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Article

Tailoring Cationic Helical Polypeptides for Efficient Cytosolic Protein Delivery

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Abstract Protein delivery is of central importance for both diagnostic and therapeutic applications. However, protein delivery faces challenges including poor endosomal escape and thus limited efficiency. Here, we report the facile construction and screening of a small library of cationic helical polypeptides for cytosolic protein delivery. The library is based on a random copolymer poly(γ-{2-[2-(2-methoxyethoxy)ethoxy]ethoxy}esteryl-*L*-glutamate)-randompoly(γ-6-chlorohexyl-*L*-glutamate)[P(EG_3-r-ClC_6)Glu], which is then modified with various pyridine derivatives and alkyl thiols. Flow Cytometry, confocal laser scanning microscopy, and viability assay collaboratively identify two leading polymers, showing efficient delivery of enhanced green fluorescent protein(eGFP) and low cytotoxicity. This finding is further validated by the cytosolic delivery of RNase A and cytochrome C(Cyt C) to HeLa cells in the viability assay. Together, this work demonstrates that high-throughput screening is an effective and viable approach to the selection of cationic helical polypeptides for the cytosolic delivery of functional proteins.

Keywords Cationic helical polypeptide; Protein delivery; Cytosolic protein

1 Introduction

Proteins are intriguing macromolecules with specific biological functions, making them promising candidates for drug discovery[1—5]. While extracellular biomarkers are intensively exploited, more importantly, the pursue of intracellular targets can stimulate new disease treatment options and even change the whole repertoires of protein therapy[6—8]. However, almost all protein therapeutics on market, including those monoclonal antibodies[9] and cytokines[10], act exclusively on extracellular markers, and the use of protein drugs regulating intracellular targets remain elusive in clinic. As such, many strategies for intracellular protein delivery have been developed including fusing expression of protein transduction domains(PTD)[11—14], cationic liposomes[15—17] and polymers[18—30], and receptor mediated endocytosis using bacterial toxins[31,32] or viral components[33]. However, proteins-of-interest(POI) delivered by those vehicles often remain trapped and/or degraded in lysosome. Moreover, some delivery materials have limited solubility in water and/or are too toxic, leading to unsatisfactory therapeutic window. Recently, Cheng and colleagues[34—38] developed several fluorinated amphiphilic, boronic acid-rich, polymers for protein delivery and achieved outstanding cytosolic efficiency. Despite those advances, there are still pressing needs for a robust and general platform accessing highly efficient delivering materials with low toxicity.

The surfaces of proteins are composed of discretely distributed hydrophobic, hydrophilic, cationic, and anionic patches. This unique surface pattern facilitated protein interactions with certain random amphiphilic polymers *via* cooperative weak interactions, such as hydrogen bonding, electrostatic forces, and van der Waals interactions. Harnessing this heterogenic surface feature, Xu *et al.*[39] elegantly achieved the preservation of protein activity under non-native conditions by simply mixing semi-rationally designed statistical random polymers with the POI. Inspired by this, we seek to make protein-delivering materials using a similar design principle. More specifically, we hypothesize that effective delivery carriers can be identified from libraries of random copolymers that simultaneously contain hydrophobic, aromatic, hydrophilic, and charged, preferably cationic in this case, domains.

We choose synthetic polypeptides prepared *via* the ring-opening polymerization(ROP) of amino acid *N*-carboxyanhydrides(NCAs) as the scaffold. Previously, Cheng *et al.*[40—44] developed various cationic helical polypeptides, which afforded extraordinary transfection efficiency for both plasmid genes and siRNAs. However, the ability of delivering protein using such helical polypeptides is yet to be interrogated. Herein, we report the facile and modular construction of a small library of cationic helical polypeptides, from which highly efficient protein carriers are identified. The polymers are found to deliver various proteins including enhanced green fluorescent

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protein(eGFP), RNase A, and cytochrome C(Cyt C) into cytosol of HeLa cells, where the proteins execute their functions effectively.

