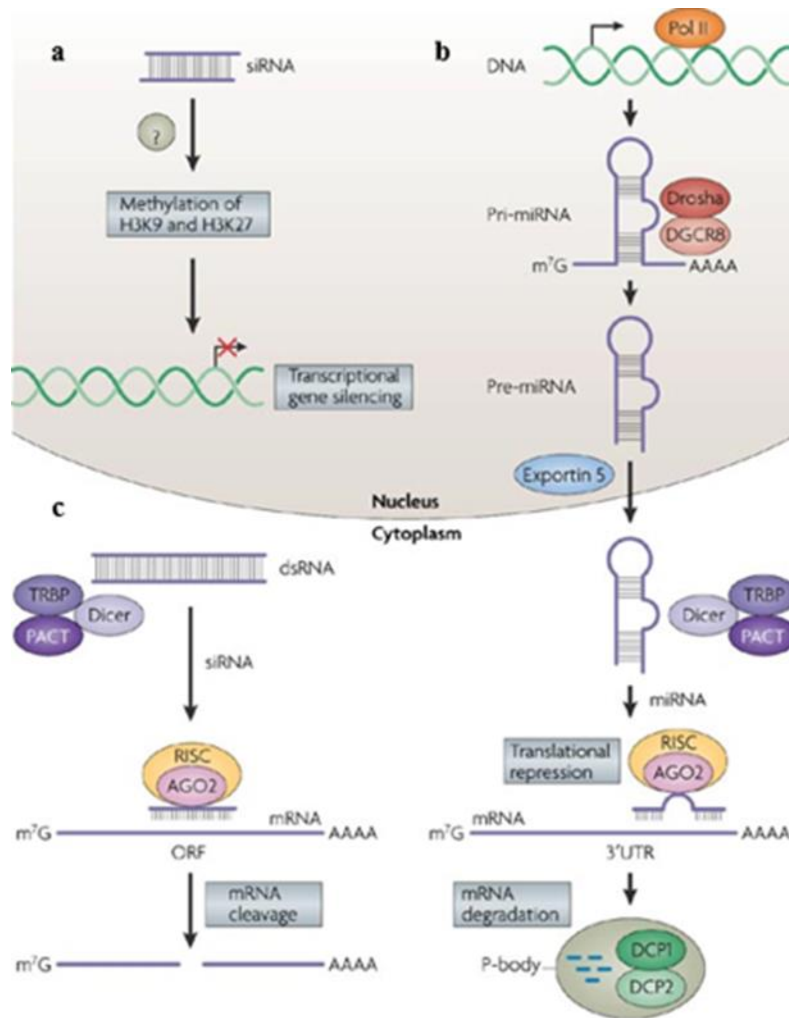

Figure 2.2. Mechanism of RNAi (a) transcriptional gene silencing mechanism of siRNA (b) miRNA mediated gene silencing (c) siRNA mediated gene silencing mechanism (Source: Kim and Rossi, 2007)

2.1.1.1. siRNA

siRNAs are typically ~21 nucleotide long, double stranded RNA molecules with 2 nucleotides overhanging at 3'-end (Figure 2.3). They are the mediator of RNAi pathway and can trigger the RNAi mechanism without processing by dicer (Elbashir et al., 2001). They are less immunogenic than long dsRNA molecules and can be generated by chemical synthesis (Elbashir et al., 2001). They can be produced easily in a cost-effective manner. Although pharmacokinetic properties of siRNA are convenient for the delivery to various organs (Braasch et al., 2004), their blood stability is poor, delivery in general is a big challenge and their gene silencing effect is transient (the gen

silencing duration in proliferating mammalian cells are reported between 3 and 7 days) (Leung & Whittaker, 2005; Yang, Tutton, Pierce, & Yoon, 2001).

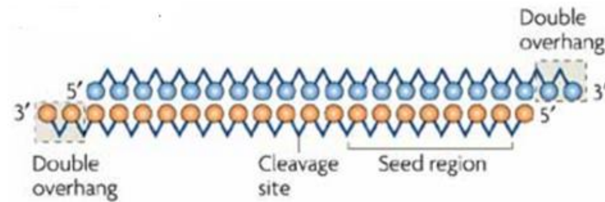

Figure 2.3. The structure of siRNA
(Source: Fougerolles et al., 2007)

Regarding the therapeutic application of siRNA, there are some limitations like off-targeting, saturation of endogenous RNAi pathway components and induction of immune responses (Aagaard & Rossi, 2007). It was reported that partial match (for ‘seed region’ as little as 6-7 nucleotide) between siRNA and mRNA was adequate for gene silencing and therefore one introduced siRNA sequence might affect the expression of many genes beside the target gene (off-target) (Lin et al., 2005). This non-specific silencing of genes may cause vital consequences (Birmingham et al., 2006).

Other than this, it is also known that bioactive drugs that use the natural pathways in living systems might obstruct ‘the natural processes’ via ‘saturation phenomena’ (Aagaard & Rossi, 2007). Exogenously introduced siRNAs and short hairpin RNAs (shRNAs) (longer RNA hairpin structures) follow the similar pathway with endogenous miRNAs and they occupy RNAi machinery (D. H. Kim & Rossi, 2007). It is known that this ‘exogenous’ occupation might prevent the ‘natural process’ and this may cause to morbidity (Aagaard & Rossi, 2007).

Grimm et al. reported the dose-dependent liver damage and death at high amounts of shRNA expression in mice (Grimm et al., 2006). The authors showed that the reduced expression of liver specific miRNA caused to the morbidity. This was attributed to the saturation of miRNA pathway (Grimm et al., 2006).

Another problem associated with the application of siRNAs is induction of immune response. It is stated in the literature that although siRNAs are less immunogenic than long dsRNAs, they can be detected by toll-like receptors and as a result, an interferon response may be induced (Seth, Sun, & Chen, 2006). Moreover it is

also reported that cellular helicase RIG-1 can also detect siRNA and results in ‘interferon-independent immune response’ (Grimm, 2009).

Although the problems mentioned above limit the therapeutic application of siRNA, it is often stated in the literature that, rational design of siRNA sequences, usage of appropriate doses and development of new approaches in siRNA design and delivery can help to overcome these limitations (Aagaard & Rossi, 2007; D. H. Kim & Rossi, 2007; Sibley, Seow, & Wood, 2010).

Especially in recent years, significant progress has been made in application of siRNA therapeutics and it is understood that among the other gene silencing strategies, siRNA therapy has huge potential (Aagaard & Rossi, 2007; Khan et al., 2004; Elizabeth R Rayburn & Zhang, 2008).

Zimmermann et al. showed in their study that the anti-ApoB siRNAs can safely be delivered in therapeutic doses using liposomes in Chimpanzees (Zimmermann et al., 2006). Their work proved the potential of safe and effective siRNA delivery in primates (Zimmermann et al., 2006).

Schwarz et al. reported that mutant alleles of SOD1 and the huntingtin (HTT) genes which are related to amyotrophic lateral sclerosis (ALS) and Huntington disease respectively, can be selectively treated with siRNAs (Schwarz et al., 2006). Their findings showed that rationally designed siRNA therapeutics can distinguish single nucleotide polymorphisms and selectively silence the mutant alleles without perturbing the wild-type (Schwarz et al., 2006).

Hu-Lieskovan et al. developed a non-viral siRNA carrier system against metastatic Ewing’s sarcoma (Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005). Their work showed that transferrin containing cyclodextrin nanoparticles can effectively deliver siRNA to metastatic Ewing's sarcoma cells and the systemic delivery of siRNA complex can inhibit the tumour growth selectively (Hu-Lieskovan et al., 2005).

2.2. siRNA Delivery

It is well-known that the biggest struggle in the development of siRNA based gene silencing therapies is the delivery of these molecules (Aagaard & Rossi, 2007). Even though some tissues such as lung and eye are convenient for the delivery of ‘naked’ or chemically modified siRNA therapeutics, it is often reported in the literature

that additional systems for efficient delivery of siRNAs to other tissues are required (DiFiglia et al., 2007; Whitehead et al., 2009).

2.2.1. Delivery Challenges

siRNA can be administered to a living system in two ways: via localized delivery and systemic delivery (Whitehead et al., 2009). Direct application of siRNA -localized delivery- provides proximity of therapeutic to the target tissue leading to higher bioavailability. Also this type of administration results in reduced adverse effects compared to intravenous injection –systemic delivery- (Whitehead et al., 2009).

Localized therapy is suitable for treatments in some tissues like eye, skin and lungs. Bitko et al. have reported that intranasally applied siRNAs against respiratory syncytial virus (RSV) in mouse can inhibit the replication of RSV (Bitko, Musiyenko, Shulyayeva, & Barik, 2005). Also, DeVincenzo et al. have shown in their study that when the siRNA showing anti-RSV activity (ALN-RSV01) have administered intranasally to humans, no adverse effects have been reported different than placebo –normal saline- treatments (DeVincenzo et al., 2008). They have stated that ALN-RSV01 is well-tolerated and can safely be used in clinical applications (DeVincenzo et al., 2008).

Although intravenous administration is necessary for many other tissues and organs, this type of administration requires to overcome many additional obstacles (Whitehead et al., 2009). When systemic delivery is the subject; serum nuclease stability, immune system induction, aggregation with serum components and other non-spesific interactions, renal clearance, cell uptake, endosomal escape and triggering of RNAi mechanism are the important steps and issues that must be considered (Alexis, Pridgen, Molnar, & Farokhzad, 2008; R. L. Kanasty, Whitehead, Vegas, & Anderson, 2012; Whitehead et al., 2009) .

When naked siRNA is introduced into the bloodstream, it faces with the risk of degradation by serum nucleases (Layzer et al., 2004). One widely used approach against this challenge includes the chemical modification of the siRNA. For this aim, fluorine or methoxy groups are usually incorporated into ribose sugar to obtain 2'-fluoro and 2'-O-methyl groups respectively or phosphodiester bonds at the backbone of the siRNA are substituted with phosphorothioate (Deleavey & Damha, 2012) . It is reported that

this kind of modifications also can prevent the induction of immune response and off-target effects besides providing protection against nucleases (Jackson & Linsley, 2010; Nguyen et al., 2012). Other than chemical modifications, delivery systems are also used to overcome the degradation problem (A. Z. Wang, Langer, & Farokhzad, 2012).

Aggregation with serum components is another problem especially when cationic delivery systems are utilized. The positive charges at the surface of the carrier particles can cause undesired aggregation with red blood cells (Malek et al., 2009). Also delivery systems can be ‘labelled’ via serum opsonin proteins and afterwards removed by mononuclear phagocyte system (MPS) (Alexis et al., 2008). It is reported that adsorbed proteins on the surface of delivery particles raise the risk of opsonization and subsequent aggregation (Owens & Peppas, 2006). Following the aggregation, the phagocytic cells such as macrophages and monocytes causes the rapid clearance of the delivery systems without allowing them to show any therapeutic effect (Whitehead et al., 2009). A very well-known strategy ‘PEGylation’ is widely utilized to overcome these challenges. Highly hydrophilic polyethylene glycol (PEG) reduces the interaction with serum proteins and phagocytic cells via ‘shielding effect’ and it increases the circulation time of therapeutics (Alexis et al., 2008; Bazile et al., 1995).

Once naked siRNA is administered into the bloodstream, it encounters ‘renal clearance’ challenge. Naked siRNA can rapidly be removed from the bloodstream by kidneys without showing any therapeutic effect. (Bumcrot, Manoharan, Koteliansky, & Sah, 2006). It is well-known that while smaller molecules (typically <6 nm) rapidly pass through the glomerulus and accumulate in urine, larger molecules (>8 nm) are retained in circulation (Jarad & Miner, 2009; Longmire, Choyke, & Kobayashi, 2008). Santel et al. have administrated the naked siRNA in mice and showed the accumulation in urine within 5 minutes after injection and this is attributed to ‘size related’ excretion of naked siRNAs (Santel et al., 2006). It is often reported in the literature that when delivery systems are used, renal clearance of siRNAs is avoided (H. Lee et al., 2012). However Zuckerman et al. stated that if the assembly of siRNA and delivery system is due to electrostatic interactions, the complex can disassemble at the kidney glomerular basement membrane and this may result in the rapid elimination of siRNA from circulation (Zuckerman, Choi, Han, & Davis, 2012).

In order to reach the target tissues, delivery systems should leave the bloodstream across the endothelium (R. Kanasty et al., 2013). It is mentioned that if the diameter of a molecule is larger than 5 nm, it cannot easily cross the capillary

endothelium therefore it stays in circulation until it is eliminated from the body (Whitehead et al., 2009). However some tissues such as liver, spleen and many tumours which have discontinuous endothelia allow the diffusion of molecules up to 200 nm in diameter (R. L. Kanasty et al., 2012; Wisse, Jacobs, Topal, Frederik, & De Geest, 2008).

Extracellular matrix which consists of fibrous proteins and polysaccharides serves as another biological barrier. Upon leaving the bloodstream, siRNA delivery particles must diffuse through this dense network. It is reported that during this diffusion process, delivery systems can be taken up by macrophages and delivery process may be slow or even terminated (J. Zamecnik, Vargova, Homola, Kodet, & Sykova, 2004).

Cellular uptake is another challenging step in siRNA delivery. Due to its' negative charge, large size (~ 13 kDa) and highly hydrophilic nature; siRNA therapeutics show poor cellular uptake (Zhang et al., 2007). Additionally, it is well known that the internalization mechanism for most siRNA delivery systems is endocytosis (R. Kanasty et al., 2013). In accordance with this mechanism, the delivery system is internalized in a vesicle which fuses with early endosomes. The pH inside the vesicle begins to drop as the vesicle matures into late endosomes (R. Kanasty et al., 2013). If the delivery system cannot escape from the endosome, it is exposed to lysosome where highly degradative conditions exist (Di Guglielmo, Le Roy, Goodfellow, & Wrana, 2003). Different strategies have been developed to increase the efficacy of cell uptake and endosomal escape (Rozema et al., 2007; Semple et al., 2010). One strategy to overcome the poor cell uptake problem of siRNAs is the incorporation of various targeting ligands to trigger receptor-mediated endocytosis (Yu, Zhao, Lee, & Lee, 2009). To overcome the endosomal escape problem cell penetrating peptides have been used in formulations to increase the cytoplasmic release of siRNAs (Bolhassani, 2011). Inclusion of pH-selective membrane disruptive groups to delivery systems is also another method that facilitates endosomal escape (Rozema et al., 2007; Shim & Kwon, 2012).

Lastly, the released siRNA in cytosol should trigger RNAi mechanism (R. Kanasty et al., 2013; Whitehead et al., 2009).

2.2.2. Delivery Systems

To overcome the obstacles that prevent the clinical applications of siRNA therapeutics various delivery systems have been developed. Although some promising delivery systems have been successfully introduced, the need for safe and effective delivery system is still reported to be a major goal in siRNA therapy (R. Kanasty et al., 2013; Whitehead et al., 2009).

Many viral and non-viral delivery systems have been developed for gene delivery applications. Although viral vectors have been more efficient and can provide long-term gene silencing, they have been considered as toxic and immunogenic (Merdan et al., 2002; D. Xu et al., 2005). Because of this safety concerns, gene therapy applications of non-viral vectors have gained importance (Merdan et al., 2002).

Lipid based systems, cationic polymers, and various siRNA conjugates have been widely used as synthetic non-viral siRNA carriers (R. Kanasty et al., 2013). Although the vectors that have been used in plasmid DNA delivery have received attention in siRNA delivery applications, it has been reported that the short and rigid structure of siRNA might prevent the direct usage of vectors designed for DNA delivery (De Smedt, Demeester, & Hennink, 2000). Therefore, development of carriers specific to siRNA has been stated to be necessary for success in siRNA treatment strategy. (Spagnou, Miller, & Keller, 2004).

Liposomes -lipid bilayer spherical structures - are one of the successfully used delivery vehicles in drug delivery applications (Torchilin, 2005). It is reported in the literature that liposomes can protect oligonucleotides against nucleases, avoid renal clearance, facilitate cellular uptake and endosomal escape which make them highly attractive in the delivery of DNA based drugs (Y. Xu & Szoka, 1996). In addition to DNA delivery applications, liposomes have also been used as siRNA carriers in various *in vitro* and *in vi*vo studies (Zhang et al., 2007).

Intravenously injected siRNA incorporated DOTAP liposomes have been shown to inhibit the expression of green fluorescent protein (GFP) in mice. Moreover they have also reported that lipopolysaccharide-induced tumor necrosis factor α (TNF-α) gene expression was inhibited, when anti-TNF-α siRNA-DOTAP complexes were injected intraperitoneally (Sørensen, Leirdal, & Sioud, 2003).

Galactosylated cationic liposomes-siRNA complexes have been intravenously administrated in mice. Selective delivery of siRNA to liver parenchymal cells (PC) have resulted in 80% knock-down in the interest of gene when the dose of the complex was 0.29 nmol/g (Sato, Takagi, Shimamoto, Kawakami, & Hashida, 2007).

'Stable nucleic acid-lipid particles (SNALPs)' which are composed of lipid bilayer encapsulating siRNA have been also introduced (Morrissey et al., 2005). The non-toxic and effective gene silencing studies with SNALPs have been reported in several studies by various groups (Geisbert et al., 2006; Zimmermann et al., 2006).

Although many successful results have been obtained with liposomal carriers, liposomes have been reported to enhance the interferon response, change the cell metabolism and moreover show toxic effects (Barreau, Dutertre, Paillard, & Osborne, 2006; Ma et al., 2005).

Cylodextrin polymers have been used successfully in siRNA delivery by various groups. They have been tested in clinical trials in a short time after their introduction (Davis et al., 2010; R. Kanasty et al., 2013).

Hu-Lieskovan et al have developed cylodextrin nanoparticles that include a targeting ligand transferrin and an siRNA specific to EWS–FLI1 gene which is associated with Ewing's sarcoma. They have shown the inhibition at tumor growth in a mouse model of metastatic Ewing's sarcoma (Hu-Lieskovan et al., 2005).

Various conjugates (polymers, peptides, antibodies, aptamers, lipids) of siRNA have been reported by different groups (Jeong, Mok, Oh, & Park, 2008; Rozema et al., 2007; Wolfrum et al., 2007) as a siRNA delivery tool.

Soutschek et al. have reported for the first time that cholesterol conjugated siRNA could silence the gene encoding ApoB when intravenously administrated in mice (Soutschek et al., 2004). The authors have shown that cholesterol groups increased the stability of siRNA and the plasma level of ApoB protein decreased with administration of the conjugate (Soutschek et al., 2004).

Cell penetrating peptides (CPPs) are another class of carriers that have been used for delivery of macromolecular therapeutics such as proteins, plasmid and antisense DNA (Meade & Dowdy, 2007). Their ability to pass directly from the cell membrane bypassing the endocytotic pathway made them attractive as delivery agents (J. Wang, Lu, Wientjes, & Au, 2010). Muratovska et al. have prepared siRNA-CPPs (penetratin and transportan) conjugates via disulphide bonds and shown that compared

to liposome carriers these siRNA-CPPs showed similar or better knock down in the corresponding genes in various cell lines (Muratovska & Eccles, 2004).

