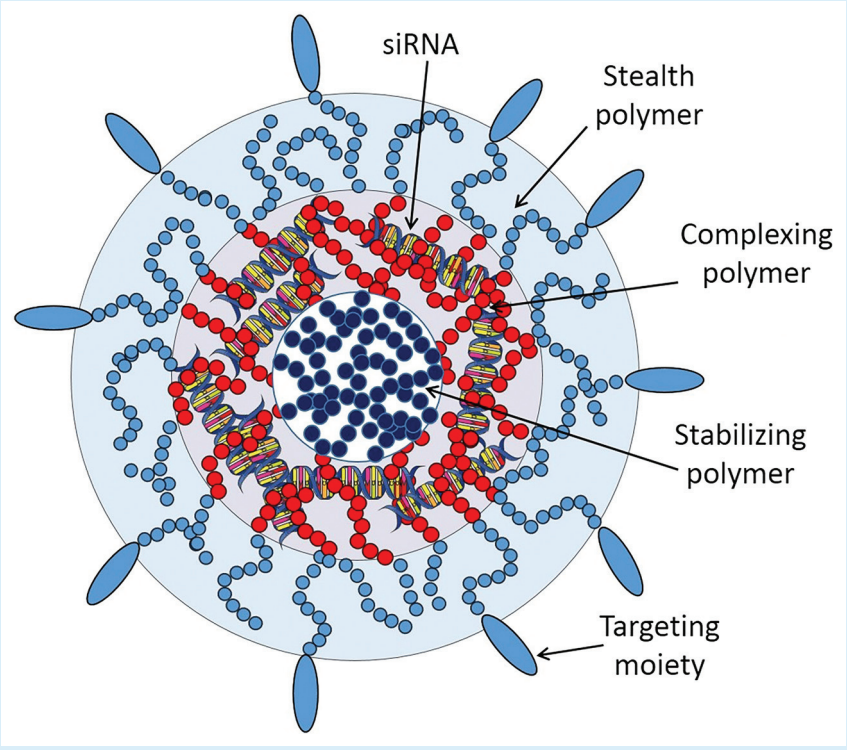
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Control of Amphiphile Self-Assembling at the Molecular Level: Supra-Molecular Assemblies with Tuned Physicochemical Properties for Delivery Applications



Marc A. Ilies

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Chapter 2

Designing Polymer Micelles of Controlled Size, Stability, and Functionality for siRNA Delivery

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Small interfering Ribonucleic acid (siRNA) is at the forefront of a universal therapeutic approach based on gene silencing for diseases ranging from viral infections to cancers and genetic disorders. The main challenge to realizing this remarkable goal is our ability to deliver siRNA to the cytoplasm of the cell where it can perform the mRNA knockdown and downregulate gene expression. Naked siRNA degrades in the blood stream within minutes. Delivery of naked siRNA suffers from severe limitations of clearance through liver and renal systems, degradation by enzymes in the blood and the extracellular environment, low cellular uptake and poor endosomal escape, thus leading to inefficient siRNA delivery and gene silencing. To overcome these challenges and effectively deliver siRNA, carrier vehicles are necessary. Polymer micelles based on block polyelectrolytes are proving to be effective systems due to the range of complex materials available, their ease in complexation with siRNA and forming particles of desirable size, and their ability to incorporate other therapeutics for synergistic siRNA and drug deliveries. Not only are the block polyelectrolytes used alone for delivering siRNA, but they have been used in conjunction with peptides, nanoparticles, dendrimers, and lipids to promote the effectiveness of the siRNA delivery.

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This paper focuses on factors relevant to designing polymer micelle systems of controlled size and stability. Polyelectrolyte complexation with siRNA which is the basic building block for the micelle, factors influencing the micelle size, variations in block copolymers selected as the building block, and the use of polymer conjugates with peptides, nanoparticles, dendrimers, and lipids to increase siRNA stability and its cellular uptake are discussed.

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1. Introduction

RNA interference (RNAi), the inhibition of gene expression, was discovered in 1998 by Fire and Mello using double stranded RNA segments in a *Caenorhabditis elegans* model (*1*). They demonstrated that a short strand of RNA is able to induce the degradation of a complementary sequence of mRNA, thereby giving RNAi great potential in its ability to modulate and silence virtually any gene. Since this discovery, research on the mechanism for RNAi and the usefulness of short interfering RNA (siRNA), has led to significant advancements in biomedical therapeutics (*2–6*). For example, siRNA has shown benefits for cancer therapies, viral infections, wound healing, and diagnostics (*7–11*). Within the last ten years, studies have produced encouraging results for siRNA treatment in Huntington's disease (*12–14*), ovarian cancer (*15–17*), leukemia (*18–21*), and prostate cancer (*22*, *23*). Over 21 siRNA therapeutics have been developed with at least 14 diseases being targeted in clinical trials, demonstrating the rapid transition from siRNA discovery to its clinical application (*24*).

The growing interest in pursuing therapeutics involving siRNA is due to its ability to inhibit specific genes influencing a disease's progression. For example, two biomarkers for breast cancer include increased activity of matrix metalloproteinase (MMP) and overexpression of the folate receptor. siRNA specific to these proteins and receptors have been developed in order to "turn off" the genes responsible for causing the increased activity and overexpression, respectively (*25*). This mechanism of action makes siRNA useful for many diseases and contributes to the universal nature of the therapeutic approach.

1.1. Mechanism of siRNA Action

siRNA is a double stranded RNA molecule carrying a high negative charge and for gene silencing applications has a relevant size range of 19-23 nucleotides in length (*26*, *27*). The shape of the double stranded siRNA can be described by an end to end distance of about 8 nm and a cross-sectional area of about 6.5 nm^2. For siRNA to cause gene silencing, it must target the messenger RNA (mRNA), which is located inside of a cell. For this to occur, siRNA must go through the systemic circulation, reach targeted cells, enter into the cell's extracellular matrix, and then taken up by the cell in order to be available to function in the cytosol. Since naked siRNA is susceptible to degradation by serum endonucleases and causes an innate immune response, carrier vehicles for effective delivery of siRNA are needed (*28*).

One of the main mechanisms by which carrier vehicles with siRNA as cargo are taken up by a cell is endocytosis (Figure 1).

The use of a carrier can protect siRNA from degradation before reaching the cell. It can also help overcome the hindrance to cellular uptake of the negatively charged naked siRNA by the similarly charged cellular membrane. The main mechanism by which the siRNA carrying vehicle is taken up by a cell is through endocytosis. Of the three main types of endocytosis - phagocytosis, pinocytosis, and receptor-mediated endocytosis - siRNA nanocarrier uptake is typically by receptor-mediated endocytosis (*29*). Thus, most carriers will have specific targeting moieties (e.g. antibodies, small ligands, and aptamers) in order to achieve siRNA uptake by target cells. Once siRNA nanocarrier is taken up by the cell via endocytosis, it then requires escape from the endosome prior to reaching the lysosome stage, which will degrade the siRNA. Also the cellular uptake of siRNA could be significantly reduced because of recycling through endosomal recycling pathways (*30*).

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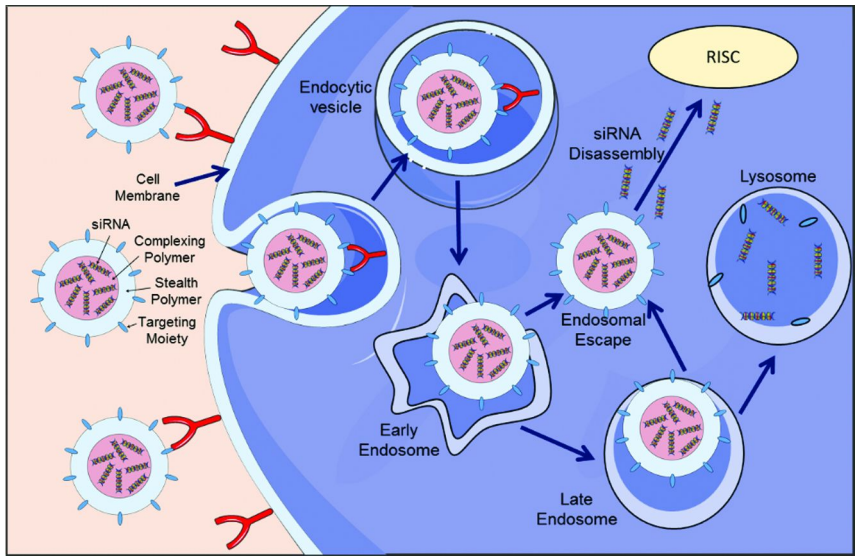


Figure 1. Cellular uptake of nanocarriers with siRNA as cargo is frequently by receptor mediated endocytosis. siRNA is shown incorporated within a polymer nanocarrier. Targeting moieties are added to the nanocarrier surface to facilitate receptor mediated endocytosis by target cells. siRNA nanocarrier must escape endosomes before reaching lysosomal degradation. If siRNA nanocarrier escapes during the early endosome or late endosome cycles, then it is free in the cytoplasm of the cell where it can disassemble and release siRNA. If the nanocarrier cannot escape, all its contents including the siRNA will be degraded in the lysosomes.

After release from the endosomes, siRNA promotes RNA interference in a cell's cytoplasm through the siRNA pathway (Figure 2). Briefly, once siRNA is released into the cytoplasm, it becomes incorporated with RNAi-induced silencing complex (RISC) which is a multiprotein complex including the enzyme Argonaute 2 (AGO2) (*29*, *31*, *32*). The enzyme separates the double stranded siRNA into its sense and anti-sense strands. The anti-sense strand remains with the complex while the sense strand leaves the complex and is freed into the cytoplasm. The RISC complex, now containing the antisense strand, can recognize messenger RNA (mRNA) which is transported from the nucleus to the cytoplasm. If the antisense strand has a complementary sequence that the mRNA can recognize, they will bind. The siRNA is short and foreign to the attached mRNA, which causes the cleavage and degradation of mRNA by AGO2.

