# Polymeric micelles for siRNA delivery

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*Post-transcriptional gene silencing mechanism using small interfering RNA (siRNA) as a potential therapeutic modality has generated great interest in recent years. However, significant hurdles such as off-target effects, possible immunogenicity, in vivo instability and effective delivery to the cellular and intracellular site of action following systemic administration should be overcome before synthetic siRNA can be used as an effective therapeutic agent. Development of several siRNA delivery systems as well as chemical modification of the siRNA structure has been pursued to combat problems associated with therapeutic use of siRNA. Among different structures, polymeric micelles have attracted tremendous attention in recent years as promising carriers for targeted siRNA delivery. The aim of this manuscript is to review different polymeric micelles of siRNA developed to date and provide an overview on the extent of in vitro/in vivo success in siRNA delivery, targeted gene silencing or therapeutic activity by these siRNA delivery systems.*

Key words: *siRNA - Polymeric micelles - Polyion complex micelles - Block copolymer - Gene silencing.*

RNA interference (RNAi) is a sequence specific, evolutionary mechanism where double stranded RNA (dsRNA) is used for the regulation of gene expression. In this post-transcriptional gene silencing mechanism, discovered by A. Fire and C. Mello in *Caenorhabditis elegans* [1, 2], sequence-specific double-stranded small interfering (siRNA) selectively degrades complementary messenger RNAs (mRNA) [3, 4]. Degradation of mRNA occurs when the antisense or guide strand of the siRNA directs the RNA-induced silencing complex (RISC) to cleave the target mRNA [5, 6]. siRNA can be produced either synthetically or from vectors expressing short hairpin RNA (shRNA) that are cytoplasmically cleaved to siRNA. The synthetic siRNA acts in a way similar to natural microRNAs that are used by the cells to regulate many protein-coding genes [7].

siRNA has created much interest as a research tool with high degree of specificity for temporary silencing of the gene of interest to identify their biological function. Therapeutically, RNAi mechanism can be employed to silence any specific gene at will, rendering it more advantageous than typical drugs that may cause widespread toxicity as a result of activity on undesired molecular targets. However, the development of this technology as a ground-breaking therapy with a high degree of specificity for disease related genes has been relatively slow, as compared to its role as a research tool. The slow progress in therapeutic use of siRNA has been mainly due to problems associated with its safe and effective delivery in a clinical setting (*Figure 1*) [8].

The objective of RNAi-based therapeutics is to selectively stimulate targeted mRNA cleavage for effective gene silencing. With the shRNA approach, an oligonucleotide containing the siRNA sequence followed by a 9-nucleotide loop and a reverse complement of the siRNA sequence is cloned into expression vectors to endogenously express hairpin loops with the desired sequence, which is subsequently processed by the dicer enzyme into the desired siRNA [9]. Viral vectors are the most effective shRNA delivery systems due to their innate ability to protect the genetic information in the extracellular environment and to bind and cross plasma membranes in order to deliver their genetic cargo efficiently. The common viral delivery systems used for this purpose include adenoviruses, adeno-associated viruses (AAV), lentiviruses and retroviruses. The attractive aspect of viral delivery is their good ability to transfect clinically-relevant primary cells and mediation of repeatable, long-term protein knockdown [6]. However, high immuno-genicity, potential of saturating the endogenous microRNA pathways, difficulty in constructing selective and effective hairpin RNAs and a
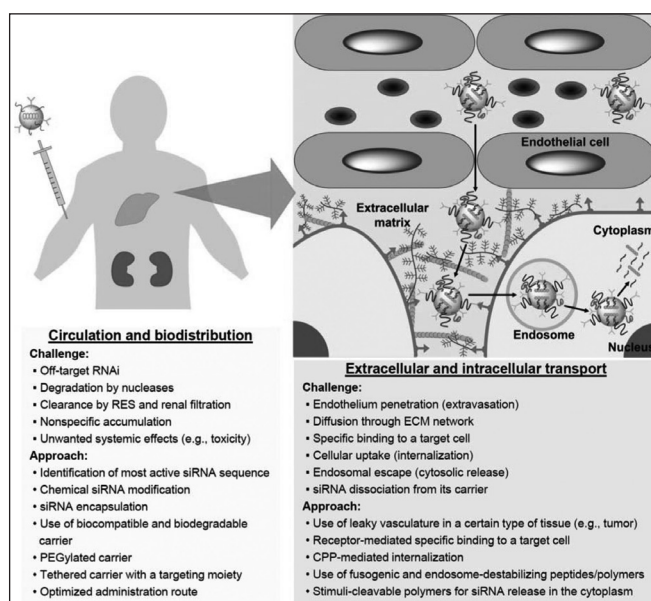


**Figure 1-** Problems associated with the *in vivo* use of siRNA and their possible solutions (reprint from [8] with permission). CPP, cell penetrating peptide; ECM, extracellular matrix.

negative view on the safety of viral vectors, limit application of these vehicles in clinical settings [10-12].

Synthetic siRNAs are less likely to interfere with endogenous microRNA pathways and are less prone to non-specific off-target effects if used at reasonable quantities. They have been successfully used to knockdown targets in different malignancies such as ovarian carcinoma [13], bone tumors [14] and prostate cancer [15]. However, significant hurdles also remain with siRNA for its effective employment as a therapeutic agent (*Figure 1*). In addition to potential off-target effects and instability in serum, the potential for activation of innate immune response by siRNA duplex is a major obstacle [16, 17]. Furthermore, siRNA delivery to the site of action represents a major challenge in the development of RNAi therapeutics [9]. siRNA has a large molecular weight (~13 kDa; ~50 times larger than small molecule drugs [9]) and a very short half-life owing to degradation by nucleases, uptake by the reticuloendothelial system and rapid renal

excretion, all of which can lead to early elimination of the siRNA [18]. The polyanionic nature of siRNA makes it impossible to cross the cell membrane for access to intracellular target [19]. In order to address these problems, chemical modifications on siRNA molecule and/or the development of lipid as well as polymer based carriers of siRNA has been examined.

Chemical modifications on the sugars, backbone or bases of the oligoribonucleotides have been employed to alleviate problems associated with siRNA stability and immunogenicity [20-26]. An important approach to enhance the accessibility of siRNA to its cellular or intracellular targets include conjugation of a variety of small molecules to siRNA [27]. Some of these molecules include cholesterol [28], α-tocopherol [29], lipid [30], TAT [31], penetratin [32], poly(ethylene glycol) (PEG) [33], and antibodies [34]. These modifications are intended to improve the pharmacokinetic behavior, cellular uptake or protein binding of siRNA [35].

The more common approach has been to employ nanocarriers made from lipids or polymers for siRNA delivery. Most lipid-based siRNA delivery systems make use of cationic lipids to form effective complexes with negatively charged siRNA by electrostatic interactions [36]. Cationic lipids are composed of three domains: a positively charged head group, a hydrophobic chain and a linker which connects polar and non-polar groups [37]. Solid lipid nanoparticles [38], liposomes [39-41] and lipoplexes [42, 43] are some of the configurations of lipid-based nanoparticles used in siRNA delivery.

Cationic polymers can also be used to electrostatically bind and entrap siRNA in nanoparticles. Polymeric systems are advantageous compared to their lipid counterparts since their structure can be easily changed and modified in order to obtain desirable physiochemical properties [44]. Natural cationic polymers used for siRNA delivery include chitosan [45,46], atelocollagen [14,47] and cationic polypeptides [48]; the synthetic ones consist of branched or linear polyethyleneimine (PEI) [49-51], poly-L-lysine (PLL) [52] and cyclodextrin [53, 54]. Introducing molecules such as PEG stabilizes these nanoparticles and usually improves their pharmacokinetic profile (i.e., prolongs their circulation time). Introduction of targeting ligands such as RGD peptide or folate to these nanoparticles, makes them more effective in targeting cells expressing their specific receptors.