2 Results and Discussion

We started our library generation by making a precursor random copolymer poly(γ-{2-[2-(2-methoxyethoxy)ethoxy]-ethoxy}esteryl-*L*-glutamate)-random-poly(γ-6-chlorohexyl-*L*-glutamate)[P(EG_3-r-ClC_6)Glu] from the ROP of two glutamate NCA monomers. Briefly, γ-{2-[2-(2-methoxyethoxy)ethoxy]-ethoxy}esteryl-*L*-glutamate-*N*-carboxyanhydride(EG_3-*L*-GluNCA) and γ-(6-chlorohexyl)-*L*-glutamic-*N*-carboxyanhydride (ClC_6-*L*-GluNCA) were polymerized at monomer-to-initiator ratios of 34/1 and 66/1, respectively[Fig.1(A)]. EG_3-*L*-GluNCA was selected here to increase the solubility and biocompatibility of the polymer. Previously, we have shown that *L*-P(EG_3Glu) is an outstanding antifouling polymer providing prolonged circulation half-life for protein conjugates and nanoparticles[45,46]. ClC_6-*L*-GluNCA was selected for several reasons: (1) it had a comparable copolymerization rate to EG_3-*L*-GluNCA to afford a statistical polymer, (2) the long hydrophobic C_6 linker could help maintaining cationic helical polypeptides[47], (3) the terminal alkylchloride could be substituted by nucleophiles to introduce other necessary components, such as cationic charges and aromatic moieties[48]. Analysis of P(EG_3-r-ClC_6)Glu by size exclusion chromatography(SEC) gave a molar mass(M_n) of 26000 and the dispersity($Đ$) was measured to 1.05(Fig.S1, see the Electronic Supplementary Material of this paper). ^{1}H NMR spectrum indicated that the obtained polymer had a EG_3/ClC_6 molar ratio of 34/70, very close to the feeding molar ratio of EG_3-*L*-GluNCA/ ClC_6-*L*-GluNCA(Fig.S2, see the Electronic Supplementary Material of this paper). To derivatize P(EG_3-r-ClC_6)Glu, the polymer was incubated at 80 °C with various pyridine derivatives(P1—P7) and alkyl thiols(T1—T3) in a 96-well plate to generate a small library denoted as P(EG_3-r-P*x*-r-T*y*)Glu, where *x* and *y* were the serial numbers of the pyridine and thiol species used in the study, respectively[Fig.1(B)]. We chose pyridine derivatives rather than alkyl amines because after the S_N1 reaction, the formers could provide not only cations but also aromatic moieties in one-step without potential risk of crosslinking. Alkyl thiols were chosen to fine-tune the hydrophobicity of the polymers.