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1.2. Requirements for a siRNA Delivery System

It is evident that siRNA faces many barriers prior to being able to induce gene silencing. The challenges include clearance through the liver and renal systems, degradation by enzymes in the blood and extracellular environment, and inefficient gene silencing (*33*, *34*). In order to negate these challenges, many nanocarrier systems have been developed (*5*, *28*, *29*, *32*).

An effective nanocarrier system for siRNA delivery must first encapsulate siRNA with high efficiency to ensure effective utilization of the siRNA as a drug.

The nanocarrier should be capable of maintaining structural integrity in the in vivo environment and protect the siRNA from interactions with plasma proteins or degrading plasma nucleases. Naked siRNA is completely degraded in 30 min into the bloodstream after recognition by plasma enzymes. Plasma proteins that are negatively charged can form aggregates with nanocarriers with positive charges. Such aggregates can be recognized by the innate immune system of macrophages and can be eliminated from circulation. Typical size of these particles eliminated by macrophage mechanism is above 200 nm.

The nanocarrier should be protected from clearance by liver, spleen or kidneys. Typical renal clearance removes particles below approximately 5 nm while the hepatic clearance removes particles in the 10 to 20 nm size range. Very large particles above 200 nm accumulate in liver and spleen. Particles in the 50 to 200 nm range prove long lasting in circulation and escape filtration by liver and spleen. Therefore delivery systems in the size range of 50 nm to 200 nm can limit their elimination by these different mechanisms and increase circulation time in the bloodstream.

The nanocarrier should be capable of crossing endothelia to reach target tissues. Nanocarriers in the blood stream first interact with vascular endothelium before they may cross or pass the endothelial cells and enter the tissue. Vascular endothelium in different tissues has distinctive properties including the surface glycoproteins, surface receptors, intercellular junctions or rate of production of transport vesicles. Non-specific binding of the nanocarrier to the endothelium is a key element in absorptive endocytosis which precedes trans-endothelial transport.

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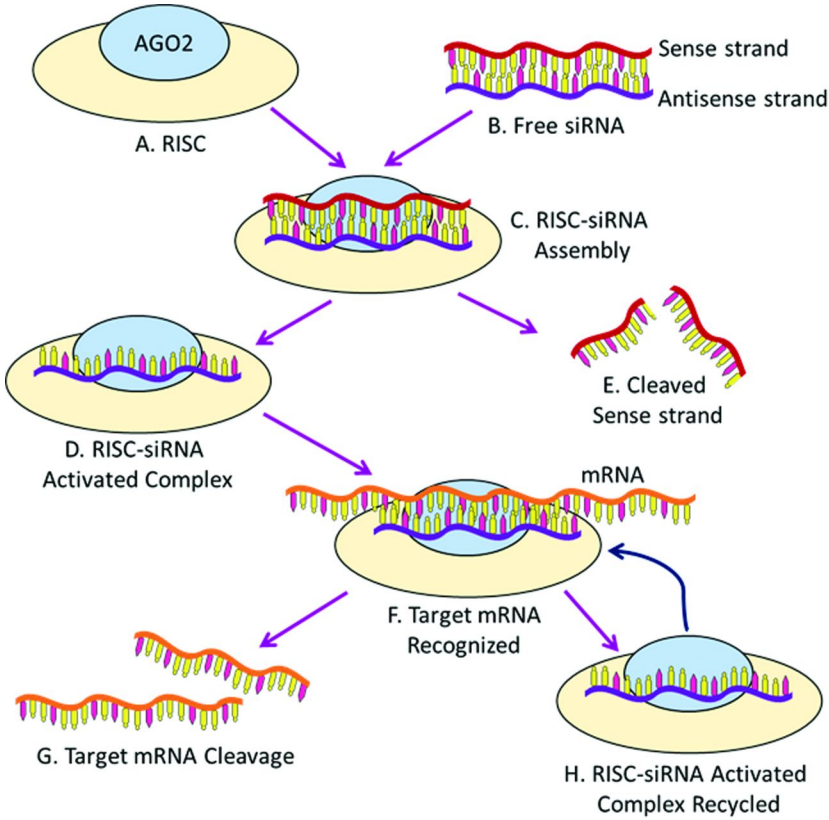


Figure 2. Schematic of siRNA pathway leading to mRNA degradation. (A) RNAi-induced silencing complex (RISC) which incorporates the enzyme Argonaute 2 (AGO2). (B) Intact double stranded siRNA, typically with 19 to 23 nucleotides in length. (C) siRNA assembles with RISC. (D) AGO2 separates the strands of the siRNA duplex, leaving the anti-sense strand with the complex and discarding the sense strand. (E) The sense strand is no longer situated with the RISC complex and is free in the cytoplasm. (F) The antisense strand guides the activated RISC complex to recognize target sites on mRNA. (G) mRNA is cleaved and degraded. (H) The activated RISC complex with the antisense strand is recycled for many rounds of degradation of the same mRNA target.

The nanocarrier should be equipped with features to facilitate efficient internalization into the cell via endocytosis. While there are many mechanisms of cellular uptake, receptor mediated internalization generally occurs via receptors associated with the ligands used for the active targeting.

The nanocarrier should be designed to facilitate endosomal escape following internalization into the cell. After internalization, early endosomal vesicles are formed and mature into late endosomes characterized by an acidic pH and the presence of active enzymes that can degrade siRNA. One proposed mechanism of endosomal escape is the proton sponge effect, that is, pH buffering induced by

the protonation of entrapped molecules in the acidic pH medium that results in osmotic swelling allowing the endosomal membrane to rupture. Recently it has been shown (*35*) that even without the change in pH, proton sponge effect may occur as represented by increased influx of protons. The other mechanism is for the nanocarrier components to interact with the endosomal membrane and perturb the bilayer organization causing membrane disruption possibly by inducing pores.

Following endosomal escape, there should be disassembly and exposure of the siRNA in the cytosol for RISC formation and gene silencing action.

siRNA delivery systems actively explored currently include using polymers, nanoparticles, dendrimers, and lipids (*36*). This paper focuses on the use of polymers, specifically block polyelectrolytes forming micelles, and how the synergistic incorporation of other delivery systems listed above have been utilized to create hybrid systems and enhance the stability, transport, and targeting. The goal of this paper is to focus on how the polymer micelle delivery systems are being designed to control their size and stability and promote enhanced delivery of siRNA to the cytoplasm for action with the RISC machinery and gene silencing.

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2. Constructing Polymer Micelles with siRNA

2.1. siRNA Complexation with Block Polyelectrolyte and Micellization

Developing polymer micelles for siRNA delivery is generally achieved using the self-assembly phenomenon intrinsic to amphiphilic block copolymers. The classical system involves the use of a double hydrophilic block copolymer, one block being neutral and the second block being a cationic polyeletrolyte (*36*, *37*). Because of its hydrophilicity, this block copolymer will not self-assemble to create micelles. However, when siRNA is present, electrostatics allows the cationic polymer block to complex with the anionic siRNA to create a charge neutralized complex. This complex is hydrophobic and transforms the double hydrophilic block copolymer into an amphiphilic block copolymer capable of self-assembly as micelles (Figure 3).

In the presence of the double hydrophilic block copolymer poly(ethylene glycol)-poly(L-lysine), PEG-b-PLL, siRNA complexes with the cationic block PLL and forms the core of the micelle. The presence of the PEG block in the corona region enhances micellar stability, namely it prevents aggregation of micelles and also any aggregation with proteins thereby allowing for extended circulation time.

The typical diameter of polymeric micelles range from 20-100 nm in order for them to achieve endocytic cellular uptake. The ease in assembling block copolymers into micelles is one of the advantages in their use as delivery vehicles. However, controlling the amount of siRNA complexed with each block copolymer is important to ensure proper loading. Determining the amount of polymer necessary per desired amount of siRNA in a delivery vehicle is essential in order to produce effective loading and establish a desired charge on the polymer micelle. Typically, targeting moieties can be added to the outside of the micelle in order to allow for receptor mediated endocytosis.

2.2. Factors Influencing Micelle Formation and Properties

Consider the complexation between the anionic siRNA and the double hydrophilic block copolymer BC made up of uncharged block B and cationic block C. For the cationic polymer that is a polyamine, the number of charges is equal to the number of nitrogen atoms N_C in the polymer block, if all of the amine groups are in the dissociated state contributing to the charge. The extent of dissociation would depend on the pH of the environment and there could be less than complete dissociation. The number of negative charges on the siRNA are determined by the number of phosphate units P_A.

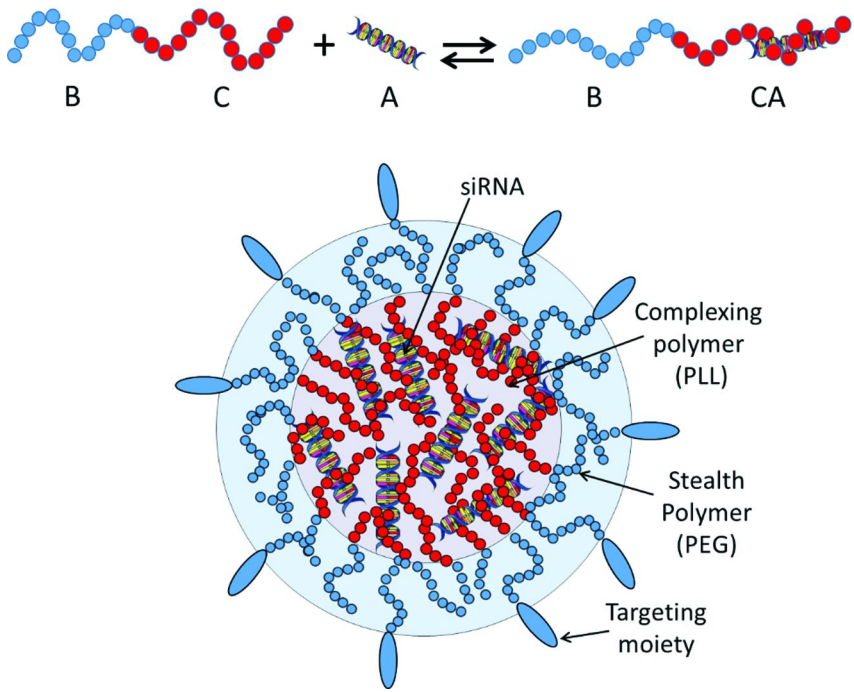


Figure 3. Schematic of complexation between a double hydrophilic block copolymer (BC) and siRNA (A) and the resulting formation of micelle. Block B is neutral while block C is cationic. The association between cationic block C and anionic siRNA gives rise to a hydrophobic complex (CA). Because of this hydrophobicity, self-assembly as micelles occurs. Schematic of micelle formed from the complexation of siRNA with the diblock copolymer, poly(ethylene glycol)-poly (L-lysine), PEG-b-PLL. One or more siRNA can be associated with each block copolymer depending on the ratio of total cationic charges to anionic charges in solution. Targeting moieties are added to the surface of the micelle (in this case by conjugation to PEG terminus) to promote receptor-mediated endocytosis of the siRNA carrying micelle.

