Protein Delivery

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Peptide Brush Polymers for Efficient Delivery of a Gene Editing Protein to Stem Cells

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Abstract: *The scarcity of effective means to deliver functional proteins to living cells is a central problem in biotechnology and medicine. Herein, we report the efficient delivery of an active DNA-modifying enzyme to human stem cells through high-density cell penetrating peptide brush polymers. Cre recombinase is mixed with a fluorophore-tagged polymer carrier and then applied directly to induced pluripotent stem cells or HEK293T cells. This results in efficient delivery of Cre protein as measured by activation of a genomically integrated Cre-mediated recombination reporter. We observed that brush polymer formulations utilizing cell penetrating peptides promoted Cre delivery but oligopeptides alone or oligopeptides displayed on nanoparticles did not. Overall, we report the efficient delivery of a genome-modifying enzyme to stem cells that may be generalizable to other, difficult-to-transduce cell types.*

Efficient biomolecule transduction is a persistent problem in chemical biology, biotechnology, and medicine.[1–4] DNA and RNA delivery using synthetic or viral means allows controlled production of specific gene products (RNA and proteins), but these approaches do not allow pre-formation of biomolecular complexes or precise stoichiometric control among gene products. Further, direct protein delivery affords precise control over protein levels, timing, and chemical modifications such as tagging with radioisotopes or bright organic fluorescent molecules.[5] It is expected that off-target effects caused by genome editing enzymes can be limited through temporal control of their activity,[6,7] which highlights the need for efficient protein delivery techniques in the context of cellular therapies such as stem cell or cellular immunotherapies. As a result, methods that mediate efficient protein delivery are needed both in the context of basic research and in therapeutics.[1,8]

Many conventional protein delivery techniques rely on the physical conjugation of the protein of interest to a cell penetrating peptide (CPP).[9,10] However, CPPs are susceptible to proteolytic degradation and protein–CPP conjugates often cannot escape endosomes, which prevents access to crucial targets in the interior of a cell.[11] Alternative approaches involving charged protein fusions necessitate the inclusion of large tags that can affect protein activity.[12] Cationic transfection reagents that do not require covalent tagging of the delivered cargo provide an alternative but can be toxic.[13,14] Liposome-based methods can promote efficient protein delivery but must be individually optimized for the physical properties of each protein load.[15] As such, new protein delivery methods must be developed that do not require physical modification or conjugation of the protein of interest and do not cause any additional toxicity to the cells.

Stem cells pose a particular challenge for biomolecule delivery as they are typically intransigent to most transduction methods but are promising targets for protein delivery because of their utility in research and cellular therapies.[16] As a result, the delivery of genome-modifying proteins to stem cells is of major interest. Success in protein delivery to primary and cancer cell lines has been demonstrated using dimers of cell penetrating peptides[17] and by using polymers with guanidinium-functionalized side chains.[18] We hypothesized that higher-order oligomers of CPPs would enhance the delivery capabilities of these materials and support delivery to stem cells, which has not been reported with high efficiency. Utilizing a polymerization technique that affords uniquely high-density oligomerization of peptides, we set out to determine whether these materials would support delivery of a genome-modifying enzyme to stem cells.

We have previously described a robust method for directly polymerizing peptides into high-density brush polymers through a simple graft-through technique using ring opening metathesis polymerization (ROMP).[19] The resulting peptide brush polymers are well-defined and exhibit narrow disper-

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sities.[19–21] CPPs polymerized in this manner are resistant to proteolytic degradation and maintain their inherent cell penetrating capabilities even in the presence of a protease.[20,21] Moreover, the ROMP technique allows for the incorporation of fluorophore tags at the termini of the polymers[22] and for formulation as higher-valency nanoparticles by synthesis and self-assembly of amphiphillic block copolymers.[21,23] In this paper, we examine how the identity of the peptide, and its configuration, influence the ability of these materials to transduce Cre protein into living cells.