In recent years, significant progress has been made in the design of polymeric siRNA carriers mimicking structural aspects of viruses. An ideal carrier for systemic siRNA administration should have the following properties: a) be non-toxic and non-immunogenic for systemic human administration; b) condense siRNA efficiently; c) maintain integrity of its content before reaching the target site and avoid rapid elimination from blood circulation; d) reach diseased tissue and specifically interact and get internalized by target cells; and finally e) dissociate in intracellular compartments of the target cell to release the entrapped siRNA, making it accessible to mRNA. Polymeric micelles are one of those carriers, which have a nanoscopic size, are easy to prepare, have the ability to deliver their siRNA cargo to cytoplasm and are versatile enough to be equipped with targeting ligands on the surface and/or fusogenic molecules for enhanced cytoplasmic siRNA delivery in selective cell populations (*Figure 2*). The aim of this review is to provide an overview on the development of viral mimetic polymeric micelles of siRNA and evaluate the success of different designs and structures in this category in *in vitro/in vivo* gene silencing.

## POLYMERIC MICELLES FOR siRNA DELIVERY

Polymeric micelles of siRNA developed to date can be classified under two categories based on their structure: a) polymeric micelles formed through direct conjugation of PEG via degradable or non-degradable linkages to siRNA and further condensation of PEG-siRNA with an siRNA condensing agent (e.g., polycations) to micellar structure (*Figure 3A*); b) polymeric micelles formed by complexation of an amphiphilic block copolymer containing a polycation (and/or lipid)
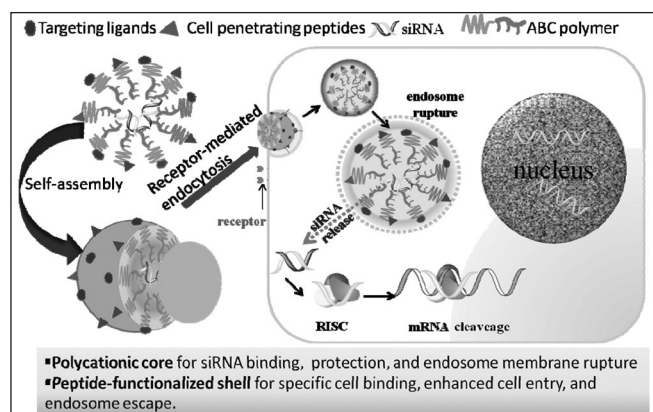


**Figure 2 -** Proposed model for the cellular interaction of ideal polymeric micellar systems for siRNA delivery.
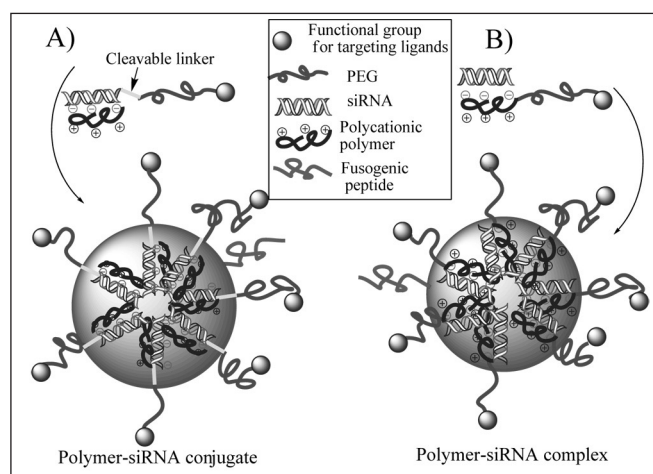


**Figure 3 -** Models of different polymeric micellar structures for siRNA delivery. A) Polymer-siRNA conjugates complexed with polycations. B) Block copolymers complexed with siRNA.

segment with siRNA followed by micellization of block copolymer/siRNA complex (*Figure 3B*). Incorporation of targeting ligands on the surface of carriers and/or fusogenic peptides under each category has also been tried.

## 1. Polymeric micelles based on PEG-siRNA conjugates

Several micelle-like structures that use polymer-siRNA conjugates and siRNA condensing agents have been reported in the literature (*Table I*). In most cases, conjugation of PEG to siRNA is accomplished via disulfide linkages. The use of PEG is expected to reduce the adsorption of proteins (including nucleases) to siRNA *in vivo* and provide means for its stabilization. Disulfide linkages ensure *in vivo* degradability of the siRNA-polymer conjugate inside cells where reducing enzymes cleave the linkage leading to intracellular siRNA release.

One of the siRNA delivery systems under this category has been developed by Kim *et al*. who conjugated vascular endothelial growth factor (VEGF) siRNA to PEG (5 kDa) via a disulfide linkage and interacted these conjugates with PEI (25 kDa), as the siRNA condensing agent, to form polyion complex (PIC) micelles. VEGF is established as a key regulator of angiogenesis, an important stage in the process of tumor growth and metastasis [55, 56]. The PIC micelles formed from siRNA-SS-PEG and PEI were shown to protect siRNA against degradation in serum within 48 hs of incubation. This was in contrast to unmodified siRNA or siRNA-SS-PEG conjugates alone which were degraded within 8 and 16 h after incubation, respectively. The study also provided evidence for the release of free siRNA from the delivery

**Table I -** Polymeric micelles containing PEG-siRNA.

| Carrier composition | Targeted gene | Down regulation of gene compared to control | Dose of si RNA | Efficacy following *in vivo* study | Ref. |
|---|---|---|---|---|---|
| PEG-SS-siRNA/PEI | VEGF | ~ 96 % | 100 nM | NR | [33] |
| PEG-SS-siRNA/PEI | VEGF | ~ 75 % after intratumoral inj., ~ 86 % after intravenous inj. | Multiple intratumoral inj. 500 pmol/multiple intravenous inj. 1.5 nmol | Significant inhibition of tumor growth compared to no treatment | [51] |
| LHRH-PEG-SS-siRNA/PEI | VEGF | ~ 63 % | 50 nM | NR | [57] |
| PEG-SS-siRNA/PEI/ KALA | VEGF | ~ 90 % | 200 pmol/mL | NR | [59] |
| PEG-SS-siRNA/PEI | VEGF | ~ 50 % | 200 pmol | NR | [60] |
| 6PEG-siRNA-Hph1/ KALA | GFP | ~ 69 % | 75 pmol | NR | [61] |
| Lactose-PEG-siRNA/ PLL | Luciferase | ~ 70 % ~ 60 % | 100 nM 10 nM | NR | [62] |

PEG, polyethylene glycol; PEI, polyethyleneimine; LHRH, luteinizing hormone-releasing hormone; 6PEG-siRNA-Hph1, siRNA conjugated to a six-arm polyethylene glycol (PEG) functionalized with a cell penetrating peptide Hph1; PLL, poly-L-lysine; VEGF, vascular endothelial growth factor; Inj., injection; NR, not reported.

system through breakdown of disulfide linkage under reductive conditions using *in vitro* glutathione (GSH) at concentrations analogues to GSH intracellular levels. Whether the breakdown of the disulfide bond follows similar kinetics in cells is not known, however. The PIC micelles showed a siRNA dose dependent (10-100 nM) VEGF gene suppression. A maximum of 96 % inhibition in the expression of VEGF was shown in prostate carcinoma cells (PC-3) at 100 nM of VEGF siRNA. In the absence of the serum, siRNA/PEI complexes were as efficient as PIC micelles in gene silencing. However, a superior gene silencing was observed with micelles in the presence of 10 % fetal bovine serum. The presence of the PEG chains on the corona of the PIC micelles was shown to decrease the adsorption of serum proteins and particle aggregation, and enhance the stability of the formulation [33]. In further studies, these PIC micelles were assessed for *in vivo* silencing of VEGF expression after intravenous and intratumoral administration in a xenograft tumor model (human prostate cancer PC3 cells in nude mice). siRNA formulations containing 0.5 nmol of siRNA were intratumorally administered on days 0, 6, and 15 as well as intravenously at a much higher dose of 1.5 nmol per mouse on days 0, 4, 10, 18, and 28. Upon intratumoral administration, the siRNA-SS-PEG/PEI PIC micelles inhibited the expression of VEGF protein by 75 % compared to no treatment control. This formulation was shown to inhibit the formation of intratumoral microvessles effectively, leading to a significant reduction in tumor size compared to no treatment group (up to 13 % of the size of the tumor in the no treatment group 36 days after treatment). Systemic administration of the PIC micelles resulted in a significant suppression of VEGF protein (up to 86 %) compared to no treatment control. An inhibition of 78 % for microvessel formation was reported for this formulation compared to no treatment control. Tumor volume at 36 days after intravenous treatment with PEG-SS-siRNA PIC micelles was reduced to 25 % of tumor volume in non-treatment control group. Minor antitumor effects were observed for scrambled and naked siRNA. No detectable IFN-α response for the PIC micelle formulation was observed both *in vitro* and *in vivo*, indicating a lack of siRNA mediated immune-stimulation. The results suggested a potential for siRNA-SS-PEG/PEI PIC micelles for suppression of VEGF expression in anti-angiogenic cancer therapy, although off-target effects might be also important in the observed responses [51].

