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Pendant Polymer:Amino-β-Cyclodextrin:siRNA Guest:Host Nanoparticles as Efficient Vectors for Gene Silencing

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Supporting Information

ABSTRACT: A novel siRNA delivery vector has been developed, based on the self-assembly of monosubstituted cationic β-CD derivatives with a poly(vinyl alcohol)-MW27kD (PVA) main-chain polymer bearing poly(ethylene glycol)MW2000 (PEG) and acid-labile cholesterol-modified (Chol) grafts through an acid-sensitive benzylidene acetal linkage. These components were investigated for their ability to form nanoparticles with siRNA using two different assembly schemes, involving either precomplexation of the pendant Chol-PVA-PEG polymer with the cationic β-CD derivatives before siRNA condensation or siRNA condensation with the cationic β-CD derivatives prior to addition of Chol-PVA-PEG to engage host:guest complexation. The pendant polymer:-amino-β-CD:siRNA complexes were shown to form nanoparticles in the size range of 120−170 nm, with a slightly negative zeta potential. Cell viability studies in CHO-GFP cells shows that these materials have 10^3-fold lower cytotoxicities than 25 kD bPEI, while maintaining gene-silencing efficiencies that are comparable to those of benchmark transfection reagents such as bPEI and Lipofectamine 2000. These results suggest that the degradable Chol-PVA-PEG polymer is able to self-assemble in the presence of siRNA and cationic-β-CD to form nanoparticles that are an effective and low-toxicity vehicle for delivering siRNA cargo to target cells.

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism arising from degradation or translation arrest of target RNA. The ability of 21−23 nucleotide RNAs (siRNA) to mediate RNAi in mammalian cells has enormous therapeutic potential for the treatment of viral infections, cancer, and neurological disorders.[1] The use of siRNA has several advantages over conventional chemotherapy in that the high specificity nucleic acid drug acts “upstream” from most conventional chemotherapeutic agents, conferring the ability to target any protein and the capacity to potentially evade drug resistance.[2] The safe and efficient delivery of siRNA specifically to target cells, however, remains a major challenge.[3−6] A variety of viral and nonviral vectors have been developed for this purpose. Although viral vectors have shown promise, they suffer from scalability, immunogenicity, and safety issues. Nonviral vectors have attracted considerable attention due to their modest host immunogenicity and manufacturability. Many nonviral vectors such as cationic liposomes, Lipofectamine 2000 (L2k), polypeptides, and inorganic nanoparticles have been studied for this purpose.[7,8] A variety of cationic polymers also have been investigated as nonviral vectors, including polyethylenimines (PEI),[9] poly-(L-lysine),[10] PAMAM dendrimers,[11,12] poly(lactic-*co*-glycolic acid) (PLGA),[13,14] chitosan,[15,16] PEI-alginate nanoparticles,[17] and cyclodextrin (CD) oligomers.[6,18−22] All these polymer vectors are capable of condensing siRNA to form positively charged particles that enter cells via nonspecific uptake mechanisms; however, most of these materials either display significant cytotoxicity at the concentrations needed for effecting nucleic acid cargo bioactivity or suffer from poor efficiency due to insufficient endosomal escape.

β-CD has well-known host−guest interactions with a vast array of compounds with binding constants in the $10^{0.5}$−10^5 M^{-1} range in aqueous media.[26] This property has led to a wide variety of biomedical applications ranging from drug solubilization to their use as a building block for nonviral vector construction. Davis and co-workers reported a class of CD oligomers[6,23−26] as vectors for delivery of siRNA in a clinical trial for melanoma therapy with encouraging results. The fixed cationic groups on the relatively rigid oligomeric backbone, however, may be responsible for the high N:P ratios required for nucleic acid compaction and delivery in this case.

