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Gene Delivery

Reactive and Bioactive Cationic α-Helical Polypeptide Template for Nonviral Gene Delivery**

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Polypeptides were the first set of materials considered for use as nonviral gene delivery vectors. With its ability to bind and condense anionic plasmid DNA, cationic poly-L-lysine (PLL) was one of the most well studied of the early gene delivery polypeptides.[1,2] Unfortunately, as a DNA delivery vector, unmodified PLL suffered from low transfection efficiency. Although there have been tremendous efforts to increase the efficiency of PLL-mediated gene delivery by incorporating various motifs such as saccharide,[3,4] imidazole,[5] and guanidinium[6] groups, the improvement has been limited. As such, enthusiasm for PLL and its modified variants as transfection agents has dwindled. As an alternative, many basic gene delivery studies now utilize a more efficient material such as polyethylenimine (PEI).[7]

As the use of PLL in gene delivery studies declined, the function of peptide-based materials gradually shifted to other roles relevant to transfection. For example, through covalent conjugation to existing vectors, peptides found use as bioactive agents capable of adding functionality such as cell targeting,[8,9] nuclear localization,[10–12] or membrane destabilization[13] to existing gene delivery materials. Membrane destabilization, in particular, has been a large area for peptide use in nonviral gene delivery systems. The cell-penetrating peptides (CPPs) penetratin,[14,15] transportan,[16,17] melittin,[18–20] GALA,[21–23] TAT,[24–26] and oligoarginine[27–30] are some of the commonly used peptide-based materials for membrane destabilization. When incorporated into delivery vectors, these CPPs have been shown to lead to increased internalization, improved endocytic escape, and overall better transfection efficiency.[31] While effective in promoting membrane destabilization as part of a larger vector, CPPs are often too small or lack an adequate cationic charge density to function as stand-alone gene delivery agents.

All cationic polypeptides (PLL, modified PLL, or other polypeptide electrolytes) adopt random coil structures because strong intramolecular charge repulsion between side chains prohibits helix formation.[32,33] However, a shared feature among many CPPs is a helical secondary structure that allows them to interact with and destabilize lipid bilayers such as the cell and endosomal membranes.[34,35] Because of this discrepancy in secondary structure, there has been no report of cationic polypeptides that can function as both a gene delivery vector with comparable or better transfection efficiency than some of the leading nonviral delivery vectors, and a CPP that destabilizes cellular membranes.

We recently reported a strategy for the facile generation of cationic and helical polypeptides.[36] Typically, cationic polypeptides such as PLL are unable to adopt helical conformations at physiological pH because of charge disruption with the side chains.[32,33] However, our findings revealed that the helical structure of cationic polypeptides can be stabilized by increasing the distance between the charged groups of the side chains and the backbone of the polypeptide, thus minimizing the effect of charge repulsion by reducing the charge density on the helix surface (Scheme 1A). Stable helical structures with very high helical content (> 90%) can be achieved by maintaining a minimum separation distance of 11 σ bonds between the peptide backbone and the charged side-chain for a polypeptide having completely charged side chains and a reasonable length (degree of polymerization of 60).[36] By following this general strategy, it is possible to generate polypeptide materials that are sufficiently large and positively charged to bind and condense DNA, but also retain the helical structure seen in many CPPs. The unique combination of material properties allows us to examine helicity as a functional motif in the backbone of gene delivery vectors and evaluate its impact on transfection efficiency.

