ACCOUNTS
of chemical research

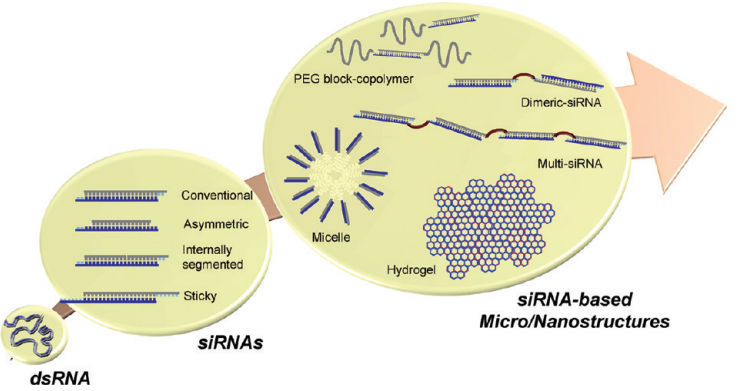
Small-Interfering RNA (siRNA)-Based Functional Micro- and Nanostructures for Efficient and Selective Gene Silencing

SOO HYEON LEE,[‡] BONG HYUN CHUNG,[∥] TAE GWAN PARK,[‡] YOON SUNG NAM,[*,§] AND HYEJUNG MOK[*,†]

[†]*Department of Bioscience and Biotechnology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea,* [‡]*Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea,* [∥]*BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea, and* [§]*Department of Materials Science and Engineering, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*

RECEIVED ON AUGUST 30, 2011

CONSPECTUS



Because of RNA's ability to encode structure and functional information, researchers have fabricated diverse geometric structures from this polymer at the micro- and nanoscale. With their tunable structures, rigidity, and biocompatibility, novel two-dimensional and three-dimensional RNA structures can serve as a fundamental platform for biomedical applications, including engineered tissues, biosensors, and drug delivery vehicles. The discovery of the potential of small-interfering RNA (siRNA) has underscored the applications of RNA-based micro- and nanostructures in medicine.

Small-interfering RNA (siRNA), synthetic double-stranded RNA consisting of approximately 21 base pairs, suppresses problematic target genes in a sequence-specific manner via inherent RNA interference (RNAi) processing. As a result, siRNA offers a potential strategy for treatment of many human diseases. However, due to inefficient delivery to cells and off-target effects, the clinical application of therapeutic siRNA has been very challenging. To address these issues, researchers have studied a variety of nanocarrier systems for siRNA delivery.

In this Account, we describe several strategies for efficient siRNA delivery and selective gene silencing. We took advantage of facile chemical conjugation and complementary hybridization to design novel siRNA-based micro- and nanostructures. Using chemical crosslinkers and hydrophobic/hydrophilic polymers at the end of siRNA, we produced various RNA-based structures, including siRNA block copolymers, micelles, linear siRNA homopolymers, and microhydrogels. Because of their increased charge density and flexibility compared with conventional siRNA, these micro- and nanostructures can form polyelectrolyte complexes with poorly charged and biocompatible cationic carriers that are both more condensed and more homogenous than the complexes formed in other carrier systems. In addition, the fabricated siRNA-based structures are linked by cleavable disulfide bonds for facile generation of original siRNA in the cytosol and for target-specific gene silencing. These newly developed siRNA-based structures greatly enhance intracellular uptake and gene silencing both in vitro and in vivo, making them promising biomaterials for siRNA therapeutics.

Published on the Web 03/13/2012 www.pubs.acs.org/accounts
10.1021/ar2002254

1. Introduction

RNA has been used as an informative building block to create diverse geometric structures of micro- and nanosize through complementary hybridization, ligation, chemical conjugation, and loop−loop interactions.[1,2] Despite its chemical vulnerability, the RNA double helix is physically more stable than the DNA double helix and the DNA/RNA hybrid duplex,[2] making double-stranded RNA very attractive as a rigid structural building block. Rationally designed self-assembly can generate finely tuned RNA-based 2D and 3D structures with diverse morphologies and sizes including the tecto-square nanopattern,[1] microfilaments,[3] and nanorings.[4] Nature-inspired RNA constructs, originating from bacteriophage phi29 and human immunodeficiency virus (HIV), have also been applied for the design of dimeric, trimeric, and hexameric RNA nanostructures.[5−7] Recently, these types of research in the field of RNA-based micro- and nanostructures have been greatly boosted for biomedical application with therapeutic siRNA.

RNAi involves post-transcriptional gene suppression in a target-specific manner following cleavage of long double-stranded RNA (dsRNA) of several hundred base pairs into pieces of approximately 21 base pairs.[8] Not only biologically transcribed long dsRNA but also chemically synthesized dsRNA with 21 base pairs, also known as siRNA, can elicit RNAi in mammalian cells.[9] Based on its high specificity and potent silencing effect, siRNA has been viewed as the newest medical breakthrough following antibody/protein drugs to overcome life-threatening diseases.[10] Currently, around a dozen RNA-based drugs are under clinical evaluation.[11] For RNAi processing, siRNA should be taken up into cells and escape from endosomes to cytoplasm where it encounters intracellular RNAi machinery such as endoribonuclease Dicer and RNA induced silencing complex (RISC). However, siRNA cannot be delivered alone due to its negative charge. To overcome these delivery hurdles, properly designed carriers, such as cationic polymers, lipids, and nanoparticles, have been used for enhanced intracellular uptake of siRNA and subsequent escape from the endosome. One of the conventional siRNA delivery systems involves formation of polyelectrolyte complexes via ionic interactions between anionic phosphate groups in the siRNA backbone and cationic molecules in carriers. To form compact and homogeneous siRNA polyelectrolyte complexes, the use of highly positively charged carriers is inevitable despite their severe side effects, which include cytotoxicity and immune activation via nonspecific interactions with the cell membrane and intracellular organelles.[12] More potent carriers could elicit an even higher cytotoxicity, which could offset the advantage of newly designed carriers. Therefore, safe and potent delivery systems are still elusive for the clinical application of siRNA drugs.

This Account introduces recent interesting work regarding the design of novel siRNA-based structures via facile chemical conjugation and molecular hybridization, which could make siRNA more suitable to be successfully incorporated into less toxic and safer cationic carriers by increasing charge densities and molecular flexibilities. First, the physicochemical properties of siRNA to be considered for the design of siRNA-based structures are described. Then, synthetic schemes of diverse siRNA-based structures, including siRNA block copolymers, micelles, siRNA homopolymers, and microhydrogels, are introduced. The focus is on the rational design of novel siRNA-based structures to increase charge density and intermolecular flexibility as well as the consequential biological benefits such as improved efficiency and specificity of gene silencing. Lastly, some perspectives regarding the challenges of siRNA-based micro- and nanostructures for clinical application are provided.

2. Physicochemical Characteristics of siRNA

The symmetric dsRNA with a 19 + 2 structure (19 nucleotides comprising complementary RNA sequences to target mRNA composed of adenine (A), guanine (G), cytosine (C), and uracil (U); 2 nucleotides comprising a 3′ overhang usually composed of thymine (T)) is a conventional siRNA (Figure 1A).[9] Considering that the length of a single base pair and the persistence length of dsRNA are approximately 2.7 Å and 70 nm (approximately 260 base pairs), respectively, conventional siRNA with 21 base pairs is an innately rigid and rodlike structure with a size of around 5.67 nm and a molecular weight of approximately 13 400 g/mol.[13,14] The pK_a value of the phosphate groups in phosphodiester bonds is around zero, which makes siRNA negatively charged under physiological conditions. Accordingly, this negatively charged siRNA may not be taken up into cells without assistance, unlike chemical drugs.

