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Sequence segregation improves non-covalent protein delivery

Federica Sgolastra[a], Coralie M. Backlund[a], E. Ilker Ozay[b], Brittany M. deRonde[a], Lisa M. Minter[b,c], Gregory N. Tew[a,b,c,*]

[a] Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, United States
[b] Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA 01003, United States
[c] Department of Veterinary & Animal Sciences, University of Massachusetts, Amherst, MA 01003, United States

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ABSTRACT

The impermeability of the plasma membrane towards large, hydrophilic biomolecules is a major obstacle in their use and development against intracellular targets. To overcome such limitations, protein transduction domains (PTDs) have been used as protein carriers, however they often require covalent fusion to the protein for efficient delivery. In an effort to develop more efficient and versatile biological vehicles, a series of PTD-inspired polyoxanorbornene-based synthetic mimics with identical chemical compositions but different hydrophobic/hydrophilic segregation were used to investigate the role of sequence segregation on protein binding and uptake into Jurkat T cells and HEK293Ts. This series was composed of a strongly segregated block copolymer, an intermediately segregated gradient copolymer, and a non-segregated homopolymer. Among the series, the block copolymer maximized both protein binding and translocation efficiencies, closely followed by the gradient copolymer, resulting in two protein transporter molecules more efficacious than currently commercially available agents. These two polymers were also used to deliver the biologically active Cre recombinase into a *loxP*-reporter T cell line. Since exogenous Cre must reach the nucleus and retain its activity to induce gene recombination, this *in vitro* experiment better exemplifies the broad applicability of this synthetic system. This study shows that increasing segregation between hydrophobic and cationic moieties in these polymeric mimics improves non-covalent protein delivery, providing crucial design parameters for the creation of more potent biological delivery agents for research and biomedical applications.

1. Introduction

Intracellular delivery of bioactive proteins constitutes a convenient and valuable alternative to DNA and siRNA transfections. The large number of recombinant proteins currently available offer many opportunities to study cellular mechanisms and to discover new therapeutic candidates for intracellular targets [1,2]. The various intracellular compartments, however, are often inaccessible to many exogenous biomolecules. The low permeability of the cell membrane and endosomal entrapment generally limit intracellular availability, thus posing major challenges to their wide-spread use. As a consequence, there has been rising interest in the development of more efficient delivery systems for mammalian cells capable of overcoming such challenges. Among the different strategies employed in the last few decades, the use of protein transduction domains (PTDs) is one of the most promising solutions [3,4].

PTDs are a class of short peptides, mostly cationic, that can traverse the cell membrane. Although their exact mechanism of entry has not been fully elucidated [5], they have been shown to transport a wide range of biomolecules (plasmid DNA, siRNA, proteins, and peptides) into a variety of cell types and *in vivo* models [6–9]. In the majority of cases PTDs, like TAT, require covalent attachment to their cargo for protein delivery [10]. Although covalent conjugation offers stability advantages for *in vivo* applications, it presents several limitations including the risk of altering the biological activity of the cargo [11]. Additionally, they can be time consuming and experimentally restrictive [12] since every new cargo needs to be developed into a fusion protein to be used. To this end, a non-covalent protein delivery strategy is often preferred, in which the cargo is delivered as part of a supramolecular complex with the PTD [13,14]. This non-covalent strategy was first introduced by Heitz, Divita, and coworkers who developed a short primary amphiphilic peptide, Pep-1, able to form stable nanoparticles with proteins [15]. Pep-1 is a chimera obtained by the fusion of a hydrophilic translocation moiety, the nuclear localization sequence (NLS) of the SV40 large T-antigen (KKKRKV), to a tryptophan-rich hydrophobic motif through a short linker [16]. The

* Corresponding author at. Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, United States.
E-mail address: tew@mail.pse.umass.edu (G.N. Tew).

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NLS is required for solubility and intracellular trafficking, while the tryptophan-rich domain promotes both protein complexation and membrane interactions. Although Pep-1 has been used in a variety of cell types and has become a popular commercially available agent for protein delivery (Chariot™ by Active Motif, Inc.), challenges still remain with generally hard to transfect cell types such as primary and suspension cells. Thus, there is still a real need for more effective transporters that work well against the most challenging cell types. Following the discovery that peptide chirality and H-bonding are not critical for delivery [17,18], several research groups have developed synthetic abiotic oligomers to mimic PTD function by incorporating key functional groups onto alternative backbones [19–22]. This provides access to a much larger number of synthetic structures, compared to peptides, and the study of their activity will allow for the creation of new, more effective transporters.

In this context, we previously developed a highly efficient set of synthetic PTD mimics (PTDMs) using the polyoxanorbornene backbone [23] that combines the essential characteristics of Pep-1 and TAT. Similar to the chimeric Pep-1, they contain hydrophobic and cationic domains in a sequential arrangement (block copolymers). The cationic segment is guanidinium-rich, thus mimicking TAT or polyarginine. The hydrophobic region is composed of phenyl-functionalized repeat units, which have proved to impart better cell penetrating properties with respect to other aromatic or aliphatic groups [24,25]. We have exploited these PTDMs for the intracellular delivery of siRNA and proteins [25,26] and have recently investigated the correlation between hydrophobic content and protein delivery efficiency of these PTDMs [25,27]. Now, in this paper, we use constitutional macromolecular isomers [28] to determine the role of hydrophobic/hydrophilic segregation in non-covalent protein delivery.