The authors continued this work and generated targeted siRNA PIC micelles through conjugation of luteinizing hormone releasing hormone (LHRH) peptide analogue as a targeting moiety to the PEG end of siRNA-SS-PEG (PEG, 3400 Da) conjugate, followed by mixing this

conjugate with PEI (25 kDa). LHRH-modified PIC micelles exhibited a 50 % increase in uptake when compared to non-targeted micelles in LHRH receptor overexpressing ovarian cancer cells (A2780). As a result, LHRH-modified PIC micelles containing 50 nM siRNA reduced VGEF expression by 63 %, significantly better than their unmodified counterpart, i.e., siRNA-PEG/PEI that reduced VGEF expression by 50 %. In contrast, in SK-OV3 cells that are LHRH receptor negative, the same level of VEGF expression was observed after treatment with LHRH-modified and unmodified PIC micelles containing VGEF siRNA [57]. The results showed the specificity of LHRH modified PIC micelles for LHRH receptor and the superiority of actively targeted formulation over non-targeted one in selective siRNA transfection.

In a separate study, the same group prepared PIC micelles by conjugating siRNA to PEG via disulfide linkage and further condensing it with cationic fusogenic peptide (KALA). The core of the PIC micelles was proposed to contain siRNA/KALA complex, while the surface was surrounded by a PEG shell. KALA is an amphipathic 30 amino acid peptide undergoing conformational change from pH 5.0 to 7.5, resulting in endosomal membrane disruption [58]. Cytotoxicity of KALA peptide was compared to other condensing agents, such as PEI (25 kDa) and PLL (28 kDa). KALA peptide exhibited the lowest cytotoxicity among three cationic species in PC-3 cells. siRNA-PEG/ KALA micelles were used for down-regulation of VEGF in PC-3 cells making comparisons with siRNA-PEG micelles that used PEI or PLL as siRNA condensing agents. Overall, at optimum N/P ratios, siRNA-PEG/PEI micelles exhibited better VEGF down-regulation compared to siRNA-PEG/KALA and siRNA-PEG/PLL micelles, but were also more toxic than the latter two delivery systems. siRNA-PEG/PEI PIC micelles at an optimum N/P ratio of 16 exhibited almost complete inhibition of VEGF expression, while siRNA-PEG/PLL micelles formulated at the same N/P ratio of 16 inhibited less than 40 ± 0.7 % of VEGF expression. siRNA-PEG/KALA PICs at optimum N/P ratio of 6 demonstrated 90 ± 6.6 % of gene inhibition. Silencing of VEGF by siRNA-PEG/KALA PIC micelles was also examined as a function of siRNA concentration in further studies in this paper. At the fixed N/P ratio of 6, the optimum siRNA concentration was 100 nM, which suppressed the VEGF expression by 70 %. The authors suggested the use of KALA (instead of PEI used in a previous paper, ref 51) as a less toxic and effective fusogenic peptide for siRNA condensation and delivery [59].

PIC micelles developed from siRNA-SS-PEG/PEI were also used by Al-Abd *et al.* to study the silencing of VEGF expression in multi cellular layer (MCL) culture of human colorectal cancer using DLD-1

cells. The micelles were prepared by conjugating VEGF-targeted siRNA to PEG (5 kDa) via a disulfide bond followed by simply mixing this conjugate with cationic PEI (25 kDa) as explained in the previous studies [33, 51]. The aim of this study was to mimic the *in vivo* condition of solid tumors and investigate special distribution of siRNA delivery system developed by Kim *et al*. within multiple layers of tumor cells *in vitro*. Naked siRNA was quickly (within 24 h) and evenly distributed in MCL, but distribution of PIC micelles of siRNA was limited to the upper layers of MCL within 24 h and full penetration in a punctuate (rather than uniform) pattern was only seen after 48 h. A dose dependent decrease in VEGF expression at mRNA and protein level was observed in the MCL only after exposure to PIC-siRNA micelles for 72 h. This was in contrast to MCLs treated with naked siRNA which did not show a significant down-regulation of VGEF [60]. The observation was contributed to the instability of naked siRNA in comparison to PIC micelles of siRNA.

Choi *et al*. prepared a GFP-specific siRNA conjugated to a six-arm PEG (6PEG-siRNA) derivative and functionalized it with cell-penetrating peptide Hph1. The 6PEG-siRNA-Hph1 was then electrostatically complexed with cationic self-crosslinked fusogenic KALA peptide (cl-KALA). Naked siRNA and siRNA/cl-KALA complexes degraded in serum after 6 h of incubation, while 6PEG-siRNA, and 6PEG-siRNA-Hph1 conjugates, as well as their complexes with cl-KALA stayed intact. Significant inhibition of GFP expression in GFP transfected MDA-MB-435 cells was observed for 6PEG-siRNA-Hph1/cl-KALA micelles (69 % inhibition at siRNA dose of 75 pmol). In comparison, siRNA/cl-KALA and 6PEG-siRNA/cl-KALA complexes suppressed GFP expressions by 19 and 62 % at the same dose, respectively. As positive controls, both 6PEG-siRNA-Hph1/PEI and 6PEG-siRNA-Hph1/Lipofectamine2000TM suppressed GFP expression by 30 %. The presence of KALA and, particularly, 6PEG in these micelles proved to be valuable in enhancing stability of siRNA and improving gene silencing efficiency of the siRNA delivery system. The cell penetrating peptide Hph1 enhanced intracellular uptake, causing an increase in gene silencing as well [61].

Oishi *et al*. prepared pH-sensitive PIC micelles for effective gene silencing in HuH-7 human hepatoma cells, possessing asialoglycoprotein (ASGP) receptors. The siRNA was conjugated to lactosylated PEG through an acid-labile linkage of β-thiopropionate (targeted for endosomal degradation) followed by complexation with PLL (degree of polymerization of 40). Compared to lactose-PEG-siRNA conjugate alone, lactosylated PIC micelles showed much better RNAi transfection efficacy. The 50 % inhibitory concentration (IC50) of 1.3 nM against expression of firefly luciferase was seen for siRNA formulated in lactosylated PIC micelles in comparison to IC50 of 91.4 nM for lactose-PEG-siRNA conjugates alone. No silencing activity was detected for free siRNA at 100 nM, while lactosylated PIC micelles and lactose-PEG-siRNA conjugates demonstrated 70 and 55 % inhibition of luciferase at the same dose, respectively. At 10 nM siRNA concentration, lactosylated PIC micelles showed the same level of silencing activity to that of oligofectAMINE (around 60 % inhibition); however, after pre-incubation with 50 % serum for 30 min, silencing by oligofectAMINE was decreased (reaching 27 % inhibition), but it remained the same for PIC micelles. Addition of asialofetuin, an inhibitor for ASGP receptor-mediated endocytosis and nigericin, an inhibitor for the endosomal acidification to the cultured media, decreased silencing activity of lactosylated PIC micelles proving the role of lactose and acid-labile linkage contribution in RNAi activity [62].