2.2.2.1. Cationic Polymers

Cationic polymers are one of the most widely used nucleic acid carriers. They can be produced easily and inexpensively. They can form complexes with nucleic acids via simple electrostatic interactions. Moreover they can be tailored and modified for specific applications (Merdan et al., 2002). All of these features of cationic polymers have made them attractive in gene delivery applications. However it is necessary to improve their transfection efficiency and lower toxicity (Merdan et al., 2002).

Due to their positive charges, cationic polymers can form 'polyplexes' with negatively charged nucleic acids via electrostatic interactions (Agarwal, Unfer, & Mallapragada, 2005). As a result of this complexation, siRNA could be protected against nuclease degradation. Depending on the polmer/siRNA amount, polyplexes may show different size and charge properties and as a result different cell uptake and pharmacokinetic profiles (J. Wang et al., 2010). Also some polyplexes may induce nonspecific endocytosis and endosomal escape (Akhtar & Benter, 2007).

The endosomal escape mechanism for certain cationic polymers has been suggested as 'proton sponge effect' (Figure 2.4) (Boussif et al., 1995). If the polymer has groups protonable between pH 5 and 7, it behaves as a 'proton sponge' during endosomal acidification (Merdan et al., 2002). The buffering effect of the polymer causes the increased influx of protons into the vesicle to reach the desired pH. To maintain electroneutrality, as a counter ion the influx of chloride ions also increases (Wong et al., 2007). Subsequently water diffuses into the vesicle due to increased osmotic pressure. Eventually, the vesicle swells and disintegrates (Merdan et al., 2002). Although the proton sponge effect has been accepted as a major mechanism for endosomal escape of certain cationic polymers, the contrary findings have been reported by some authors (Benjaminsen, Mattebjerg, Henriksen, Moghimi, & Andresen, 2013).

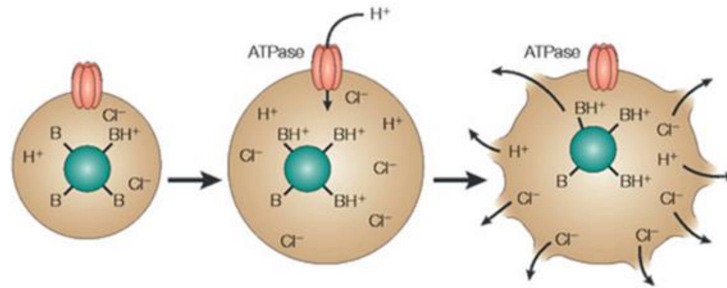

Figure 2.4. Proton sponge hypothesis
(Source: Pack et al., 2005)

Among cationic polymers polyethylenimine (PEI) has been known as ‘gold standard’ in gene delivery (Figure 2.5) (Merdan et al., 2002). Different molecular weights of linear (LPEI) and branched PEI (BPEI) has been investigated *in vitro* and *in vivo* (W. J. Kim & Kim, 2009). It is reported that PEI can condense large DNA molecules and form stable complexes (Godbey, Barry, Saggau, Wu, & Mikos, 2000; Marschall, Malik, & Larin, 1999). It is also known that high buffering capacity of PEI provides efficient endosomal escape via possible ‘proton sponge effect’ (Boussif et al., 1995). All of these properties make PEI relatively effective transfection reagent (Marschall et al., 1999). However the toxicity of PEI especially at high molecular weights has been often reported as a major drawback (Kichler, 2004; Kircheis, Wightman, & Wagner, 2001; Merdan et al., 2002).

PEI has been investigated also as a siRNA delivery agent both *in vitro* and *in vivo*. Recently low molecular weight or lineer PEI has been introduced as a less toxic delivery agent (Hassani et al., 2005; Niola et al., 2006; Thomas et al., 2005).

Werth et al. have fractionated 25kDa BPEI and obtained low molecular weight PEI (PEI F25-LMW) (pooled fractions: 4kDa-10kDa) with gel permeation chromatography. They have reported that their PEI F25-LMW can effectively form complexes with siRNA and protect it against nuclease degradation. Moreover superior transfection efficiency and low toxicity has been obtained with PEI F25-LMW (Werth et al., 2006).

Urban-Klein et al. have reported that when the siRNA complexes formed with commercially available linear low molecular weight PEI (JetPEI) (22kDa) have been administrated intraperitonally in mouse, tumor growth has been reduced via the ‘siRNA

mediated downregulation' of HER-2 (Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner, 2005) .

Grayson et al. have evaluated different PEI structures (BPEI of 25 kDa and 800 Da molecular weights, LPEI of 22 kDa molecular weight) for *in vitro* siRNA delivery and they have reported that the successful delivery was only obtained with BPEI 25kDa in certain conditions (Grayson, Doody, & Putnam, 2006).

Besides PEI, many other cationic polymers have been tested in siRNA delivery. PAMAM (polyamidoamine) dendrimers, chitosan, poly(TETA/CBA) and some PEI derivatives, are a few reported examples which have shown varying efficacies in siRNA delivery (Figure 2.5) (Breunig et al., 2008; Howard et al., 2006; Jeong et al., 2007; Patil et al., 2008).

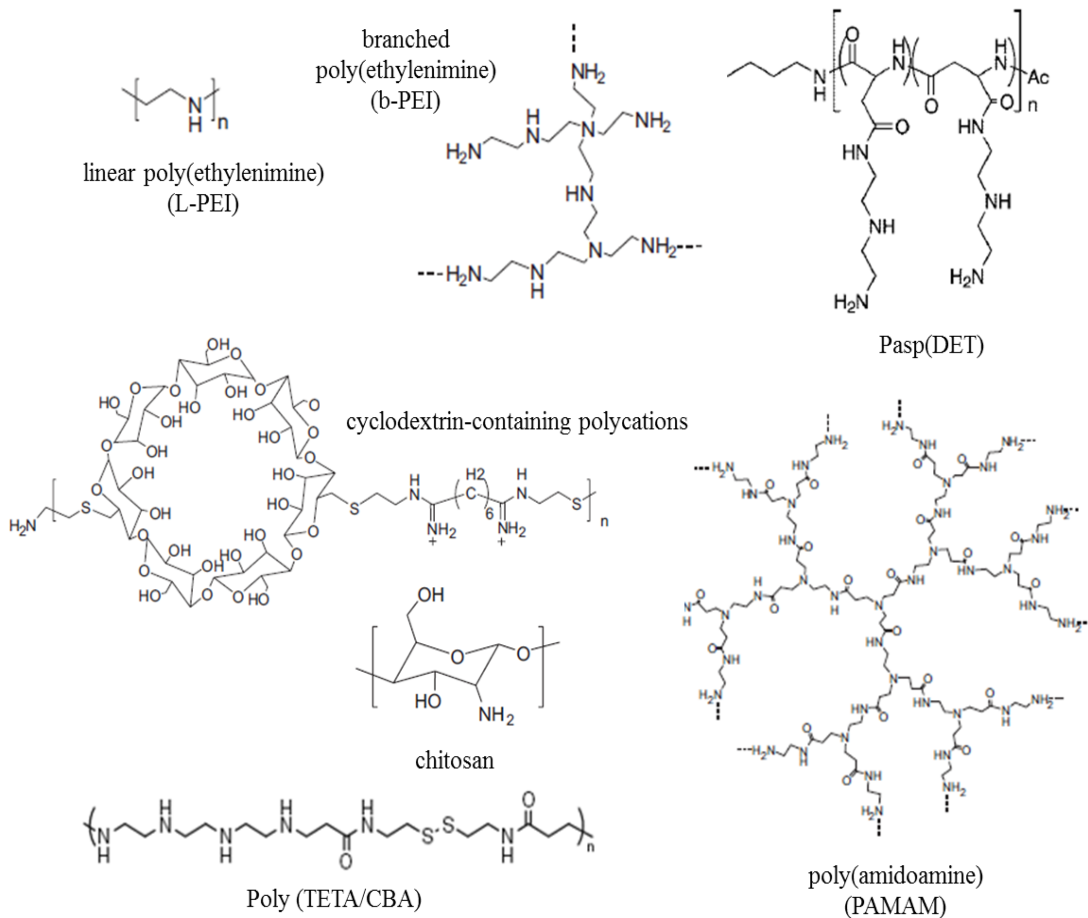

Figure 2.5. Some cationic polymers used as nucleic acid carriers
(Source: Wong et al., 2007; Wagner, 2011)

2.2.2.1.1. Diaminoethane Motif

It has been reported that diaminoethane motif in carrier systems can increase the transfection efficiency due to pH-dependent membrane destabilization activity (Wagner, 2011). While diaminoethane motif is partially protonated at physiological pH, once it is at acidic pH inside the endosomes it becomes fully protonated (Miyata et al., 2008) (Figure 2.6). This pH-dependent protonation provides high endosomal proton sponge capacity and membrane destabilization capability during endosomal acidification (Miyata et al., 2008). The high transfection efficiency of PEI and some other transfection reagents has been attributed to this 'unique' behaviour of the motif (Wagner, 2011).

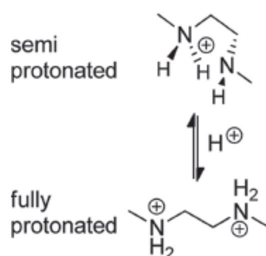

Figure 2.6. Two-step protonation of diaminoethane motif
(Source: Wagner, 2011)

Kataoka and his group have reported the *in vitro* and *in vivo* applications of poly(N-(N-(2-aminoethyl)-2-aminoethyl)aspartamide) [PAsp(DET)] (Figure 2.5) structure in their several studies (Hayakawa et al., 2015; Itaka et al., 2007; Kanayama et al., 2006; H. J. Kim et al., 2010; Miyata et al., 2008). This poly(aspartamide) derivative bearing diaminoethane motif as a pendant group have shown highly promising results (Hayakawa et al., 2015; Miyata et al., 2008).

Kanayama et al. have prepared PEG-*b*-P[Asp(DET)] block polymer and reported the pDNA transfection and cytotoxicity results (Kanayama et al., 2006). They have shown the high transfection efficiency of the polymers without toxicity in several cancer cells and mouse primary osteoblast cells (Kanayama et al., 2006).

Miyata et al. have evaluated and compared the physicochemical and biological properties of PAsp(DET) and as a control PAsp(DPT) (bearing 1,3-diaminopropane as a side chain) (Miyata et al., 2008). They have shown that PAsp(DET) in contrast to

PAsp(DPT) is much less toxic and more efficient at pDNA transfection. There was no significant difference in particle size and zeta-potential between the polyplexes formed from two different polymers. However hemolysis and lactate dehydrogenase (LDH) assays revealed that PAsp(DPT) strongly destabilizes the membranes due to high toxicity while PAsp(DET) destabilizes the membrane minimally in physiological pH (Miyata et al., 2008).

Itaka et al. have used PEG-b-P[Asp(DET)]/pDNA polyplex micelles to introduce osteogenic factor-expressing genes in a bone defect mouse model (Itaka et al., 2007). They have reported important bone formation on the surface of the implant without inflammation(Itaka et al., 2007). They have stated that the results represents the first *in vivo* successful application of polyplex nanomicelles in gene transfer (Itaka et al., 2007).

Kim et al. have evaluated the gene silencing ability of stearic acid conjugated PAsp(DET) (H. J. Kim et al., 2010). They have shown that the hydrophobic stearoyl moiety increases the stability of the PAsp(DET)/siRNA complex and it facilities the cellular internalization (H. J. Kim et al., 2010). They have reported that stearic acid conjugated PAsp(DET) shows more efficient gene knockdown than commercially available reagents and these structures are promising candidates for in vivo applications of siRNA (H. J. Kim et al., 2010).

Yuan et al. have synthesized a triblock copolymer containing poly(2-(2-aminoethyl amino) ethyl methacrylate) (P(AEAEMA)) block as diaminoethane motif (Yuan, Li, Ma, Cheng, & Zhuo, 2011). They have reported that the length of P(AEAEMA) has no significant effect on DNA-binding capacity and also size and zeta potential properties however, the buffering capacity was correlated with the chain length of P(AEAEMA) (Yuan et al., 2011). They also showed that the polymers showed better transfection efficiency and lower toxicity when compared with 25 kDa BPEI in the presence of serum (Yuan et al., 2011).

Salcher et al. have prepared sequence-defined four-arms structures consisting of different repeats of artificial amino acids containing 1,2-diaminoethane motif (Salcher et al., 2012). They have evaluated the complex formation and transfection efficiency of the structures with plasmid DNA and siRNA (Salcher et al., 2012). The results indicated that these structures could show up to 50-fold better transfection efficiency than LPEI (Salcher et al., 2012).

Jin et al. have introduced PAM-DET structure which consists of PAMAM dendrimers with diaminoethane groups on the surface (Jin et al., 2011). They have proved the high buffering capacity and pH-dependent membrane destabilization activity of PAM-DET with LDH assay and titration (Jin et al., 2011). They have shown that the superior transfection ability of PAM-DET is highly related to the presence of diaminoethane groups on the surface (Jin et al., 2011).

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

RAFT polymerization, a living radical polymerization technique, has been reported for the first time in 1998. This technique uses chain transfer agents with thiocarbonylthio groups. By using RAFT polymerization polymers with controlled molecular weight and low polydispersity index (PDI <1.2) can be obtained (Bulmus, 2011; Moad, Rizzardo, & Thang, 2009). The technique is suitable for various monomers. Polymers with different molecular structures and functional ends can be obtained in mild conditions (Moad et al., 2009). It is stated that the RAFT polymerization is one of the most appropriate polymerization technique for synthesis of well-defined vinyl polymers for biological applications (Boyer, Bulmus, et al., 2009; Bulmus, 2011; Moad et al., 2009).

Many polymers having various structures and compositions have been synthesized via RAFT polymerization for the delivery of nucleic acids. Nelson et al. have prepared pH-responsive diblock copolymers from a PEGylated macro-chain transfer agent [poly[(ethylene glycol)-*b*-[(2-(dimethylamino)ethyl methacrylate)-*co*-(butyl methacrylate)] [PEG-(DMAEMA-*co*-BMA)] using RAFT polymerization technique. They have evaluated siRNA delivery potential of the polymers and reported that all the polymers with varied compositions of BMA could be synthesized with low PDI (≤ 1.1) via RAFT polymerization (Nelson et al., 2013).

Duvall et al. have synthesized diblock copolymer composed of N-(2-hydroxypropyl) methacrylamide (HPMA) as first block and equimolar quantities of DMAEMA, propylacrylic acid (PAA) and butyl methacrylate (BMA) as second block (poly[HPMA]-b-[(PAA)(BMA)(DMAEMA)]). Pyridyl disulfide end-functionalized

polymers with controlled molecular weight (Mn) and low PDI were obtained via RAFT polymerization (Duvall, Convertine, Benoit, Hoffman, & Stayton, 2010).

York et al. have prepared a folat conjugated block copolymer via aqueous RAFT polymerization for siRNA delivery. For this aim N-(2- hydroxypropyl)methacrylamide) (HPMA) has been copolymerized with N-(3-aminopropyl)methacrylamide (APMA) and the obtained polymer chain extended with a cationic block poly(N-[3-(dimethylamino)propyl]methacrylamide). The polymers with narrow PDI values have been obtained and used for the targeted delivery studies of siRNA (York et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

N-Hydroxyethylethylenediamine (99% purity), di-tert-butyl dicarbonate and methacryloyl chloride were purchased from Aldrich for synthesis of 2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino)ethyl)amino)ethyl methacrylate (BocAEAEMA). For polymerizations, chain transfer agent, 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) was purchased from Aldrich. The initiator 2,2′-Azobis(2-methylpropionitrile) (AIBN) was used after recrystallization twice in methanol.

Linear polyethyleneimine (PEI; Mn: 8 kDa) and poly(ethylene glycol) methyl ether methacrylate (PEGMA; Mw:467 g/mol) were purchased from Sigma-Aldrich. Aluminum oxide (Geduran® Al 90) for column chromatography was obtained from Merck. Silica gel (pore size 60 Å, 70-230 mesh) was purchased from Fluka. Toluene, ethyl acetate, hexane, dichloromethane (DCM), trifluoroacetic acid, deuterium oxide (D_2O), deuterium chloroform ($CDCl_3$), triethylamine (TEA), hexylamine, diethylether, methanol and N'N-dimethylacetamide (DMAc, HPCL grade ≥ %99.9) were purchased from Sigma. Dialysis membrane (MWCO= 500-1000 Da) was purchased from Spectrum® Laboratories.

DMEM (Dulbecco's Modified Eagle's Medium) and L-glutamine were purchased from Lonza. FBS (Fetal Bovine Serum) was purchased from Sigma. Pen-strep, non-essential amino acids and Trypsin-EDTA were obtained from Biological Industries. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS) were purchased from Sigma. Skov-3-Luc cell line was kindly provided by Prof. Dr. Sacide ALTINKAYA. Luciferase assay kit was purchased from Promega. Commercial transfection reagent Lipofectamine RNAimax was purchased from Invitrogen. Standard desalted siRNA (sense: 5'GAUUAUGUCCGGUUAUGUA-UU3'; antisense: 5'UACAUAACCGGACAUAAUC-UU 3') was purchased from IDT DNA. Agarose and

ethidium bromide were purchased from Sigma. 6X loading dye solution and DNA markers (100bp and 1kb) were purchased from Fermantas.

3.2. Instruments

3.2.1. Gel Permeation Chromatography

The molecular weight and molecular weight distribution of polymers synthesized throughout the study were 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 (410-67000 g/mol) was used. The mobile phase was DMAc containing 0,05 % w/v lithium bromide.

3.2.2. Nuclear Magnetic Resonance Spectroscopy

^{1}H 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 (D_2O), chloroform ($CDCl_3$) and dimethyl sulfoxide ($(CD_3)_2SO$) were used as NMR solvents. For NMR analysis, samples were dissolved at 10 mg/ml concentration in deuterium oxide (D_2O), chloroform ($CDCl_3$) or dimethyl sulfoxide ($(CD_3)_2SO$) depending on the solubility of the samples.

3.2.3. Dynamic Light Scattering and Zeta Potential Analysis

The hydrodynamic diameter and surface charge of the polyplexes were determined using a NanoPlus DLS Nano Particle Size and Zeta Potential Analyzer (measurement range for size: 0.1 nm to 12.30 µm for zeta -500 to +500 mV; laser source: diode laser; laser wavelength: 660 nm; laser power: dual laser 30 mW + 70 mW).

Dynamic Light Scattering (DLS) measurements were performed after an equilibration time of 120 s. 2 x 70 runs were carried out at 25 °C. The counts were detected at an angle of 165 °. Each measurement was performed in duplicate.

