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Multifunctional Dendronized Peptide Polymer Platform for Safe and Effective siRNA Delivery

Hanxiang Zeng, Hannah C. Little, Timothy N. Tiambeng, Gregory A. Williams, and Zhibin Guan*

Department of Chemistry, 1102 Natural Sciences 2, University of California, Irvine, California 92697-2025, United States

Supporting Information

ABSTRACT: In this study, we designed and synthesized a biodegradable dendronized polypeptide (denpol) platform for delivery of small interfering RNA (siRNA). The novel denpol architecture combines the multivalency of dendrimers and conformational flexibility of linear polymers for optimal siRNA binding. Multifunctional amino acids were incorporated onto the dendrons and the structure was tuned both systematically and combinatorially to select optimal vectors. By screening a focused library, we identified several denpols that can effectively deliver siRNA to NIH 3T3 cells in vitro and exhibit minimal toxicity. For comparison, the best-performing denpol showed significantly improved transfection efficiency over Lipofectamine in serum-containing media. Fluorescence intracellular trafficking studies indicated that amphiphilicity is important for cell uptake and that the buffering capacity of histidine facilitates endosomal membrane rupture and therefore enhances the transfection efficiency. The combination of high delivery efficiency in serum and low cytotoxicity suggests the denpol system as a promising new carrier for siRNA delivery.

Since its discovery, small interfering RNA (siRNA) has been investigated for the treatment of several diseases, including cancer, inflammation, diabetes, and neurodegenerative diseases.[1] Despite its potential, therapeutic application of siRNA has been greatly hindered by the lack of safe and effective delivery vectors.[2] Both viral and nonviral vectors have been studied intensively in the last decades. Viral vectors, although with higher efficiency in general, have safety concerns related to their infectious nature and immunogenicity.[3] On the other hand, synthetic nonviral vectors offer versatile and precise structure control and are promising candidates for siRNA delivery.[4] Among different synthetic vectors, cationic lipids,[5] polymers,[6] dendrimers,[7] peptides,[8] and nanoparticles (NPs)[9] have been shown to be effective for siRNA delivery in vitro. However, many suffer from low efficiency as well as toxicity or immunogenicity in vivo, and few have progressed into clinical trials; to date, there are no FDA-approved siRNA delivery vectors.[2]

For successful siRNA delivery, multiple barriers have to be overcome at different stages,[10] including strong siRNA binding for complex formation, biocompatibility, serum stability, and tissue penetration for systemic delivery and effective cell uptake, endosomal escape, and siRNA dissociation in the cytoplasm at the cellular level.[11] Several important lessons related to overcoming these barriers have been learned in previous studies. First, because of the short, rigid structure of siRNA,[12] a highly branched architecture[12b] and chain flexibility[12c] are important for stable complex formation. Second, amphiphilic molecules can help both cellular uptake and endosomal escape through enhanced membrane permeability.[13] Furthermore, pH-responsive moieties can facilitate endosomal membrane rupture[14] through either the "proton sponge" effect[15] or increased amphiphilicity at lower pH.[13e] Lastly, to take advantage of the reducing environment in the cytoplasm, disulfide bonds have been introduced into the polymer for efficient unpacking of the siRNA.[16]

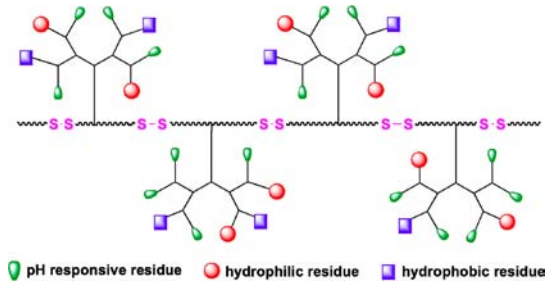

Figure 1. Concept of multifunctional amphiphilic dendronized polypeptide vectors for siRNA delivery.