In general, PEG-siRNA micelles prepared through condensation of PEG-siRNA conjugates with a polycation (e.g., PLL, PEI or KALA) were shown to be more effective siRNA delivery agents than PEG-siRNA conjugates alone in studies described above. It is, however, unclear whether this increase in siRNA transfection by PIC micelles is merely due to the incorporation of polycationic condensing agents

in the micellar structure leading to better uptake/cytoplasmic delivery of siRNA by cells and/or better protection of siRNA by the micellar carrier compared to the PEG-siRNA conjugates against degradation. Effective down-regulation of targeted proteins by PIC micelles, has generally been achieved at relatively high siRNA concentrations (~100 nM). This may enhance the chance of off-target effects by siRNA. Incorporation of cell targeting ligands as well as fusogenic peptides is shown to be an effective strategy for increasing the transfection efficiency and/or selectivity of the polymeric micellar siRNA delivery systems reducing the required dose of siRNA for effective down-regulation of desired genes. Finally, despite extensive *in vitro* studies reflecting a potential for polymeric micellar delivery systems based on PEG-siRNA conjugates, reported data on the *in vivo* efficiency of polymeric micelles under this category is still limited.

## 2. Polymeric micelles based on block copolymer/siRNA complexes

Among polycationic polymers, PEI and PLL are promising synthetic polymers that are able to form non-covalent complexes with siRNAs and effectively deliver siRNA [63] [64]. While the unprotonated amines of PEI create an opportunity for endosomal escape due to the "proton sponge effect", the high density of positive charge on PEI facilitates strong binding to siRNA, which in turn can create a strong protection against enzymatic degradation. Although PEI and PLL have been extensively used in siRNA delivery formulations *in vitro* and *in vivo*, the toxicity caused by nonspecific interactions attributed to the cationic charge of these polymers has been a hurdle for their clinical use. To prevent non-specific interactions, conjugation of hydrophilic or amphiphilic macromolecules to PLL and PEI has been widely investigated and reported in the literature (*Table II*). Most studies have used PEGylted PEI or PLL for siRNA delivery [65],[66],[67],[68]. Chemical modifications of PEI and PLL with cell/tissue-specific ligands were also explored to increase specificity and efficacy for *in vivo* delivery [69].

### 2.1. PEG-PEI

PEGylation of PEI has been used extensively for the development of effective siRNA delivery systems (*Table II*). In this context, Mao *et al*. studied the effect of PEG chain length and graft density on the stability of PEI-PEG polyplexes with siRNA. PEI(25 kDa)-g-PEG copolymers had different PEG chain lengths (550 Da or 2, 5 and 20 kDa), which were substituted on PEI from high to low density (30, 10, 4, and 1 number of PEG blocks per PEI, respectively). PEI(25 kDa)/siRNA polyplexes exhibited the highest stability in heparin displacement assay indicating a strong interaction between PEI and the siRNA. A decline in the stability of polyplexes after PEGylation was observed. The protection was, however, increased with increasing PEG chain length. siRNA/PEI did not provide sufficient protection against RNAase digestion, particularly when compared to PEI(25 kDa)-g-PEG(5 kDa) and PEI(25 kDa)-g-PEG(20 kDa) with respective 4 and 1 number of PEG block per PEI. These results show binding by PEI still exposes the siRNA to nuclease attack and that PEG addition (at relatively higher PEG lengths and low substitution density) is a worthwhile strategy to protect the siRNA. β-galactosidase silencing experiments in NIH/3T3 fibroblasts using the above polymers were conducted. In knockdown experiments, silencing efficiency increased with increasing the PEG chain length up to 5 kDa and simultaneously lowering its substitution density. This polyplex showed 70 % down-regulation of the targeted gene. Introduction of 20 kDa PEG did not exhibit any change in silencing activity in comparison to PEI-PEG(5 kDa) polyplexes. Lipofectamine 2000 exhibited the highest knockdown efficiency in this study. PEI/siRNA showed lower knockdown compared to PEGylated polymers perhaps due to better release of siRNA with PEGylated PEIs [70].

Same group later studied these PEG grafted PEI formulations consisted of either PEG 2 kDa grafted at high density on PEI (25 kDa)

**Table II -** Polymeric micelles containing complexed siRNA.

| Carrier composition | Targeted gene | Down regulation of gene compared to control | Dose of si RNA | *In vivo* study | Ref. |
|---|---|---|---|---|---|
| PEG-PEI | β-galactosidase | ~ 70 % | 50 pmol | NR | [70] |
| PEG-PEI | EGFP | ~ 42 % (*in vivo*) | 50 µg | Intratracheal instillation | [67] |
| PEI-g-PEG | sCLU | ~ 60 % | 400 pmol | NR | [74] |
| PEG-PEI | CD44v6 | ~ 60 % | 20 pM | NR | [77] |
| PEG-PEI | EGFP | ~ 75 % (*in vivo*) | 35 µg | Intratracheal instillation | [78] |
| PEI8.3(C16-C18-EO25)1.4 | EGFP | ~ 69 % (*in vivo*) | 35 µg | Intratracheal instillation | [78] |
| CS-g-(PEI-b-mPEG) | IKKβ | ~ 40 % | 50 nM | NR | [81] |
| PEG-PEI | VEGF | 68 % | 50 nM | NR | [82] |
| PEI-PEG-PCP | VEGF | 79 % | 50 nM | NR | [82] |
| PEI-PEG-FOL | GFP | ~ 75 % | 0.5 µg | NR | [83] |
| PLL/PEG/DMMAn-Mel | Luciferase | ~ 90 % | 500 ng | NR | [84] |
| PEI/PEG/DMMAn-Mel | Luciferase | ~ 60 % | 500 ng | NR | [84] |
| PLL-g-PEG | NR | NR | NR | Increase in siRNA half life upon intravenous inj. | [86] |
| PEG-b-(PLL-IM) | Pp-Luc | ~ 80 % | 100 nM | NR | [87] |
| FA-PEG-PGA/PEI-PCL | Bcl-2 | ~ 90 % | 20 nM | NR | [105] |
| mPEG-b-PCL-b-PPEEA | GFP | ~ 70 % | 150 nmol/L | NR | [89] |
| PDMAEMA-PCL-PDMAE-MA | GFP, VEGF | ~ 70 % for GFP and ~ 85 % for VEGF | 0.5 µg/mL | NR | [90] |
| PEO-b-PCL-SP | P-gp | ~ 50 % | 300 nM | NR | [19] |
| RGD4C/TAT decorated PEO-b-PCL-SP | P-gp | ~ 55 % | 100 nM | NR | [93] |
| pDMAEMA-b- pDbB/pSMA | Plk1 | ~ 60 % | 50 nM | NR | [98] |
| PAMAM/PEG-b-P(PrMA-co-MAA) | Bcl-2 | 60 % | 25 nM | NR | [99] |
| PEG-b-P(PrMA-co-MAA)/PAMAM | Bcl-2 | ~ 60 % | 50 nM | NR | [100] |
| PMAA-b-PEG/PLL | EGFP | Qualitative down-regulation was observed | 2.5 nM | NR | [101] |
| poly[(DMAEMA)-b-(BMA)-co-(DMAEMA)-co-(PAA)] | GAPDH | ~ 90 % | 100 nM | NR | [102] |
| PAsp(-SS-siRNA)/PAsp(DET) | Luciferase | ~ 80 % | 100 nM | NR | [103] |
| siRNA-SS-PE/PEG-PE | GFP | ~ 28 % | 84 nM | NR | [104] |

