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REVIEW

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Peptide/protein-based macrocycles: from biological synthesis to biomedical applications

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Living organisms have evolved cyclic or multicyclic peptides and proteins with enhanced stability and high bioactivity superior to their linear counterparts for diverse purposes. Herein, we review recent progress in applying this concept to artificial peptides and proteins to exploit the functional benefits of these macrocycles. Not only have simple cyclic forms been prepared, numerous macrocycle variants, such as knots and links, have also been developed. The chemical tools and synthetic strategies are summarized for the biological synthesis of these macrocycles, demonstrating it as a powerful alternative to chemical synthesis. Its further application to therapeutic peptides/proteins has led to biomedicines with profoundly improved pharmaceutical performances. Finally, we present our perspectives on the field and its future developments.

Introduction

Cyclization is a simple modification of a linear polymer chain by intramolecular chemical ligation, forming macrocycles with certain changes in properties.[1–4] Depending on the sites of ring closure (head-to-tail *vs.* side-chain ligation), macrocycles may exist in different types. The categorization and variation of macrocycles is a subject of study in chemical topology which concerns the invariant properties of a molecule during continuous deformations such as bending, twisting, and stretching, or simply put, the connectivity and spatial relationship of a molecule.[5–12] To illustrate this concept, we take the molecular graphs of three macrocycle variants shown in Fig. 1A as an example.[8,13] Graph I has a connectivity completely different from the other two. Although Graph II and III have identical connectivity, the latter has an additional entwined spatial relationship in 3D space. As a result, all of them have distinct topology from each other. To provide a broad coverage of complex cyclic structures, the concept of macrocycles is stretched in this review to cover a broad range of cyclic variants with different topologies as shown in Fig. 1B. Specifically, when

cyclization occurs on non-entangling polymer chains, (multi)cyclic and cyclic-branched macrocycle variants are obtained; when cyclization occurs on knotted or entangled precursors, macrocyclic knots and links with nonplanar molecular graphs (molecular graphs which could only be embedded in 3D space) are usually obtained where the molecular segments are mechanically interlocked (*i.e.*, there is an entanglement between molecular entities such that they cannot be separated without breaking or distorting chemical bonds). In both cases, cyclization plays a vital role in defining connectivity and preventing the disentanglement of polymer chains, which could help reshape the conformational space of the polymer and endow properties distinct from its linear counterpart. In peptides and proteins, cyclization exerts significantly more conformation restriction on the unfolded state rather than the folded state, thereby increasing the stability of the folded state.[14–16] Eliminating the free termini of polypeptide chains also reduces the possibility of enzymatic degradation by exopeptidases.[17–19] The effects are perhaps even more significant for those with nonplanar molecular graphs, where tight coupling between molecular segments and dynamic transition between different states are anticipated.[20,21] Therefore, over the past decades, peptide/protein-based macrocycles have emerged as a unique class of macromolecular therapeutics.[22–30]
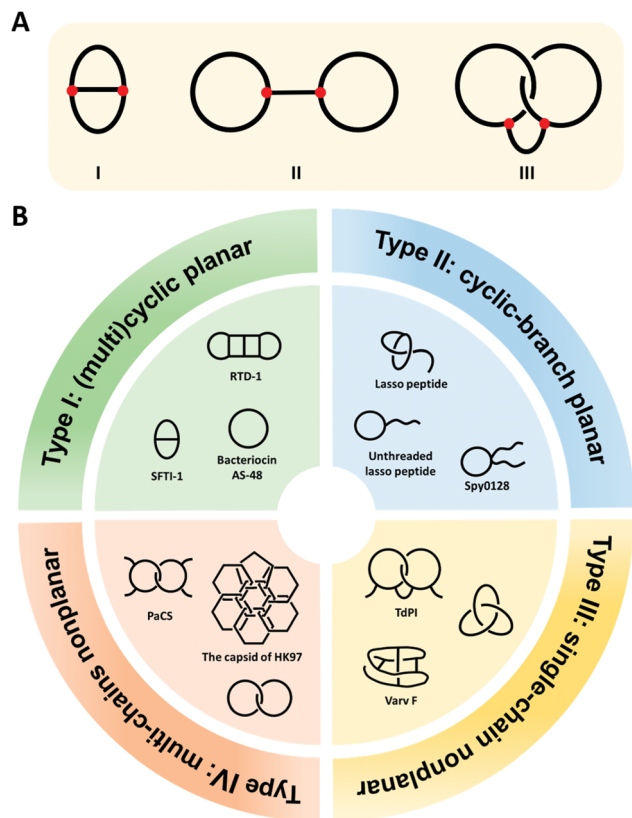
Peptide/protein macrocycles are not uncommon in nature. Although nascent proteins are strictly linear due to the template polymerization mechanism of ribosomal synthesis,[31,32] living organisms have evolved diverse cyclic peptides/proteins *via* post-translational processing to combat diseases or as part of their defense mechanisms. The most common form is simple macrocycles formed by seamlessly linking the N- and C-termini after expression.[33] Up to now, more than 1400 sequences

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Fig. 1 (A) The molecular graphs of three macrocycles with different chemical topologies. The line represents a polymer chain with a red vertex being a branch point. (B) General illustration and typical examples of the four categories of the naturally occurring peptide/protein-based macrocycles with distinct topologies. Type I: sunflower trypsin inhibitor 1 (SFTI-1) with planar θ-curve topology, rhesus-θ defensin-1 (RTD-1) with multicyclic topology,[51] and bacteriocin AS-48 with pure cyclic topology; Type II: lasso peptide and Spy0128 domain with cyclic-branch topologies; Type III: cyclotide varv F with $K_{3,3}$ graph topology, tick-derived protease inhibitor (TdPI) with interchain linked topology,[52] and trefoil knot; Type IV: Pyrobaculum aerophilum citrate synthase (PaCS) with [2]catenane or Hopf link topology and the capsid of HK97 with "chain-mail" topology.

