Published in final edited form as:
Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2013 ; 5(5): 449–468. doi:10.1002/wnan.1233.

Bioengineered Nanoparticles for siRNA delivery

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Abstract

Short interfering RNA (siRNA) has been an important laboratory tool in the last two decades and has allowed researchers to better understand the functions of non-protein-coding genes through RNA interference (RNAi). Although RNAi holds great promise for this purpose as well as for treatment of many diseases, efforts at using siRNA have been hampered by the difficulty of safely and effectively introducing it into cells of interest, both *in vitro* and *in vivo*. To overcome this challenge, many biomaterials and nanoparticles (NPs) have been developed and optimized for siRNA delivery, often taking cues from the DNA delivery field, although different barriers exist for these two types of molecules. In this review, we discuss general properties of biomaterials and nanoparticles that are necessary for effective nucleic acid delivery. We also discuss specific examples of bioengineered materials, including lipid-based NPs, polymeric NPs, inorganic NPs, and RNA-based NPs, which clearly illustrate the problems and successes in siRNA delivery.

Introduction

Since its discovery in 1998,[1] the RNA interference (RNAi) gene silencing pathway has been a focus of many major areas of research. Chief among these are the natural mechanism of viral defense in plants and insects,[2–4] the discovery of endogenous protein function by reducing its production,[5] and the treatment of diseases that are caused by overproduction of a specific gene.[6, 7] The last of these applications in particular requires safe and effective methods for siRNA delivery in order to maximize the clinical potential of RNAi.

The natural RNAi pathway begins with double-stranded RNA (dsRNA) that is cleaved by the protein Dicer into ~21-base pair dsRNA known as short interfering RNA (siRNA). The antisense strand of siRNA is incorporated into the RNA-induced silencing complex (RISC), which binds and subsequently cleaves, in a catalytic fashion, strands of mRNA complementary to the siRNA. This prevents protein production and results in sequence-specific gene knockdown (for review, see Hannon[8]). Early methods to non-virally induce RNAi included direct injection of dsRNA[9–11] or mechanical agitation of cells in the presence of dsRNA.[12] However, these methods are not clinically translatable, and introduction of dsRNA longer than 30 base pairs has been shown to induce an interferon (IFN) response.[13] As a result, delivery of siRNA to circumvent IFN is more frequently employed.

Although very effective, viral methods for nucleic acid delivery have been associated with immunogenicity and tumorigenicity.[14] Non-viral nucleic acid delivery systems are

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traditionally less effective[15] but can be designed to avoid issues typical of viruses. Several types of materials have been used for delivery of nucleic acids and of siRNA in particular. Because many early efforts at siRNA delivery used materials that were already well-studied in the context of DNA delivery, we first discuss properties of these materials that make them suitable for nucleic acid delivery in general and then describe their utility for overcoming barriers to siRNA delivery (Figure 1) in particular.

General properties of nucleic acid delivery nanoparticles

Nucleic acid binding or encapsulation ability

The biomaterial transfection agent must have sufficient ability to bind to or encapsulate the nucleic acid. Because nucleic acids are negatively charged, positively charged biomaterials are commonly used. Poly(L-lysine) (PLL)'s ability to bind DNA was discovered through studies of DNA-histone binding and conformation,[16] and it was later explored for delivery of exogenous DNA.[17, 18] PLL, however, lacks the ability to escape the endosome, an essential step in intracellular delivery through the endocytic internalization route.[19, 20] Other biomaterials such as liposomes are able to encapsulate nucleic acids within their aqueous interior. These artificial vesicles can be made with tight control over physical properties by changing the amphiphilic lipids that compose them as well as by varying fabrication methods.[21–23] Nucleic acid binding or encapsulation is generally a necessary, but not sufficient, biomaterial attribute to prevent degradation of the nucleic acid and to facilitate the entry of such highly negatively charged molecules into the cell.

Shielding

Liposomes, as well as other types of nanoparticles (NPs), often suffer from quick clearance from the circulation by cells of the mononuclear phagocyte system (MPS).[24, 25] Chemical structure of specific materials aside, most NPs for nucleic acid delivery rely at least partially on ionic interactions for cellular uptake and carry charge on their surface. Although electrostatic repulsion contributes to NP colloidal stability in aqueous suspension, physiological salt[24, 25] and serum[26] conditions are often enough to coat the NPs non-specifically with proteins, and cause aggregation, leading to decreased delivery efficiency and increased clearance by MPS cells. One way to overcome this potential problem is to coat the NP surface with a shielding molecule, commonly poly(ethylene glycol) (PEG), whose hydrated structure can prevent non-specific interactions with biomolecules.[27] This strategy, dubbed "PEGylation," has been employed in a number of gene delivery systems, including NPs based on chitosan,[28] gelatin,[29] cationic polymers,[30] lipids,[31] and metallic NPs.[32]

It should be noted that PEGylation has also been found to prevent desirable interactions, such as those leading to intracellular delivery to target cells. As a result, researchers have explored the use of cleavable PEG chains on NP surfaces to increase circulation time but also allow effective transfection.[33]

Targeting and cellular internalization

A number of methods can be used to promote uptake of nucleic acids by cells of interest. For example, taking advantage of the properties of the lipid bilayer of cell membranes, cationic lipids can facilitate cellular uptake by interacting with anionic cell surface molecules.[34–36] Similarly, other cationic materials, such as polymers, also show high uptake into cells compared to neutral or anionic materials due to interactions with the relatively negative cell surface.[37] Unfortunately, these interactions with NPs and the cell membrane can also contribute to cytotoxicity.[38]

Various chemical moieties and biological ligands can also be incorporated into the material or into the NP as an alternative or additional method of increasing cellular internalization. For instance, the amphipathic peptide GALA (30 residue polypeptide containing 4 Glu-Ala-Leu-Ala repeats) forms an alpha-helical structure that promotes interaction with lipid bilayers,[39] as does the KALA peptide (30 residue polypeptide containing 3 Lys-Ala-Leu-Ala repeats),[40] which has the additional advantage of being positively-charged at neutral or low pH for binding to nucleic acids. The transition from random coil to alpha helix, and therefore the ability to disrupt membranes, is pH-dependent for both of these peptides, making them potentially useful specifically for environmentally triggered membrane fusion.[41, 42] Other cell-penetrating peptides (CPPs) used for this purpose include the trans-activating transcriptional activator (TAT) peptide derived from the human immunodeficiency virus (HIV).[43, 44] This strategy can be combined with a disassembly approach by synthesizing a high molecular-weight form of TAT peptide, linked with disulfide bridges, that can be degraded by bioreduction to reduce potential cytotoxicity.[45]

In addition to general, non-specific cellular uptake, it is often desirable to be able to deliver siRNA to a specific cell or tissue type. Ligands such as sugars, aptamers, peptides, and proteins for cell- or tissue-specific cell surface proteins can also be coated on[46, 47] or conjugated to[48, 49] a biomaterial or NP to enhance uptake and delivery in a targeted manner.