To increase the delivery efficiency of siRNA, RNA-based nanostructures have been investigated using natural RNA structures, such as packaging RNA (pRNA) originating from bacteriophage phi29.[5] Synthetic cationic carriers can also be used to employ siRNA by forming siRNA polyelectrolyte complexes. However, compared with plasmid DNA, siRNAs are not easily condensed and attach to carriers such as

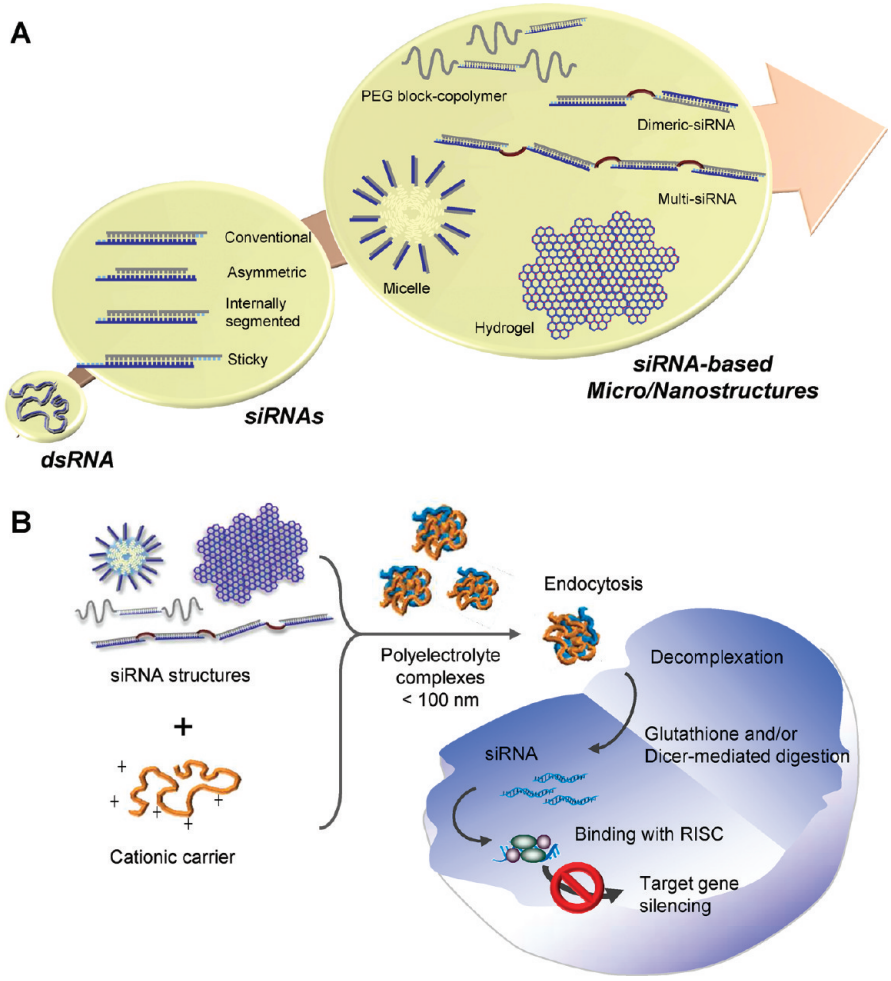


FIGURE 1. (A) Diverse monomeric siRNA structures and siRNA-based micro- and nanostructures to improve delivery efficiency and reduce off-target effects. (B) Intracellular fate of siRNA-based micro- and nanostructures following their complexation with cationic carriers.

cationic polymers, lipids, and inorganic nanoparticles with poor binding affinity due to their stiff structure and low spatial charge density. Stability of siRNA−carrier complexes has been increased by the design of sticky siRNA with 5−8 extra single strand nucleotides of A and T at both 3′ ends (Figure 1A), which increased the target gene suppression by 10-fold in vitro when compared with conventional siRNA due to stabilization of the nanosized polyelectrolyte complexes via external A-T hybridization.[15]

After intracellular uptake, siRNA should be incorporated into RISCs in the cytosol for RNAi processing, where the thermodynamic characters of the siRNA duplex are crucial for efficient gene inhibition.[16,17] The first nucleotide of the antisense strand at the 5′ end for complementary base-pairing (e.g., A-U, C-G, and G-U) significantly affects the internal strand stability of siRNA. Gibbs free energy (ΔG, kcal/mol) of helix formation for the first base pair at the 5′ end of the antisense strand in potent siRNAs is higher than that of nonfunctional siRNAs, suggesting that an unstable 5′ end of an antisense strand is preferred for unwinding and RISC incorporation over a stable one. Thus, chemical or genetic modification at the 5′ end of antisense siRNA, which might alter the intrinsic strand stability, can significantly manipulate gene knock-down efficiency and off-target effects by the undesired incorporation of the sense strand into the RISC. The adoption of the sense strand into the RISC can tremendously decrease the intended sequence specific gene silencing due to the limited RNAi machinery in cells. To avoid that, asymmetric siRNA structures with a short sense strand and an internally segmented sense strand have been reported (Figure 1A). An asymmetric dsRNA with 21 nucleotides of antisense strand and a shorter sense strand showed reduced nonspecific gene silencing without loss of biological activity compared with conventional siRNA.[18,19] Internally segmented siRNA composed of two segmented sense strands with 10−12 nucleotides and a complementary intact antisense

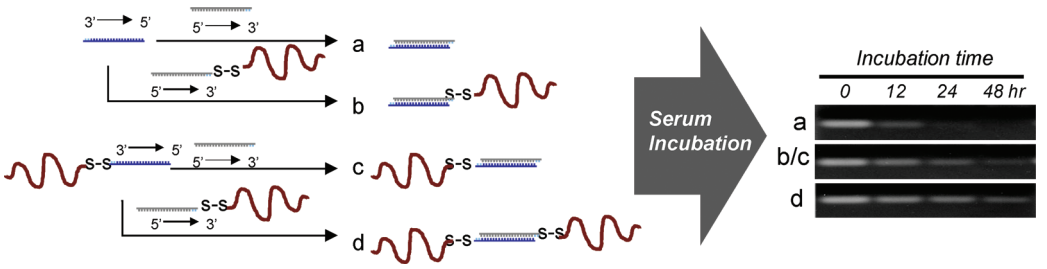


FIGURE 2. Synthetic strategy for the preparation of siRNA–PEG block copolymers via complementary base pairing and their enhanced serum stability. (a) Monomeric siRNA, diblock siRNA–PEG linked at the 3′ end of (b) sense strand or (c) antisense strand, and (d) triblock siRNA–PEG linked at 3′ end of both sense and antisense strands. Adapted with permission from ref 23. Copyright 2011 Wiley-VCH.

strand with 21 nucleotides also exhibited target-sequence specific gene suppression.[20] Only the antisense strand with 21 nucleotides efficiently participated in silencing of the target gene while gene inhibition by the short sense strands was negligible, probably due to the minimum length of nucleotides required for successful incorporation into the RISC.

Hydroxyl groups at the 2′-, 3′-, and 5′-positions of the pentose sugar of siRNA could be modified with versatile functional groups, such as amine and thiol groups, which could be exploited for chemical conjugation to construct diverse siRNA-based structures as shown in Figure 1A. Employment of functional polymers and chemical linkers with different sizes and shapes at the end of siRNA terminals could enable fabrication of new siRNA-based structures including micelles, block copolymers, and hydrogels. The resulting siRNA-based micro- and nanostructures are expected to complement the inherent weaknesses of conventional siRNA, such as stiffness and low charge density, for efficient intracellular delivery (Figure 1B).

