Protein transduction domain mimics (PTDMs) enable cellular uptake of macromolecular cargo such as proteins and nucleic acids. The presence of hydrophobic domains in PTDMs has been shown to enhance cargo uptake, but the role of hydrophobicity in PTDM-binding of the desired cargo is not fully understood. Herein, block copolymer PTDMs composed of varying hydrophobic monomers were synthesized via ring-opening metathesis polymerization (ROMP) to probe the effect that increasingly hydrophobic side chains had on binding enhanced green fluorescent protein (EGFP). PTDM-facilitated cellular uptake of EGFP into Jurkat T cells was performed to assess the correlation between binding, hydrophobicity, and delivery. Binding studies demonstrated that all PTDMs bound EGFP similarly despite a five log difference in monomer $K_{\text{sw}}$ (octanol-water partition coefficient) and that immediately hydrophobic PTDMs facilitated higher cellular uptake of EGFP. Taken as a whole, hydrophobicity of the PTDM is a better predictor of effective delivery in Jurkat T cells than cargo binding.

Cell penetrating peptides (CPPs) like HIV-1 TAT, along with their associated protein transduction domains (PTDs), have been proven to facilitate the transport of biologically relevant, macromolecular cargoes across the cellular membrane. Using the membrane translocation capabilities of CPPs and PTDs, researchers can access intracellular targets and explore new biochemical pathways, potentially enabling innovative therapeutics. Mimics of PTDs (PTDMs) and CPPs (CPPMs) represent the most recent development in the field. Their synthetic nature engenders de novo design and the synthesis of optimized and efficacious delivery systems. Through custom-made synthetic mimics, precise polymeric architectures, like block copolymers, can be obtained and used to build broader structure-activity relationships. Hydrophobic components, in conjunction with standard guanidinium-rich domains, have been shown to impart enhanced performance in terms of cellular uptake and delivery for both CPPs and their mimics. CPPMs and PTDMs gain an edge over their natural counterparts because the hydrophobic domain can be systematically optimized thereby maximizing performance. The Tew group has previously shown that precise optimization of the hydrophobic domain led to enhancements in siRNA delivery to a non-trivial target, Jurkat T cells, while the presence of a hydrophobic block boosted EGFP delivery to HeLa cells. Varying the segregation of the hydrophobic domain has also been shown to impact EGFP binding and delivery. Since Tew and coworkers have demonstrated that the likely and prevalent mode of internalization for these ROMP-based PTDMs is endosomal uptake and given that hydrophobicity initiated fusion into the endosomal membrane is a method of escape, further modification of PTDM hydrophobicity could be a mechanistically advantageous way to improve delivery. Likewise, hydrophobicity can aid in membrane adsorption and subsequent endocytosis thereby justifying its careful optimization.

In general, additional hydrophobicity has proven important to the performance of synthetic transfection materials especially low molecular weight polyethylenimine (PEI) which is preferred due to its diminished cytotoxicity; however, careful modulation of hydrophobicity in PTDMs, as it correlates to protein binding and delivery, has not been thoroughly explored. In this vein, the general relationship between hydrophobicity-based cargo binding and subsequent intracellular delivery has not been fully elucidated in the field of CPP-mediated delivery, although cargo binding and delivery have been studied together. In the field of gene transfection, DNA cargo binding and subsequent delivery using hydrophobically-modified low molecular weight PEI was studied. It was shown that modifying PEI with varying amounts of hydrophobicity had no effect on the ability of hydrophobically-enhanced PEI to bind DNA. They observed a reduction in binding when too many of the primary, cationic amines were eliminated by the conjugation chemistry used to attach the hydrophobic groups. While this study was concerned with whether or not modifying PEI would affect its cargo binding, it did not specifically draw conclusions about the relationship between hydrophobicity, binding, and delivery. Given that protein delivery deals with a more heterogeneous cargo and that proteins are susceptible to hydrophobicity driven inter-
facial interactions, it is more critical to resolve the question about how hydrophobicity affects PTDM-protein binding in a non-covalent protein delivery strategy.

Herein, a series of variably hydrophobic PTDMs accessed through controlled polymerization were used to probe how hydrophobicity modulates cargo binding and how both parameters affect EGFP delivery in Jurkat T cells. Being able to access intracellular targets in vital human immune cells, such as T cells, will aid researchers studying new biochemical pathways and immunotherapies.

To probe the effect of varied PTDM hydrophobicity on EGFP binding and delivery, a series of oxanorbornene derived monomers were synthesized using previously described methods. The monomers featured one or two hydrophobic aromatic R groups with variable numbers of methyl substituents permitting control over hydrophobicity (Scheme 1). The monomers were polymerized via ROMP to form block copolymers whose theoretical degree of polymerization is shown in Scheme 1. The architecture and block ratio were chosen due to their previous use in EGFP delivery. Ten equivalents of each hydrophobic monomer from 1–7 were first homopolymerized using one equivalent of Grubb’s 3rd generation catalyst and then chain extended with five equivalents of the diguanidine monomer, shown in its Boc-protected form in Scheme 1. All polymers were deprotected with trifluoroacetic acid (TFA) to form P1–P7 which display cationic, guanidinium side chains in their hydrophilic block.

The monomers were designed to achieve incrementally different degrees of hydrophobicity which were then confirmed through experimental and theoretical measurements. Figure 1 details the hydrophobic characterization of monomers 1–7 where monomers are arranged by number from left to right in order of their increasing hydrophobicity.

The monomer shown in red was proposed to be the most hydrophobic but could not be isolated due to the formation of the retro Diels-Alder product during the synthesis (Figure S6). The HPLC retention time (RT) on a hydrophobic C8 stationary phase as well as the theoretical Log Kow value is listed below each monomer where greater values indicate increased hydrophobicity. All of the synthesized monomers are shown, both experimentally and theoretically, to have verifiably different levels of hydrophobicity. The theoretical and experimental measurements correlate linearly when plotted against each other, indicating their corroborative nature.

