ACCOUNTS
of chemical research

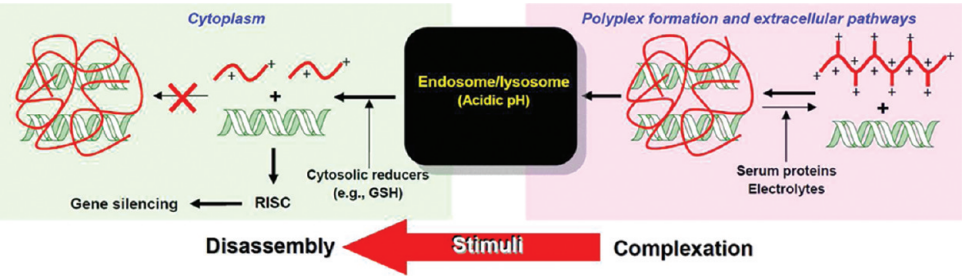
Before and after Endosomal Escape: Roles of Stimuli-Converting siRNA/Polymer Interactions in Determining Gene Silencing Efficiency

YOUNG JIK KWON*

Department of Pharmaceutical Sciences, Department of Chemical Engineering and Materials Science, Department of Biomedical Engineering, and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697, United States

RECEIVED ON SEPTEMBER 15, 2011

CONSPECTUS



Silencing the expression of a target gene by RNA interference (RNAi) shows promise as a potentially revolutionizing strategy for manipulating biological (pathological) pathways at the translational level. However, the lack of reliable, efficient, versatile, and safe means for the delivery of small interfering RNA (siRNA) molecules, which are large in molecular weight, negatively charged, and subject to degradation, has impeded their use in basic research and therapy. Polyplexes of siRNA and polymers are the predominant mode of siRNA delivery, but innovative synthetic strategies are needed to further evolve them to generate the desired biological and therapeutic effects.

This Account focuses on the design of polymeric vehicles for siRNA delivery based on an understanding of the molecular interactions between siRNA and cationic polymers. Ideal siRNA/polymer polyplexes should address an inherent design dilemma for successful gene silencing: (1) Cationic polymers must form tight complexes with siRNA via attractive electrostatic interactions during circulation and cellular internalization and (2) siRNA must dissociate from its cationic carrier in the cytoplasm before they are loaded into RNA-induced silencing complex (RISC) and initiate gene silencing. The physicochemical properties of polymers, which dictate their molecular affinity to siRNA, can be programmed to be altered by intracellular stimuli, such as acidic pH in the endosome and cytosolic reducers, subsequently inducing the siRNA/polymer polyplex to disassemble. Specific design goals include the reduction of the cationic density and the molecular weight, the loss of branched structure, and changes in the hydrophilicity/hydrophobicity of the polymeric siRNA carriers, via acid-responsive degradation and protonation processes within the endosome and glutathione (GSH)-mediated reduction in the cytoplasm, possibly in combination with gradual stimuli-independent hydrolysis.

Acetals/ketals are acid-cleavable linkages that have been incorporated into polymeric materials for stimuli-responsive gene and drug delivery. Tailoring the ketalization ratio and the molecular weight of ketalized branched PEI (K-BPEI) offers molecular control of the intracellular trafficking of siRNA/polymer polyplexes and, therefore, the gene silencing efficiency. The ketalization of linear PEI (K-LPEI) enhances gene silencing in vitro and in vivo by improving siRNA complexation with the polymer during circulation and cellular internalization, supplementing proton buffering efficiency of the polymer in the endosome, and facilitating siRNA dissociation from the polymer in the cytoplasm, in a serum-resistant manner. Spermine polymerization via ketalization and esterification for multistep intracellular degradations provides an additional polymeric platform for improved siRNA delivery and highly biocompatible gene silencing. The chemistry presented in this Account will help lay the foundation for the development of innovative and strategic approaches that advance RNAi technology.

Published on the Web 11/21/2011 www.pubs.acs.org/accounts
10.1021/ar200241v

1. Introduction

Nucleic acids, such as DNA and RNA, are employed to induce a broad range of desired and controlled biological effects at a molecular level without nonspecific toxicity. They can introduce, strengthen, or silence target genes and represent a promising class of emerging therapeutics. Among them, RNA interference (RNAi) using small interfering RNA (siRNA) has not only become a powerful tool in targeting cell signaling pathways for basic research but also silencing pathological events for treatment of diseases.[1−3] Selective targeting of specific biological/pathological pathways at the translational level can be a convenient, flexible, and safe mode of therapy, compared to the more challenging endeavor of delivering a completely new set of transgene for stable expression. While continual advancements in genomic and proteomic profiling will reveal additional information about the molecular blueprints of the human body, it will concurrently enable the rational design of siRNA as well.

Unlike what its name may suggest, siRNA is a large double stranded molecule in consisting of about a couple dozen base pairs (∼15 kDa). With properties such as high molecular weight, strong anionic charge, potential recognition by the immune system, and degradation by nucleases, the development of effective and efficient in vivo delivery of siRNA is required.[4,5] Considering the fact that cost-effective manufacturing of siRNA at a large scale is no longer a technological obstacle,[6,7] developing effective and safe means of delivery (administration) is a key challenge in bringing RNAi technology a step closer to becoming a widely acceptable and reliable option in both basic research and clinical medicine. Medicinal chemistry approaches to directly modifying the structure of siRNA molecules have been attempted in order to obtain improved pharmacokinetic (PK) properties and biodistribution.[8,9] However, a promising, possibly inevitable, strategy is to reformulate siRNA in the form of nanoparticles that complex siRNA and shield it from destructive/inactivating forces. Examples include siRNA/polymer polyplexes, siRNA-encapsulating liposomes, and siRNA-encoding viruses.[5,10,11]

This Account focuses on the complexation of siRNA with various polymeric materials (i.e., siRNA/polymer polyplexes) as a predominant mode of siRNA delivery and also addresses the need for innovative chemical (synthetic) approaches to developing efficient, safe, and versatile vehicles for siRNA delivery. Particularly, converting interactions of siRNA with its carrier polymers in opposing modes (from complexation to disassembly) during intracellular delivery pathways are considered as the most important, fundamental, and indispensible factor in designing novel siRNA delivery carriers. Liposomes, another major vehicle for siRNA delivery, rely on the interactions between liposomes and the cell membrane for siRNA release; this is relatively independent of the interactions with siRNA, and requires design considerations that are distinctively different from considerations for siRNA/polymer polyplexes.

2. Barriers in siRNA Delivery

RNAi is an end-point result that requires the successful, yet complex, extracellular and intracellular processing of siRNA (Figure 1). First of all, siRNA should be complexed by its delivery vehicle (carrier) and shielded from degradation by nucleases and immune recognition. Complexed siRNA should circulate in the body for a desired period of time and accumulate in the target tissue while avoiding (1) premature disassembly via interactions with serum proteins, (2) activation of innate immune effectors, and (3) clearance by the reticuloendothelial system (RES).[12−14] Polyplexes of siRNA/polymer form nanoparticles that are more efficiently taken up than free, negatively charged siRNA by a target cell via faciliated nonspecific adhesion on the cell surface. Uninternalized polyplexes are disassembled via various interactions with serum proteins and electrolytes, and the released siRNA is eventually degraded and removed.

siRNA must free itself from its shielding polymers into the cytoplasm, while surviving the degradative intracellular processes (e.g., low-pH and acid-activated enzymes in the endosome). In the cytoplasm, only fully dissociated siRNA can bind to RNA-induced silencing complex (RISC) and the downstream mRNA. Therefore, disassembly of siRNA/polymer polyplexes is a key rate-limiting step in siRNA delivery and is closely related to overall gene silencing efficacy.[15−17] Transnuclear localization of siRNA, either in its complexed or dissociated form, reduces the availability of siRNA for binding to RISC and mRNA in the cytoplasm, lowering gene silencing efficiency.[16,18]

Figure 1 clearly highlights two key aspects in siRNA delivery: (1) what happens in the endosome and/or cytoplasm plays determining roles in intracellular trafficking of siRNA by converting siRNA/polymer interactions from complexation to disassembly, and (2) ideal siRNA carriers should be able to convert their interactions with siRNA in response to stimuli provided in the endosome and/or cytoplasm, suggesting crucial parameters in designing novel polymers for efficient siRNA delivery.