Based on these lessons, we have developed a novel amino acid-based biodegradable dendronized polymer (denpol) platform for effective siRNA delivery (Figure 1). Although both individual dendrons[12c] and dendrimers[7c,17] have been investigated for siRNA delivery, the denpol architecture represents a novel design with a number of advantages. Several studies have indicated that the rigidity of high-generation dendrimers makes them nonideal for siRNA binding and delivery[12c,18] and that introducing flexibility into dendrimers can significantly improve their transfection efficiency.[19] Lower-generation dendrimers or single dendrons, on the other hand, lack the multivalency needed for strong siRNA binding.[20] In contrast, *the denpol architecture combines the mulivalency of dendrimers and the conformational flexibility of linear polymers* and thus represents a conceptual advancement in siRNA carrier design. Furthermore, the facile synthesis of lower-generation denpols allows for easy structural permutation and optimization, and the designed denpols are fully composed of natural amino acids to ensure biodegradability and low toxicity.

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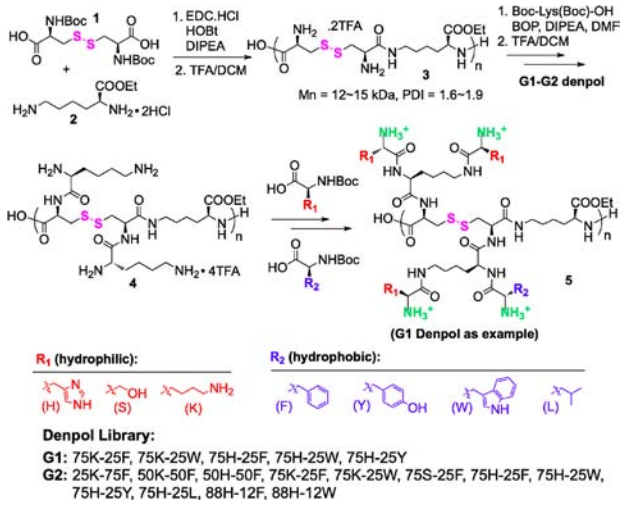
Scheme 1. Synthetic Route for the Denpol Library



To demonstrate our concept, we constructed a small focused library of amino acid-based denpols using a "graft-from" approach (Scheme 1). First, the polymer backbone (**3**) was synthesized by step-growth polymerization of dicysteine (**1**) and L-lysine (**2**) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the coupling reagent (polymer $M_n \approx 15$ kDa, PDI ≈ 1.8 by gel-permeation chromatography). Dicysteine was introduced into **3** as an environmentally responsive motif to facilitate siRNA release in the cytoplasm.[16] An L-lysine-based dendron was then grown generation by generation onto **3** through solution-phase peptide coupling. Finally, after the desired generation was reached, the outer layer of the dendron was functionalized with one hydrophilic and one hydrophobic amino acid in various ratios, giving the denpol **5**. Three hydrophilic amino acids [lysine (K), serine (S), and histidine (H)] and four hydrophobic amino acids [tryptophan (W), phenylalanine (F), tyrosine (Y), and leucine (L)] were included in our combinatorial library. Enabled by the highly efficient peptide coupling reaction, a small focused library of G1 and G2 amphiphilic denpols were quickly generated (Scheme 1). For simplicity, the denpols were named using the dendron generation and the percentages and one-letter codes of the amino acids on the outer layer. For example, G2 75H-25W represents a denpol with multivalent second-generation dendrons having 75 mol% histidine and 25 mol% tryptophan incorporated on the outer layer. Denpol synthesis and characterization details can be found in the Supporting Information (SI).

The siRNA binding capabilities of the denpols were initially assayed by gel electrophoresis, and most of the denpols could completely complex with siRNA at denpol primary amine/siRNA phosphate (N/P) molar ratios of 10−30. The binding strengths were further evaluated by competitive binding assays using an anionic polymer, dextran sulfate (DS) (MW = 25 kDa) as the challenger (Figure 2A−C). For this purpose, polyplexes prepared at N/P = 40 were incubated with different amount of DS to compete with siRNA. A number of trends were observed: (1) The dendron generation affects the strength of binding to siRNA. The G2 denpols bind much more strongly than the G1 denpols (Figure 2A,B) because of their larger number of multivalent binding sites. (2) The composition of the amino acids on the outer layer also has a significant impact on the siRNA binding capability. In our small library, denpols incorporating W