We began by evaluating the ability of various configurations of CPPs to promote Cre protein delivery in HEK293T cells (Figure 1). Specifically, we looked for changes in the uptake proficiencies of Tat-Cre recombinase[24] and observed minimal uptake of Tat-Cre (referred to as Cre protein, hereafter) in the absence of carrier in the cell lines used. We compared Cre delivery by individual peptide strands and different high-density configurations of arrayed peptides, including brush homopolymers and spherical amphiphilic peptide copolymer nanoparticles (See Figure 1 A,B, Supporting Information Figures S1–S4 and Tables S1 and S2 for details on the synthesis and characterization of all materials). First, we examined the viability of HEK293T cells subjected to various brush polymers and observed little impact on the health of the cells with the materials being largely nontoxic at the relevant concentrations (see the Supporting Information for description of cytotoxicity assays, Figure S5). Next, comparisons of protein uptake ability were conducted by monitoring a green fluorescent genomically integrated reporter that operates as a Cre reporter switching from dsRed expression to expression of EGFP in the presence of Cre recombinase in HEK293T cells (Figure 1 C and Figure S6). The Tat peptide was used in this initial study as it has been shown to promote protein delivery as a dimer[17] and is widely known to promote protein uptake through direct fusions,[25] including fusions to cyclic variants.[26] We combined rhodamine-tagged materials with Cre protein in serum-free media and immediately applied these mixtures to HEK293T cells. Evaluation of GFP expression 24 h later by fluorescence microscopy (Figure 1 D) or flow cytometry (Figure 1 E) revealed that only the Tat peptide arranged as a brush polymer promoted significant levels of Cre delivery to HEK293T cells, which correlated well with the relative uptake efficiencies of the three configurations (Figure S7) and the cytosolic distribution of the polymer (Figure S8). As a verification of the reporter system, a reduction in red fluorescence (dsRed expression) was observed after 1–2 weeks of incubation with the polymer and protein (Figure S9). As a result, all subsequent studies used the successful brush polymer configuration.

We next assessed whether other peptide brushes were capable of promoting Cre protein delivery in the HEK293T model system (Figure 2). We observed that Tat peptide-derived polymers promoted the greatest delivery of Cre protein as measured by calculating the fraction of GFP-positive cells. We found that brush polymers of other established cell-penetrating peptides CPP30[27] and Arg8[9,10] also promoted GFP expression in large fractions of the measured populations while uncharged polymers such as those containing a peptide with the sequence GSGSG (GS Poly.) or an oligoethylene glycol (OEG Poly.) unit did not promote protein delivery. The lower degree of Cre delivery exhibited by Cpp44 polymers could reflect the smaller number of arginine residues in the CPP44 amino acid sequence[27] since Arg content has been demonstrated to be a key determinant of cellular uptake of similar peptide polymers.[20] A tumor-penetrating version of an integrin binding peptide (iRGD; sequence: CRGDKGPDC, with a disulfide bond between the two cysteine residues) also did not promote protein delivery relative to the Cre-only control but this may be due to a lack of integrin and/or neurophillin receptors on the HEK293T cells, which are necessary for uptake of this peptide.[28] The necessity of the rhodamine label was also tested using an unlabeled variation of the Tat polymer. The unlabeled polymer was not as efficient at promoting Cre delivery compared to the rhodamine labeled