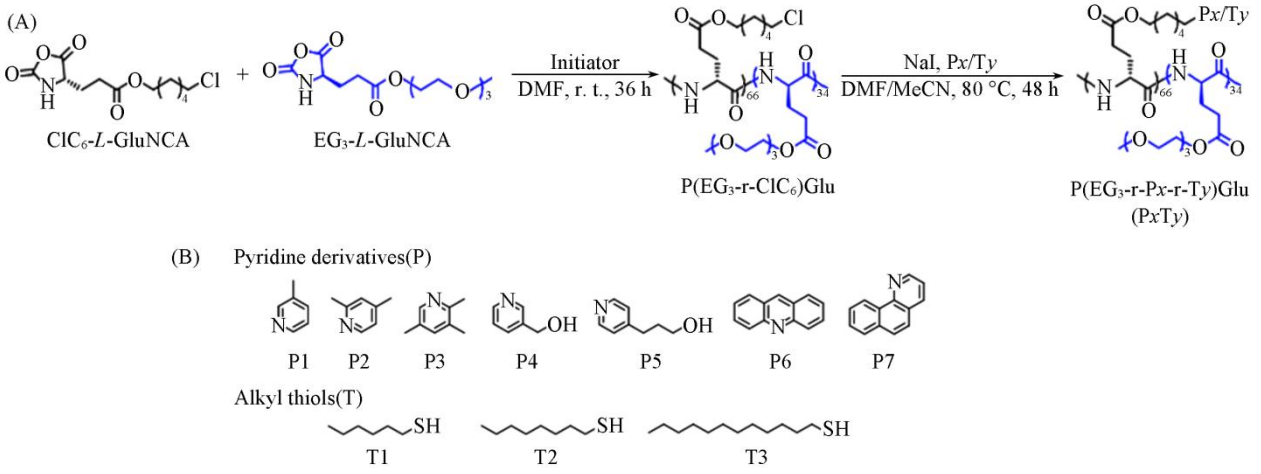

Fig.1 Synthesis of a library of cationic helical polypeptides P(EG_3-r-Px-r-T*y*)Glu(A) and structures of pyridine derivatives(P1—P7) and alkyl thiols(T1—T3) used in the study(B)

To set up the initial high throughput screening assay, we mixed the polymer library with a model protein, enhanced green fluorescent protein(eGFP, pI=5.81), at a fixed molar ratio of 3/1. In parallel, branched polyethylene imine(PEI, M_w=25000), and a classic PTD, TAT peptide(sequence: GRKK-RRQRRRPPQY)[49], were selected as positive controls. The polymer-eGFP complexes were incubated with HeLa cells for 4 h before being analyzed by a flow cytometer equipped with a high throughput sampler(HTS). The delivery efficiency of each polymer was assessed by the mean fluorescence intensity(MFI) in GFP channel. Fig.2 shows the MFI values of all polymers in the format of thermograph, where a darker color represented a higher transfection efficiency. It was found that combinations, such as P2/T1, P3/T1 and P3/T2 gave MFI values relative higher than PEI does. These polymers were therefore selected for next round examination. Of note, TAT showed negligible delivering efficiency even after optimization.

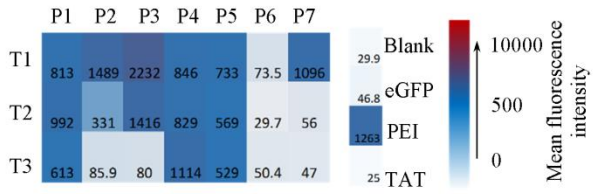

Fig.2 Thermograph of flow cytometry mean fluorescence intensity of cell transfected with cationic helical polypeptides mix with eGFP

Because flow cytometry cannot give us information on subcellular location of the protein, next, we proceeded to validate the four selected polymers from flow cytometry by carefully monitoring the cellular internalization and trafficking using confocal laser scanning microscopy(CLSM). Essentially, a dot-like and punctate pattern of green fluorescence indicates entrapment of the GFP in compartments, such as endosome and

lysosome, whereas a pattern with smear fluorescence signal implies successful delivery of the protein into the cytosol. Both PEI[Fig.3(A)] and P(EG_3-r-P*3*-r-T1)Glu[Fig.S3(A), see the Electronic Supplementary Material of this paper] showed considerable high toxicity seen from the cell morphology observed in CLSM. On the other hand, the polymers based on combination of P2/T1 and P3/T2 were found to show higher transfection efficiency than PEI at 500 nmol/L[polymer/eGFP=1/1 (molar ratio), Fig.3(A)]. Both P(EG_3-r-P2-r-T1)Glu and P(EG_3-r-P3-r-T2)Glu showed considerable smear green fluorescence, indicating eGFPs were partially escaped from endosome[Fig.3(A)]. When the polymer and protein concentrations were both increased to 750 nmol/L, P(EG_3-r-P2-r-T1)Glu and P(EG_3-r-P3-r-T2)Glu showed further improved cytosolic delivery efficiency, with almost fluorescent signals lighted up all over the cells[Fig.3(B)]. However, PEI killed all the cells at the same concentration(data not shown). TAT peptides were found completely ineffective at both 500 and 750 nmol/L(Fig.3).