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The strength of the binding between A and C is determined by the number of charges that are being neutralized by complexation and therefore, the intrinsic equilibrium constant for binding has an exponential dependence on N_C or P_A, depending on whichever one is smaller. Further, the ionic strength of the medium also influences the magnitude of the electrostatic attractions and the extent of complexation.

The complex CA is hydrophobic. Consequently, the water soluble block copolymer BC is transformed into an amphiphilic molecule B-CA which is capable of self-assembly as micelles. The strength of the complex CA and hence its intrinsic stability is influenced by the smaller of N_C or P_A and the ionic strength of the medium. In turn, these factors influence the hydrophobicity of the complex formed which is the driving force for the self-assembly to occur.

If we can construct an explicit model for the standard free energy change on formation of the B-CA complex micelle, we can predict how the size and composition of the micelles can be controlled by the molecular properties of siRNA and the block copolymer BC. The standard free energy change can be expressed in a form analogous to what has been used to describe the formation of neutral block copolymer micelles in our earlier work (*38*).

$$\Delta\mu_g^o = \left(\Delta\mu_g^o\right)_{\text{CA Transfer}} + \left(\Delta\mu_g^o\right)_{\text{C Deform}} + \left(\Delta\mu_g^o\right)_{\text{Interface}} + \left(\Delta\mu_g^o\right)_{\text{B Osmotic}} + \left(\Delta\mu_g^o\right)_{\text{B Deform}} \quad (1)$$

The various contributions appearing in the expression for the standard free energy change on aggregation represent all physicochemical changes accompanying complexation between siRNA (A) and the cationic polymer block (C) and the resulting micelle formation. The first term in eq.(1) represents the transfer of the hydrophobic CA complex from water into the micelle core. This is a negative term and is the sole driving force for micelle formation to occur. The second term accounts for the elastic deformation of the polymer block C within the micelle core so that the micelle core remains hydrophobic. The third term accounts for the interface generated between a core region of the CA complex and the corona region which is a solution of the polymer block B in water. The fourth term represents the free energy change due to osmotic effects when the polymer block B is transferred from its initial state in the BC copolymer to the corona region of the micelle. The last contribution accounts for elastic deformation free energy for the polymer block B to remain within the corona region. Expressions for each of these free energy contributions can be written in terms of molecular properties of the block copolymer BC and the properties of siRNA (A) and such modeling will be described in detail in a later paper.

In a system with mole fractions X_{BC} of the block copolymer and X_A of siRNA, the ratio between the total number of cationic charges N to the anionic charges P is determined by both the solution composition and the intrinsic charge numbers N_C and P_A, that is $N/P = (X_{BC}N_C)/(X_AP_A)$. The N/P ratio is an experimental variable in forming the complexes and along with the factors mentioned previously, controls the size of the siRNA containing micelles. For siRNA delivery, the typical number

of phosphate units are in the range 20 to 30 and N/P ratios of 1 and higher are normally used. Under these conditions the complex CA may be entirely neutral or neutral in region where the complexation occurs and have residual positive charges on the block C.

Based on the free energy contributions summarized in eq.(1), we can make many qualitative predictions even without a detailed free energy model. If the N/P ratio deviates from unity, one can expect the neutralization of the charges to be less effective and the presence of net cationic charges in the block copolymer. As a result, the size of the micelle would decrease. Further, because of the simultaneous reduction in the magnitude of the driving force (complex hydrophobicity) the concentrations at which micelle can form would increase and micellization may not occur at all beyond certain N/P ratio.

Further, as for neutral block copolymers, an increase in the length of the uncharged hydrophilic block B will cause the micelle size to decrease while an increase in the hydrophobicity of the CA complex will lead to an increase in the micelle size. Therefore, through control of the chain lengths of polymer blocks B and C, the molecular parameters P_A and N_C representing the charged states of siRNA and the polycation C, and the solution composition as represented by the N/P ratio, one can design micelles of desired size and intrinsic stability.

2.3. Various Polymer Systems Used for siRNA Carrying Micelles

Many variations in the copolymer architectures have been explored in the literature and some structural examples are schematically shown on Figure 4. Early studies on siRNA delivery were designed around cationic polymers that have been among the major types of non-viral carriers for DNA delivery and gene therapy investigated in the past thirty years (*39*). They formed complexes with siRNA analogous to the polyplexes formed with DNA, but not micelle structures. These cationic polymers have demonstrated attractive features, however, the delivery efficiency still needs to be improved for more effective gene therapy.

The first structure shown on Figure 4 is the cationic branched poly(ethylene imine), BPEI which complexes with siRNA to form a nanostructure analogous to polyplexes but not micelles. High-molecular weight BPEIs have been found to deliver siRNAs more efficiently than low-molecular weight BPEIs, as their high charge density creates a strong attractive interactions between BPEI and siRNA, providing more efficient protection of siRNA against enzymatic degradation. The degree of branching in the BPEI structure has also been shown to impact the efficiency and toxicity of BPEI. The BPEI has shown enhanced efficacy of nucleic acid delivery compared to linear poly(ethylene imine), LPEI (*40*).

There has been significant concern regarding the cytotoxicity of PEI and other cationic polymers, which are believed to increase the permeability of the cell membrane by forming transient nanopores in the membrane (*41*). Further, while the cationic polymers allow for siRNA complexation, their unneutralized positive charge can promote aggregation with proteins in the biological fluids upon systemic administration. The adhesion of proteins on the polymer-siRNA complex nanostructures greatly affects their circulation and biodistribution.

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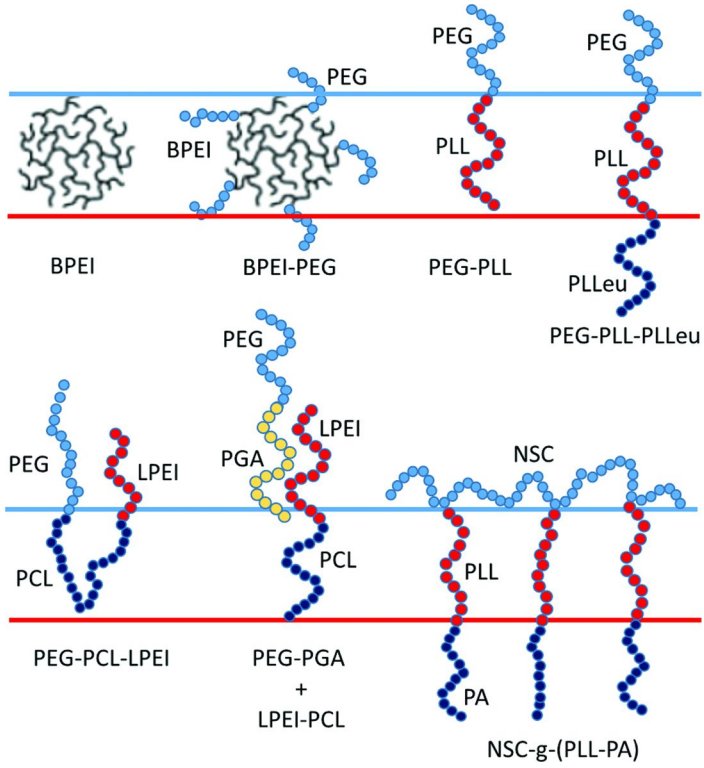


Figure 4. Molecular architectures of polymers that have been considered for complexation with siRNA to enable siRNA delivery. Three regions are marked to describe how these polymers would organize themselves following complexation with siRNA. The region that always remains hydrophilic is where PEG polymer block is located. The region that always remains hydrophobic is where the PCL, PLLeu, PA blocks of different copolymers shown are located. The intermediate region is where the polycations PLL, BPEI, and LPEI are located and on complexation with siRNA or PGA as shown, the region becomes hydrophobic to different degrees. For BPEI and BPEI-PEG, the complexes constitute nanostructures but not micelles. For all other copolymers shown, micelles are generated. Polymer abbreviations are explained in the text as they appear and they have been also collected at the end of the paper.

To overcome these problems, the standard practice has been to introduce neutral polymers as part of the complex. For example, as shown on Figure 4, a number of PEG molecules can be grafted onto the surface of BPEI (*42*). The PEG moieties, form a protective outer layer over the BPEI-siRNA complex, shielding the polyplex core, giving rise to the steric stabilization of the polyplex structure against undesirable interactions with surroundings.

The stability and nuclease resistance efficiency of PEGylated polyplexes has been shown to depend on the PEG chain length (*42*). The stability was compared for a series of siRNA/BPEI(25 kDa)-g-PEG(X)$_Y$ polyplexes, where the molecular

weight of the PEG chains, X, were 0.55, 2, 5 and 20 kDa, and the grafting densities Y (number of PEG molecules per BPEI) were 30, 10, 4 and 1, respectively. It was found that a high density of short PEG chains, BPEI (25 kDa)-g-PEG(0.55 kDa)$_{30}$ did not provide sufficient protection of siRNA whereas formulations with larger PEG chain molecular weights of 2, 5 and 20 kDa protected siRNA against RNase at realistic enzyme concentrations.