3. siRNA Block Copolymers

3.1. siRNA–PEG Block Copolymer. RNA is vulnerable to enzymatic attack, which can significantly reduce the therapeutic potential of siRNAs in vivo. To address this issue, diverse chemical modifications in siRNA backbones, such as phosphorothioate linkage and modification of the 2′-hydroxyl group of the pentose sugar, have been investigated.[21] As an alternative strategy to protect siRNAs from surrounding proteins, poly(ethylene glycol) (PEG) can be conjugated at both terminal ends to produce siRNA–PEG block copolymers. PEG is well-known to improve serum stability and blood circulation time of various therapeutic biomolecules including interferon-α, interleukin-6, and tumor necrosis factor.[22]

In this study, amine-functionalized PEG (methoxy PEG-NH_2, molecular weight, M_w = 5000) was coupled with the thiol group at the 3′ end of each sense and antisense siRNA using a heterofunctional cross-linker, SPDP (*N*-succinimidyl 3-(2-pyridyldithio)-propionate).[23] Using siRNA as a building block, two types of block copolymer, siRNA–PEG diblock copolymer (A–B type (A = PEG; B = siRNA)) and PEG–siRNA–PEG triblock copolymer (A–B–A type), were prepared by a different combinatorial hybridization step (Figure 2). These block copolymers were linked via reducible disulfide bonds, which could be easily cleaved to regenerate inherent siRNA for RNAi processing in a reductive environment, cytoplasm containing around 5–10 mM of glutathione.[24] In our previous study, PEG was completely dissociated from siRNA block copolymers within 15 min under reductive conditions.[25] Interestingly, the siRNA block copolymers made via cleavable linkage showed sequence-specific inhibition of the target gene, while those made via noncleavable covalent linkage were demonstrated to elicit nonspecific gene suppression as well as elevated immune response, suggesting that cleavability of siRNA block copolymers was crucial for biological function.[26] After serum incubation, most of conventional siRNA was completely degraded within 24 h while approximately half of the initial siRNA in siRNA–PEG triblock copolymers still remained intact (Figure 2, right panel). Considering that the radius of gyration of PEG (M_w = 5000) is about 3.08 nm, triblock copolymers with two PEG blocks at the ends of single siRNA might shield siRNA more safely from enzymatic attacks than diblock copolymers.[27] However, excessive PEG molecules on siRNA can also disturb the interaction of siRNA–PEG block copolymers with the target cell membrane, resulting in diminished uptake into the cells.[28] Hydrodynamic radius and proportion of PEG molecules in total siRNA–PEG block copolymer can affect the surface coverage by PEG. Thus, it is necessary to determine the optimal PEG amount and molecular weight that will not inhibit cellular uptake while protecting the siRNA from serum proteins before in vivo application of siRNA–PEG block copolymers is considered.

Recently, siRNA-grafted block copolymer made via chemical conjugation has also been studied to increase spatial

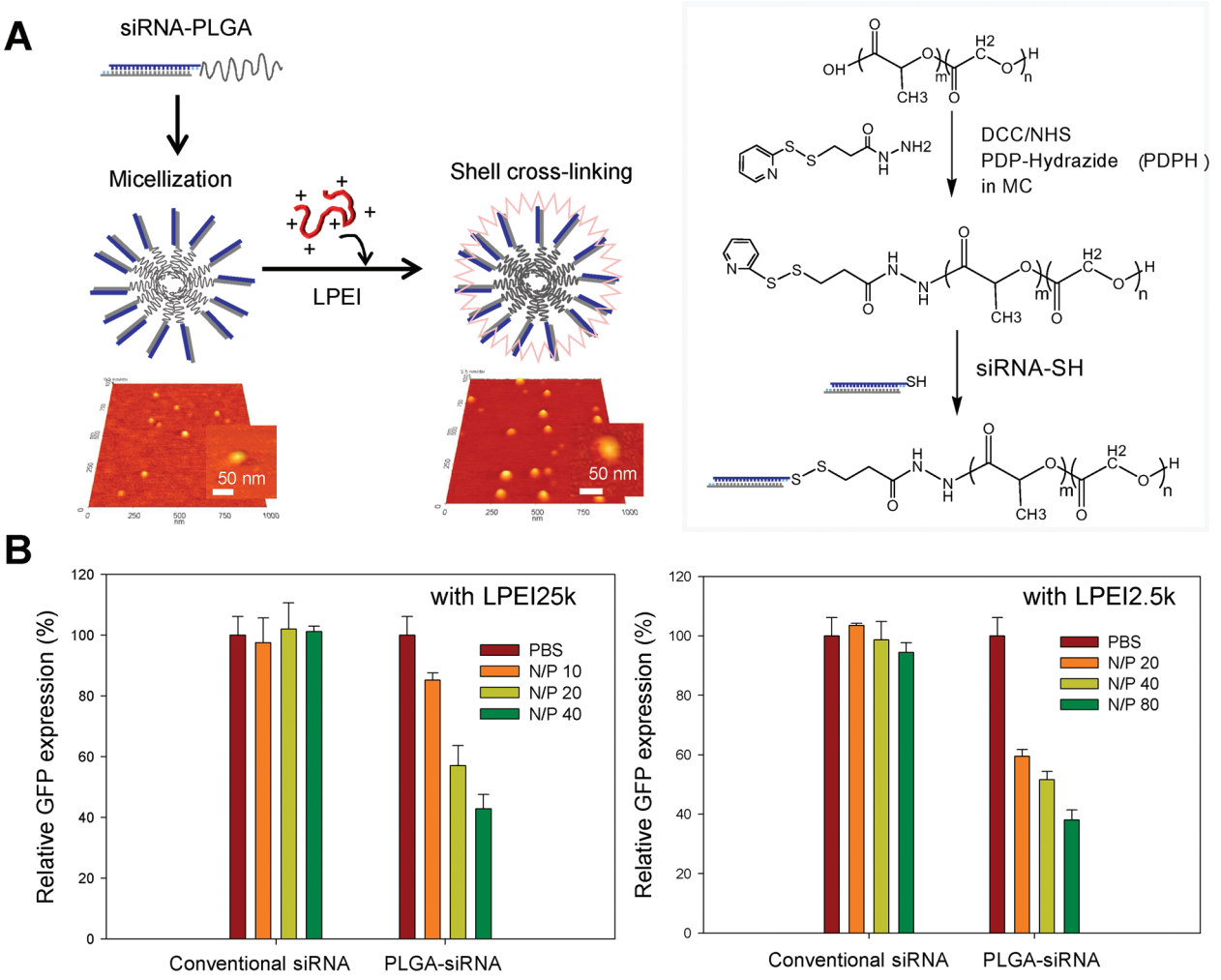


FIGURE 3. (A) Schematic diagram for the preparation of siRNA–PLGA micelles and their AFM images of before/after surface-cross-linking with LPEI. (B) Gene silencing efficiencies of siRNA–PLGA micelles against GFP. LPEI (25000 and 2500)-coated siRNA–PLGA micelles were applied to GFP-expressing MDA-MB-435 cells. Adapted with permission from ref 30. Copyright 2011 Elsevier.

charge density.[29] Multiple siRNAs grafted on poly(aspartic acid) (approximately four siRNAs per single polymer) exhibited much enhanced binding affinity with a cationic carrier, poly-{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), which resulted in improved stability of polyelectrolyte complexes and delivery efficiency compared with conventional siRNAs.