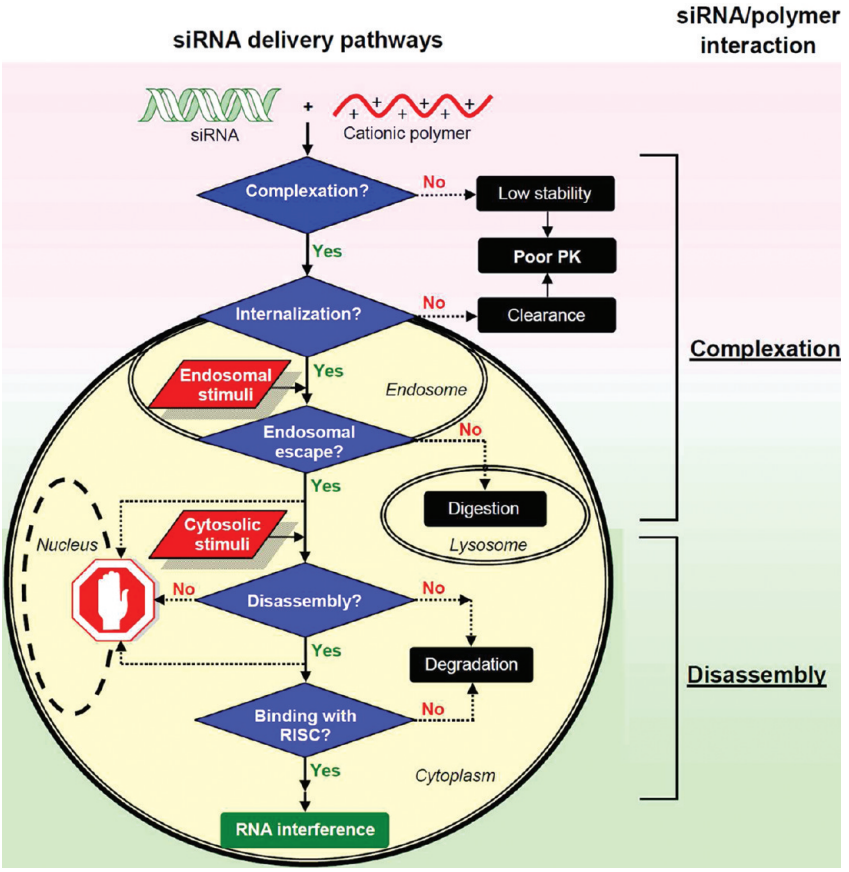


FIGURE 1. Efficiency-determining extracellular and intracellular steps (barriers) in siRNA delivery. siRNA should remain complexed with its carrier until reaching the endosome under a mildly acidic condition, followed by efficient release of siRNA in the cytoplasm in order to interact with RISC and target mRNA. Therefore, the endosome is the transition point for siRNA/polymer polyplexes to convert their interactions from a complexed to a disassembled mode. Reducible environment in the cytoplasm can further contribute to siRNA release from its polymeric carrier. Another important aspect for successful RNAi is cytosolic localization of siRNA. The sizes of free siRNA and its complex allow them to be transported to the nucleus through nuclear pore complexes (NPCs), reducing the available number of siRNA and overall gene silencing efficiency. Therefore, minimizing transnuclear localization of siRNA is a key design parameter in designing efficient siRNA carriers.

3. Dilemmatic siRNA/Polymer Interactions: Conversion from Complexation to Disassembly

Ideal siRNA carriers should be able to overcome the extracellular and intracellular barriers which require dilemmatic interactions of siRNA with its carriers in two opposing manners: complexation and disassembly. Consequently, it is crucial to incorporate converting physicochemical properties, particularly in response to intracellular stimuli, into polymeric siRNA carriers, in order to achieve desired gene silencing efficiency. Molecular interactions between siRNA and its carriers are comprehensively determined by electrostatic interactions, van der Waals forces, and nonpolar salvation.[19] Since siRNA structure is relatively consistent, except variations in its base sequence, physicochemical properties of polymers, such as cationic density, molecular weight, morphology, and hydrophilicity/hydrophobicity, dictate the molecular affinity of siRNA to its carrier which should be substantially reduced after endocytosis (Figure 2).

As discussed in Figure 1, siRNA must be complexed with its polymeric carrier to circulate for a prolonged period of time while avoiding premature inactivation and degradation. Cationic polymers, such as polyethylenimine (PEI), poly-L-lysine (PLL), and polyamidoamine (PAMAM) dendrimers, are popularly employed for siRNA complexation because electrostatic interactions between anionic siRNA and cationic polymers play the greatest roles in forming siRNA/polymer polyplexes.[20] Polyplexes of siRNA/cationic polymers form cationic nanoparticles that are efficiently taken up by a target cell via attractive electrostatic interactions of a cationic polyplex with the slightly negatively charged cell membrane. Amines of the cationic polymers are protonated in the acidic endosome and used to destabilize the membrane by water influx after counterions (e.g., Cl^-) are

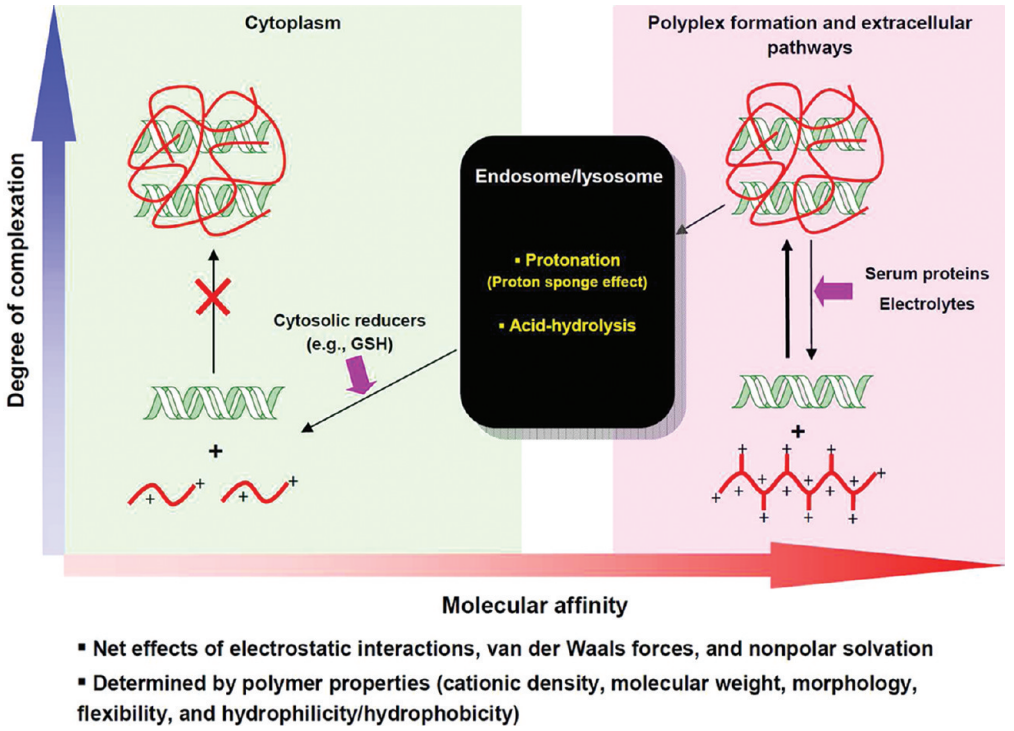


FIGURE 2. Converting interactions between siRNA and its polymeric carriers from complexation to disassembly, before and after endosomal escape. The physicochemical properties of polymeric carriers must be converted, in response to intracellular stimuli (e.g., mildly acidic pH and cytosolic reducers), reducing the molecular affinity with siRNA. To effectively bind to RISC, followed by initiating RNA interference, siRNA should remain in the cytoplasm without being recomplexed with its polymeric carriers. Therefore, irreversible reduction in molecular affinity between siRNA and its polymeric carriers is desirable.