of naturally occurring simple cyclic peptides/proteins have been documented.[34,35] The largest group of cyclic peptides were discovered from plants, named cyclotides.[36–38] Lasso peptides are perhaps the second largest group with a cyclic peptide threaded by its tail (Fig. 1B).[39,40] The disulfide bond formation leads to even more complex topologies, such as cysteine knots and ladders (embedded rings that are threaded by the disulfide bonds)[33,41] and mechanically interlocked, multicyclic protein links (Fig. 1B).[20] Occasionally, the cyclic structures can also be fixed by other covalent linkages, such as thioester, ester, or isopeptide bonds. The beautiful molecular 'chain-mail' structure of bacteriophage HK97 capsid is composed of mechanically interlocked protein rings that are covalently closed by isopeptide bonds (Fig. 1B).[42–44] While their cyclization mechanism differs from case to case, it seems that folding and assembly are all critical in pre-organizing the protein precursors for ring closure.[35,40,43,45,46] Cyclization confines the conformational space of peptides/proteins and thus, imparts diverse functions while enhancing their stability.[33,35] For example,

cyclotide Kalata B1 exhibits cytotoxicity, anti-microbial, and anti-virus activities, as well as excellent proteolytic resistance.[33,47,48] A larger cyclic bacteriocin AS-48, isolated from Enterococcus faecalis S-48, has a remarkably high melting temperature (T_m) of 93 °C (Fig. 1B).[49,50] The protein catenane Pyrobaculum aerophilum citrate synthase (PaCS) displays a T_m about 10 °C higher than the linear control without disulfide bonds (Fig. 1B).[20] The HK97 capsid can even tolerate up to 5 M of guanidinium hydrochloride.[42,43]

Inspired by the functional benefits observed in these naturally occurring peptide/protein-based macrocycles, much effort has been directed to synthesizing artificial peptide/protein-based macrocycles as well as their variants. While nature evolves these compounds for optimal fitness, artificial systems can fully unleash the power of cyclization in engineering their properties. To date, there has been considerable success in their syntheses, with a rapidly expanded toolbox of broad ranges of efficient technologies for chemical cyclization.[53–60] In this review, we shall focus on their biological synthesis and biomedical applications. Unlike organic synthesis, biosynthesis relies on the cellular machinery and is often genetically encoded to allow programming of the products' structures. It is usually highly efficient, specific, and capable of generating considerable complexity with high bioactivity both in vitro and in vivo.[59,60] By relating genotype to phenotype, biosynthesis also provides a facile approach for the rapid discovery of bioactive leads.[61–63] The chemical space could be even further expanded by incorporating non-canonical amino acids.[64–66] The biosynthesis of diverse peptide/protein-based macrocycles and their variants thus provides a rich resource for exploring their biomedical applications.

Overview of peptide/protein-based macrocycles

As shown in Fig. 1B, we categorize peptide/protein-based macrocycles into four types. On one hand, (multi)cyclic and cyclic-branched macrocycles with planar molecular graphs (molecular graphs which enable to be embedded in 2D space) are classified as Type I and Type II. The former results from main-chain cyclization (such as the protein bacteriocin AS-48) and the latter has at least one terminal of the chain remaining (such as the Spy0128 domain originated from Streptococcus pyogenes[67]). For example, sunflower trypsin inhibitor 1 (SFTI-1) belongs to Type I and possesses a θ-curve topology which is an embedding of the Greek letter θ in the plane.[68] Lasso peptides and proteins usually belong to Type II. On the other hand, macrocycles with nonplanar molecular graphs are classified either as Type III (single-chain) or Type IV (multi-chain). Their formation could arise from multiple covalent linkages.[41] For example, cyclotide varv F has been found to possess a $K_{3,3}$ graph (a prototypical nonplanar graph that cannot be embedded in the plane in such a way that its edges intersect only at their end points).[69] When folding and assembly are involved, highly complex structures, such as knots and links, emerge. Knots are the embeddings of a circle in 3D space. Although proteins are

typically linear, knotted proteins do exist with entanglements upon folding. In nature, the topologies of such macrocycles are often further reinforced by disulfide bonds from cysteine residues. Sulkowska and coworkers[70,71] have systematically looked into this problem and identified many nonplanar topologies in the protein data bank. Catenanes or links comprise two or more mechanically interlocked rings, while [2]catenane or Hopf link consists of two rings linked together exactly once. The existence of link-form macrocycles is rare in nature. *Pyrobaculum aerophilum* citrate synthase (PaCS) is a branched catenane since the disulfide bond only closes a relatively small ring. The capsid of HK97 possesses a "chain-mail" topology which is composed of multiple mechanically interlocked rings.[42–44] In view of all these macrocycle variants, it is apparent that chemical ligation tools are of primary importance owing to their determinant role in ring closure, whereas folding and assembly further increase the overall structural complexity. Below, we summarize various ligation tools for cyclization and then present "assembly-reaction" synergy as an effective strategy for creating diverse peptide/protein-based macrocycle variants.