The mechanism of nucleic acid complex disassembly and escape from the endosome of cells that have internalized them is still unclear in the case of most nonviral vector systems. A variety of ion-exchange,[27] endosomolytic, and degradative processes[28] have been proposed; however, the diversity of proposals is likely a reflection of the multiple internalization pathways[29] and vast array of nucleic acid nanoparticle formulations employed. Zhang et al. have shown that the presence of cholesterol-conjugated lipids induces conversion of membrane lipids from the L_α to the H_{II} phase, thereby causing disruption of the endosomal membrane.[31] Since the presence of amino-cholesterol derivatives may promote disruption of biological membranes under endosomal pH conditions to facilitate intracellular siRNA delivery, we designed a delivery vector for siRNA based on the self-assembly of cationic β-CD derivatives with a pendant polymer[32] comprised of cholesterol-modified (Chol) poly(ethylene glycol)-poly(vinyl alcohol) (PEG-PVA), whose Chol units are linked through an acid-sensitive acetal motif (Figure 1). It was anticipated that siRNA compaction could be achieved via complexation with self-assembled Chol-PVA-PEG:amino-β-CD guest:host pendant polymer complexes via multivalent electrostatic interactions.

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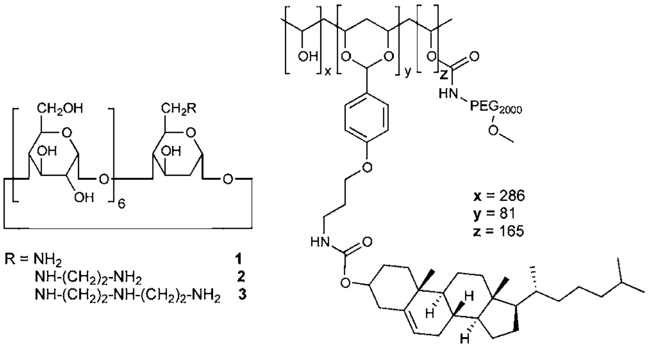
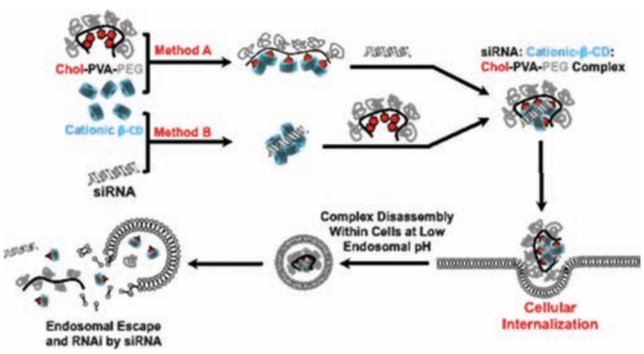


Figure 1. Structures of amino-β-CDs **1**–**3** (left) and Chol-PVA-PEG (right).

This approach enables the compaction of the siRNA cargo into stable nanometer-size particles that can then be internalized by target cells into acidic endosomes. Endosomal degradation of the polymer acetal linkage should promote release of the cholesterol pendant groups and decondensation of the cationic CDs and siRNA cargo (Scheme 1), thereby facilitating endosomal escape of the cargo. Three cationic β-CD derivatives (Figure 1) were synthesized to test this concept: mono-6-(amino)-6-deoxy-β-cyclodextrin (**1**), mono-6-(N,N'-dimethyl-ethane-1,2-diamine)-6-deoxy-β-cyclodextrin (**2**), and mono-6-(N'-(2-aminoethyl)ethane-1,2-diamine)-6-deoxy-β-cyclodextrin (**3**). PVA (27 kD) was used to prepare Chol-PVA-PEG with 13.2 mol% Chol acetal modifications and 26.9 mol% PEG carbamate modifications based on ^{1}H NMR analysis (Supporting Information (SI)).

Scheme 1. Conceptual Diagram of Chol-PVA-PEG:Amino-β-CD:siRNA Complexation and Endosomal Escape