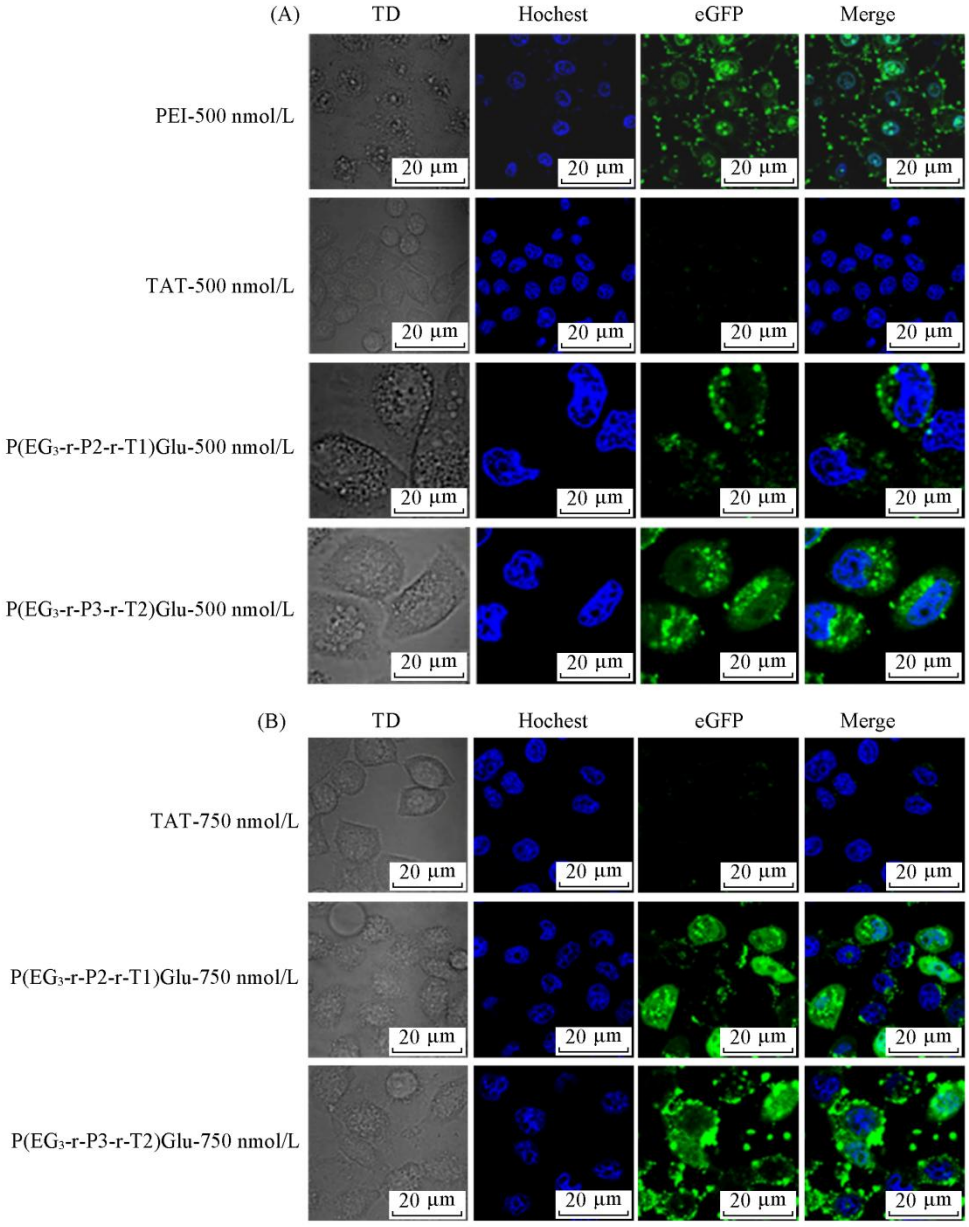

Fig.3 eGFP delivery to HeLa cells by various polymers monitored by CLSM

Cells were incubated with eGFP and polymers at 500(A) or 750(B) nmol/L for 4 h.