Chemical tools for main-chain cyclization

Main-chain cyclization in biological synthesis relies either on genetically encoded protein ligation chemistry or enzymatic ligation chemistry with high sequence specificity. The former includes intein-mediated protein splicing or split-intein-mediated trans-splicing reactions. The latter involves many enzymes such as Sortase A, Butelase 1, OaAEP, *etc.*

Expressed protein ligation is a strategy for protein ligation or cyclization based on an intein-mediated splicing reaction.[72] It was first reported independently by Muir[73] and Xu[74] groups in 1998. The intein-bearing protein precursor generates a C-terminal α-thioester group *via* S/O acyl transfer, which then undergoes reversible trans(thio)esterification with the N-terminal residue (Cys, Ser, or Thr) of another protein precursor to form a branched oxy(thio)ester intermediate. After succinimide formation and subsequent S/O to N shift, the intermediate converts to a conjugate ligated with a native peptide bond.[75] The process is similar to that of native chemical ligation,[76] but both precursors are produced by recombinant technique, not chemical synthesis. It has been successfully employed for protein backbone cyclization where the reactive Cys and intein are appended at the N- and C-termini of the target protein, respectively (Fig. 2A). The first case was reported by Camarero and Muir[77] on the cyclization of Src homology 3 domain from the c-Crk adaptor protein which increases its ligand binding affinity by 6-folds. Afterward, a series of peptides (such as a brain-binding peptide, complementary determining region H3/C2)[78] and proteins (such as β-lactamase (BLA),[79] thioredoxin, maltose binding protein[74]) were cyclized by the same method. More interestingly, it could also be applied in living cells.[80]

Split-intein-mediated ligation takes advantage of the fact that intein domains may be split into two parts, named N-intein
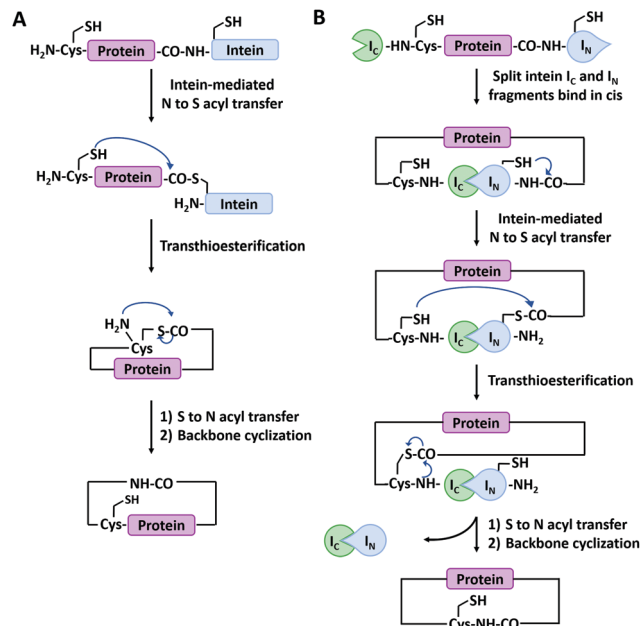

Fig. 2 Schematic illustration of protein head–to–tail cyclization by expressed protein ligation (A), and split–intein mediated reaction (B).

(I_N) and C-intein (I_C), which can spontaneously reconstitute to form a native peptide bond linkage between their fusion proteins while excising themselves off the fusion.[81,82] This protein splicing event can be used for protein cyclization (Fig. 2B). Benkovic and co-workers[83] first used split *Ssp* inteins to produce cyclic peptides and proteins by fusing I_N and I_C at the C- and N-termini of target protein, respectively. The obtained circular dihydrofolate reductase (DHFR) exhibited improved thermal and proteolytic stability over its linear counterpart. It has later been used on other proteins as well, including green fluorescence protein (GFP),[84] xylanase,[85] and a peptide HIV inhibitor.[86] Notably, this strategy is also applicable in cultured cells. For example, Camarero and co-workers[87] synthesized cyclotide MCoTI-I by both expressed protein ligation and split-intein-mediated ligation, the latter of which outperformed the former in the *in vivo* expression. Besides *Ssp* inteins, a lot of split inteins with high reactive activity and robust extein tolerance have been developed by genomic sequencing and rational engineering (*e.g.*, gp41-1, gp41-8, NrdJ-1, and IMPDH-1).[72] Among them, a split intein derived from *Nostoc puntiforme* PCC73102 (*Npu*), which possesses robust trans-splicing activity with an efficiency of 98%, has been widely used.[88] By varying the positions of split sites in *Npu* intein, two mutually orthogonal split intein pairs (102 residues for I_{N1} and 36 residues for I_{C1}; 15 residues for I_{N2} and 123 residues for I_{C2}) have been developed, which greatly enhances our capability to synthesize complex protein macrocycle variants.[89,90]

Sortase A is a kind of transpeptidase enzyme that could anchor proteins onto the cell wall surface. It was first reported by Schneewind and co-workers[91] in 1999 and has received wide applications since then. It usually recognizes a pentapeptide

