

# Review

## Development of Protein Mimics for Intracellular Delivery

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Received 19 December 2014; revised 24 March 2015; accepted 30 March 2015

Published online 9 April 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bip.22658

### ABSTRACT:

Designing delivery agents for therapeutics is an ongoing challenge. As treatments and desired cargoes become more complex, the need for improved delivery vehicles becomes critical. Excellent delivery vehicles must ensure the stability of the cargo, maintain the cargo's solubility, and promote efficient delivery and release. In order to address these issues, many research groups have looked to nature for design inspiration. Proteins, such as HIV-1 trans-activator of transcription (TAT) and Antennapedia homeodomain protein, are capable of crossing cellular membranes. However, due to the complexities of their structures, they are synthetically challenging to reproduce in the laboratory setting. Being able to incorporate the key features of these proteins that enable cell entry into simpler scaffolds opens up a wide range of opportunities for the development of new delivery reagents with improved performance. This review charts the development of protein mimics based on cell-penetrating peptides (CPPs) and how structure-activity relationships (SARs) with these molecules and their protein counterparts ultimately led to the use of polymeric scaffolds. These scaffolds

deviate from the normal peptide backbone, allowing for simpler, synthetic procedures to make carriers and tune chemical compositions for application specific needs. Successful design of polymeric protein mimics would allow researchers to further understand the key features in proteins and peptides necessary for efficient delivery and to design the next generation of more efficient delivery reagents. © 2015 Wiley Periodicals, Inc. *Biopolymers (Pept Sci)* 104: 265–280, 2015.

**Keywords:** cell-penetrating peptides; delivery; guanidinium-rich molecular transporters; peptidomimetics; polymers; protein mimics

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of any preprints from the past two calendar years by emailing the *Biopolymers* editorial office at [biopolymers@wiley.com](mailto:biopolymers@wiley.com).

### INTRODUCTION

Proteins are large, complex biomolecules that perform numerous biological functions.<sup>1</sup> They contain both secondary and tertiary structure, which helps them arrange and fold into specific and functional conformations. While nature has developed efficient ways to generate correctly folded and functional proteins, it is substantially more difficult to recreate these structures synthetically. Many research groups have successfully developed peptidomimetics that mimic conformations of short peptide sequences, but to date, mimicking larger protein surfaces or

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Contract grant sponsor: The NIH

Contract grant number: T32 GMO8515

Contract grant sponsor: NSF

Contract grant number: CHE-0910963

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entire protein functions represent more significant challenges.<sup>2</sup> The field of proteomimetics looks to specifically address these challenges by moving away from naturally occurring amino acids and developing non-peptidic materials that can capture key secondary structures found in proteins.<sup>2</sup>

An elegant example of synthetic protein mimic development is from Andrew Hamilton and coworkers in which they were able to mimic part of the protein helix from the myosin light chain kinase using a terphenyl scaffold.<sup>3</sup> These synthetic mimics operate on the premise that the critical residues needed for efficient protein–protein interactions lie along one face of the  $\alpha$ -helix.<sup>3,4</sup> Using this scaffold, they were also able to assess binding affinities for calmodulin, which is a calcium-binding messenger that aids in cell signaling.<sup>3</sup> Specifically, they were able to prove that the synthetic  $\alpha$ -helix had similar binding properties to the myosin light chain kinase, thus demonstrating the protein mimicking capacity of these materials.<sup>3</sup> Other excellent work has also been published in this field.<sup>5–8</sup>

In addition, many researchers have explored foldamers, which are chains of molecules that can fold into organized structures, such as  $\alpha$ -helices and  $\beta$ -sheets, in solution.<sup>9–22</sup> Foldamers differ from other proteomimetics in that they require non-covalent interactions, such as hydrogen bonding,  $\pi$ -interactions, electrostatic interactions, van der Waal's interactions, and solvophobic effects with non-adjacent surfaces.<sup>9,10,12,15,16,18,20,21</sup> These molecules have been used to mimic the folding of proteins, polysaccharides, and nucleic acids.<sup>9,10,12,15,16,18,20,21</sup> One specific subset of foldamers, referred to as abiotic foldamers, aim to capture key features of proteins, such as secondary structure, with non-natural materials.<sup>9–17,19–22</sup>

While mimicking protein secondary structure is an impressive feat and can aid the future development of entirely synthetic protein mimics, it remains difficult to generate these scaffolds and to predict the proper folding or assembly processes. Protein mimics that capture key features using simpler scaffolds without secondary structures can more easily be attained with tunable, synthetic platforms.<sup>23,24</sup> One prominent example involves mimicking antimicrobial peptides (AMPs). AMPs are potent antibacterial agents that are part of the innate immune systems for many organisms.<sup>25–28</sup> Magainin 2, which is one of the many AMPs currently found in nature, and other similar antimicrobial agents have been shown to be effective against both Gram positive and Gram negative bacteria.<sup>25–27,29,30</sup> These peptides have facially amphiphilic topologies, enabling segregation of hydrophilic (cationic) and hydrophobic residues for improved membrane activity and antimicrobial properties.<sup>25,31</sup> Much effort has been devoted to understanding these peptides and the mechanisms by which

they kill bacteria, both experimentally and computationally.<sup>25,32–49</sup> Despite the level of controversy surrounding their mode of action, hydrophobic and electrostatic interactions between these peptides and the bacterial cell wall play prominent roles in pore formation, which ultimately leads to bacterial cell death.<sup>44,45</sup>

Given the rise in antibacterial resistance, researchers have turned to AMPs as a source of inspiration. Incorporating key features of these peptides into synthetic scaffolds offer more structural options for tuning chemical compositions for improved performance.<sup>15,25,45,50–54</sup> These molecules are often referred to as synthetic mimics of antimicrobial peptides (SMAMPs). One example from DeGrado and coworkers<sup>55</sup> demonstrated the use of  $\beta$ -peptides, which mimic the  $\alpha$ -helical structures and amphiphilicity of many AMPs. Although many of these molecules contained  $\alpha$ -helical structures, linear  $\beta$ -peptides made by Gellman and coworkers and  $\beta$ - and  $\Upsilon$ -peptides made by Seebach and coworkers demonstrated that  $\alpha$ -helical structures were not necessary for potent antimicrobial properties.<sup>56,57</sup> These studies paved the way for the development of synthetic mimics with completely abiotic scaffolds. Such molecules, designed by Tew and coworkers,<sup>58–61</sup> used facially amphiphilic triaryl scaffolds in which the hydrophobic content and cationic charge content (ammonium or guanidinium groups) could be tuned for improved antimicrobial properties and selectivities. By converting AMP designs to simple, synthetic scaffolds, production time and costs are considerably reduced.<sup>51</sup> In addition, peptide *in vivo* limitations, such as proteolysis, tissue distribution, and toxicity, are overcome and robust *in vivo* antibacterial activity against drug resistant infections has been demonstrated, specifically with a compound in phase II clinical trials.<sup>51</sup> An important new development was the demonstration that polymers could be designed with AMP-like biological activity.<sup>15,17,23,50,53,54,62–72</sup> Unlike proteins and peptides, which typically have a single, exact molar mass, synthetic polymers, even when termed monodisperse, are characterized by a distribution of molecular weights. Although it is unclear how antimicrobial activity trends with dispersity, this opens a wider range of molecules and chemistries that can be used for the development of synthetic antimicrobial mimics.