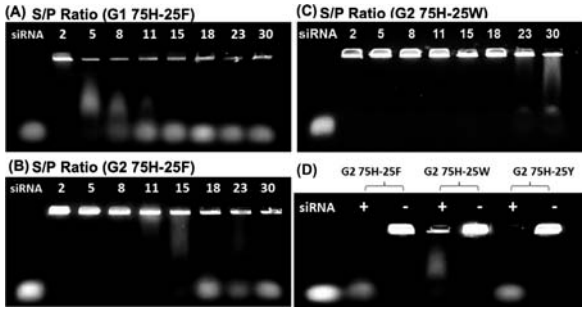


Figure 2. Gel electrophoresis study of denpol/siRNA complexation. (A−C) DS competition with different siRNA/denpol polyplexes prepared at N/P = 40: (A) G1 75H-25F; (B) G2 75H-25F; (C) G2 75H-25W. (D) siRNA release from polyplexes prepared at N/P = 40 upon treatment with GSH (5 mM) at r.t. for 30 min (+, treated; −: control).

showed the strongest binding to siRNA. For example, no appreciable siRNA release could be observed for G2 75H-25W at S/P (molar ratio of DS sulfate to siRNA phosphate) up to 30 (Figure 2C). The indole ring on tryptophan can intercalate into nucleotide base pairs,[21] which could contribute to the increased siRNA binding affinity of W-functionalized denpols. Because of the dicysteine building block in the polymer backbone, the denpols are responsive to reducing agents for release of complexed siRNA. For this assay, different denpol/siRNA polyplexes were treated with glutathione (GSH) at a concentration close to physiological conditions (5 mM).[22] Gel assays showed that complete siRNA release occurred for most of the denpols after GSH treatment; for G2 75H-25W, which has the strongest siRNA binding affinity, the binding strength was significantly reduced (Figure 2D). Presumably, this is a result of reduction-triggered degradation of the denpol, which decreases the multivalency for binding. We envision that such GSH-triggered release of siRNA should be beneficial for intracellular siRNA delivery.

The size of the polyplexes was investigated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The DLS results showed that most of the denpols were able to condense siRNA into particles smaller than 100 nm in diameter and that the polyplexes had moderate positive charge (ζ potential ≈ 15 mV) (Figure S3). Figure 3C shows a representative DLS curve for polyplexes prepared from G2 75H-25W at N/P = 40 (z_{avg} = 80 nm, PDI = 0.317). Direct visualization of the polyplexes by TEM showed spherical NPs with diameters of 20−80 nm (Figures 3A and S5). After GSH treatment, however, no discrete NPs could be observed under TEM (Figure 3B), confirming their responsiveness to the reducing agent as observed by gel assay (Figure 2D).

The efficiency of siRNA transfection was first screened using an engineered NIH 3T3 cell line expressing enhanced green fluorescent protein (GFP). siRNA against GFP was complexed with different denpols at N/P = 20−120 and transfected to 3T3 cells cultured in a 96-well plate for 4 h. After incubation for 48 h, the GFP fluorescence of each well was measured using a plate reader and the cell viability was determined by MTT assay. The GFP fluorescence was then normalized by the percent viability to eliminate toxicity-related GFP reduction. Two benchmark vectors, branched poly(ethylene imine) (PEI) (MW ≈ 25 kDa) and Lipofectamine, were used as positive controls. The screening results for the transfection efficiencies of G2 denpols at the optimal N/P ratios are summarized in Figure S6 (the

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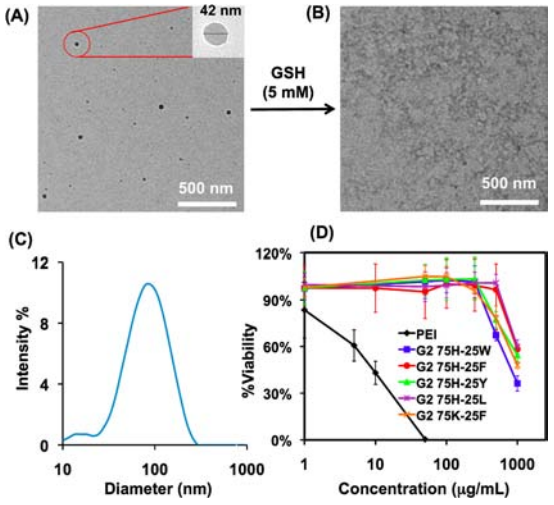

Figure 3. Basic characterizations of the denpol/siRNA polyplexes. (A,B) TEM images of siRNA and G2 75H-25W polyplexes at N/P = 10 (A) before and (B) after GSH treatment. (C) Size distribution measured by DLS for G2 75H-25W/siRNA polyplexes at N/P = 40. (D) MTT assay of selected denpols using the NIH 3T3 cell line.