The more promising approach to provide protection to siRNA has been to create core-shell type micelle structures from amphiphilic copolymers. In these systems, the size of the polymer micelle created can be controlled so that the amount of siRNA carried is optimized and the siRNA is protected from the environment until it is delivered at the site of interest. The diblock copolymers PEG-PLL (*36*) and poly(ethylene glycol)-poly(spermine), PEG-b-PS (*37*) were the first block copolymers to be considered for the low molecular weight antisense oligodeoxynucleotides (ODN) delivery using micelles in contrast to delivery methods for high molecular weight DNA using polymer-DNA complexes (polyplexes). The ODNs are much closer to siRNA in their sizes compared to plasmid DNA which are orders of magnitude larger. The PLL or the PS block, by virtue of its complexation with anionic ODN, provided the driving force for micelle formation while the PEG block provided stability for the micelle in solution against aggregation and also limited interaction with proteins in the biological environment.

Other variations of the simple double hydrophilic block copolymer have been developed and a few examples are shown on Figure 4. The micelle structures generated from typical block copolymers are schematically shown on Figure 5. The triblock copolymer poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine), PEG-b-PLL-b-PLLeu has an hydrophobic block PLLeu at the end of the double hydrophilic polymer (*43*). The presence of this block can promote micelle formation, independent of the nature of PLL complexing with siRNA (Figure 5a). In this manner the ability to form the micelles could be controlled independently from the complexation of siRNA with the polycationic block.

PEG-b-PLL-b-PLLeu triblock copolymers when dissolved in water, spontaneously formed self-assembled micelles (*43*). The hydrodynamic diameter of the micelles were tunable and correlated with the length of PLL and PLLeu segments. The stability of PEG-PLL-PLLeu micelles can be attributable to the high charge density on their surface and the presence of PEG layer. Further, since the secondary structures such as α-helices, β-sheets, and random coils, within polypeptides, are known to contribute to the structural stability of the biopolymers, the predominantly rigid α-helical conformation of PLLeu segments within the micelles could also enhance the stability of micelles. The concentration of the triblock copolymer at which micelles form (the critical micelle concentration, CMC) value of micelles was primarily related to the length of hydrophobic blocks (PLLeu) and mass ratio of hydrophilic blocks (PEG-PLL) to hydrophobic blocks (PLLeu).

Another triblock polymer shown on Figure 4 is poly(ethylene glycol)-b-poly(ε-caprolactone)-b-linear poly(ethylene imine), PEG-b-PCL-b-LPEI (*44*, *45*). In this case the PCL block is hydrophobic and it is now the middle block. The choice of PCL is influenced by it being a degradable polymer. Again, the

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presence of the hydrophobic block promotes micelle formation independent of siRNA complexation with the cationic LPEI. However, the size of micelles formed will be different because of the placement of the hydrophobic PCL in the middle rather than in the end as for PEG-b-PLL-b-PLLeu shown on Figure 5a.

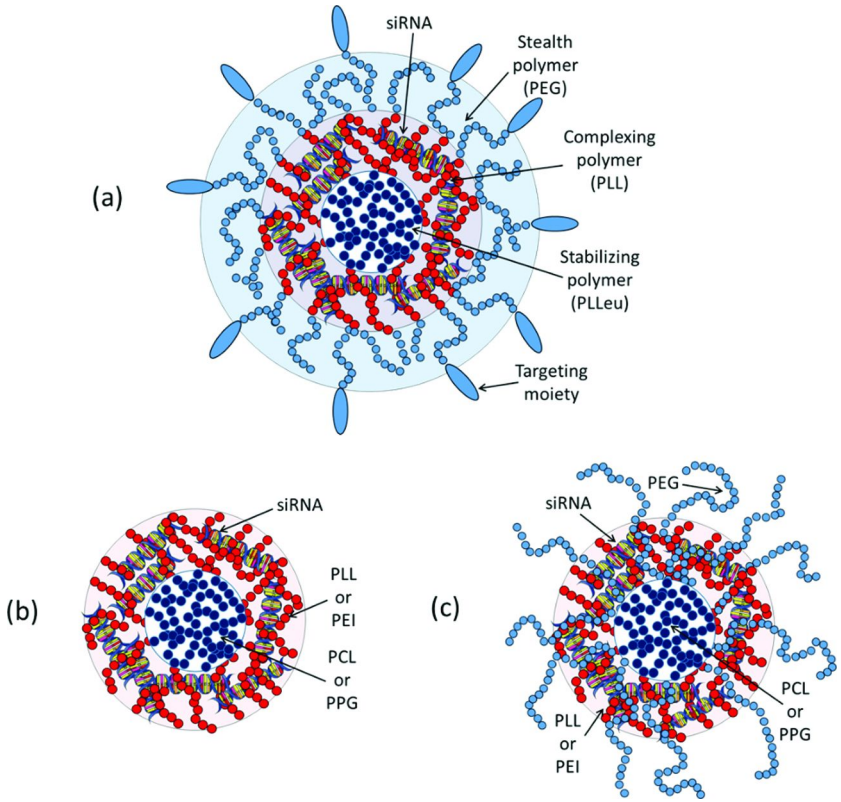


Figure 5. (a) Assembly based on PEG-b-PLL-b-PLLeu triblock copolymer with the hydrophobic PLLeu as an end block. (b) Assembly based on triblock PEG-b-PCL-b-LPEI copolymer with the hydrophobic PCL as the middle creating a different structure compared to that shown on (a). (c) Assembly based on a diblock copolymer with hydrophobic PCL or PPG and ionic PLL or LPEI. See text for discussion of structural variations.

A structurally analogous triblock copolymer system that has been developed is monomethoxy poly(ethylene glycol)-b-poly(ε-caprolactone)-b-poly(2-amino ethyl ethylene phosphate) (*46*), denoted as $mPEG_{45}$-b-PCL_{100}-b-$PPEEA_{12}$ where the subscripts indicate the number of monomer units in each polymer block. These polymer micelles took uniform spherical morphology and allowed siRNA loading after the micelle formation without change in the uniformity of micelle sizes. The average diameter of the polymer micelles after siRNA complexation ranged from 98 to 125 nm depending on N/P ratios.

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Another micelle forming polymer system shown on Figure 4 involves a mixture of two block copolymers (*47*), the amphiphilic LPEI-b-PCL and the double hydrophilic poly(ethylene glycol)-poly(glutamic acid), PEG-b-PGA. LPEI-b-PCL is capable of forming micelles by itself and the micelles with cationic LPEI in the shell can complex with siRNA (Figure 5c). To provide protection to the siRNA complex, the second block copolymer PEG-b-PGA is introduced, where PGA is an anionic polymer. The overall micelle structure involves a hydrophobic core of PCL surrounded by a weaker hydrophobic domain involving the complexation of cationic LPEI, anionic PGA and the anionic siRNA and finally an outer neutral shell containing PEG.

The last example shown on Figure 4 is a graft copolymer with a backbone of N-succinyl chitosan (NSC) to which are grafted the cationic PLL end functionalized with palmitic acid (*48*). The palmitic acid is hydrophobic and allows the formation of micelles. The complexation of PLL with siRNA further contributes to the micellization behavior. The hydrophilic NSC provides stability for the micelles and also reduces the cytotoxic presence of PLL on the micelle surface. The copolymer NSC-g-(PLL-PA) was complexed with siRNA for co-delivery of doxorubicin (DOX) and P-glycoprotein siRNA for synergistic cancer treatment. The hydrophobic domain formed by PA serves as the locus for incorporating hydrophobic chemotherapeutic drugs.

In Table 1, many examples of polymer systems used for generating siRNA containing micelles are presented along with some relevant features.

Table 1. Examples of polymer systems for siRNA micelle formation*

Polymer system	*Some relevant features*	*Ref*
BPEI; BPEI-g-(PEG); PEI-g-(PEG-RGD)	Three forms of polyplexes were prepared based on the three forms of branched poly(ethylene imine), BPEI, a PEGylated BPEI and a PEGylated BPEI with an RGD peptide (ACRGDMFGCA) at its distal end. The nanostructures formed were not micelles. Approximately 40 PEGs were attached to each BPEI. BPEI contributes to proton sponge effect for endosomal release into the cytosol.	(*7*)
LPEI-b-PCL	LPEI allows the formation of micelle structures in contrast to the polyplexes formed with BPEI. The hydrophobicity of PCL induces micellar core formation and stability. PCL is biodegradable.	(*47*)
BPEI; BPEI-g-(PEG-Folate)	Two forms of polyplexes were prepared based on the branched poly(ethylene imine), BPEI and the PEGylated form containing a folate at its distal end. The nanostructures formed were not micelles. siRNA complexed with PEI-PEG-FOL exhibited the most dose-effective and fastest gene silencing effect for FOL receptor overexpressing KB cells (human epidermal carcinoma).	(*49*)

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Table 1. (Continued). Examples of polymer systems for siRNA micelle formation*

Polymer system	*Some relevant features*	*Ref*
PEG-b-PLL; PLL-b-PEG-b-PLL	The double hydrophilic diblock copolymer cannot form micelles by itself. PLL complexes with siRNA and the hydrophobicity of the complex promotes core-shell type micelle formation. PEG in the shell acts as the stealth polymer allowing for increased systemic circulation of the micelle. PLL-b-PEG-b-PLL is a triblock version of PEG-b-PLL.	(*50*)
PLL-b-PPG-b-PEG-b-PPG-b-PLL	A penta-block version of PEG-b-PLL. The added PPG block is hydrophobic and can provide a domain to solubilize therapeutic drugs allowing co-delivery of drugs and siRNA. By varying arrangements of blocks of PEG, PPG, and PLL, the study showed that one can effectively deliver siRNA to silence their target (GFP) as effective as a common liposome (Lipofectamine 2000) with low cytotoxicity effects.	(*50*)
PEG-b-PLL-b-PLLeu	The hydrophobicity of PLLeu facilitates formation of micelles independent of the extent of complexation of PLL with the oligonucleotide and also controls the disassembly of the oligonucleotide in the physiological environment. The PEG-PLL-PLLeu micelles were of tunable sizes ranging from 40 to 90 nm depending on the length of PLL and PLLeu. Compared with PEG-PLL, PEG-PLL-PLLeu micelles demonstrated significantly higher transfection efficiency and less cytotoxicity. The transfection efficiency and biocompatibility of the micelles can be simultaneously improved by tuning the length of PLL and PLLeu.	(*43*)
PEG-b-PLL-b-PLLeu	Tumor-associated dendritic cells (DCs) are less matured with poor responsiveness to Toll-like receptor (TLR) stimulation, which has been related with STAT3 hyperactivity. In this study, Poly I:C (PIC, a TLR3 agonist), STAT3 siRNA and OVA antigen were co-encapsulated by PEG-PLL-PLLeu micelles to generate a nanovaccine aimed to effectively overcome DC dysfunction in vivo by deleting STAT3 gene in situ.	(*51*)
NSC-g-(PLL-PA)	Polymer system designed for co-delivery of siRNA and DOX. N-succinyl chitosan (NSC) forming the hydrophilic shell was designed to increase the half-life of micelle and decrease the toxicity of PLL. The cationic PLL was designed to electrostatically complex with siRNA. The palmitic acid (PA) forming the hydrophobic core was used to encapsulate DOX.	(*48*)

