Peptide Mimics

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Self-Activation in De Novo Designed Mimics of Cell-Penetrating Peptides**

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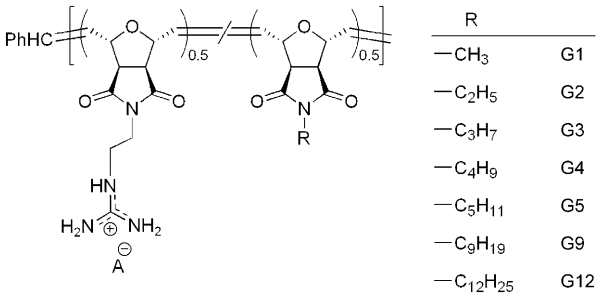
The unique ability of cell-penetrating peptides (CPPs), also known as protein transduction domains, to navigate across the nonpolar biological membrane has been under intense investigation.[1] In vitro studies have shown that multiple mechanisms are available, with the precise details being dependent on the peptide and cell line studied. The several clearly demonstrated pathways include various forms of endocytosis,[2] macropinocytosis,[2e] lipid-raft-dependent macropinocytosis,[3] and protein-dependent translocation.[4] In addition, an energy-independent pathway, or spontaneous translocation, has also been illustrated.[1i,5]

Perhaps the clearest example of an energy-independent pathway is the ability of CPPs, and their synthetic mimics, to cross model phospholipid bilayer vesicle membranes.[1g,h,6] General consensus in the literature suggests that hydrophobic counterions play an essential role in this transduction by complexation around the guanidinium-rich backbone, thus 'coating' the highly cationic structure with lipophilic moieties. For example, an octamer of arginine in the presence of sodium laurate partitioned into octanol versus water with better than 95% efficiency.[7] Separately, it was shown that the simple peptide nonaarginine ($(Arg)_9$) does not in fact transverse membranes very effectively on its own.[1h] However, the presence of hydrophobic counterions "activates" this molecule, thus turning it into a potent transduction peptide. It was shown that *n*-alkyl chain surfactants were good "activators" and thus efficient at promoting the transport of oligo- and polyarginines across biological membranes.[1g,i,8]

After the initial discovery that CPP-like behavior could be emulated in simple norbornene-based polymers,[6b,9] we wondered if the presence of covalently attached hydrophobic residues would increase their translocation activity. To evaluate this hypothesis, we designed and synthesized a series of norbornene-based guanidine-rich polymers, where the hydrophobic groups were introduced through a side chain rather than as counterions (Scheme 1). Remarkably, the guanidine polymers containing certain alkyl side chains exhibited significantly enhanced activity (by three orders of magnitude) without the need for any "counterion activator".



Scheme 1. Guanidino copolymers G1–G12.

Monomers were prepared by either Mitsunobu coupling or nucleophilic substitution reactions (see the Supporting Information). Random copolymers G1–G12 with 50:50 mol% monomer distribution were targeted at two molecular weights (M_n) using ring-opening metathesis polymerization (ROMP; low M_n 2.9–3.9 kDa and high M_n 11.4–13.6 kDa of the *tert*-butyloxycarbonyl (Boc)-protected polymers were obtained). Gel-permeation chromatography gave monomodal signals and narrow molecular-weight indices (1.05–1.15). The Boc-protected polymers were deprotected to obtain G1–G12, and their activities were studied in vesicle assays.

Using the standard biophysical assay well-accepted in the CPP literature, the transport activities of G1–G12 were determined.[6b] Specifically, 5(6)-carboxyfluorescein (CF) was used as a fluorescent probe in egg yolk phosphatidylcholine large unilamellar vesicles (EYPC-LUVs). The activity of G1–G12 transporters increased with increasing polymer content at a constant vesicle concentration as detected by CF emission intensity, yielding plots of fluorescence intensity versus polymer concentration for the series G1–G12 (Supporting Information, Figure S1). Fitting the Hill equation ($Y \propto (c/EC_{50})^n$) to this data for each individual polymer revealed a nonlinear dependence of the fractional fluorescence intensity Y on the polymer concentration c. This analysis gave Y_{max} (maximal CF release relative to complete release by Triton X-100), EC_{50} (effective polymer concentration needed to reach $Y_{max}/2$), and the Hill coefficient n (Supporting Information, Figure S2, Tables S1 and S2). For direct comparison, it is worth mentioning that the CPPs heptaarginine and polyarginine were inactive under these conditions; it is known that polyarginine needs counterions for activation.[1g,h,8]

Figure 1 collects the EC_{50} values for this series of copolymers. Polymers with lower EC_{50} values are said to be

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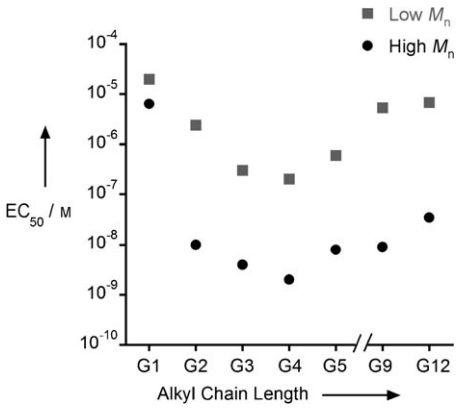


Figure 1. Effective concentrations (EC_{50}) of low-molecular-weight and high-molecular-weight copolymers G1–G12 as a function of alkyl side-chain length.