By using the same process that gave rise to SMAMPs, researchers have used proteins and peptide sequences as inspiration for the design of the next generation of delivery reagents (Figure 1).<sup>23</sup> This is an area of particular interest since delivery of therapeutic agents is an ongoing challenge. Although there is an increasing demand for new treatments and treatment options, the field lacks a clear understanding of how to efficiently and reliably deliver bioactive molecules across cellular membranes, especially as therapies move increasingly toward more complex biologics.<sup>73–79</sup> Nature, however, is already

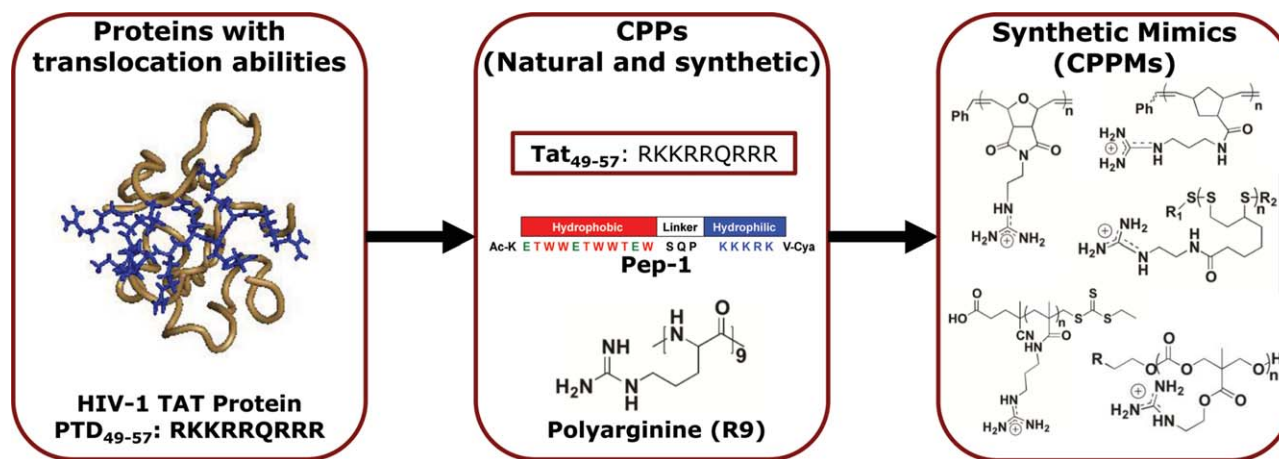


FIGURE 1 The progression from proteins and peptides to guanidinium-rich CPPMs.

capable of designing proteins that can perform these functions. One example is HIV-1 TAT (trans-activator of transcription) protein, which is responsible for the spread of the virus<sup>80,81</sup> This protein, along with others, contain a region referred to as a protein transduction domain (PTD) that is responsible for their abilities to enter cells.<sup>82–84</sup> The study of TAT and other PTDs have subsequently led to the development of cell-penetrating peptides (CPPs), which are peptides that are capable of delivering cargo, such as small molecules, siRNA, pDNA, antibodies, and proteins, into cells via covalent or non-covalent interactions.<sup>85–90</sup> Two examples of such molecules include TAT<sub>49–57</sub>, which is a guanidinium-rich sequence, and Pep-1, which has a segregated architecture similar to a block copolymer.<sup>83,84,90–92</sup> While both peptides are cation-rich, Pep-1 also incorporates a hydrophobic segment that is thought to further aid in cellular uptake.

Despite the development of many CPPs such as Pep-1, which is now commercially available through Active Motif as Chariot<sup>TM</sup>, moving away from the peptide scaffold offers distinct advantages. Most peptides are prepared by solid phase synthesis, which is both costly and time consuming because amino acids need to be sequentially added using a series of deprotection, addition, wash, and protection steps. Switching to a completely abiotic backbone allows delivery agents to be made cheaper and potentially in larger quantities. In addition, a non-peptide-based system offers many more structural options in terms of chemical compositions and molecular arrangements because it is not restricted to the incorporation of natural amino acids.<sup>23</sup> This expanded chemistry toolset is expected to generate more efficient structures than their natural peptide analogs.<sup>23</sup>

This review aims to document the very early development of cell-penetrating peptide mimics (CPPMs), in particular

ones based on polymeric scaffolds. A number of recent reports suggest this area will develop similarly to SMAMPs and will provide new tools for biology and perhaps new delivery opportunities for society.<sup>23,24</sup> A summary of the early CPP work is highlighted followed by an overview of polymeric CPPMs developed to date.

### CELL-PENETRATING PEPTIDES (CPPS)

CPPs are a class of peptides that can facilitate the delivery of various cargoes into cells.<sup>85–90</sup> These peptides are generally 7–30 amino acid residues in length and cation-rich, usually containing multiple arginine and/or lysine residues.<sup>85</sup> In general, CPPs can be broken down into three broad categories: protein-derived, chimera-derived, and synthetic. These classes of peptides are summarized in the following subsections and in Table 1.<sup>85</sup>

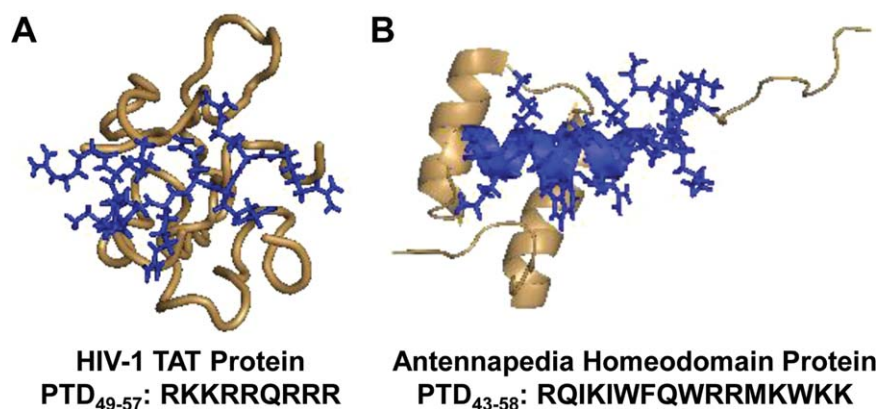
#### Protein-derived CPPs

Protein-derived CPPs are based on known sequences from naturally occurring proteins.<sup>85</sup> The specific sequences used for these CPPs are generally derived from the PTDs of these molecules. Disruptions in these sequences often lead to partial or complete uptake inhibition.<sup>82,84,109</sup> Two examples of protein-derived CPPs are TAT<sub>49–57</sub> and the Antennapedia homeodomain protein derivative Penetratin. These proteins are modeled in Figure 2 with their PTDs highlighted in blue.