Endosomal Escape

For successful intracellular delivery of nucleic acids, it is critical that that the NP be able to reach the cytoplasm safely and efficiently. A common method of overcoming the endosomal escape barrier is the proton sponge mechanism, in which a reversibly protonated material is believed to act as a buffer. This not only protects the cargo from acidification of the endosomal compartment but also causes a water influx that leads to endosome lysis and release of the cargo into the cytosol. This mechanism was proposed to explain the effectiveness of polyethylenimine (PEI) for gene transfer, as PEI contains reversibly protonated tertiary amines,[50] and while its validity has not been incontrovertibly proven and has been challenged,[51] it nonetheless remains the most widely believed hypothesis.[52, 53] Other materials like PLL can be modified to contain titratable amines, such as by substituting histidine or arginine for lysine residues,[54, 55] leading to improved transfection. Other materials like poly(amidoamine) (PAA) dendrimers are also reversibly protonated at physiologically relevant pH.[56] However, while these materials increased DNA delivery efficacy, some, like PAAs, bind siRNA less tightly, compromising their ability to act as siRNA delivery agents without additional modifications.[57]

Other methods for endosomal escape include hydrophobic biomaterials that can fuse with the membrane, such as dioleoylphosphatitylethanolamine (DOPE) in lipid-based particles.[58, 59] Polyanionic biomaterials have also been employed to promote membrane destabilization, often in a pH-dependent manner that allows triggered endosomal escape when NPs are in certain environments.[60] Membrane-disruptive peptides, such as GALA and KALA, can also be conjugated to or non-covalently associated with NPs to cause endosomal escape.[18, 41, 42] KALA in particular maintains α-helical character even at low pH (4.5), thus making it capable of membrane disruption and leakage even in lower pH environments comparable to late endosomes. KALA was shown to cause nearly 100% leakage of endosomal contents over pH range 4.5–8.[40]

Degradability and nucleic acid release

Release of a NP into the cytoplasm after endosomal escape is not necessarily sufficient for biological effect. It was shown that DNA plasmids must unbind sufficiently from a delivery vehicle for transfection to occur.[61, 62] However, while DNA must be trafficked to the

nucleus, siRNA must be released in the cytoplasm, its principal site of action.[63] A number of factors can affect material degradation and cargo release rate; for example, poly(beta-amino ester)s (PBAE)s are hydrolytically degradable,[64] with their degradation and complex disassociation rate dependent on the local pH and the polymer conformation.[65] Because hydrolytic degradation of free PBAEs in solution takes place in hours at pH 7 at 37°C, these nanoparticles can diffuse or be convected to cells and release their cargo shortly thereafter. Moreover, the polymers showed slowed degradation at the lower pH (5–6) found in endosomal compartments, which could provide some protection for nucleic acids until after endosomal escape to the cytoplasm.

Aside from hydrolysis, as is characteristic of PBAEs and other polyesters, another mode of degradation useful in siRNA delivery is bioreduction due to disulfide linkages (for review, see Son *et al.*[66]). The latter mechanism takes advantage of the reducing cytoplasmic environment to cause quick, environmentally-triggered degradation and siRNA release into the correct cellular compartment.

NPs that do not have a mechanism to stimulate siRNA release may cause low knockdown. Gold NPs, for example, without an siRNA release mechanism may achieve lower knockdown or require higher siRNA doses[67] versus similar delivery vehicles that contain an efficient release mechanism.[68]

Biocompatibility

It is critical that materials administered to a patient be biocompatible. Although cationic lipids have been studied often for nucleic acid delivery, their disruption of the membrane, while an advantage for cellular uptake, can also cause excessive cytotoxicity.[69] Biomaterial degradation is one mechanism of increasing biocompatibility. Degradation of a biomaterial can reduce cytotoxicity, as molecular weight of cationic polymers has been positively correlated with cytotoxicity.[70] For instance, poly[alpha-(4-aminobutyl)-l-glycolic acid] (PAGA), a hydrolytically degradable PLL analogue containing ester bonds in place of the amide bond of the polypeptide, showed not only increased transfection efficacy but also negligible toxicity *in vitro* compared to unmodified PLL.[71] A hydrolytically degradable form of branched PEI showed a similar increase in biocompatibility[72] compared to the typically high cytotoxicity of unmodified PEI.[73, 74]

Stability

Although many of the above biomaterials have been explored for siRNA delivery, and most continue to be investigated for this purpose, several challenges remain to be overcome. Many of these biomaterials and NPs were initially developed for DNA delivery. Aside from differences in the site of action of these two nucleic acids, there are also biophysical differences between them. Although both are composed of similar anionic bases, siRNA molecules are much smaller in size than DNA plasmids. In addition, dsRNA is stiffer than dsDNA,[75, 76] and segments as short as siRNA act as rigid rods. This has important implications for nucleic acid binding properties[77, 78]: there is less multivalency in siRNA than in DNA when interacting with a cationic polymer because of fewer binding sites per molecule. In addition, the stiff RNA molecule, which is expected to condense very little, may not be able to bend to the conformations ideal for high affinity binding and encapsulation, and this can lead to weak stability of siRNA-containing NPs.

In addition to low binding affinity, instability of the RNA cargo itself is a major problem in many of the delivery systems currently investigated. siRNA is more prone to enzymatic degradation than DNA, though less so than single-stranded RNA. Therefore, protection from the extracellular and endosomal environments is necessary for successful delivery. Stability

is a challenge for lipid-based materials as well since lipid-based colloids often exhibit low stability.[79]

Examples of broad classes of biomaterials used for siRNA delivery are presented in Figure 2. Below and in Table 1, we will discuss illustrative examples of next-generation materials that address many of the challenges listed here, including siRNA binding and protection, particle stability, cellular internalization and targeting, material biocompatibility and efficacy, endosomal escape and intracellular targeting, and siRNA release.

Lipid-based nanoparticles

Lipid-based materials are the most widely-used biomaterials for nanoparticulate siRNA delivery. Of over 20 siRNA phase I clinical trials, nearly half use NPs as the delivery vehicle, and almost all of these are lipid-based.[80] Many of the leading commercially available reagents are lipid-based, including Lipofectamine™ 2000,[81] 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP),[82] RNAifect,[82] and TransIT-TKO and TransIT-siQuest.[83] It has become clear, however, that careful controls are necessary for the successful interpretation of results, as siRNA in combination with cationic lipids can cause off-target effects. For example, an *in vivo* study in mice showed that intravenous injection of naked siRNA had no measurable effect; however, injection of liposomal siRNA NPs based on 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a commonly used cationic lipid for nucleic acid delivery, induced a potent IFN response, including the upregulation of downstream molecule STAT1 (signal transducer and activator of transcription 1).[82] Although the greatest effect was seen with DOTAP/siRNA complexes, including several different siRNA sequences, DOTAP alone did cause an IFN and STAT1 response, emphasizing the importance of careful design of the material used for lipid-based NPs to avoid potentially adverse side effects as well as careful interpretation of results. Some researchers have focused on overcoming this problem in particular; for example, Chono *et al.*[84] used the additional components of hyaluronic acid, a polysaccharide with low immunogenicity, PEG, and a targeting ligand, and found that their liposome elicited no significant immunotoxicity, measured by cytokine expression, within their therapeutic range. Other strategies have been employed to reduce the off-target immunological response to siRNAs, particularly when delivered within liposomes; for instance, 2′-O-methyl-modified siRNAs were designed and delivered in stable nucleic acid lipid particles (SNALPs)[85] with less unwanted immune stimulation observed.