Next, the two polymers P(EG_3-r-P2-r-T1)Glu and P(EG_3-r-P3-r-T2)Glu were carefully characterized. ^{1}H NMR spectra confirmed the identity and purity of the polymers [Fig.4(A, B)]. The relative ratios of EG_3/P/T were 34/56/10 for P(EG_3-r-P2-r-T1)Glu and P(EG_3-r-P3-r-T2)Glu according to the integration of selected peaks, respectively. Circular dichroism(CD) spectra indicated that both polymers adopted typical α-helical conformation[Fig.4(C)], similar to many other cationic helical polypeptides reported previously[40]. Viability assay using HeLa cells further confirmed the low cytotoxicity of P(EG_3-r-P2-r-T1)Glu and P(EG_3-r-P3-r-T2)Glu, which gave half inhibition concentrations(IC_{50}'s) of 2.61 and 1.80 μmol/L, respectively[Fig.4(D)]. As such, the two polypeptides were more than 200 fold less toxic than PEI, which showed a IC_{50} of 0.0069 μmol/L. P(EG_3-r-P3-r-T1)Glu was found to show an IC_{50} of 0.033 μmol/L, which was 80 and 60 fold more toxic

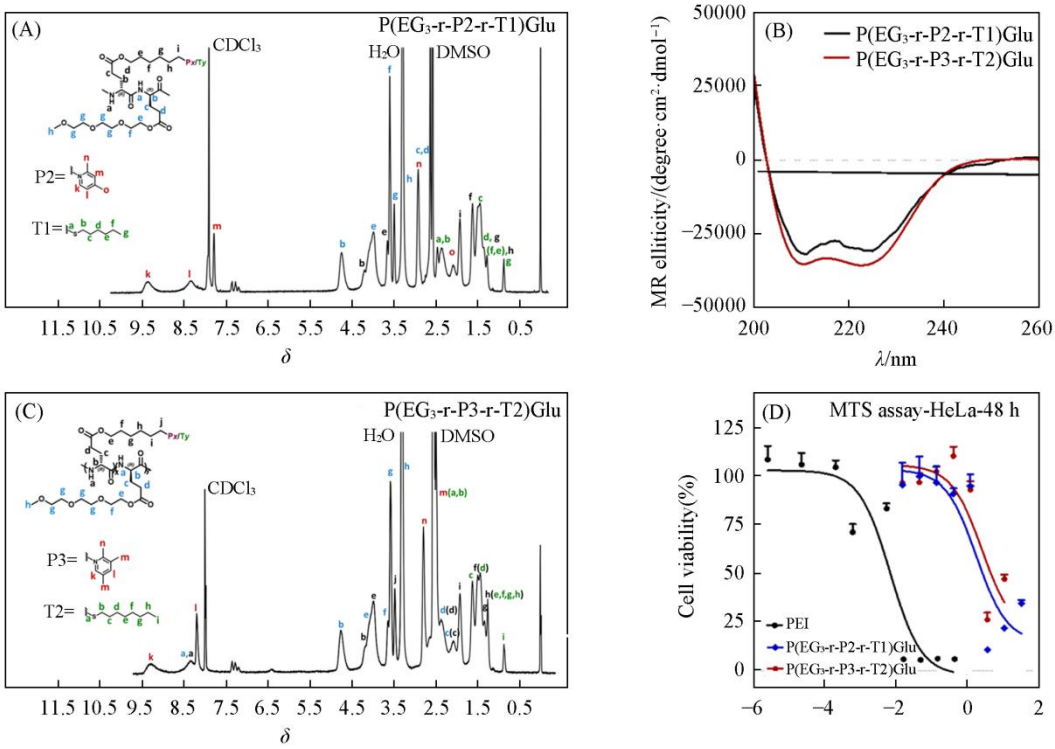

Fig.4 Characterization of P(EG$_3$-r-P2-r-T1)Glu and P(EG$_3$-r-P3-r-T2)

(A) ^{1}H NMR spectrum of P(EG$_3$-r-P2-r-T1)Glu in CDCl$_3$/DMSO-d$_6$; (B) CD spectra of P(EG$_3$-r-P2-r-T1)Glu and P(EG$_3$-r-P3-r-T2) in PBS; (C) ^{1}H NMR spectrum of P(EG$_3$-r-P3-r-T2) in CDCl$_3$/DMSO-d$_6$; (D) cytotoxicity of P(EG$_3$-r-P2-r-T1)Glu and P(EG$_3$-r-P3-r-T2) in HeLa cells.

than P(EG$_3$-r-P2-r-T1)Glu and P(EG$_3$-r-P3-r-T2)Glu[Fig.S3(B), see the Electronic Supplementary Material of this paper]. The results also corroborated previous confocal observation shown in Fig.S3(A).