3.2. siRNA–PLGA Block Copolymer for Micelle Formation. By adopting a hydrophobic moiety at the end of siRNA, a self-assembled nanostructure with siRNA was fabricated as shown in Figure 3. Poly(D,L-lactic-*co*-glycolic acid) (PLGA) was selected as the hydrophobic moiety due to its superior biocompatibility and biodegradability in vivo.[30] After activation of the carboxyl group of PLGA with 3-(2-pyridyldithio) propionyl hydrazide (PDPH), thiol-modified siRNAs were reacted with the pyridyl disulfide-functionalized PLGA in a DMSO (dimethyl sulfoxide)/PBS (phosphate-buffered saline)

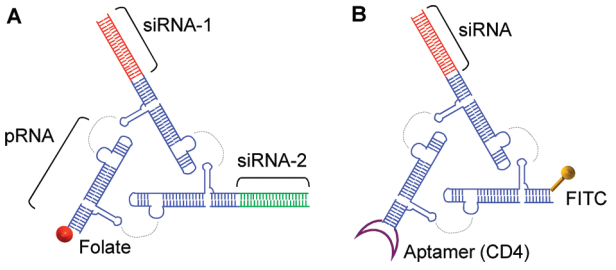


FIGURE 4. Design of trimeric RNA structures containing (A) two kinds of siRNAs and folate as a targeting ligand and (B) siRNA, fluorescein isothiocyanate (FITC), and aptamer using pRNA as a vector. Chimeric RNA molecules were biologically constructed by incorporating various functional moieties at distal ends of phi29 pRNA vector. Adapted with permission from ref 5. Copyright 2005 American Chemical Society.

cosolvent (DMSO/PBS (volume ratio) = 10:1) to produce siRNA–PLGA block copolymers. Amphipathic siRNA–PLGA block copolymers formed a micellar structure, where hydrophobic PLGA moieties were located in the core and

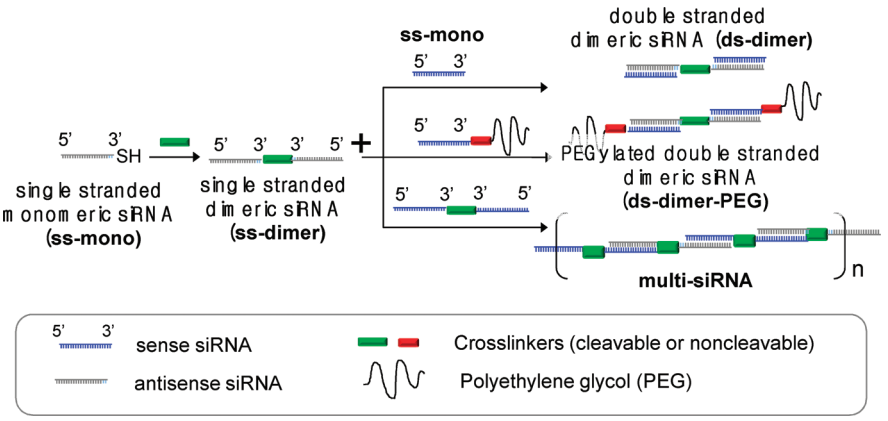


FIGURE 5. Schematic illustration for synthesis of dimeric siRNA, PEGylated dimeric siRNA, and multimeric siRNA.

hydrophilic siRNAs were exposed on the shell via a hydrophobic interaction between PLGA blocks. In the siRNA–PLGA micelle, siRNA molecules could be closely located to each other on the shell surface due to strong hydrophobic interactions despite intermolecular charge repulsion between the negatively charged siRNAs. The critical micelle concentration (CMC) of spherical siRNA–PLGA micelles with a size of 40.5 ± 5.8 nm was around 5 mg/L, which also supported that the siRNA–PLGA conjugates were indeed self-assembled as micelles. Polymeric micellar structures via noncovalent interactions remain transient only above their CMC. To stabilize micelle structure even below the CMC, as well as alter surface charge from negative to positive, linear polyethylenimine (LPEI) was physically coated onto the shells of the micelles, which could also contribute to the enhanced cellular uptake of siRNAs. The micellization of siRNA–hydrophobic polymers augments spatial charge density of siRNA, which allows far more facile ionic interactions with weakly charged cationic carriers like low molecular weight LPEI (M_w 25000 and 2500) than conventional siRNA. Accordingly, siRNA–PLGA micelles were successfully delivered into cells and inhibited target green fluorescence protein (GFP) expression, while conventional siRNA did not suppress (Figure 3B). After intracellular uptake, siRNA–PLGA micelles would be immediately disintegrated by cleavage of disulfide bonds between siRNA and PLGA, as well as by hydrolytic degradation of PLGA blocks in the acidic endosome, which release innate siRNAs for RNAi processing. This siRNA-based micellar structure could be further exploited for simultaneous multiple drug delivery by employing hydrophobic chemical drugs in the micelle core as well as therapeutic siRNAs on the shell.

4. siRNA Homopolymers

4.1. Dimeric siRNA. Previously, two distinct siRNAs were incorporated into a phi29-originated pRNA structure through complementary hybridization between left- and right-hand loops, genetically engineered to contain a specific siRNA sequence by a computer-based sequence design (Figure 4).[5] These RNA based chimeric nanostructures employing therapeutic siRNAs exhibited biological activity, probably due to fragmentation of long dsRNA into small RNAs via Dicer-mediated processing. Long dsRNAs should be cleaved into 19–25 base pair sequences by Dicer, an RNaseIII-class endonuclease, in the cytoplasm for RNAi processing, which may elicit expression of cytokines related to the innate immune response.[31] Chimeric nanostructures could also employ targeting ligands such as RNA-based aptamer and folate for efficient and selective intracellular delivery.

Diverse siRNAs can be incorporated not only using biologically derived RNA structures but also using facile chemical conjugations of the ends of siRNAs via a cross-linker (Figure 5).[32] Dimeric sense strand siRNAs were prepared by direct coupling of two thiol groups at the 3′ ends of each sense strand using a cross-linker, dithio-bismaleimidoethane (DTME), which were sequentially hybridized with a complementary antisense single-strand siRNA for the preparation of a dimeric siRNA structure. Unlike biologically prepared chimeric siRNA structures,[5] chemically linked dimeric siRNA via disulfide linkage could easily regenerate monomeric siRNA in reductive cytosol without any enzymatic reaction. Accordingly, the chemically fabricated dimeric structure might avoid Dicer-mediated immune response after intracellular uptake.

