Sophistication of foldamer form and function *in vitro* and *in vivo*

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Advances in the foldamer field in recent years are as diverse as the backbones of which they are composed. Applications have ranged from cellular penetration and membrane disruption to discrete molecular recognition, while efforts to control the complex geometric shape of foldamers has entered the realm of tertiary and quaternary structure. This review will provide recent examples of progress in the foldamer field, highlighting the significance of this class of compounds and the advances that have been made towards exploiting their full potential.

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Introduction

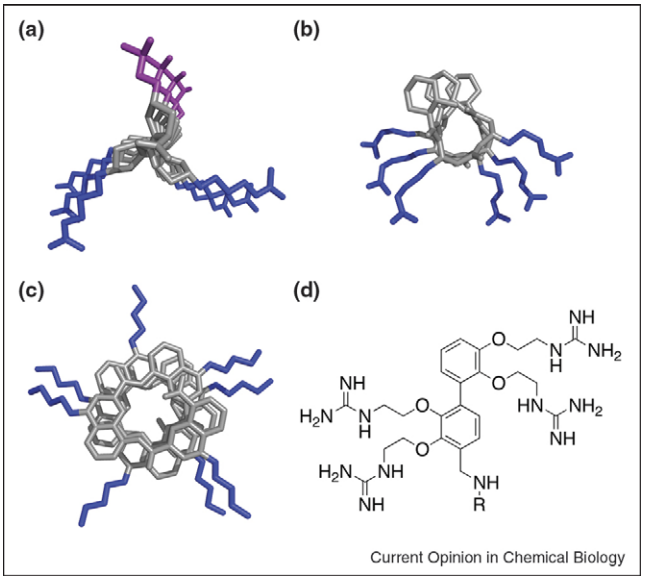
Life has evolved for billions of years in large part due to the sophisticated functions of an elite group of biological polymers. Unique in their historical position, biopolymers were long thought to be alone in the world of macromolecules that assume complex three-dimensional structures based solely on their primary sequence. A new field has emerged and flourished thanks to the realization that chemical moieties with unique, non-biological backbones, more popularly known as foldamers, are also capable of higher-ordered structure and function. This often begs the question as to why Nature chose nucleic acids and polypeptides as the vehicles of life. Advances in the foldamer field in recent years are as diverse as the backbones of which they are composed. Applications have ranged from cellular penetration and membrane disruption to discrete molecular recognition, while efforts to control the complex geometric shape of foldamers has entered the realm of tertiary and quaternary structure. This review will provide recent examples of progress in the foldamer field, highlighting the significance of this class of compounds and the advances that have been made towards exploiting their full potential.

Gaining entry – all in the charge

Cell-penetrating peptides (CPPs) have emerged as promising tools for the intracellular delivery of molecules ranging in size from small molecules and peptides to proteins and quantum dots [1–5]. Previously reported CPPs, such as the 9-aa HIV-1 Tat peptide [6], penetratin (16-aa) [7], and HSV-1 VP22 (34-aa) [8] are cationic and/or amphipathic, often with high α-helical propensity [9]. Sequences rich in arginine are particularly effective – in fact, short oligo-arginine peptides are among the most efficient encodable CPPs [10]. By virtue of their predictable secondary structures and proteolytic resistance [11], foldamers represent a burgeoning new CPP family. A host of foldamer CPPs have recently been reported or characterized, including those based on peptoid [10], β-peptide [12], and oligocarbamate [13] backbones.