PEG, polyethylene glycol; PEI, polyethyleneimine; EGFP, enhanced green fluorescent protein; sCLU, signalling peptide of secretory clusterin; PEI8.3(C16-C18-EO25)1.4, low molecular weight PEI 8.3 kDa with a fatty acid modification, namely palmityl-stearyl-(C16-C18-) modified hydrophobic PEG; CS-g-(PEI-b-mPEG), linear poly(ethyleneimine) blocked with polyethylene glycol and grafted onto a chitosan; IKKb, IkB kinase subunit b; VEGF, vascular endothelial growth factor; PEI-PEG-PCP, prostate cancer binding peptide (PCP) conjugated with polyethylenimine (PEI) via a poly(ethylene glycol); PEI-PEG-FOL, PEI-poly(ethylene glycol)-folate; GFP, green fluorescent protein; PLL, polylysine; DMMAn, dimethylmaleic anhydride; Mel, melittin; PLL-g-PEG, poly(L-lysine)-graft-poly(ethylene glycol); PEG-b-(PLL-IM), iminothiolane-modified poly(ethylene glycol)-block-poly(L-lysine); Pp-Luc, photinus pyralis luciferase; FA-PEG-PGA, folic acid-poly(ethylene glycol)-block-poly(glutamic acid); PEI-PCL, poly(e-caprolactone) (PCL) and linear poly(ethylene imine); mPEG-b-PCL-b-PPEEA, monomethoxy poly(ethylene glycol)-b-poly(3-caprolactone) and poly(2-aminoethyl ethylene phosphate); PDMAEMA-PCL-PDMAEMA, 2-(N,Ndimethylaminoethyl) methacrylate-poly caprolactone-2-(N,N-dimethylaminoethyl) methacrylate; P-gp, P-glycoprotein; PEO-b-PCL, poly(ethylene oxide)-block-poly(e-caprolactone); SP, spermine; TP, tetraethylenepentamine; DP, N,N-dimethyld-ipropylenetriamine; pDMAEMA, dimethylaminoethyl methacrylate; pDbB, butyl methacrylate; pSMA, poly(styrene-alt-maleic anhydride); plk1, polo-like kinase 1; PEG-b-P(PrMAco-MAA), poly(ethylene glycol)-b-poly(propyl methacrylate-co-methacrylic acid); PAMAM, poly(amido amine); PMAA-b-PEG, polymethacrylic acid-b-polyethylene oxide; polyDMAEMA, poly(N,N-dimethylaminoethyl methacrylate); BMA, butyl methacrylate; PAA, proplylacrylic acid; GADPH, glyceraldehydes phosphate dehydrogenase; PAsp, poly(aspartic acid); PAsp(DET), poly(aspartamide) having 1,2-diaminoethane side chains; PE, phosphothioethanol; NR, not reported.

or PEG 20 kDa grafted at low density on PEI on the knockdown of enhanced green fluorescence protein (EGFP) by 25/27mer 2'-OMe siRNA after intratracheal instillation in mice. Stability of polyplexes in terms of siRNA release in the presence of mucin and lung surfactant (Alveofact) was measured to see if the polyplexes can penetrate the airway lineaging fluid as intact nano-carriers. Similar to the results of previous studies, PEI complexes were found to be more stable and released less siRNA in the presence of Alveofact or mucin compared to PEG-PEI complexes *in vitro*. Between the two PEG-PEI complexes, the complex containing 2 kDa PEG at higher grafting density on PEI provided less siRNA release compared to the one with 20 kDa PEG with lower grafting density on PEI. In mice administered with siRNA/radiolabelled PEG2 kDa-PEI polyplexes, the radioactive signal was still present in the mouth and upper trachea, 24 h post treatment. The radioactive labelled polymer was excreted in the kidneys at 48 h but the strongest signal was still observed in the lung and radioactive material was still present in lower trachea. Interestingly, for labelled polymers the residence time in the trachea was decreased with PEGylation. In comparison, when siRNA was labelled no accumulation in trachea was seen. The complexed siRNA seemed to be released from the carriers, especially the PEI/siRNA complexes, and cleared in a similar fashion to that of free siRNA. These results suggest that PEI/siRNA complexes were less stable compared to PEG-PEI/siRNA and behaved similar to free siRNA *in vivo*. To confirm the effectiveness of intratracheal siRNA delivery of PEG-PEI/siRNA complexes, actin-EGFP expressing mice were treated with polyplexes containing 50 μg of anti-EGFP siRNA. The EGFP expression was down-regulated by 42 % by PEG-PEI complexes of 2 kDa PEG compared to the same formulation containing control irrelevant siRNA. No comparison to PEI/siRNA complexes in EGFP down-regulation studies was conducted, so that the beneficial effect of PEG on efficacy cannot be evaluated[67]. Upon intravenous (i.v.) administration, radiolabeled PEI polyplexes have shown high degree of liver uptake, and PEGylation lowered the uptake of polyplexes by the liver [71]. An inverse relationship between the liver accumulation of radiolabeled siRNA by its polyplexes and siRNA release from the carriers was observed.

Sutton *et al*. synthesized a series of graft copolymers of PEI(10 kDa)-g-PEG(2 kDa) with grafting PEG densities of 5.7, 9.7 and 15.4 per PEI chain (polymers named 2 k5, 2 k10, and 2 k15, respectively) for siRNA delivery. They targeted the signalling peptide of secretary clusterin (sCLU) in human breast cancer MCF-7 cells. sCLU is cytoprotective protein, overexpressed in different cancers and functions to protect cells from ionizing radiation (IR) injury, as well as chemotherapeutic agents [72, 73]. Among these copolymers, 2 k10 exhibited the highest siRNA binding forming the smallest complexes. Although no significant toxicity was detected in MCF-7 cells after exposure to 2 k10, or 2 k10-siRNA complexes at 5 N/P ratio, N/P ratios of 7 and 14 gave significant toxicities (75 % and 50 % cell viability, respectively). A siRNA semi-dose dependent decrease (200-800 pmol) up to 60 % in sCLU expression has been detected for nano-complexes containing siRNA-sCLU, with similar inhibition at 400 and 800 pmol. The 2 k10/siRNA-sCLU nano-complexes exhibited an increase in IR lethality (> 3-fold) over complexes containing scrambled siRNA [74].

Wu *et al*. used PEG(2 kDa)-PEI 25(kDa) with a PEG graft density of 10 as siRNA carriers to target CD44v6 in gastric carcinoma SGC7901 cells. CD44 is a cell adhesion molecule involved in extracellular matrix binding, cell migration, and lymphocyte homing [75]. One of its isoforms, CD44v6 plays an important role in tumor progression and metastasis of gastric cancer [76]. PEG-PEI complex exhibited lower cytotoxicity compared to PEI. The cytotoxicity was slightly increased with increasing N/P ratios. At an N/P ratio of 15 the SGC7901 cell viability was 80 %, similar to what was observed for Lipofectamine 2000/siRNA complexes. Transfection efficiencies of PEG-PEI/siRNA at N/P 15 and 30, was slightly higher than that of Lipofectamine 2000/siRNA and PEI/siRNA. The expression of CD44v6 was decreased up

to 60 % when using PEG-PEI/siRNA at N/P ratio of 15 compared to no treatment and negative control siRNA/PEG-PEI complex group [77].

Beyerle *et al*. investigated the *in vivo* efficacy and lung toxicity of two different types of PEI complexes for siRNA in mice after intratracheal administration. One of the polymers was composed of 25 kDa PEI grafted with hydrophilic 2 kDa PEG (1:1 ratio). The other polymer consisted of low molecular weight PEI (8.3 kDa) with a fatty acid modification, i.e., a mixture of palmityl (C16)/stearyl (C18) modified 1.4 kDa PEG [PEI8.3(C16-C18-EO25)1.4]. The hydrophilic PEG modification reduced the cytotoxicity, but increased the immune response and caused pro-inflammatory effects. Increased levels of IgM in broncho-alveolar fluid (BALF) have been observed with PEG-PEI. The PEI8.3(C16-C18-EO25)1.4 exhibited acute proinflammatory effects as well. In comparison, both high and low molecular weight PEI caused mild inflammation despite of high toxicity. *In vivo* knockdown efficiency with 35 μg siRNA was investigated against EGFP in comparison to unmodified PEI complexes. Although an EGFP knockdown of 75, 66 and 69 % was achieved for PEG-PEI, 8.3 kDa PEI and PEI8.3(C16-C18-EO25)1.4, respectively, these polyplexes also caused a non-specific knockdown of EGFP with non-specific siRNA against luciferase (GL3) [78].