The dimeric siRNA has a 2-fold higher molecular weight than monomeric siRNA as well as an additional flexible spacer between the siRNAs, which was greatly favorable

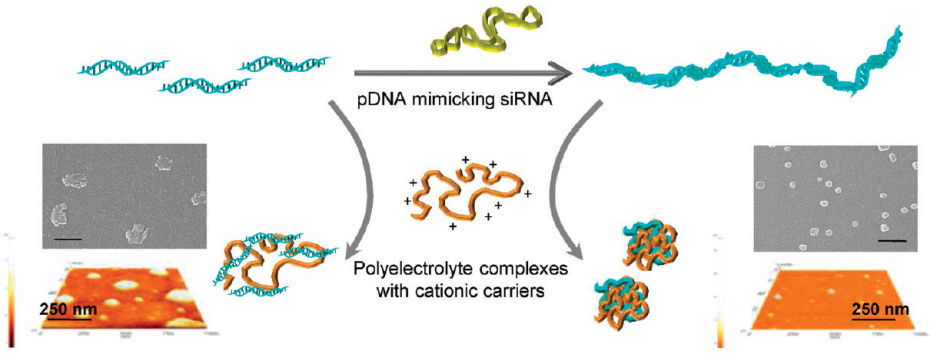


FIGURE 6. Schematic illustration of siRNA multimerization to improve binding affinity with low-charge cationic carriers. SEM and AFM images show LPEI polyelectrolyte nanocomplexes with multi-siRNA (right panel) and conventional siRNA (left panel). Adapted with permission from ref 33. Copyright 2010 Nature Publishing Group.

for ionic complexation with LPEI and cellular uptake compared with conventional siRNAs. The dimeric siRNA formed homogeneous and compact nanocomplexes with a size of approximately 100 nm and exhibited higher gene silencing, while conventional siRNA showed negligible gene inhibition. Furthermore, by this synthetic strategy, diverse moieties including PEG, polymer, dyes, and lipids could be adopted at both ends of the dimeric siRNAs as shown in Figure 5.

4.2. Multimeric siRNA. Using siRNA as a building block, linear siRNA homopolymers, multimeric siRNAs (multi-siRNA), were prepared by complementary hybridization between a sense strand dimeric siRNA (sense dimer) and an antisense strand dimeric siRNA (antisense dimer) (Figure 5).[33] Around 40% of the overall multi-siRNAs formed linear siRNA homopolymers with more than five siRNAs despite their heterogeneous population with different lengths. In multi-siRNA, the hybridization between the complementary 19 nucleotides of the siRNA monomer was strong enough to tolerate the charge repulsion between siRNA blocks, maintaining a multimeric structure under physiological conditions.

It was thought that siRNAs with higher molecular weight can bind to cationic carriers much more efficiently due to their increased charge density and flexibility of the intermolecular spacer. As expected, multi-siRNAs interacted much more efficiently with the cationic carrier, LPEI (M_w 25000), than conventional siRNAs, and formed a stable and compact polyelectrolyte complex. In addition, each terminal end of multi-siRNA, which remained as an unannealed form, was likely to elicit the inter- and intramolecular annealing/unannealing for the stabilization of polyelectrolyte complexes. Compared with loose and heterogeneous complex structures of conventional siRNA/LPEI, multi-siRNA/LPEI was observed as homogeneous and spherical nanoparticles about 100 nm in size (Figure 6). It is well-known that the size and morphology of nanoparticles determines the route and efficiency of intracellular uptake. Accordingly, the intracellular uptake by multi-siRNA/LPEI was much higher than that of conventional siRNA/LPEI, which consequently allowed efficient gene suppression in vitro and in vivo via release of siRNA from polyelectrolyte complexes after the cleavage of multi-siRNAs in cytoplasm.[33] With increasing siRNA length, the efficiency of target gene silencing was significantly enhanced, which might be attributed to the fact that increased charge density might elicit far more enhanced binding efficiencies with cationic carriers.

Fabrication of linear siRNA homopolymer via chemical conjugation allows multi-siRNA to employ diverse chemical linkage with different flexibility, length, and cleavability within polymers, which is a big advantage over biologically derived methods, such as complementary hybridization and enzyme-catalyzed ligation. Cleavable multi-siRNAs might be favorable for subsequent RNAi after intracellular uptake because of facile and prompt regeneration of homogeneous monomeric siRNAs compared with noncleavable multi-siRNAs (Figure 7A). For example, cleavable multi-siRNA linked by a disulfide linkage could easily produce monomeric and biologically active siRNAs through the degradation of disulfide bonds in a reductive cytoplasmic environment without any loss of siRNA units. However, noncleavable multi-siRNA is likely to be a substrate for Dicer, which could produce diverse siRNA fragments of different lengths through enzymatic digestion. We prepared both cleavable and noncleavable multi-siRNA using two types of cross-linkers, a cleavable cross-linker, DTME, and a noncleavable cross-linker, 1,8-bis(maleimido)di(ethylene glycol) (BM(PEG)$_2$). Two different multi-siRNAs exhibited similar physicochemical characteristics

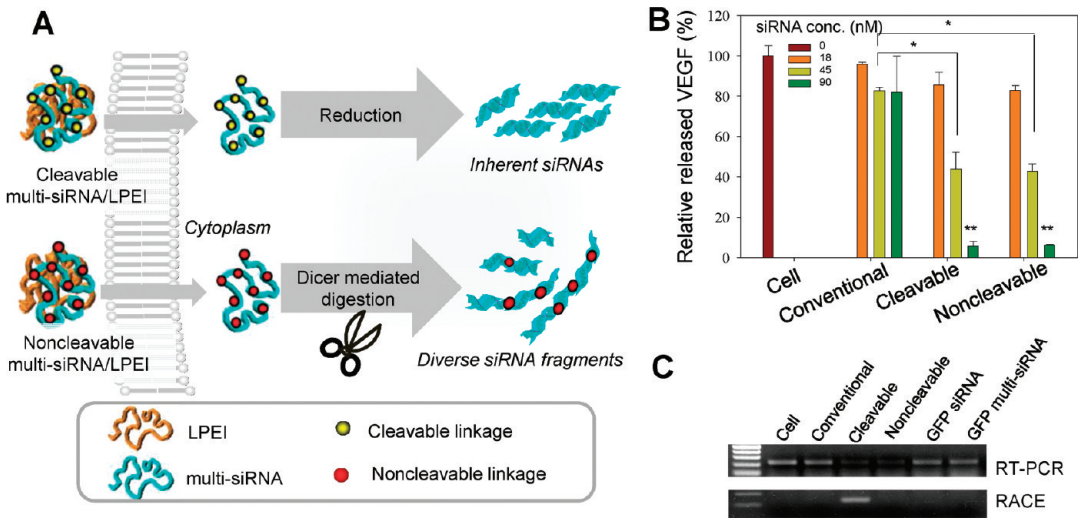


FIGURE 7. (A) Different intracellular fates of two types of multi-siRNA. The cleavable multi-siRNA generates intact siRNA monomers by reduction with cytoplasmic glutathione (GSH) while the noncleavable multi-siRNA can produce a mixture of fractionated siRNA species by Dicer-mediated cleavage. (B) Target VEGF silencing at the protein level and (C) mRNA inhibition (upper panel) and site-specific mRNA cleavage (lower panel) by conventional siRNA, cleavable multi-siRNA, and noncleavable multi-siRNA after LPEI complexation in PC-3 cells. (RT-PCR, semiquantitative reverse transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends). Panels B and C adapted with permission from ref 33. Copyright 2010 Nature Publishing Group.

and gene knock-down against endogenous vascular endothelial growth factor (VEGF) at the protein and mRNA level after complexation with LPEI (Figure 7B,C). However, RACE has demonstrated that cleavable multi-siRNAs could indeed bind and degrade their target mRNA at the specific site via RNAi only (Figure 7C). In the case of noncleavable multi-siRNAs, the Dicer-mediated random fragments might elicit target protein silencing via microRNA-related translational repression although a portion of them could participate in RNAi processing.[34] This silencing mechanism could potentially induce nonspecific gene silencing by randomly fragmented oligomers containing off-target sequences. In addition, it is well-known that long dsRNAs could be recognized by intracellular inflamasomes, which subsequently induce nonspecific immune responses.[35] Exogenous dsRNA could induce the activation of endosomal toll-like receptors (TLR), especially TLR-7, and cytoplasmic inflamasomes, including RIG-I, TLR-3, MDA5, and PKR, by recognition of specific sequence and chain length, respectively. Even though the length-extended multi-siRNAs could potentially act as immune stimulants, cleavable multi-siRNAs could escape from the PKR-mediated immune response because their intrinsic structure could be restored immediately in the reductive cytoplasm. Noncleavable multi-siRNA, however, remains as long dsRNA before Dicer-mediated degradation, which might augment an intracellular immune response.[35] In our study, noncleavable multi-siRNA elicited a 2.5-fold higher release of interferon-α, a cytokine related to the immune response for human peripheral blood monocyte cells, than cleavable multi-siRNA and conventional siRNA.