In 1988, Green and Loewenstein, as well as Frankel and Pabo, independently reported that HIV-1 TAT had the unique ability to translocate into and out of cells.<sup>80,81</sup> Then, in 1994, Fawell et al.<sup>110</sup> tested the delivery efficiencies of two truncated HIV-1 TAT sequences: TAT<sub>1–72</sub> and TAT<sub>38–72</sub>. The former was

**Table 1** Summary of Cell-Penetrating Peptides (CPPs) Color-Coded Based on Classification Into Three Categories: Protein-Derived (Top, Pink), Chimera (Middle, Blue), and Synthetic (Bottom, Yellow)

Peptide	Sequence	Derivation	Total # of Charged Residues	Cargo	Alternative Classification
HIV-1 TAT <sup>74,93</sup> <sub>49-57</sub>	RKKRRQRRR	Human Immunodeficiency Virus	8	protein, peptides, siRNA	Non-amphipathic
Penetratin <sup>94</sup>	RQIKWFAQRRMKWKK	<i>Drosophila</i> homeoprotein antennapedia	7	protein, PNA, siRNA	Secondary amphipathic
pVEC <sup>95</sup>	LLIILRRRIRKQAHASHK	Murine VE-cadherin	6	protein, peptides	Secondary amphipathic
VP22 <sup>96</sup>	NAKTRRHERRRKLAIER	Herpes simplex virus	8	protein	Secondary amphipathic
Pep-1 <sup>90-92,97</sup>	KETWWETWTEWSQPKKRRKV	NLS from SV40 T-antigen and a Tryptophan-rich domain	5	protein, peptides	Primary amphipathic
MPG <sup>91,98,99</sup>	GALFLGLGAAGSTMGA WSQPKKRRKV	Hydrophobic domain from fusion sequence of HIV gp41 and NLS of SV40 T-antigen	5	siRNA, plasmids	Primary amphipathic
Transportan (TP10) <sup>100,101</sup>	AGYLLGKINLKALAALAKKIL	Galanin and mastoparan	4	protein, PNA, siRNA	Primary amphipathic
M918 <sup>102</sup>	MVTVLFRRLRIRACGPPRRRV	Tumor suppressor protein p14ARF	7	Proteins, PNA	Secondary amphipathic
YTA2 <sup>103</sup>	YTAIAWVVKAFIRKLRK	MMP cleavage site	5	small molecules	Secondary amphipathic
YTA4 <sup>103</sup>	IAWVKAFIRKLRKGPLG	MMP cleavage site	5	small molecules	Secondary amphipathic
MAP <sup>104,105</sup>	KLALKLALKALKAAALKLA	Synthetic	5	small molecules, plasmids	Secondary amphipathic
Polyarginine <sup>83,84,89,106</sup>	R <sub>5-15</sub>	HIV-1 TAT	5-15	protein, peptides, siRNA	Non-amphipathic
CADY <sup>107,108</sup>	GLWRALWRLRLSLWRLIWRRA	Derived from PPTG1 peptide	5	protein, peptides, siRNA	Secondary amphipathic



**FIGURE 2** Models of HIV-1 TAT and antennapedia homeodomain protein with their PTDs modeled in blue.

examined because it was thought to be involved in protein binding and cellular uptake and the latter was examined because it lacked the cysteine-rich region (residues 22–37).<sup>110</sup> Both sequences were able to deliver proteins, demonstrating that the entire protein sequence was not required for efficient delivery.<sup>110</sup> This study also compared these sequences to shorter peptides, Tat<sub>37–58</sub> and Tat<sub>47–58</sub>, which were also able to deliver proteins to cells.<sup>110</sup> Following this study, in 1997, Vives et al.<sup>82</sup> studied four HIV-1 TAT-derived sequences to test the effects of altering the  $\alpha$ -helical and basic regions on cellular uptake. Uptake data illustrated that the  $\alpha$ -helix was not required for cellular uptake but that there could be no interruptions in the basic domain.<sup>82</sup>

In 2000, Wender et al. also studied the TAT peptide, as well as polyarginine. They confirmed that the basic region, amino acids 49–57, must be completely preserved in order for it to maintain its function.<sup>82,84</sup> Truncating the sequence or substituting individual amino acids with alanine residues all resulted in a reduction in uptake efficiency. By studying Tat<sub>49–57</sub> sequences that were synthesized with D-amino acids and/or in reverse, they also illustrated that backbone chirality, hydrogen bonding, and overall peptide backbone were not critical for efficacy.<sup>84</sup>

Similar studies were also performed for the antennapedia homeodomain protein. This protein contains approximately 60 amino acids, which fold to give three  $\alpha$ -helices, and is a transcription factor that aids in DNA binding in *Drosophila*.<sup>111</sup> In 1991, it was discovered that this antennapedia homeodomain protein could translocate into cells and that the third  $\alpha$ -helix was important for its cellular uptake ability.<sup>111</sup> Similar studies to those performed on HIV-1 TAT, demonstrated that the entire protein sequence was not required for cellular uptake, just the third  $\alpha$ -helix and that backbone chirality, hydrogen bonding, and peptide secondary structure were not

also required for uptake.<sup>109,112</sup> This sequence of amino acids required for uptake has since been referred to as Penetratin.<sup>109</sup> Other CPPs inspired by proteins include pVEC and VP22.<sup>95,96</sup>

Through studying HIV-1 TAT, antennapedia homeodomain protein, and their structural derivatives, it became apparent that full protein sequences, protein secondary structures, and peptide-based backbones are not necessarily required for efficient cellular uptake but that the cationic charge content was absolutely critical.<sup>82–84,94,109,110,112</sup> These results opened up the possibility of designing synthetic mimics.