Electrophoretic Light Scattering was used to determine surface charge (ζ-potential) of the particles. Smoluchowski equation was used to calculate zeta potential from the mobility. 5 runs were carried out for each measurements. Each experiment was performed in triplicate at 25 °C.

3.2.4. Microplate Reader

Thermo Electron Corporation Varioskan microplate reader was used to measure absorbance and luminescence for MTT and luciferase assays, respectively.

3.2.5. Agarose Gel Electrophoresis

The electrostatic complex formation ability of polymers with siRNA was investigated via agarose gel electrophoresis using a Thermo Scientific Owl™ EasyCast™ B1 mini gel system.

3.3. Methods

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

The diaminoethane motif containing monomer 2-((tert-butoxycarbonyl) (2-((tert-butoxy carbonyl) amino) ethyl) amino) ethyl methacrylate (BocAEAEMA) was synthesized according to the procedure reported by Kurtulus et al. (Figure 3.1) (Kurtulus et al., 2014). First, the amine groups of the starting compound N-(2-hydroxyethyl)ethylenediamine were protected according to the procedure reported by Moura et al. (Moura et al., 2006). Briefly, N-(2-hydroxyethyl)ethylenediamine (0.024 mol) and di-*tert*-butyl dicarbonate (0.048 mol) was separately dissolved in dry DCM (40 ml) at -10 °C. Di-tert-butyl dicarbonate solution was added dropwise into N-(2-

hydroxyethyl)ethylenediamine solution at -10 °C. The solution was purged with nitrogen for 3 h and stirred for 24 h at room temperature under nitrogen atmosphere. After the reaction, the precipitate was removed by filtration, Water-DCM extraction was performed to remove unreacted starting compound. Tert-butyl-2-(((tert-butoxycarbonyl)amino)ethyl)(2-hydroxyethyl)carbamate (BocAEAE) was obtained after collecting the organic phase and evaporating the solvent using a rotary evaporator.

At the second step, BocAEAE was reacted with methacryloyl chloride to obtain methacrylate derivative of tert-butyl-2-(((tert-butoxycarbonyl)amino)ethyl)(2-hydroxyethyl)carbamate (BocAEAEMA). Briefly, BocAEAE (0.0154 mol) was dissolved in dry DCM at 0 °C. Under nitrogen, triethylamine (0.043 mol) was added dropwise to the solution and the solution was allowed to stir for 30 minutes. Afterwards methacryloyl chloride (0.029 mol) was dropped into the solution and the final solution was stirred at 0 °C for 4 h under nitrogen. The solution was further stirred for 15 h at room temperature. The final product was purified by filtration, extraction and hexane-ethylacetate silica gel column chromatography as reported elsewhere (Kurtulus et al., 2014). Additionally basic alumina chromatography was performed using hexane and ethyl acetate at a volume ratio of 1:1 to remove remaining methacrylic acid. The purified monomer was characterized by ^{1}H-NMR spectroscopy.

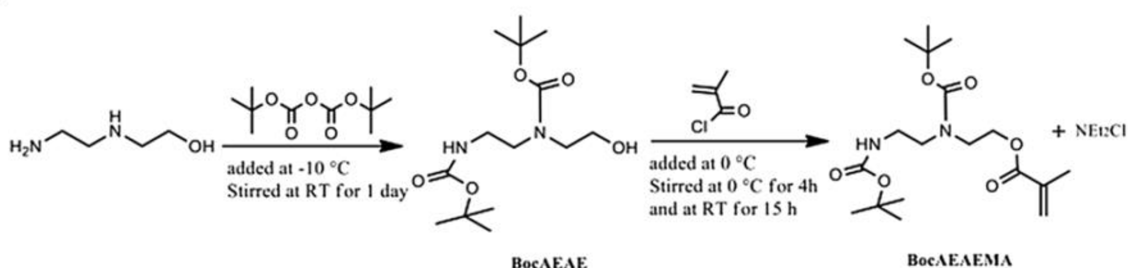

Figure 3.1. The scheme of BocAEAEMA synthesis.
(Source: Kurtulus et al., 2014)

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

BocAEAEMA was polymerized via a living radical polymerization technique namely RAFT polymerization (Figure 3.2). Briefly, corresponding amounts of monomer (BocAEAEMA), RAFT agent (CPADB) and initiator (AIBN) were dissolved

in toluene. The solution was purged with nitrogen for at least 15 minutes then immersed in an oil bath at 65 °C. At the end of the polymerization time, the reaction solution was cooled in an ice bath and exposed to air. Polymers were purified by precipitating the polymerization solution in hexane at least three times. The number average molecular weight (M_n) and molecular weight distribution (PDI) of the polymers were determined by GPC. Monomer conversions were determined by ^{1}H-NMR spectroscopy.

The conditions used for RAFT polymerization of BocAEAEMA throughout the study are given in Table 3.1.

Table 3.1. Reaction conditions for the polymerizations of BocAEAEMA. *(P(BocAEAEMA)[19] was obtained after fractionation of the polymer that was synthesized according to the condition given in this table).

Polymers	[M] (M)	[M]/[R]/[I]	Time (h)
P(BocAEAEMA)$_{41}$	1.2	100/ 1/ 0.25	16
***P(BocAEAEMA)$_{19}$**	0.7	100/ 1/ 0.25	7

After polymerizations, (*tert*-butyloxycarbonyl) (Boc) groups were removed from the polymers by using trifluoroacetic acid (TFA) according to the procedure reported by Kurtulus et al. (Kurtulus et al., 2014). Briefly; polymer (4.35 μmol) was dissolved in DCM (1 ml) and TFA (0.5 ml) was added into the solutions at 0 °C. The solution was allowed to stir for 30 min at room temperature. After the solvent was evaporated, the reaction mixture was washed with chloroform and diethyl ether. The deprotected polymer P(AEAEMA) was dried in vacuum oven and characterized by ^{1}H-NMR spectroscopy in DMSO.

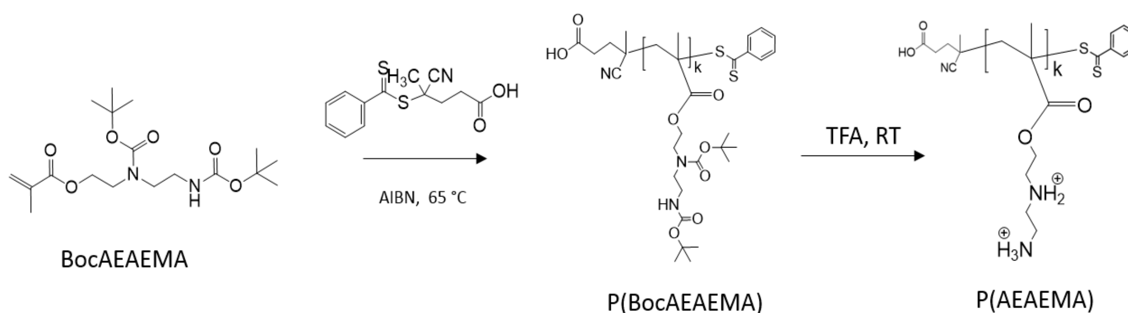

Figure 3.2. The scheme of P(AEAEMA) synthesis.

Thiocarbonylthio RAFT-end group of P(AEAEMA) was also removed from the polymer in order to prevent possible cytotoxic effects of the group by following a well-known protocol reported in the literature (Boyer, Granville, Davis, & Bulmus, 2009). Briefly, P(AEAEMA) was reacted with methyl methacrylate (MMA) in the presence of hexylamine (HEA) and triethylamine (TEA) for 3 hours under nitrogen atmosphere at room temperature ([P(AEAEMA)]/ [HEA]/ [TEA]/ [MMA] = 1/ 10/ 10/ 3). The polymer was precipitated in diethyl ether and further purified by dialysis against water (MWCO 1000 Da). The polymer was dried using freeze-dryer and characterized by ^{1}H-NMR spectroscopy in D_2O.

3.3.3. RAFT Copolymerization of Poly(ethylene glycol) Methyl Ether Methacrylate and 2-((Tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl Methacrylate

To synthesize poly(poly(ethylene glycol) methyl ether methacrylate)-*b*-poly(2-((tert-butoxycarbonyl) (2-((tert-butoxycarbonyl) amino) ethyl) amino) ethyl methacrylate) block copolymer (P(PEGMA)-*b*-P(BocAEAEMA)); first, poly(ethylene glycol) methyl ether methacrylate (PEGMA) was polymerized by RAFT polymerization and the obtained polymer P(PEGMA) was used as a macroRAFT agent for copolymerization (Figure 3.3). Briefly; PEGMA (M_n: 467 g/mol, repeating unit: 7-8), CPADB and AIBN were dissolved in acetonitrile ([PEGMA] = 1 M and ([PEGMA]/ [CPADB]/ [AIBN] ratio was 50/ 1/ 0.25). The reaction solution was purged with nitrogen, then immersed into an oil bath at 65 °C and polymerized for 100 min. After the polymerization was terminated, the reaction medium was purified by dialysis (MWCO 3500 Da) against water.

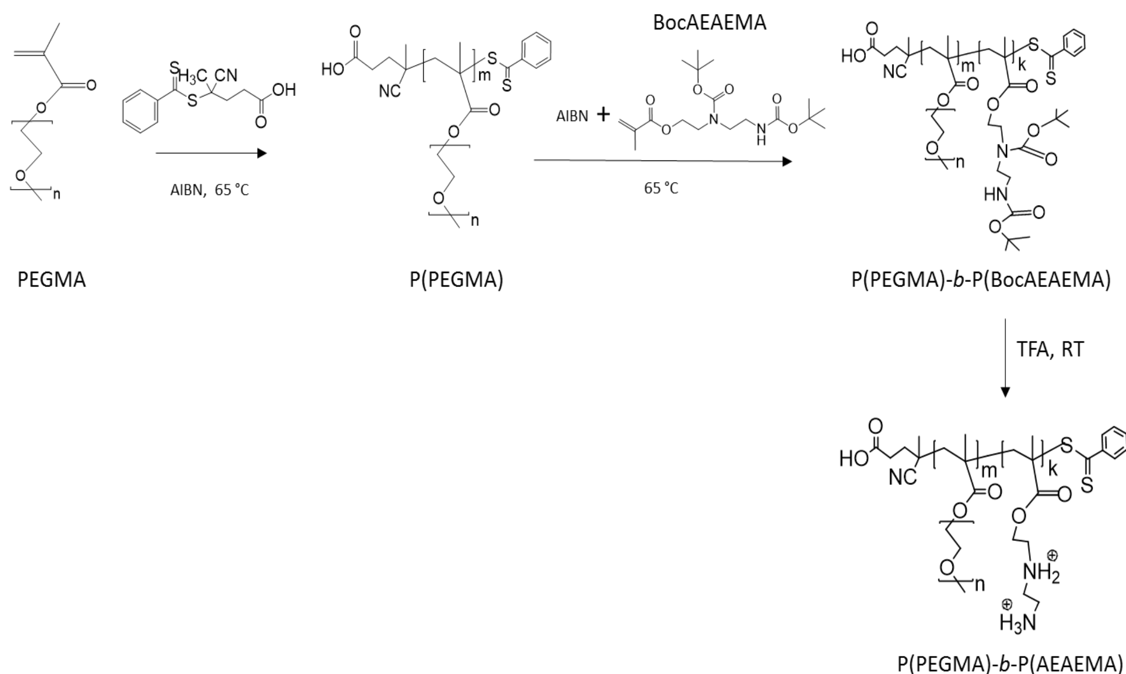

Figure 3.3. The scheme of P(PEGMA)-b-P(AEAEMA) synthesis.

The purified P(PEGMA) was used as a macro chain transfer agent. BocAEAEMA, P(PEGMA), AIBN were dissolved in acetonitrile ([BocAEAEMA] = 1 M and ([BocAEAEMA]/ [P(PEGMA)]/ [AIBN] ratio was 100/ 1/ 0.25) The mixture was purged with nitrogen, then immersed into oil bath at 65 °C and polymerized for 16 h. After polymerization was stopped, the copolymer was purified by precipitating in hexane. The block copolymer P(PEGMA)-*b*-P(BocAEAEMA) was further characterized by ^{1}H-NMR and GPC analyses.

The Boc groups of P(BocAEAEMA) block and the thiocarbonylthio end of P(PEGMA)-*b*-P(AEAEMA) was removed in a similar way described in Section 3.3.2.

3.3.4. Polyplex Formation and Characterization

3.3.4.1. Determination of Polyplex Formation by Agarose Gel Electrophoresis

The siRNA binding ability of P(PEGMA)-*b*-P(AEAEMA) and P(AEAEMA) with different molecular weights were investigated by agarose gel electrophoresis.

The polymers were dissolved in 10 mM phosphate buffer at pH 6 to yield a polymer stock solution of 10^{-1} mM. Separately, siRNA was dissolved in RNase-free water at a concentration of 10 µM. Corresponding amounts of polymers were added to 0.02 nmol siRNA to obtain complexes at varying nitrogen/phosphate (N/P) ratios (1, 2, 3, 4, 5, 10 and 30). The siRNA-polymer solutions were incubated at room temperature for 15 minutes. The solutions were then mixed with 6X loading dye and loaded into %3 agarose gel stained with 0.5 µg/ml ethidium bromide. The gel was run at 100 V for 25 minutes in 1X TAE running buffer and analyzed with UV illumination.

3.3.4.2. Investigation of Serum Stability of the Polyplexes

The siRNA protection ability of the polymers against serum components was investigated by agarose gel electrophoresis.

The polyplexes were prepared at an N/P of 2 or 10 as described above and incubated with equal volume of fetal bovine serum at 37 °C for predetermined times (30 min, 2 h, 4 h, 6 h and 24 h). At the end of the corresponding incubation time the aliquots were taken and immediately treated with 0.5 M EDTA to stop the degradation. The aliquots were frozen and stored at -80 °C until the analysis. SDS (% 2) was added in equal volume to the polyplex solution in order to displace the siRNA from the complex and the final solution (20 µl, 0.04 nmol siRNA) was analyzed by % 3 agarose gel electrophoresis. The gel was run at 100 V for 25 min.

0.5 M EDTA treated serum (inactivated serum) was used in control experiments. The serum stability of naked siRNA was also determined by applying the same procedure.

3.3.4.3. Determination of Size and Surface Charge of the Polyplexes

The particle size and zeta potential of the polyplexes were determined by NanoPlus DLS Nano Particle Size and Zeta Potential Analyzer.

The polyplexes at different N/P ratios (2, 5, 10, 30, 50) were prepared as described in Section 3.3.4.1. The siRNA concentration in this case was 4 µM. The hydrodynamic volume of the polyplexes was measured in phosphate buffer at pH 6.0 using quartz micro volume cell (~ 30 µl). The zeta potential value of the same

polyplexes was also determined using micro volume disposable cell (~130 µl) using the same instrument.

The diameter of the polyplexes prepared at N/P ratio of 2 was also determined after incubating the complexes in DMEM with 10% FBS for 15 min, 2h, 4h and 24h.

The results are expressed as mean ± standard deviation.

3.3.5. Cell Culture Experiments

Stably firefly luciferase expressing Skov-3-luc human ovary cancer cell line was cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM MEM Non-Essential Amino Acids, 2 mM L-glutamine and 1% penicillin-streptomycin at 37 ºC and 5% carbon dioxide in a humidified atmosphere. Subculture was performed when the cells had ~ 90% confluency. All experiments have been performed within the passage range of 3-10.

3.3.5.1. Determination the Effect of Polymers on Cell Viability via MTT Assay

The effect of the polymers on cell viability was determined via MTT assay. This colorimetric method relies on the detection of insoluble formazan which is formed with the conversion of soluble MTT by actively respiring cells (Mosmann, 1983).

One day prior to sample exposure, the human ovary cancer cell line Skov-3-luc cells were seeded at a density of 10^4 cells/well in a 96 well plate. The polymer stock solutions were prepared in PBS at predetermined concentrations. 5 µl of polymer stocks in PBS were added to the wells in triplicate. In positive control experiments 5 µl of PBS only was added into the wells. The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere for 24 or 72 h. After the incubation period, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution prepared in PBS at a concentration of 5 mg/ml was added to wells by adjusting the final concentration of dye to be 10% (v/v). The cells were further incubated for 4 h in dark and humidified atmosphere at 37 °C. Afterwards the plates were centrifuged at 1800 rpm for 10 minutes. The supernatants were removed by tapping gently and the formazan

crystals were dissolved in DMSO. The absorbance at 570 and 690 nm was determined using a microplate reader.

3.3.5.2. Optimization Studies of the Luciferase Assay

In order to determine the transfection ability of the polymers synthesized in this study the luciferase assay was optimized using Promega luciferase assay kit. To perform optimization experiments, a commercial transfection reagent lipofectamine RNAimax was used according to the manufacturer's protocol. An siRNA sequence synthesized against firefly luciferase gene was used to selectively inhibit the expression of luciferase gene (J.-S. Lee et al., 2009). The luciferase expression level in stably luciferase expressing Skov-3-luc cell line was evaluated using a luminescence microplate reader.

To determine the appropriate incubation time with complexes, Skov-3-luc cells were seeded in a 96 well-plate at a density of 15 x 10^3 cells/well. After 24 h incubation at 37 °C 5% CO_2, the medium was removed and fresh complete medium (90 µl) was added to wells. The cells were transfected with 10 µl of lipofectamine RNAimax-siRNA complexes at an siRNA dose of 100 nM. Samples were tested in duplicate. As a control, 10 µl of DMEM was added into wells. Cells were incubated with complexes for 4h (plus further 20 h incubation with fresh medium -without complexes-) or 24 h at 37 °C in an atmosphere containing 5% CO_2. After the incubation period, the medium was removed and cells were rinsed with 1X PBS. After removing PBS, 20 µl of 1X lysis reagent was dispensed into the wells and the cells were incubated with the lysis reagent for 10 min. The luminescence in each well was recorded during 10 sec upon introducing 100 µl luciferase assay reagent.

To determine the optimum siRNA dose and the total assay time, the procedure described above was followed using three different siRNA doses (10, 50 and 100 nM). The complexes were incubated with the cells for 24 h. The results were obtained either directly at the end of the incubation time or after an additional 24 h incubation with fresh medium –without complexes- (total 48 h incubation). The effect of the complexes on cell viability was also investigated via MTT assay. To do this, the complexes were incubated with cells for 24 h and the method described in Section 3.3.5.1 was performed.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Synthesis and Characterization of BocAEAEMA

The procedure reported by Kurtulus et al. was followed to synthesize the diaminoethane motif containing monomer, BocAEAEMA (Kurtulus et al., 2014).

In the first step of the synthesis, the amine groups of the starting compound *N*-hydroxyethylethylenediamine were protected using Boc anhydride. The signals of the Boc groups were observed at 1.42-1.47 ppm in the ^{1}H-NMR spectrum of BocAEAEMA (Figure 4.1).