Foldamers that function as CPPs must tolerate multiple cationic groups along their backbone. Although proline itself is not easily modified in this way, Chmielewski and co-workers [14,15] reported previously that oligomers of hydroxyproline bearing precisely arrayed hydrophobic and cationic appendages gained entry into MCF-7 cells (Figure 1a). One molecule, P14LRR, was 35-fold more cell-permeable at 15 μM concentration than the HIV-1 $Tat_{47–57}$ peptide. Confocal microscopy confirmed that P14LRR was present in both the cytosol and nucleus. Mechanistic investigations suggested a temperature-dependent, energy-independent endocytotic mechanism as the primary entry pathway. In related work, Gellman and co-workers [16,17] demonstrated that amphipathic β-peptides (Figure 1b) gained entry into HeLa cells at 8 μM with a distribution pattern similar to that of P14LRR in MCF-7 cells. Further studies revealed that β-peptides enter HeLa cells via a dynamin-independent, ameloride-sensitive endocytotic entry mechanism similar to macropinocytosis. While both polyproline type II helices and β-peptides achieve the goal of cell permeability, the latter possesses the advantage of small size (MW, P14LRR = 2979 Da; Gellman β-peptide decamer 1 = 1485 Da) as well as demonstrated compatibility with microwave synthesis [18]. In addition, the synthesis of β^3-amino acid monomers [19] is more straightforward than the pre-decoration of hydroxyproline. Most recently, Olsen and co-workers [20] have described an antiplasmodic α-peptide/β-peptoid that enters HeLa cells at a concentration of 10 μM. Further details on the mechan-

Figure 1



Cell-permeable foldamers bearing hydrophobic (purple) and/or cationic (blue) side chains. **(a)** Amphipathic hydroxyproline oligomers [14,15]; **(b)** cationic β-peptide 14-helices [16,17]; **(c)** aromatic amide polymer [21•]; **(d)** biaryl 4G-SMoC [22••].

ism of entry of this molecule are currently under investigation.

Cell-permeable foldamers have also been constructed from monomers that differ significantly from α-amino or β-amino acids. Huc and co-workers [21•] reported aromatic amide oligomers functionalized with peripheral cationic side chains that deliver fluorescein to HeLa cells with efficiencies similar to HIV-1 $Tat_{47–57}$ (Figure 1c). Like polyhydroxyproline and β-peptides, these oligomers localize to the cytosol and nucleus of live cells. Unlike these molecules, however, the aromatic amides do not require sequestration of the positively charged groups along a specific region of the helix.

Selwood and co-workers [22••] explored strategies toward fully synthetic delivery agents known as SMoCs (small molecule carriers) comprised of a biphenyl core functionalized with guanidinium groups in an arrangement that mimics the display of side chains on an α-helix (Figure 1d) [23]. SMoCs mediate the internalization of both dyes and recombinant proteins into a variety of live cells with efficiencies that equal or exceed than of $Tat_{47–57}$. After conjugation to a carrier containing four guanidinium groups (4G-SMoC), Alexa Fluor 488-labeled DNA replication repressor geminin (23.5 kDa) was effectively delivered to WI-38 human diploid fibroblasts (HDF), human U20S cells, and mouse NIH-3T3 fibroblasts, as detected by confocal fluorescence microscopy. In an impressive demonstration of potential future utility, 4G-SMoC-geminin effectively suppressed DNA synthesis in NIH-3T3, WI-38 HDF, HeLa S3 adenocarcinoma cells, and MOLT-4 leukemic lymphoblasts *in vitro*.

Though encouraging, much work remains toward the application of these non-natural CPP mimics as functional drug delivery vectors. Kodadek and co-workers [24••] have made significant progress toward this goal by developing a clever assay to evaluate cell permeability in non-natural peptidomimetics. This method was used to identify a peptoid that inhibits the activity of the proteasome 19S regulatory particle in living cells [25••]. In related work, peptoid-containing constructs have been discovered that activate gene expression without the assistance of transfection agents (vide infra) [26••,27].