Duan *et al*. synthesized a ternary cationic copolymer composed of linear blocks of PEI and PEG, grafted onto chitosan, chitosan-g-(PEI-b-mPEG). The effect of siRNA mediated down-regulation of IκB kinase subunit β (IKKβ) on the proliferation of human Tenon's capsule fibroblasts (HTFs) was investigated. IκB kinase (IKK) protein has been shown to regulate the activation of transcription factor NF-κB that has a regulatory role in fibroblasts proliferation [79, 80]. Chitosan-g-(PEI-b-mPEG) exhibited significantly higher *in vitro* cell viability compared to 25 kDa PEI at concentrations of 10 μg/mL and above. Transfection with IKKB-siRNA complex (5-50 nM of siRNA) resulted in suppression of mRNA transcription for IKKβ in the HTFs in a dose-dependent manner up to 55 % compared to cells treated with scrambled siRNA. IKKB-siRNA complexes targeting IKKβ decreased HTF proliferation in a dose-dependent manner up to 40 % at a siRNA dose of 50 nM. The authors concluded that the use of these cationic complexes may be a safe and effective anti-scarring method following glaucoma filtration surgery [81].

Second generation of PEG-PEI polyplexes containing targeting ligands on the PEG end has also been developed and studied for siRNA delivery. Prostate cancer-binding peptide (PCP) was conjugated to PEI via a PEG linker (PEI-PEG-PCP) for VEGF gene silencing in PC3 cells. Positively charged PEI-PEG-PCP conjugate successfully formed polyplexes with negatively charged siRNA. The VEGF silencing efficiency was dependent on both N/P ratio and siRNA concentration with complete suppression achieved using polyplexes at N/P ratio of 24 and ≥ 50 nM of VEGF siRNA. In the absence of serum, the highest VEGF silencing was achieved by siRNA/PEI polyplexes, exhibiting 83 % down-regulation. PEI-PEG-PCP/siRNA and PEI-PEG/siRNA exhibited 79 and 60 % down-regulation under serum free condition, respectively. In the presence of serum, the highest down-regulation of VEGF was achieved with PEI-PEG-PCP/siRNA showing 68 % silencing followed by PEI/siRNA and PEI-PEG/siRNA with 51 and 48 % gene silencing, respectively [82].

Using a PEI-g-PEG-folate (PEI-PEG-FOL) polymer, Kim *et al*. compared the silencing abilities of an oligodeoxynucleotide (ODN), siRNA, and siRNA expressing plasmid. The PEI and PEI-PEG-FOL complexes exhibited comparable sizes of ~ 110 nm and surface charge of 1.5 mV regardless of the type of the incorporated nucleic acid. Target-specific inhibition of GFP expression was measured for the complexes in folate receptor overexpressing KB cells. At a siRNA dose of 0.5 μg, PEI-PEG-FOL complexes of siRNA exhibited highest level of GFP down-regulation (75 % down-regulation at an N/P ratio of 16). PEI-PEG-FOL complexes with 2 μg ODN and 2 μg

plasmid siRNA reduced GFP expression by 69 and 59 %, respectively. Comparatively, PEI complexes of the same dose of siRNA, ODN and plasmid siRNA exhibited 22, 26 and 20 % down-regulation, respectively. These results indicated the superiority of PEI-PEG-FOL over PEI complexes. Mechanistic studies as well as comparisons between targeted and non-targeted PEI-PEG siRNA complexes that can shed light on the reason behind this observation were not reported in this study [83].

In general, different studies on the effect of PEGylation of PEI on the stabilization of siRNA, its release from the delivery system and the cytotoxicity of polyplexes points to a positive impact for PEGylation of PEI for siRNA delivery. The final outcome, however, will very much be dependent on the density and length of conjugated PEG as well as PEI architecture and N/P ratio in the PEG-PEI/siRNA complexes. On the other hand, the protective effect of PEG-PEI seems to compensate for the negative impact of PEG on the condensation of siRNA and its cellular uptake, positively influencing the overall transfection efficiency and silencing activity of the PEG-PEI delivery systems of siRNA in comparison to PEI complexes. Systematic studies are required to achieve optimized PEG/PEI based delivery systems of siRNA for maximum effect as well as minimum toxicity for *in vivo* use.

**2.2. PEG-PLL**

Similar to PEI, PLL have been the subject of extensive studies for siRNA delivery. PLL based carriers may be advantageous over PEI because of better biocompatibility and biodegradability profile. Similar to observations with PEI, PEGylation of PLL have produced positive impact on transfection efficiency of complexed siRNA perhaps by providing a better siRNA protection.

In this context, PLL (32 kDa) and PEI (branched, 25 kDa) were PEGylated (5 kDa) and further coupled with dimethylmaleic anhydride-melittin (DMMAn-Mel) via the N-terminal cystein. DMMAn acts as a protective group for melittin peptide which is a pH responsive peptide with lytic activity. At endosomal pH, the DMMAn protecting groups are cleaved and lytic activity of melittin is exposed. siRNA silencing ability of these conjugates was measured in mouse neuroblastoma cell line stably expressing luciferase (Neuro 2A-eGFPLuc). At 500 ng of siRNA, neither PLL nor PEI (with or without PEG) exhibited any siRNA knockdown activity, while silencing was achieved with PLL-PEG-DMMAn-Mel/siRNA (90 %) and PEI-PEG-DMMAn-Mel/siRNA (60 %). Conjugation with DMMAn-Mel not only lowered the acute toxicity of PLL but combined with PEG increased the siRNA delivery ability of the polycation [84]. The same group also attached the siRNA to PLL backbone by a disulfide bond. This pH and redox sensitive PEG-PLL-DMMAn-Mel-siRNA conjugate was compared to the analogous electrostatic polyplex. The covalent binding of the siRNA improved the stability against natural occurring polyanions like heparin. Luciferase silencing activity of the conjugate as well as the viability of treated cells was evaluated using Neuro2A-eGFPLuc-cells. A siRNA dose dependent silencing activity was observed for both conjugate and polyplexes up to 90 %. However, MTT assay showed higher cytotoxicity for polyplex compared to conjugate. *In vivo* studies in mice indicated a high systemic toxicity for the siRNA conjugate upon systemic or even intratumoral administration, with visible liver damage, limiting the formulation applicability [85]. No comparison was made with the siRNA complexes in *in vivo* studies.

Sato *et al.* synthesized a series of cationic comb-type copolymers (CCCs) with a PLL backbone and PEG side chains for prolonged blood circulation. Distinct polymers, one with 10 wt. % PLL and 90 wt. % PEG, and another with 30 wt. % PLL and 70 wt. % PEG were prepared. Cationic comb-type copolymers with a higher density of PEG chains exhibited stronger interaction with siRNA than the one with lower density. The CCC/siRNA complex with a higher PEG density exhibited resistance to nucleases for 24 h *in vitro*. This stability was

lower for lower PEG density polyplexes and jet PEI. *In vivo* stability for CCC/siRNA complexes was evaluated in mice as well. Both naked siRNA and jet PEI/siRNA injected into mice disappeared at 5 min after injection. In contrast, CCC/siRNA complex with long cationic backbone and higher PEG content retained the siRNA in bloodstream by ≥100-fold. Higher stability of CCC complex with 27-bp dsRNA over complex with 21-bp siRNA has been observed, perhaps due to increased electrostatic interaction between the cationic backbones (PLL) and the longer siRNA [86]. Interestingly, the half-life of siRNA upon preinjection of CCC was increased by more than 60 fold compared to siRNA alone, but this increase did not reach the level achieved by CCC/siRNA complexes.