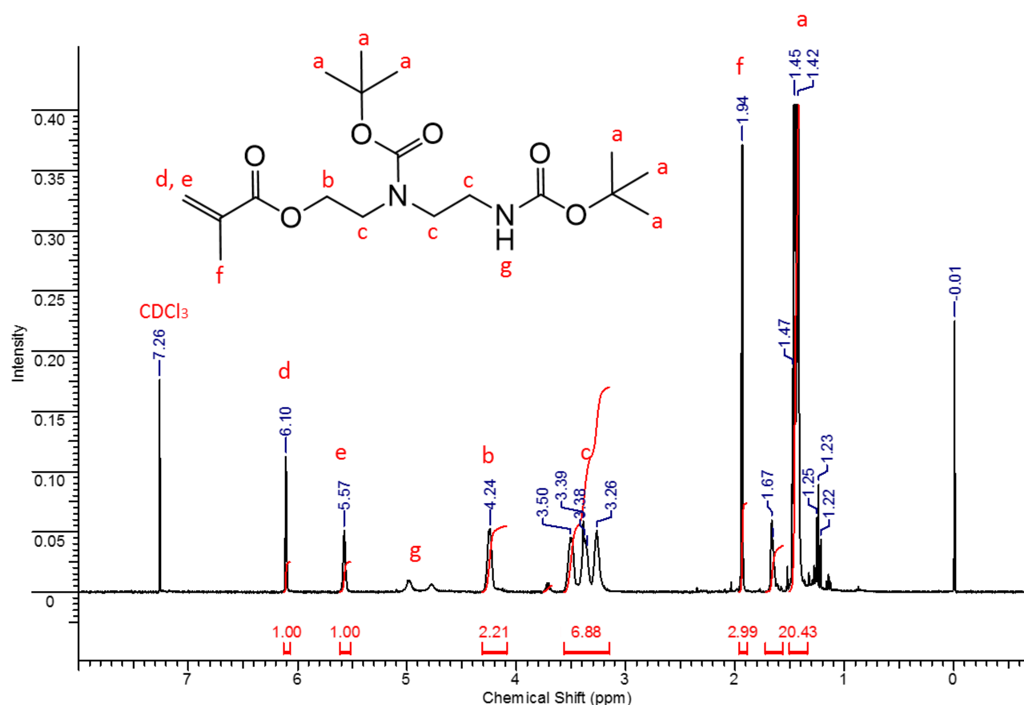

Figure 4.1. ^{1}H-NMR spectrum of pure BocAEAEMA

In the second step of the synthesis, Boc protected product (BocAEAE) was reacted with methacryloyl chloride to obtain the final methacrylate monomer BocAEAEMA. The signals of the vinyl protons were observed at 6.10, 5.57, and 1.94

ppm in the ^{1}H-NMR spectrum of the methacrylate monomer obtained after purification via column chromatography (Figure 4.1). The shift (from 3.71 to 4.24 ppm) in the signal of the protons of -CH_2- adjacent to OH group in BocAEAE was observed upon the formation of the ester bond upon methacrylation.

4.2. Synthesis and Characterization of P(AEAEMA)

The synthesized monomer BocAEAEMA was polymerized via RAFT polymerization. CPADB and AIBN were used as RAFT agent and initiator, respectively. The polymers were characterized by NMR and GPC analysis (Figure 4.2 and 4.3).

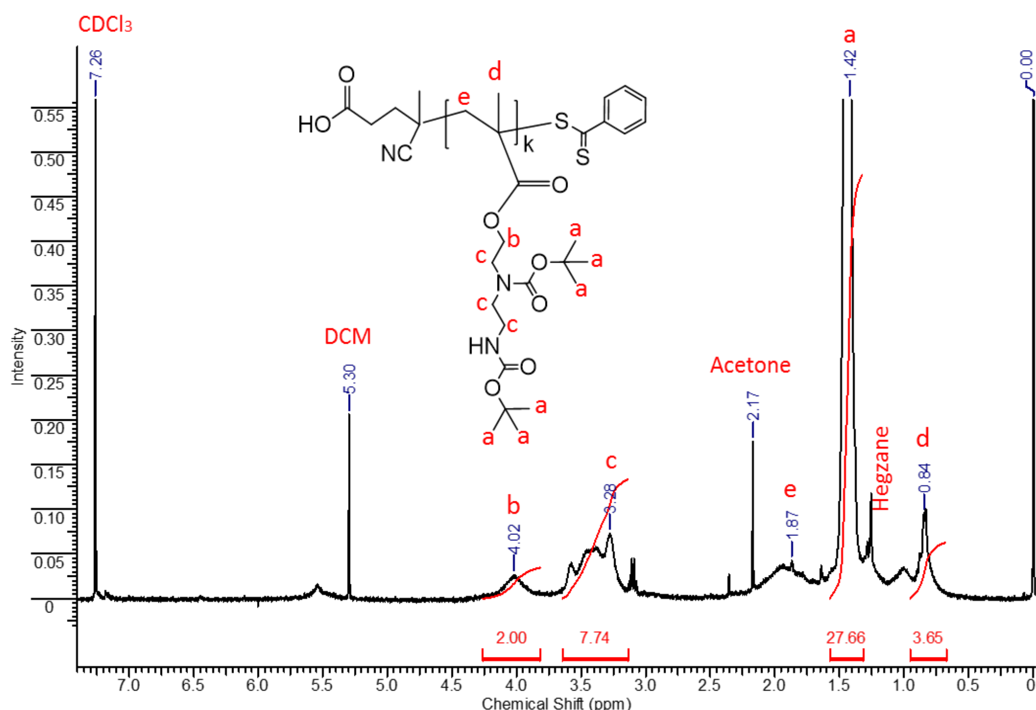

Figure 4.2. Representative ^{1}H-NMR spectrum of purified P(BocAEAEMA)

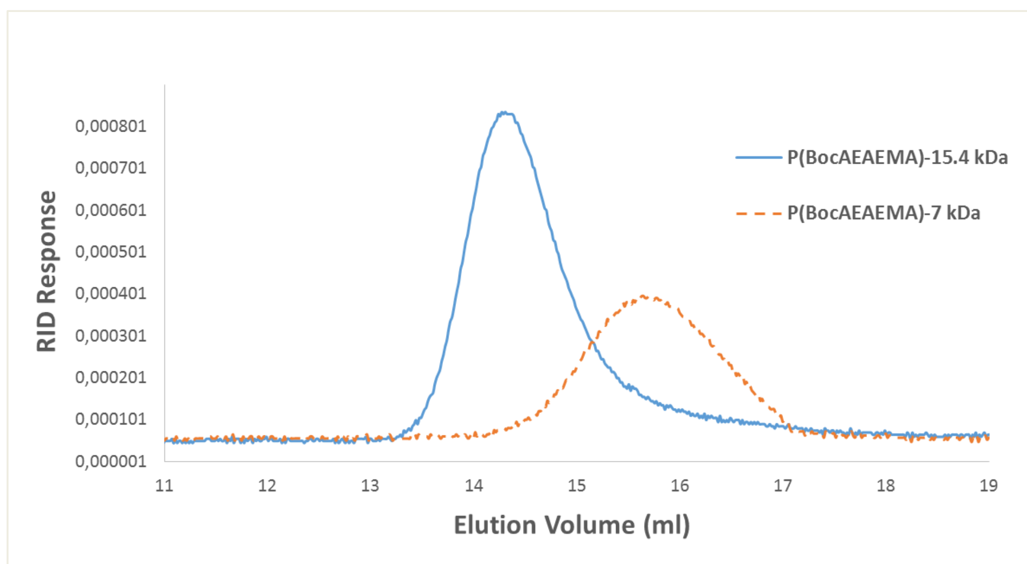

Figure 4.3. GPC chromatogram of P(BocAEAEMA)s

Obtained polymers were deprotected and aminolyzed to yield poly(2-((2-aminoethyl)amino)ethyl methacrylate) P(AEAEMA). Deprotection of amino groups of P(AEAEMA) was verified by ^{1}H-NMR spectroscopy (Figure 4.4). The molecular weight of the polymers after deprotection was calculated theoretically assuming all Boc groups were removed.

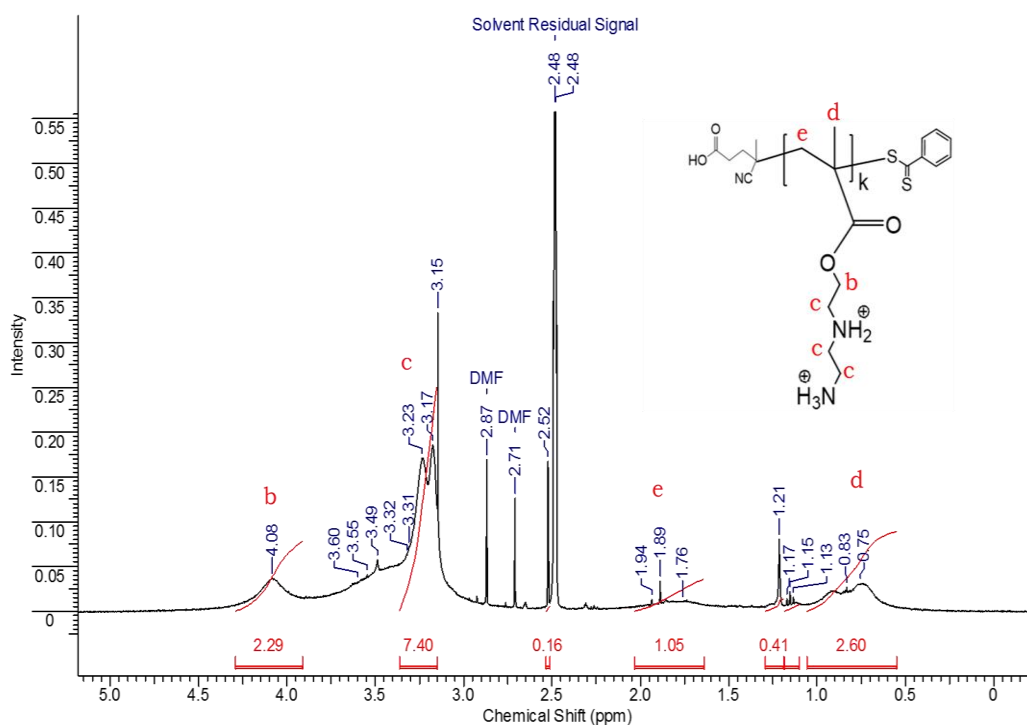

Figure 4.4. Representative ^{1}H-NMR spectrum of P(AEAEMA)

The molecular weight before and after deprotection, polydispersity indices (PDI) and degree of polymerizations (DP) of the two polymers used throughout this study are given in Table 4.1.

Table 4.1. The properties of the homopolymers synthesized and used in this study. (M_n^{GPC}: number average molecular weight determined by GPC, Mn_{theo}: number average molecular weight calculated theoretically assuming all Boc groups were removed, DP: average degree of polymerization calculated from GPC, PDI: polydispersity indices determined by GPC)

Polymer No	**M_n^{GPC} (before deprotection)**	**Mn_{theo} (after deprotection)**	**DP**	**PDI**
1	7000 Da	3500 Da	19	1,23
2	15400 Da	7700 Da	41	1,40

4.3. Synthesis and Characterization of P(PEGMA)-b-P(AEAEMA)

The block copolymer P(PEGMA)-b-P(BocAEAEMA) was synthesized to potentially improve the compatibility of the polycationic carrier with the biological system. It was intended to shield the cationic charge and potentially improve the stability and toxicity of the cationic polymer by incorporating a neutral, hydrophilic and non-immunogenic polymer block. The properties and efficiency of the block copolymer was compared with those of homopolymers throughout the study.

First, PEGMA (Mn: 467 g/mol, repeating unit: 7-8) was polymerized via RAFT polymerization. Poly(PEGMA) was then used as a macro chain transfer agent for synthesis of P(PEGMA)-b-P(BocAEAEMA). The obtained polymers were characterized by NMR and GPC analysis (Figure 4.5 and 4.6).

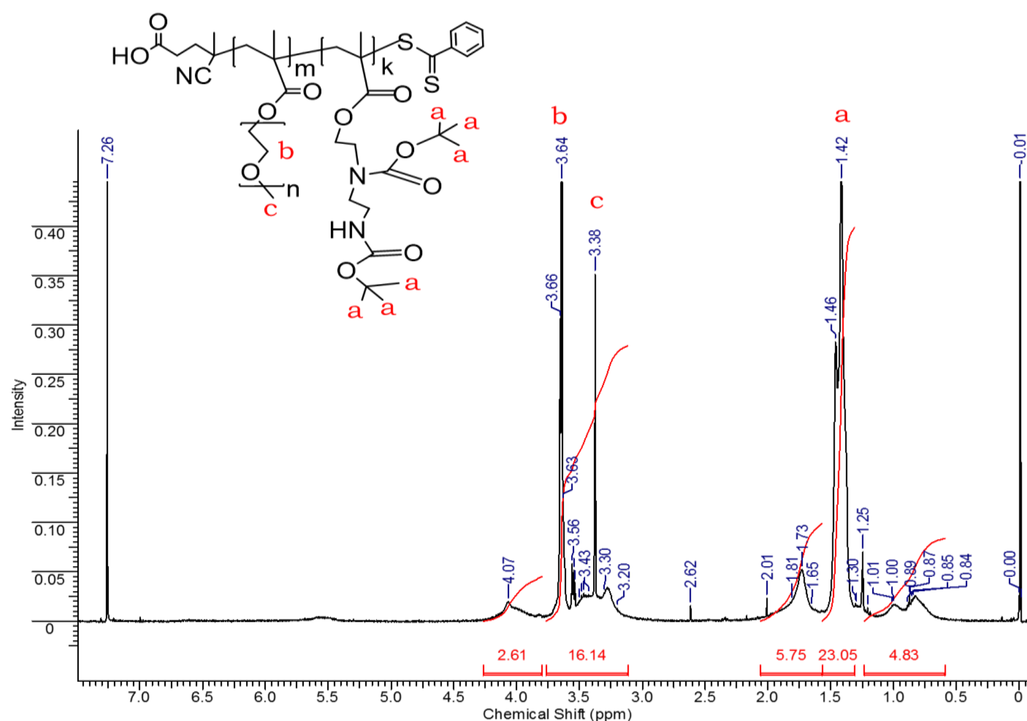

Figure 4.5. ^{1}H-NMR spectrum of purified P(PEGMA)-b-P(BocAEAEMA)

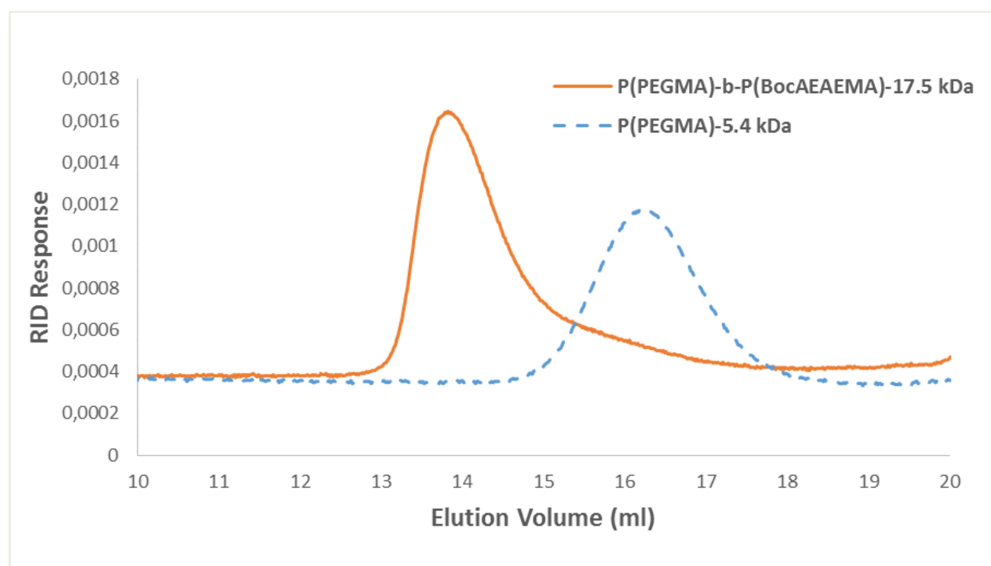

Figure 4.6. GPC chromatogram of P(PEGMA) and P(PEGMA)-b-P(BocAEAEMA)

The block copolymer was deprotected and aminolyzed to yield P(PEGMA)-*b*-P(AEAEMA) (Figure 4.7). The molecular weight of the polymer after deprotection was calculated theoretically assuming that all Boc groups were removed. The specifications

of the P(PEGMA) macro chain transfer agent and the block copolymer are given in Table 4.2.

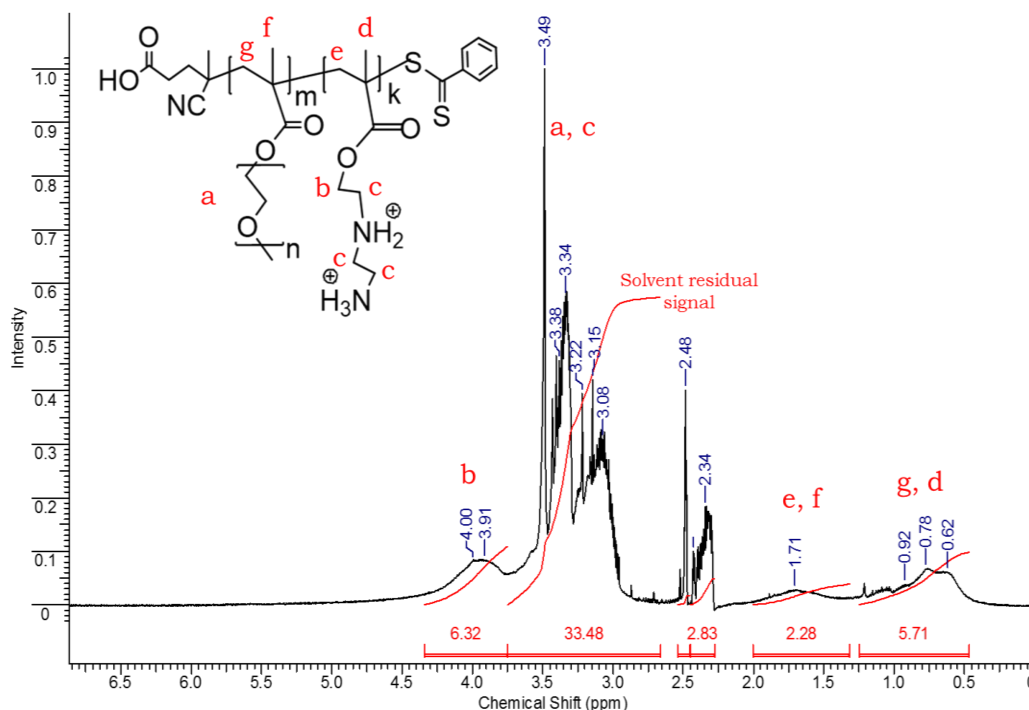

Figure 4.7. ^{1}H-NMR spectrum of P(PEGMA)-*b*-P(AEAEMA)

Table 4.2. The properties of the macroCTA and P(PEGMA)-b-P(BocAEAEMA) (Mn^{GPC}: number average molecular weight determined by GPC, Mn_{theo}: number average molecular weight calculated theoretically assuming all Boc groups were removed, DP: average degree of polymerization, PDI: polydispersity indices)

Polymers	M_n^{GPC} (Da)	Mn_{theo} (af dep.) (Da)	DP_{PEGMA}	$DP_{BocAEAEMA}$	PDI
P(PEGMA)	5400	-	12	0	1,15
P(PEGMA)-b-P(BocAEAEMA)	17500	11500	12	32	1,60

4.4. Effect of Polymers on Cell Viability

The effect of polymers ($P(AEAEMA)_{19}$-3.5 kDa, $P(AEAEMA)_{41}$-7.7 kDa, $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-11.5 kDa) on cell viability was investigated using Skov-3-luc human ovary cancer cell line via MTT assay. The results of polymers were

compared with those of linear PEI-8 kDa. PEI is a commonly used polycation for the delivery of siRNA and plasmid DNA (Merdan et al., 2002; Niola et al., 2006; Thomas et al., 2005). Although the most common used PEI in transfection studies is 25 kDa branched PEI, linear PEI-8 kDa was used in this study as its macromolecular structure resembles more to linear polycation synthesized in this study when compared with branched PEI having 25 kDa molecular weight.