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Table 1. (Continued). Examples of polymer systems for siRNA micelle formation*

Polymer system	*Some relevant features*	*Ref*
BPEI-g-(PCL-b-PEG-Folate)	The nanostructure resembles a combination of a polyplex because of BPEI and also a polymer micelle, because of PCL. The hydrophobicity of PCL induces micellar core formation and stability. PCL is biodegradable. Folate acts as the recognition element.	(*52*)
FA/mPEG-ss-b-P(LG-Hyd-DOX)-b-PDMAPMA	Polymer system was designed for the co-delivery of DOX and siRNA into breast cancer cells. DOX was covalently conjugated onto the poly-(LG-Hyd) blocks via a pH-labile hydrazone linkage. The fast payload release rates were closely related to both the glutathione-triggered detachment of PEG blocks (breaking the ss linkage) and the pH-sensitive cleavage of hydrazone linkages.	(*53*)
TAT/RGD-PEO-b-P(CL-Hyd-DOX) + PEO-b-P(CL-g-SP)	Polymer system designed for traceable co-delivery of siRNA and DOX. The prepared micelles have a versatile core that can stably complex siRNA and conjugate DOX and imaging probes and have a shell for cell-specific recognition and efficient cellular uptake. These micelles could release DOX by pH-triggered mechanism. The RGD/TAT-micelles demonstrated significant cellular uptake and improved DOX penetration into nuclei. Incorporation of fluorescent probes in the micellar core allows for in vitro and in vivo tracking of micelles.	(*54*)
mPEG-b-PCL-b-PPEEA	The cationic polymer block PPEEA is biocompatible and degradable and is used to both complex with siRNA and also to facilitate the release.	(*46*)
Mal-b-PEG-b-PCL	Cyclic RGD was conjugated to DOX loaded PEG-b-PCL micelles by a post-micelle modification method involving the maleimide functional group on the PEG. Attachment of the cyclic RGD ligand greatly enhanced internalization of the micelles in tumor endothelial cells that overexpress specific integrins, apparently through receptor-mediated endocytosis.	(*55*)
mPEG-b-PCL-TAT	The cell penetrating peptide also acts as the cationic moiety that complexes with siRNA. The hydrophobic PCL ensures micelle formation stabilized by the PEG. The size of mPEG-PCL-TAT/siRNA complex micelles ranged from 60 to 200 nm, and the particle size decreased with an increase in the N/P ratio. These micelles were tested as carrier for nose-to brain delivery of siRNA and anticancer drugs. The study compared the effects of siRNA with and without a delivery vehicle on a rat model of malignant glioma. Shrinkage of glioma tissue was substantial when siRNA was encapsulated in a polymer micelle carrier in comparison to glioma tissue treated with naked siRNA.	(*56*)

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Table 1. (Continued). Examples of polymer systems for siRNA micelle formation*

Polymer system	*Some relevant features*	*Ref*
Lac-PEG-ss-siRNA + PLL	Instead of the diblock copolymer PEG-PLL complexing with siRNA, in this case a PEG-siRNA conjugate complexes with PLL. pH-sensitive and targetable micelles of siRNA were developed based on the conjugation of siRNA with lactosylated PEG through acid-labile linkage of β-thiopropionate (Lac-PEG-siRNA), followed by the complexation with poly(L-lysine). Note that β-thiopropionate linkage is readily cleaved at the pH corresponding to that of the intracellular endosomal compartment (pH of 5.5). The micelle with disulfide cross-linked core was prepared by using thiolated PLL	(*57*)
LPEI-b-PPG-b-LPEI	$LPEI_{I50}$-PPG_{36}-$LPEI_{I50}$ and $LPEI_{14}$-PPG_{68}-$LPEI_{14}$ were considered for the delivery of siRNA (21 b). The first copolymer was found almost inactive for siRNA delivery while the second was very effective. The first copolymer has a small hydrophobic PPG middle block and very long charged PEI end blocks. Therefore it is unable to form micelles making it inefficient for siRNA delivery. The second copolymer has a larger PPG middle block and much smaller PEI end blocks. It is able to form micelles with the coronal PEI blocks complexing with siRNA. Hence it is effective for siRNA delivery.	(*58*)
PEG-b-(DMAEMA-co-BMA)	The carrier was designed with the positively charged block of dimethylaminoethyl methacrylate (DMAEMA) to allow siRNA binding and the butyl methacrylate (BMA) block to control the hydrophobicity. The relative mole % of DMAEMA and BMA was varied to optimize cationic and hydrophobic content in the core based on siRNA packaging, cytocompatibility, blood circulation half-life, endosomal escape, and in vivo bioactivity.	(*59*)
poly[(DMAEMA)-b-(BMA)-co-(DMAEMA)-co-(PAA)]	The carriers were composed of a positively charged block of dimethylaminoethyl methacrylate (DMAEMA) to facilitate siRNA binding and a second pH-responsive endosome releasing block composed of DMAEMA and propylacrylic acid (PAA) in roughly equimolar ratios and butyl methacrylate (BMA). This design incorporates a longer endosomolytic block with increased hydrophobic content to induce micelle formation and the pH-responsive, membrane-destabilizing block copolymers. These polymers respond to changes in pH by transitioning from an ionized, hydrophilic structure at physiologic pH (~7.4) to a hydrophobic, membrane-destabilizing conformation at endosomal pH values (<6.6). Poly(propyl-acrylic acid) (PPAA) in particular has been shown to be effective at endosomal release. This new generation of siRNA delivery polymers based on this design exhibit enhanced transfection efficiency and low cytotoxicity.	(*60*)

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Table 1. (Continued). Examples of polymer systems for siRNA micelle formation*

Polymer system	*Some relevant features*	*Ref*
PDMAEMA-b-PCL-b-PDMAEMA	Biodegradable cationic micelles were prepared from PDMAEMA–PCL–PDMAEMA triblock copolymers and applied for the delivery of siRNA and paclitaxel into cancer cells. PDMAEMA was selected due to its relatively low toxicity and high buffer capacity. The biodegradation and low molecular weight of PDMAEMA was expected to further reduce the in vitro and in vivo toxicity of the micelle. The combinatorial delivery of VEGF siRNA and paclitaxel showed an efficient knockdown of VEGF expression.	(*61*)
mPEG-b-P(CL-co-DLLA)-g-(PDMAEMA)	The contribution of polyester segments in siRNA delivery in vitro was investigated by introducing different ratio of DLLA moieties in PCL segments. Differing compositions of the polyester cores showed different endocytosis mechanisms thus influencing the cellular uptake efficiency. The core composition affected capabilities in escaping from endosome/lysosome, entering the RNA-induced silencing complex (RISC) and cutting the target mRNA efficiently. The different siRNA release profile was dominated by the degradation rate of polyester segments. Thus this study showed that variations in hydrophobic core of micelles can significantly affect their transfection behavior.	(*62*)

* The abbreviations for various polymers are listed at the end of the main text. In the abbreviations, 'b' refers to block, 'g' refers to graft and 'co' refers to co-monomer that is polymerized, 'ss' refers to disulfide bridge.

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While various block copolymers as described above are being actively used for developing polymer micelles for siRNA delivery, polymers not capable of forming micelles such as chitosan and gelatin have also been used to derive specific benefits by formation of polymer-siRNA complex nanoparticles. Chitosans are naturally occurring and used for their improvement in intracellular uptake. For example, Holzerny et al. recently demonstrated the ability of intracellular uptake at low N/P chitosan/siRNA complexations (*63*). Results from this study showed that the smaller molecular weight chitosan polymers exhibited faster cellular uptake of the siRNA carriers. Modified potato starch, or Q-starch, has been developed for delivering specific siRNA in order to silence P-glycoprotein in human ovarian adenocarcinomas. Amar-Lewis et al. demonstrated complexes that were able to fully encapsulate siRNA, were safe, and effective in showing significant gene silencing (*64*). Gelatin nanocarriers have also had significant and advantageous results. Youngren et al. studied gelatin nanocarriers for complexing with siRNA (STAT6) to increase the stability and biocompatibility of the delivery vehicle (*65*). These nanocarriers are unique because they already have FDA approval and are biodegradable and stable.

3. Enhancing Delivery with Polymer Nanocarriers

Block copolymers complexing with siRNA to create micellar structures provides the basic functionality for these nanocarriers required for siRNA therapeutics. The block copolymers are advantageous nanocarriers, because of their ability to incorporate other moieties, forming hybrid carriers adding additional functionalities that could benefit siRNA delivery. The use of four classes of hybrids incorporating nanoparticles, peptides, dendrimers and lipids as siRNA carrier systems is briefly described here.

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3.1. Polymer-Nanoparticle Conjugates

Nanoparticles (NPs) have been used for a variety of drug delivery applications (*66–72*). The ability to synthesize precisely controlled sizes of nanoparticles plays a role in their use where critical size requirements must be met for siRNA delivery. Nanoparticles with a size that is smaller than 50 nm can efficiently accumulate in tumor tissues and therefore the size of nanocarriers is important for enhancing siRNA accumulation in a variety of tumor tissues. Gold nanoparticles (AuNPs) are promising biocompatible nanotemplates, as their size can be precisely controlled with a narrow distribution, and also because polymers or biomolecules can be conjugated to gold through thiol chemistry. Such thiol bonding is relatively stable under extracellular conditions, but these conjugated molecules can be removed from AuNP with glutathione (GSH), which is abundant in the cytosol.