### Chimera-derived CPPs

As an alternative to using shortened TAT sequences to overcome some of the limitations of using the full TAT-protein, amphiphilic peptides with improved stability were developed based on a chimeric scaffold.<sup>90</sup> Chimera-derived CPPs are fusions of two or more naturally occurring protein or peptide sequences.<sup>85</sup> Most often, they are the combination of sequences that enable specific protein functions, such as nuclear localization sequences (NLSs) and signaling sequences.<sup>85</sup> One of the first chimera-derived CPPs reported in the literature was Transportan (AGYLLGKINLKALAALAKKIL-NH<sub>2</sub>), which was a fusion of the neuropeptide galanin1–13 and the wasp venom peptide mastoparan.<sup>100,101,113,114</sup>

One of the most popular examples of a chimera-derived CPP is Pep-1.<sup>90–92</sup> This peptide has a block copolymer-like structure with a tryptophan-rich hydrophobic domain that is segregated from a lysine-rich hydrophilic domain by a linker and is based on the NLS of the simian virus 40 (SV40) large T antigen, as well as on the reverse transcriptase of HIV.<sup>90–92</sup> Pep-1 is considered to be a primary amphipathic peptide because the hydrophilic/hydrophobic segregation is not dependent on the secondary structure of



the peptide.<sup>90–92</sup> Although stability is often an issue with peptide-based carriers, Pep-1 has an acetylated N-terminus and a cystamide C-terminus to improve shelf life. Since the development of Pep-1, structural variations have been made to try to enhance delivery of proteins, peptides, and peptide nucleic acids.<sup>91</sup> In addition, Divita and coworkers<sup>91,98,99</sup> have developed MPG for nucleic acid delivery, which is structurally similar CPP with a primary amphipathic and alpha-helical structure. This CPP is commercially available as DeliverX™ through Panomics.<sup>91,98,99</sup>

In addition to their amphiphilicity, what set Transportan, Pep-1, and MPG apart from others CPPs was their ability to deliver cargo using non-covalent attachment.<sup>90–92,99–101,113,114</sup> Many CPPs, such as TAT<sub>49–57</sub> and Penetratin, require covalent attachment for efficient cargo delivery.<sup>81,82,110–112</sup> By developing a non-covalent system, the carrier can simply be mixed with its cargo to form a transient complex that can dissociate upon entry into cells. Although this presents in vivo limitations because of the high risk of non-specific binding, it could mean less complex synthetic procedures and fewer experiments to ensure activity is not lost upon chemical conjugation.

### Synthetic CPPs

Synthetic CPPs are based on a peptide backbone but are not derived from naturally occurring protein or peptide sequences.<sup>85</sup> CPPs that fall into this category include polyarginine; Amphiphatic Model Peptide (MAP), which is a lysine-rich secondary amphiphatic peptide; and CADY, which is arginine-rich and self-assembles to yield a facially amphiphilic structure.<sup>104,115</sup> While polyarginine does not require secondary structure or self-assembly for delivery, the delivery activities of MAP and CADY are highly dependent on their secondary structures.<sup>104,115</sup> Disruptions in the helices or self-assembly lead to diminished delivery efficiencies.<sup>104,115</sup> The primary focus will be placed on polyarginine due to its importance in the development of synthetic guanidinium-rich molecular transporters.

“Polyarginine” and “oligoarginine” broadly define a series of peptide sequences that only contain arginine residues and, depending on length, may be synthetically easier to prepare than TAT<sub>49–57</sub>.<sup>80–82,84,110</sup> As part of Wender et al.’s<sup>84</sup> study in 2000, polyarginine (lengths = five to nine residues) was shown to outperform TAT<sub>49–57</sub>, with longer sequences performing better than shorter ones. Polyarginine sequences were also compared to their D enantiomers and their corresponding peptoids.<sup>84</sup> Both sets led to better cellular uptake. This indicated that cellular uptake was not dependent on hydrogen bonding present in the peptide

backbone and that the peptide backbone may not be necessary.<sup>84</sup> Another study from Mitchell et al. in 2000 showed that guanidinium groups led to superior uptake efficiencies compared to other possible cationic residues.<sup>83</sup> They also demonstrated that cellular uptake increased as the number of arginine residues increased up to 15 and that further increases in arginine content led to decreased cellular uptake.<sup>83</sup> This suggested that there is a critical number of arginine residues required for efficient uptake/delivery.

Unlike other hydrophobic-containing CPPs such as Pep-1, MPG, MAP, and CADY, which contain hydrophobic components to aid in cellular uptake, polyarginine is purely hydrophilic. To further explore polyarginine’s internalization efficiencies, Matile and coworkers<sup>106</sup> studied the effect counterions have on cellular uptake. In 2005, they reported that pairing polyarginine with bulky, aromatic activators, such as pyrenebutyrate, led to better peptide activity. This study suggested that there was some intrinsic benefit to having a hydrophobic component in addition to cationic charge as opposed to just having cationic charge.<sup>106</sup>

Through systematic studies with polyarginine and other synthetic peptides, it became clear that efficient delivery could be achieved without restricting structures to naturally occurring sequences.<sup>83,84,106</sup> Not only did structure-activity relationships (SARs) reveal that these molecules could be optimized for more efficient delivery through altering molecular weights and/or structural compositions, but also that the peptide backbone was not necessarily essential for successful delivery. This set the stage for the development of tunable delivery vehicles based on novel, abiotic scaffolds.

### Alternative Classification of CPPs

As an alternative to the aforementioned CPP classification system, CPPs can be classified based on their distribution of hydrophilic/cationic amino acids and hydrophobic amino acids.<sup>116</sup> These categories include primary amphipathic, secondary amphipathic, and non-amphipathic CPPs.<sup>116,117</sup> A summary of CPPs that fall into these categories can be found in the last column of Table I. For primary amphipathic peptides, segregation of cationic and hydrophobic amino acids is based on the sequential order of amino acids. Examples of these peptides include Pep-1, MPG, and Transportan.<sup>90–92,98–100</sup> Secondary amphipathic peptides, such as penetratin, MAP, pVEC, and M918, achieve the segregation of cationic and hydrophobic amino acids through their secondary structures, which enable them to either form  $\alpha$ -helices or  $\beta$ -sheets.<sup>95,102,104,105,109,118</sup> Lastly, non-amphipathic peptides contain mostly cationic residues, with no cationic/hydrophobic amino acid segregation including polyarginine and TAT<sub>49–57</sub>.<sup>82–84,89,110</sup>

## SURVEY OF CELL-PENETRATING PEPTIDE MIMICS

Although a large body of work focused on the development of peptide and peptidomimetic scaffolds for intracellular delivery,<sup>79,83,84,119–127</sup> moving away from naturally occurring amino acid residues and peptide backbones offers many advantages. Solid phase peptide synthesis can be avoided, which saves both time and money in producing the desired delivery vehicles. Additionally, a non-peptidic system, such as a polymeric scaffold, permits the use of different chemistries and allows the chemical compositions and polymer architectures to be tailored for efficient cargo delivery.<sup>23</sup> As a direct result of the easily tunable scaffolds, these materials are expected to be more potent and provide new models for fundamental studies. One prominent example of this is Tew and coworkers<sup>128</sup> oxanorbornene-based mimics of R<sub>9</sub>, referred to as GMe<sub>9</sub> and dG<sub>9</sub>. These polymeric-based mimics both contain nine repeat units, but dG<sub>9</sub> has double the guanidine density (18 charges) than GMe<sub>9</sub> (nine charges).<sup>128</sup> Both of these mimics perform better than R<sub>9</sub>, with dG<sub>9</sub> outperformed GMe<sub>9</sub>, suggesting that higher guanidinium density yields better cellular uptake.<sup>128</sup> Branched peptide scaffolds have been developed,<sup>127</sup> which increase the guanidinium density. These molecules have similar uptake efficiencies to their linear analogs; however, they are synthetically more difficult to access and develop, making it harder to tune uptake and delivery efficiencies.<sup>127</sup> Figure 3 charts the development and progress of guanidinium-rich molecular transporters. While this review highlights the use of guanidinium-rich, polymeric materials, other non-polymeric scaffolds have been developed and extensively studied, including guanidinioglycosides, dendrimers/branched materials, and carbohydrates.<sup>129–137</sup>