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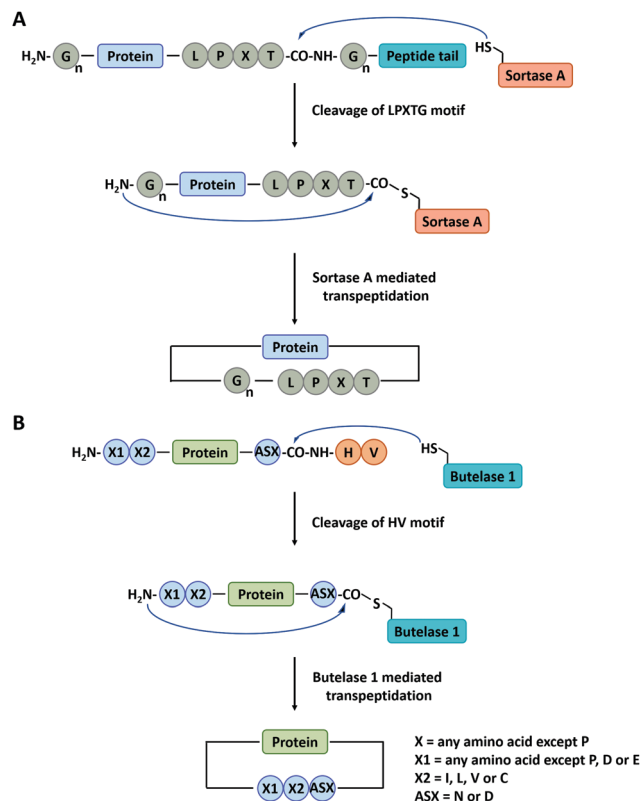*RSC Chem. Biol.*, 2022, **3**, 815–829 | **817**

Fig. 3 Schematic illustration of protein head-to-tail cyclization by Sortase A mediated reaction (A), and Butelase 1 mediated reaction (B).

sequence of LPXTG (X represents any amino acid) at the C-terminus and an oligoglycine sequence at the N-terminus of the substrate and catalyzes the cleavage of the amide bond between T and G to generate an acyl-enzyme thioester intermediate, which was subsequently attacked by the amine group at the N-terminal oligoglycines for ligation or cyclization (Fig. 3A).[92] Boder and co-workers first used Sortase A to cyclize a bifunctional precursor protein, Gly$_3$-GFP-LPETG-His$_6$.[93] The final products were characterized as a mixture of both monomer and dimer. The more specific cyclization of GFP was then realized by Ploegh group[94] and Gao group[23] and further extended to biomedical applications. Since then, a series of cyclic proteins, such as histatin-1[95] and interferon-α (IFN-α),[22,24,25] were produced by this method. These cyclic peptides and proteins showed enhanced stability and improved biological activity compared with their linear counterparts. Nowadays, Sortase A has been engineered to be independent of Ca^{2+} and capable of recognizing different pentapeptide sequences, greatly enriching this toolbox.[75] A Ca^{2+}-independent Sortase A discovered from *Streptococcus pyogenes* has been successfully applied to cyclize GFP *in vivo*.[96,97] Compared with intein-mediated cyclization, Sortase A requires fewer recognition sequences in the precursor protein. However, its application *in vivo* is quite limited due to the complication of intracellular nucleophiles such as the ε-amino group of lysine.[98] Sortase A also suffers from low turnover rates (the molar ratio of the enzyme to protein substrate is usually from 0.1 to 1) and undesirable reverse reaction.[94] It thus requires a lot of enzymes

and a large excess of one substrate to promote the reaction, which is not ideal for protein cyclization.

Apart from Sortase A, other enzymes have also been developed to produce peptide/protein-based macrocycles. The most representative case is a class of peptide ligases in the asparaginyl endoproteases (AEP) family, which requires very short recognition motifs to ligate a range of targets.[99] Butelase 1 is the first AEP peptide ligase purified from the cyclotide-producing plant *Clitoria ternatea*.[100,101] This enzyme recognizes the C-terminal D/N-HV sequence of polypeptide precursor and catalyzes the cleavage of the amide bond between the D/N and H to generate an acyl-enzyme intermediate. Then, target cyclization proceeds *via* ligation of the D/N residue to the N-terminal amino acid residue (Fig. 3B).[102,103] Tam and co-workers[102] have constructed cyclic GFP, human growth hormone (hGH), and interleukin-1 receptor antagonist *via* Butelase 1 mediated ligation. Compared with Sortase A, Butelase 1 shows exceptionally high efficiency (>90% yield, low enzyme to substrate molar ratio: 0.001 to 0.01, and short reaction time) and better tolerance to recognition amino acids (almost all amino acids at the N-terminus).[102,104] However, until now, Butelase 1 still cannot be recombinantly expressed in an active form on a large scale, which limits its availability and wide use.[104]

OaAEP1b is another kind of AEP peptide ligase isolated from the plant *Oldenlandia affinis* with a similar catalytic mechanism to that of Butelase 1 but different recognition sequences at the C-terminus of protein precursor (NGL, NAL, or NCL).[105,106] Despite usually lower catalytic efficiencies than Butelase 1, OaAEP1b could be expressed in *E. coli* and activated under acidic conditions.[107] Based on the structure-based mutagenesis of OaAEP1b, Wu and co-workers[107] engineered an OaAEP1b variant which shows hundreds of times faster catalytic kinetics than the wild-type. This variant was demonstrated to be highly efficient for the ligation and cyclization of peptides and proteins. By now, a lot of peptide/protein-based macrocycles have been synthesized by OaAEP1b, including GFP and its variants,[108] intrinsically disordered malarial vaccine candidate *Plasmodium falciparum* merozoite surface protein 2,[109] and several cyclotides (MCoTI-II, Kalata B1, a kallikrein-related peptidase 5 inhibitor based on the SFTI-1 scaffold and a potent α-conotoxin from *Conus victoriae*).[105,110,111]

