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ROMP- and RAFT-Based Guanidinium-Containing Polymers as Scaffolds for Protein Mimic Synthesis

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Abstract: Cell-penetrating peptides are an important class of molecules with promising applications in bioactive cargo delivery. A diverse series of guanidinium-containing polymeric cell-penetrating peptide mimics (CPPMs) with varying backbone chemistries was synthesized and assessed for delivery of both GFP and fluorescently tagged siRNA. Specifically, we examined CPPMs based on norbornene, methacrylate, and styrene backbones to determine how backbone structure impacted internalization of these two cargoes. Either charge content or degree of polymerization was held constant at 20, with diguanidinium norbornene molecules being polymerized to both 10 and 20 repeat units. Generally, homopolymer CPPMs delivered low amounts of siRNA into Jurkat T cells, with no apparent backbone dependence; however, by adding a short hydrophobic methyl methacrylate block to the guanidinium-rich methacrylate polymer, siRNA delivery to nearly the entire cell population was achieved. Protein internalization yielded similar results for most of the CPPMs, though the block polymer was unable to deliver proteins. In contrast, the styrene-based CPPM yielded the highest internalization for GFP (≈40% of cells affected), showing that indeed backbone chemistry impacts protein delivery, specifically through the incorporation of an aromatic group. These results demonstrate that an understanding of how polymer structure affects cargo-dependent internalization is critical to designing new, more effective CPPMs.

Introduction

Delivery of bioactive cargo to living cells is a challenging problem with promising opportunities in many fields, including therapeutics, cancer treatment, and vaccines.[1,2] The plasma membrane, however, limits internalization of large biomolecules like nucleotides and proteins.[3] Numerous strategies, namely electroporation, microinjection, and viral transfection, have been suggested to overcome this problem, but solutions are often hampered by issues such as low efficacy and high cellular toxicity.[4–6] Cell-penetrating peptides (CPPs) and their synthetic mimics (CPPMs) represent a viable alternative to traditionally used methods for transfection.[7]

CPPs are a class of short, cationic polypeptides able to deliver large molecules across the cell membrane.[8] The field of CPPs essentially began with the discovery of HIV-1 TAT, a protein with the ability to cross cell membranes with ease, alone or covalently attached to cargo.[9] By studying translocation with smaller segments of the TAT protein, it was later determined that a short, cationic domain (RKKRRQRRR), corresponding to residues 49–57, within the TAT sequence was responsible for cellular internalization.[10] Generating the same amino acid sequence with a different chirality resulted in peptides still capable of membrane translocation, indicating that secondary structure is not crucial for CPP function;[11] however, when residues within this sequence were modified or replaced with other amino acids, such as alanine, uptake into cells was decreased.[12] These studies highlight the importance of cationic residues for translocation.

Rothbard and co-workers performed a systematic study investigating the importance of the cationic charge chemistry, as the transduction domain of TAT contains both lysine and arginine.[13] By measuring the cellular uptake of arginine, lysine, histidine, and ornithine oligomers, it was found that the guanidinium groups in arginine promote significantly more uptake than either imidazolium or ammonium groups. Due to these, and subsequent findings, guanidine has become a cornerstone functional group within the field of CPPs and their mimics.[14–18] This has been proposed to be related to the lower acidity of the guanidinium cation, resulting in ion pair repulsion facilitating CPPM translocation.[19] This mechanism has been shown to operate through direct translocation, which is highly advanta-

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geous over other modes of delivery because it is an energy independent process.[20] Alternatively, the guanidinium cation is softer and more delocalized than its amine counterpart which may also have a role. Reports demonstrate that guanidine itself is not necessarily required for efficient delivery; glycosylated sulfonium cations can be similarly effective[21,22] and phosphonium cations have shown delivery properties as well.[23,24] Regardless, CPPMs are generally oligmeric in nature, take inspiration from efficacious biological CPPs, and are subsequently refined and improved through continuous structure activity relationship studies.