To bypass problematic toxicity and particle instability and to maximize siRNA delivery and gene knockdown, several strategies can be employed. Akinc *et al.* used a combinatorial library to link acrylate esters or acrylamides containing 9- to 18-carbon hydrocarbon tails via one of many different amine-containing small molecules,[86] thereby creating a wide range of lipidoid materials with slightly varying structure (Figure 3). These lipidoid molecules were mixed with cholesterol and a PEG-lipid (polyethylene glycol conjugated to a lipid moiety for incorporation into liposomes via hydrophobic interactions) and loaded with siRNA. Using this method, the authors optimized nanocomplex stability and efficacy *in vitro*, then used top materials to achieve >90% knockdown *in vivo* after two daily intravenous injections of 2 mg siRNA/kg mouse. A follow-up study showed increased efficacy of their material to nearly 100% when the siRNA dose was increased to 10 mg/kg, though high toxicity associated with cationic lipids necessitated a lower lipid concentration to be tolerable. The authors improved the biocompatibility by maximizing siRNA loading, thereby reducing the total lipid content delivered, and optimized the PEG chain length to improve *in vivo* particle stability.[87]

Lipid material optimization and selection has also been done to examine very specific aspects of transfection. For example, one reason for the use of cholesterol in liposome formulations is to increase fusion with the cell membrane for internalization,[88, 89] in addition to affecting the fluidity or rigidity of the liposome bilayer. Other lipids have also been found to promote fusion of the liposome with the endosomal membrane because their structure requires less energy to transition to a non-lamellar phase, forming tubular structures with hydrophobic tails exposed and promoting siRNA release into the cytoplasm.[90, 91] While many groups have incorporated fusogenic lipids into their NP formulations and some trends had been implicated in increasing fusogenicity, Heyes *et al.* specifically studied the effect of saturation and chain length on fusogenic phase transition as well as on transfection efficacy.[92] They and others have investigated siRNA delivery from SNALPs by selecting the best fusogenic lipid for the endosomal escape step. Semple *et al.* used *in vivo* screening in mice to develop siRNA-containing SNALPs that could be administered i.v. to non-human primates and cause 80% knockdown of a target gene.[93]

The *in vivo* studies described, while showing high efficacy, required ~1 mg/kg of siRNA to achieve knockdown. By using a similar combinatorial method to that mentioned above, Love *et al.* identified a linker molecule, *N*-(2-(4-(2-aminoethyl)piperazin-1-yl)ethyl)ethane-1,2-diamine, that, after reaction with the 12-carbon epoxide 1,2-epoxydodecane, formed lipid-based NPs that were primarily taken up by macropinocytosis rather than endocytosis, as with their previously-studied materials.[95] They were able to achieve one to two orders of magnitude better efficiency (0.03–0.3 mg/kg dose) in non-human primates with up to ~75% knockdown at the lowest dose, possibly because their internalization route allowed their particles to avoid the endosome. The authors note that lower siRNA doses, while causing the same initial effect, had lower knockdown duration compared to high siRNA doses. This high nucleic acid efficiency allowed the authors to reduce the total amount of lipidoid material needed for therapeutic benefit, lowering potential toxicity, and also allowed them to knock down several genes in a single combined dose.

In addition to nanomaterial composition, siRNA delivery efficacy *in vivo* can also depend heavily on the route of administration. Leconet *et al.*, using commercially available reagents (the lipid-based Invivofectamine 2.0 and the polymeric JET-PEI), achieved transient knockdown in diabetic mice, with a single injection of siRNA/lipid-based carrier able to knock down a target gene for at least seven days in their system.[96] In particular, they found that pancreatic immune cells were transfected with much greater efficacy after intraperitoneal injection compared with intravenous injection. All of these various factors, therefore--specific siRNA sequence and chemical modification, material composition, dose of siRNA and/or material, additives into the liposome, route of cellular entry, and route of *in vivo* administration--can each have enormous effects on RNAi mediated by lipid-based nanocarriers.

The ability of lipid-based materials to stably encapsulate siRNA has led to their prolific use in ongoing clinical trials relative to other siRNA delivery technologies (for review, see Forbes and Peppas[80]). In particular, the decreased immunogenicity and enhanced *in vivo* stability and shielding promoted by the PEG layers surrounding SNALPs has enabled multiple clinical trials involving SNALPs. Drug TKM-080301 sponsored by Tekmira Pharmaceuticals Corporation and Alnylam Pharmaceuticals is a SNALP containing siRNA targeting polo-like kinase-1 (PLK-1). This drug has completed a Phase I trial administered via hepatic arterial infusion for primary or secondary liver cancer, and is currently undergoing an additional Phase I trial targeting solid tumors via intravenous infusion.[97, 98] SNALP-based siRNA drug ALN-TTR01, also sponsored by Alnylam Pharmaceuticals, has completed a Phase I trial using siRNA targeting transthyretin in an effort to treat

Transthyretin-Mediated Amyloidosis.[99] Cationic lipid nanoparticle-based siRNA drug Atu027 sponsored by Silence Therapeutics AG has completed a dose-escalation, Phase I clinical trial to assess toxicity.[100] These and other lipid-based siRNA delivery technologies represent the class of material nearest to reaching the clinic for siRNA delivery.

Polymeric nanoparticles

Although lipid-based nanoparticles have been historically more widely used for nucleic acid delivery, polymer-based nanoparticles are very promising newer class of materials for the delivery of nucleic acids and are an active subject of ongoing research. Because many of the polymers used for siRNA delivery were originally investigated as DNA delivery materials, many of them have gone through iterations of modification as various hurdles specific to siRNA delivery were discovered. Key classes of materials are described below, along with examples of the bottlenecks encountered and the strategies used to sidestep them.

Poly(l-lysine)- and polyethylenimine-based materials

PLL and PEI, as mentioned above, were early candidate materials for siRNA delivery, with PEI still used frequently in research today. Derivatives of both polymers continue to be developed and optimized and are the basis of many of the polymeric systems being studied for siRNA delivery. Because PLL/nucleic acid complexes can be taken up by cells but cannot efficiently escape the endosome, Meyer *et al.* developed PLL- and PEI- based materials by conjugating the polycation to an endosomolytic mellitin peptide sequence modified with pH-labile protecting groups (dimethylmaleic anhydride), which restrict the lytic activity of the peptide until they are cleaved at acidic pH.[101] However, because the peptide was negatively charged and was necessary in high number for effective endosomal escape, this covalent modification destabilized the polymer/siRNA complex, precluding the formation of sufficiently small NPs for cell uptake. As a solution for this, the authors grafted PEG to the polycation before modification with the peptide, which increased the stability of the complex and caused gene knockdown *in vitro* in human neuroblastoma cells.

Miyata *et al.* used a previously-developed[102, 103] PEG-*b*-PLL copolymer to deliver nucleic acids.[104] By modifying some of the lysine sidechains with thiol-containing functional groups, they could form micelles via electrostatic interactions with anionic DNA and then oxidize the complexes, forming disulfide bridges to stabilize the particles.[104] This method was also effective for *in vitro* siRNA delivery to Huh7 human hepatoma cells, with ~80% knockdown seen after delivery of 100 nM siRNA to Huh7 cells in the presence of serum. Because of weaker binding seen in siRNA polyplexes compared with DNA polyplexes, the authors further modified their polymer with 2-iminothiolane to increase the cationic character and thus strengthen siRNA binding.[105] Because of the difference in redox potential between the cytoplasm and extracellular environment, this system carries with it the advantage of quick siRNA release once in the desired cellular compartment. Further improvements on this system included the conjugation of cyclic RGD peptide to the PEG block, thereby providing a method to target tissues *in vivo* (Figure 4a).[106] These siRNA-containing micelles accumulated in tumor tissue and the surrounding vasculature in a subcutaneous HeLa tumor model. Delivery of 24 μg siRNA per mouse (~0.5–1 mg/kg) against vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) two times every four days for 12 days showed ~50% decreased in measured VEGF mRNA levels in the tumor and significantly slowed tumor growth (~60–70% smaller tumor volume after 12 days compared to control) without apparent toxic side effects on the animal. Similar strategies have also been employed with PEI, using PEGylated siRNA against VEGF to form PEI-based micelles that caused VEGF knockdown in an *in vivo* PC-3 prostate cancer model.[107]

With toxicity as a major problem limiting the use of PEI, forms of degradable PEI have been studied extensively for siRNA as well as DNA delivery. Breunig *et al.* linked low-molecular weight linear PEI segments with disulfide bridges to form a bioreducible PEI.[108] Combining the efficacy of high-molecular PEI with the biocompatibility of shorter polymer chains, they delivered 100 nM siRNA *in vitro* to Chinese hamster ovary cells (CHO-K1) in serum-free medium and were able to achieve *in vitro* RNAi of green fluorescent protein (GFP) expression (~50% knockdown) that was comparable in efficacy to that of high-molecular weight branched PEI (bPEI) while being less cytotoxic. This design carried with it the additional benefit of targeted siRNA release into the cytoplasm, as disulfide bridges would be expected to be reduced to thiols.