The percent viability of the cells was determined with respect to the control wells containing untreated cells. The data were presented as mean (n=3) ± standard deviation. Student's *t* test (two-tailed) was used to analyze the data and $p<0,05$ was considered statistically significant.

Figure 4.8 shows the percent viability of Skov-3-luc cell line after treatment with at various doses of polymers (2.5, 5, 10, 20 μM) for 24 or 72 hours of incubation period.

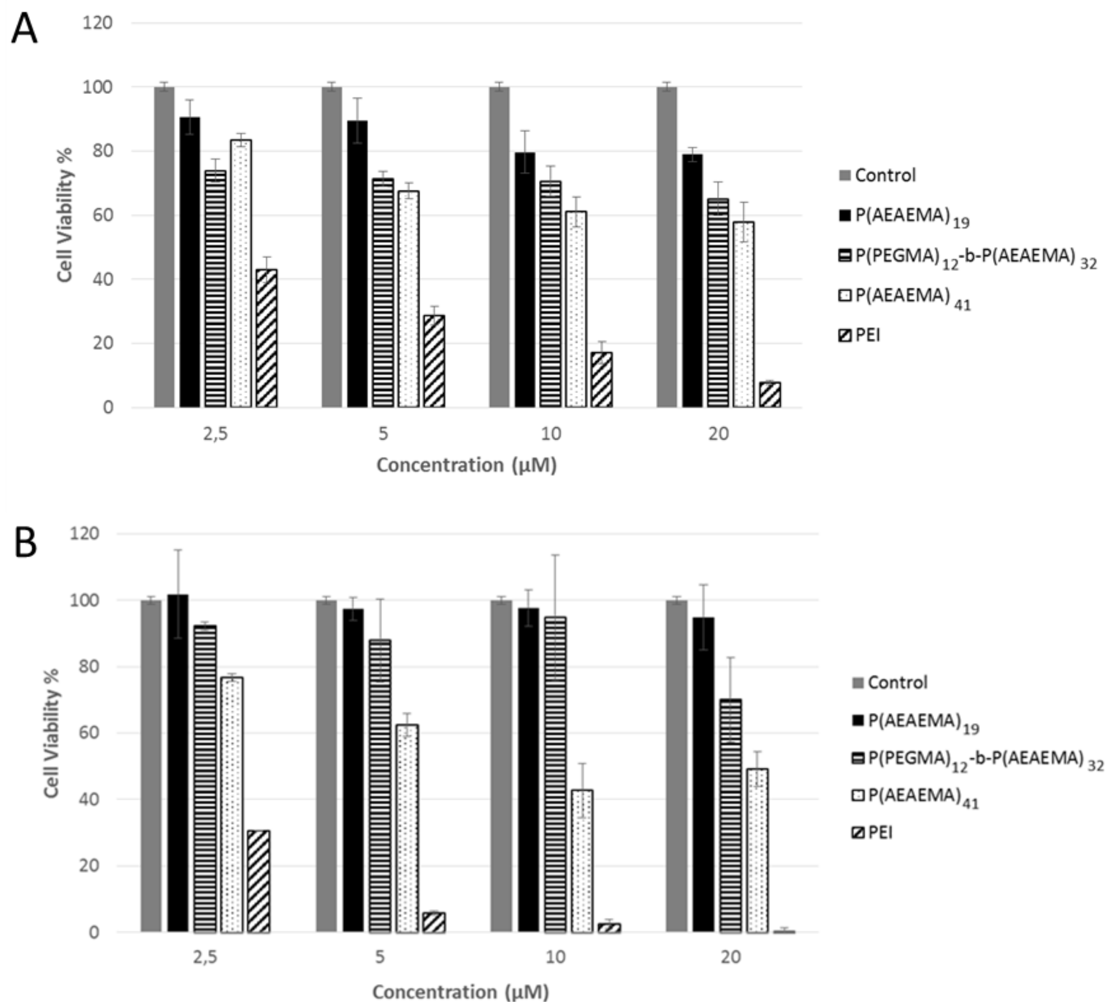

Figure 4.8. The percent cell viability of Skov-3-luc cell line after incubation with polymers for (A) 24 hours and (B) 72 hours.

The cell viability decreased slightly with increasing polymer concentration however, the polymers $P(AEAEMA)_{19}$-3.5 kDa, $P(AEAEMA)_{41}$-7.7 kDa and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-11.5 kDa did not reduce the cell viability less than 50% after 24 h incubation. In contrast to this, linear PEI-8kDa (which is known to have less cytotoxicity compared to branched and high molecular weight PEI (Deng et al., 2009)) was toxic at all polymer concentrations tested. The dose dependent increase in cytotoxicity was observed for PEI.

With the increase in molecular weight, significant decrease in cell viability was observed for P(AEAEMA) at the polymer concentrations of 5, 10 and 20 µM (at 24h incubation) ($p<0,05$). Although the positive effects of PEGMA block in cationic block containing copolymers on cell viability are known from the literature (Beyerle, Merkel, Stoeger, & Kissel, 2010; W. Wang et al., 2009), there was no meaningful difference between $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ and $P(AEAEMA)_{41}$ for 24 h incubation. It is possible that longer P(PEGMA) block is needed to shield the cationic polymer block.

At longer incubation period (72 h) the effect of polymer molecular weight and dose was more profound. $P(AEAEMA)_{19}$ did not show any toxic effect even at 20 µM concentration (cell viability > 94%). The differences in viability of cells treated with $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ and $P(AEAEMA)_{41}$ for 72 h were found to be statistically insignificant. $P(AEAEMA)_{41}$ was toxic at 10 and 20 µM polymer concentrations (cell viability < 50%). The viability of cells treated with PEI was found to be very low or almost 0% for all concentrations tested.

Overall, P(AEAEMA) homopolymers showed dose-, molecular weight and time-dependent toxicity on Skov-3 cell line. Surprisingly PEGMA block did not improve the cytotoxic profile of the cationic polymer. It is possible that a longer P(PEGMA) block or longer PEG grafts are needed to shield the cationic polymer block. However the AEAEMA polymers displayed much less cytotoxicity compared to linear PEI-8 kDa which is known to have very little toxicity when compared with branched PEI (25 kDa) which is widely used in nucleic acid delivery applications.

4.5. Determination of Polyplex Formation

The ability of the cationic polymers to form polyplexes with siRNA was investigated via gel electrophoresis. The electrostatic interactions between the positively charged amine groups of P(AEAEMA) and negatively charged phosphate groups of siRNA lead to the formation of complexes (polyplexes).

In order to determine the amount of polymer required to fully complex with siRNA, varying amounts of polymers were complexed with siRNA at a fixed concentration. Complexes prepared at varying nitrogen/ phosphate (polymer/ siRNA) ratios were analyzed by gel electrophoresis. The complete disappearance of siRNA band at the corresponding N/P ratio indicated that all siRNA molecules in the media were complexed with the added polymer through electrostatic interactions.

The gel electrophoresis results revealed that $P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ can efficiently form complexes with siRNA at an N/P ratio of 5, 2 and 2, respectively (Figures 4.9, 4.10 and 4.11).

The effect of polymer molecular weight on the complex formation could be clearly observed when the complexation of $P(AEAEMA)_{19}$ and $P(AEAEMA)_{41}$ was compared (Figures 4.9 and 4.10). Low molecular weight $P(AEAEMA)_{19}$ was able to form complex with siRNA at an N/P ratio of 3, however a smear was observed until the N/P of 5 indicating that all negative charges on siRNA molecules were fully complexed with positively charged polymer only at an N/P of 5. On the other hand, $P(AEAEMA)_{41}$ could efficiently bind siRNA at the N/P of 2. The complex forming ability of $P(AEAEMA)_{41}$ at lower N/P ratios compared to $P(AEAEMA)_{19}$ was attributed to the higher molecular weight of $P(AEAEMA)_{41}$. It is likely that high molecular weight P(AEAEMA) could interact with the negative charges of siRNA more efficiently due to its flexible structure compared with the low molecular weight P(AEAEMA) which has less flexible and more rigid structure.

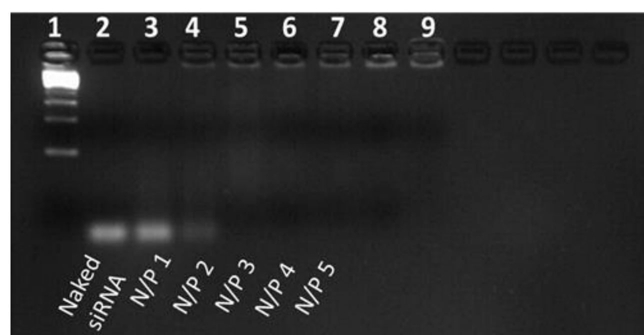

Figure 4.9. Agarose gel electropherogram of $P(AEAEMA)_{19}$-siRNA complexes. Lane 1: DNA marker; Lane 2: naked siRNA; Lanes 3-9: Complexes prepared at N/P of 1, 2, 3, 4, 5, 10 and 30, respectively.

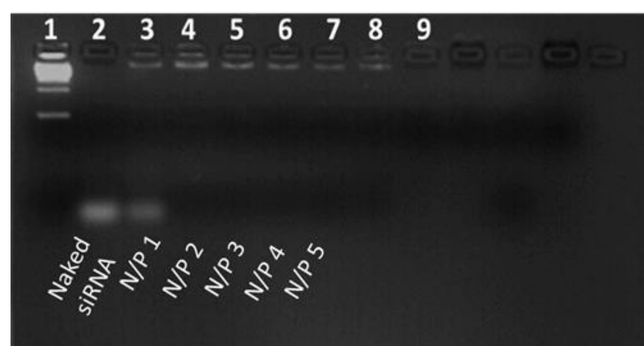

Figure 4.10. Agarose gel electropherogram of $P(AEAEMA)_{41}$-siRNA complexes. Lane 1: DNA marker; Lane 2: naked siRNA; Lanes 3-9: Complexes prepared at N/P of 1, 2, 3, 4, 5, 10 and 30, respectively.

The block copolymer $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ was able to bind siRNA completely at an N/P ratio of 2, suggesting that neutral PEGMA block didn't interfere with the complexation of P(AEAEMA) block and siRNA (Figure 4.11).

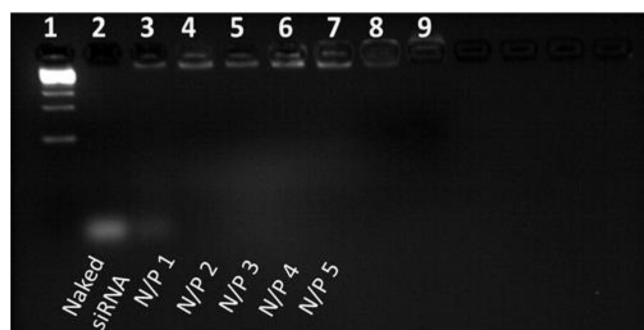

Figure 4.11. Agarose gel electropherogram of $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ -siRNA complexes. Lane 1: DNA marker; Lane 2: naked siRNA; Lanes 3-9: Complexes prepared at N/P of 1, 2, 3, 4, 5, 10 and 30, respectively.

The complex forming ability of PEI was also investigated (Figure 4.12). PEI could completely bind siRNA at an N/P ratio of 5.

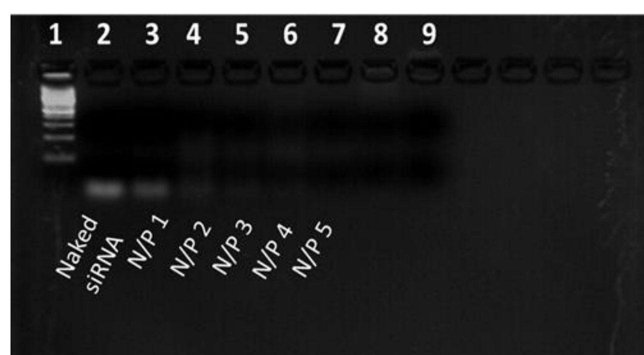

Figure 4.12. Agarose gel electropherogram of PEI-siRNA complexes. Lane 1: DNA marker; Lane 2: naked siRNA; Lanes 3-9: Complexes prepared at N/P of 1, 2, 3, 4, 5, 10 and 30, respectively.

In summary, the gel electrophoresis results showed that all the polymers tested ($P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$) can bind siRNA and form complexes at relatively low N/P ratios. This suggest the potential of these new cationic polymers to be candidate for siRNA carriers.

4.6. Serum Stability of the Polyplexes

One of the important features that an ideal carrier should have is the ability to protect siRNA against nucleases (Gary, Puri, & Won, 2007). siRNA protecting ability of $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ against serum nucleases was investigated by agarose gel electrophoresis (Figures 4.14 and 4.15). The data were quantified using ImageJ gel analysis software.

The electropherogram showing the degradation of naked siRNA in serum with time is presented in Figure 4.13. As it can be seen in the Figure 4.13, degradation was not observed when siRNA was incubated in inactivated (EDTA treated) serum (Figure 4.13 Lanes 6-9). In contrast to this, active serum degraded the naked siRNA at increasing extents with increasing incubation time. According to ImageJ analysis results, 54% of the siRNA was degraded in the first 30 minutes. In 4 hours almost complete degradation of siRNA was observed (80% degradation).

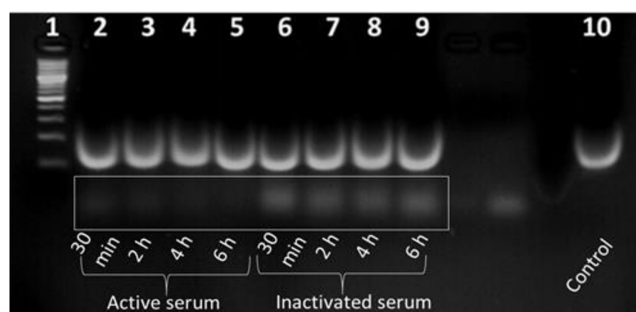

Figure 4.13. Agarose gel electropherogram of naked siRNA in serum. Lane 1: DNA marker; Lanes 2-5: siRNA incubated in serum at 37 °C during 30 min., 2 h, 4 h and 6 h, respectively; Lanes 6-9: siRNA incubated in inactivated serum at 37 °C during 30 min., 2 h, 4 h and 6 h, respectively; Lane 10: control (includes all components in the experiment except siRNA)

The siRNA protection ability of $P(AEAEMA)_{41}$ was investigated at two different N/P ratios (2 and 10) via gel electrophoresis (Figure 4.14). According to the electropherogram analysis results, at both N/P ratios ~30% of the siRNA was degraded in the first 30 min. The degradation percentage of the siRNA did not increase up to 6 h. However ~74% degradation of siRNA was observed in 24 h incubation at both N/P ratios (Table 4.3).

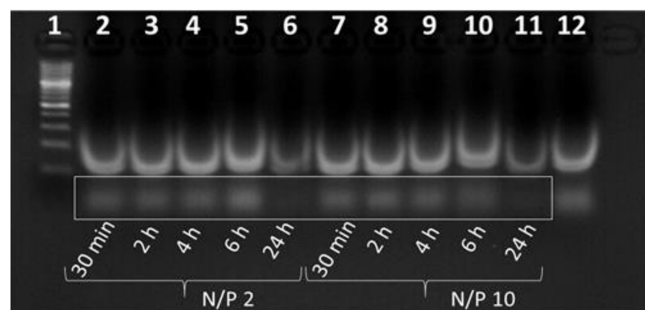

Figure 4.14. Agarose gel electropherogram of $P(AEAEMA)_{41}$-siRNA in serum. Lane 1: DNA marker; Lanes 2-6: complexes prepared at N/P of 2 and incubated in serum at 37 °C during 30 min., 2 h, 4 h, 6 h and 24h, respectively; Lanes 7-11: complexes prepared at N/P of 10 and incubated in serum at 37 °C during 30 min., 2 h, 4 h, 6 h and 24h, respectively; Lane 12: control (complex prepared at N/P of 10 and loaded in inactivated serum)

Table 4.3. The percent degradation of siRNA with respect to time

ImageJ Analyses Results				
		Degradation of siRNA (%)		
	N/P	**30 min.**	**4 h**	**24 h**
Homopolymer	**2**	30	32	74
	10	30	27	74
Block copolymer	**2**	45	47	73
	10	20	18	55
Naked siRNA	**-**	54	80	-

The serum stability of $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ block copolymer was also investigated and its gel electrophoresis is presented in Figure 4.15. According to the electropherogram analysis results, ~45% degradation of the siRNA was observed in the first 30 min. when the complexes at an N/P ratio of 2 were used. The degradation ratio of the siRNA did not change up to 6 h. Similar to the results of $P(AEAEMA)_{41}$, ~73% degradation of siRNA was observed in 24 h incubation at N/P of 2. When the complexes at an N/P ratio of 10 were used, ~20% degradation of the siRNA was observed in first 30 min. The degradation ratio of the siRNA did not change up to 6 h. 55% of siRNA was degraded in 24 h at an N/P of 10 (Table 4.3).