Function realized

Mixed α/β-peptides

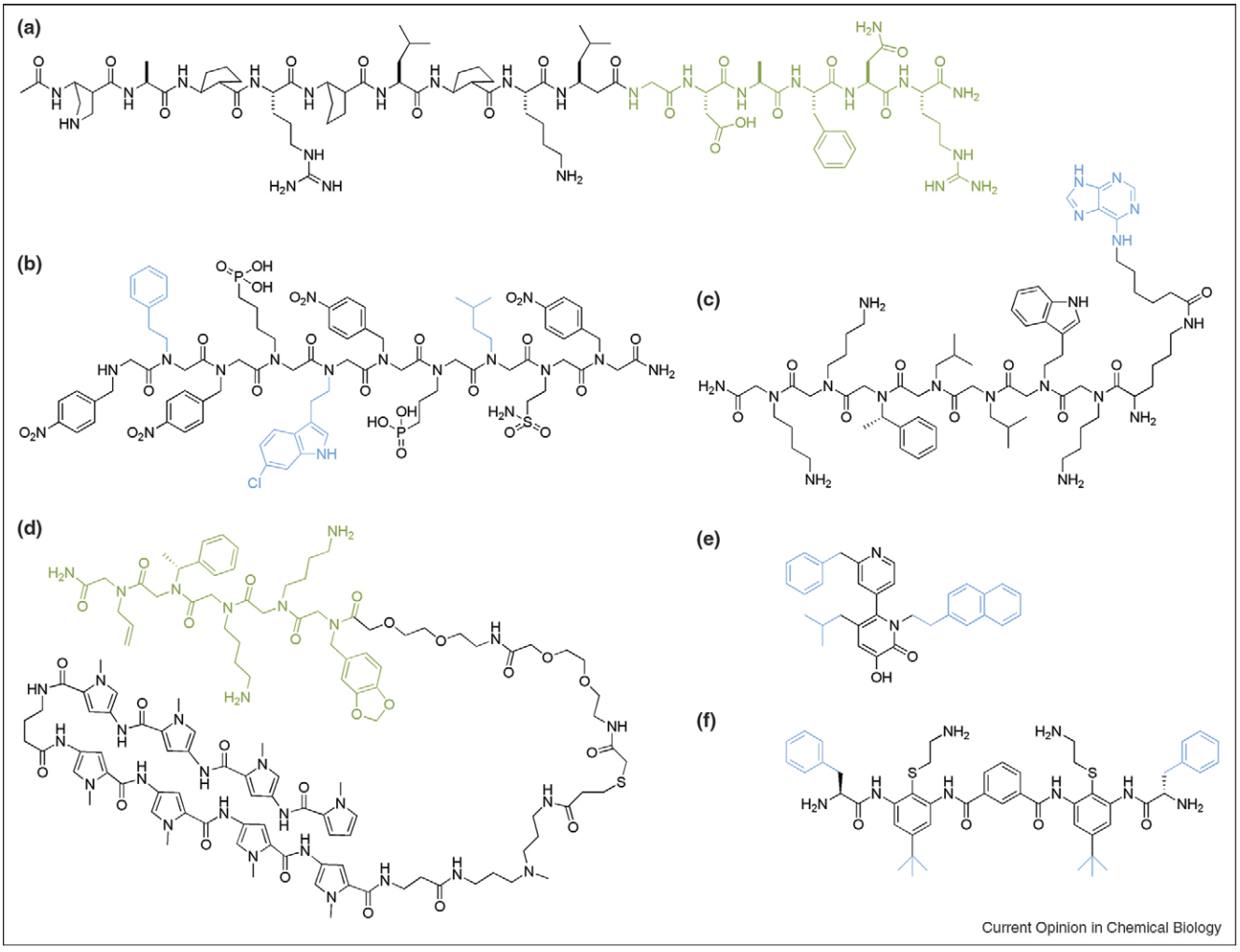
Several recent reports have documented the utility of β-peptides in biological applications, highlighting their potential as inhibitors of intramolecular or intermolecular protein–protein interactions. The substitution of β-amino acids into α-peptides has been reviewed previously [28], but new work by Muller and co-workers [29] expands the idea to the incorporation of aza-β^3-amino acids, which contain nitrogen in the backbone, into a model peptide that mimics a CD4 T cell epitope. These peptides were recognized by T cells generated to the parent peptide and were also able to generate T cells with modified cytokine production, indicating their immunogenic activity.