Other classes of polymers

Also capitalizing on bioreducible linkages for targeted release, Jeong *et al.* described the use of a bioreducible PAA, designated SS-PAEI [poly(amido ethylenimine)], for siRNA delivery.[109] Having found previously that SS-PAEI had higher buffering capacity than PEI,[110] they further demonstrated that their bioreducible polymer could cause significant knockdown of vascular endothelial growth factor (VEGF) expression from human prostate cancer cells *in vitro* with 30 nM siRNA delivered in serum-free medium. Their polymer was more effective (up to ~80% less mRNA expression) and less toxic than the linear PEI used as a comparison. Because there was not a significant increase in cellular uptake of SS-PAEI/siRNA particles compared to PEI/siRNA, the authors concluded that intracellular events, likely the disulfide reduction step, was the main reason for the increased efficacy. The siRNA binding efficiency of SS-PAEI is lower than that of PEI; therefore, SS-PAEI was copolymerized with a polymer block containing ethylene diamine.[57, 111] The increased amine content and therefore increased positive charge caused tighter siRNA binding and, at optimized ratios, better *in vitro* knockdown efficiency in human head and neck carcinoma cells (UM-SCC-14C) and non-small-cell lung carcinoma (H1299).

Further optimization of this system included PEGylation by adding PEG-amine into the SS-PAEI synthesis, then mixing PEG-containing SS-PAEI with PEG-free SS-PAEI in order to improve particle stability in the presence of blood components like serum.[113] However, while particles with higher PEG content were more stable against aggregation in serum or salt, they were also more easily dissociated in the presence of heparin, suggesting less stable binding interactions between siRNA and highly PEGylated polymers. Toxicity in erythrocytes as well as H1299 cells decreased with increasing PEG content, although this came at the cost of strongly compromised knockdown efficacy as well.

Similar to PAAs are poly(ester amine)s (PEAs) or poly(beta-amino ester)s (PBAEs), which are hydrolytically degradable through their ester groups. Initially studied primarily for DNA delivery, some early attempts to use PBAEs for siRNA delivery fell short without additionally conjugating siRNA to solid particles.[114] As with other cationic polymers, this class of materials faced obstacles in polymer-siRNA binding efficiency and intracellular release. By increasing the ratio of PBAE to siRNA (100:1 or greater PBAE:nucleic acid ratio by weight, compared to ~50:1 for DNA delivery), better particle formation and siRNA complexation was achieved, resulting in successful knockdown (~70%) in human umbilical vein endothelial cells when 60 nM siRNA was delivered in medium with 2% serum.[115]

Other groups were able to show ~70% *in vitro* knockdown in hepatoma cells in serum-free medium with lower PBAE:siRNA weight ratios when using PBAEs of similar chemical structure.[116] Interestingly, the PBAE molecular weight reported was higher than that of the previous two studies cited and this may have enabled the increased efficacy at lower PBAE:siRNA weight ratios. Trends in PBAE structure with siRNA delivery efficacy have since been further examined,[65] confirming the general trend of increasing knockdown

efficacy with increasing PBAE molecular weight, as well as other polymer properties like hydrophobicity. This study also showed that the siRNA dose could be decreased by a factor of twelve (as low as 5 nM) without negatively affecting the knockdown efficacy (~90% knockdown in serum-free medium) as long as the total polymer concentration was not altered. Transfection in 10% serum-containing medium was lower (~70%), which the authors believed was due to some destabilization of the nanoparticles in the presence of serum proteins. In particular, this was observed with the top PBAEs in the study, most of which contained a primary amine terminal functional group that could be cleaved in a reducing environment. By utilizing disulfide bonds, this study and others[117, 118] have found bioreducible linkages can be a key attribute for successful non-viral siRNA delivery. In another study, poly(ethylene oxide) (PEO)-*b*-PBAE NPs were made for siRNA delivery in combination with PEO-*b*-poly(ε-caprolactone) (PCL) NPs loaded with paclitaxel.[7] This study showed successful knockdown of MDR-1, a cause of multiple drug resistance in cancer cells, and consequently increased efficacy of a chemotherapeutic *in vitro* in SKOV3 ovarian cancer cells.

The combination of PEGylation with disulfide linkages has also been applied to polypeptides, including KALA.[119] Mok *et al.* conjugated PEG to siRNA via a disulfide bridge. Cysteine-containing KALA was then self-crosslinked to form a high-molecular weight, but bioreducible polymer, which electrostatically complexed with siRNA-PEG. This system was able to achieve nearly 50% gene knockdown in MDA-MB-435 melanoma cells, cultured *in vitro* in the presence of 10% serum with siRNA concentration of ~60 nM.

Woodrow *et al.* had an interesting finding while investigating the effectiveness of poly(lactic-*co*-glycolic acid) (PLGA) NPs at siRNA-mediated silencing *in vivo*.[120] Although a positively-charged component, spermidine, was included in the encapsulation formulation, it was used as an excipient rather than as a primary delivery agent. The PLGA/siRNA NPs themselves were small enough for cellular internalization, and siRNA released over time from the particles could be isolated and delivered separately without loss of function. Using these particles, the authors delivered siRNA across the vaginal mucosal barrier and effected knockdown in cells in the vaginal tract. A major advantage of this system is the biocompatibility of PLGA and related polyesters, which have been used in a number of FDA-approved devices. In addition, being solid particles, this delivery system does not suffer from unstable binding interactions as do many polymeric electrostatic complexes. Researchers are interested in adding cationic materials, such as chitosan or the above spermidine, as part of PLGA-based NPs to increase siRNA loading into anionic PLGA NPs and increase knockdown efficacy.[121]

A study by Zhou *et al.*[122] provides an example of how several components can be combined in a NP design to incorporate many different functions and properties. The authors used as its base material a PEG-grafted PLGA-*b*-PLL block copolymer previously developed for fabrication of surface-functionalized NPs.[123, 124] DNA or siRNA was loaded into the NPs during synthesis, along with palmitoyl-avidin, allowing biotinylated peptides to be conjugated to the NP surface. In this way, the authors were able to design NPs containing polycations for nucleic acid complexation; PLGA as a biocompatible matrix; PEG to improve stability and circulation time; two encapsulated drugs to enhance delivery efficacy and siRNA processing; and three peptides to promote cellular uptake, endosomal escape, and tissue homing (Figure 5). While this type of drug delivery vehicle is more complex than some of the other examples described here, it provides flexibility in formulation and design details and was effective *in vitro* for ~91% knockdown in A549 lung cancer cells in culture medium.

Other elegant polymer designs for siRNA delivery, including a multifunctional, cyclodextrin-based vehicle, have had successful siRNA delivery preclinical studies and have moved forward to clinical testing.[125] This formulation, denoted CALAA-01, is a self-assembled complex of siRNA with a multi-component delivery vehicle, consisting of (i) a cyclodextrin-containing polymer, which also contains positive charges for siRNA binding; (ii) adamantane-conjugated PEG, with PEG added for particle stability and adamantine used to bind to cyclodextrin; and (iii) an adamantine-PEG-transferrin conjugate for targeting. This polymeric siRNA formulation, designed for good *in vivo* stability and site-specific delivery, is currently in a phase I trial.

Inorganic NPs

Calcium Phosphate

Calcium phosphate (CaP) crystals were originally developed for DNA delivery and were fabricated by highly saturated solutions of DNA and CaP that resulted in spontaneous coprecipitation.[126–128] In an effort to limit the rapid and often difficult-to-control growth of CaP crystals, Kakisawa *et al.* developed an inorganic-organic NP hybrid for DNA delivery in which polyaspartate segments of polyaspartate-PEG block copolymers could adsorb onto the expanding crystal surface, thereby limiting continued growth.[129] The PEG segments impart biocompatibility and particle stability.