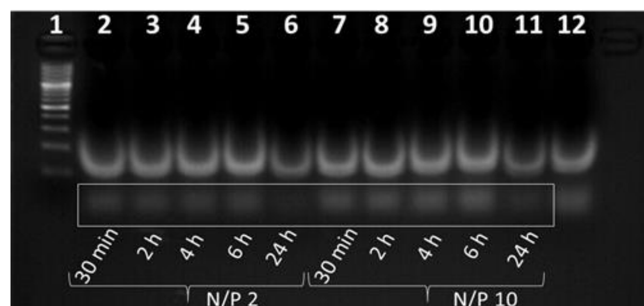

Figure 4.15. Agarose gel electropherogram of $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA in serum. Lane 1: DNA marker; Lanes 2-6: complexes prepared at N/P of 2 and incubated in serum at 37 °C during 30 min., 2 h, 4 h, 6 h and 24h, respectively; Lanes 7-11: complexes prepared at N/P of 10 and incubated in serum at 37 °C during 30 min., 2 h, 4 h, 6 h and 24h, respectively; Lane 12: control (complex prepared at N/P of 10 and loaded in inactivated serum)

According to gel electrophoresis results, when compared with naked siRNA, siRNA in complexes with $P(AEAEMA)_{41}$ or $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ show resistence to serum for longer times. Block copolymer at an N/P ratio of 10 could protect siRNA longer (for up to 24 h) when compared with the homopolymer. This was attributed to the protein-repellent effect of PEGMA block. It is likely that PEGMA enhanced the protection of siRNA by reducing non-specific interactions with serum components (Ogris et al., 1999).

4.7. Size and Surface Charge of the Polyplexes

The size and surface charge of the polyplexes are important physicochemical properties that strongly affect the pharmacokinetic, cellular uptake and transfection efficiency (Gratton et al., 2008; D.-M. Xu et al., 2007). Thus size and zeta potential of the $P(AEAEMA)_{41}$-siRNA and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA complexes at varying N/P ratios were investigated in phosphate buffer at pH 6.0 (10 mM) (Figures 4.16, 4.17; Tables 4.4 and 4.5). Additionally the size of the complexes at an N/P ratio of 2 was determined after incubating the complexes in DMEM with 10% FBS during 15 min., 2 h, 4 h, 24 h (Figures 4.18, 4.19 and Table 4.6).

Table 4.4. The volume average diameter of $P(AEAEMA)_{41}$-siRNA and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA complexes at N/P 2, 5, 10, 30, 50.

	Volume Average Diameter (nm) of the Complexes	
N/P	**$P(AEAEMA)_{41}$-siRNA**	**$P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA**
2	109,7 ± 45,8	71,7 ± 31,1
5	88,4 ± 39,3	Aggregation was observed
10	99,7 ± 40,9	Aggregation was observed
30	61,4 ± 32,4	Aggregation was observed
50	75,7 ± 38,5	Aggregation was observed

As shown in Figure 4.16 and Table 4.4 the volume average diameter of the $P(AEAEMA)_{41}$-siRNA complexes was found to be lower than 110 nm at all N/P ratios tested. Similar to literature studies, the average diameter of the complexes were laid between a certain range (110-75 nm) with the increase in N/P ratio (Kanayama et al. 2006; Kim et al., 2011). Unimodal size distribution was observed for all $P(AEAEMA)_{41}$-siRNA complexes.

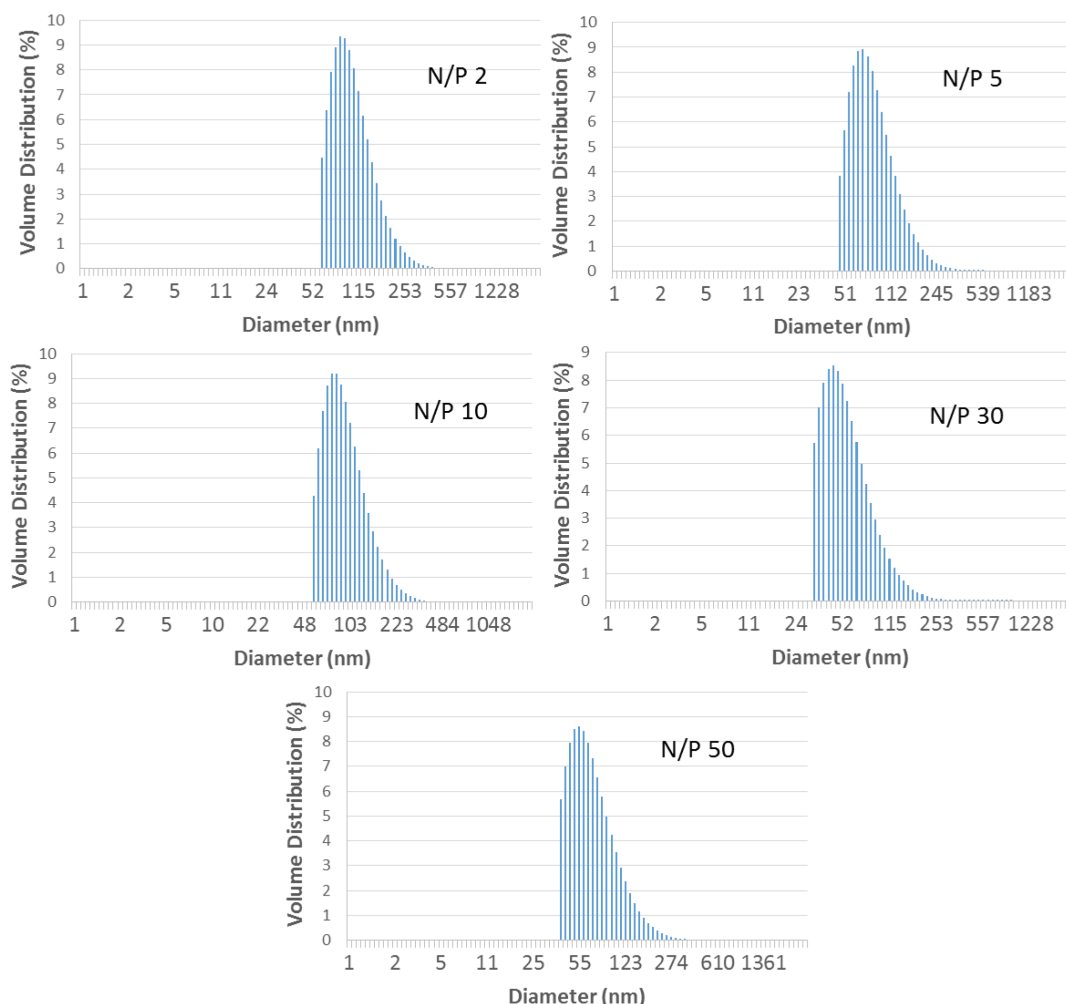

Figure 4.16. The volume distributions of the P(AEAEMA)41-siRNA complexes at varying N/P ratios.

As shown in Figure 4.17 and Table 4.4 the diameter of the P(PEGMA)$_{12}$-b-P(AEAEMA)$_{32}$-siRNA complexes at an N/P of 2 was found to be approximately 70 nm. However when the N/P ratio was increased, aggregation was observed. With the increasing amount of the polymer at high N/P ratios, the higher amount of PEGMA block which is neutral and amphiphilic might have led to the aggregation of the complex particles. Although the stabilizing effects of P(PEGMA) have been reported in literature (Ogris, Brunner, Schüller, Kircheis, & Wagner, 1999), it is known that the length of the PEG segment is an important issue (Glodde, Sirsi, & Lutz, 2006; Petersen et al., 2002). Incorporation of longer PEG segments into the copolymeric structure or the use of a longer P(PEGMA) block would need to be investigated in future to improve the stability and size of the particles.

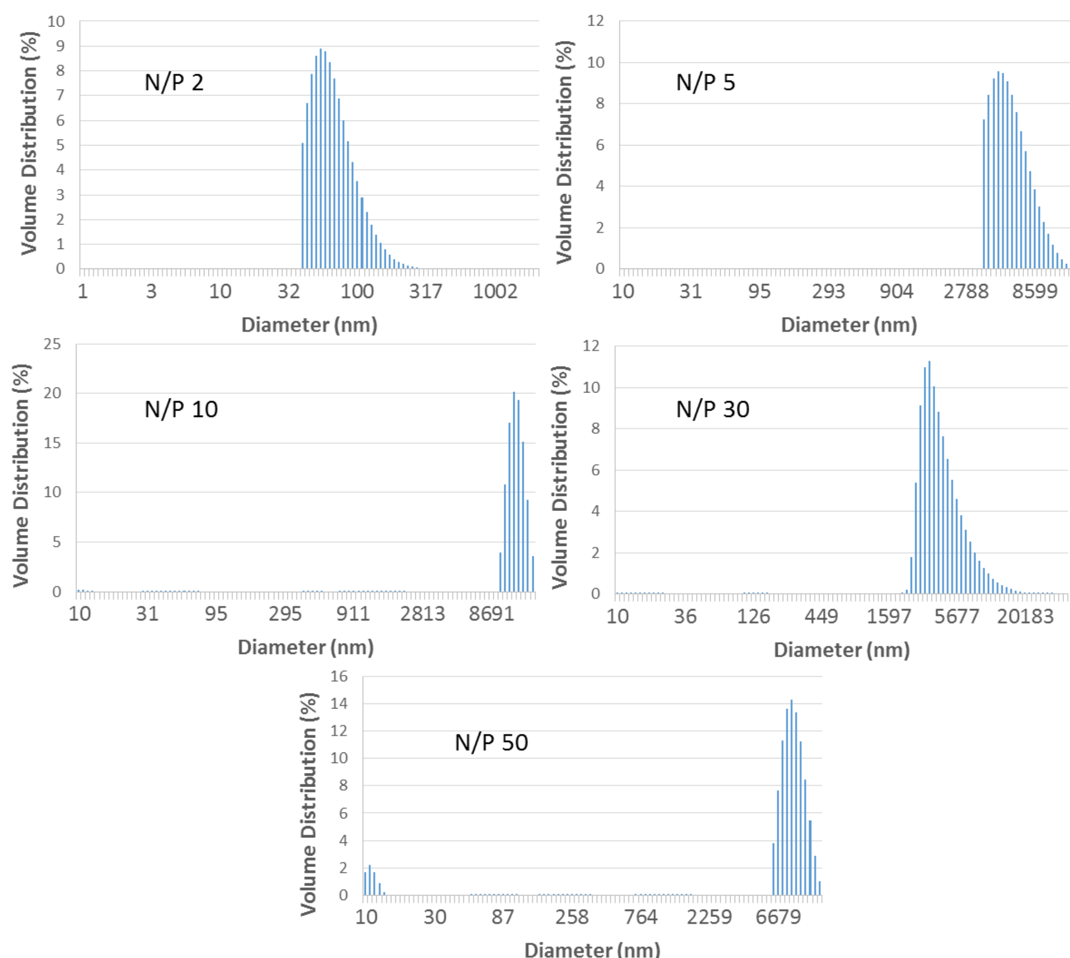

Figure 4.17. The volume distributions of the P(PEGMA)$_{12}$-b-P(AEAEMA)$_{32}$-siRNA complexes at varying N/P ratios.

Zeta potential values of polymer-siRNA complexes are given in Table 4.5. All polyplexes displayed positive zeta potential which is anticipated to enhance the cellular uptake of the complexes. However, the positive zeta potential would also be expected to lead non-specific interaction with serum components and aggregation of complexes in serum containing media. The zeta potential of the homopolymer was found to be higher than the copolymer at the N/P ratio of 2. As expected, PEGMA block could shield the positive charge of the cationic block in the copolymer to some extent. Similar to size results, the zeta potential values were laid between a certain range (3.76 – 8.77 mV) at all N/P ratios.

Table 4.5. Zeta potential values of the complexes at varying N/P ratios. (The data are presented as mean (mV) ± st. deviation).

POLYMERS	N/P 2	N/P 5	N/P 10	N/P 30	N/P 50
$P(AEAEMA)_{41}$	8,64± 1,3	8,39± 3,2	3,76± 2,3	5,07± 1,2	8,77± 3,4
$P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$	4,31± 0,1	-	-	-	-

When the $P(AEAEMA)_{41}$-siRNA complexes were incubated in serum containing DMEM, a significant increase in the size of the polyplexes was observed possibly due to interactions of negatively charged serum proteins with the positively charged complexes (Figure 4.18 Table 4.6). The size of the $P(AEAEMA)_{41}$-siRNA complexes dramatically increased at longer incubation times (2 h and 4 h) indicating the aggregation of the complexes in the presence of serum components. After 24 h incubation in serum, bimodal distribution was observed which was attributed to the dissociation of the siRNA and the polymer.

Table 4.6. The average diameter (nm) of the polyplexes at N/P 2 after incubation in 10% serum containing DMEM for 15 min, 2 h, 4 h and 24 h.

POLYPLEXES	15 min.	2 h	4 h	24 h
$P(AEAEMA)_{41}$-siRNA	372,0± 258,7	972,1± 463,7	1637,5± 557,3	4,0± 0,6 282,1± 134,0
$P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA	10,6± 2,8 90,2± 42,5	10,7± 4,9 132,7± 76,8	11,5± 4,5 136,5± 89,0	7,5± 4,3 379,7± 202,3

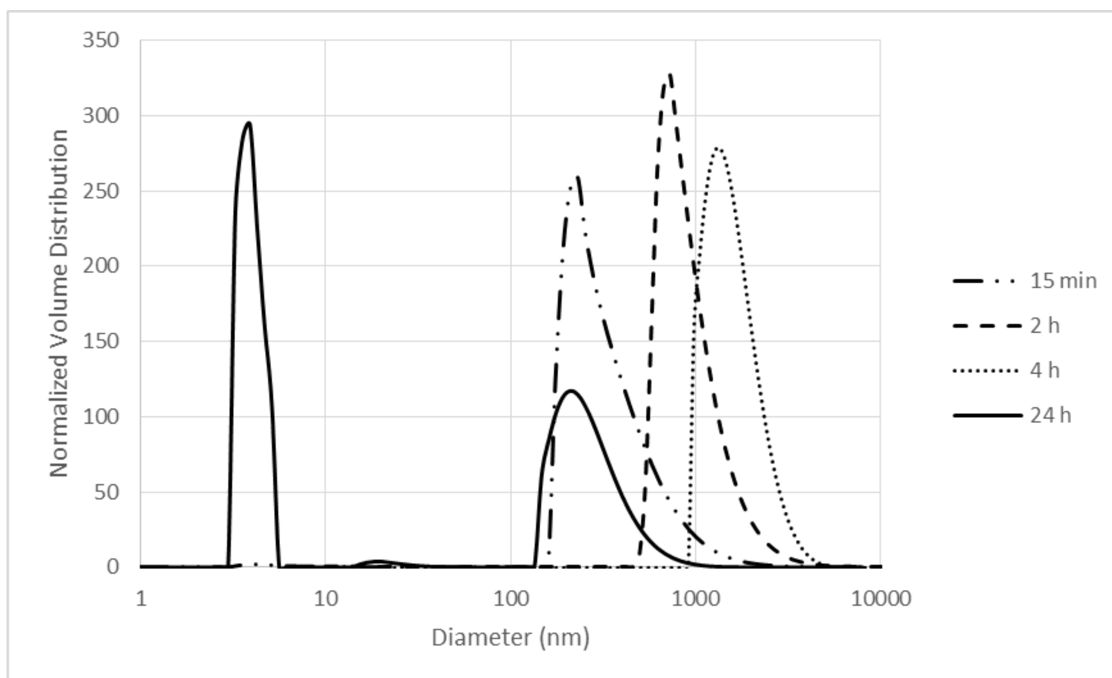

Figure 4.18. The volume distribution of $P(AEAEMA)_{41}$-siRNA complexes at N/P ratio of 2 after incubating the complexes in DMEM with 10% FBS for 15 min., 2 h, 4 h or 24 h.

In the case of the polyplexes prepared from $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ copolymer, the size of polyplexes was much lower compared to that of $P(AEAEMA)_{41}$-siRNA complexes. This result suggested that P(PEGMA) block indeed reduced to some extent the non-specific interactions of the polyplexes with serum proteins (Figure 4.19 Table 4.6). However, bimodal distribution was also observed for all incubation times indicating partial dissociation of the complexes.

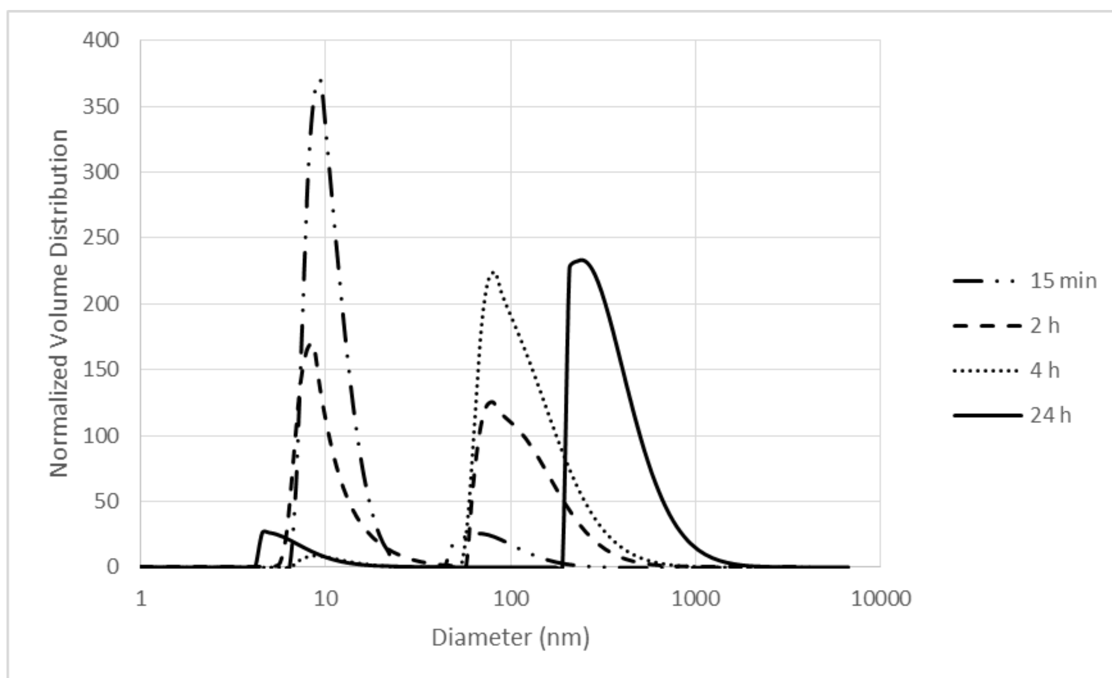

Figure 4.19. The volume distribution of P(PEGMA)$_{12}$-b-P(AEAEMA)$_{32}$-siRNA complexes at N/P ratio of 2 after incubating the complexes in DMEM with 10% FBS for 15 min., 2 h, 4 h or 24 h.

4.8. Luciferase Assay

In order to determine the transfection efficiency of the polymers that were synthesized in this study, the optimization of the luciferase assay was performed in stably luciferase expressing Skov-3-luc cell line. An anti-luciferase siRNA sequence was used to selectively inhibit the expression of luciferase gene (J.-S. Lee et al., 2009). A commercial reagent lipofectamine RNAimax was used as a transfection agent. The reduction in protein expression level was determined by measuring the luminescence using Promega luciferase assay kit.