Foldamers composed of mixtures of α- and β-amino acids have also been evaluated as potential protein interaction inhibitors use. Libraries designed by Gellman and coworkers were screened to identify a 15-mer (α/β + α)-peptide that selects among antiapoptotic proteins (Bcl-x_L ~ Bcl-w > Bcl-2 ≫ Mcl-1) and inhibits Bcl-x_L–BH3 interactions in cell lysates as judged by cytochrome *C* release (Figure 2a) [30••]. Although these and related peptides are likely not cell permeable, their design provides a useful framework for similar experiments targeting other protein-binding pockets [31].

β-Peptides

A variety of smaller molecules composed entirely of β-amino acids have been identified as inhibitors of protein-protein interactions and viral fusion. Previously, Schepartz and co-workers described a series of 10-mer β-peptide 3_{14}-helices that inhibit the p53–hDM2 interaction *in vitro* [32,33] as well as HIV-1 gp41-mediated syncytia formation [34]. Recently, significantly improved affinity for hDM2 has been achieved using non-natural side chains, producing β-peptides that activate p53 in tumor cells with IC_{50} values below 5 μM (EA Harker, *et al.* unpublished). Gellman and co-workers [35] have used 2.5_{12}-helical β-peptides to block human cytomega-

Figure 2



Foldamers that function as protein interaction inhibitors. Key side chains and segments in each molecule are highlighted in blue and green, respectively. **(a)** (α/β+α)-peptide that binds Bcl-x_L with IC_{50} = 29 nM [30••]. **(b)** Peptoid that binds hDM2 with IC_{50} = 6.6 μM [39•]. **(c)** Peptoid that inhibits the proteasome 19S regulatory particle with $IC_{50} \sim 3$ μM in a protein unfolding assay [25••]. **(d)** Synthetic transcription factor mimic with K_d = 11.6 μM for GST-KIX fusion protein [26••]. **(e)** Biaryl scaffold that binds the estrogen receptor with IC_{50} = 4.2 μM [44••]. **(f)** Arylamide derivative that binds calmodulin with K_i = 7.10 nM [47].

lovirus (HCMV) entry, building upon the ability of this structure to closely mimic α-helical segments within the HCMV gβ glycoprotein. A series of 13-mer peptides showed reasonable potency ($IC_{50} \sim 30$ μM), selectivity over other viral glycoproteins in a cell-based infectivity assay, and inhibition of virus-cell membrane fusion in a virion content delivery assay. Recently, glycosylated β-peptides were shown using surface plasmon resonance to bind lectins [36], an application that has been further extended to the formation of molecular assemblies using cyclic tri-β-peptides [37].

Peptoids

A related class of molecules consisting of *N*-substituted glycines, or peptoids, has also been explored as protein interaction inhibitors. Notably, oligomers of these building blocks feature a non-natural backbone and form helical structures in solution [38]. Appella and co-workers identified peptoids that inhibit the interaction between p53 and hDM2 *in vitro* (Figure 2b) [39•,40]. Modifications aimed at improving aqueous solubility and affinity revealed that residues beyond the recognition epitope are important for binding hDM2 and demonstrated that structural characterization is still needed. Kodadek and co-workers [25••] reported another class of peptoid inhibitors with affinity for the 19S regulatory particle (RP) of the proteasome (Figure 2c). A library of peptoids, each capped with a purine analog to bias selection towards the proteasomal ATPases of the RP, identified a molecule that inhibited protein folding *in vitro* and induced

accumulation of p27, a target of the ubiquitin-proteasome pathway, in live cells.