Using block copolymers, siRNA has been tethered to NP surfaces, with major advantages being increased circulation in the blood and siRNA release. This method differs from the traditional micelle formulation, because instead of siRNA being encapsulated within micelle core, the siRNA is conjugated to the outside of the particle. While traditional micelles are designed to allow for the siRNA to be released, conjugation to the outside has its advantages for siRNA release. An example of the arrangement of these nanocomplexes is shown in Figure 6.

siRNA has been delivered by conjugation to the surface of nanoparticles (NPs). Gold NPs are commonly used for drug and gene delivery due to their intrinsic properties and biocompatible behavior. Recently, the addition of polyions, such as PEG-b-PLL have been used with gold nanoparticles in order to enhance longer blood circulation (*71*). Kim et al. showed that compared to polyions or gold nanoparticles alone, the siRNA with PEG-b-PLL conjugated to gold NPs demonstrated an increase in stability, release of siRNA, and efficient luciferase gene silencing in cervical cancer models (*71*).

Another nanoparticle system that has been extensively developed and investigated by the Mirkin lab is the spherical nucleic acid (SNA) nanoparticle (*70*, *72*, *73*). SNAs are gold nanoparticles with the gold cores densely functionalized with highly oriented, thiolated siRNA duplexes. The SNA surface can be passivated with oligoethylene glycol for colloidal stability. Unlike linear nucleic acids, SNAs rapidly enter mammalian cells, despite their negative charge, through class A scavenger receptor-mediated endocytosis and have been used to regulate the expression of genes in vitro and in vivo (*73*).

Spherical nucleic acid nanoparticles developed on 13 nm gold nanoparticles dispersed in Aquaphor have been shown to penetrate the epidermal barrier of both intact mouse and human skin, enter keratinocytes, and efficiently down-regulate gene targets (*72*). Ganglioside-monosialic acid 3 synthase (GM3S) is a known target that is overexpressed in diabetic mice and responsible for causing insulin resistance and impeding wound healing (*72*).

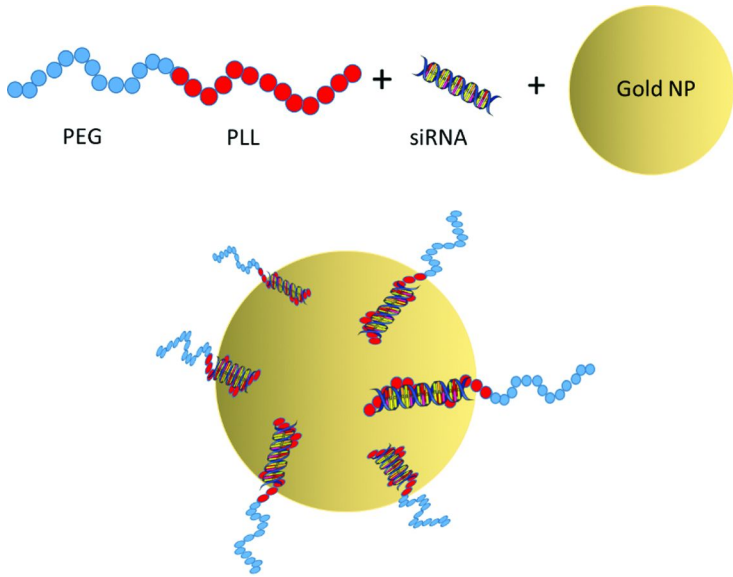


Figure 6. Process and arrangement of siRNA assembling with cationic portion of block copolymer. Thiol moiety is attached to the end of the PLL block. The complex of siRNA with PEG-PLL block copolymer attached to the gold nanoparticle surface via thiol bonding. Conjugation to surface of nanoparticles allows for long circulation time with PEG on the outside. The nanocarrier size is principally determined by the size of the template gold nanoparticle.

It has also been found that the chemical environment of siRNA on the nanoparticle surface can alter the recognition of siRNA by serum nucleases and change the inherent stability of the nucleic acid (*73*). This has made possible chemical modifications that significantly enhance the lifetime of siRNA against nucleases. The study found that the sites of nuclease-catalyzed hydrolysis of the oligonucleotides bound to the AuNP are different from those for the free oligonucleotide. Consequently, the study concluded that the development of strategies for enhancing the serum stability of oligonucleotide–nanoparticle conjugates must consider the context and influence of the nanoparticle surface and should not rely solely on strategies developed from the investigation of siRNA free in solution (*73*).

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3.2. Polymer-Peptide Conjugates

Receptor specific peptides are advantageous for tumor-targeted delivery of siRNA (*74*). The advantage of polymeric micelles is not only their ability to incorporate siRNA into the core, but also conjugate advantageous peptides to their outer shell (*75*). Peptides have previously been conjugated to polymeric micelles for many tumor targeting applications (*7*, *56*, *76*, *77*).

The adaptation of peptides for siRNA carriers has demonstrated advantages for cell penetration. As previously noted, one of the major issues with siRNA delivery is uptake by the cell. The peptide-polymer conjugates forming micelles for siRNA delivery can enhance their cell penetration (*78*). Additionally, the incorporation of peptides can also help form disulfide cross linkages, which is one method to increase endosomal escape likelihood (*79*). Conjugation of peptides to one of the block copolymers, most often PEG, has been shown to increase not only improved cellular uptake, but also increase gene silencing efficacy, higher subcellular distribution, and the improvement of accumulation to their target site (*80*).

For example, the TAT peptide, which aids in cell penetration, has been used to develop a modified block copolymer mPEG-b-PCL-TAT. A schematic of the micelles formed from this polymer system is shown on Figure 7. Results demonstrated that peptide and siRNA uptake alone was significantly lower than uptake with the polymer-peptide and siRNA complex (*56*).

Another study by Christie et al. used a PEG-*b*-PLL block copolymer modified with 2-iminothiolane (2IT) for siRNA delivery for cancer treatment (*80*). Alone, this block copolymer and modification increased serum stability. However, with the addition of cyclic-Arg-Gly-Asp (cRGD) peptide, bioactivity was improved (*80*).

Schileffers et al (*7*) developed polymer nanoparticles with siRNA complexed with branched poly(ethylene imine), BPEI, the branched cationic polymer PEGylated, BPEI-g-PEG, and BPEI that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol, BPEI-g-(PEG-RGD), as a means to target tumor neovasculature expressing integrins and used to deliver siRNA inhibiting vascular endothelial growth factor receptor 2 (VEGF R2) expression and thereby tumor angiogenesis. This BPEI-g-(PEG-RGD) conjugate allows individualized optimization or chemical replacement for each functional domain.

Ren et al (*74*) described a novel molecular design approach to developing peptide based siRNA delivery system. The cell penetrating peptides such as TAT rapidly penetrate the cell membrane but they lack receptor specificity. Ren et al proposed a tandem peptide with two complementary characteristics: it should efficiently penetrate tissues and cross cellular membranes, but it should also be cell type-specific by targeting only tumor cells while sparing normal cells. They developed tandem peptide sequences bearing a tumor-penetrating domain, LyP-1 (CGNKRTRGC) and variable cell-penetrating/siRNA-binding domains by systematic design. They synthesized N-terminally myristoylated versions of all tandem peptides, since myristoylation has been shown to enhance hydrophobic interactions and peptide affinity to lipid bilayers such as the cell membrane. To

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reduce the risk that siRNA complexation interfering with receptor targeting, the cell-penetrating and tumor-targeting domains were separated by a four-glycine spacer. They demonstrated that a number of the nanocomplexes prepared by such design achieved functional delivery of siRNA in a cell type-specific manner. Two properties, the valence of the tumor-specific ligand on the nanocomplex and the peptide charge were found to be key considerations when designing a siRNA delivery system to knock down genes in a receptor-specific manner.

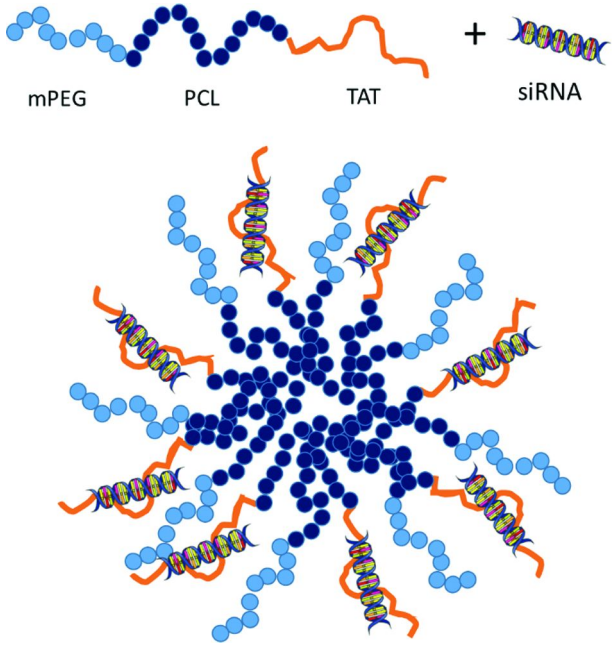


Figure 7. Complexation of siRNA with mPEG-b-PCL-TAT micelles. The PCL domain is hydrophobic and allows for incorporation of hydrophobic drugs. The TAT peptide facilitates cell recognition and entry while PEG provides serum stability of the micelle and the nuclease stability of siRNA.