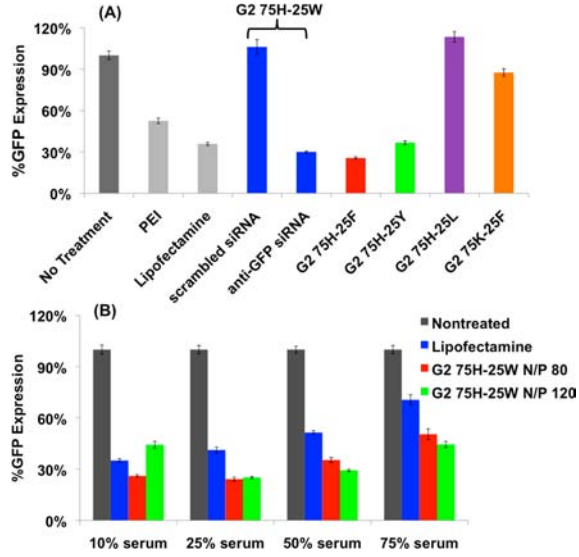

Figure 4. Flow cytometry analysis of transfected NIH 3T3 cells. (A) Transfection summary of selected G2 denpols at their optimal N/P ratios (80 for G2 75H-25W and 75K-25F and 120 for the rest) in serum-free media. (B) Comparison of in vitro transfection efficiencies of Lipofectamine and G2 75H-25W at different serum concentrations.

complete data set is shown in Figures S7 and S8). An important trend can be generalized from the data: *both aromatic groups and histidine are critical for effective silencing*. Denpols carrying both histidine and aromatic amino acids (75H-25F, 88H-12W, 75H-25Y) showed very high transfection efficiency and low cytotoxicity. Without aromatic groups (75H-25L), no transfection was observed, and without histidine, substantial silencing occurred only at a very high percentage of hydrophobic amino acid (25K-75F), which caused significant cytotoxicity.

Based on the initial screening results, several denpols were selected for more detailed investigation. The dose-dependent toxicities were first determined by MTT assay. All of the denpols are 2 orders of magnitude less toxic than PEI (Figure 3D), suggesting that the current denpols are a very safe platform for siRNA delivery. The transfection of selected denpols was then repeated and analyzed by flow cytometery for more accurate measurement. The results agreed well with the initial screening results and confirmed that both histidine and aromatic groups are critical for successful GFP silencing (Figure 4A). Denpols with either K−F (lacking H) or H−L (lacking aromatic residue) combinations did not show any significant gene knockdown. Scrambled siRNA/G2 75H-25W was also transfected and showed a minimal effect on GFP expression, indicating high specificity and a low off-target effect (Figure 4A).

To serve as a successful in vivo delivery system, the polyplexes must be able to protect the siRNA from RNase in the serum and should also have minimum aggregation with negatively charged proteins in serum. To study the serum compatibility of the current system, transfection of denpol/siRNA was carried out in DMEM solution containing 10−75% fetal bovine serum. G2 75H-25W was chosen because it showed the highest siRNA binding affinity and a high transfection efficiency under serum-free conditions. At all serum concentrations tested, this denpol showed a significantly higher transfection efficiency than Lipofectamine (Figure 4B). Even at a serum concentration of 75%, >50% knockdown could still be observed for this denpol.

To gain insights into the role of different functional groups, we conducted an intracellular fluorescence trafficking study using a Cy3-labeled siRNA. Three amphiphilic analogues of G2 denpols were chosen for comparative studies: G2 75H-25F (with both H and an aromatic residue), G2 75K-25F (aromatic residue but no H), and G2 75H-25L (H but no aromatic residue). Various Cy3-labeled polyplexes were exposed to 3T3 cells for 4 h in serum-free media and then placed in normal media with 10% serum. Confocal fluorescence images taken at different time points after the transfection (Figure 5) showed that the aromatic residue (F) is critical for cellular uptake. While no siRNA internalization was observed with the H−L-functionalized denpol, both the H−F and K−F combinations showed very effective cell uptake. On the other hand, the buffering capacity of H is also critical for