The natural translation process in ribosome is limited to 20 canonical amino acid building blocks, however, genetic code reprogramming (GCR) provides a new opportunity to incorporate hundreds of non-canonical amino acids, especially side chain-substituted amino acids, into the sequence of peptides/proteins. In general, GCR is based on the "mis-acylation" of tRNA molecules with non-canonical amino acids for subsequent incorporation into polypeptide chains during translation.[112] Suga and co-workers[113] have shown that GCR could generate backbone-cyclized polypeptides during ribosomal synthesis using recombinant elements for peptides synthesis (Fig. 4). They introduced a Cys-Pro-glycolic acid (C-P-^{HO}G) sequence into the peptide chain *via* GCR, which could self-rearrange to form a diketopiperazine-thioester intermediate, and then react with an N-terminal Cys
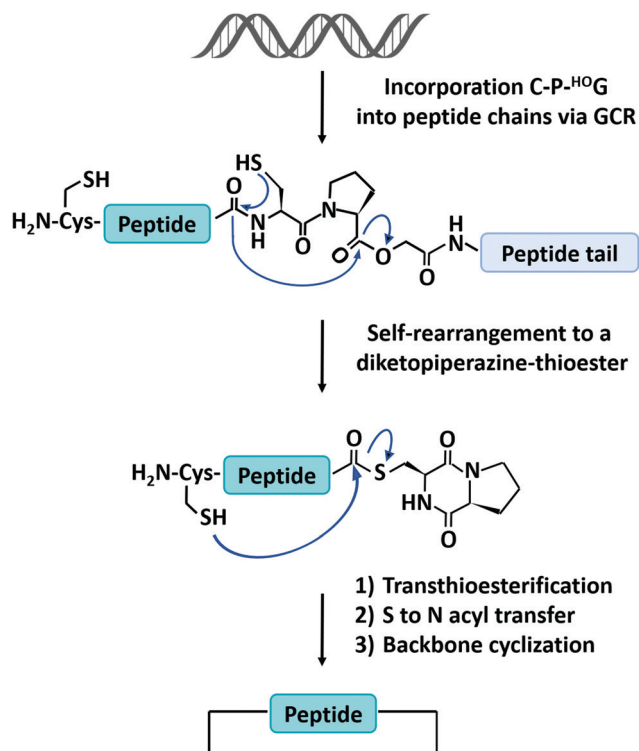
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Fig. 4 Schematic illustration of the head-to-tail cyclization of peptides by genetic code reprogramming.

residue in the same way as native chemical ligation. This elegant strategy not only enables the synthesis of several naturally occurring backbone-cyclized peptides, such as eptidemnamide, scleramide, RTD-1, and SFTI-1 but also provided another option for the generation of cyclic peptide libraries and screening of such libraries *in vitro* to identify potential lead drugs. The involvement of non-canonical amino acids often leads to lower production yields. Once a target is identified, the short cyclic peptide may also be synthesized using solid-phase peptide synthesis, obviating the problem of scale-up in the recombinant ribosomal synthesis. It should be noted that since GCR typically needs flexizyme-mediated tRNA acylation,[114] it is challenging to use this system in cells for making large-sized protein molecules.

Chemical tools for side-chain cyclization

In nature, there are many types of side-chain covalent linkages, such as disulfide bond, isopeptide bond, or ester/thioester bond.[67,115–118] Despite wide existence in natural proteins, the disulfide bond is unstable under reducing conditions. Thus, we mainly focus on other more stable covalent linkages, such as isopeptide bonds which is an amide bond formed between the side chains of amino acids like Lys and Asn/Asp.[115,119] It has commonly been found in microbial protein domains, such as the Spy0128 and fibronectin-binding protein (FbaB) from *Streptococcus pyogenes*.[67,115,119] Notably, these protein domains

can be split to develop chemical reaction tools and have been widely used.[118,120–124]

Howarth and co-workers[125] first dissected Spy0128 into two types of peptide/protein reactive pairs, termed as pilin-C (residues 18–229)/isopeptag (16 amino acids at the C-terminal) and pilin-N/isopeptag-N. The two parts of each reactive pair can reconstitute and spontaneously form an isopeptide bond irreversibly between Lys and Asn *via* an autocatalysis process. However, the inefficient reaction (60% yield) and relatively large molecular weight (∼35 kDa) limit their application. Howarth and co-workers[126] then divided the CnaB2 domain (the second immunoglobulin-like collagen adhesin domain)[119] derived from FbaB into SpyTag (the C-terminal β-strand composed of 13 amino acid residues) and SpyCatcher (138 residues at the N-terminal), giving rise to another peptide/protein reactive pair. The SpyTag/SpyCatcher chemistry shows high reactivity with a second-order rate constant (k) of $\sim 1.4 \times 10^3$ M^{-1} s^{-1}. It was further optimized into the second and third generations with exceptional reactivity *via* rational engineering and directed evolution ($k \sim 2.0 \times 10^5$ M^{-1} s^{-1} for Spy-Catcher002 and $k \sim 5.5 \times 10^5$ M^{-1} s^{-1} for SpyCatcher003).[127,128] Meanwhile, the reaction is broadly applicable both *in vitro* and *in cellulo*. As a supplement, the SnoopTag/SnoopCatcher pair was also created by splitting the D4 domain of RrgA adhesin from *Streptococcus pneumoniae*, which shows comparable efficiency but mutually orthogonal reactivity to SpyTag/SpyCatcher ligation.[129] Up to now, many peptide/protein reactive pairs have been developed, constituting a rich toolbox for side-chain ligation.[118]

Compared to main-chain ligation, the genetically encoded peptide/protein reactive pairs, such as SpyTag/SpyCatcher chemistry, are advantageous in producing cyclic proteins and their variants. First, the reaction is highly efficient and robust. Second, since tag and catcher react not only at the termini but also inside the protein chain, they can enable domain-selective cyclization with intact N-and C-termini for further modification.[130] For example, by genetically fusing SpyTag and SpyCatcher to the N- and C-termini of the target protein, Howarth and co-workers[131,132] successfully realized the side-chain cyclization of a series of enzymes, including BLA, DHFR, and phytase, leading to the cyclic-branched structures as we categorized in Type II (Fig. 5). These enzymes exhibit remarkably enhanced thermal stability in many regards: (1) improved anti-aggregation behavior; (2) excellent thermal resilience, tending to refold and restore the catalytic activity better. These functional benefits are presumably attributed to the cyclic-branched topology as well as the extraordinary stability of the SpyTag/SpyCatcher complex. Besides, other proteins such as firefly luciferase[133] and lichenase[134] have also been cyclized by SpyTag/SpyCatcher chemistry to improve their performance in bioactivity and stability.