This NP formulation was eventually used for *in vitro* siRNA delivery to human embryonic kidney 293 cells and was able to achieve roughly 50% gene knockdown using a 125 nM siRNA dose in serum-containing media.[130] However, these particles required chloroquine for efficient endosomal escape, so the same researchers replaced polyaspartate with poly(methacrylic acid) (PMA), as PMA becomes more hydrophobic in pH 4–6 and could disrupt the endosomal membrane. To assess the siRNA delivery of this CaP-PMA-PEG system to 293 cells in a more physiologically relevant way *in vitro*, the group incubated all siRNA transfection agents in serum-containing media for 30 min prior to transfection. They were able to show significantly lower toxicity than Lipofectamine™ 2000 and a significantly higher gene knockdown of 65% versus RNAifect™ at 42%.[130]

siRNA loading into CaP based NPs was improved five-fold versus previously described systems by Zhang *et al.* via the covalent linkage of PEG to siRNA before precipitation with CaP. These NPs achieved roughly 60% gene knockdown in HeLa cells with 100 nM siRNA in serum-containing media.[131]

Gold

Gold-based nanoparticles (AuNPs) are attractive drug delivery candidates due to their low cytotoxicity,[132] easily tunable physical properties,[133] and readily functionalizable surface chemistry.[134–136] Elbakry *et al.* used AuNPs and the ionic layer-by-layer (LbL)[137] surface modification process for siRNA delivery. These researchers took advantage of sulfur-gold binding to coat AuNPs with 11-mercaptoundecanoic acid (MUA), then used electrostatic interactions to coat the particles with a layer of PEI, followed by siRNA and another layer of PEI. *In vitro* delivery to CHO-K1 cells using 290 nM siRNA in serum-containing media resulted in approximately 80% knockdown.[67] However, this particular NP formulation required a high siRNA dose most likely because it included no siRNA release mechanism. Cytoplasmically-targeted siRNA release was achieved by Lee *et al.* in a AuNP formulation in which siRNA was attached to AuNPs via a PEG linker and bioreducible disulfide bonds, then coated with PBAEs. This formulation achieved near-complete gene knockdown in HeLa cells when a 90 nM dose of siRNA was delivered in serum-containing media.[68] Giljohann *et al.* have also demonstrated transfection agent-free delivery of siRNA through

the use of AuNPs densely functionalized with RNA oligonucleotides.[138] In this study, ~100 nM of RNA duplex (3 nM AuNPs) caused 73% knockdown in HeLa cells. In an intriguing recent development, spherical nucleic acids, consisting of 13 nm AuNP cores and 30 siRNAs per particle densely functionalized around the surfaces, were able to penetrate skin and cause functional knockdown in hairless mice following topical treatment *in vivo*.[139]

Silica

As NP-based siRNA therapeutics are developed for clinical use, an important obstacle will be to create optimal dosing regimens, ideally those that minimize dosing frequency. Multistage release vectors were developed to address this issue by using nanosized lipid-based siRNA carriers loaded into micron sized, porous silicon particles.[140] In an *in vivo* study of orthotopic mouse ovarian carcinoma, this systemic delivery system extended gene knockdown from a few days to more than three weeks and effectively reduced angiogenesis, cell proliferation, and tumor burden. Another formulation was developed in which porous silicon nanoparticles were loaded with the chemotherapeutic doxorubicin and siRNA targeting a drug efflux transporter, and then coated with siRNA. *In vitro* delivery of these particles to multidrug-resistant KB-V1 cells showed knockdown of the drug transporter, resulting in increased intracellular and intranuclear doxorubicin levels.[141]

Quantum Dots

Quantum dots (QDs) are inorganic semiconductor nanoparticles and can be used as fluorophores. QDs with easily tunable emission properties, such as CdSe/ZnS nanoparticles, are promising candidates for imaging purposes in that they are brighter,[142, 143] less susceptible to photobleaching,[144] are easier to detect amongst *in vivo* background fluorescence versus most fluorescent dyes,[142] and are able to be made biocompatible enough to be used with cells.[145, 146] Chen *et al.* first codelivered siRNA and QDs in order to trace the delivered siRNA *in vitro*.[147] This concept was extended to make QDs part of the delivery system itself by Derfus *et al.*, in which QDs were covalently modified with PEG, siRNA, and a tumor homing peptide (F3) to deliver siRNA to HeLa cells.[148] They were able to show that cell uptake was facilitated by F3 conjugation, and the NPs achieved approximately 30% gene knockdown in serum conditions with a 50 nM dose of siRNA. Although the NPs could not improve upon the knockdown achieved by Lipofectamine™ 2000, this delivery system showed an exciting proof-of-concept for a method to deliver and specifically track siRNA molecules using QDs.

Engineered RNA-based NPs

As gene knockdown via direct delivery of siRNA is a transient, dose-dependent process, siRNA loading into the delivery system is critical for complete and long-lasting gene suppression. Delivery systems in which siRNA is conjugated directly to a biomaterial or in which engineered RNA is the delivery material itself present exciting strategies to maximize loading. An example of increased loading was examined earlier in this review when siRNA was directly conjugated to PEG and coprecipitated with CaP to improve siRNA loading five-fold.[131] Additionally, particle stability can be enhanced with the use of multimeric siRNAs, a class of material created by either covalently or noncovalently linking siRNA molecules. These multimeric siRNAs promote multivalent biomaterial-nucleic acid interactions similar to those seen with plasmid DNA, resulting in improved NP stability with RNA. Strategies for modifying siRNA while maintaining its biological activity are discussed below.

siRNA Conjugates

Important considerations for chemically modifying siRNA include assuring that the modification will not interfere with RNAi,[149] and, for modifications where multiple siRNAs are attached to each other, that the long chain of RNA will not induce an IFN response.[13] Singh *et al.* examined the former issue using the PEGylated siRNA-QD conjugates described earlier in this review. The authors found that decreasing the length of the siRNA-QD linker resulted in less RNAi, while lengthening this linker promoted more RNAi. They also found that the site of conjugation on the siRNA molecule, 5′ versus 3′, sense versus antisense, did not seem to effect RNAi.[150] These results confirmed what an earlier study had concluded with conjugation to magnetic NPs.[151]

Another investigation into PEG-siRNA conjugation utilized a disulfide linker and formed NPs using PEG-siRNA and PEI. These NPs were able to achieve 80% and 96.5% gene knockdown in serum and serum-free conditions, respectively, in human prostate carcinoma PC-3 cells using a 100 nM siRNA dose.[152] The same material in an *in vivo* study using subcutaneous PC-3 tumors and IV injection of NPs with 1.5 nmol VEGF-targeting siRNA showed 86 ± 4% decreased VEGF expression, resulting in a 78 ± 9% reduction in microvessel formation and a ten-fold decrease in tumor volume.[107]

An alternative siRNA conjugation strategy involves conjugating the 3′ end of the sense strand of siRNA to cholesterol. Such a system can cause 50% knockdown in HeLa cells with a ~200 nM siRNA dose without using any additional transfection agent or nanoparticle.[153] Cholesterol-siRNA, but not unmodified siRNA, has improved pharmacokinetic properties and can mediate knockdown in mice following i.v. administration. Other lipophilic modifications to siRNA can also enhance *in vivo* knockdown.[154]

Multimeric siRNA

While most strategies focus on tuning vector material properties to make DNA delivery vehicles work for siRNA delivery, another approach is to adjust the siRNA itself to make it physically more like DNA. Multimeric siRNA allows for increased multivalent interactions in addition to lending the geometric flexibility that is favorable for higher affinity binding. An interesting method to form multimeric siRNA was introduced by Bolcato-Bellemin *et al.* in which they synthesized siRNA strands with 5–8 bp overhangs to yield “sticky siRNA ends” that would reversibly concatemerize to form long repeats of siRNA.[155] When delivered with linear PEI to A549 human lung carcinoma cells in serum-free conditions, these NPs achieved 80% gene knockdown with 50 nM siRNA and 70% knockdown with as little as 20 nM siRNA. The authors also showed that this material triggered no IFN response. This concept was later expanded upon by covalently linking siRNA strands with either a bioreducible or non-degrading linkage.[156] When complexed with linear PEI, both the cleavable and non-cleavable siRNA NPs achieved near complete gene knockdown in PC3 cells with a 90 nM siRNA dose in serum-free conditions. However, when using rapid amplification of cDNA ends to look for the specific cleaved target mRNA, the authors only found a significant concentration of the expected fragment in the samples treated with the disulfide-linked siRNA.