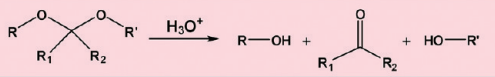
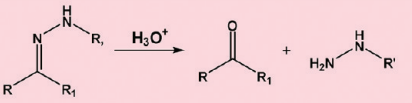
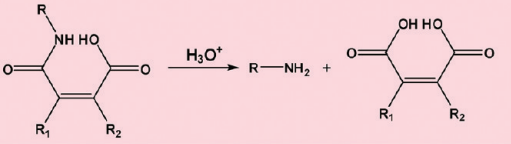
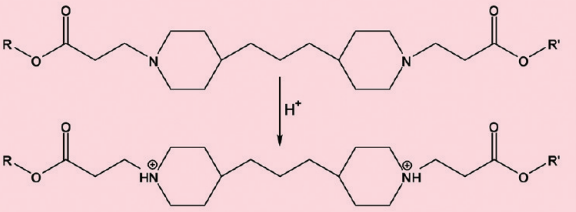
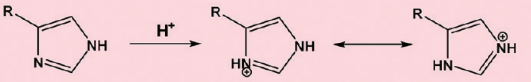
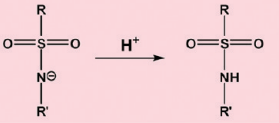
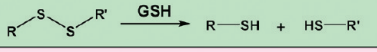
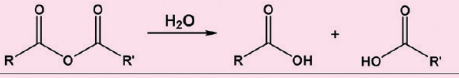
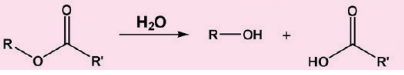
recruited.[21] However, the existence and effectiveness of this hypothetical "proton sponge effect" is debatable.[22] Another way to induce endosomal escape is to destabilize the endosome upon polymer degradation which result in dramatic polyplex swelling and increased osmotic pressure by generating small molecules.[23,24] Thus, it is reasonable to expect to achieve optimal endosomal escape (cytosolic release) of siRNA or siRNA/polymer polyplexes by combining both proton sponge effect and polymer degradation.

In the cytoplasm, only free forms of siRNA can specifically bind to RISC before interfering the translation of the target mRNA,[25,26] which requires cationic polymeric siRNA carriers to release siRNA into the cytoplasm. It has been observed that sulfated glycosaminoglycans (GAGs) such as heparan sulfate and chondroitin sulfate relax nucleic acid/polymer complexes and release nucleic acids in vitro.[27−29] This process is likely to be very slow because the anionic macromolecules have very limited access to nucleic acids complexed with polymers. Therefore, cationic polymeric siRNA carriers should be able to convert their interactions with siRNA (molecular affinity) from complexation to disassembly in the endosome and/or cytoplasm, which can be triggered by intracellular stimuli, for efficient gene silencing.

4. Considerations in Designing siRNA Carriers

siRNA delivery is most distinctively different from plasmid DNA delivery in intracellular trafficking. While it is beneficial for plasmid DNA to remain complexed for facilitated diffusion in the cytoplasm and transnuclear localization,[30] siRNA should be dissociated from its carriers in the cytoplasm for efficient gene silencing.[16−18,31] This obviously implies a design benefit that transnuclear localization is unnecessary for successful siRNA delivery. However, this raises a question in designing siRNA carriers: Where should siRNA/polymer polyplexes be disassembled for efficient gene silencing, the endosome or cytoplasm? One successfully delivered copy of plasmid DNA might be able to produce a sufficient number of proteins, generating new biological effects at a desired level. In contrast, the interference of mRNA translation is proportional to the number of delivered siRNA in the cytoplasm and maximum gene silencing efficiency is only obtained with delivery of large amounts of siRNA. In addition, RNA is more rapidly digested and hydrolyzed by nucleases and the acidic environment of endosomes/lysosomes than DNA. Therefore, an effective polymeric carrier should be able to tightly complex siRNA at least until reaching the

TABLE 1. Chemical Libraries Demonstrated for Stimuli-Responsive, Converting siRNA/Polymer Interactions

		Name	Stimuli-triggered conversion
Responsive to mildly acidic pH in the endosome	Acid-cleavable	Acetal (ketal) (acetal: R_1 = H)	
		Hydrazone	
		Maleic amide (including *cis*-aconitylamide)	
	Acid-protonating	β-Amino esters	
		Imidazole	
		Sulfonamide	
Cytosolic reducible		Disulfide	
Hydrolytic (stimuli-independent)		Acid anhydride	
		Ester	

endosome of a cell and rapidly release the maximum number of siRNA into the cytoplasm.

Stimuli provided in the endosome and/or cytoplasm can be utilized to convert the physicochemical properties of polymeric siRNA carriers before and after endosomal escape (i.e., stimuli-converting interactions of siRNA with polymers). The stimuli-converting siRNA/polymer interactions should reduce the molecular affinity and release siRNA into the cytoplasm more rapidly and efficiently than gradual and limited disassembly of siRNA complexed with stimuli-independent (i.e., not highly responsive to a specific biological stimulus) cationic polymers (e.g., PEI) via replacement by anionic biomacromolecules (e.g., heparan sulfate). Too early of a siRNA dissociation from its carriers in the endosome can result in premature acid-hydrolysis and enzymatic degradation. Therefore, the maximum amount of structurally intact and functionally active siRNA can be delivered into the cytoplasm if it is fully protected by its carriers in the digested endosome and rapidly dissociated by a cytosolic reducer upon endosomal escape. However, there are very limited number of distinct cytoplasmic stimuli that have been identified (Table 1). In fact, disulfide reducible by glutathione (GSH), whose intracellular concentration is substantially higher than its extracellular ones, might be the only linkage that is well-characterized and proven for efficiency and specificity for cytosolic release of free siRNA.[15,32−34] Therefore, the flexibility of designing polymeric carriers for efficient dissociation of siRNA specifically in the cytoplasm is also limited.

On the contrary, a broad range of acid-responsive chemistry not only provides efficient siRNA release from its carrier into the cytoplasm but also enhances endosomal escape (Table 1), including acid-cleavable linkers and

acid-protonating groups. It should be noted that the endosomal disruption/escape by utilizing acid-responsive linkers and groups should be fast enough in order to avoid degradation of dissociated siRNA from its carriers in the endosome. Cationic amine-bearing branches that complex siRNA to form a polyplex can be conjugated to a polymer via an acid-cleavable linker (e.g., acetal/ketal[31,35] and hydrazone[36]) (Table 1). siRNA is more efficiently complexed with polymers containing cationic branches than linear cationic polymers and the resulting polyplexes are efficiently taken up by cells.[37] At a mildly acidic pH, the cationic termini of the polymers are protonated and destabilize the endosome via proton sponge effect. Simultaneously, loss of cationic branches upon acid-hydrolysis greatly reduces the attractive interactions between siRNA and the polymer, releasing siRNA, preferably into the cytoplasm. Efficient cleavage of anionic maleic amide and *cis*-aconitylamide groups of a polymer by an acidic pH regenerates amine groups but the acid-triggered charge conversion can strengthen the attractive interactions with siRNA. However, acid-cleavage of anionic maleic amide and *cis*-aconitylamide groups generates small maleic and aconityl acids, and effectively destabilizes the endosome, releasing siRNA into the cytoplasm.[38] β-amino ester,[24,39] imidazole,[40,41] and sulfonamide[42] are protonated at an endosomal pH and strengthen the attractive interactions with siRNA. In the cytoplasm at a neutral pH, the attractive interactions are reduced and siRNA is released, resulting in efficient gene silencing.