Herein we report our efforts to develop a library of cationic α-helical polypeptides with CPP-like properties for gene delivery through the well-known ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCAs).[37] The ROP of γ-(4-vinylbenzyl)-L-glutamate *N*-carboxyanhydride (VB-Glu-NCA) was used to form poly(γ-(4-vinylbenzyl)-L-glutamate) (PVBLG; Scheme 1B).[36,38] PVBLG served as a reactive template that, through subsequent ozonolysis and reductive amination, allowed us to create a library of cationic polypeptides (PVBLG$_n$-X, where *n*

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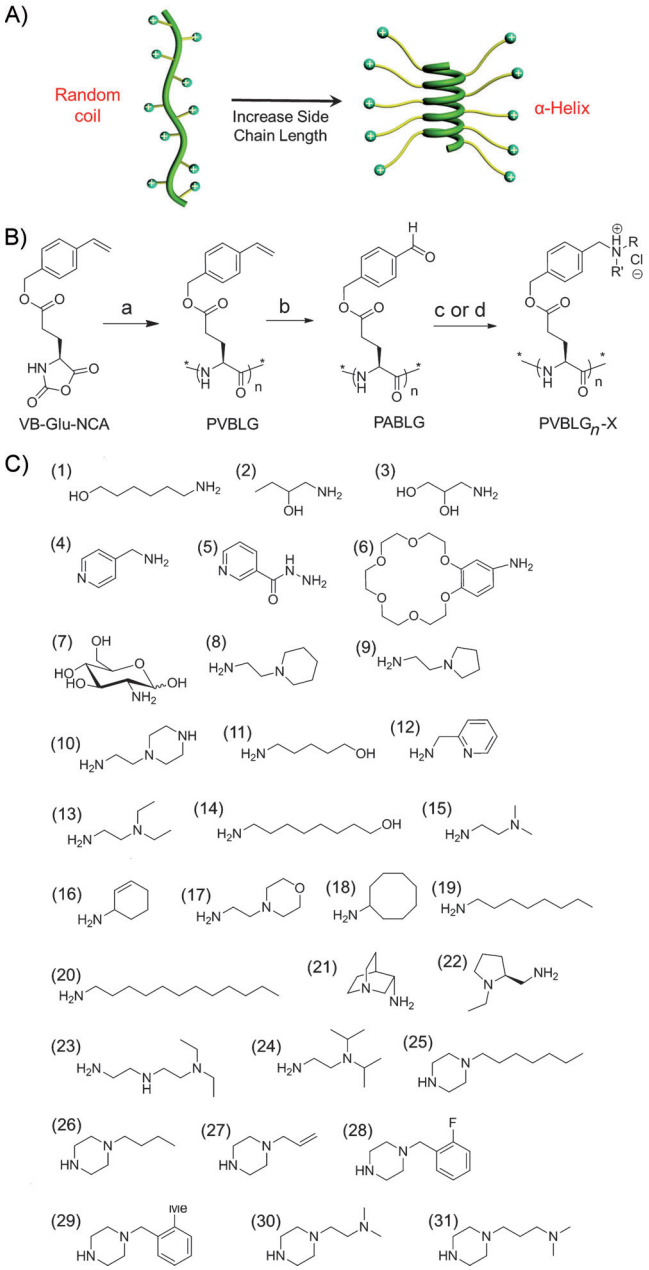

Scheme 1. A) Polypeptide with charged side chains and the random coil to helix transformation in response to elongated side chains. B) Reaction Scheme for the synthesis of PVBLG$_n$-X polypeptides. a) 1. HMDS/TBD/DMF/nitrobenzene; 2. benzyl chloroformate/TBAF/DIPEA, 2 h; b) 1. O_3/$CHCl_3$, −78 °C, 1-5 min; 2. PPh_3, RT, 2 h; c) 1. RNHR′, $NaBH(OAc)_3$, DMF/HOAc, 60 °C, 24 h; 2. HCl; a) 1. RNHR′, DMF/HOAc, 60 °C, 16 h; 2. BH_3 pyridine complex, 8 h; 3. HCl. C) Amine groups used to synthesized PVBLG$_n$-X. DIPEA = diisopropylethylamine, DMF = *N*,*N*′-dimethylformamide, HMDS = hexamethyldisilazide, TBD = 1,5,7-triazabicyclo[4.4.0]dec-5-ene, TBAF = tetra-*n*-butylammonium fluoride.