Since monomers were shown to be hydrophobically distinct, it was presumed that the incorporation of 1–7 into their respective blocks with equal degrees of polymerization would result in a series of polymers that also exhibited correspondingly different levels of hydrophobicity. The full characterization data for the Boc-protected polymers (P1’–P7’) can be found in the supporting information. All polymers were determined to have desirable molecular weight distributions (dispersity (Ð) < 1.1) and targeted block compositions by GPC and 1H NMR. It was critical to determine that the polymers had similar compositions and sizes so that the assumption about distinct degrees of hydrophobicity translating from monomer to polymer could be made. The characterization data confirmed that the targeted structures were achieved rendering seven polymers that could be appropriately compared.

The binding of polymers P1–P7 with EGFP was assessed with a fluorescence quenching assay. EGFP’s fluorescent properties made it an excellent choice for both a self reporting
cargo and a signaler for the formation of a PTDM-EGFP complex.[28] When the PTDMs interacted with EGFP, the inherent protein's fluorescence was quenched as a function of PTDM concentration allowing a binding constant to be determined.[29] Figure 2a shows the PTDM binding curves for P1-P4 and P6-P7.

Binding constants were obtained by employing a linear binding equation

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F_0/F = K_b \frac{[\text{Polymer}]}{[\text{Polymer}]+1}
\]

adapted from the Stern-Volmer law[30] for fluorescence quenching, where \(F_0\) is the fluorescence of the unperturbed EGFP at 200 nM, \(F\) is the measured fluorescence, and \([\text{Polymer}]\) is the concentration of the PTDM in solution. This form of the equation[31] provides a binding constant (\(K_b\)) through the slope of the linear fit. All of the polymer binding data was modeled according to this equation and the values of \(K_b\) obtained were tabulated in Figure 2c. By visual inspection of Figure 2a, it is evident that the PTDMs bind EGFP similarly which was further substantiated once the \(K_b\) values for all PTDMs were calculated to be at or around 1 \(\text{mM}\). P5 was excluded from Figure 2a because it was not soluble in the experimental conditions and thus did not bind EGFP (Figure S23). The insolubility was most likely due to the pentamethylphenyl functional groups present in P5, which are capable of forming radical cations in the presence of TFA; furthermore, two pentamethylphenyl aromatic rings can associate and share the delocalized radical cation resulting in either aggregation[32] or potential cross-linking through a radical cation initiated side reaction. Consequently, P5 was removed from all data sets.

While the variability in hydrophobicity had no discernible effect on the ability of the PTDM to bind EGFP, despite a five log difference in monomer \(K_{ow}\), cellular uptake experiments were carried out to ascertain the effect of PTDM hydrophobicity on the uptake of EGFP into Jurkat T Cells. After incubation with the PTDM-EGFP complexes, uptake was assessed using flow cytometry to quantify the percentage of EGFP positive cells (Figure 3a) and median fluorescence intensity (MFI) of the total, live cell population (Figure 3b). The MFI is a measure of the performance of each PTDM, whereby large MFI values indicate a greater median fluorescence of internalized cargo.

Figure 3 shows the performance of the PTDMs as a function of EGFP percent positive cells (Figure 3a) and MFI (Figure 3b), where P1[11] and P4[33] are positive controls. It demonstrates that PTDMs P1-P4 affected the highest percentage of cells in the population treated with the complexes while P6-P7 impacted significantly fewer cells. When examining MFI, a narrower range of effective PTDMs is observed with only P3 and P4 exhibiting substantial performance, whereas the least hydrophobic (P1-P2) and most hydrophobic (P6-P7) PTDMs performed worse. Figure 3c shows MFI versus monomer retention time to highlight the relationship between hydrophobicity and EGFP internalization; it further illustrates that the two PTDMs with the highest MFI possess intermediate hydrophobicity. Given that all PTDMs bind EGFP with similar binding constants, but that P3 and P4 have higher MFIs than the others, it appears that PTDM-cargo binding constants do not predict internalization within the range of values studied here.

This work demonstrates that modulating the hydrophobicity of PTDMs optimizes the delivery of EGFP into difficult to transfect T cells. Surprisingly, there was no connection between PTDM-protein binding constant and the amount of internalized EGFP as measured through MFI despite monomer hydrophobicity varying by five orders of magnitude in Log \(K_{ow}\). Although only one cargo was explored, PTDMs with a range of hydrophobicities bound it with the same \(K_b\). This implies that...
within some range of transporter-cargo binding constants, the binding constant yields little information regarding intracellular delivery ability. This work further highlights the complexity of the relationships present in the intracellular delivery landscape. Although $K_b$ shows no correlation with internalization, this study does suggest that there is an optimal, intermediate hydrophobicity for protein delivery into T cells.

Figure 2. A) EGFP binding curves for P1-P4 and P6-P7. B) Binding data fit for P4 using Eq. 1. C) Summary table of $K_b$ values for P1-P4 and P6-P7.

Figure 3. A) The percentage of EGFP positive cells after transducing Jurkat T cells using P1-P4 and P6-P7. B) MFI of the live cell population. C) Correlation plot between HPLC retention time and EGFP MFI. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ns = not significant.

Associated Content

Experimental methods and full characterization data can be found in the supporting information along with supplementary...
figures. Supporting information for this article is given via a link at the beginning of the document.

**Author Contributions**

This manuscript was prepared with contributions from all authors who have all approved of the final version of this manuscript.

**Conflict of Interest**

The authors claim no competing, financial conflicts of interest.

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