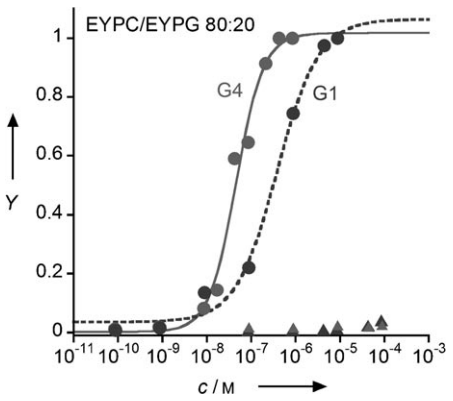


Figure 2. Hill plot for copolymers G1 and G4 in EYPC/EYPG⊃CF vesicles with fits to the Hill equation (circles). G1 $EC_{50} = 0.4$ μM, G4 $EC_{50} = 0.04$ μM. G1 and G4 remained inactive against EYPC/EYPG⊃ calcein vesicles (triangles).

"more active", because the concentration needed to reach 50% activity is lower. In Figure 1, EC_{50} values are plotted against the alkyl chain length of the copolymers G1–G12 with low and high molecular weights. Two trends are evident from this data. First, within both molecular-weight series, a comparison of the EC_{50} values shows that the activity of the copolymers increases as the length of the hydrophobic side chain is increased up to butyl (G4). For longer side chains, the activity decreases.

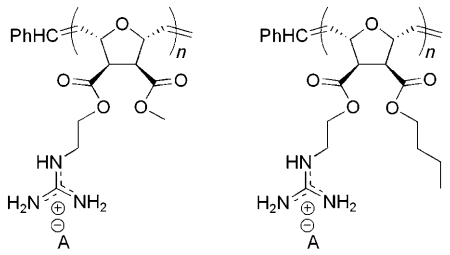
Although it is not entirely clear why the more hydrophobic side chains are less active, it is likely that aggregation of these relatively nonpolar polymers plays some role, as G9 and G12 are significantly less soluble than G1–G5. This hypothesis is also supported by the Y_{max} values for G9 and G12, which are significantly smaller than those for copolymers G1–G5 (Supporting Information, Tables S1 and S2).

The second trend is that higher-molecular-weight samples are more active across the entire series, in agreement with the previously observed "polymer effect".[6b] For example, G1 has an $EC_{50} = (20.0 \pm 0.9)$ μM and (6.4 ± 0.2) μM for the low- and high-molecular-weight samples, respectively. Similarly, G4 has EC_{50} values of (0.20 ± 0.06) μM and (0.0030 ± 0.0005) μM. In all cases, the Hill coefficient generally ranged between $n = 1$ and $n = 3$, implying poor cooperativity, which supports transduction[6b] and no requirement for multichain structures being involved in the transport activity. These results strongly support the proposed hypothesis that the presence of hydrophobic side chains can be used for "self-activation". At the same time, this strong support assumes the mechanism of action is transduction and not some type of general pore formation. To investigate this aspect further, G1 and G4 were evaluated against EYPC/EYPG (EYPG = egg yolk phosphatidylglycerol) vesicles containing either CF or calcein. Calcein-loaded vesicles are routinely used to demonstrate pore formation induced by antimicrobial peptides and their synthetic mimics.[10]

Figure 2 shows that both G1 and G4 induced nonlinear increases in the fractional fluorescence from EYPC/EYPG⊃CF vesicles as a function of concentration in a manner similar to that discussed previously. However, and in sharp contrast, when EYPC/EYPG⊃calcein vesicles were used, no fluorescence increase was observed (see triangles in Figure 2). These experiments strongly support the hypothesis that these new polymers exhibit transduction activity and that they are "self-activated" by the presence of the alkyl substituent.

Even further support for transduction comes from reports of similar studies conducted on CPPs. These studies similarly investigated calcein release for classical CPPs, including R8,[11] R9,[12] and TAT[11,12] in various lipid systems. At very low peptide-to-lipid (P/L) ratios of 0.05, R9 exhibited 7% leakage from EYPC⊃calcein vesicles and was inactive against EYPC/EYPG⊃calcein vesicles.[12] TAT_{48-60} showed 15 and 2% leakage from EYPC⊃calcein and EYPC/EYPG⊃calcein vesicles, respectively.[12] Various P/L ratios were not reported. Similarly, the ability of R8 and TAT_{48-61} to induce leakage of DMPC/DMPG⊃calcein (DMPC = dimyristoyl phosphatidylcholine; DMPG = dimyristoyl phosphatidylglycerol) vesicles was examined as a function of the P/L ratio.[11] Consistent with the previous findings,[12] at low P/L ratios, little to no leakage was observed; however, at P/L = 1.2, greater than 10% leakage was observed (R8 ca. 18% and TAT_{48-61} ca. 10%).[11] As shown in Figure 2, G1 and G4 induced no change in calcein emission, despite very high P/L (here: polymer-to-lipid) ratios above 20 (Supporting Information, Figure S5). These experiments clearly demonstrate that the novel polymers reported herein are able to induce increases in CF emission but not in calcein emission (even at very high P/L ratios), completely consistent with the numerous reports on CPP⊃CF transduction.