is the degree of polymerization and X refers to the grafted amine side chain shown in Scheme 1C). As a result of its glutamate residues, PVBLG has a propensity to adopt an α-helical secondary structure.[32,33,39,40] By maintaining a minimum separation distance of 11 σ bonds between the peptide backbone and the charged side chains, the PVBLG$_n$-Xs synthesized for this study have a helical structure which is stable over a broad range of pH values and salt concentrations, and is also stable when mixed with anionic plasmid DNA (see Figure S1 in the Supporting Information).[36] By synthesizing and screening a library of materials, we hoped to identify amine side chains that yielded helical molecules with the appropriate balance of hydrophilicity (i.e., DNA binding strength) and hydrophobicity (i.e., endosomolysis) to mimic the membrane-disruptive capabilities of CPPs yet also mediate efficient gene delivery without the addition of extraneous lytic materials.

The synthetic scheme shown in Scheme 1B was used to generate PVBLG$_n$-X having 31 different amine side chains. The degree of polymerization was varied between 10 and 300 for the top-performing amines. Of the various side chains, 15 showed improved performance relative to the 22 kDa PLL and two (X = 1 and 8) showed improved performance relative to the 25 kDa branched polyethylenimine (PEI) in COS-7 cells (Figure 1A). Generally speaking, transfection efficiency increased with increasing molecular weight of PVBLG$_n$-X. The top-performing material, PVBLG$_{267}$-8, resulted in the highest transfection efficiency—a 12-fold improvement over PEI. The superior performance of PVBLG$_{267}$-8 was confirmed in three additional cell lines (HEK293, MDA-MB-231, and HeLa; see Figure S3a in the Supporting Information). Moreover, in sharp contrast to PEI, PVBLG$_{267}$-8 showed low toxicity in COS-7 cells (Figure 1B). Circular dichroism analysis (CD) confirmed that PVBLG$_{267}$-8 maintained its helical conformation at physiological pH as well as the acidic pH encountered within endosomes and lysosomes (Figure 1C).

Since the PVBLG$_n$-Xs were designed to have an α-helical architecture similar to that found in CPPs, we examined the ability of the polymers to cause pore formation in cell membranes. COS-7 cells were incubated with 250 μM calcein, a fluorescent dye, in the presence of various concentrations of PVBLG$_{267}$-8. Calcein is unable to cross intact membranes. As such, in the absence of an agent capable of pore formation, calcein is taken up by cells in a pinocytic fashion, thus resulting in the appearance of small punctate intracellular fluorescent spots (Figure 2A, 0 μg mL^{-1}). However, as the amount of PVBLG$_{267}$-8 in the extracellular medium is increased, the intracellular fluorescent signal becomes more diffuse, thus indicating membrane permeation and non-endocytic calcein uptake (Figure 2A, 50 μg mL^{-1}). Although PVBLG$_{267}$-8 can function as an effective CPP when present in the medium at 50 μg mL^{-1}, such a high polypeptide concentration does not correspond with the optimum transfection formulation. Thus, we also tested calcein uptake at an intermediate PVBLG$_{267}$-8 concentration (15 μg mL^{-1}), which corresponds to the concentration of PVBLG$_{267}$-8 used in the optimum transfection formulation. As indicated by the punctate fluorescent signals, 15 μg mL^{-1} PVBLG$_{267}$-8 is unable to cause cell membrane pore formation. Thus, it would seem that the complexes formed between PVBLG$_{267}$-8 and plasmid DNA enter cells by endocytosis and not by direct membrane penetration. This finding is supported by flow cytometry data, which shows reduced complex uptake in the presence of an inhibitor of caveolae-mediated endocytosis