Initial CPPMs were closely related to polyarginine. Incorporation of guanidine groups into a modified peptide-like backbone directly improved cellular uptake of oligomers based on β-peptide or peptoid backbones.[12,25] Natural degradation of CPPs can be circumvented by applying synthetic polymer chemistry to tune the backbone and overall structure. Moreover, arduous synthesis and purification of peptide-based CPPMs can be bypassed when utilizing well-established synthetic polymerization techniques. In this light, several groups have developed polymerization platforms that incorporate guanidine groups for translocation.[26–29]

A particularly early example of a synthetic CPPM platform developed by Kiessling and co-workers utilized post-polymerization addition of an amino-guanidine to an activated ester functionalized polymer made through ring-opening metathesis polymerization (ROMP).[27] The resulting polynorbornene derivatives were able to localize into cells, confirming uptake when functionalized with a dye. This platform has been extended to allow for the synthesis of block polymers with varying functionality.[30] Other examples of CPPMs include a guanidine bearing oligocarbonate[28] and oligophosphoester[31] scaffold developed by Wender and co-workers through ring opening polymerization, as well as a guanidine containing polymethacrylamide system developed by McCormick and co-workers.[32] These systems also demonstrate cellular internalization, reinforcing the critical importance of guanidinium incorporation. It is important to note that polymeric guanindium methacrylates similar to some molecules studied in this report have also been synthesized by Locock et al., and examined as highly effective antimicrobials.[33–36]

In addition to the examples given in Figure 1, our own group has developed and studied a broad range of polymers derived from oxanorbornene open-ester monomers.[37–41] Early molecules include dye-functionalized polymers bearing either one or two guanidinium units per monomer. These polymers, similar to the aforementioned CPPMs, were found to cross cell membranes, demonstrating very high uptake.[37] The impact of hydrophobic incorporation, as inspired by numerous chimeric CPPs,[42,43] has resulted in copolymers capable of coordinating and delivering biologically relevant cargo, such as siRNA, protein, and antibody.[40,44,45] The sequence segregation of the hy-

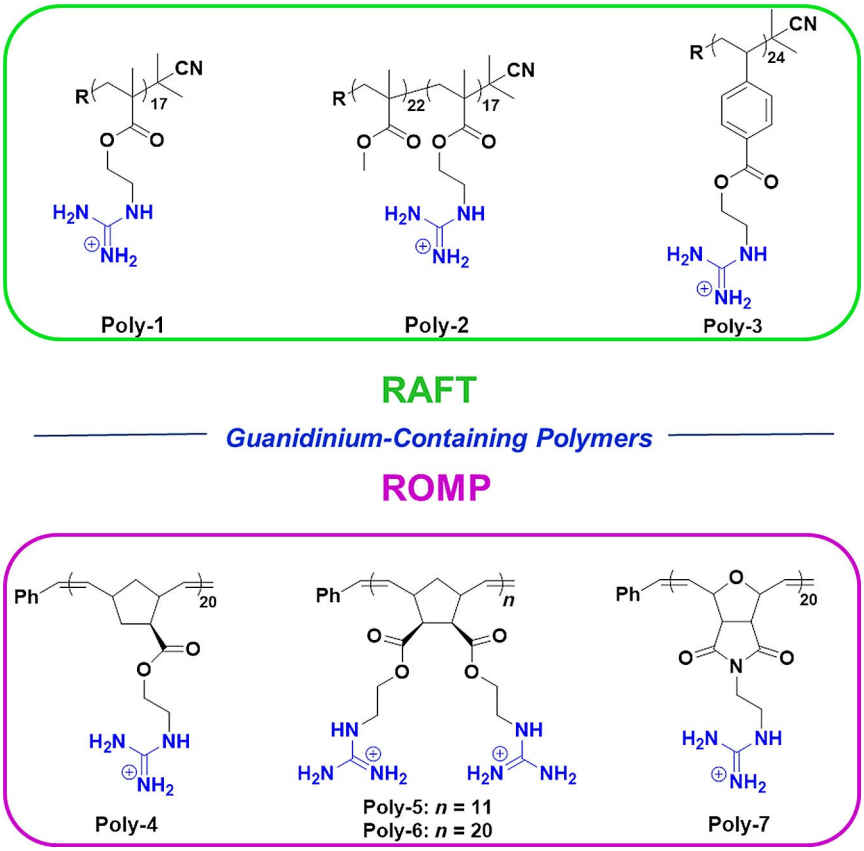

Figure 1. Guanidinium-containing polymers used in this study. Polymers were synthesized by RAFT (green) and ROMP (purple) to vary backbone composition. R = –C(CH$_3$CN)(CH$_2$)$_2$COOH.