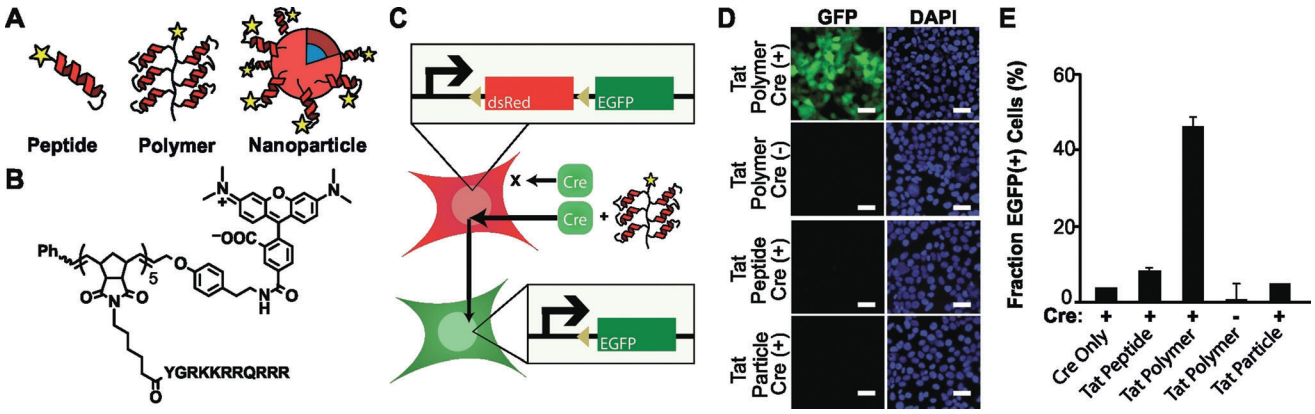

Figure 1. Mono- and polyvalent configurations of Tat peptide (sequence: YGRKKRRQRRR) cause varying degrees of Tat-Cre recombinase protein delivery. A) Configurations of monomeric peptides, brush polymers and nanoparticles with rhodamine label depicted as a yellow star. B) Chemical structure of Tat brush polymer with rhodamine end-label. C) A genomically integrated fluorescence reporter is used to monitor the cytosolic delivery of functional Cre protein to living human cells. D) Fluorescence microscopy images of HEK-293T cells treated with Cre protein and the materials depicted in (A); scale bars = 20 μm. E) Measurement of the EGFP(+) HEK-293T cells treated with Cre protein and rhodamine-labeled materials by flow cytometry as a proxy for Cre protein delivery.

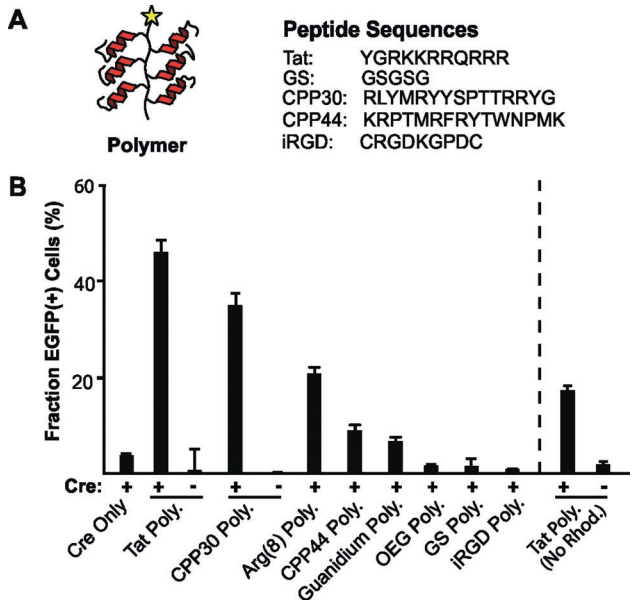

Figure 2. Investigation of the effect of fluorophore and peptide identity on efficacy of polymer brushes as delivery agents. A) Amino acid sequences of peptides associated with various peptide brush polymers end-labeled with rhodamine (yellow star). B) Influence of the identity of the peptide used on Tat-Cre recombinase delivery to HEK293T cells as measured by flow cytometry. Note that the CPP30, CPP44, guanidinium, and iRGD polymers were prepared as block copolymers with an OEG block to ensure solubility in aqueous media (See Supporting Information, Figure S1 and Table S2.) All materials before the dotted line are end-labeled with rhodamine. The Tat polymer data after the dotted line was obtained from a variation of the Tat polymer that does not contain a rhodamine label.

version (less than 50% as effective), suggesting a significant role for the rhodamine fluorophore. We note that previous protein delivery work with a Tat dimer also included a rhodamine label.[17] We also note that that the degree of polymerization of the Tat polymer (DP = 5) cannot be optimized further as it is essentially at the maximum value achieved by this polymerization technique for this particular peptide sequence (Figure S10).

Guanidium moieties have been used extensively elsewhere to promote cell uptake when polymerized,[29] and we observed some degree of protein delivery using this brush polymer configuration although less than that of the Tat brush polymer. We note that the Tew lab has demonstrated that guanidinum containing-norbornyl polymers are more effective at delivering proteins to Jurkat cells when accompanied by hydrophobic moieties,[14,15] and so the guanidinium polymers utilized in our study may not represent optimized structures. Collectively, these results indicate that simple association of the peptide brush with the cell surface or gross physical properties such as a large number of positive charges (i.e., the guanidinium polymers) are not alone sufficient to promote maximal protein delivery. Electron microscopy (EM) studies (Figures S11–13) were conducted to ascertain whether the Cre protein physically associates with the polymers. Dry-state and cryo-EM reveal that the protein and Tat polymer (with or without a rhodamine label) aggregate into micron-scale structures when mixed in OPTI-MEM medium. This suggests that association of the protein and polymer is important for uptake or transport to the cytosol.