The interactions between siRNA and its polymeric carriers can be converted without changes in chemical structure of the polymers. For example, block copolymers can undergo conformational changes from a hydrophilic structure to a membrane-destabilizing hydrophobic form at an endosomal pH or facilitated disassembly by the abruptly decreased intracellular calcium concentration, resulting in efficient siRNA delivery.[43,44] Fragmentation of cationic polymers into small pieces (reduction in molecular weight), via acid-hydrolysis in the endosome and/or reduction in the cytoplasm, also greatly decreases the interactions with siRNA, releases siRNA into the cytoplasm, and enhances gene silencing.[32,34,35,44,45] Incorporation of linkers that are cleavable in a stimuli-independent manner (e.g., ester and acid anhydride) also can contribute to reducing the molecular weight and inducing changes in hydrophilicity/hydrophobicity of polymeric siRNA carriers, consequently dissociating siRNA.[46−48]

Toxicity of siRNA carriers should not be neglected at any level, particularly when aiming to obtain fully sequence-specific gene silencing. It is anticipated that many parameters that determine the toxicity of DNA carriers, such as molecular weight, cationic density, morphology, polymer to nucleic acid ratio (N/P ratio), and biodegradability, are also prevalent in siRNA carriers. Efficient siRNA complexation necessitates the use of polymers with a high molecular weight, high cationic density, and branched morphology, at a high N/P ratio, increasing the possibility of inducing toxicity and nonspecific gene silencing.[31] Therefore, stimuli-converting reduction in molecular weight and cationic density of siRNA carriers, as well as morphological changes from branched to relatively linear forms, not only greatly facilitates siRNA release for efficient gene silencing but also lowers toxicity and nonspecific gene silencing. The optimum dose of siRNA carriers that are required to achieve a desired level of gene silencing with minimal toxicity, particularly in vivo, heavily depends on the molecular characterizations of each carrier.

5. Ketalized Polymers as Stimuli-Converting siRNA Carriers

Acetals/ketals are one of the most acid-cleavable linkages and have been popularly employed in stimuli-responsive gene and drug delivery.[49,50] For example, the half-life of dimethyl ketal linkage at an endosomal pH is as fast as few minutes.[23] The proton-catalyzed hydrolysis kinetics of acetal/ketal linkages is tunable depending on side groups as well as hydrophilicity of the polymer backbone.[51] The regenerated groups after acetal/ketal hydrolysis are hydroxyls that are neutral and more hydrophilic than the unhydrolyzed linkages. It needs to be noted that hydrolysis of acetals generates aldehydes that can react with amines of proteins, potentially causing adverse effects, while ketones are the hydrolysis products of ketals. Efficient acid-triggered, converting interactions between siRNA/polymer can be achieved by incoporating the polymers with branches carrying an amine group at the terminus linked via acetal/ketal (amino acetal/ketal branches), internal acetal/ketal linkages, or both into the polymeric siRNA carrier. Upon acid-hydrolysis, amino acetal/ketal branches convert to neutral hydroxyl overhangs and siRNA is readily released due to weakened interactions with its carrier. Simultaneously, endosomal escape contributed by proton buffering is supplemented by the dramatic swelling of polyplexes due to weakened siRNA/polymer interactions and increased osmotic pressure in the endosome by acid-hydrolyzed

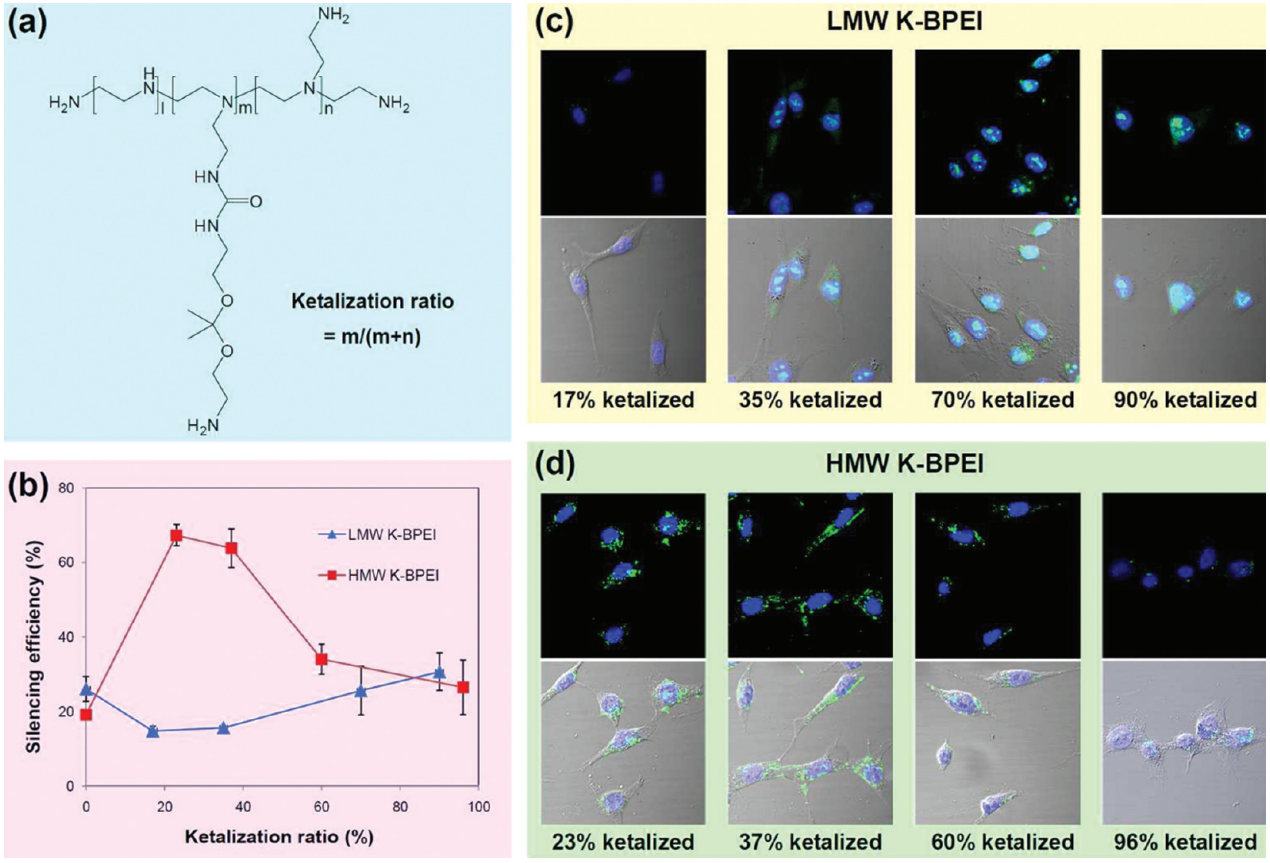


FIGURE 3. Molecular weight-dependent gene silencing efficiency of ketalized branched PEI (K-BPEI) due to differential intracellular localizations of siRNA/K-BPEI polyplexes. (a) Primary amine-bearing branches of low molecular weight (LMW, 800 Da) and high molecular weight (HMW, 25 kDa) BPEI were ketalized.[16] (b) Distinct effects by LMW and HMW K-BPEI on gene silencing in NIH 3T3 cells were observed.[18] Moderate ketalization (23−37%) of HMW BPEI resulted in dramatically enhanced gene silencing efficiency, while even minimal ketalization (17%) of LMW BPEI reduced its gene silencing capability. High ketalization (>60%) did not improve the gene silencing capability of both LMW and HMW BPEI. (c and d) Confocal fluorescence microscopy demonstrated that siRNA/LMW K-BPEI polyplexes were mainly localized in the nucleus and were not able to encounter mRNA, while siRNA/HMW K-BPEI polyplexes were exclusively found in the cytoplasm.[18] The polyplexes prepared with siRNA and minimally ketalized (17%) LMW K-BPEI and maximally ketalized (96%) HMW K-BPEI were not efficiently internalized by the cells, due to poor complexation. Figures were modified with permission from the American Chemical Society (a) and Elsevier (b−d).

byproducts of acetal/ketal linkages. The reduced cationic density of acid-hydrolyzed cationic polymers limits the interactions with endogenous genes, lowering cytotoxicity,[52] and, more notably, prevents recomplexation of siRNA in the cytoplasm. Fragmentation of cationic polymers with internal acetal/ketal linkages significantly weakens siRNA complexation and the fragmented polymers are rapidly cleared from the body, enhancing biocompatibility of the carriers.