To determine the optimum incubation time of the complexes with cells, the prepared lipofectamine-siRNA complexes were incubated for 4 h (plus further 20 h incubation with fresh medium –without complexes-) or 24 h with cells. Gene silencing effect could not be observed when the complexes were incubated with cells only for 4 h. In contrast to this ~70% reduction in gene expression was observed after 24 h incubation of the complexes with cells (Figure 4.20). Based on this result, an incubation

time of would be necessary for cells to be incubated with polyplexes for efficient transfection.

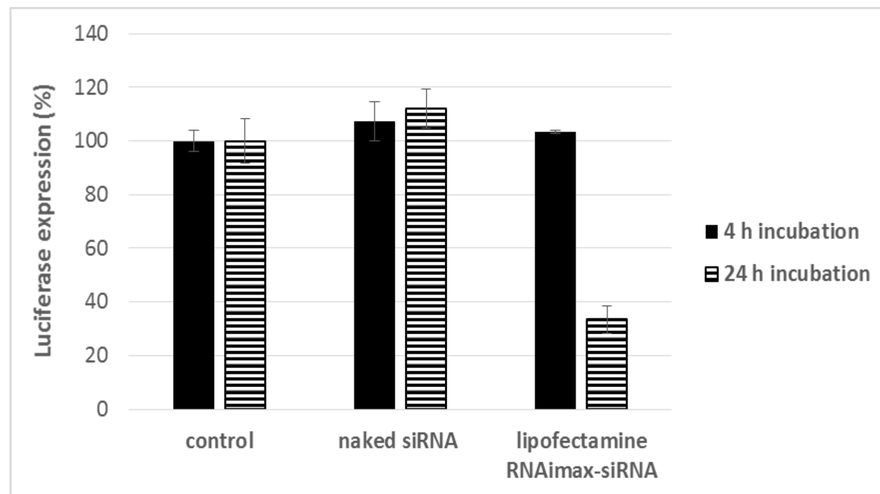

Figure 4.20. The percent luciferase expression after incubation with lipofectamine RNAimax-siRNA complexes (including 100 nM dose of siRNA) for 4 hours and for 24 hours.

To determine the optimum (non-toxic and effective) siRNA doses and the required time to observe protein expression, lipofectamine-siRNA complexes having three different doses of siRNA were prepared and incubated with the cells for 24 h. The results were obtained either directly at the end of the incubation time or after an additional 24 h incubation with fresh medium –without complexes- (total 48 h incubation) (Figure 4.21). In parallel, the cytotoxicity of the complexes was also investigated by MTT assay (Figure 4.22). The complex having 50 nM dose of siRNA was found non-toxic (cell viability ~100%) and moreover reduced the gene expression level (approximately 70% knockdown) as efficiently as the complex having 100 nM dose of siRNA after 48 h incubation. The results also showed that the gene silencing could be observed 24 h after the exposure of cells to the complexes and the silencing effect lasted at least for another 24 h.

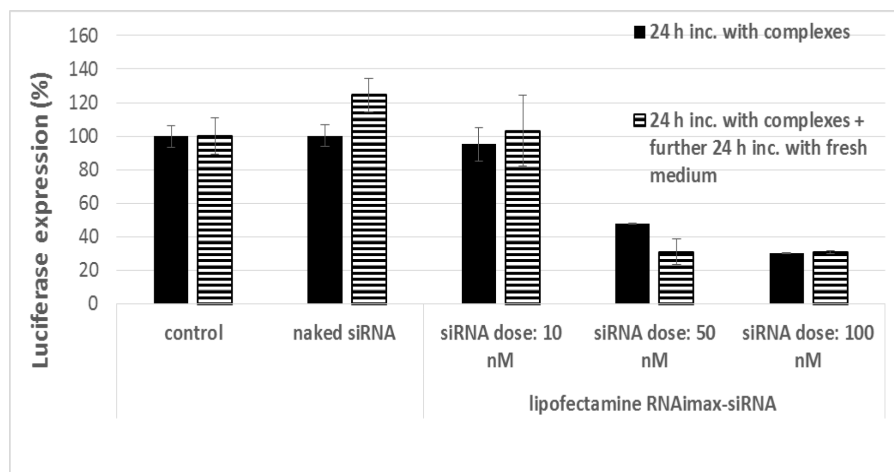

Figure 4.21. The percent luciferase expression after incubation with lipofectamine RNAimax-siRNA complexes for total 24 hours and 48 hours.

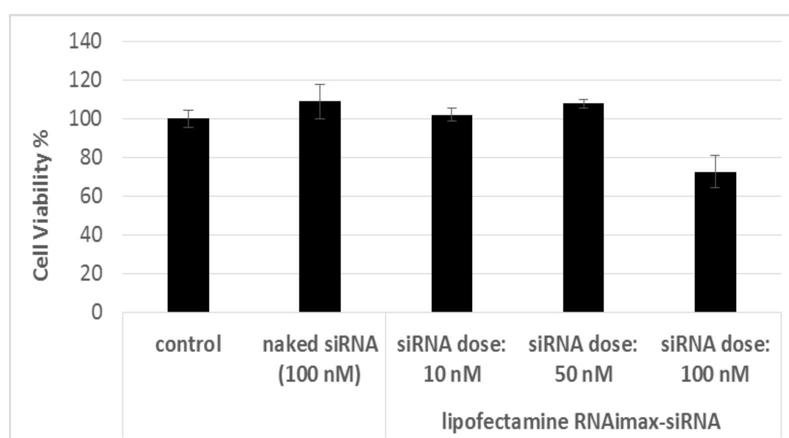

Figure 4.22. The percent cell viability after incubation with lipofectamine RNAimax-siRNA complexes for 24 h.

In this thesis study, after the optimization of transfection assay parameters using a commercially available transfection agent, lipofectamine RNAimax, transfection ability of P(AEAEMA) homo- and co- polymers together with PEI 8 kDa was investigated. However, the results with the polyplexes were found to be non-reproducible. The reasons for non-reproducible results would need to be investigated in detail in future studies.

CHAPTER 5

CONCLUSION

In this thesis work, poly(2-((2-aminoethyl)amino)ethyl methacrylate) (P(AEAEMA)), a new polymer bearing diaminoethane motif, was evaluated as a potential siRNA carrier.

The diaminoethane containing monomer 2-((tert-butoxycarbonyl) (2-((tert-butoxy carbonyl) amino) ethyl) amino) ethyl methacrylate (BocAEAEMA) was synthesized following the procedure reported by Kurtulus et al. (Kurtulus et al., 2014). The homopolymers (P(AEAEMA)) having molecular weights of 3.5 kDa (DP:19) and 7.7 kDa (DP: 41) and the copolymer (P(PEGMA)-b-P(BocAEAEMA)) ($DP_{P(PEGMA)}$: 12, $DP_{P(AEAEMA)}$: 32) having molecular weight of 11.5 kDa were prepared via RAFT polymerization and characterized by Nuclear Magnetic Resonance Spectroscopy (NMR) and Gel Permeation Chromatography (GPC).

The cytotoxicity of the polymers was investigated via MTT assay using human ovary cancer cell line, Skov-3-luc. The polymers did not alter significantly the viability of cells after incubation for 24 h (cell viability > 50%). In contrast, PEI was toxic at all polymer concentrations tested (cell viability < 50%). There was no significant difference in the cytotoxic character of $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ and $P(AEAEMA)_{41}$ indicating that a longer P(PEGMA) block would possibly be needed to reduce the cytotoxicity of cationic block P(AEAEMA).

The ability of the cationic polymers to form polyplexes with siRNA was investigated via gel electrophoresis. $P(AEAEMA)_{19}$, $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ could efficiently form electrostatic complexes with siRNA at an N/P ratio of 5, 2 and 2, respectively. The complex forming ability of $P(AEAEMA)_{41}$ at lower N/P ratios compared to $P(AEAEMA)_{19}$ was attributed to the higher molecular weight of $P(AEAEMA)_{41}$. The block copolymer $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ was able to complex with siRNA completely at an N/P ratio of 2, suggesting that the neutral PEGMA block didn’t interfere with the complexation of cationic P(AEAEMA) block and siRNA.

siRNA protecting ability of $P(AEAEMA)_{41}$ and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$ against serum nucleases was investigated by agarose gel electrophoresis. According to ImageJ analysis results, when naked siRNA was incubated in serum, while 54% of siRNA was degraded in the first 30 minutes, in 6 h 80% degradation of siRNA was observed. When $P(AEAEMA)_{41}$-siRNA complexes at an N/P ratio of 2 or 10 were incubated in serum, only 30% of siRNA was degraded in 6 h. siRNA degradation reached 74% in 24 h at both N/P ratios. On the other hand block copolymer at an N/P ratio of 10 could protect siRNA longer (only 55% degradation in 24 h) when compared with the homopolymer. This was attributed to the shielding and protein-repellent effect of PEGMA block. It is likely that PEGMA enhanced the protection of siRNA by reducing non-specific interactions with serum components.

The size and zeta potential of the $P(AEAEMA)_{41}$-siRNA and $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA polyplexes at varying N/P ratios were investigated in phosphate buffer at pH 6.0 (10 mM). The diameter of the $P(AEAEMA)_{41}$-siRNA complexes was found to be lower than 110 nm at all N/P ratios tested. However in the case of $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA polyplexes aggregation was observed at all N/P ratios except N/P ratio of 2. Incorporation of a longer P(PEGMA) block and/or a lineer PEG segment into the copolymeric structure would need to be investigated in future to improve the stability and size properties of the polyplex particles.

The surface charge of the homopolymer was found to be higher than the copolymer at the N/P ratio of 2. The results are suggesting that PEGMA block could shield the positive charge of the cationic block in the copolymer.

When the polyplexes were incubated in serum containing cell culture medium, increase in the hydrodynamic sizes was observed possibly due to interactions of negatively charged serum proteins with the positively charged polyplexes. Compared to the size of $P(AEAEMA)_{41}$-siRNA polyplexes (between 372-1637 nm in first 4 h), $P(PEGMA)_{12}$-b-$P(AEAEMA)_{32}$-siRNA complexes incubated in serum were much smaller (between 90-136 nm in first 4 h) suggesting that the PEGMA block reduced the non-specific interactions of the cationic P(AEAEMA) with serum proteins.

Finally, the optimization of the luciferase assay was performed using a commercial transfection agent. The results showed that 24 h incubation with complexes was found to be necessary for the cellular uptake of the complexes and the silencing effect could be observed after the first 24 h incubation. Investigations on the transfection efficiency of the polymers would be the focus of the future studies.

In conclusion, diaminoethane motif bearing methacrylate based polymers were synthesized via RAFT polymerization and their siRNA delivery potential was evaluated. The results revealed that the cationic P(AEAEMA) can efficiently complex with siRNA through electrostatic interactions. The polyplex formation between the cationic homopolymer and siRNA is dependent on the molecular weight of the polymer. While incorporation of a neutral polymer block, P(PEGMA), to the cationic polymer does not interfere with the interactions between siRNA and P(AEAEMA), it provides an advantage in terms of siRNA protection ability. On the other hand, polyplexes formed from block copolymers surprisingly displayed aggregation tendency at N/P ratios higher than 2, whereas polyplexes of homopolymers had hydrodynamic diameters less than 110 nm at all N/P ratios tested. Nevertheless, block copolymer polyplexes at N/P ratio of 2 were stable in serum containing medium. In contrast, homopolymer polyplexes formed giant aggregates after incubation in serum containing medium.

Further investigations that should be carried out to determine the siRNA delivery potential of these promising polymers, include the followings:

1. The transfection ability of the polymers needs to be investigated using optimized Luciferase Assay conditions.

2. Linear and/or longer PEG segments can be incorporated to the cationic block to improve the physicochemical properties of the particles.

3. Hydrodynamic size and zeta potential of the polyplexes needs to be investigated in physiological pH using formulations prepared in a proper buffer.

4. Cellular uptake of the polyplexes can be investigated.

REFERENCES

Aagaard, L., & Rossi, J. J. (2007). RNAi therapeutics: principles, prospects and challenges. *Advanced drug delivery reviews, 59*(2), 75-86.

Agarwal, A., Unfer, R., & Mallapragada, S. K. (2005). Novel cationic pentablock copolymers as non-viral vectors for gene therapy. *Journal of Controlled Release, 103*(1), 245-258.

Aigner, A. (2006). Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. *Journal of biotechnology, 124*(1), 12-25.

Akhtar, S., & Benter, I. F. (2007). Nonviral delivery of synthetic siRNAs in vivo. *The Journal of clinical investigation, 117*(12), 3623.

Alexis, F., Pridgen, E., Molnar, L. K., & Farokhzad, O. C. (2008). Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Molecular pharmaceutics, 5*(4), 505-515.

Barreau, C., Dutertre, S., Paillard, L., & Osborne, H. B. (2006). Liposome-mediated RNA transfection should be used with caution. *Rna, 12*(10), 1790-1793.

Bartel, D. P., & Chen, C.-Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nature reviews genetics, 5*(5), 396-400.

Bartolome, J., Castillo, I., & Carreno, V. (2004). Ribozymes as antiviral agents. *Minerva medica, 95*(1), 11-24.

Bazile, D., Prud'homme, C., Bassoullet, M. T., Marlard, M., Spenlehauer, G., & Veillard, M. (1995). Stealth Me. PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system. *Journal of pharmaceutical sciences, 84*(4), 493-498.

Benjaminsen, R. V., Mattebjerg, M. A., Henriksen, J. R., Moghimi, S. M., & Andresen, T. L. (2013). The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Molecular Therapy, 21*(1), 149-157.

Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature, 409*(6818), 363-366.

Beyerle, A., Merkel, O., Stoeger, T., & Kissel, T. (2010). PEGylation affects cytotoxicity and cell-compatibility of poly (ethylene imine) for lung application: structure–function relationships. *Toxicology and applied pharmacology, 242*(2), 146-154.

Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., . . . Karpilow, J. (2006). 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nature methods, 3*(3), 199-204.

Bitko, V., Musiyenko, A., Shulyayeva, O., & Barik, S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. *Nature medicine, 11*(1), 50-55.

Bolhassani, A. (2011). Potential efficacy of cell-penetrating peptides for nucleic acid and drug delivery in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1816*(2), 232-246.

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.

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.

Boyer, C., Granville, A., Davis, T. P., & Bulmus, V. (2009). Modification of RAFT-polymers via thiol-ene reactions: A general route to functional polymers and new architectures. *Journal of Polymer Science Part A: Polymer Chemistry, 47*(15), 3773-3794.

Braasch, D. A., Paroo, Z., Constantinescu, A., Ren, G., Öz, O. K., Mason, R. P., & Corey, D. R. (2004). Biodistribution of phosphodiester and phosphorothioate siRNA. *Bioorganic & medicinal chemistry letters, 14*(5), 1139-1143.

Breunig, M., Hozsa, C., Lungwitz, U., Watanabe, K., Umeda, I., Kato, H., & Goepferich, A. (2008). Mechanistic investigation of poly (ethylene imine)-based siRNA delivery: disulfide bonds boost intracellular release of the cargo. *Journal of Controlled Release, 130*(1), 57-63.

Bulmus, V. (2011). RAFT polymerization mediated bioconjugation strategies. *Polymer Chemistry, 2*(7), 1463-1472.

Bumcrot, D., Manoharan, M., Koteliansky, V., & Sah, D. W. (2006). RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nature chemical biology, 2*(12), 711-719.

Cullen, B. R. (2004). Transcription and processing of human microRNA precursors. *Molecular cell, 16*(6), 861-865.

Davis, M. E., Zuckerman, J. E., Choi, C. H. J., Seligson, D., Tolcher, A., Alabi, C. A., . . . Ribas, A. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature, 464*(7291), 1067-1070.

De Smedt, S. C., Demeester, J., & Hennink, W. E. (2000). Cationic polymer based gene delivery systems. *Pharmaceutical research, 17*(2), 113-126.

Deleavey, G. F., & Damha, M. J. (2012). Designing chemically modified oligonucleotides for targeted gene silencing. *Chemistry & biology, 19*(8), 937-954.

Deng, R., Yue, Y., Jin, F., Chen, Y., Kung, H.-F., Lin, M. C., & Wu, C. (2009). Revisit the complexation of PEI and DNA—How to make low cytotoxic and highly efficient PEI gene transfection non-viral vectors with a controllable chain length and structure? *Journal of Controlled Release, 140*(1), 40-46.

DeVincenzo, J., Cehelsky, J. E., Alvarez, R., Elbashir, S., Harborth, J., Toudjarska, I., . . . Vaishnaw, A. K. (2008). Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). *Antiviral research, 77*(3), 225-231.

Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., & Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-β receptor signalling and turnover. *Nature cell biology, 5*(5), 410-421.

DiFiglia, M., Sena-Esteves, M., Chase, K., Sapp, E., Pfister, E., Sass, M., . . . Rajeev, K. G. (2007). Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proceedings of the National Academy of Sciences, 104*(43), 17204-17209.

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 pharmaceutics, 7*(2), 468-476.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature, 411*(6836), 494-498.

Gary, D. J., Puri, N., & Won, Y.-Y. (2007). Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. *Journal of Controlled Release, 121*(1), 64-73.

Geisbert, T. W., Hensley, L. E., Kagan, E., Yu, E. Z., Geisbert, J. B., Daddario-DiCaprio, K., . . . Phelps, J. R. (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *Journal of Infectious Diseases, 193*(12), 1650-1657.

Geiser, T. (1990). Large-Scale Economic Synthesis of Antisense Phosphorothioate Analogues of DNA for Preclinical Investigations. *Annals of the New York Academy of Sciences, 616*(1), 173-183.

Glodde, M., Sirsi, S. R., & Lutz, G. J. (2006). Physiochemical properties of low and high molecular weight poly (ethylene glycol)-grafted poly (ethylene imine) copolymers and their complexes with oligonucleotides. *Biomacromolecules, 7*(1), 347-356.

Godbey, W., Barry, M. A., Saggau, P., Wu, K. K., & Mikos, A. G. (2000). Poly (ethylenimine)-mediated transfection: a new paradigm for gene delivery. *Journal of biomedical materials research, 51*(3), 321-328.

Grassi, G., Dawson, P., Guarnieri, G., Kandolf, R., & Grassi, M. (2004). Therapeutic potential of hammerhead ribozymes in the treatment of hyper-proliferative diseases. *Current pharmaceutical biotechnology, 5*(4), 369-386.

Gratton, S. E., Ropp, P. A., Pohlhaus, P. D., Luft, J. C., Madden, V. J., Napier, M. E., & DeSimone, J. M. (2008). The effect of particle design on cellular internalization pathways. *Proceedings of the National Academy of Sciences, 105*(33), 11613-11618.

Grayson, A. C. R., Doody, A. M., & Putnam, D. (2006). Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro. *Pharmaceutical research, 23*(8), 1868-1876.

Grimm, D. (2009). Small silencing RNAs: state-of-the-art. *Advanced drug delivery reviews, 61*(9), 672-703.

Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., . . . Kay, M. A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature, 441*(7092), 537-541.

Hamilton, A. J., & Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science, 286*(5441), 950-952.

Hammond, S. M., Bernstein, E., Beach, D., & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature, 404*(6775), 293-296.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., & Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science, 293*(5532), 1146-1150.

Hannon, G. J. (2002). RNA interference. *Nature, 418*(6894), 244-251.

Hassani, Z., Lemkine, G. F., Erbacher, P., Palmier, K., Alfama, G., Giovannangeli, C., . . . Demeneix, B. A. (2005). Lipid-mediated siRNA delivery down-regulates exogenous gene expression in the mouse brain at picomolar levels. *The journal of gene medicine, 7*(2), 198-207.

Hayakawa, K., Uchida, S., Ogata, T., Tanaka, S., Kataoka, K., & Itaka, K. (2015). Intrathecal injection of a therapeutic gene-containing polyplex to treat spinal cord injury. *Journal of Controlled Release, 197*, 1-9.

Howard, K. A., Rahbek, U. L., Liu, X., Damgaard, C. K., Glud, S. Z., Andersen, M. Ø., . . . Besenbacher, F. (2006). RNA interference in vitro and in vivo using a chitosan/siRNA nanoparticle system. *Molecular Therapy, 14*(4), 476-484.

Hu-Lieskovan, S., Heidel, J. D., Bartlett, D. W., Davis, M. E., & Triche, T. J. (2005). Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer research, 65*(19), 8984-8992.

Hutvágner, G., & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science, 297*(5589), 2056-2060.

Itaka, K., Ohba, S., Miyata, K., Kawaguchi, H., Nakamura, K., Takato, T., . . . Kataoka, K. (2007). Bone regeneration by regulated in vivo gene transfer using biocompatible polyplex nanomicelles. *Molecular Therapy, 15*(9), 1655-1662.

Jackson, A. L., & Linsley, P. S. (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nature reviews Drug discovery, 9*(1), 57-67.

Jarad, G., & Miner, J. H. (2009). Update on the glomerular filtration barrier. *Current opinion in nephrology and hypertension, 18*(3), 226.

Jens, K. (2003). Antisense technologies Improvement through novel chemical modification. *Eur J Biochem, 270*, 1628-164420.

Jeong, J. H., Christensen, L. V., Yockman, J. W., Zhong, Z., Engbersen, J. F., Kim, W. J., . . . Kim, S. W. (2007). Reducible poly (amido ethylenimine) directed to enhance RNA interference. *Biomaterials, 28*(10), 1912-1917.

Jeong, J. H., Mok, H., Oh, Y.-K., & Park, T. G. (2008). siRNA conjugate delivery systems. *Bioconjugate chemistry, 20*(1), 5-14.

Jin, G.-w., Koo, H., Nam, K., Kim, H., Lee, S., Park, J.-S., & Lee, Y. (2011). PAMAM dendrimer with a 1, 2-diaminoethane surface facilitates endosomal escape for enhanced pDNA delivery. *Polymer, 52*(2), 339-346.

Kanasty, R., Dorkin, J. R., Vegas, A., & Anderson, D. (2013). Delivery materials for siRNA therapeutics. *Nature materials, 12*(11), 967-977.

Kanasty, R. L., Whitehead, K. A., Vegas, A. J., & Anderson, D. G. (2012). Action and reaction: the biological response to siRNA and its delivery vehicles. *Molecular Therapy, 20*(3), 513-524.

Kanayama, N., Fukushima, S., Nishiyama, N., Itaka, K., Jang, W. D., Miyata, K., . . . Kataoka, K. (2006). A PEG-Based Biocompatible Block Catiomer with High Buffering Capacity for the Construction of Polyplex Micelles Showing Efficient Gene Transfer toward Primary Cells. *ChemMedChem, 1*(4), 439-444.

Khan, A., Benboubetra, M., Sayyed, P. Z., Wooi Ng, K., Fox, S., Beck, G., . . . Akhtar, S. (2004). Sustained polymeric delivery of gene silencing antisense ODNs, siRNA, DNAzymes and ribozymes: in vitro and in vivo studies. *Journal of drug targeting, 12*(6), 393-404.

Kichler, A. (2004). Gene transfer with modified polyethylenimines. *The journal of gene medicine, 6*(S1), S3-S10.

Kim, D. H., & Rossi, J. J. (2007). Strategies for silencing human disease using RNA interference. *Nature reviews genetics, 8*(3), 173-184.

Kim, H. J., Ishii, A., Miyata, K., Lee, Y., Wu, S., Oba, M., . . . Kataoka, K. (2010). Introduction of stearoyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp (DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown. *Journal of Controlled Release, 145*(2), 141-148.

Kim, W. J., & Kim, S. W. (2009). Efficient siRNA delivery with non-viral polymeric vehicles. *Pharmaceutical research, 26*(3), 657-666.

Kircheis, R., Wightman, L., & Wagner, E. (2001). Design and gene delivery activity of modified polyethylenimines. *Advanced drug delivery reviews, 53*(3), 341-358.

Kurtulus, I., Yilmaz, G., Ucuncu, M., Emrullahoglu, M., Becer, C. R., & Bulmus, V. (2014). A new proton sponge polymer synthesized by RAFT polymerization for intracellular delivery of biotherapeutics. *Polymer Chemistry, 5*(5), 1593-1604.

Layzer, J. M., McCaffrey, A. P., Tanner, A. K., Huang, Z., Kay, M. A., & Sullenger, B. A. (2004). In vivo activity of nuclease-resistant siRNAs. *Rna, 10*(5), 766-771.

Lee, H., Lytton-Jean, A. K., Chen, Y., Love, K. T., Park, A. I., Karagiannis, E. D., . . . Jayaraman, M. (2012). Molecularly self-assembled nucleic acid nanoparticles for targeted in vivo siRNA delivery. *Nature nanotechnology, 7*(6), 389-393.

Lee, J.-S., Green, J. J., Love, K. T., Sunshine, J., Langer, R., & Anderson, D. G. (2009). Gold, poly (β-amino ester) nanoparticles for small interfering RNA delivery. *Nano letters, 9*(6), 2402-2406.

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell, 75*(5), 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., . . . Kim, S. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature, 425*(6956), 415-419.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., & Kim, V. N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *The EMBO journal, 23*(20), 4051-4060.

Leung, R. K., & Whittaker, P. A. (2005). RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacology & therapeutics, 107*(2), 222-239.

Lin, X., Ruan, X., Anderson, M. G., McDowell, J. A., Kroeger, P. E., Fesik, S. W., & Shen, Y. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucleic acids research, 33*(14), 4527-4535.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., & Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature cell biology, 7*(7), 719-723.

Longmire, M., Choyke, P. L., & Kobayashi, H. (2008). Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E., & Kutay, U. (2004). Nuclear export of microRNA precursors. *Science, 303*(5654), 95-98.

Ma, Z., Li, J., He, F., Wilson, A., Pitt, B., & Li, S. (2005). Cationic lipids enhance siRNA-mediated interferon response in mice. *Biochemical and biophysical research communications, 330*(3), 755-759.

Malek, A., Merkel, O., Fink, L., Czubayko, F., Kissel, T., & Aigner, A. (2009). In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI (–PEG)/siRNA complexes. *Toxicology and applied pharmacology, 236*(1), 97-108.

Marschall, P., Malik, N., & Larin, Z. (1999). Transfer of YACs up to 2.3 Mb intact into human cells with polyethylenimine. *Gene therapy, 6*(9), 1634-1637.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P., & Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell, 123*(4), 607-620.

Matzke, M. A., & Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. *Nature reviews genetics, 6*(1), 24-35.

McManus, M. T., & Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. *Nature reviews genetics, 3*(10), 737-747.

Meade, B. R., & Dowdy, S. F. (2007). Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Advanced drug delivery reviews, 59*(2), 134-140.

Merdan, T., Kopeček, J., & Kissel, T. (2002). Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Advanced drug delivery reviews, 54*(5), 715-758.

Miyata, K., Oba, M., Nakanishi, M., Fukushima, S., Yamasaki, Y., Koyama, H., . . . Kataoka, K. (2008). Polyplexes from poly (aspartamide) bearing 1, 2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity. *Journal of the American Chemical Society, 130*(48), 16287-16294.

Moad, G., Rizzardo, E., & Thang, S. H. (2009). Living radical polymerization by the RAFT process–a second update. *Australian Journal of Chemistry, 62*(11), 1402-1472.

Morrissey, D. V., Lockridge, J. A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., . . . Jadhav, V. (2005). Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nature biotechnology, 23*(8), 1002-1007.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods, 65*(1), 55-63.

Moura, C., Vítor, R. F., Maria, L., Paulo, A., Santos, I. C., & Santos, I. (2006). Rhenium (v) oxocomplexes with novel pyrazolyl-based N 4-and N 3 S-donor chelators. *Dalton Transactions*(47), 5630-5640.

Muratovska, A., & Eccles, M. R. (2004). Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS letters, 558*(1), 63-68.

Nelson, C. E., Kintzing, J. R., Hanna, A., Shannon, J. M., Gupta, M. K., & Duvall, C. L. (2013). Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo. *ACS nano, 7*(10), 8870-8880.

Nguyen, D. N., Mahon, K. P., Chikh, G., Kim, P., Chung, H., Vicari, A. P., . . . Krieg, A. M. (2012). Lipid-derived nanoparticles for immunostimulatory RNA adjuvant delivery. *Proceedings of the National Academy of Sciences, 109*(14), E797-E803.

Niola, F., Evangelisti, C., Campagnola, L., Massalini, S., Buè, M. C., Mangiola, A., . . . Ciafrè, S. A. (2006). A plasmid-encoded VEGF siRNA reduces glioblastoma angiogenesis and its combination with interleukin-4 blocks tumor growth in a xenograft mouse model. *Cancer biology & therapy, 5*(2), 174-179.

Ogris, M., Brunner, S., Schüller, S., Kircheis, R., & Wagner, E. (1999). PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene therapy, 6*(4), 595-605.

Orban, T. I., & Izaurralde, E. (2005). Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *Rna, 11*(4), 459-469.

Owens, D. E., & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International journal of pharmaceutics, 307*(1), 93-102.

Patil, M. L., Zhang, M., Betigeri, S., Taratula, O., He, H., & Minko, T. (2008). Surface-modified and internally cationic polyamidoamine dendrimers for efficient siRNA delivery. *Bioconjugate chemistry, 19*(7), 1396-1403.

Petersen, H., Fechner, P. M., Martin, A. L., Kunath, K., Stolnik, S., Roberts, C. J., . . . Kissel, T. (2002). Polyethylenimine-graft-poly (ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjugate chemistry, 13*(4), 845-854.

Preall, J. B., & Sontheimer, E. J. (2005). RNAi: RISC gets loaded. *Cell, 123*(4), 543-545.

Rand, T. A., Petersen, S., Du, F., & Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell, 123*(4), 621-629.

Rayburn, E. R., Wang, H., & Zhang, R. (2006). Antisense-based cancer therapeutics: are we there yet? *Expert Opin Emerg Drugs, 11*(2), 337-352. doi: 10.1517/14728214.11.2.337

Rayburn, E. R., & Zhang, R. (2008). Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? *Drug discovery today, 13*(11), 513-521.

Rozema, D. B., Lewis, D. L., Wakefield, D. H., Wong, S. C., Klein, J. J., Roesch, P. L., . . . Blokhin, A. V. (2007). Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proceedings of the National Academy of Sciences, 104*(32), 12982-12987.

Salcher, E. E., Kos, P., Fröhlich, T., Badgujar, N., Scheible, M., & Wagner, E. (2012). Sequence-defined four-arm oligo (ethanamino) amides for pDNA and siRNA delivery: Impact of building blocks on efficacy. *Journal of Controlled Release, 164*(3), 380-386.

Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Fisch, G., . . . Arnold, W. (2006). A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene therapy, 13*(16), 1222-1234.

Sato, A., Takagi, M., Shimamoto, A., Kawakami, S., & Hashida, M. (2007). Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice. *Biomaterials, 28*(7), 1434-1442.

Schwarz, D. S., Ding, H., Kennington, L., Moore, J. T., Schelter, J., Burchard, J., . . . Zamore, P. D. (2006). Designing siRNA That Distinguish between Genes That Differ by a Single Nucleotide. *PLoS Genetics, 2*(9), e140. doi: 10.1371/journal.pgen.0020140

Semple, S. C., Akinc, A., Chen, J., Sandhu, A. P., Mui, B. L., Cho, C. K., . . . Yaworski, E. (2010). Rational design of cationic lipids for siRNA delivery. *Nature biotechnology, 28*(2), 172-176.

Seth, R. B., Sun, L., & Chen, Z. J. (2006). Antiviral innate immunity pathways. *Cell research, 16*(2), 141-147.

Shim, M. S., & Kwon, Y. J. (2012). Stimuli-responsive polymers and nanomaterials for gene delivery and imaging applications. *Advanced drug delivery reviews, 64*(11), 1046-1059.

Sibley, C. R., Seow, Y., & Wood, M. J. (2010). Novel RNA-based strategies for therapeutic gene silencing. *Molecular Therapy, 18*(3), 466-476.

Sørensen, D. R., Leirdal, M., & Sioud, M. (2003). Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *Journal of molecular biology, 327*(4), 761-766.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., . . . Harborth, J. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature, 432*(7014), 173-178.

Spagnou, S., Miller, A. D., & Keller, M. (2004). Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. *Biochemistry, 43*(42), 13348-13356.

Sullenger, B. A., & Cech, T. R. (1994). Ribozyme-mediated repair of defective mRNA by targeted trans-splicing.

Sullenger, B. A., & Cech, T. R. (1994). Ribozyme-mediated repair of defective mRNA by targeted trans-splicing. *Nature, 371*(6498), 619-622.

Thomas, M., Lu, J. J., Ge, Q., Zhang, C., Chen, J., & Klibanov, A. M. (2005). Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proceedings of the National Academy of Sciences of the United States of America, 102*(16), 5679-5684.

Tijsterman, M., Ketting, R. F., & Plasterk, R. H. (2002). The genetics of RNA silencing. *Annual Review of Genetics, 36*(1), 489-519.

Torchilin, V. P. (2005). Recent advances with liposomes as pharmaceutical carriers. *Nature reviews Drug discovery, 4*(2), 145-160.

Urban-Klein, B., Werth, S., Abuharbeid, S., Czubayko, F., & Aigner, A. (2005). RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene therapy, 12*(5), 461-466.

Wagner, E. (2011). Polymers for siRNA delivery: inspired by viruses to be targeted, dynamic, and precise. *Accounts of chemical research, 45*(7), 1005-1013.

Wang, A. Z., Langer, R., & Farokhzad, O. C. (2012). Nanoparticle delivery of cancer drugs. *Annual review of medicine, 63*, 185-198.

Wang, J., Lu, Z., Wientjes, M. G., & Au, J. L.-S. (2010). Delivery of siRNA therapeutics: barriers and carriers. *The AAPS journal, 12*(4), 492-503.

Wang, W., Xiong, W., Wan, J., Sun, X., Xu, H., & Yang, X. (2009). The decrease of PAMAM dendrimer-induced cytotoxicity by PEGylation via attenuation of oxidative stress. *Nanotechnology, 20*(10), 105103.

Wartiovaara, J., Öfverstedt, L.-G., Khoshnoodi, J., Zhang, J., Mäkelä, E., Sandin, S., . . . Skoglund, U. (2004). Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *Journal of Clinical Investigation, 114*(10), 1475.

Werth, S., Urban-Klein, B., Dai, L., Höbel, S., Grzelinski, M., Bakowsky, U., . . . Aigner, A. (2006). A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. *Journal of Controlled Release, 112*(2), 257-270.

Whitehead, K. A., Langer, R., & Anderson, D. G. (2009). Knocking down barriers: advances in siRNA delivery. *Nature reviews Drug discovery, 8*(2), 129-138.

Wisse, E., Jacobs, F., Topal, B., Frederik, P., & De Geest, B. (2008). The size of endothelial fenestrae in human liver sinusoids: implications for hepatocyte-directed gene transfer. *Gene therapy, 15*(17), 1193-1199.

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.

Wong, S. Y., Pelet, J. M., & Putnam, D. (2007). Polymer systems for gene delivery—past, present, and future. *Progress in Polymer Science, 32*(8), 799-837.

Xu, D.-M., Yao, S.-D., Liu, Y.-B., Sheng, K.-L., Hong, J., Gong, P.-J., & Dong, L. (2007). Size-dependent properties of M-PEIs nanogels for gene delivery in cancer cells. *International journal of pharmaceutics, 338*(1), 291-296.

Xu, D., McCarty, D., Fernandes, A., Fisher, M., Samulski, R., & Juliano, R. (2005). Delivery of MDR1 small interfering RNA by self-complementary recombinant adeno-associated virus vector. *Molecular Therapy, 11*(4), 523-530.

Xu, Y., & Szoka, F. C. (1996). Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry, 35*(18), 5616-5623.

Yang, S., Tutton, S., Pierce, E., & Yoon, K. (2001). Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Molecular and Cellular Biology, 21*(22), 7807-7816.

York, A. W., Zhang, Y., Holley, A. C., Guo, Y., Huang, F., & McCormick, C. L. (2009). Facile Synthesis of Multivalent Folate-Block Copolymer Conjugates via Aqueous RAFT Polymerization: Targeted Delivery of siRNA and Subsequent Gene Suppression†. *Biomacromolecules, 10*(4), 936-943.

Yu, B., Zhao, X., Lee, L. J., & Lee, R. J. (2009). Targeted delivery systems for oligonucleotide therapeutics. *The AAPS journal, 11*(1), 195-203.

Yuan, Z. F., Li, F., Ma, M., Cheng, S. X., & Zhuo, R. X. (2011). Synthesis and characterization of poly (ethylene glycol)-b-poly (ε-caprolactone)-b-poly (2-(2-aminoethyl amino) ethyl methacrylate) triblock copolymers as efficient gene delivery vectors. *Journal of Applied Polymer Science, 121*(2), 666-674.

Zamecnik, J., Vargova, L., Homola, A., Kodet, R., & Sykova, E. (2004). Extracellular matrix glycoproteins and diffusion barriers in human astrocytic tumours. *Neuropathology and applied neurobiology, 30*(4), 338-350.

Zamecnik, P. C., & Stephenson, M. L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proceedings of the National Academy of Sciences, 75*(1), 280-284.

Zhang, S., Zhao, B., Jiang, H., Wang, B., & Ma, B. (2007). Cationic lipids and polymers mediated vectors for delivery of siRNA. *Journal of Controlled Release, 123*(1), 1-10.

Zimmermann, T. S., Lee, A. C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., . . . John, M. (2006). RNAi-mediated gene silencing in non-human primates. *Nature, 441*(7089), 111-114.

Zuckerman, J. E., Choi, C. H. J., Han, H., & Davis, M. E. (2012). Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. *Proceedings of the National Academy of Sciences, 109*(8), 3137-3142.