Using chemical fabrication, diverse siRNAs against different target genes could be easily incorporated into a single multi-siRNA for combinatorial RNAi (co-RNAi) therapy.[36] Biological systems are homeostatically regulated by multiple feedback mechanisms. For example, functional loss by suppression of one disease-related gene could be compensatorily replaced by other genes, which might significantly lessen the therapeutic effect of siRNA. Indeed, death of cancer cells was not significantly induced by suppression of a single anti-apoptotic gene.[37] Co-RNAi is a new therapeutic strategy that inhibits multiple problematic genes simultaneously by treating with siRNA mixtures against different genes. In recent studies, simultaneous treatment with multiple siRNA cocktails in the nanocomplexes has greatly increased therapeutic effect without repeated injections.[38] To harbor two different kinds of siRNAs into one multi-siRNA, two different types of double-stranded siRNAs with terminal thiol groups at both 3′ ends were concatenated using chemical cross-linkers (Figure 8A). Dual gene targeted multi-siRNAs (DGT multi-siRNAs) are composed of two types of siRNAs against anti-apoptotic genes, survivin and bcl-2, which are usually overexpressed in cancer cells making them resistant to intra/extracellular death signals via dysregulation of the apoptotic signal cascade. Interestingly, DGT multi-siRNA complexes with LPEI were delivered into the cells more efficiently than single gene

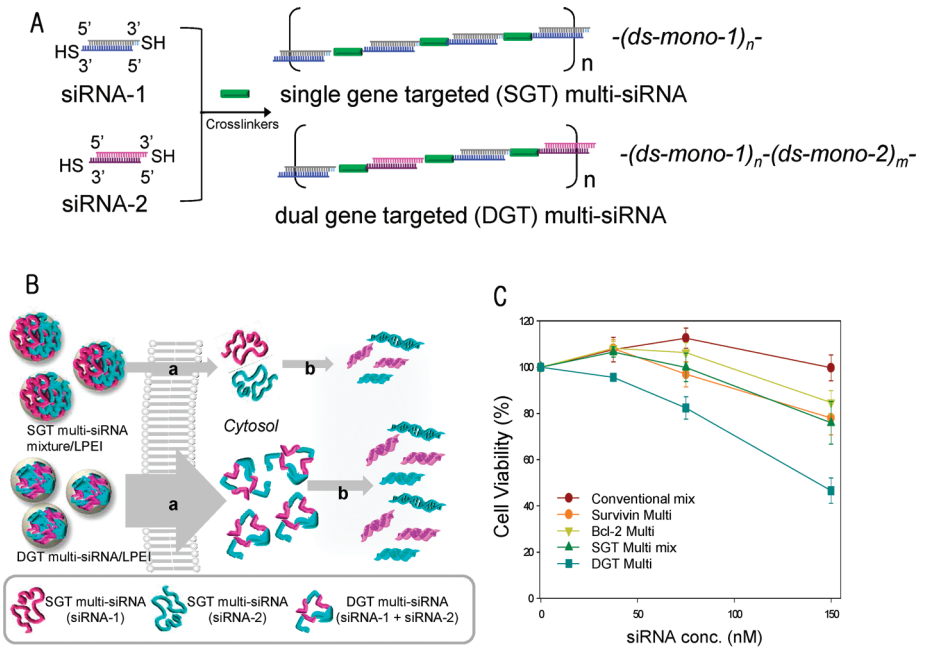


FIGURE 8. (A) Schematic diagram for preparation of SGT multi-siRNA and DGT multi-siRNA. (B) Putative comparison of intracellular uptake efficiencies derived from different morphological properties. (a) Endocytosis mediated intracellular uptake and (b) spontaneous cleavage of multi-siRNAs in a reductive cytoplasm. (C) The relative cell viability of HeLa cells after treatment of conventional siRNA mixture (Conventional mix), survivin multi-siRNA (Survivin Multi), bcl-2 multi-siRNA (Bcl-2 Multi), SGT multi-siRNA mixture (SGT Multi mix), and DGT multi-siRNA (DGT Multi). The viability of mocked cells was assigned as 100%. Panel C adapted with permission from ref 36. Copyright 2011 Elsevier.

targeted multi-siRNA (SGT multi-siRNAs) complexes with LPEI, which was demonstrated by a direct measurement of intracellular siRNA amounts. It is conceivable that the heterogeneous sequence composition in DGT multi-siRNAs assigns higher structural flexibility for compact ionic complexation than homogeneous sequence composition in SGT multi-siRNAs because the sequence heterogeneity could affect thermodynamic parameters of nucleotide structures, such as persistent length, free energy for condensation, and contour length (Figure 8B).[39,40] The DGT multi-siRNA against survivin/bcl-2 genes complexed with LPEI suppressed much more efficiently target genes at the mRNA and protein level than the mixture of SGT multi-siRNAs/LPEI and the physical mixture of conventional siRNAs/LPEI complexes. More importantly, only DGT multi-siRNA/LPEI complexes induced significant apoptotic cell death of cancer cells (Figure 8C). This co-RNAi system using DGT multi-siRNAs could be exploited as a novel approach for the treatment of multigene related diseases.

4.3. siRNA Microhydrogels. It is conceivable that branched siRNA polymer can have favorable properties for ionic complexation with cationic carriers over linear siRNA polymer, considering branched polymer has superior binding capacity with counterionic molecules compared with linear polymer.[41] To prepare branched siRNA polymer, Y-shaped single strand siRNAs were prepared by reacting thiol-terminated sense or antisense single strand siRNAs with tris-2-maleimidoethyl-amide (TMEA), a trifunctional cross-linker containing three reactive maleimide groups (Figure 9). Dimeric linear single-strand siRNAs were also synthesized using $BM(PEG)_2$. Using the resulting trimeric branched units, dimeric linear units, and monomeric unit, siRNA microhydrogels were fabricated via spontaneous complementary hybridization.[42] By mixing different combinatorial siRNA units, two types of micrometer-sized siRNA hydrogels were produced: moderately branched siRNA hydrogels (DY-siRNA) by mixing dimeric sense and Y-shaped antisense strand siRNAs and highly branched siRNA hydrogels (YY-siRNA) by mixing Y-shaped sense and Y-shaped antisense strand siRNAs with a size of around 5−15 μm. In this study, the size and cross-linking density in hydrogels were mainly controlled by the molecular ratio of trimeric single strands in the total population, considering the functionality of dimeric and trimeric single strand siRNAs as a chain extension and three-way cross-linker, respectively. As expected, DY-siRNA hydrogel exhibited a significantly larger overall size and higher porosity than YY-siRNA hydrogel, which was attributed to the preferential length extension in DY-siRNA hydrogel due to the higher extent of dimeric units. The pore size in DY-siRNA hydrogel was approximately 8-fold larger than