5.1. Improved Complexation, Biocompatibility, and Molecularly Tunable Intracellular Localization of siRNA by Ketalized Branched PEI (K-BPEI). PEI is the most commonly used cationic polymer in the field of nucleic acid delivery, due to its strong interactions with nucleic acids and efficient proton-buffering capacity, attributed to its high cationic density. Branched PEI (BPEI) has primary amine-bearing branches as well as secondary and tertiary amines in the backbone, buffering protons at various endosomal acidification stages. Primary amine-bearing short branches of low molecular weight (LMW, 0.8 kDa) and high molecular weight (HMW, 25 kDa) BPEI were conjugated with amino ketals at varying ratios (>96%), resulting in LMW and HMW ketalized PEI (K-PEI) (Figure 3a). It was clear that moderate ketalization slightly reduced the gene silencing capability of LMW BPEI (Figure 3b) despite the efficient complexation of siRNA by LMW K-BPEI, as supported by small siRNA/LMW K-BPEI polyplex sizes.[16] Confocal fluorescence microscopy demonstrated exclusive nuclear localization of siRNA/LMW K-BPEI polyplexes (Figure 3c), accounting for the low gene silencing efficiency. On the other hand, moderately ketalized (23 and 37%) HMW B-PEI showed substantially enhanced gene silencing efficiency (Figure 3b). It was also

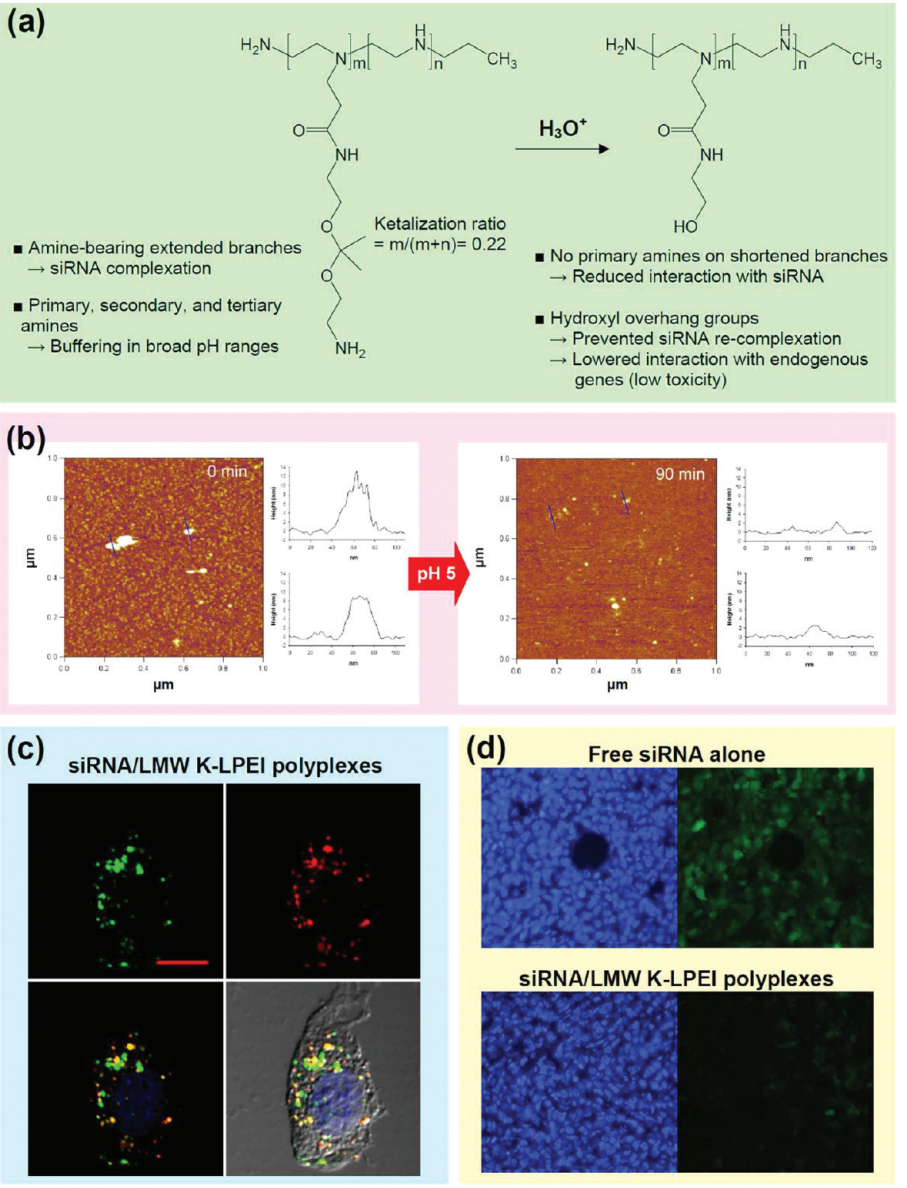


FIGURE 4. Ketalized linear PEI (K-LPEI) for efficient endosomal escape and siRNA release into the cytoplasm. (a) Secondary amines (22%) in the low molecular weight (LMW, 3.4 kDa) LPEI backbone were ketalized with primary amine-bearing branches.[31] The resulting LMW K-LPEI resembles B-PEI in structure as well as proton buffering capability over a broad pH range by primary, secondary, and tertiary amines. Upon acid-hydrolysis in the endosome, all primary amine-bearing branches convert to neutral hydroxyl overhangs and siRNA is efficiently dissociated from hydrolyzed LMW K-LPEI without being recomplexed in the cytoplasm. (b) siRNA/LMW K-LPEI polyplexes in differentially acidifying buffers to an endosomal pH of 5.0 were observed by in situ AFM.[17] The polyplexes were efficiently disassembled after 90 min of incubation under an endosomal condition. An expanded view of the AFM images is also shown in Figure S1 in the Supporting Information. (c) siRNA (labeled with Cy3, red) was efficiently released from LMW K-LPEI (labeled with Alexa Fluor 488, green), represented by distinct red dots from green ones with minimal overlapping of the two signals, after selective localization in the cytoplasm of NIH 3T3 cells.[31] Polyplexes of siRNA/unketalized LPEI were found in the cytoplasm and nucleus without noticeable dissociation.[31] (d) C57BL/6 mice carrying an E.G7-OVA/GFP subcutaneous tumor were intravenously injected with free anti-GFP siRNA or siRNA/LMW K-LPEI polyplexes (10 μg siRNA per mouse). After 3−5 days, tumors were harvested from euthanized animals and sliced sections were observed under a fluorescence microscope (left, DRAQ5 (blue)-stained nuclei; right, GFP-expressing cells). siRNA complexed with LMW K-LPEI silenced GFP expression more efficiently than free siRNA. Figures were modified with permission from the American Chemical Society (a and c) and Wiley (b).

observed that most siRNA/HMW K-BPEI polyplexes were localized in the cytoplasm where target mRNA resides (Figure 3d). High ketalization (>60%) interfered with cellular uptake of siRNA/HMW K-BPEI polyplexes, resulting in low gene silencing efficiency (Figure 3d). In addition, ketalization completely diminished the unacceptably high cytotoxicity of HMW BPEI.