3.3. Polymer-Dendrimer Conjugates

Dendrimers are synthetic branched polymers with wide applications in drug and gene delivery (*81–84*). Their size and shape are well controlled, which leads to reproducible pharmacokinetics. Additionally, they exhibit controlled multivalency, which allows for well-defined attachment of drug molecules. The positive surface charges on dendrimers interact with the anionic siRNA allowing the dendrimer to be densely complexed with siRNA. The highly condensed spherical structure has been shown to protect siRNAs from nuclease degradation compared to bare siRNA (*85*). This is possibly due to the dense structure that reduces access of RNase to the siRNA through steric barriers. Excess amine groups on the dendrimers confer the dendrimer-siRNA complex a positive surface charge to promote their cellular interactions, but it also can

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cause severe cytotoxicity and may not lead to a high transfection efficiency. The complexation parameters need to be optimized to promote cellular entry while diminishing cytotoxicity. The structural flexibility of dendrimers has been shown to affect the transfection efficiency and this is achieved either through partial degradation of the dendritic structure or the choice of chemical moieties like flexible triethanolamine core to enhance the delivery of siRNA (*85*).

Recent studies have demonstrated that including dendrimers with block copolymers to develop triblocks allows the creation of a more stable and effective complex with siRNA. Poly(amido amine) (PAMAM) has been used as a dendrimer of interest. A triblock polymer comprised of PAMAM-b-PEG-b-PLL was complexed with siRNA (Figure 8).

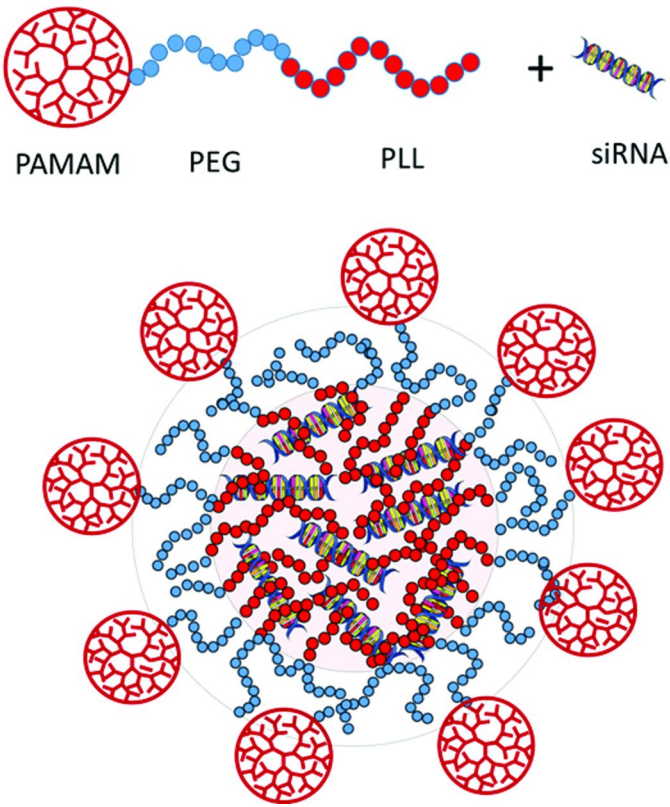


Figure 8. siRNA complex with PAMAM dendrimer-b-PEG-b-PLL triblock copolymer. The PAMAM dendrimer was partially acetylated leaving 6 primary amine groups that served for conjugation with PEG. PAMAM does not serve as the polycation complexing with siRNA. The PAMAM dendrimer provides tertiary amines for endosomal escape. PEG shields siRNA protecting it from enzymatic degradation, while PLL offers cationic amine groups for electrostatic interaction with negatively charged siRNA.

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PAMAM-PEG-PLL was synthesized through three sequential steps: first, the PAMAM dendrimer was partially acetylated to create PAMAM-[$(NHAc)_{58}(NH_2)_6$] dendrimer, with six primary amine groups available for conjugation but distributed over the surface of the dendrimer. Second, this was reacted with one of the acid groups of bis-(2-carboxyethyl)polyethylene glycol. Mostly the product had a single PEG conjugated to the dendrimer. The carboxylic end of PEG was then conjugated to PLL.

Each component in the triblock copolymer plays a significant role. The tertiary amine groups in the PAMAM dendrimer work as a proton sponge and play a vital role in the endosomal escape and cytoplasmic delivery of siRNA. The PEG block renders nuclease stability and protects siRNA in human plasma. The PLL block provides primary amines to complex with siRNA and also acts as penetration enhancer. The conjugation of PLL to PEG and PAMAM reduced toxicity of PLL. This PAMAM-b-PEG-b-PLL delivery vehicle showed stability in human plasma as well as effective uptake and gene silencing (*33*).

Amphiphilic dendrimers have been used to deliver small interfering RNA (siRNA) (*86*, *87*). In one case, the dendrimer is conjugated with a lipid with two alkyl chains. This amphiphilic lipid-dendrimer conjugate self-assembles into vesicle-like structures, which, in the presence of siRNA, spontaneously undergo structural rearrangement into smaller, spherical micelles. The other type of dendritic amphiphile was obtained from the conjugation of an hydrophobic alkyl chain to the dendrimer (*87*) and the length of the alkyl chain was varied to optimize performance. This dendrimer amphiphile forms micelles before and after complexation with siRNA. Both of these nanocarriers successfully delivered Hsp27 siRNA in vitro and in vivo in a prostate cancer model, which led to pronounced gene silencing and anticancer activity (*86*, *87*).

3.4. Polymer-Lipid Conjugates

Lipids have been used in many drug delivery applications, primary in the form of liposomes, where they have shown a high ability of passing through hydrophilic barriers (*88–90*). While lipids self-assemble to form liposomes for drug entrapment, the polymer conjugated lipid forms micelles (*91*) (Figure 9). This conjugation has been shown to increase circulation, stability, and enhanced permeability and retention (EPR) effect (*92*).

For example, one recent study used BPEI conjugated with the lipids, 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (DOPE) and dipamitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (DPPE) (*93*). The BPEI-DOPE and BPEI-DPPE particles showed how the performance of BPEI was drastically altered when conjugated to a lipid (*93*). The conjugation of low molecular weight PEI to the lipid phosphoethanolamine (DOPE and DPPE) was shown to provide good siRNA delivery whereas the low molecular weight PEI by itself was not effective. The complexation of PEI with siRNA was done at higher N/P ratios to retain still positive charges on the micelle, thereby allowing for the stability of the micelles. The micellar structures also permit the incorporation of hydrophobic drugs in the lipid core, making possible the co-delivery of siRNA and poorly soluble drugs combinations in a single carrier.

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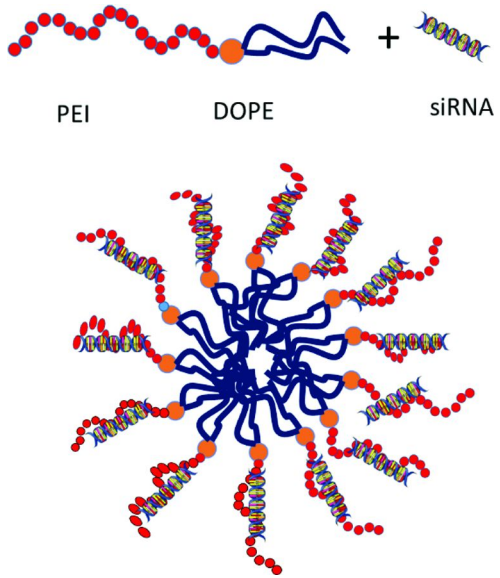


Figure 9. Schematic of the micellar structure formed with linear PEI-lipid conjugate. The lipid which by itself forms liposomes facilitates the formation of micelle structures when conjugated with PEI. Similar qualitative behavior is expected with branched PEI conjugated to the lipid.

Figure 10 shows the lipid modified triblock polymer system with PAMAM (generation G4) dendrimer, PEG and the lipid DOPE, creating siRNA encapsulated PAMAM-b-PEG-1,2-dioleoyl-*sn*-glycero-3-phophoethanolamine (PAMAM G(4)-b-PEG-DOPE) nanovehicle (*94*).

In this study, a mixed micellar system with a 1:1 molar ratio of PAMAM G(4)-b-PEG-DOPE and PEG-DOPE was also investigated. Biswas et al. determined that both the PAMAM G(4)-b-PEG-DOPE and mixed micellar siRNA systems demonstrated the ability to decrease gene expression with higher efficacy than using only the dendrimer. However, the mixed micellar system was able to deliver a higher amount of DOX chemotherapeutic. Due to the mixed micellar system's ability in drug loading and co-delivery of siRNA and DOX, it may be useful for therapeutics such as multi-drug resistant cancers (*94*).

Recently, hybrid nanostructures readily coassembled from amphiphilic dendrimers and a naturally occurring phospholipid have been proposed as effective drug delivery vehicles (*95*), although no work with siRNA was presented in this study. Amphiphilic dendrimers were obtained by the conjugation of a hydrophobic alkyl chain to a dendrimer. Both concentration and generation of the dendrimers have direct impacts on the structural and biophysical properties of the assembled structures. Raising the dendrimer concentration increases the hybrid bilayer stability, while changes in the generation and the concentration of the embedded dendrimers impact the fluidity of the coassembled systems. Multivalent dendrimer amine terminals allow for nondestructive in situ derivatization,

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allowing facile functionalization and control over the local environment of the hybrid aggregate. The lipid/dendrimer hybrid systems with modular and controllable behavior have potential for siRNA delivery.