Although SpyTag/SpyCatcher chemistry has been successful for biologically inspired synthesis of protein macrocycles, especially those with complex topological structures,[130] it is not traceless. The large SpyTag/SpyCatcher complex (∼15 kDa) after cyclization might unexpectedly alter the structure and function of the original protein due to the increased steric
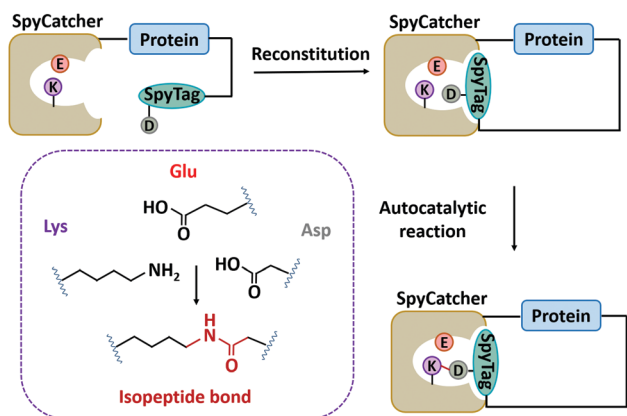
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Fig. 5 Schematic illustration of protein side-chain cyclization by SpyTag/SpyCatcher chemistry.

Fig. 6 Schematic illustration of biosynthesis processes of protein knots.

hindrance and potential immunogenicity. To reduce the size of the ligation scar, Howarth and co-workers[135,136] have developed "SpyTag/KTag/SpyLigase" and "SnoopTagJr/DogTag/SnoopLigase" systems by further splitting the SpyTag/SpyCatcher and SnoopTag/SnoopCatcher pairs into three parts. The SpyLigase and SnoopLigase catalyze the reaction of SpyTag/KTag and SnoopTagJr/DogTag, respectively, forming an isopeptide bond with a scar of only 5–6 kDa molecular weight. Recently, Zhang and co-workers[137] have found an alternative way to split SpyCatcher, leading to BDTag (25 amino acids), and SpyStapler (~ 8 kDa). SpyStapler can efficiently catalyze the isopeptide bonding between SpyTag and BDTag. This tool has later been adapted to develop an active-template synthesis of protein catenanes.[138] The small size of fused tags and higher coupling efficiency make these tools attractive for researchers in the field of chemical biology, synthetic biology, protein engineering, and biomaterials.

"Assembly-reaction" synergy

Compared with Type I and Type II macrocycles, Type III or Type IV macrocycles possess nonplanar molecular graphs and potentially remarkable functional benefits, such as enhanced thermal/chemical stability and proteolytic resistance. To address the challenge in their syntheses, the "assembly-reaction" synergy has been developed, which utilizes entwined protein domains as a template to guide the chain entanglement and combines with genetically encoded ligation chemistry to provide a module way for producing diverse topological peptide/protein macrocycles (knots, links, branched links, *etc.*).[139–141]

Recently, Iwaï and co-workers[142] have reported the production of a mathematical protein trefoil knot with closed ends. The trefoil knot is the simplest knot that could not be embedded in the plane. A deeply trefoil-knotted YibK from *Pseudomonas aeruginosa* was cyclized by split intein and Sortase A, respectively, to generate the trefoil knot exhibiting increased thermal stability and reduced aggregation propensity (Fig. 6). In comparison, protein catenanes contain two or more mechanically interlocked rings with higher structural complexity. Based on the "assembly-reaction" synergy, Zhang and
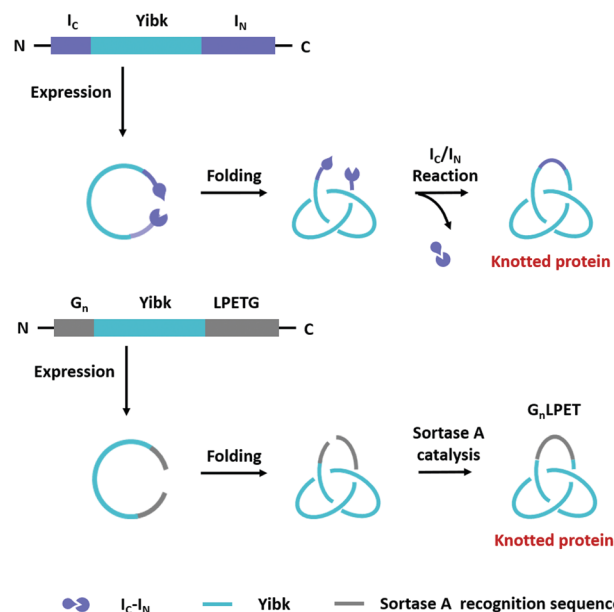
co-workers[143,144] have synthesized a series of protein catenanes using the entwined p53dim homodimerization domain[57] and SpyTag/SpyCatcher chemistry *in cellulo*. The expressed nascent protein precursors first pre-organize into an entangled intermediate assembly as guided by p53dim followed by ring closure through SpyTag/SpyCatcher reaction to lock in the catenane topology (Fig. 7A).[143]