We first compared PTDMs of identical chemical compositions but different degrees of hydrophobic/hydrophilic segregation (Fig. 1b) based on their ability to interact with and deliver enhanced green fluorescent protein (EGFP) into Jurkat T cells, a non-trivial suspension human leukemic T cell line. PTDM-mediated cellular protein delivery efficiency was also compared with popular commercially available agents. Additionally, HEK293T cells were chosen as a representative adherent cell type, in contrast to the suspension Jurkat T cells. Bovine serum albumin (BSA), avidin, and a recombinant neutral form of streptavidin were also tested to evaluate the influence of protein isoelectric point (pI). Furthermore, the ability of our PTDMs to deliver biologically active proteins was demonstrated with the enzyme Cre recombinase, thus confirming the potential of these novel molecules as protein transporters for biological applications. The diversity of cell lines and cargoes used highlights the versatility of this approach. Exploring the ability of PTDM's to non-covalently bind and deliver protein into cells highlighted sequence segregation as an important structural design parameter.

2. Materials and methods

2.1. Materials

EGFP was purchased from *BioVision*. Chariot™, Xfect™, and SAINT-PhD™ were purchased from *Active Motif*, *Clontech*, and *Synvolux Therapeutics*, respectively. Cre recombinase (HNC form) was received from *Excellgen*. Cre/LoxP reporter human T cell line (ABP-RP-CGFPLoxT) was obtained from *Allele Biotech*.

2.2. PTDM synthesis

The synthesis of each monomer and corresponding PTDM was reported by us [28]. Briefly, the monomers were obtained by ring-opening a Diels-Alder anhydride adduct with a desired alcohol to obtain a mono-functional intermediate, followed by EDC coupling with another equivalent of alcohol, the same type or different, to introduce the second functionality. The desired polymers were subsequently obtained by ring-opening metathesis polymerization (ROMP) using the Grubbs' third generation catalyst [29] in dichloromethane (CH_2Cl_2), with polydispersity indices under 1.1 ($Đ = M_w/M_n$). The final products were purified by dialysis against RO water and recovered by lyophilization. Further characterization of these polymers, including the reaction kinetics elucidating the random character of PTDM **2** is available in our previous report [28].

2.3. EGFP fluorescence titration

In a 96-well plate, increasing concentrations of PTDM were added to 200 nM of EGFP in 200 μL PBS at pH 7.2 and left undisturbed for 30 min at room temperature in order for the components to bind and reach equilibrium. Samples with buffer only or EGFP only were included for background measurements. Changes in fluorescence were measured at 25 °C using a fluorescence plate reader (Biotek SynergyMx) at 507 nm following excitation at 488 nm. Binding parameter values (K_d and ΔG) were obtained by fitting the titration curves to the following equations: [30,31]

$$F = F_0 - (F_0 - F_s)\frac{(P + c + K_d) - \sqrt{(P + c + K_d)^2 - 4Pc}}{2P} \quad (1)$$

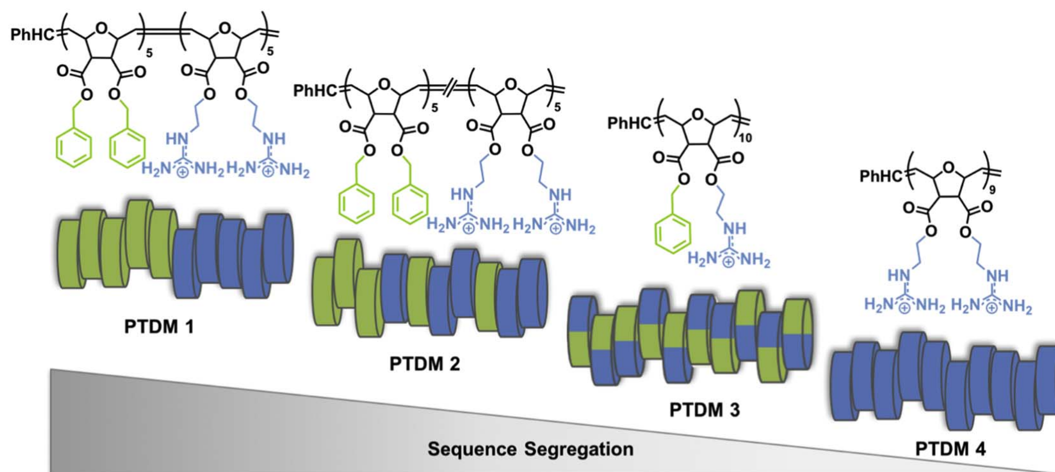

Fig. 1. PTDMs series used in this study. a) PTDM **1** chemical structure; b) cartoon of PTDM sequence variation analyzed in this study. Hydrophobic (phenyl-containing) units are represented in green and cationic (guanidinium-containing) groups are represented in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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