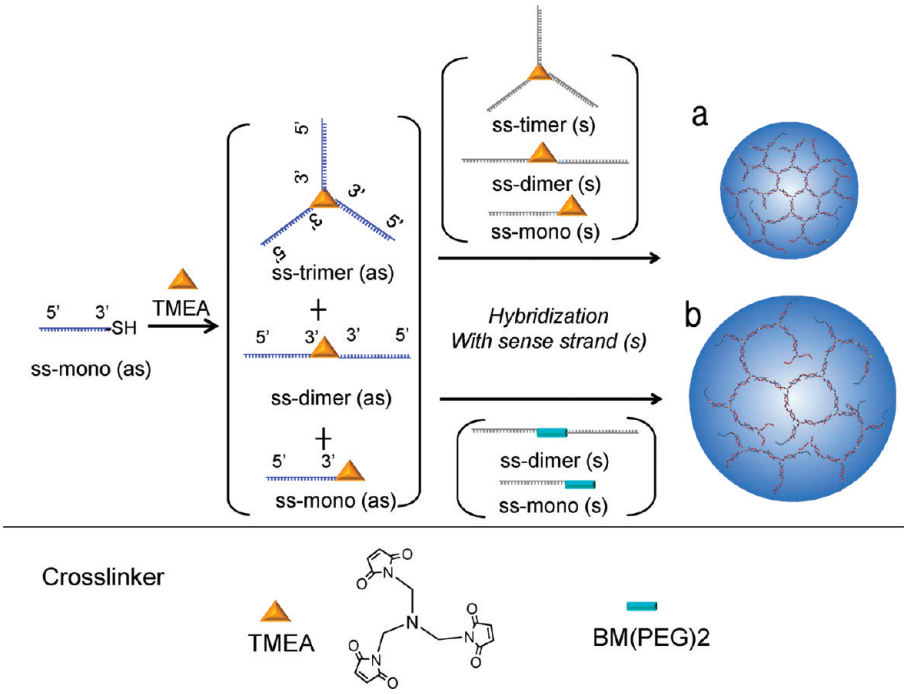


FIGURE 9. Schematic illustration for the preparation of siRNA microhydrogels. Highly branched hydrogels (a, YY-siRNAs) and moderately branched ones (b, DY-siRNAs) were synthesized via complementary base pairing of Y-shaped antisense strand siRNA (as) with Y-shaped sense strand siRNAs (s) and dimeric sense strand siRNAs, respectively. Adapted with permission from ref 42. Copyright 2011 American Chemical Society.

that of the YY-siRNA hydrogel. Due to dramatically expanded structures and flexibility, DY- and YY-siRNA hydrogels exhibited highly improved binding affinities with low molecular weight LPEI (M_w 2500) compared with conventional siRNAs, which resulted in formation of compact and stable nanocomplexes with a size below 120 nm. Both DY- and YY-siRNA hydrogel/LPEI complexes were even smaller, by approximately 2-fold, than multi-siRNA/LPEI complexes probably due to the much higher charge density, flexibility, and complexity derived from preformed 3D geometric structure of siRNA hydrogels than those of multi-siRNAs. Likewise, DY- and YY-siRNA hydrogels were more efficiently taken up into the cells and sequentially silenced the target gene expression than multi-siRNA in vitro.

Interestingly, DY- and YY-siRNA hydrogels showed highly efficient gene silencing despite noncleavable intermolecular thioether bonds, suggesting that the hydrogels might be dissociated via Dicer processing into biologically active siRNAs in the cytoplasm. In this study, as a proof-of-concept, dimeric and trimeric building units for micrometer-sized hydrogels were synthesized via noncleavable thioether bonds, which can be replaced with cleavable linker to avoid undesirable immune response and nonspecific gene silencing as well as increase sequence specific gene suppression.

5. Conclusion and Perspectives

Despite the great potential of siRNA drugs, initial vigorous developments by pharmaceutical industries and academia have been slowing down due to several challenges such as delivery hurdles and off-target effects. To address these critical issues, not only a specialized carrier system but also optimized siRNA structures should be developed. Early studies focused on chemical and physical modifications based on monomeric siRNA structures. Currently, as an alternative novel strategy, siRNA based micro- and nanostructures have been fabricated by facile chemical conjugation and complementary base pairing to complement the physicochemical weakness of monomeric siRNAs, resulting in formation of stable complexes with carriers and enhanced intracellular delivery. The new siRNA structures should be designed to endow favorable characteristics for siRNA delivery over conventional siRNA while maintaining intrinsic biological activity and evading undesired effects, such as elevated immune response and off-target effects. Newly designed siRNA structures, including block copolymers, micelles, linear siRNA homopolymers, and hydrogels, endow increased spatial charge density and flexibility for facile complexation with low-charged and biocompatible cationic carriers. Additionally, by introducing a flexible and cleavable spacer between

siRNAs and polymer blocks, the siRNA micro- and nanostructures could be easily dissociated and generate innate siRNAs in a reductive environment such as the cytoplasm.

Chemically fabricated siRNA structures have several advantages over biologically derived siRNA structures including facile synthesis using only siRNAs without any unessential RNA strands and employment of diverse intermolecular spacers with unique characters including long spacing, cleavability, and stimuli-sensitivity, which might enable avoidance of off-target effects and nonspecific immune responses. However, it is still challenging to produce finely tuned structures only using limited synthetic strategy with siRNA sequences and chemical cross-linkers. In addition, fabricated siRNA based structures should be complexed with cationic carriers for facile intracellular delivery. Thus, development of chemically prepared siRNA-based structures that elicit comparably efficient intracellular delivery to cationic carriers might be needed. Future studies should be focused on rational design from a new viewpoint to make up for the weaknesses of current siRNA-based structures by combining a chemically fabricable structure with biologically derived structures and adopting new devices/materials. Finally, a rigorous biological characterization of newly fabricated siRNA-based structures in vivo is needed to elucidate any physiological side effects for their clinical translation.

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant 2011-0023201), the Pioneer Research Program, and KRIBB Research Initiative Program.

BIOGRAPHICAL INFORMATION

Soo Hyeon Lee is a postdoctoral associate in the group of Prof. Jean-Christophe Leroux at the ETH Zurich. Her research interests are novel delivery systems of nucleic acid drugs based on biomaterials.

Bong Hyun Chung is a director of the BioNanotechnology Research Center at KRIBB and a chief professor of BioNano Major at the University of Science and Technology (UST). His main research interests include bionano interfacing science, biochip/biosensor, and nanomedicine.

Tae Gwan Park (1957−2011) served as a professor at KAIST, published over 252 papers in SCI journals, and was the recipient of numerous honors and awards during his academic career including the Clemson Award.

Yoon Sung Nam is an assistant professor of Materials Science and Engineering at KAIST. His research group focuses on biological interfaces, particularly related to functional nanomaterials for practical applications in catalysis and medicine.

Hyejung Mok is an assistant professor of Bioscience and Biotechnology at Konkuk University. Her main research interests include biomaterials-based gene and drug delivery systems, gene therapy, and nanomedicine.

FOOTNOTES

*Corresponding authors. E-mail addresses: hjmok@konkuk.ac.kr (H.M.); yoonsung@kaist.ac.kr (Y.S.N.).

The authors declare no competing financial interest.

REFERENCES

1 Chworos, A.; Severcan, I.; Koyfman, A. Y.; Weinkam, P.; Oroudjev, E.; Hansma, H. G.; Jaeger, L. Building programmable jigsaw puzzles with RNA. *Science* **2004**, *306*, 2068–2072.