PIC micelles were prepared from iminothiolane-modified PEG-b-PLL [PEG-b-(PLL-IM)] where the mercaptopropyl groups were used to prepare disulfide cross-linked micelles. The presence of both disulfide cross-links and amidine groups was suggested to increase the stability of the micelles. These PIC micelles exhibited good stability at physiological salt conditions contributing to the protection of micellar structure compared to PEG-b-PLL/siRNA system. Dose-dependent siRNA transfection efficiencies were evaluated in Huh7 cells; at a dose of 100 nM siRNA concentration, PIC micelles exhibited 80 % down-regulation of luciferase while the non-cross-linked PIC assemblies with PEG-b-PLL exhibited 20 % silencing [87]. The superior transfection efficiency of PIC micelles was attributed to the efficient protection of incorporated siRNA by the cross-linked micellar structure.

**2.3. Polymeric micelles based on other amphiphilic copolymers for siRNA complexation**

In addition to PEI and PLL, other polycations have also been incorporated in the structure of amphiphilic block copolymers and used for siRNA condensation and delivery. For instance, Cao *et al.* developed PEI-poly(ε-caprolactone) (PEI-PCL) micelles that were used for co-delivery of doxorubicin (DOX) and siRNA against anti-apoptotic Bcl-2 protein to human hepatic cancer cells Bel-7402 [88]. PEG-b-poly(glutamic acid) conjugated to folic acid (FA-PEG-PGA) was then added to coat the nanocarrier through electrostatic interactions between exposed PEI and PGA. A coating of FA-PEG-PGA on the surface of cationic micelles decreased the cytotoxicity compared to cationic micelles alone. The FA formulation showed a 2-fold increase in cell uptake and a 3-fold decrease in the level of Bcl-2 mRNA expression when compared to unmodified formulation. The FA modified formulation containing Bcl-2 siRNA decreased the level of Bcl-2 mRNA by 10-fold compared to formulation containing scrambled siRNA. The FA modified formulation containing both Bcl-2 siRNA and DOX showed the highest cell apoptosis (84 %) compared to formulations containing either Bcl-2 siRNA or DOX alone (23 % and 43 % apoptosis, respectively) indicating the synergistic effect of delivering both DOX and Bcl-2 siRNA.

Sun *et al.* synthesized an amphiphilic triblock copolymer consisting of monomethoxy PEG, PCL and poly(2-aminoethyl ethylene phosphate) denoted as mPEG-b-PCL-b-PPEEA and used it for siRNA delivery. The micellar structures exhibited prolonged stability when incubated with bovine serum albumin due to PEG segment preventing particle aggregation. Fluorescent microscope images indicated partial internalization of micelles, followed by the dissociation of internalized micelles in the cytoplasm and release of siRNA. This was attributed to the deprotonization polyphosphoester block in cytoplasm leading to siRNA release. Delivery of siRNA at 150 nM and N/P ratio of 50:1 significantly inhibited GFP expression (by 70 %) in HEK293 cells. This level of gene silencing was similar to that by Lipofectamine 2000 delivery at 20 nM siRNA. Interestingly, the micelles at N/P ratio of 100:1 exhibited ≥ 90 % silencing that surpassed what observed with Lipofectamine 2000. The advantage of this triblock copolymer based delivery system was its superior cytocompatibility compared to Lipofectamine 2000 rendering it useful for siRNA delivery [89].

Zhu *et al*. prepared cationic micelles from dimethylaminoethyl methacrylate-PCL-dimethylaminoethyl methacrylate (PDMAEMA-PCL-PDMAEMA) triblock copolymers for simultaneous delivery of siRNA and paclitaxel. The triblock copolymer was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization of dimethylaminoethyl methacrylate (DMAEMA) using CPADN-PCL-CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent. PDMAEMA-PCL-PDMAEMA micelles, particularly those with lower PDMAEMA molecular weights, exhibited lower toxicity compared to PEI 25 kDa. Micelles containing the shortest PDMAEMA chains (2.7 kDa), showed the highest transfection efficiency reducing GFP expression up to 70 % at siRNA dose of 0.5 ug/mL, compared to PEI which only inhibited GFP expression by 40 %. PDMAEMA homopolymer did not show any silencing efficiency. Co-delivery of VEGF siRNA and paclitaxel in PC-3 cells caused 85 % silencing efficiency. This level of silencing was higher than that of micelles/VEGF siRNA alone which reduced the gene silencing by 70 %. The effect of combination therapy on the *in vitro/in vivo* cell viability was not reported. Nevertheless, the results showed a potential in combination cancer therapy with siRNA and chemotherapeutic drug [90].

Xiong *et al*. derivetized the biodegradable PEG-b-PCL with polyamine side chains spermine (SP), tetraethylenepentamine (TP) and N,N-dimethyldipropylenetriamine (DP) for siRNA delivery. These polymers were able to effectively bind siRNA, self-assemble into micelles, protect siRNA from degradation by nuclease and release siRNA efficiently in the presence of low concentrations of anionic heparin. siRNA formulated in PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) micelles showed efficient cellular uptake through endocytosis by drug resistant MDA435/LCC6/MDR cells which over express MDR-1 gene encoding for P-glycoprotein (P-gp). P-gp is the most abundant protein pump involved in ATP dependent efflux of chemotherapeutic drugs in cancer [91, 92]. PEO-b-P(CL-g-TP) and PEO-b-P(CL-g-SP)/MDR-1 siRNA complex micelles were able to down-regulate the P-gp expression up to 60 % and 50 %, respectively, at a dose of 300 nM. This is a relatively high dose, consistent with the intended use of these micelles (i.e., systemic application) [19].

In the continuation of this study, PEO-b-PCL micelles containing polyamines in their core were decorated with integrin αvβ3 targeting peptide (RGD4C) and/or cell penetrating peptide (TAT) on the PEO shell. An increase in cellular uptake and effective endosomal escape of delivered siRNA has been observed with RGD/TAT micelles compared to unmodified micelles in MDA435/LCC6/MDR cells. RGD/TAT micelles caused a 70 % decrease in mdr-1 mRNA expression and a 55 % reduction of P-gp protein levels at dose of 100 nM siRNA. A 3-fold lower dose of siRNA was required by the RGD/TAT modified micelles compared to unmodified ones to cause similar level of P-gp down-regulation. As a result of P-gp down-regulation by RGD/TAT micelles delivering mdr-1 siRNA, a 2-fold increase in the intracellular levels of DOX in MDA435/LCC6/MDR cells has been observed compared to unmodified micelles. Assessment of the effect of P-gp silencing on cytotoxicity of DOX showed a 3-fold increase in the cytotoxicity to DOX obtained with RGD/TAT micelles delivering mdr-1 siRNA compared to unmodified micelles [93].

More recently, micelles were prepared from PEO-b-P(CL-SP) and a PEO-b-PCL block copolymer with pendent DOX attached on PCL backbone through pH sensitive hydrazone linkages PEO-b-P(CL-DOX) [94]. The PEO-b-P(CL-SP) and PEO-b-P(CL-DOX) were partially modified with TAT and RGD4C peptides on the PEO end. The prepared micelles had a versatile core that could stably complex siRNA and conjugate DOX via a pH sensitive linkage, and a virus-like shell for cell specific recognition and efficient cellular uptake. The peptide-functionalized micelles demonstrated significant cellular uptake, pH-triggered DOX release, improved DOX penetration into nuclei and enhanced DOX cytotoxicity in the DOX resistant MDA-

MB-435/MDR cells when compared to unmodified micelles. Incorporation of fluorescent probes in the micellar core through covalent linkages allowed for *in vivo* tracking of micelles providing evidence for tumor-targeted delivery of RGD decorated micelles and incorporated siRNA in animal models following intravenous administration of this system.