## Ring-opening Metathesis Polymerization-based Scaffold

In 2008, Tew and coworkers, as well as Kiessling and coworkers independently demonstrated that they could use ring-opening metathesis polymerization-based scaffolds to design materials with CPP-like activity. Tew and coworkers have used oxanorbornene-based systems for the design of their materials and Kiessling and coworkers have primarily used a norbornene-based system. Both groups selected ROMP because it offers fast, efficient, and functional group tolerant polymerizations.<sup>138–146</sup> These polymerizations are often living, allowing for good control over molecular weights and polydispersities and allowing for the formation of more advanced architectures, such as block copolymers.<sup>23,129,147,148</sup> Both groups' work is summarized below.

**Oxanorbornene-based CPPMs.** The first ROMP-based CPPM developed by Tew and coworkers<sup>149,150</sup> was polyguanidinium oxanorbornene (PGON). This molecule (Figure 4) was originally designed as an alternative to their amine-based antimicrobial agents.<sup>149</sup> Although it had good antimicrobial activity against *E. coli* and *S. aureus* and was non-hemolytic, it was also not membrane-disruptive, suggesting it could also be used as a molecular transporter.<sup>149</sup> Preliminary dye release experiments using model vesicle systems confirmed that PGON (degree of polymerization (DP) = 5–41) was able to induce dye release in a non-linear, molecular weight dependent fashion, further indicating it was membrane active and could be used as a molecular transporter.<sup>150</sup>

In an effort to improve PGON's activity, hydrophobic monomers, which contained alkyl chains ranging from methyl to dodecyl, were copolymerized with guanidinium-rich monomers to obtain a new series of CPPMs (Figure 4). Inspiration for this came from a Matile and coworkers<sup>106</sup> study, which showed that hydrophobic counter ion activators improve cellular uptake of polyarginine. The goal was to develop CPPMs that were "self-activating", meaning they did not need external activators for improved efficiency.<sup>151</sup> CPPM activity improved with increasing alkyl chain length up until the incorporation of the butyl chain (Figure 4)<sup>151</sup> Beyond this, CPPM insolubility led to a reduction in polymer activity.<sup>151</sup> All CPPMs, however, performed better than PGON, indicating the importance of incorporating hydrophobic moieties.<sup>149–151</sup> As a follow up to this study, CPPMs were designed in order to assess the role of aromaticity.<sup>152</sup> Aromatic groups were selected for study because they are common structural features in CPPs, such as Pep-1 and Penetratin, and because some of the best CPP activators contain aromatic groups.<sup>90–92,106,109,112</sup> Also, many cellular components contain aromatic groups, such as transmembrane proteins, which use aromatic amino acids to stabilize the interface between the hydrophilic and hydrophobic portions of the protein.<sup>153,154</sup> In addition, it has been shown that the flat, rigid shape of aromatic rings along with their quadrupole moments can aid in membrane interactions.<sup>155</sup> The CPPMs containing phenyl rings were the most active in the series, thus indicating the importance of aromaticity for CPPM activity.<sup>152</sup> Further studies looked to probe the role of  $\pi$ -electronics in these phenyl ring systems through the incorporation of electron donating and electron withdrawing groups. All CPPMs, regardless of the electron rich or electron poor ring incorporated, maintained similar membrane activities.<sup>156</sup> This demonstrated both the limits of the structural tuning that could be performed in this system and the number of structural options available without loss of membrane activity.<sup>156</sup>