2 Guo, P. The emerging field of RNA nanotechnology. *Nat. Nanotechnol* **2010**, *5*, 833–842.

3 Nasalean, L.; Baudrey, S.; Leontis, N. B.; Jaeger, L. Controlling RNA self-assembly to form filaments. *Nucleic Acids Res.* **2006**, *34*, 1381–1392.

4 Grabow, W. W.; Zakrevsky, P.; Afonin, K. A.; Chworos, A.; Shapiro, B. A.; Jaeger, L. Self-assembling RNA nanorings based on RNAI/II inverse kissing complexes. *Nano Lett.* **2011**, *11*, 878–887.

5 Khaled, A.; Guo, S.; Li, F.; Guo, P. Controllable self-assembly of nanoparticles for specific delivery of multiple therapeutic molecules to cancer cells using RNA nanotechnology. *Nano Lett.* **2005**, *5*, 1797–1808.

6 Zhang, F.; Lemieux, S.; Wu, X.; St-Arnaud, D.; McMurray, C. T.; Major, F.; Anderson, D. Function of hexameric RNA in packaging of bacteriophage phi 29 DNA in vitro. *Mol. Cell* **1998**, *2*, 141–147.

7 Chang, K. Y.; Tinoco, I., Jr. Characterization of a "kissing" hairpin complex derived from the human immunodeficiency virus genome. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8705–8709.

8 Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **1998**, *391*, 806–811.

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

10 Elbashir, S. M.; Martinez, J.; Patkaniowska, A.; Lendeckel, W.; Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **2001**, *20*, 6877–6888.

11 Watts, J. K.; Corey, D. R. Clinical status of duplex RNA. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 3203–3207.

12 Hunter, A. C. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv. Drug Delivery Rev.* **2006**, *58*, 1523–1531.

13 Shah, S. A.; Brunger, A. T. The 1.8 Å crystal structure of a statically disordered 17 base-pair RNA duplex: principles of RNA crystal packing and its effect on nucleic acid structure. *J. Mol. Biol.* **1999**, *285*, 1577–1588.

14 Kebbekus, P.; Draper, D. E.; Hagerman, P. Persistence length of RNA. *Biochemistry* **1995**, *34*, 4354–4357.

15 Bolcato-Bellemin, A. L.; Bonnet, M. E.; Creusat, G.; Erbacher, P.; Behr, J. P. Sticky overhangs enhance siRNA-mediated gene silencing. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16050–16055.

16 Khvorova, A.; Reynolds, A.; Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **2003**, *115*, 209–216.

17 Schwarz, D. S.; Hutvagner, G.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P. D. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **2003**, *115*, 199–208.

18 Sano, M.; Sierant, M.; Miyagishi, M.; Nakanishi, M.; Takagi, Y.; Sutou, S. Effect of asymmetric terminal structures of short RNA duplexes on the RNA interference activity and strand selection. *Nucleic Acids Res.* **2008**, *36*, 5812–5821.

19 Sun, X.; Rogoff, H. A.; Li, C. J. Asymmetric RNA duplexes mediate RNA interference in mammalian cells. *Nat. Biotechnol.* **2008**, *26*, 1379–1382.

20 Bramsen, J. B.; Laursen, M. B.; Damgaard, C. K.; Lena, S. W.; Babu, B. R.; Wengel, J.; Kjems, J. Improved silencing properties using small internally segmented interfering RNAs. *Nucleic Acids Res.* **2007**, *35*, 5886–5897.

21 Manoharan, M. RNA interference and chemically modified small interfering RNAs. *Curr. Opin. Chem. Biol.* **2004**, *8*, 570–579.

22 Harris, J. M.; Chess, R. B. Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery* **2003**, *2*, 214–221.

23 Lee, S. H.; Mok, H.; Park, T. G. Di- and triblock siRNA-PEG copolymers: PEG density effect of polyelectrolyte complexes on cellular uptake and gene silencing efficiency. *Macromol. Biosci.* **2011**, *11*, 410–418.

24 Schafer, F. Q.; Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biol. Med.* **2001**, *30*, 1191–1212.

25 Kim, S. H.; Jeong, J. H.; Lee, S. H.; Kim, S. W.; Park, T. G. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. *J. Controlled Release* **2006**, *116*, 123–129.

26 Jung, S.; Lee, S. H.; Mok, H.; Chung, H. J.; Park, T. G. Gene silencing efficiency of siRNA-PEG conjugates: effect of PEGylation site and PEG molecular weight. *J. Controlled Release* **2010**, *144*, 306–313.

27 Devanand, K.; Selser, J. C. Asymptotic-behavior and long-range interactions in aqueous-solutions. *Macromolecules* **1991**, *24*, 5943–5947.

28 Mishra, S.; Webster, P.; Davis, M. E. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur. J. Cell Biol.* **2004**, *83*, 97–111.

29 Takemoto, H.; Ishii, A.; Miyata, K.; Nakanishi, M.; Oba, M.; Ishii, T.; Yamasaki, Y.; Nishiyama, N.; Kataoka, K. Polyion complex stability and gene silencing efficiency with a siRNA-grafted polymer delivery system. *Biomaterials* **2010**, *31*, 8097–8105.

30 Lee, S. H.; Mok, H.; Lee, Y.; Park, T. G. Self-assembled siRNA-PLGA conjugate micelles for gene silencing. *J. Controlled Release* **2011**, *152*, 152–158.

31 Aliyari, R.; Ding, S. W. RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol. Rev.* **2009**, *227*, 176–188.

32 Chung, H. J.; Hong, C. A.; Lee, S. H.; Jo, S. D.; Park, T. G. Reducible siRNA dimeric conjugates for efficient cellular uptake and gene silencing. *Bioconjugate Chem.* **2011**, *22*, 299–306.

33 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.

34 Martin, S. E.; Caplen, N. J. Mismatched siRNAs downregulate mRNAs as a function of target site location. *FEBS Lett.* **2006**, *580*, 3694–3698.

35 Whitehead, K. A.; Dahlman, J. E.; Langer, R.; Anderson, D. G. Silencing or stimulation? siRNA delivery and the immune system. *Annu. Rev. Chem. Biomol. Eng.* **2011**, *2*, 77–96.

36 Lee, S. H.; Mok, H.; Jo, S.; Hong, C. A.; Park, T. G. Dual gene targeted multimeric siRNA for combinatorial gene silencing. *Biomaterials* **2011**, *32*, 2359–2368.

37 Holcik, M.; Korneluk, R. G. XIAP, the guardian angel. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 550–556.

38 Tai, W.; Qin, B.; Cheng, K. Inhibition of breast cancer cell growth and invasiveness by dual silencing of HER-2 and VEGF. *Mol. Pharmaceutics* **2010**, *7*, 543–556.

39 Travers, A. A. The structural basis of DNA flexibility. *Philos. Trans. R. Soc., A* **2004**, *362*, 1423–1438.

40 Scipioni, A.; Anselmi, C.; Zuccheri, G.; Samori, B.; De Santis, P. Sequence-dependent DNA curvature and flexibility from scanning force microscopy images. *Biophys. J.* **2002**, *83*, 2408–2418.

41 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.

42 Hong, C. A.; Lee, S. H.; Kim, J. S.; Park, J. W.; Bae, K. H.; Mok, H.; Park, T. G.; Lee, H. Gene silencing by siRNA microhydrogels via polymeric nanoscale condensation. *J. Am. Chem. Soc.* **2011**, *133*, 13914–13917.