Another powerful demonstration of the biological application of peptoid oligomers from Kodadek and co-workers is their use as synthetic transcriptional activation domains. Peptoid libraries were screened for binding to the CBP (CREB-binding protein) KIX (KID-interacting; KID = kinase-inducible domain) domain and the winners linked to the DNA-binding hairpin polyamide ImPy7 (Im = *N*-methyl-imidazole; Py = *N*-methylpyrrole) to generate a compound that could bind both DNA and KIX *in vitro* (Figure 2d) [26••,41]. In cells, this construct was recruited to a luciferase reporter gene whose promoter contained six ImPy7 binding sites, and induced a dose-dependent increase in luciferase expression. Most significantly, it was able to activate endogenous genes with multiple ImPy7 binding sites in their promoter regions as verified by mRNA upregulation. These results collectively illustrate the potential for biological applications of peptoids and provide useful insights into the optimization and screening of these types of molecules.

Additional structured scaffolds

A number of other non-natural oligomers with preorganized structure have been examined for their ability to recognize target peptides or proteins. One notable example are oligomers of pyrimidine hydrazide synthesized by Konig and co-workers [42] that bind peptides through complementary hydrogen bonding. These ligands lack the specificity elements of other rigid scaffolds that enable accurate mimicry of α-helical side chain display [43]. This strategy was adopted by Hamilton and co-workers [44••] using a conserved leucine-rich coactivator motif to interact with pockets on the estrogen receptor (ER) ligand-binding domain (Figure 2e). Several potent inhibitors based on a substituted biaryl pyridyl-pyridone scaffold directly compete with the coactivator estradiol for its ER binding site in a competitive radioligand assay.

An alternative rigid scaffold based on arylamide derivatives, previously used as a mimic of membrane-binding helical peptides [45], was adapted by DeGrado and co-workers [46••] to identify molecules that inhibit interaction of type I collagen with the α_2 I-domain of $\alpha_2\beta_1$ integrin. The most interesting molecule identified inhibited the collagen/integrin $\alpha_2\beta_1$ interaction in a platelet adhesion assay in whole cells (IC_{50} = 3.4 μM). The versatility of this arylamide scaffold was further highlighted in the design of calmodulin inhibitors that emulate a known α-helical calmodulin-binding peptide (smMLCK, smooth muscle myosin light-chain kinase) (Figure 2f) [47•]. Altogether, this recent data clearly illustrates the applicability of non-natural, well-structured scaffolds in diverse biological applications. Moreover, continued advances in the study of α-peptides, recently exemplified by DeGrado and co-workers [48] in targeting transmembrane helices, continue to extend the goals and potential of foldamer research.

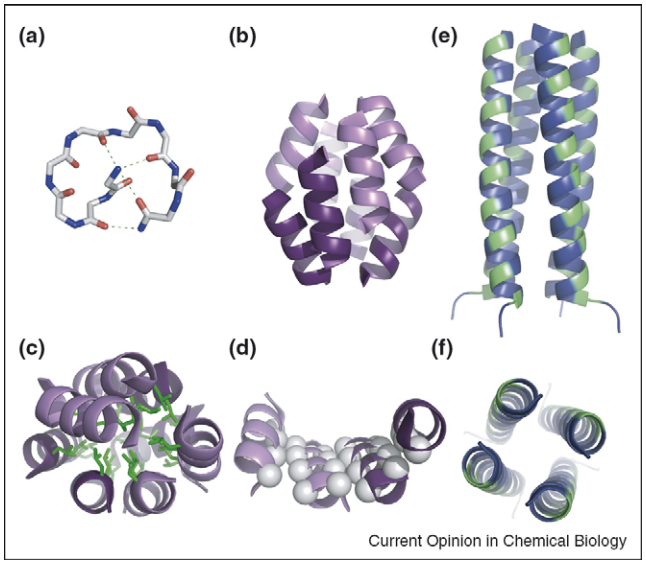
Foldamers take shape

Natural biopolymers fold with fidelity, can exist as oligomers or discrete complexes, and possess kinetic and thermodynamic signatures that distinguish them from non-biological polymers and smaller molecules. Significant progress has been made in the past year towards foldamers that assemble cooperatively into discrete tertiary and quaternary structures, paving the way towards foldamers with sophisticated function.