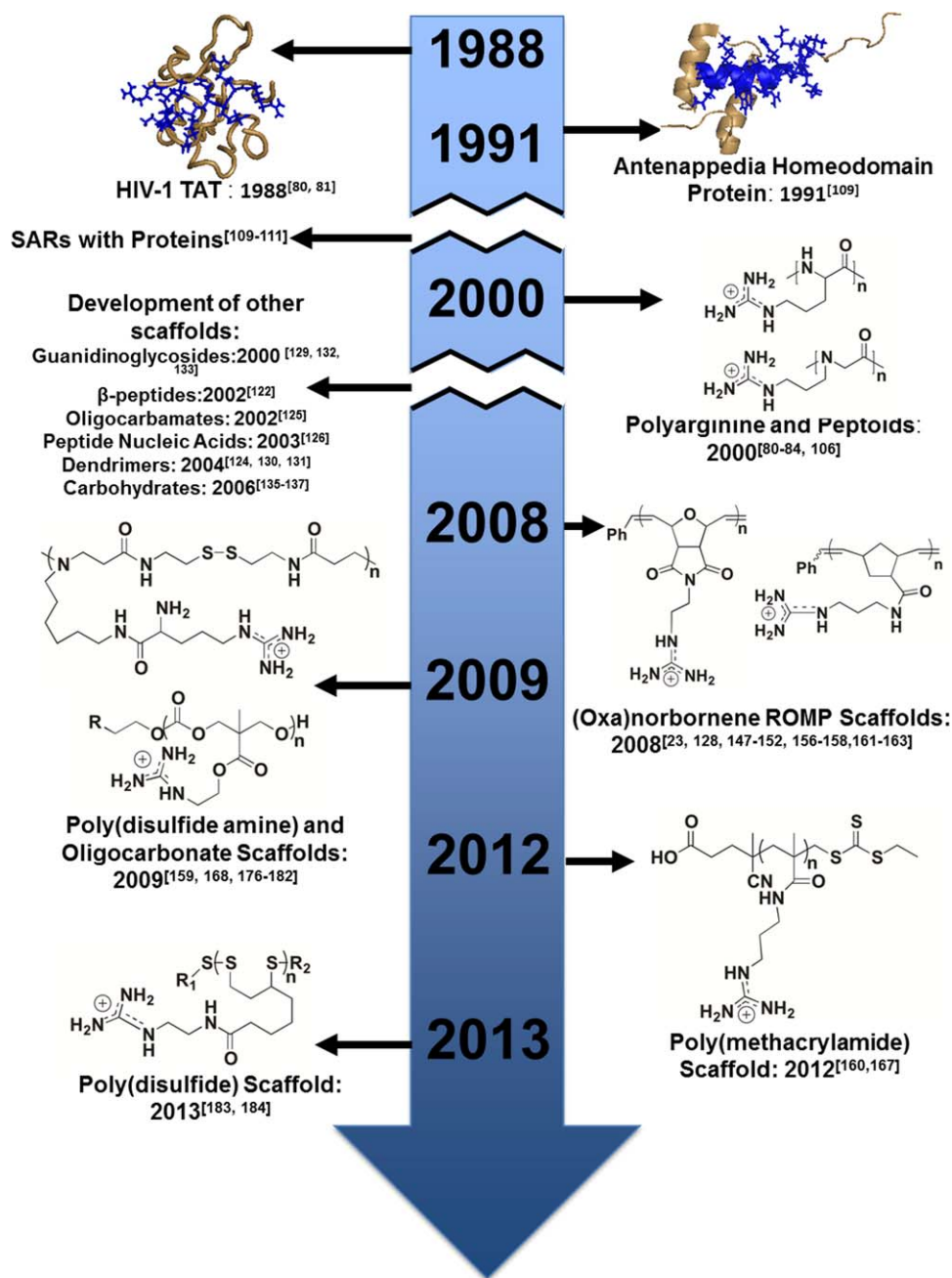
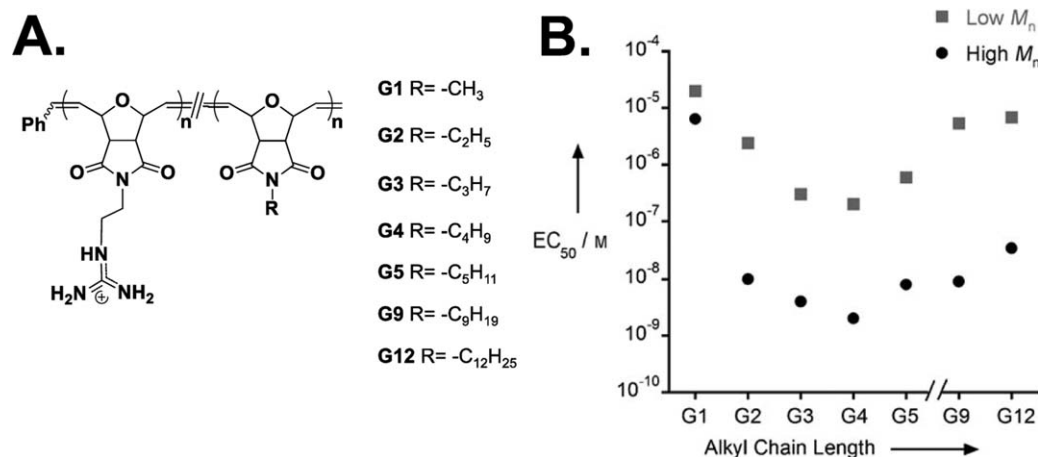


FIGURE 3 Development timeline for key guanidinium-rich CPPM scaffolds.

Tew and coworkers also designed CPPMs using di-armed oxanorbornene monomers. This system added more synthetic versatility as each monomer contains two functionalities that can be independently tuned.<sup>23</sup> Homopolymers containing one guanidine group and one hydrophobic group (aliphatic, aromatic, electron rich/electron poor aromatic systems) per monomer were designed as a direct comparison to their imide counterparts. These polymers had similar activities compared

to the imide system.<sup>151,156</sup> In addition, further studies were aimed at understanding the role of functional group segregation. The results indicated improved delivery efficiencies of a block copolymer scaffold without loss of activity in the presence of serum compared to its homopolymer counterparts (Figure 5A).<sup>157</sup> This block copolymer was designed to capture the guanidinium-rich nature of TAT<sub>49-57</sub> and the amphiphilicity of Pep-1.<sup>82-84,90-92</sup> Follow-up studies explored the



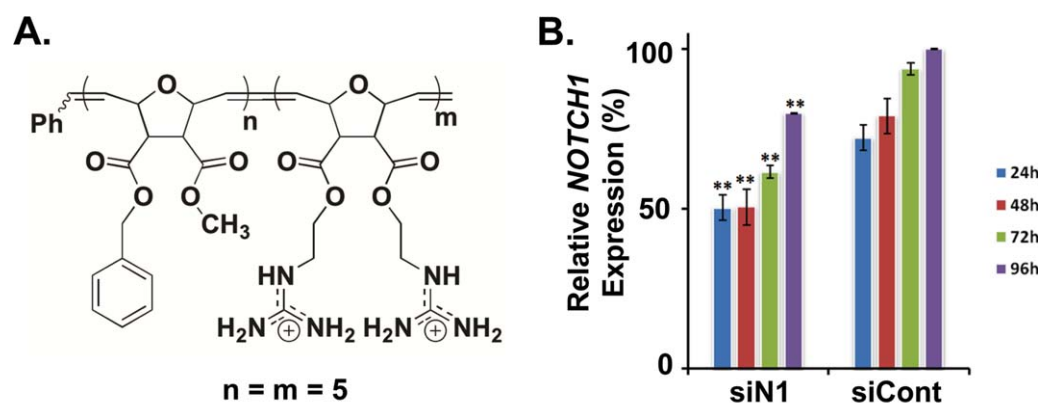


**FIGURE 4** Oxanorbornene-based “self-activating” CPPMs. (A) Molecular structures and (B) Plot of EC<sub>50</sub> vs. alkyl chain length.

functional group distribution using constitutional macromolecular isomers, which are polymers of the same DP but different arrangements of their pendent groups, ranging from completely segregated to completely mixed.<sup>158</sup> These arrangements were accessed through block (completely segregated), gradient (partially segregated), and homopolymerizations (mixed distribution).<sup>158</sup> Although studies indicated that the homopolymers enabled the best cellular uptake of the polymers, it is likely that block copolymers will deliver cargo more efficiently, as suggested by the literature.<sup>148,157,159,160</sup>

While early studies with PGON strictly used model vesicle systems to assess membrane activity, di-armed homopolymers were used to assess the effect of guanidinium density on cellular uptake in three different cell lines: HEK293T, CHO, and Jurkat T cells.<sup>128</sup> Although these polymers could enter all cell types with low cell death, uptake efficiencies

were highly cell line dependent.<sup>128</sup> In all cases, these CPPMs outperformed polyarginine (R9).<sup>128</sup> These studies were extended to explore the constitutional macromolecular isomers, wherein cellular uptake was the best for the homopolymer tested and uptake efficiencies were found to be cell-type dependent. To further these studies and probe the effect of architecture when delivering biologically relevant cargo, a block copolymer was synthesized containing a 1:1 ( $n=m=5$ ) ratio of hydrophobic to cationic monomers (Figure 4).<sup>157,161</sup> This block copolymer was shown to deliver FITC-tagged siRNA and bioactive siRNA to NOTCH1 (siN1) to Jurkat T cells and human peripheral blood mononuclear cells (hPBMCs), respectively, with >90% viability.<sup>157</sup> Delivery efficiencies were not altered by the presence of serum proteins in the media. The NOTCH1 knockdown results were noteworthy because 50% knockdown of an