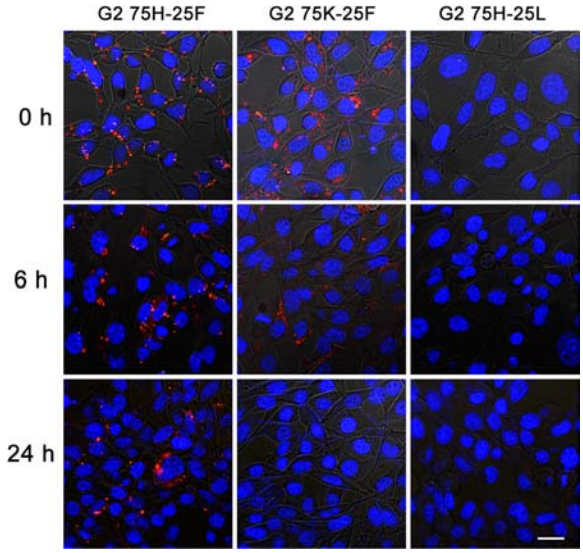

Figure 5. Intracellular fluorescence trafficking of transfected NIH 3T3 cells. Cells were incubated with Cy3-labeled siRNA (red) complexed with different denpols for 4 h. The medium was changed back to fresh DMEM with 10% serum. Fluorescence images were taken at 0, 6, or 24 h after the transfection. Cell nuclei were counter-stained with DAPI (blue). Scale bar: 20 μm.

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successful delivery. In G2 75K-25F transfected cells, the siRNA fluorescence greatly diminished after 6 h, and no siRNA could be observed 24 h after transfection. In contrast, siRNA remained present in G2 75H-25F transfected cells for up to 24 h. The buffering capacity of H could aid in endosomal membrane disruption[14] through either the "proton sponge" mechanism[15] or increased amphiphilicity.[13] Without the pH-responsive groups (G2 75K-25F), endocytosed siRNA would likely be transported to lysosome and undergo enzymatic degradation and fast clearance.

Based on the transfection and fluorescence trafficking results, we hypothesize that the amphiphilicity of aromatic amino acids and the buffering capacity of H work synergistically for effective siRNA delivery in the current denpol system. Amphiphilicity has been shown to be important for cellular membrane interaction in several peptide and polymer vectors.[13] More specifically, Liu and co-workers recently reported that aromatic amino acid (Trp, Phe, Tyr)-functionalized gold NPs had higher cellular uptake than NPs with hydrophobic alkyl amino acids (Val, Leu, Ile).[13c] Our results are similar: denpols with aromatic amino acids (F, W, Y) showed effective cellular uptake and transfection, while no cellular uptake or silencing was observed with the L-functionalized denpol (G2 75H-25L). Presumably, the relatively large aromatic hydrophobic groups enhance cell membrane interactions for the denpol complexes.

Efficient endosomal escape is also critical for successful siRNA delivery, as most vectors are internalized by endocytosis. As noted above, the buffering capacity of H is known to aid endosomal membrane rupture.[14] Therefore, H-containing denpols require only a relatively low percentage of aromatic groups for effective delivery, while denpols without H functionalization need a high percentage of aromatic amino acid incorporation, which also induces cytotoxicity by disrupting the cell membrane. The pH responsiveness ensures the biocompatibility of denpols at neutral pH and increased membrane lysis at acidic pH to facilitate endosomal escape.

In summary, we have introduced a novel denpol architecture for the design of siRNA delivery vectors. We synthesized a focused library of multifunctional amphiphilic dendronized polypeptide for siRNA delivery. MTT assays indicated that the denpols generally have very low cytotoxicity. Through 96-well fluorescence screening, we quickly identified several candidates that can effectively transfect siRNA to NIH 3T3 cells. More quantitative cell flow cytometry studies confirmed the screening data and showed significantly improved transfection efficiency of the best-performing denpol relative to Lipofectamine in serum-containing media. Furthermore, a detailed intracellular fluorescence trafficking study revealed that *the combination of histidine and aromatic residues is critical for successful delivery*: while aromatic residues enhance cell uptake, the buffering capacity of H facilitates endosomal membrane rupture. The high delivery efficiency and low toxicity suggest denpols as a promising platform for siRNA delivery. The basic structure–property information obtained in this study should also be applicable to the design of other types of synthetic vectors for gene delivery.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, additional data, and complete ref 5. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

zguan@uci.edu

Notes

The authors declare no competing financial interest.

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