To further explore the molecular design of these CPP-like polymers, we designed and synthesized another series of polymers (G1′ and G4′, Scheme 2). Unlike the statistically random copolymers G1–G12, these new homopolymers contain a precise sequence of guanidinium and alkyl side chains on every repeat unit. The monomers for G1′ and G4′ were synthesized in three steps and polymerized by ROMP (Supporting Information, Scheme S6). G4′, with an EC_{50} value of (0.0010 ± 0.0004) μM, exhibited three orders of magnitude better activity than G1′ ($EC_{50} = (1.3 \pm 0.2)$ μM), which is similar to the trend observed for G4 ($EC_{50} =$



Scheme 2. Guanidino homopolymers G1′ and G4′.

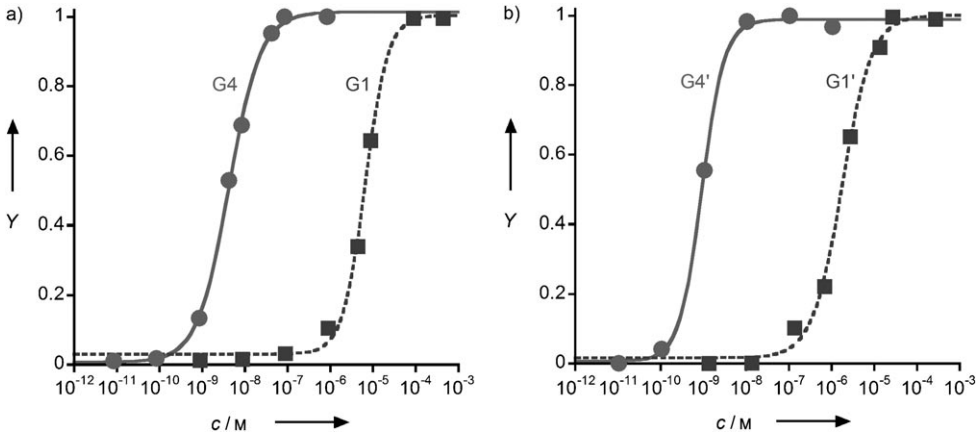


Figure 3. Hill plot of a) copolymers G1, G4 and b) homopolymers G1′, G4′ in EYPC⊃CF vesicles with fits to the Hill equation.

(0.0030 ± 0.0005) μM) and G1 ($EC_{50} = (6.4 \pm 0.2)$ μM; all shown in Figure 3). These data confirm the findings of "self-activation" reported in Figure 1 and Figure 2. Moreover, the precise sequence of these homopolymers coupled with the similar behavior of the copolymers implies that the exact molecular sequence along the backbone is not critical for transport activity as measured by these assays.

We have established that "self-activation" is possible for these polymeric mimics of CPPs, or protein transduction domain mimics (PTDMs). In addition, the butyl side chain appears to optimize transport activity, with the maximum degree of self-activation being achieved for copolymer G4 and homopolymers G4′. Upon increasing or decreasing the length of the side chain, transport activity decreased. The closest arginine-rich CPP analogues of low- and high-molecular-weight PTDMs, R9 and polyarginine (pR), remained inactive across the concentration range in which G1 and G4 exhibited concentration-dependent activity (Supporting Information, Figure S6). In addition, the hydrophobic counteranion pyrenebutyrate (PB) efficiently "activated" G1 with a low EC_{50} (3 μM) compared to pR–PB activation ($EC_{50} =$ 40 μM; Supporting Information, Figure S7a). 'Inactivation' was also observed at a fixed concentration of G1 (at 15 μM, $Y = 0.8$) with the commonly used hydrophilic counteranion Cascade Blue pyruvate with an IC_{50} of 12.5 μM (Supporting Information, Figure S7b). The fact that this PTDM demonstrated activation and inactivation properties similar to classical CPPs is further evidence that these novel polymers can replicate the essential biochemical features of CPPs.

This study reports new cost-effective, highly efficient molecular transporters. Biophysical studies clearly demonstrate CPP-transport-like activity as opposed to general pore formation for these polymeric PTDMs. There is at least a three-fold activity enhancement upon moving from methyl to butyl side chains. Furthermore, we observed that the location of the alkyl substituents along the polymer backbone did not influence transport activity, as both designs (random copolymer or homopolymer) yielded similar EC_{50} values. This study also shows that the complexation between the guanidinium and anionic functionality of the "activators" does not play a central role, as in the studies presented herein we did not "neutralize" the guanidinium cationic charge but simply added "lipophilicity" to the structures. We continue to design new synthetic polyguanidines to expand the structure–activity relationships. At the same time, it will be critical to see how these synthetic mimics compare to natural peptide sequences in cellular uptake studies for the improved delivery of cargo into cells.

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