A unique method to create an siRNA-based material was recently suggested by Lee *et al.*[157] The group employed rolling circle transcription[158] to make connected repeats of hairpin RNAs, which then crystallized into growing sheets to eventually form microsponges. From these microsponges, micron-sized, spherical particles could be pinched off and coated with bPEI to compact them into roughly 200 nm sized particles. These particles achieved such incredibly high siRNA loading that the authors were able to show comparable knockdown to commercially available siRNA delivery systems using three orders of magnitude fewer

particles. *In vitro* delivery of these particles to T22 cells achieved nearly 60% knockdown with 100 nM siRNA, and an *in vivo* intratumoral injection to T22 cells showed significant gene knockdown at 4 days post-transfection.

An additional class of three-dimensional nucleic acid-only structures for RNA delivery are polyvalent nucleic acid nanostructures. These have been synthesized by Chad Mirkin and his research group by constructing intra-crosslinked spherical nucleic acids with an inorganic nanoparticle core and then subsequent dissolution of this core.[159] These polyvalent nucleic acid nanostructures share many of the characteristics of spherical nucleic acids including being able to cause comparable efficient gene knockdown.

Conclusion and outlook

Despite its clinical potential, use of siRNA as a therapeutic has been hampered by a lack of effective and safe methods of delivery. Many different materials have been explored in the laboratory, with some, mostly lipid-based, having been translated to the clinic in early-phase trials. It is important to note, however, that both the siRNA molecule itself and also the delivery vehicle can have off-target effects that may confound results and affect the translation of this technology.

As each biomaterial is developed, challenges in siRNA delivery are illuminated, aiding in the design of improved carrier NPs. New biomaterials have now been developed to fit the chemistry, biophysical structure, and biological function of siRNA. Many research groups are exploiting the benefits of increased siRNA binding affinity, for example, by increasing the positive charge of a biomaterial or by synthesizing multimeric siRNA molecules, to improve stability and delivery efficacy. Similarly, other researchers are investigating triggered release properties, such as disulfide bonds to cause siRNA release upon entering the cytoplasm, to improve delivery efficacy and reduce cytotoxicity. These next-generation materials have shown promise in the laboratory both *in vitro* and *in vivo*. As better ways for siRNA delivery are discovered, the wide range of therapeutic benefits to using siRNA will more fully come to light.

Acknowledgments

This work was supported in part by the NIH (1R01EB016721). KLK thanks the NIH Cancer Nanotechnology Training Center (R25CA153952) at the JHU Institute for Nanobiotechnology for fellowship support and SYT thanks the National Science Foundation for fellowship support.

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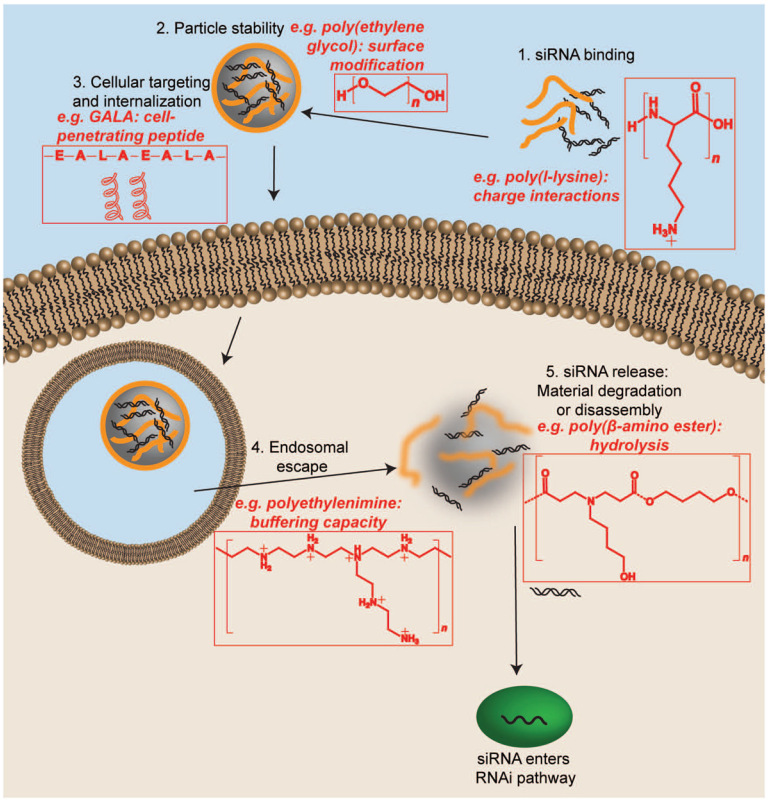

Figure 1.

siRNA faces several barriers during intracellular delivery. Representative biomaterials that are able to overcome these barriers are shown above, along with the particular strategy employed by that material.