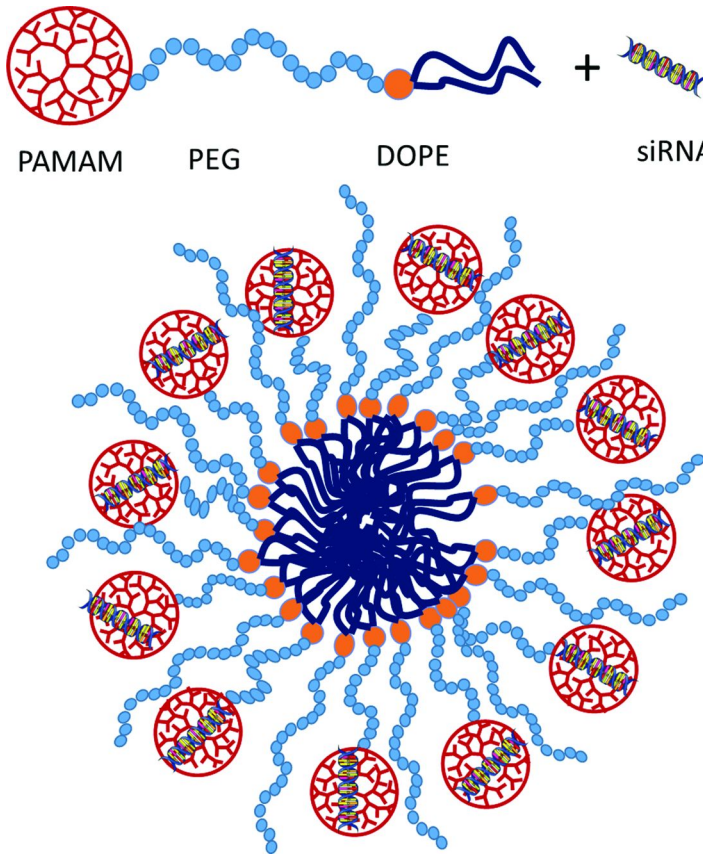


Figure 10. Micelles of PAMAM G(4)-b-PEG-DOPE triblock copolymer mixed with a PEG-DOPE diblock copolymer having a higher molecular weight of PEG. The use of mixtures where the PEG chain length can be modified allows the creation of micelles with different properties with respect to siRNA stability.

4. siRNA Disassembly from the Micelle

4.1. Monitoring Disassembly

In order to gain quantitative information on the delivery of siRNA, fluorescence resonance energy transfer (FRET) labelled siRNA probes were recently used, especially to understand intracellular trafficking. Identical siRNA strands were labelled with either an AlexaFluor594 or AlexaFluor647 molecule. When the siRNA were together in micelles complexed of lipidoids nanoparticles, b-PEI, and Lipofectamine, the donor siRNA with the AlexaFluor594 molecule was able to transfer some of its energy to the acceptor AlexaFluor647 molecule

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(*96*, *97*). When micelles were degraded or siRNA was released, this energy transfer was weakened or "turned-off," resulting in a FRET peak shift.

In another approach, the delivery of siRNA using quantum dot-mediated fluorescence resonance energy transfer (QD-FRET) allows for a more sensitive imaging technique. Endres et al. delivered siRNA via a cationic triblock of poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene imine), PEG-b-PCL-b-PEI, *in vitro* (*45*). The best performing nanocarrier PEG(500Da)-PCL(10kDa)-PEI(2.5kDa) were studied for FRET capability using QDs, which resulted in an approach to measure the displacement of siRNA from their complexes (*45*).

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4.2. Designing Polymers To Promote Disassembly

The design of polymer molecules can be done incorporating responsive features that will allow the polymer micelle to disassemble and release siRNA in the cytoplasmic environment for subsequent RISC process. Convertine et al (*60*) reported a new diblock copolymer poly(DMAEMA)-b-(DMAEMA-co-BMA-co-PAA). The poly(N,N-dimethylaminoethyl methacrylate) (polyDMAEMA) block is cationic and complexes with siRNA. This second block, poly(DMAEMA-co-BMA-co-PAA) is composed of a mixture of positively charged DMAEMA monomers, negatively charged propylacrylic acid (PAA) monomers, and hydrophobic butyl methacrylate (BMA) monomers. This polymer block is pH-responsive, hydrophobic and capable of pH dependent membrane disruption. The mixture of positive and negative charged groups throughout the second block reduces the overall net charge of this polymer block at physiological pH. Under the acidic condition of endosomal compartments, protonation of the carboxyl residues occurs, rendering a net positive charge to this second block. This change in charge allowed the polymer to interact with endosomal membranes and was found to efficiently delivery siRNA to the cellular cytoplasm. These polymers become strongly hemolytic at endosomal pH (5.8-6.6), with increasing hemolytic activity as the percentage of BMA in the second block is increased.

The above polymer micelle system displayed excellent delivery activity, but the cationic segment could contribute adverse toxicity. To overcome that a similar block copolymer was developed by replacing the cationic DMAEMA segment with (HPMA-*co*-PDSMA), consisting of a hydrophilic block of N-(2-hydroxypropyl)methacrylamide (HPMA) and a disulfide conjugatable monomer N-(2-(pyridin-2-yldisulfanyl)ethyl)-methacrylamide (*98*). This system has the promising potential for future targeted siRNA delivery schemes using pH-responsive polymeric micelles for therapeutic use.

4.3. Use of Disulfide Bridges To Control Disassembly

In designing polymer systems for siRNA based polymer micelles, the incorporation of disulfide bridge in the molecules has been of significant interest since this allows one to control when and where the siRNA will be released. Disulfide bridging couples two thiol groups, creating a covalent bond. Disulfide bonds can be cleaved with specific concentrations of glutathione, which is present

in the cellular environment, and cause the release of siRNA from the micelle carrier.

Previous literature on polymeric micelles shows that at extracellular concentrations of glutathione (10 μM) the siRNA complex remains intact; however, at intracellular concentrations of glutathione (10 mM) the disulfide bonds are cleaved, releasing siRNA (*99*). This is of interest due to the difference in the intracellular and extracellular conditions, which allows the complex to remain intact until it reaches the intracellular environment. Dextran conjugated siRNA particles were utilized via disulfide bonds that have the ability to dissociate in the cytoplasm (*100*). These particles resulted in high serum stability due to both the disulfide bonds as well as the anti-fouling properties of dextran (*100*).

Another study conducted by Li et al. used a ternary copolymer, mPEG-*b*-PLL-*g*-(ss-LPEI), which has also been conjugated using disulfide bonds in order to deliver siRNA. Both in vitro (Skov-3 cells) and in vivo (Female nude mice BALB/c) studies showed the copolymer featured low cytotoxicity, easy degradability, and high siRNA transfection (*101*). Specifically, the disulfide bonds aided in carrier biodegradability and siRNA release. Neutral polymers, including [(HPMA-co-PDSMA)-b-(DMAEMA-co-PAA-co-BMA)], have also recently been developed using disulfide bonds (*98*). This polymer construct demonstrated low cellular toxicity with effective uptake in vitro (HeLa cells), resulting in the knock down of mRNA (GAPDH) via the reducibility of the nanocarrier when in glutathione. It is the specificity of when the siRNA is released that makes disulfide bridging attractive; however, other limitations including polymer degradation, endosomal escape, and renal clearance cannot be achieved through disulfide bridging.

It is important to note that not all polymeric nanocarriers utilize disulfide bridges. Controlled release and degradation mechanisms are currently being investigated that do not rely on a concentration change of glutathione. However, the addition of disulfide bridging during the nanocarrier construction has been of considerable interest for many polymeric vehicles as a mechanism to aid siRNA release.

5. Conclusions

This review discussed block copolymers for their use in assembling with siRNA to facilitate siRNA delivery. The typical building block based on diblock and triblock copolymers showed an overall increase in stability, transport, and gene silencing is possible with polymeric nanocarriers compared to naked siRNA delivery. Additionally, combining block copolymers with nanoparticles, peptides, dendrimers, and lipids added additional functionalities beyond siRNA payloads and demonstrated further advantages for siRNA delivery. Even with promising results using block copolymers and with some clinical trials in progress, siRNA therapeutics still has many limitations that need to be addressed with regard to the challenges faced throughout the delivery process including stability, cellular uptake, endosomal escape and siRNA release to the cytoplasm. New techniques and complex assemblies utilizing block copolymers and polymer

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hybrid nanocarriers show promise towards achieving advancements for siRNA therapeutics to be effective in the clinic.

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Abbreviations Used

AGO2, argonaute-2 protein; AuNP, gold nanoparticle; BMA, butyl methacrylate; BPEI, branched poly(ethylene imine); CMC, critical micelle concentration; cRGD, cyclic-Arg-Gly-Asp; DC, dendritic cells; DLLA, D,L-lactide; DMAEMA, dimethyl aminoethyl methacrylate; DOPE, 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl); DOX, doxorubicin; DPPE, dipamitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl); EPR, enhanced permeability and retention; FA, folic acid; FITC, fluorescein isothiocyanate; FRET, Fluorescence Resonance Energy Transfer; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; GSH, glutathione; GM3S, ganglioside-monosialic acid 3 synthase; HPMA, N-(2-hydroxy propyl) methacrylamide; Lac PEG, Lactosylated Poly(ethylene glycol); LPEI, Linear poly(ethylene imine); Mal, maleimide; MMP, matrix metalloproteinase; mPEG, methoxy poly(ethylene glycol); mRNA, messenger RNA; NSC, N-succinyl chitosan; ODN, oligodeoxynucleotides; P(LG-Hyd), poly(L-glutamate-hydrazide); PA, palmitic acid ; PAA, propylacrylic acid; PAMAM, poly(amido amine); PAsp(DET), poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}; PCL, poly(ε-caprolactone); PDLLA, poly(D,L-lactide); PDMA, poly(2-dimethyl aminoethyl methacrylate); PDMAPMA, poly(N,N-dimethyl aminopropyl methacrylamide); PDPA, poly(2-diisopropyl aminoethyl methacrylate); PDSMA, poly(N-(2-(pyridin-2-yldisulfanyl)ethyl) methacrylamide); PEG, poly(ethylene glycol); PEI, poly(ethylene imine); PGA, poly(glycolide); PGA, poly(glutamic acid); PHPMA, poly(N-(2-hydroxypropyl)methacrylamide); PLGA, poly(lactic-co-glycolic acid); PLL, poly(L-lysine); PLLeu, poly(L-leucine); PPAA, poly(propyl acrylic acid); PPEEA, poly(2-aminoethyl ethylene phosphate); PPG, poly(propylene glycol); PPI, polypropylene imine; PS, poly(spermine); QD-FRET, quantum dot-mediated FRET; RES, reticuloendothelial system; RISC, RNA-induced silencing complex; RNA, ribonucleic acid; RNAi, RNA interference; siRNA, small interfering RNA; ss, disulfide linkage; SNA, spherical nucleic acid; STAT3, Signal transducer and activator of transcription 3; STAT6; Signal transducer and activator of transcription 6; TAT, TAT peptide (CGRKKRRQRRR); TLR, Toll-like receptor; VEGF, vascular endothelial growth factor.

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