drophobic moieties, commonly a phenyl or diphenyl containing oxanorbornene monomer, was also shown to affect uptake efficiency, with gradient and block copolymers outperforming alternating hydrophobic-guanidinium polymers.[39,46]

Recently, a systematic study was conducted to determine the optimum hydrophobic window for the delivery of siRNA into Jurkat T cells.[41] In a similar system, it was found that an important interplay exists between cationic block length and delivery efficiency of siRNA into Jurkat T cells, with 20 repeat units of diguanidinium monomer being optimal.[40,47] In all of these cases, delivery was accomplished without covalent conjugation of cargo to the CPPM; a marked contrast to the CPPs, like TAT 49–57. These structure–property studies are strong examples of progress that is enabled by new synthetic platforms and approaches.

While work by our group on oxanorbornene ROMP polymers has elucidated a range of design principles for CPPMs, there is still a general lack of understanding regarding the impact of polymer backbone on the ability to deliver cargo. To address this point, a series of polymers containing a variety of backbones synthesized by either ROMP or reversible addition fragmentation chain transfer polymerization (RAFT) were developed and characterized for their ability to deliver cargo. By utilizing multiple polymerization techniques, maintaining cationic content across polymers, and subjecting each resulting CPPM to the same performance tests, we can identify how particular backbones impact delivery capabilities. Additionally, multiple cargo types (siRNA and protein) were tested for delivery using these polymers, which highlights important cargo-dependent differences that should be considered when designing CPPMs. These findings contribute to the overall design paradigm of CPPMs, and in fact potentially add new molecules to the current repertoire of CPPMs.

Results and Discussion

Design and synthesis

To effectively probe backbone chemistry as a CPPM variable, a variety of polymer structures were targeted (Figure 1). **Poly-1–Poly-3** were generated using RAFT, a technique sharing many of the benefits of ROMP, such as controlled nature, broad functional group tolerance, and accessibility to block architectures.[48] Utilizing RAFT polymerization allowed for the synthesis of polymers with distinct backbones compared to their ROMP counterparts.[35] Methacylate and styryl-derived monomers were synthesized by the EDC coupling of a Boc-guanidine alcohol to an appropriate carboxylic acid (Figure S1). It should be noted that **Poly-2** includes a hydrophobic methyl methacrylate block, mimicking previous blocky CPPMs that incorporate hydrophobicity to improve delivery of cargo. This polymer tests whether or not that particular design principle is universal across backbones. The styryl monomer **2**, in contrast to all other monomers, includes a hydrophobic aromatic group, which was expected to impact both cargo coordination and delivery.

Table 1. Summary of molecular weight characterization of guanidinium containing.

Polymer	Theoretical DP	M_n[a]	M_w[a]	$Đ$[a]	Conversion [%][b]
Poly-1	40	8300	9800	1.18	47
Poly-2[c]	20	9700	11 600	1.20	97
Poly-3	40	9500	12 000	1.26	50
Poly-4	10	10 800	11 800	1.09	>99
Poly-5	20	7900	9000	1.14	>99
Poly-6	20	12 900	15 400	1.20	>99
Poly-7	20	7800	8100	1.05	>99

[a] Molecular weights and dispersity ($Đ$) as determined using gel permeation chromatography with THF as the eluent at a flow rate of 1 mL min^{-1} and toluene as the flowrate marker. PS standards were used for **Poly-3**, and PMMA standards were used for all other polymers. [b] Conversion, as determined by ^{1}H NMR spectroscopy. [c] Initiated from a 2200 g mol^{-1} PMMA macro-CTA.

When compared directly to ROMP, however, dispersities obtained through RAFT are slightly broader, conversions are lower, and polymerization times are often longer. These drawbacks are observed here as well (Table 1), with dispersities of 1.18–1.26, conversions near 50%, and polymerization times of roughly six hours. It should be noted that conversions were intentionally kept below 50%, as higher conversions were associated with significantly increased dispersities (Figure S3) and loss of polymerization control. Through these conversion studies, rate constants were determined for both monomer **1** and **2**, with the methacrylate monomer polymerizing faster (Figure S4). Polymers synthesized through RAFT also retained the dithiobenzoate moiety responsible for chain transfer. In order to deprotect the Boc-protected guanidine groups, a 1:1 TFA/DCM treatment was used (excess TFA removed through azeotropic distillation), which would hydrolyze the dithiobenzoate to a thiol. End-functionalized thiol polymers are capable of dimerizing, which convolutes the effective molecular weights of the CPPMs. Thus, an end-group removal step was performed for all RAFT polymers, using 20 equiv. of AIBN in 70 °C and eliminating the opportunity for thiol functional polymers after deprotection (Figure S2).[49] In spite of these drawbacks (low conversion, long polymerization time, end group removal), RAFT is catalyst free, and also allows for the inclusion of a plethora of monomers, both commercial and tailored, into CPPM materials that are otherwise not accessible through ROMP.