The ability of these non-covalent pendant polymer assemblies to condense siRNA was then evaluated. Two different complexation methods (Scheme 1) were used to evaluate the relative capacity of Chol-PVA-PEG:amino-β-CDs guest:host polymer assemblies toward siRNA condensation. In method A, Chol-PVA-PEG was pre-associated with amino-β-CDs before addition to the siRNA solution. In method B, the siRNA was first complexed with amino-β-CDs, followed by addition of Chol-PVA-PEG. Zeta potentials were measured for both types of complexes to determine the surface charge of the resulting transfection particles (SI). We observed that complexes formed by both methods had slightly negative zeta potentials ($\zeta < -8$ mV). As the N/P ratio increases from 10 to 20, the ζ-potential approaches neutrality. Method B ($\zeta = -16$ to -12 mV) particles were shown to be more negatively charged than those produced by method A ($\zeta = -10$ to -8 mV). Among the CD variants, particles formulated from **1** had the lowest observed ζ, followed by **2** and **3**, respectively. The absence of a positive charge on the surface could be due to the high loading of PEG on the polymer backbone, which is able to effectively shield the positive charges arising from the cationic CDs. These results are encouraging since a positive surface charge is considered to be one of the major reasons for nanoparticle opsonization or macrophage uptake.[34] Gabizon and Papahadjopoulos have previously shown that liposomes with a slight negative charge have prolonged circulation times and enhanced tumor uptake due to RES evasion.[35]

Dynamic light scattering (DLS) showed that the complex sizes produced by these different materials and methods were in the 120–170 nm range, with higher N/P ratios producing smaller particles (SI). In general, the method of formulation did not significantly affect the size of the particles. Compound **3** was able to generate smaller particles than **2**, which formed particles smaller than **1**, suggesting that an increase in CD charge leads to smaller particle formation. DLS measurements as a function of pH revealed that the particles were stable at pH 7.4 for up to 24 h; however, at pH 5.5, the polydispersity of the particles started increasing after about 4 h, yielding multiple particle sizes by 48 h. We attribute these observations to pendant group hydrolysis at low pH, leading to destabilization of the Chol-PVA-PEG:amino-β-CD:siRNA complexes (SI).

AFM images of Chol-PVA-PEG:amino-β-CD samples revealed the presence of particles (Figure 2A) of average diameters 33 ± 6 nm and heights of 1.5 ± 0.6 nm. Upon addition of siRNA at N/P = 10, larger particles were formed that were of an average diameter of 51 ± 8 nm and height of 5 ± 1.7 nm (Figure 2B) (SI). The low heights may be due to deformation of the particles during the sample preparation for AFM. The sizes determined by AFM are smaller than those measured by DLS due to the dry nature of the AFM samples (i.e., polymer solvent swelling is absent). These results support the conclusion that supramolecular complexation of Chol-PVA-PEG with amino-β-CD produces a non-covalent assembly that is capable of condensing siRNA into compact and unimodal particles.

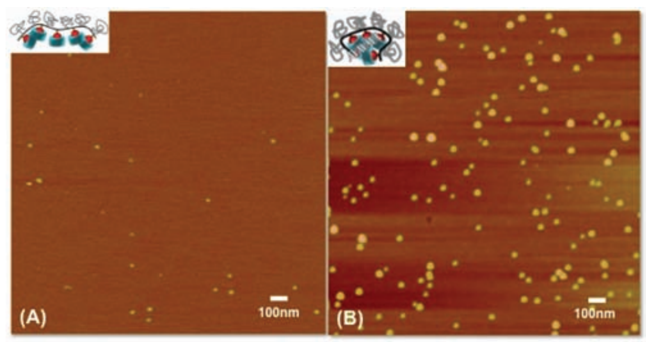


Figure 2. AFM images of (A) Chol-PVA-PEG:**3** and (B) Chol-PVA-PEG:**3**:siRNA at N/P = 10 (inset showing high resolution image). Scale bar = 100 nm.

The *in vitro* cytotoxicity of amino-β-CDs, Chol-PVA-PEG, and their host:guest complexes are an extremely important factor for their consideration as a safe nonviral vector. Figure 3 shows that Chol-PVA-PEG, all of the amino-β-CDs, and the Chol-PVA-PEG:**1** host:guest complex were nearly at least 3 orders of magnitude less cytotoxic than bPEI (i.e., the LD50's of bPEI, Chol-PVA-PEG, and 1:1 Chol-PVA-PEG:**1** were 0.01,