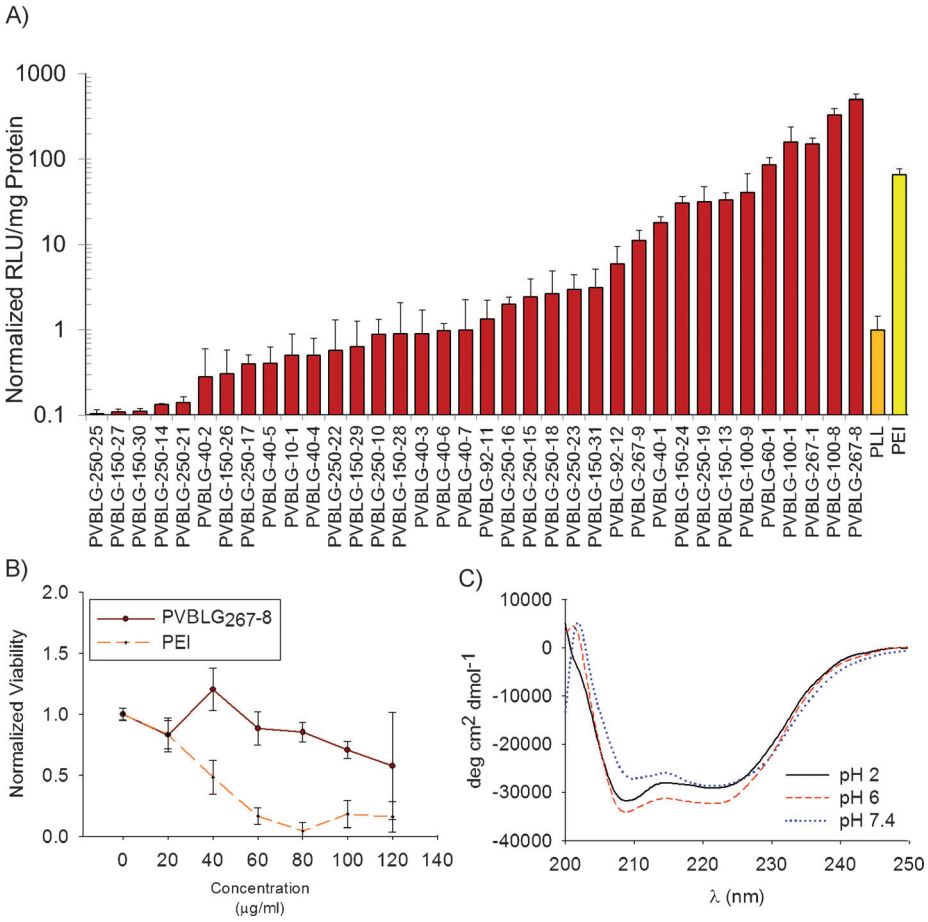

Figure 1. A) In vitro transfection of COS-7 cells with PVBLG$_n$-X polypeptides. 22 kDa PLL and 25 kDa PEI were included as controls. RLU = relative light units. B) Viability of PVBLG$_{267}$-8 and PEI in COS-7 cells. C) CD analysis of PVBLG$_{267}$-8 at pH values of 2, 6, and 7.4.

(see Figure S6a in the Supporting Informtion). Similar results for calcein and complex uptake were observed for analogous experiments conducted in HEK293 cells (Figures S6c and S9).