**FIGURE 5** Delivery of siRNA to NOTCH1 (siN1) into hPBMCs. (A) Block copolymer structure used for delivery. (B) Percent relative protein expression as a function of time for PBMCs that received siN1 and PBMCs that received a scrambled, negative control (siCont).

active gene was reported (Figure 5).<sup>157</sup> This siRNA study represents proof of concept work that explored the delivery capabilities of this type of CPPM. Additional SARs studies are underway to develop design parameters for efficient siRNA delivery using these molecular transporters. These studies will also be expanded in order to develop design parameters for other biologically relevant cargo.<sup>23</sup>

**Norbornene-based CPPMs.** Kiessling and coworkers<sup>147,148</sup> developed a norbornene-based scaffold. Single-armed norbornene monomers used for these materials initially contained succinimidyl ester moieties, which provided handles for post-polymerization functionalization. Polymeric materials could be reacted with N-(3-aminopropyl)guanidine in the presence of N-methyl morpholine to achieve near complete replacement of the succinimidyl ester pendent groups. This scaffold is based on previously published work.<sup>162</sup> The guanidinium-rich scaffold builds on this initial work by terminating the polymers with an enol-ether, which allows for dye-labeling the polymer chains for easy tracking of the materials during cellular experiments.<sup>147</sup> Uptake of these labeled molecules in HeLa cells was monitored using fluorescence imaging and results indicated that the polymer was trapped in endosomes, as noticed by punctate fluorescence, with some polymer dispersed in the cytosol.<sup>147</sup> Greater than 95% viability was observed for all polymer concentrations tested (up to 5  $\mu$ M).<sup>147</sup>

Given that after post-polymerization functionalization only homopolymer and random copolymer architectures could be accessed, follow-up work looked at the formation of block copolymers.<sup>147,148</sup> Two monomers were developed that could be modified post-polymerization by chemospecific methods, one succinimidyl ester-containing monomer and one alpha chloroacetamide-containing monomer.<sup>148</sup> These monomers were polymerized sequentially in order to yield a block copolymer template. Based on the different chemistries involved, this template could be modified post-polymerization using separate reactions to yield segregation of attached functional groups.<sup>148</sup> High degrees of conversion were demonstrated for both starting functional groups.<sup>148</sup> Internalization of dye labeled block copolymers was demonstrated using HeLa cells, with cellular uptake following similar patterns to their previously reported homopolymer counterparts.<sup>148</sup>

More recently, Kiessling and coworkers<sup>163</sup> developed a completely degradable ROMP scaffold using oxazinone-based monomers. Authors demonstrated that a wide range of functional groups could be incorporated into these scaffolds, making them potential candidates for drug delivery and biomaterials applications.<sup>163–166</sup>

## Polymethacrylamides

In 2012, McCormick and coworkers reported the synthesis of guanidinium-rich polymethacrylamides using aqueous reversible addition fragmentation chain transfer (aRAFT).<sup>160</sup> This synthetic method was advantageous for the synthesis of materials because polymerizations by this method are well-controlled and the guanidinium-containing monomers could be polymerized without protecting groups.<sup>160</sup> Homopolymers were synthesized using a N-3-(dimethylaminopropyl) methacrylamide monomer and copolymers were synthesized using a N-3-(dimethylaminopropyl) methacrylamide and N-2-(hydroxypropyl) methacrylamide monomers.<sup>160</sup> Similar to polymers generated by the Tew and Wender groups, both sets of polymers were readily able to enter KB cells, with block copolymers out performing their homopolymer counterparts.<sup>148,157,159,160</sup> Authors note that the good control over the polymerization technique could enable this platform to be used for bioactive cargo delivery.<sup>160</sup> More recently, Peneva and coworkers<sup>167</sup> developed a series of guanidinium-rich, statistical copolymers using aRAFT as potential siRNA delivery reagents. Binding strength and competitive binding were studied but, to date, no cellular uptake or viability results have been reported.<sup>167</sup>

## Oligocarbonates

Wender/Hedrick/Waymouth and coworkers developed a series of CPPMs based on oligocarbonate polymers.<sup>159,168</sup> Molecules were synthesized using metal-free, ring-opening polymerization of cyclic carbonates.<sup>159,168</sup> Initiators for these materials were designed such that the drug molecule or molecular probe to be delivered could be attached at the beginning of the polymerization to allow for simpler conjugation to various cargoes.<sup>168</sup> These molecules were shown to be biodegradable under physiological conditions, with half-lives around 8 h. Cellular uptake in Jurkat T cells revealed that these polymers entered in a charge-dependent manner, with longer polymers outperforming their shorter counterparts. To assess whether a biologically active cargo could be delivered, proof-of-concept experiments were performed in luciferin was successfully delivered and shown to luminesce in HepG2 cells.

In a follow-up study, Wender/Hedrick/Waymouth and coworkers<sup>159</sup> showed that they could tune their oligocarbonate structures through the incorporation of hydrophobic moieties for more efficient siRNA complexation and release. Experiments with a dual-functional reporter HaCaT cell line that expressed both enhanced green fluorescent protein (EGFP) and Tomato fluorescent protein (tdTOM) was utilized to show specificity of knockdown.<sup>159</sup> Delivery of siRNA to tdTOM was shown to yield efficient reduction in tdTOM protein levels but

**Table II Summary of Common Methods CPP(M) of Internalization Under Specific Conditions**

Inhibitor	Affected Pathways	Blocking Mechanism
Lowered Temperature (4 °C) <sup>193</sup>	Energy dependent	Reduces cell metabolism, inhibiting energy-dependent pathways
Sodium azide and 2-deoxy-D-glucose <sup>194</sup> Cytochalasin D <sup>119</sup>	Energy dependent Macropinocytosis	Depletes ATP Promotes disassembly of the actin cytoskeleton and blocks actin polymerization
Wortmannin <sup>195</sup>	Macropinocytosis and Clathrin-mediated endocytosis	Inhibitor of phosphatidylinositol 3-kinase inhibitor
5-(N-ethyl-N-isopropylamiloride) <sup>119</sup> Chloroquine <sup>196,197</sup> Nocodazole <sup>198,199</sup>	Macropinocytosis Endosomal Escape Macropinocytosis	Inhibitor of sodium-proton pump exchange Promoted endosomal escape Blocks actin polymerization and disrupts actin cytoskeleton
Chlorpromazine and sucrose <sup>200</sup> Dynasore <sup>201</sup> Methyl- $\beta$ -Cyclodextrin <sup>202</sup>	Clathrin-mediated endocytosis Clathrin-mediated endocytosis Clathrin-independent endocytosis	Depletes clathrin and AP2 adapter protein complex Dynamin inhibitor Cholesterol extraction from membrane