Peptoids

Although peptoid helices were described as early as 1998 [38], the development of other secondary or more complex higher-ordered structure has progressed only gradually. Recently, the rapid and efficient cyclization of peptoids greater than four residues in length allowed Kirshenbaum and co-workers [49••] to create a series of cyclic peptoids presenting a diverse array of side-chains in geometries very similar to those found in type I and III β-turns. We expect to soon see these molecules elaborated into turn mimetics with biological activity. The hairpin

Figure 3



Higher-order foldamer structures. **(a)** The threaded-loop structure of the peptoid nonamer, *N*spe$_9$, is facilitated by hydrogen bonds (green dashed lines) [51•]. **(b)** The octameric helical-bundle structure of the β^3-peptide, Zwit-1F, which resembles two hands with four fingers cupped over each other. **(c)** A side view of the Zwit-1F helical bundle depicting the packing of the hydrophobic core of β^3-hLeu residues (green sticks). **(d)** One tetramer of the helical bundle, Zwit-1F, illustrating the packing between backbone methylenes (spheres) of neighboring helices [63••]. **(e)** A side view of the α/β-peptide tetramer inspired by the α-peptide tetramer, GCN4-pLI. The α-amino acid residues are displayed in blue, while the β-amino acid residues are shown in green. **(f)** A top-down view of the α/β-peptide tetramer depicted in **(e)** [72•].

turn developed by Appella and co-workers is initiated by a 1,5-substituted triazole that allows the termini to associate favorably if hydrogen-bonding/salt-bridging entities are available [50]. A unique threaded loop conformation assumed by peptoid homo-nonamers of either (R)-*N*-(1-phenyethyl)glycine (Nrpe$_9$) or (S)-*N*-(1-phenyethyl)glycine (Nspe$_9$) has also been developed, whereby the protonated N-terminus 'threads' back into the center of the loop to form hydrogen-bonds with backbone carbonyls (Figure 3a) [51•]. In addition to exploring the effect of interactions between N-substituents and peptoid backbone carbonyl groups in altering the cis/trans tendencies of the peptoid amide [52], Blackwell and coworkers have used pentafluoroaromatic side-chains to disrupt helix-disfavoring conformers, such as the aforementioned Nspe$_9$ threaded-loop, by altering the ability of the backbone carbonyl groups to participate favorably in hydrogen bonds [53••].

β^3-Peptides

Previous efforts to rationally create higher-order β-peptide assemblies in water resulted mainly in poorly behaved aggregates with non-discrete stoichiometries [54,55]. The elegant efforts of Seebach and co-workers [56,57] towards the β-peptide equivalent of an α-peptide Zn-finger [58] came to fruition in the form of a β-hexadecapeptide that was designed to assemble into the characteristic Zn-finger fold in the presence of 1 equivalent Zn^{2+}. In the absence of structural information, ESI-MS indicates a 1:1 Zn^{2+} to peptide stoichiometry, although CD suggests that less than one equivalent of Zn^{2+} is bound per β-peptide, which may indicate oligomerization of these β-hexadecapeptides in solution (possibly in a discrete fashion) [59••].

Much progress has been made towards developing higher-ordered structure from molecules composed entirely of β^3-amino acids. Hetero-oligomeric and homo-oligomeric β^3-peptide helical bundles have been developed that illustrate both the similarities and differences between this class of foldamers and naturally occurring proteins. While some differences have been observed with respect to helical packing, mostly to accommodate eclipsed side-chains, the overarching principles that govern folding in natural proteins is maintained. In particular, the formation of salt-bridges, hydrogen bonds, and the ordered packing of a hydrophobic core, which are the hallmarks of well-characterized natural helical-bundles and coiled coils, engender discrete stoichiometries and cooperative folding in these β^3-peptide bundles.