A lot of proteins, including unstructured ELP and well-folded GFP, BLA, or DHFR have been concatenated by this modular approach.[143,144] Due to the domain-selective feature and intact termini of SpyTag/SpyCatcher, this strategy could be extended to synthesize star-like protein catenanes and protein pretzelanes.[145,146] Moreover, lasso proteins, though not included in the nonplanar molecular graphs, could also be produced by a similar strategy. The precursor protein SpyCatcher-p53dim-SpyTag-p53dim would form the cyclic-branched structure with the tail threaded in the ring due to "assembly-reaction" synergy.[147] By replacing the peptide/protein reactive pair with orthogonal split intein chemistry, Zhang and co-workers[148] recently developed an autonomous streamlined synthesis of traceless protein heterocatenanes with two distinct rings. With a sequence of multiple post-translational processes including p53dim guided chain interlacing and orthogonal intein-mediated ring closure, the nascent linear chain could be transformed into a main-chain heterocatenane directly in cells (Fig. 7B). The topological diversity has been further expanded by using an engineered p53dim heterodimer. Protein [3]catenane and [4]catenane composed of three or four interlocked rings were successfully synthesized with the combination of p53dim heterodimer for controlled multi-chain intertwining and mutually orthogonal split-intein-mediated ligation and SpyCatcher/SpyTag reaction for closing the primary and secondary rings, respectively (Fig. 7C).[149] Recently, by rewiring the connectivity of the SpyTag/
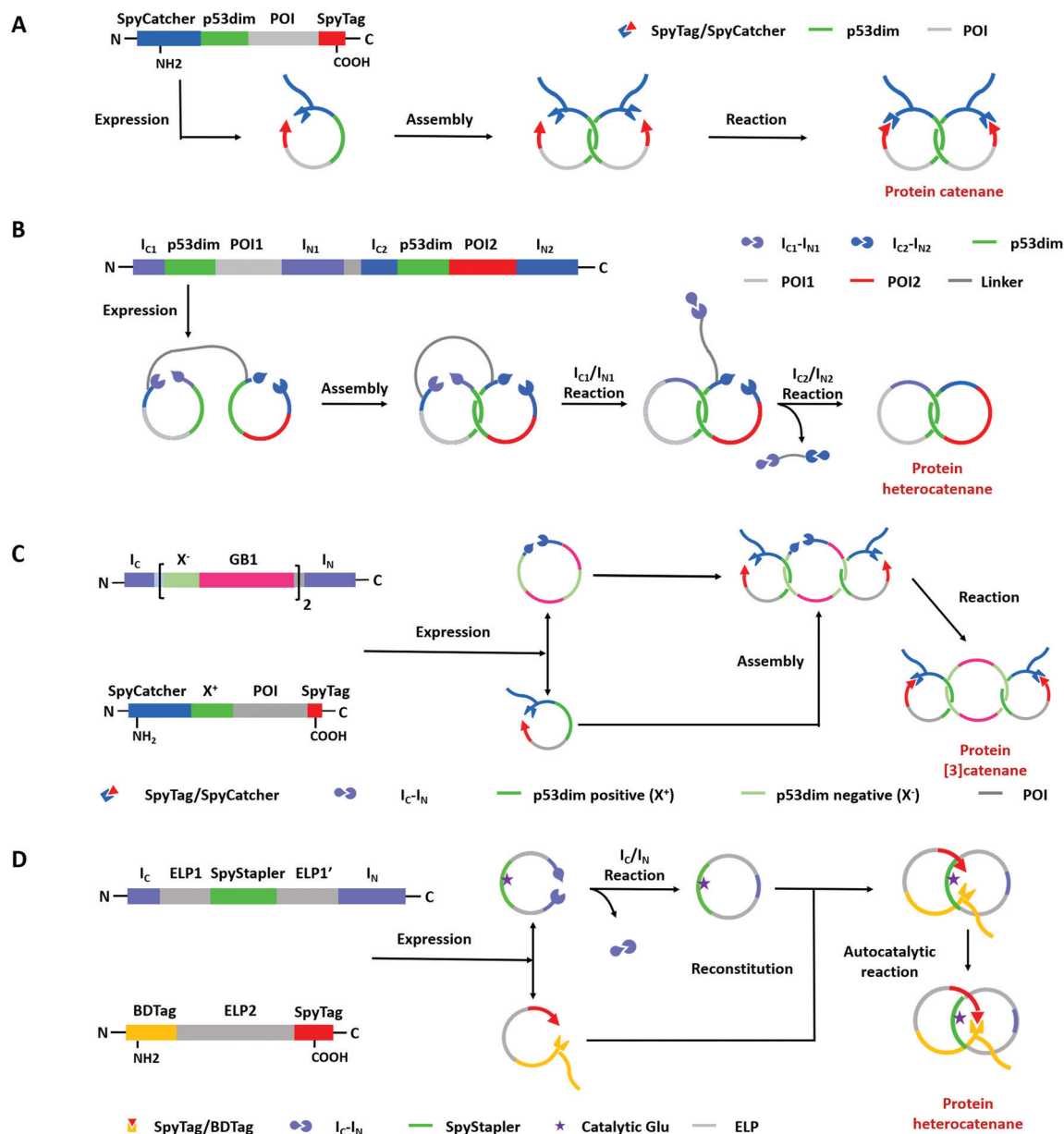
Fig. 7 Schematic illustration of biosynthesis processes of protein links *via* "assembly-reaction" synergy: (A) protein homocatenanes; (B) main-chain cyclized protein heterocatenane by streamlined synthesis approach; (C) protein [3]catenane; (D) branched protein heterocatenane based on the active template strategy (POI is short for the protein of interest).