The result shown in Figure 3 demonstrates the roles of acid-converting, molecularly tunable interactions between

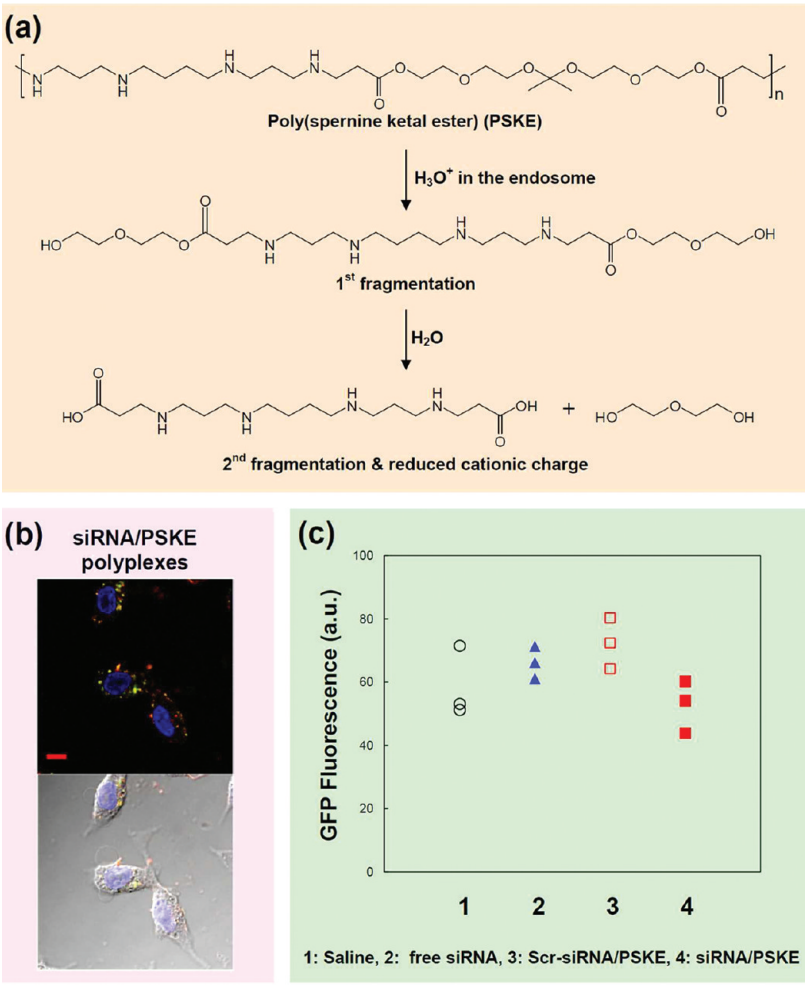


FIGURE 5. Efficient gene silencing by poly(spermine ketal ester) (PSKE).[45] (a) Small bio-oriented cation, spermine, was polymerized with two cleavable linkers: acid-cleavable ketals and hydrolytic (stimuli-independent) esters. A mildly acidic pH in the endosome degrades PSKE to spermine ester glycols. Further hydrolysis of esters generates spermine acids, offsetting the cationic charge of spermine, and ethoxy ethylene glycols. (b) The small molecular weight of hydrolyzed PSKE and reduced net charge induced rapid and efficient siRNA dissociation in the cytoplasm, which was confirmed by clear separation of siRNA (Cy3-labeled, red) from hydrolyzed PSKE (Alexa Fluor 488-labeled, green) in NIH 3T3 cells. (c) In vivo gene silencing efficiency of siRNA/PSKE polyplexes was investigated as described for the in vivo experiment in Figure 4. The results demonstrated higher gene silencing by siRNA PSKE polyplexes than free siRNA (three mice per group). All figures are modified with permission from Elsevier.

siRNA and its cationic polymeric carriers in intrcellular trafficking of siRNA and overall gene silencing efficiency. It was confirmed that cytosolic localization of siRNA after endosomal escape, which was achieved by employing moderately ketalized HMW BPEI, is a key requirement in designing efficient siRNA carriers.

5.2. Ketalized Linear PEI (K-LPEI) for Maximized siRNA Release with Minimized Recomplexation in the Cytoplasm for Efficient Gene Silencing in Vitro and in Vivo. Linear PEI (LPEI) is less toxic and more potent in in vivo gene delivery than BPEI.[53] However, lacking cationic branches limit the siRNA complexation by LPEI.[37,54] About a quarter of the secondary amines in the LPEI backbone were ketalized with amine-bearing branches. The resulting K-LPEI resembles BPEI in structure as well as extended buffering capacity over a broad pH range by primary, secondary, and tertiary amines (Figure 4a). siRNA/LMW (3.4 kDa) K-LPEI polyplexes were rapidly disassembled under a mildly acidic condition (pH 5.0), which was confirmed by in situ AFM in differentially acidifying buffers (Figure 4b). This observation was further correlated with improved intracellular trafficking of siRNA delivered by LMW K-LPEI. It was shown that siRNA/LMW K-LPEI polyplexes were selectively localized in the cytoplasm, followed by efficient siRNA release (Figure 4c). Interestingly, no obvious difference was observed between LMW and HMW (45.5 kDa) K-LPEI at the same ketalization ratio.[31] This probably indicates that ratio of acid-cleavable cationic branches conjugated to the L-PEI plays more crucial roles

than its molecular weights, unlike K-BPEI (Figure 3). In addition, K-LPEI showed high resistant to inactivation by serum proteins, while the gene silencing efficiency by B-PEI was significantly reduced in the presence of serum, despites their structural similarity. Based on the efficient in vitro gene silencing efficiency and high biocompatibility, siRNA/LMW K-LPEI polyplexes were injected into the mice carrying a GFP-expressing subcutaneous tumor. The tumors harvested after 3−5 days showed significantly reduced GFP expression (Figure 4d).

The result summarized in Figure 4 indicates that polymers with cationic branches efficiently complex siRNA and rapid loss of attractive interactions with siRNA via cleavage of cationic branches greatly enhances gene silencing efficiency.

5.3. Poly(spermine ketal ester) (PSKE): Polymerized Bio-Originated Cation for Efficient and Biocompatible siRNA Delivery via Stimuli-Responsive and Stimuli-Independent, Two Step-Degradations. Spermine involved in the eukaryotic metabolism is a small cation with four amines separated by 3−4 carbon atoms. Although the maximum interaction of siRNA with cations consisting of four carbon-bridges was anticipated,[55] the small molecular weight of spermine limits its siRNA complexation capability. This suggests that polymerized spermine via stimuli-cleavable linkages is capable of complexing siRNA and converting its interactions with siRNA to disassembly in the presence of target stimuli. Spermine was polymerized via acid-cleavable ketals and hydrolytic (stimuli-independent) esters, resulting in poly(spermine ketal ester) (PSKE) (Figure 5a). In the mildly acidic endosome, ketal linkages degrade and siRNA complexation becomes weakened by the reduced molecular weight of PSKE. In the cytoplasm, ester hydrolysis further counters the cationic charge of spermine by producing two carboxylic groups, hence, ensuring efficient siRNA release without being recomplexed. This was supported with the observation that most siRNA was dissociated from PSKE in the cytoplasm (Figure 5b). In vitro studies also demonstrated the efficient gene silencing by synergistic degradation of ketals and esters.[45] Mice injected with siRNA/PSKE polyplexes showed higher gene silencing than those injected with saline, free siRNA, and scrambled siRNA (Scr-siRNA)/PSKE polyplexes (Figure 5c).

The result shown in Figure 5 proves that converting siRNA/polymer interactions via stimuli-responsive degradations of cationic polymers is a valuable design parameter for efficient siRNA carriers. This approach can be highly desirable particularly in developing safe siRNA carriers that are rapidly removed from the body, avoiding adverse side effects for long-term and repeated administrations.