As PVBLG$_{267}$-8 complexes appear to enter cells by endocytosis and not direct membrane transduction, they must escape endocytic vesicles to mediate transfection. PVBLG$_{267}$-8 possesses secondary and tertiary amines which can act as buffering agents to aid endosomal escape by the proton sponge effect.[41] To investigate if this mechanism contributed to the gene delivery observed with PVBLG$_{267}$-8, we performed transfections in the presence of bafilomycin A1, an ATPase inhibitor that prevents endosome acidification and thus disrupts the proton sponge effect.[42] Figure 2B shows that bafilomycin A1 dramatically reduces the gene delivery efficiency of PEI vectors, which are known proton sponges, but has no negative effect on cells transfected with PVBLG$_{267}$-8 vectors.[43] This observation suggests that PVBLG$_{267}$-8 escapes from endosomes by membrane disruption. To explore this further, we also performed transfection in the presence of nocodazole. Nocodazole depolymerizes microtubules, thus preventing the active transport of endosomes along their normal progression from early endosomes to late endosomes to lysosomes.[44] As a result, endocytosed material accumulates in early endosomes. In agreement with our data indicating that the membrane disruption capabilities of PVBLG$_{267}$-8 increase with increasing polymer concentration (Figure 2A), nocodazole causes a greater than twofold increase in the transfection efficiency of PVBLG$_{267}$-8 vectors in COS-7 and HEK293 cells (Figure 2B, and see Figure S3b in the Supporting Information). Flow cytometry revealed that this increase was not due to increased complex uptake in drug-treated cells (Figure S6b). Rather, the enhanced transfection in the presence of nocodazole is likely due to the accumulation of PVBLG$_{267}$-8 complexes in endocytic vesicles. As more complexes accumulate, the effective polymer concentration becomes large enough to cause enhanced vesicle lysis. Furthermore, confocal microscopy of COS-7 cells treated with nocodazole and transfected with complexes of PVBLG$_{267}$-8 and YOYO-labeled DNA showed fluorescent aggregates in the cell cytosol, thus supporting vesicle accumulation (Figure S11).

Our results suggest that secondary structure can have a dramatic impact on the intracellular performance of polymer-based nonviral gene delivery vehicles. Specifically, the incorporation of helical architecture—a trait shared by many peptides capable of membrane disruption—into our gene delivery vector library yielded polypeptides which possess the ability to disrupt endosomes. Ultimately, this incorporation results in improved transfection performance of the polypeptides relative to random coil polymers like PLL and branched 25 kDa PEI. To directly demonstrate the importance of secondary structure, a random-coil analogue of the top-performing PVBLG$_n$-8 polymer was synthesized using D- and L- VB-Glu-NCA monomers. The racemic configuration of amino acids (1:1 ratio) was confirmed, by circular dichroism, to prevent the formation of secondary structure in the resulting PVB-DL-G$_{150}$-8 polymer (Figure 2C). For comparison, helical PVB-L-G$_{150}$-8 was also synthesized. Both polymers were used to transfect COS-7 cells over a variety of polymer/DNA weight ratios (Figure 2D). Confirming our speculations from cell penetration and drug inhibition data, the random coil PVB-DL-G$_{150}$-8 polypeptide was unable to mediate effective transfection, whereas the helical PVB-L-G$_{150}$-8 was. This result stands as direct evidence that polymer secondary structure can impact its overall performance.

To test the breadth of applicability of the helical polypeptides as gene delivery vehicles, PVBLG$_{267}$-8 was