Having identified material configurations that elicit efficient protein delivery in HEK293T cells, we next assessed whether these materials could deliver proteins to induced pluripotent stem cells (Figure 3). Stem cells were engineered to carry the same genetically integrated fluorescent reporter used in the HEK293T cells; the assay being production of fluorescence from EGFP in response to Cre activity. A smaller subset of brush polymers was compared in this context, using the Tat brush polymer to illicit uptake of Cre and the GSGSG brush polymer as a control. Consistent with experiments in HEK293T cells, the rhodamine-labeled Tat brush polymer promoted active Cre delivery to the majority of stem cells as measured by flow cytometry (Figure 3A). Furthermore, the rhodamine-labeled GSGSG-labeled polymer brush produced no appreciable delivery of Cre protein. Moreover, the materials did not affect the viability of the stem cells at the concentrations used in this study by both propidium iodide staining (Figure 3B) and Alamar blue reduction (Figure 3C).

In summary, we have described an approach employing high-density peptide brush polymers to deliver functional protein to living cells. Notably, these materials promote

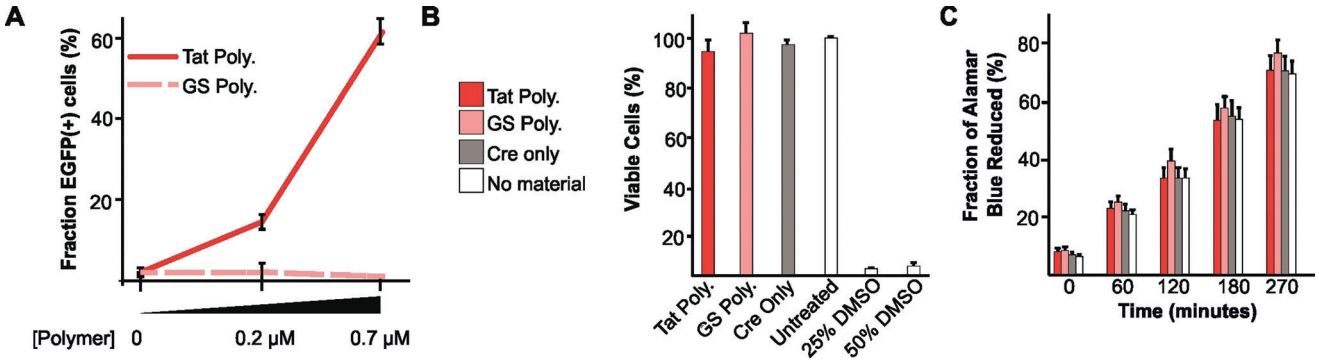

Figure 3. Efficient protein delivery to stem cells mediated by the Tat brush polymer. A) Tat-Cre recombinase protein delivery to induced pluripotent stem cells after treatment with Tat and GSGSG brush polymers as measured by flow cytometry analysis of EGFP(+). B) Viability of stem cells after treatment as measured by propidium iodide staining. C) Proliferation of stem cells after treatment as measured by Alamar blue assay.

efficient delivery of active protein to stem cells, which are typically resistant to protein transduction. The polymers used in this work also exhibit limited toxicity to cells at relevant concentrations and do not need to be physically attached to the protein of interest. Indeed, previous work from our lab has demonstrated that Tat polymers alone likely enter mammalian cells through endocytosis pathways or some other form of membrane disruption[21] and so it is possible that these complexes also traverse the cellular membrane by these mechanisms. Further development will focus on evaluating the mechanisms of uptake of these materials and their ability to deliver other genome-modifying enzymes to stem cells and to other difficult-to-transduce cell types such as neurons and other primary cell lines.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cell penetrating peptides · cellular uptake · enzyme delivery · protein delivery · stem cells

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Communications

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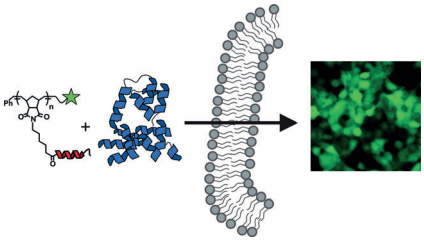
Peptide Brush Polymers for Efficient Delivery of a Gene Editing Protein to Stem Cells



(B)rush delivery: The efficient delivery of an active DNA-modifying enzyme to human stem cells through high-density cell penetrating peptide brush polymers is reported. Brush polymer formulations utilizing cell penetrating peptides could promote delivery of Cre recombinase, whereas oligopeptides alone or oligopeptides displayed on nanoparticles did not.