6. Concluding Remarks

The promise of utilizing RNAi technology in basic research and disease treatments in clinical settings requires efficient and safe delivery/administration methods. Among the many hurdles involved in successfully silencing genes, two key dilemmatic steps, complexation and disassembly of siRNA/polymer poyplexes before and after endosomal escape, were reviewed. It is clear that the siRNA/polymer interactions need to be converted by biological stimuli provided at temporally and spatially appropriate steps. There are robust and proven chemistries to meet this critical requirement for designing suitable siRNA carriers. Manipulating the physicochemical properties of siRNA, such as mulitimerization,[56] in order to achieve stimuli-converting interactions with its carrier is also a promising avenue to be continuously explored. Nonetheless, there is still a tremendous need for new and innovative synthetic strategies to better design the stimuli-responsive, converting interactions between siRNA and its carrier.

Reliable and realistic computational models would also greatly help quantitatively characterize siRNA/polymer interactions at a molecular level toward the design of novel siRNA carriers. A foreseeable challenge in the near future is whether it is possible to design siRNA carriers for differentially controlled gene silencing, which is desirable for personalized RNAi-based medical treatments.

The author is supported by a CAREER Award by the National Science Foundation (DMR-0956091) and Medical Research Award by Gabrielle's Angel Foundation for Cancer Research (Award 056). The author also thanks Dr. Jones Tsai (UC Irvine) for helpful discussions on the manuscript.

Supporting Information. An expanded view of AFM images. This material is available free of charge via the Internet at http://pubs.acs.org.

BIOGRAPHICAL INFORMATION

Young Jik Kwon is an associate professor of Pharmaceutical Sciences, Chemical Engineering & Materials Science, Biomedical Engineering, and Molecular Biology and Biochemistry at University of California, Irvine. He received his Ph.D. in Chemical Engineering from University of Southern California in 2003, followed by postdoctoral training in Chemistry at University of California, Berkeley. Since 2005, his laboratory has investigated viral and nonviral vectors for nucleic acid delivery, targeted drug delivery systems, combined imaging and therapy (theragnostics), and

cancer vaccine carriers, using stimuli-responsive polymers. His work has demonstrated the utility of molecularly tuned interactions between acid-degradable polymers with therapeutics for biomedical and pharmaceutical applications.

FOOTNOTES

*To whom correspondence should be addressed. Mailing address: 132 Sprague Hall, Irvine, CA 92697-3958, United States. Telephone: (949) 824-8714. Fax: (949) 824-4023. E-mail: kwonyj@uci.edu.

REFERENCES

1 Li, Z.; Yang, C.-S.; Nakashima, K.; Rana, T. M. Small RNA-mediated regulation of iPS cell generation. *EMBO J.* **2011**, *30*, 823–834.

2 Burnett, J. C.; Rossi, J. J.; Tiemann, K. Current progress of siRNA/shRNA therapeutics in clinial trials. *Biotechnol. J.* **2011**, *6*, 1130–1146.

3 Wang, X.; Yan, F.; Du, Q.; Liang, Z. siRNAs as potential drugs. *Drug Discovery Today: Technol.* **2010**, *7*, E125–E130.

4 Sioud, M. Advances in RNA sensing by the immune system: separation of siRNA unwanted effects from RNA interference. *Methods Mol. Biol.* **2010**, *629*, 33–52.

5 Shim, M. S.; Kwon, Y. J. Efficient and targeted delivery of siRNA in vivo. *FEBS J.* **2010**, *277*, 4814–4827.

6 Dawson, L. A.; Usmani, B. A. Design, manufacture, and assay of the efficacy of siRNAs for gene silencing. *Methods Mol. Biol.* **2008**, *439*, 403–419.

7 Rao, D. D.; Vorhies, J. S.; Senzer, N.; Nemunaitis, J. siRNA vs shRNA: similarities and differences. *Adv. Drug Delivery Rev.* **2009**, *61*, 746–759.

8 Stanton, M. G.; Colletti, S. L. Medicinal chemistry of siRNA delivery. *J. Med. Chem.* **2010**, *53*, 7887–7901.

9 Bramsen, J. B.; Kjems, J. Chemical modification of small interfering RNA. *Methods Mol. Biol.* **2011**, *721*, 77–103.

10 Gao, Y.; Liu, X.-L.; Li, X.-R. Research progress on siRNA delivery with nonviral carriers. *Intl. J. Nanomed.* **2011**, *6*, 1017–1025.

11 Kim, W. J.; Kim, S. W. Efficient siRNA delivery with non-viral polymeric vehicles. *Pharm. Res.* **2009**, *26*, 657–666.

12 Liu, Y.; Huang, L. Designer lipids advance systemic siRNA delivery. *Mol. Ther.* **2010**, *18*, 669–670.

13 Samuel-Abraham, S.; Leonard, J. N. Staying on message: design principles for controlling nonspecific responses to siRNA. *FEBS J.* **2010**, *277*, 4828–4836.

14 Ozpolat, B.; Sood, A. K.; Lopez-Berestein, G. Nanomedicine based approaches for the delivery of siRNA in cancer. *J. Intern. Med.* **2009**, *267*, 44–53.

15 Kim, S. H.; Ou, M.; Bull, D. A.; Kim, S. W. Reductive degradation behavior of bioreducible poly(disulfide amine) for enhancing siRNA efficiency. *Macromol. Biosci.* **2010**, *10*, 898–905.

16 Shim, M. S.; Kwon, Y. J. Controlled delivery of plasmid DNA and siRNA to intracellular targets using ketalized polyethylenimine. *Biomacromolecules* **2008**, *9*, 444–455.

17 Shim, M. S.; Wang, X.; Ragan, R.; Kwon, Y. J. Dynamics of nucleic acid/cationic polymer complexation and disassembly under biologically simulated conditions using in situ atomic force microscopy. *Microsc. Res. Tech.* **2010**, *73*, 845–856.

18 Shim, M. S.; Kwon, Y. J. Controlled cytoplasmic and nuclear localization of plasmid DNA and siRNA by differentially tailored polyethylenimine. *J. Controlled Release* **2009**, *133*, 206–213.

19 Ouyang, D.; Zhang, H.; Herten, D. P.; Parekh, H. S.; Smith, S. C. Structure, dynamics, and energetics of siRNA-cationic vector complexation: a molecular dynamics study. *J. Phys. Chem. B* **2010**, *114*, 9220–9230.

20 Tamura, A.; Nagasaki, Y. Smart siRNA delivery systems based on polymeric nanoassemblies and nanoparticles. *Nanomedicine* **2010**, *5*, 1089–1102.

21 Zuber, G.; Dauty, E.; Nothisen, M.; Belguise, P.; Behr, J. P. Towards synthetic viruses. *Adv. Drug Delivery Rev.* **2001**, *52*, 245–253.

22 Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans-Nieuwenbroek, N. M. E.; Crommelin, D. J. A.; Hennink, W. E. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. *Biomacromolecules* **2004**, *5*, 32–39.

23 Kwon, Y. J.; Standley, S. M.; Goodwin, A. P.; Gillies, E. R.; Fréchet, J. M. J. Directed antigen presentation using polymeric microparticulate carriers degradable at lysosomal pH for controlled immune responses. *Mol. Pharmaceutics* **2005**, *2*, 83–91.

24 Vandenbroucke, R. E.; De Geest, B. G.; Bonné, S.; Vinken, M.; Van Haecke, T.; Heimberg, H.; Wagner, E.; Rogiers, V.; De Smedt, S. C.; Demeester, J.; Sanders, N. N. Prolonged gene silencing in hepatoma cells and primary hepatocytes after small interfering RNA delivery with biodegradable poly(beta-amino esters). *J. Gene Med.* **2008**, *10*, 783–794.