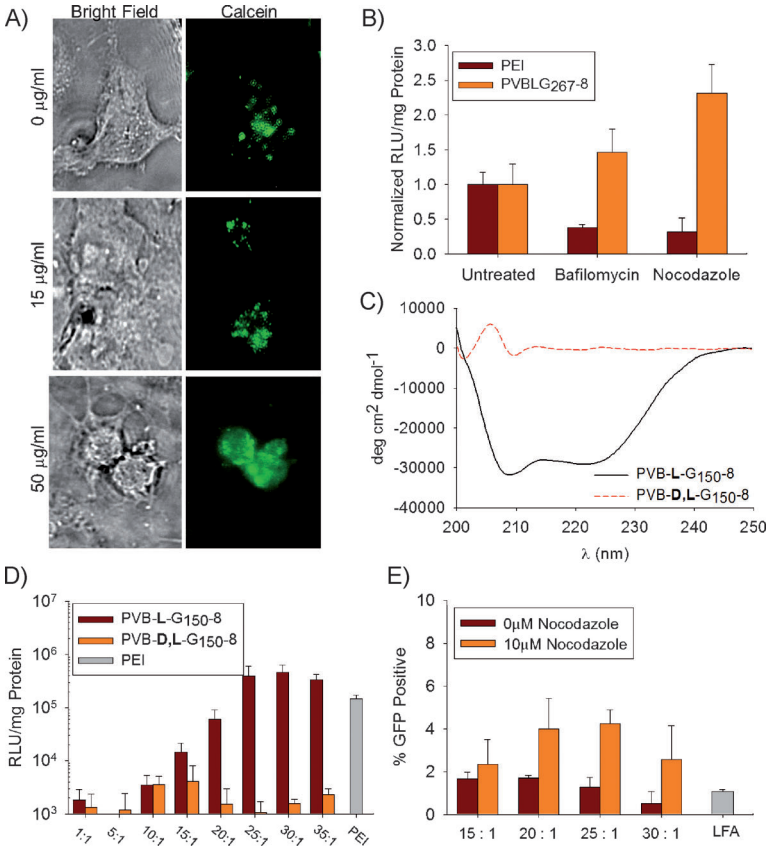

Figure 2. A) Calcein uptake in COS-7 cells treated with various concentrations of $PVBLG_{267}$-8. B) In vitro transfection of COS-7 cells transfected with complexes of 25 kDa PEI or $PVBLG_{267}$-8 in the presence of intracellular processing inhibitors. The final $PVBLG_{267}$-8 concentration was 10 $\mu g\,mL^{-1}$. C) Circular dichroism spectra of helical PVB-L-G_{150}-8 and random coil PVB-D,L-G_{150}-8 in water. d) In vitro transfection of COS-7 cells with PVB-L-G_{150}-8 and PVB-D,L-G_{150}-8 polypeptides. 25 kDa PEI was included as a control. E) In vitro transfection of H9 human embryonic stem cells with $PVBLG_{267}$-8 in the presence and absence of 10 μM nocodozole and the commercial transfection agent lipofectamine 2000 (LFA).

used to transfect the H9 human embryonic stem cell (hESC) line. hESCs are traditionally hard to transfect, with commercial agents often successfully delivering the transgene to less than 10% of the treated cells.[45] To explore if the enhanced membrane disruptive properties of $PVBLG_{267}$-8 aided transfection in hard-to-transfect cells in addition to cells more amenable to gene delivery (i.e., COS-7 and HEK293 cells), H9 hESCs were transfected with a plasmid coding for green fluorescent protein (pEGFP-N1) and assayed for gene expression 48 hours post-transfection by flow cytometry. As nocodozole treatment was observed to aid transfection with $PVBLG_{267}$-8, hESCs were also transfected in the presence and absence of nocodazole (Figure 2E). In addition to $PVBLG_{267}$-8, the commercial transfection agent lipofectamine 2000 (LFA) was also evaluated. Without the addition of nocodazole, $PVBLG_{267}$-8 at a 20:1 $PVBLG_{267}$-8/DNA weight ratio outperforms LFA by 50% and results in approximately 1.5% of all hESCs expressing the transgene. The addition of 10 μM nocodazole to the transfection media increases the percentage of cells successfully transfected with $PVBLG_{267}$-8 to roughly 4.5%. This result is approximately a threefold enhancement over the transfection efficiency of LFA. Microscopy revealed no change in phenotype after either LFA or $PVBLG_{267}$-8 transfection, although the addition of nocodazole did result in cell death (see Figure S10 in the Supporting Information).

The study reported here demonstrates the successful application of a library screening approach to the development of α-helical cationic peptides for gene delivery. To our knowledge, this is the first time a library approach has been combined with a reactive template bearing a well-defined and bioactive secondary structure. Our data indicate that certain library members retain the membrane destabilization properties commonly associated with helical peptides yet can also be used to mediate effective gene delivery in a variety of cell lines, including immortalized cancer cells and hESCs. Vector helicity appears to be an essential component in the successful use of polypeptides for gene delivery. In view of the interesting properties of the reported class of helical cationic polypeptides, current studies are focused on developing high throughput strategies to further expand the library as well as exploring the potential for the material to mediate in vivo gene delivery as well as protein and siRNA delivery.

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