Norbornene-derived monomers bearing either one or two Boc-protected guanidine groups were designed as the closest relative to previously reported oxanorbornene based CPPMs, and were synthesized through modified literature procedures.[37,38,40,41,50] Notably, the oxygen bridgehead atom present in the oxanorbornene polymers was replaced with a carbon, which in turn enabled the synthesis of monomer **3** due to the stability of the norbornene carboxylic acid Diel–Alder adduct. Monomer **4**, which contains two guanidine units, was ultimately polymerized to two different lengths (DP = 10, 20), to control for the relative number of charges contained in the polymer as well as the DP. **Poly-7**, a membrane active polymer previously reported by our group,[26] was also included to contrast the rel-

atively hydrophobic ROMP backbones of **Poly-4** through **Poly-6**.

While the CPPMs studied here were designed to isolate backbone chemistry as a variable, the polymerization routes to achieve these materials differed dramatically. Norbornene and oxanorbornene imide monomers were polymerized by ROMP, a technique known for its functional group tolerance, fast polymerization times, low dispersity (*Đ*) products, and controlled nature.[51] Polymers derived by this method from single guanidine-containing monomers (**Poly-4**, **Poly-7**) had extremely narrow dispersities and were achieved with short polymerization times (Table 1). Diguanidine polymers, **Poly-5** and **Poly-6**, required longer times, and demonstrated slightly higher dispersities, particularly with increasing degree of polymerization. Even so, all ROMP polymerizations resulted in quantitative monomer conversion in less than two hours.

Cargo delivery

CPPMs were tested for their ability to internalize two distinct types of cargo: siRNA and protein. As cargo type and composition change, CPPM-facilitated internalization can vary greatly depending on how their interactions with the cargo are altered. Additionally, in a therapeutic environment, countless biomacromolecules are present, and the delivery of a specific molecule is greatly complicated. Thus, by screening our polymers against two types of cargo, we can begin to understand how to tailor polymers towards the delivery of a specific cargo (see the Experimental Section in the Supporting Information).

Internalization of a fluorescently tagged siRNA sequence to Jurkat T cells was performed and assessed by flow cytometry (Figure 2). The median fluorescence intensity (MFI) is an indicator of the average cargo delivered to affected cells, while percent uptake represents the percentage of cells affected. The ratio of CPPM to cargo was determined experimentally through previous studies conducted by our group.[40] All polymers tested showed some level of delivery, manifesting in the histograms as a slight shift to the right of the blank cell population. CPPMs **Poly-4**, **Poly-5**, and **Poly-6**, as well as the methacrylate based **Poly-1** showed low and comparable delivery to one another. The minimal difference between **Poly-5** and **Poly-6** indicates that within this specific monomer system, degree of polymerization and overall charge content have little impact on internalization. **Poly-7** showed a much broader shoulder, indicating a cell population with a wider range of internalized siRNA. **Poly-3**, while promoting the second highest uptake of siRNA, demonstrated solubility constraints as some of the polymer precipitated out of solution upon addition to phosphate buffered saline. Given the moderate internalization demonstrated, improving the water solubility of analogous polymers with a hydrophilic block may very well improve overall delivery.

In stark contrast, **Poly-2** was able to facilitate siRNA internalization in the entire cell population. This is notable, because **Poly-2** contains the same guanidinium containing monomer as **Poly-1** in a near-identical amount, but shows substantially higher delivery, with 97% internalization versus 25%, respectively. These results clearly demonstrate that a segregated hydrophobic block improves delivery, either by enhanced interactions with the siRNA, the cell membrane, or both. Moreover, this result is in agreement with previous siRNA delivery optimization studies focusing on ROMP backbones, implying that hydrophobic block incorporation improves siRNA internalization universally.[40,41] When excluding the hydrophobically segregated sample, there seems to be very little dependence of siRNA internalization on backbone chemistry.