Initial work in the Schepartz group focused on developing β^3-peptides that associated as hetero-oligomers in a 1:1 ratio, similar to the hetero-dimer formed by Fos and Jun [60]. These β^3-peptides assembled as an octamer in the micromolar concentration range [61•], whereas previous work generated species that did not assemble appreciably at concentrations lower than millimolar [55]. Subsequently, the high-resolution structural characterization of Zwit-1F (Figure 3b), a zwitterionic homo-octamerizing β^3-peptide, revealed the intricacies of the associations that drive its formation. The overall structure resembles two hands with four fingers cupped over each other. Inter-helical interactions on the exterior of the bundle are characterized by salt-bridges, whereas the eclipsed presentation of the β^3-homoleucine residues in a 3_{14}-helix does not allow the knobs-into-holes packing found in α-protein helical bundles and coiled-coils [62], and instead results in a nestling of residues against each other (Figure 3c). Interestingly, the characteristic 3.1 residues per turn allows close helical approach whereby the backbone methylene units can come into direct contact with each other, accounting for 25–33% of the packing between a given pair of helices (Figure 3d) [63••]. Extensive solution characterization is consistent with the formation of an octamer, and indicates that these helical bundles unfold with a protein-like thermal denaturation profile, and have kinetic and thermodynamic stabilities only slightly less than those of natural proteins [64••]. Substitution of multiple residues has generated a virtually identical structure with enhanced kinetic and thermodynamic stability [65]. This robustness has likely not been fully exploited. Further alterations could enhance stability even further, or facilitate the creation of species with prescribed stoichiometries.

Mixed α/β-peptides

The development of quaternary structure from mixed α/β-peptides by Gellman and co-workers utilized the well-characterized and highly stable natural protein GCN4 [66] as a starting point. This strategy relied on the previously observed tolerance of protein secondary-structure to incorporation of β-amino acids [67–70], which were substituted into the primary sequence of GCN4-p1 and GCN4-pLI [71] (9 of the 33 residues) at positions distal to the interacting interface to avoid destabilizing the dimer or tetramer interface, respectively. The peptide based on GCN4-p1 associated as a trimer via X-ray crystallography, but was monomeric at concentrations sampled via analytical ultracentrifugation (AUC), whereas the α/β-version of GCN4-pLI associated as a trimer by AUC and a tetramer by X-ray crystallography (Figure 3e and f) [72•]. Subsequent work in the Gellman group replaced two of the four helices of the α-peptide heterotetramer that constitutes Acid-pLL/Base-pLL [60,73] with mixed α/β-peptides. Replacement of every other residue in the sequence with a β-amino acid resulted in the formation of a heterotetramer, although cyclic β-amino acids were found to be requisite; removal of even a subset of these residues completely abolished the formation of discrete species [74•].

Conclusions

Within the last year, significant advances have been made towards extending the diverse applications of foldamers

and fully understanding their utility and versatility. These molecules are of particular interest due to the unique structures formed from the monomers of which they are composed. Initial work was focused on fully characterizing their structural features, but recent reports have highlighted their ability to enter cells, to recognize protein surfaces, and to even assemble into discrete tertiary and quaternary structures. While these results are certainly remarkable, they also indicate that we have only begun to scratch the surface of this exciting area of research. For example, though foldamers have been designed to enter cells, a clear understanding of the features that will render them functional within the cell remains to be developed. Similarly, additional work is needed to determine if this class of compounds will be able to serve as a general means of disrupting protein–protein interactions or viral fusion in a cellular context. The ability of these molecules to form higher order structures further points to their potential use as synthetic mimics of biological molecules. Clearly, there are a number of opportunities for the continued expansion of this thriving field in the near future.

Acknowledgements

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This paper describes a clever method to quantify the relative cell permeability of peptides and peptidomimetics such as peptoids. In this assay, the compound is conjugated to dexamethasone and incubated with a cell containing three plasmids. One of these harbors a Gal4 DNA-binding domain and a VP16 transactivation domain that is bound and activated by dexamethasone. The other two contain a Gal4-responsive firefly luciferase reporter gene and an independently expressed *Renilla reniformis* luciferase, respectively. The concentration of compound inside the cell is measured by quantifying the ratio of luciferase output from these two reporter plasmids.

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