In another study, diblock copolymers of dimethylaminoethyl methacrylate (pDMAEMA) and butyl methacrylate (pDbB) self-assembled to form micelles with hydrophobic core and cationic shell. These micelles were electrostatically complexed with siRNA and pH-responsive poly(styrene-alt-maleic anhydride) (pSMA) in order to form ternary complexes. siRNA against Polo-like kinase 1 (Plk1) was included in this complex for delivery to the drug-resistant NCI/ADR-RES ovarian cancer cell model and in the drug-sensitive parental cell line, OVCAR8. Polo-like kinase 1 has a critical role in cell cycle-regulatory function and cell division. Its over-expression has a potential role in tumorigenesis by supporting chromosome instability and aneuploidy [95-97]. Compared with the commercially available HiPerFect™ , the ternary complexes nearly showed 2-fold higher FAM siRNA positive cells. Ternary complexes reduced the Plk1 gene expression by ~ 60 % after treatment with 50 nM siRNA, similarly in both cell lines. The level of reduction in gene expression was less (25 %) with HiPerFect, a commercial transfection reagent. However, ternary complexes showed much lower toxicity compared to HiPerFect. Plk1 knockdown by ternary complexes of PlK1 siRNA led to increased susceptibility of both sensitive OVCAR8 and resistant NCI/ADR-RES cells to DOX. The IC50 was reduced 10-fold in OVCAR8 cells and 20-fold in NCI/ADR-RES cells. Dual delivery of siRNA and DOX exhibited a less effective cell killing than when siRNA and DOX were delivered separately due to what authors described as limitations in DOX loading in micelles containing siRNA.[98]

Elsabahy *et al*. developed pH-responsive PIC micelles consisting of a poly(amidoamine) (PAMAM) dendrimer-nucleic acid core and a detachable PEG-b-poly(propyl methacrylate-co-methacrylic acid) (PEG-b-P(PrMA-co-MAA)) shell for the delivery of siRNA against Bcl-2. The shell and the core of the micelles were kept together by electrostatic interaction between cationic PAMAM and anionic MAA moieties of PEG-b-P(PrMA-co-MAA). PEG segments of the micelles were decorated with Anti-CD71 (i.e., transferrin receptor) Fab' targeting agent via disulfide linkage. Fab'-PIC micelles containing 25 nM siRNA exhibited up to 60 % down-regulation of Bcl-2 in PC-3 cells, as compared to PAMAM-siRNA or non-targeted PIC micelles which showed 10 and 20 % down-regulation, respectively [99].

The same group further functionalized micelles with anti-CD71 Fab' via a maleimide/activated ester bifunctional linker. They suggested this bond to be a more stable bond than that of disulfide linkage. Micelles exhibited relative stability in serum with more than 50 % intact siRNA after 4 h compared to naked siRNA which degraded completely within 50 min. Targeted PIC micelles showed significantly higher cellular uptake in PC-3 cells when compared to native untargeted PIC micelles. Pre-incubation of the cells with free anti-CD71 antibody caused a reduction of uptake suggesting that Fab'(CD71)-PIC are taken up via transferin receptor. Additionally, the targeted PIC micelles suppressed the expression of Bcl-2 gene (60 %) at a dose of 50 nM siRNA. However, this inhibition was less than Lipofectamine (80 %). Non-targeted PIC or targeted PIC containing mismatched siRNA sequence, both decreased Bcl-2 expression only by 20 %, indicating off-target effects by these formulations [100].

pH-sensitive polyion complex micelles, formed from a tripartite association by PMAA-b-PEG, cationic PLL and anionic siRNA have been prepared by Boudier *et al*. pH sensitivity of the siRNA loaded micelles was evaluated at pH 5 and 7.4 and the results showed complete dissociation of the micelles under acidic conditions. These micelles were successfully endocytosed by dendritic cells (DC) obtained from Balb/C transgenic mice. Using DCs from eGFP transgenic mice,

silencing of EGFP expression was observed by confocal microscopy in cells treated with polyplexes of siRNA against EGFP compared to cells treated with irrelevant siRNA loaded micelles (48 h with 2.5 nM siRNA) [101].

Convertine *et al.* synthesized a diblock copolymer consisting of a cationic PDMAEMA block, and a hydrophobic block composing of positively charged DMAEMA residues, negatively charged pro-pylacrylic acid (PAA) residues, and hydrophobic butyl methacrylate (BMA) residues. The first block is used for siRNA binding while the second block has pH-responsive membrane disruptive properties. These micelles showed hemolytic activity in red blood cells as a sign of membrane destabilizing function with reduction of the pH from extracellular to acidic endosomal pH. Formulations containing siRNA against glyceraldehydes phosphate dehydrogenase (GAPDH) exhibited up to 90 % down-regulation in Hela cells at 100 nM siRNA concentration [102].

Takemoto *et al.* used siRNA-grafted poly(aspartic acid) [PAsp(-SS-siRNA)], for polyion based siRNA delivery. siRNAs were grafted to a backbone of PAsp derivative via disulfide bond. For formation of PICs, PAsp(-SS-siRNA) was complexed with a poly(aspartamide) having 1,2-diaminoethane side chains [PAsp(DET)]. The ability of PICs in luciferase silencing was evaluated in B16F10-Luc cells at 100 nM siRNA. PAsp(-SS-siLuc)/PAsp(DET) PICs caused 80 % gene silencing compared to siLuc/PAsp(DET) PICs which only exhibited 30 % down-regulation. When ExGen500, a linear PEI-based commercial transfection reagent has been used as the polycation in PICs, similar gene-silencing efficiency was achieved. No significant decrease of cell viability was observed with PICs compared to siRNA and PAsp(-SS-siRNA) indicating the absence of toxicity. Furthermore, the absence of IFN-α production from cells treated with PICs indicated no immunogenicity elicitation by the developed delivery system [103].

An alternative structure in this category has been developed by Musacchio *et al.*, who reported conjugation of GFP-specific siRNA to a phospholipid structure, i.e., phosphothioethanol (PE), via a disulfide linkage. The PE structure was expected to facilitate the incorporation of siRNA in PEG-PE micelles. siRNA-SS-PE/PEG-PE mixed micelles were prepared by mixing the conjugate with PEG-PE. Unlike previous studies, a lipid structure (not a polycation) was used in the formation of polymeric micellar siRNA delivery system in this construct. Using C166-GFP endothelial cells no toxicity of siRNA-SS-PE/PEG-PE mixed micelles were seen, unlike siRNA formulated with the Lipo-fectamine which showed 20 % cell viability. In stability studies in the presence of RNase III from *E. coli*, no degradation products of siRNA-conjugate were detected after 24 h when it was incorporated in micelles, whereas siRNA-SS-PE degraded after 6 h. The mixed siRNA-SS-PE/PEG-PE micelles caused a 28 % GFP down-regulation, a 50-fold increase compared to naked siRNA which caused only a 0.5 % reduction in GFP expression [104]. A high molar ratio of PEG-PE to siRNA-SS-PE (750:1) was needed in the mixed micellar composition, to achieve this degree of gene silencing (28 %) by the system.

\*

The design of the efficient siRNA delivery systems has been under intense research and development over the past decade. Polymeric micelles have been focused on, due to the fact that their structure can be easily changed and modified to obtain desirable physiochemical properties for optimum biological effects. The micellar structure with PEG or other hydrophilic polymers on the surface has been shown to lead to better protection of incorporated siRNA against degradation, protein adsorption and early elimination, but it may at the same time jeopardize the carrier interaction with the desired cell population. In this regard, the density and length of PEG substitution on the carrier can be modified to provide a balance between the siRNA stability and cell uptake to achieve maximum transfection efficiency. On the other hand, while inclusion of polycations in the micellar structure is shown to enhance the condensation of siRNA and their transfection efficiency, it also negatively affects the safety of the delivery systems in both *in vitro* and *in vivo* studies. This problem may be addressed through the use of low molecular weight polycations, modification of polycation structure or application of safer siRNA condensing agents in the micellar structure. Finally, decoration of the carrier with targeting ligands may be used to enhance the interaction of the carrier with desired cell population upon systemic administration and/or compensate for the reduced cell interaction properties of the PEGylated micellar shell. Although the results of studies on several structures developed to date indicate a potential, it also dictates a need for optimization of polymeric micellar structures for their *in vivo* safety, transfection efficiency and cell selectivity.

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## MANUSCRIPT