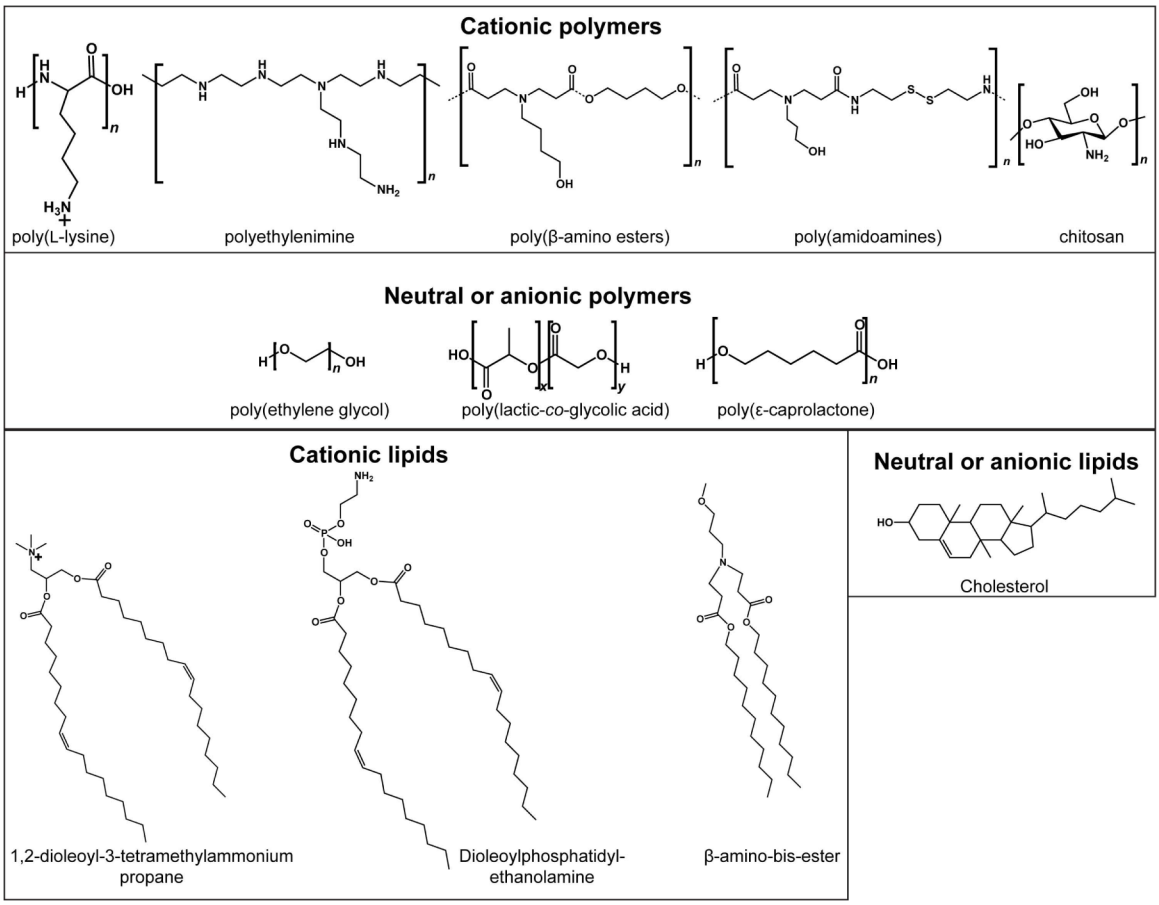

Figure 2.
Examples of classes of polymeric and lipidic biomaterials used for siRNA delivery.

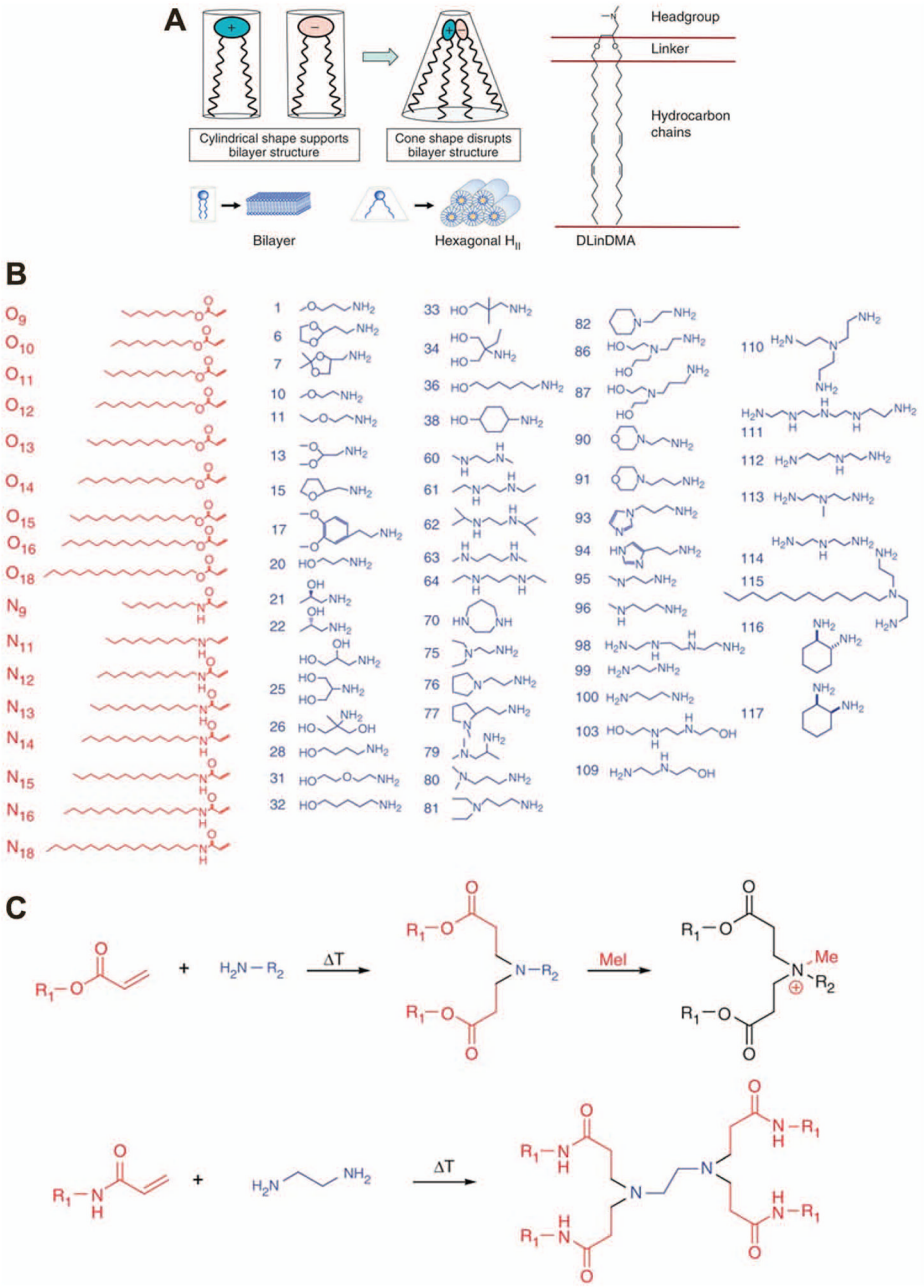

Figure 3.

Various approaches have been taken to lipid-based siRNA delivery. While some groups use rational design and focus on specific delivery aspects, such as lipid polymorphism leading to membrane fusion (A), others have employed high-throughput methods to screen through a wide array of different molecules to empirically determine the best structures (B–C). Reprinted by permission from Macmillan Publishers Ltd: *Nature Biotechnology*,[86, 94] copyright (2010) (A) and copyright (2008) (B–C).

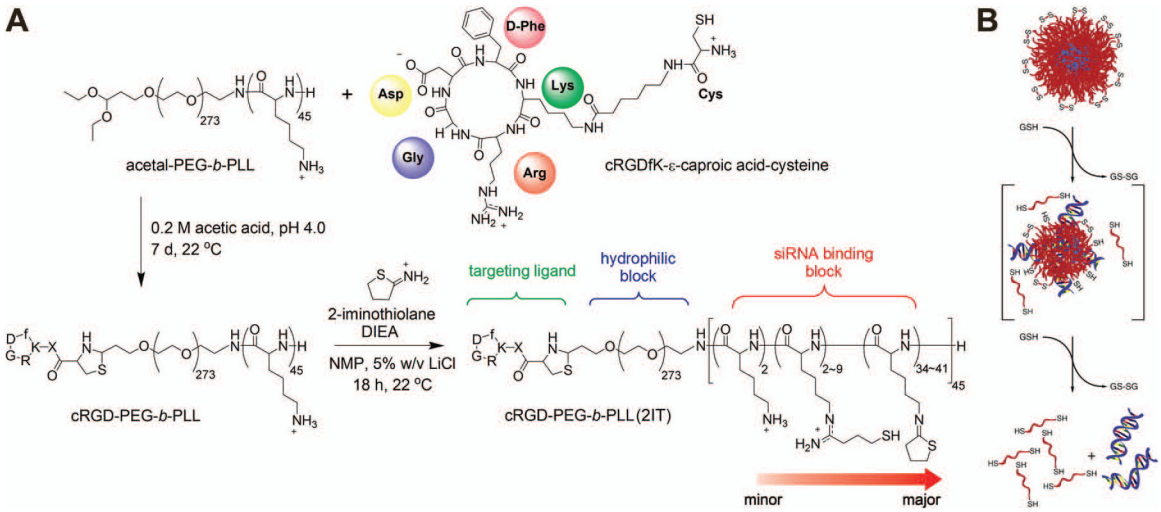

Figure 4.