Next, we moved on to test the generality of two polymers in delivering functional proteins, such as RNase A(pI=9.6) and Cyt C(pI=10.3) to the cytosol of cells. Both RNase A and Cyt C could induce cell apoptosis only if they were delivered with intact function to the cytosol. Thus, the delivery efficiency could be reflected by the viability assay. For this, P(EG$_3$-r-P2-r-T1)Glu or P(EG$_3$-r-P3-r-T2)Glu was mixed with RNase A or Cyt C at varied concentrations below the IC$_{50}$'s of free polymers(polymer/protein=1/1). P(EG$_3$-r-P2-r-T1)Glu or P(EG$_3$-r-P3-r-T2)Glu mixed with eGFP was used as a control group. As shown in Fig.5(A), P(EG$_3$-r-P2-r-T1)Glu mixed with RNase A and Cyt C showed substantially higher toxicity than free P(EG$_3$-r-P2-r-T1)Glu and P(EG$_3$-r-P2-r-T1)Glu mixed with eGFP. The results suggested that P(EG$_3$-r-P2-r-T1)Glu successfully delivered proteins into the cytosol with intact functions. Similarly, P(EG$_3$-r-P3-r-T2)Glu also showed good delivery efficacy for both RNase A and Cyt C[Fig.4(B)]. Notably, TAT failed to deliver neither RNase A nor Cyt C into the cytosol under our experimental conditions(Fig.S4, see the Electronic Supplementary Material of this paper).

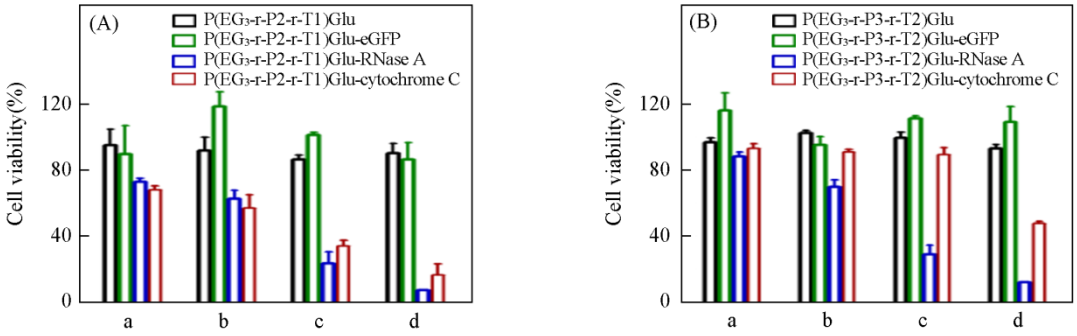

Fig.5 Relative viability of HeLa cells treated with P(EG$_3$-r-P2-r-T1)Glu(A) or P(EG$_3$-r-P3-r-T2)(B) and their complexes with various proteins

Concentration/(μmol·L^{-1}): a. 0.005; b. 0.14; c. 0.41; d. 1.23.

3 Conclusions

In summary, we designed and synthesized libraries of cationic helical polypeptides bearing hydrophobic side chains and positive charged groups in a parallel way. We established a high throughput screening approach for the facile selection of highly efficient carrier for cytosolic. Two leading cationic helical polypeptides, namely P(EG3-r-P2-r-T1)Glu and P(EG3-r-P3-r-T2)Glu, were identified to deliver functional proteins, such as eGFP, RNase A, and Cyt C into cancer cells.

Taken together, this work demonstrates that high-throughput screening is an effective and viable approach to the selection of cationic helical polypeptides for the cytosolic delivery of functional proteins.

Electronic Supplementary Material

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s40242-019-0060-z.

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