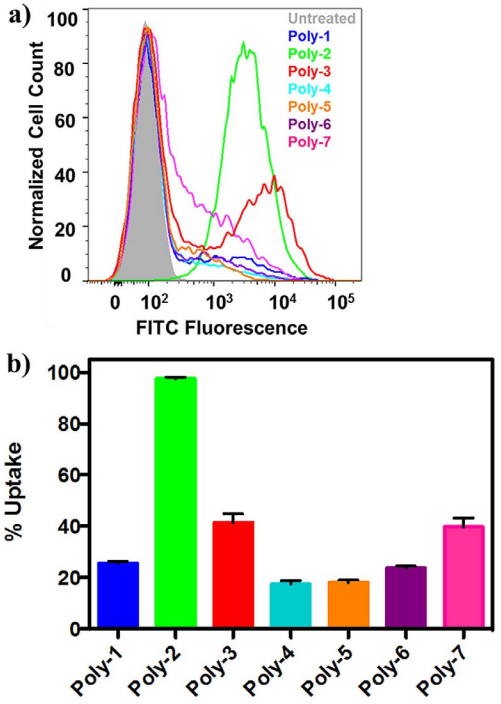

Figure 2. FITC-siRNA uptake in Jurkat T cells. a) Representative FITC-siRNA fluorescence histogram, and b) percentage of FITC-siRNA positive cells after a 4 h treatment with polymer/FITC-siRNA complexes in RPMI + 10% fetal bovine serum (FBS) using an N/P ratio of 8:1, where the FITC-siRNA concentration was held constant at 50 nM and the cell concentration was 4×10^5 cells mL^{-1}. Samples were analyzed using flow cytometry and normalized to the blank. Data points represent the mean ± SEM of at least three independent experiments.

Green fluorescent protein (GFP) was selected as the model protein delivery cargo, as it is self-reporting due to its inherent emission properties. Like the data in Figure 2, cellular internalization facilitated by CPPMs was quantified using flow cytometry (Figure 3). Similar to siRNA internalization, all polymers facilitated at least some degree of GPF delivery. **Poly-1** and **Poly-4** through **Poly-7** showed low amounts of protein internalized, which generally follows the same trend for siRNA. **Poly-2**, which was capable of delivering siRNA to the entire cell population, was unable to effectively facilitate the delivery of GFP. On first glance, this observation is surprising since, in general, the addition of a hydrophobic block increases CPPMs' delivery of cargo.[39,40,46,52] It is conceivable that **Poly-2** forms a stable microstructure in buffer that renders it unable to effectively interact with a protein that is large in comparison to siRNA. When taking previous reports into account, however, this is unlikely, as a hydrophobic block almost universally improves

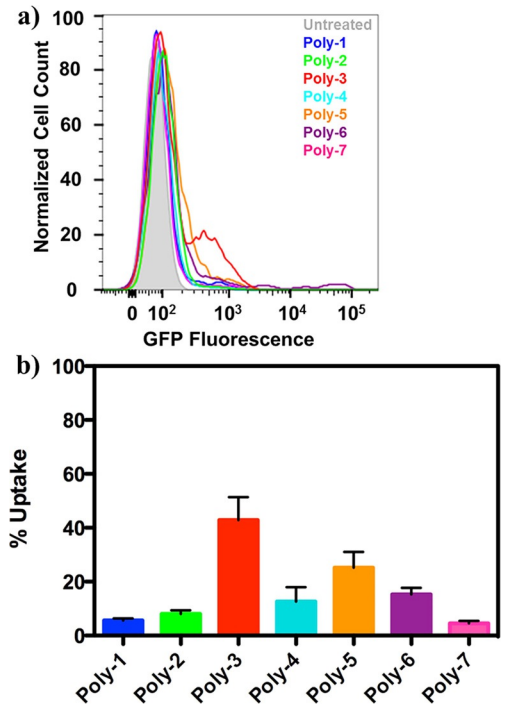

Figure 3. GFP uptake in Jurkat T cells. a) Representative GFP fluorescence histogram overlay, and b) percentage of GFP positive cells after a 4 h treatment with polymer/GFP complexes in RPMI + 10% FBS using an weight ratio of 20:1 polymer/protein, where the protein mass was held constant at 3 μg and the cell concentration was 4×10^5 cells mL^{-1}. Samples were analyzed using flow cytometry and normalized to the blank. Data points represent the mean ± SEM of at least three independent experiments.