to have a negligible effect on EGFP protein levels, thus demonstrating good knockdown specificity.<sup>159</sup> Polymers were shown to perform better in serum free media (86% knockdown) than in serum-containing media (64%).<sup>159</sup> Oligomers that incorporate longer alkyl chains (up to dodecyl) were also shown to outperform their counterparts that contained shorter alkyl chains.<sup>159</sup> Shorter oligomer lengths were also shown to outperform their longer counterparts.<sup>159</sup> Cell viability studies using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated that the most active polymers showed significant toxicity at the higher concentrations tested (100 nM) but could be greatly improved by cutting the polymer concentrations in half (50 nM).<sup>159</sup> Lastly, it was demonstrated that knockdown efficiencies and complex sizes could be tuned by mixing different oligomers.<sup>159</sup> Overall Wender/Hedrick/Waymouth and coworkers have developed a versatile, biodegradable platform for efficient delivery of biologically active siRNA. This platform has since been expanded by Wender et al.<sup>169</sup> to include glycerol-based monomers to allow for better control over oligocarbonate/siRNA complex stability.

### Poly(disulfide)s

Poly(disulfide)s are polymers that contain at least one disulfide bond in the polymer repeat unit structure.<sup>170</sup> These materials are distinctly different from proteins and vulcanized rubber, which both utilize disulfide bonds for crosslinking.<sup>170</sup> Poly(disulfide)s can serve as efficient delivery reagents for nucleic acids and proteins, releasing their cargo through reductive depolymerization in the presence of glutathione. Initially poly(amido amines) (PAAs) containing disulfide bonds were explored, which were synthesized by reacting cationic and/or hydrophobic monomers with cystaminebisacrylamide using Michael addition.<sup>171,172</sup> This platform is very functional group

tolerant, allowing for the incorporation of a wide range of functionalities, which enabled fine-tuning of PAA structures for efficient uptake and delivery.<sup>173–175</sup> S. W. Kim and coworkers, utilized a similar platform to design poly(disulfide amines) and their guanidinium-rich counterparts, which are often referred to as arginine-grafted bioreducible polymers (ABPs) and guanidinylated bioreducible polymers (GBPs).<sup>176–182</sup> The ABPs and GBPs molecules led to higher transfection efficiencies when compared to their amine counterparts.<sup>177,179–182</sup> ABPs have been explored for RNAi applications related to anti-angiogenesis gene therapy of tumors as well as ex vivo pDNA delivery vehicles for treatment of ischemic heart diseases.<sup>177,179–182</sup>

While most poly(disulfide) delivery reagents utilize non-covalent delivery strategies, Matile and coworkers<sup>183,184</sup> developed an efficient method of generating cell penetrating poly(disulfide)s that are covalently attached to their cargo utilizing surface-initiated ring-opening disulfide-exchange polymerization. Many probes, drugs, and bioactive cargoes contain or can be easily made to contain thiol moieties, which makes them convenient initiating species for this polymerization method. Molecule formation and subsequent depolymerization in the presence of dithiothreitol (DTT) were monitored using dye-loaded vesicles.<sup>183,184</sup> Fast, efficient delivery (5 min), and subsequent fast depolymerization rates to release cargo into the cytosol (1 min) were demonstrated in HeLa cells.<sup>184</sup> Low toxicities were demonstrated for all molecules tested up to 10  $\mu$ M.<sup>184</sup> Molecular uptake mechanism was independent of the cargo but could be altered based on the hydrophobic/cationic content of the materials.<sup>183</sup> The authors also suggested a thiol/counter ion-mediated uptake mechanism to explain cell entry and how chemical compositions of the delivery vehicles change their ability to enter cells efficiently.<sup>184</sup>

## MODES OF INTERNALIZATION FOR CPP(M)S

The mechanisms of CPP(M) uptake remains highly debated in the literature.<sup>79,116,119,123</sup> Early studies suggested direct translocation as the primary mode of internalization, which refers to molecules crossing membranes and directly entering the cytosol.<sup>185–187</sup> However, many of these observations were shown to be erroneous, primarily due to cell fixation, which permeabilizes cell membranes and allows extracellular cargo to be internalized.<sup>185–187</sup> Additional modes of uptake have been considered, including engulfment of particular molecules by the cell's membrane through forms of endocytosis, such as Clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and receptor-mediated endocytosis.<sup>116,119,123,187</sup> Glycosaminoglycans and lipid compositions are also thought to play a role in uptake mechanism.<sup>119</sup> Complicating matters even further, experimental conditions including, but not limited to, composition, cargo, concentration, and cell types as well as incubation temperatures and times may play distinct roles in modes of internalization.<sup>79,116,119,123</sup>

Extensive effort has been made to understand the primary modes of internalization. One common way to do so is to inhibit certain entry methods and to compare uptake and delivery efficiencies to those cells under normal conditions.<sup>188–190</sup> A summary of common methods can be found in Table II. It is important to keep in mind that inhibiting specific modes of uptake may actually cause the cell to up-regulate other modes of entry or lead to off-target effects.<sup>188–190</sup> It is also important to note that CPPs likely enter cells through multiple methods at the same time, with the different possible modes of cellular uptake being highly cell-type dependent.<sup>116,191,192</sup>

In addition, colocalization studies, which look at where polymer/cargo complexes end up in cells, and biophysical methods, which look at interactions between polymer/cargo complexes with lipid membranes, have also been utilized to elucidate uptake mechanism.<sup>106,151,152,203–211</sup> To date, no one method has been shown to clearly document all possible and prominent methods of internalization.<sup>79,116</sup> Often many methods need to be taken together in order to begin to understand what is happening in the cellular environment. This challenging problem is an area of active research.

## PERSPECTIVES AND CONCLUSIONS

Structure-activity relationships with proteins led to essential design features for CPPs and enabled protein mimics to be realized. The extension to polymers represents another critical step in our fundamental understanding and is expected to produce some of the most useful CPPMs. Delivery of proteins and

other biologics is going to be a central theme looking forward, which means delivery systems must be able to meet the challenges associated with these complex molecules. CPPMs appear to have an important role to play in this area, having already demonstrated their ability to perform in the arena of new immunological research and fundamental cell biology. Therapeutic delivery for the treatment of human disease, though a more difficult and complex issue, remains the ultimate goal. While the challenge is daunting, CPPMs are a promising technology undergoing continual refinement and offering many potential advantages.

The authors would also like to thank Ms. Coralie Backlund, Ms. Kelly McLeod, and Mr. Nicholas Posey for their feedback on early drafts of this manuscript.

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