25 Ma, J. B.; Ye, K; Patel, D. J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **2004**, *429*, 318–322.

26 Collins, R. E.; Cheng, X. Structural and biochemical advances in mammalian RNAi. *J. Cell. Biochem.* **2006**, *99*, 1251–1266.

27 Moret, I.; Peris, J. E.; Guillem, V. M.; Benet, M.; Revert, F.; Dasi, F.; Crespo, A.; Alino, S. F. Stability of PEI—DNA and DOTAP—DNA complexes: effect of alkaline pH, heparin and serum. *J. Controlled Release* **2001**, *76*, 169–181.

28 Okuda, T.; Niidome, T.; Aoyagi, H. Cytosolic soluble proteins induce DNA release from DNA-gene carrier complexes. *J. Controlled Release* **2004**, *98*, 325–332.

29 Ruponen, M.; Yla-Herttuala, S.; Urtti, A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Acta* **1999**, *1415*, 331–341.

30 Lukacs, G. L.; Haggie, P.; Seksek, O.; Lechardeur, D.; Freedman, N.; Verkman, A. S.; Size-dependent, D. N. A. mobility in cytoplasm and nucleus. *J. Biol. Chem.* **2000**, *275*, 1625–1629.

31 Shim, M. S.; Kwon, Y. J. Acid-responsive linear polyethylenimine for efficient, specific, and biocompatible siRNA delivery. *Bioconjugate Chem.* **2009**, *20*, 488–499.

32 Won, Y.-W.; Yoon, S.-M.; Lee, K.-M.; Kim, Y.-H. Poly(oligo-D-arginine) with internal disulfide linkages as a cytoplasm-sensitive carrier for siRNA delivery. *Mol. Ther.* **2011**, *19*, 372–380.

33 Manickam, D. S.; Li, J.; Putt, D. A.; Zhou, Q.-H.; Wu, C.; Lash, L. H.; Oupicky, D. Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *J. Controlled Release* **2010**, *141*, 77–84.

34 Vader, P.; van der Aa, L. J.; Engbersen, J. F. J.; Storm, G.; Schiffelers, R. M. Disulfide-based poly(amido amine)s for siRNA delivery: Effects of structure on siRNA complexation, cellular uptake, gene silencing and toxicity. *Pharm. Res.* **2011**, *28*, 1013–1022.

35 Lee, S.; Yang, S. C.; Kao, C.-Y.; Pierce, R. H.; Murthy, N. Solid polymeric microparticles enhance the delivery of siRNA to macrophages in vivo. *Nucleic Acids Res.* **2009**, *37*, e145/1–e145/10.

36 Lin, Y.-L.; Jiang, G.; Birrell, L. K.; El-Sayed, M. E. H. Degradable, pH-sensitive, membrane-destabilizing, comb-like polymers for intracellular delivery of nucleic acids. *Biomaterials* **2010**, *31*, 7150–7166.

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

38 Pittella, F.; Zhang, M.; Lee, Y.; Kim, H. J.; Tockary, T.; Osada, K.; Ishii, T.; Miyata, K.; Nishiyama, N.; Kataoka, K. Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity. *Biomaterials* **2011**, *32*, 3106–3114.

39 Jere, D.; Xu, C.-X.; Arote, R.; Yun, C.-H.; Cho, M.-H.; Cho, C.-S. Poly(β-amino ester) as a carrier for si/shRNA delivery in lung cancer cells. *Biomaterials* **2008**, *29*, 2535–2547.

40 Ghosn, B.; Singh, A.; Li, M.; Vlassov, A. V.; Burnett, C.; Puri, N.; Roy, K. Efficient gene Silencing in lungs and liver using imidazole-modified chitosan as a nanocarrier for small interfering RNA. *Oligonucleotides* **2010**, *20*, 163–172.

41 Stevenson, M.; Ramos-Perez, V.; Singh, S.; Soliman, M.; Preece, J. A.; Briggs, S. S.; Read, M. L.; Seymour, L. W. Delivery of siRNA mediated by histidine-containing reducible polycations. *J. Controlled Release* **2008**, *130*, 46–56.

42 Kang, H. C.; Bae, Y. H. Co-delivery of small interfering RNA and plasmid DNA using a polymeric vector incorporating endosomolytic oligomeric sulfonamide. *Biomaterials* **2011**, *32*, 4914–4924.

43 Convertine, A. J.; Diab, C.; Prieve, M.; Paschal, A.; Hoffman, A. S.; Johnson, P. H.; Stayton, P. S. pH-responsive polymeric micelle carriers for siRNA drugs. *Biomacromolecules* **2010**, *11*, 2904–2911.

44 Kakizawa, Y.; Furukawa, S.; Kataoka, K. Block copolymer-coated calcium phosphate nanoparticles sensing intracellular environment for oligodeoxynucleotide and siRNA delivery. *J. Controlled Release* **2004**, *97*, 345–356.

45 Shim, M. S.; Kwon, Y. J. Dual mode-degradable polyspermine for plasmid DNA and siRNA delivery in vitro and in vivo: Effects of acid-degradability. *Biomaterials* **2011**, *32*, 4009–4020.

46 Liu, J.; Jiang, Z.; Zhang, S.; Liu, C.; Gross, R. A.; Kyriakides, T. R.; Saltzman, W. M. Biodegradation, biocompatibility, and drug delivery in poly(ω-pentadecalactone-co-p-dioxanone) copolyesters. *Biomaterials* **2011**, *32*, 6646–6654.

47 Nguyen, J.; Steele, T. W. J.; Merkel, O.; Reul, R.; Kissel, T. Fast degrading polyesters as siRNA nano-carriers for pulmonary gene therapy. *J. Controlled Release* **2008**, *132*, 243–251.

48 Varkouhi, A. K.; Lammers, T.; Schiffelers, R. M.; van Steenbergen, M. J.; Hennink, W. E.; Storm, G. Gene silencing activity of siRNA polyplexes based on biodegradable polymers. *Eur. J. Pharm. Biopharm.* **2011**, *77*, 450–457.

49 Gillies, E. R.; Goodwin, A. P.; Frechet, J. M. J. Acetals as pH-sensitive linkages for drug delivery. *Bioconjugate Chem.* **2004**, *15*, 1254–1263.

50 Ko, I. K; Ziady, A.; Lu, S.; Kwon, Y. J. Acid-degradable cationic methacrylamide polymerized in the presence of plasmid DNA as tunable non-viral gene carrier. *Biomaterials* **2008**, *29*, 3872–3881.

51 Sankaranarayanan, J.; Mahmoud, E. A.; Kim, G.; Morachis, J. M.; Almutairi, A. Multi-response strategies to modulate burst degradation and release from nanoparticles. *ACS Nano* **2010**, *4*, 5930–5936.

52 Dindo, D.; Dahm, F.; Szulc, Z.; Bielawska, A.; Obeid, L. M.; Hannun, Y. A.; Graf, R.; Clavien, P.-A. Cationic long-chain ceramide LCL-30 induces cell death by mitochondrial targeting in SW403 cells. *Mol. Cancer Ther.* **2006**, *5*, 1520–1529.

53 Brissault, B.; Leborgne, C.; Guis, C.; Danos, O.; Cheradame, H.; Kichler, A. Linear topology confers in vivo gene transfer activity to polyethylenimines. *Bioconjugate Chem.* **2006**, *17*, 759–765.

54 Richards Grayson, A. C.; Doody, A. M.; Putnam, D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro. *Pharm. Res.* **2006**, *23*, 1868–1876.

55 Fischer, W.; Brissault, B.; Prévost, S.; Kopaczynska, M.; Andreou, I.; Janosch, A.; Gradzielski, M.; Haag, R. Synthesis of linear polyamines with different amine spacings and their ability to form dsDNA/siRNA complexes suitable for transfection. *Macromol. Biosci.* **2010**, *10*, 1073–1083.

56 Mok, H.; Lee, S. H.; Park, J. W.; Park, T. G. Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing. *Nat. Mater.* **2010**, *9*, 272–278.