Widely-used approaches to improving polymeric siRNA delivery include conjugation to PEG and targeting ligands (A) and introduction of disulfide bridges for controlled cytoplasmic release (B). Part A reprinted with permission from Christie, R. J.; Matsumoto, Y.; Miyata, K.; Nomoto, T.; Fukushima, S.; Osada, K.; Halnaut, J.; Pittella, F.; Kim, H. J.; Nishiyama, N.; Kataoka, K.: Targeted Polymeric Micelles for siRNA Treatment of Experimental Cancer by Intravenous Injection. *ACS Nano* **2012**, *6*, 5174–5189.[106] Copyright (2012) American Chemical Society. Part B reprinted from *Journal of Controlled Release*, 150/2, van der Aa et al, Optimization of poly(amido amine)s as vectors for siRNA delivery, 177–186,[112] Copyright (2011), with permission from Elsevier.

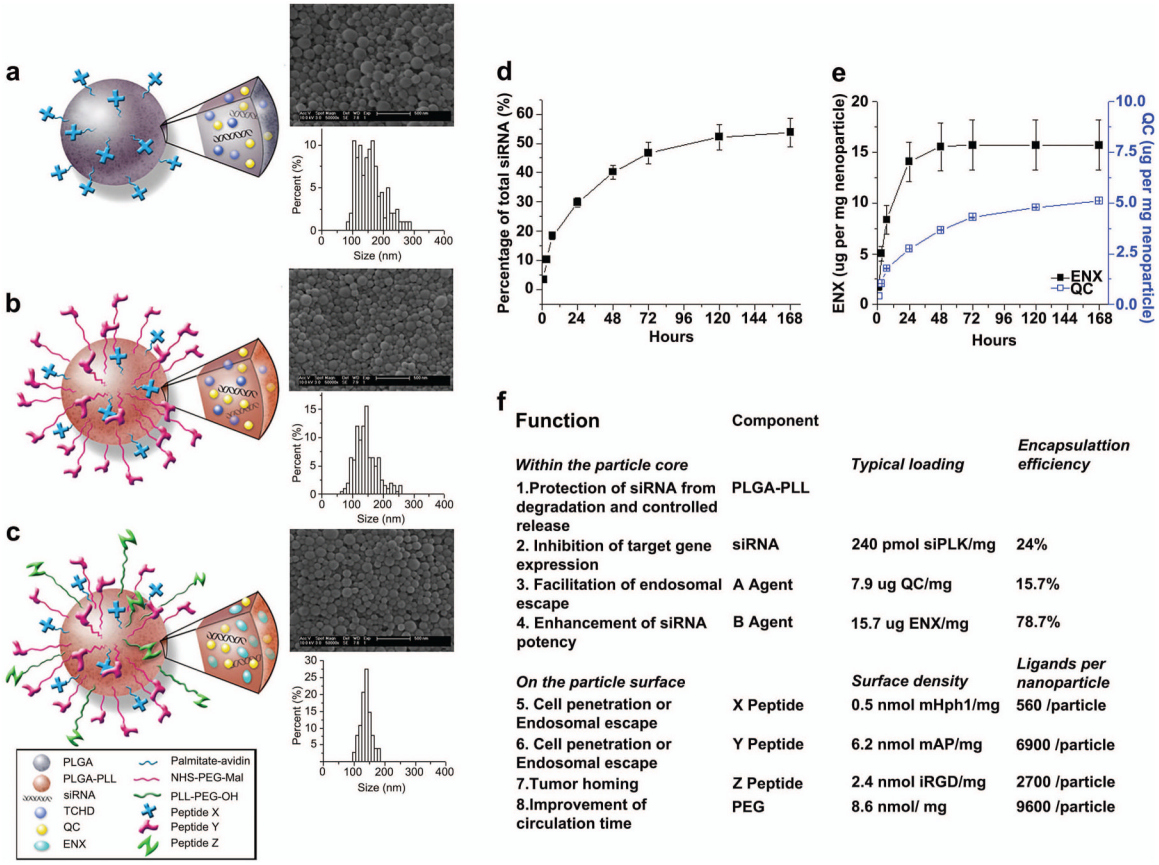

Figure 5.

PLGA-b-PLL-g-PEG NPs contain siRNA, two drugs, and three ligands for targeting, cell penetration, and trafficking. Reprinted from *Biomaterials*, 33/2, Zhou et al, Octa-functional PLGA nanoparticles for targeted and efficient siRNA delivery to tumors, 177–186,[122] Copyright (2012), with permission from Elsevier.

Table 1

Types of biomaterials used for siRNA delivery and the challenges they pose.

Biomaterial type	Major Challenges	Strategies to overcome	Examples
Lipid-based nanoparticles	Off-target immunogenicity	Surface-coating with non-immunogenic materials	DOTAP-based nanoparticles surface-modified with hyaluronic acid and PEG[84]
	Toxicity	Low lipid-to- siRNA ratio	Amine-containing lipidoid synthesized from 12-C acrylamides was complexed with siRNA at different ratios to find maximum siRNA loading efficiency[87]
		Empirical testing of different compounds	Screening of a combinatorial library of synthetic lipidoids and test for toxicity and efficacy[86]
	Cellular uptake	Fusogenic lipids	Addition of cholesterol to liposome[86–89]
			Lipid structure engineered to promote fusogenic phase transition[92]
		Other lipid interaction with membrane	Screening of lipid polar headgroups with different size, charge, and acid-dissociation constant[94]
	Liposome instability	Surface modification	Incorporation of PEG into liposome formulation[86, 87, 93]
Polymeric nanoparticles	Endosomal escape	Proton-sponge effect (buffering capacity)	Reversibly protonated tertiary amines (e.g. PEI,[50] PAA,[56] PBAE[64])
		Fusogenic peptides	PLL conjugated to pH-sensitive endosomolytic peptide[101]
			PLGA-b-PLL nanoparticles non-covalently coated with endosomolytic peptide[122]
			Membrane-penetrating peptide KALA crosslinked into an siRNA-binding polymer[119]
	siRNA binding	High polymer cationic character	Primary polymer blended or doped with polycation (e.g. chitosan[121])
			Addition of a small molecule with primary amines[57, 105, 111]
			Copolymerization with polycation[122]
		Crosslinked particles	Oxidation of thiol-functionalized polymers to form disulfides throughout nanoparticle[105, 106]
	Toxicity	Degradation	Hydrolytically-cleavable ester moieties (e.g. PBAEs)[7, 115–117]
			Bioreducible disulfide bridges[57, 108–111, 113, 115, 117, 119]
	Cellular targeting	Tissue-specific ligands	Conjugation of ligand to polymer[106]
			Non-covalent coating of nanoparticle surface with ligand[122]
Inorganic NPs	Long-term efficacy	Controlled release	Porous silica particles that can be loaded with siRNA-containing NPs[140]
	Toxicity	Gold-based NPs	Gold NPs coated with PEI and siRNA using LbL process[67, 68]
			Gold NPs functionalized with PEG and siRNA, then coated with PBAEs[68]

Biomaterial type	Major Challenges	Strategies to overcome	Examples
			Gold NPs functionalized with polyvalent siRNA[138] and made into spherical nucleic acids[139]
	Particle tracking	Quantum dot- based NPs	Quantum dots covalently modified with siRNA, PEG, and a tumor homing peptide[148]
Engineered RNA-based NPs	Particle instability	Multimeric siRNA	siRNA strands with “sticky” overhangs that reversibly concatamerize[77, 107, 150, 152]
			RNAi microsponges containing densely packed, multimeric siRNA[157]
	Interferon response	Control over RNA size	Disulfide-linked multimeric siRNA[77, 156]
			siRNA strands with “sticky” overhangs[77, 156]
	Loss of siRNA biological activity	Labile, long, or crosslinked linkages	Disulfide linkages[107, 150, 152] or long, non-labile linkages[150]
			Polyvalent nucleic acid nanostructures[159]