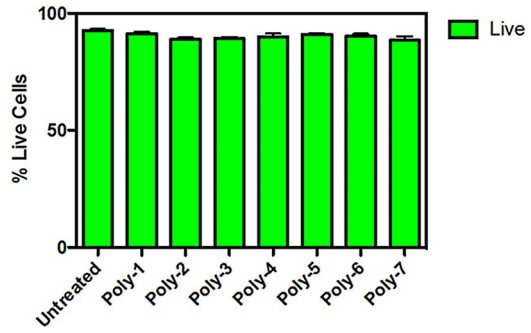

Figure 4. Jurkat T cell viability after 4 h treatment with polymer/FITC-siRNA complexes using 7-AAD and annexin V. Cells were treated with polymer/siRNA complexes (50 nM FITC-siRNA; N/P ratio = 8:1) for 4 h in RPMI + 10% FBS at a cell concentration of 4×10^5 cells mL^{-1}. An untreated sample was used for comparison. Samples were analyzed using flow cytometry and all data points represent the mean ± SEM of at least three independent experiments.

CPPM-mediated protein uptake in cells regardless of microstructure character or micelle/vesicle size.[39] Interestingly, in one report, CPPMs with hydrophobic blocks that either do or do not contain aromaticity were compared for delivery efficiency of protein, revealing that only the aromatic hydrophobic CPPMs were able to deliver.[46] In another recent study, siRNA delivery was optimized by tuning the hydrophobic content of a CPPM. A series of aromatic and aliphatic blocky CPPMs were synthesized and analyzed; in this case, aromaticity did not have an impact, and a butyl side chain had similar delivery efficiency as a phenyl side chain.[41] The delivery behavior of **Poly-2** actually agrees well with these reports; it contains a non-aromatic hydrophobic block, and is able to deliver siRNA well but not protein. Thus, taking all of these data into consideration, it seems possible that the type of hydrophobicity within a CPP or CPPM can dictate its cargo specific interactions. Specifically, aromatic hydrophobic groups are needed for robust protein internalization.[50,53] Moreover, aromatic amino acids are known to play an important role in membrane proteins, indicating that the presence of aromaticity in CPPMs may also improve membrane interactions.[54]

Along these lines, **Poly-3**, containing a benzene ring in each repeat unit, showed the highest degree of protein internalization, with roughly 45% of cells affected. This result would corroborate the need for a specific type of hydrophobicity when attempting protein delivery, notably aromatic. Again, **Poly-3** suffers from solubility constraints, where improving water solubility by copolymerization with a more hydrophilic monomer would likely improve internalization. In contrast to siRNA internalization results, backbone chemistry seems to play a larger role in protein delivery, though only through the incorporation of an aromatic hydrophobic group. This comparison should be made with care, however, because the highest performing siRNA sample was actually a block copolymer.

Viability of cells after treatment with CPPM–cargo complexes was also assessed using 7-AAD viability stain. Results of cellular viability after siRNA delivery are shown in Figure 4, while viability after protein delivery is shown in Figure S5. Cells remained largely alive after the treatment, with viabilities above 90%. Protein delivery was conducted at a higher CPPM concentration, and thus for these experiments, slightly higher toxicity was observed.

Conclusions

Generally, all polymers were able to deliver some amount of both cargos, with little backbone dependency; however, a block copolymer of methyl methacrylate and guanidinium methacrylate was able to deliver siRNA to the entire cell population, reinforcing that hydrophobic segregation is a critical design parameter for siRNA delivery. This polymer, however, was unable to facilitate the internalization of large amounts of GFP, indicating that the type or amount of hydrophobicity is important for protein delivery. Interestingly, the styrene derived CPPM was able to internalize protein to the highest proportion of cells out of all polymers tested, in spite of solubility limitations observed with this molecule. Thus, polymer backbone does have an impact on protein internalization, but only when comparing aromatic to nonaromatic polymers. With the synthesis of these polymers, we also demonstrate the potential of RAFT-based CPPMs. Given the versatility and broad monomer selection afforded by RAFT, extension of this polymerization technique can be easily employed to elucidate important CPPM design parameters as well as generate a plethora of new, more effective CPPMs.

Experimental Section

Please refer to the Supporting Information for chemical schemes, materials, methods, and experimental techniques.

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Conflict of interest

The authors declare no conflict of interest.

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