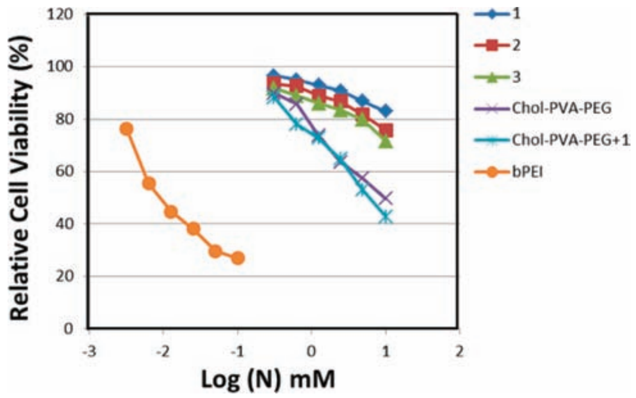


Figure 3. Cell viabilities of **1**−**3**, Chol-PVA-PEG, and Chol-PVA-PEG +**1** host:guest pendant polymer complexes in CHO-GFP cells using 25kD bPEI as control. The cells were treated with increasing amine concentrations of amino-β-CDs, Chol-PVA-PEG, Chol-PVA-PEG+**1**, and bPEI for 24 h in serum-free media before analysis by MTS assay.

9.5, and 7.9 mM, respectively, while those of **1**−**3** were all >10 mM and had negligible effect on the cell viability).

The *in vitro* gene knockdown efficiency of the complexes formed between the anti-GFP siRNA and the Chol-PVA-PEG:amino-β-CD guest:host pendant polymer system was assessed in CHO-GFP cells at N/P = 20 in the presence of serum relative to control vectors (bPEI and L2k) and negative control siRNA (Figure 4). Method A and B complexes both

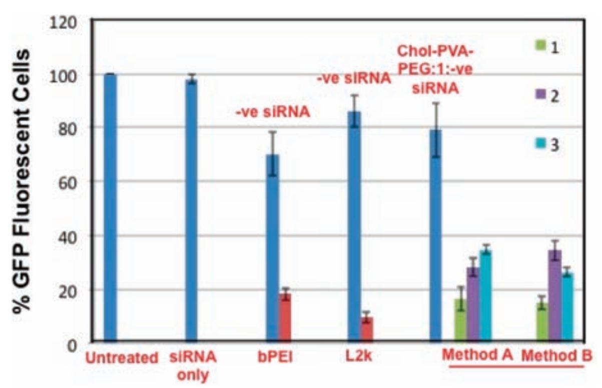


Figure 4. *In vitro* GFP knockdown efficiencies of amino-β-CD host:guest complexes (N/P = 20) with Chol-PVA-PEG and anti-GFP siRNA in CHO-GFP cells (in presence of serum) with 25kD bPEI and Lipofectamine 2000 (L2k) as controls and 100 nM anti-GFP or Allstar negative control (-ve) siRNA/well.

performed comparably to bPEI and L2k vectors. The lowest-performing guest:host pendant polymer complexes showed gene knockdown efficiencies of ~65%, and the best-performing complexes showed suppression up to ~85%, depending on the amino-β-CD type. Method A and B complexes had similar knockdown efficiencies, suggesting that the method of formulation does not appreciably affect the RNAi efficiency. Chol-PVA-PEG:**1**:siRNA complexes had the highest efficiency regardless of the formulation method used and performed similarly to L2k. This can be attributed to the lower charge density of **1** relative to **2** or **3**, thus enabling more facile dissociation of siRNA than the other two derivatives. Our studies also reveal that Chol is a more effective pendant group than adamantane with respect to the RNAi efficiencies of their guest:host pendant polymer complexes (data not shown). We attribute this enhancement to the effect that Chol has on membrane phase behavior such that endosomal escape is promoted by the pendant Chol group.

In conclusion, a novel and efficient siRNA delivery system has been developed based on the self-assembly of cationic CD derivatives with cholesterol-modified PEG-PVA. PVA, linked to Chol via a pH-sensitive acetal linkage, provides a scaffold for binding of cationic CD amines that are capable of condensing siRNA into nanoparticles less than 200 nm in size. These complexes are capable of achieving gene knockdown efficiencies in the same range as 25 kDa bPEI, L2k, while being 3−4 orders of magnitude less toxic.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures; synthesis and characterization of amino-β-CDs and Chol-PVA-PEG; DLS; zeta potential data; and FACS raw data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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