BDTag/SpyStapler complex in 3D space, an active template strategy was developed to enable selective protein heterocatenane synthesis. The chain entanglement was introduced by folding and the reconstituted complex served to catalyze the isopeptide bond formation for ring closure, leading to the heterocatenane topology (Fig. 7D).[138,150] Although still in its infancy, the biosynthesis of non-planar protein macrocycles, such as protein knots and links, is promoted by the development of genetically encoded protein entangling motifs and ligation tools. These macrocycles with nonplanar graphs often exhibit extra stability and other functional benefits, which holds great promise in protein therapeutics.

Biomedical applications of peptide macrocycles

Cyclization has been demonstrated as a simple yet effective strategy to develop therapeutic peptides, particularly as inhibitors for a wide range of protein targets.[28–30,151–153] The framework of cyclic peptides imparts defined and pre-organized conformations and enables highly specific target engagement through reducing the entropic loss upon binding.[154,155] Meanwhile, the absence of termini in cyclic peptides often leads to enhanced proteolytic resistance, which allows them to exert functions in harsh conditions.[33,35,37] Recently, the genotype–

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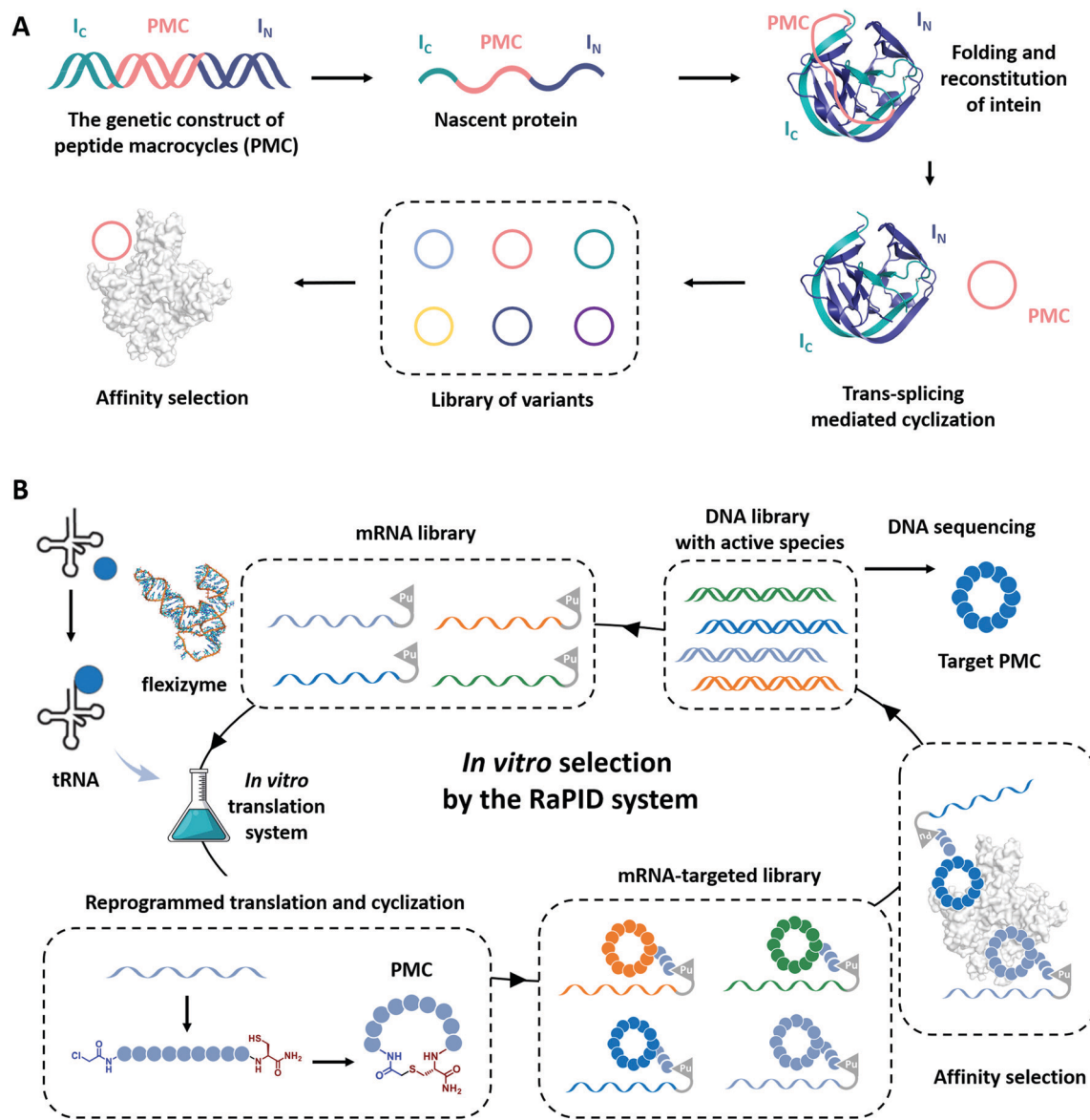*RSC Chem. Biol.*, 2022, **3**, 815–829 | 821

Fig. 8 Schematic illustration of the evolution processes of SICLOPPS (A) and mRNA display (B).

phenotype linkage of peptide macrocycles has facilitated drug discovery *via* rapid preparation and high-throughput screening of libraries consisted of hundreds of millions of peptide macrocycles with low cost.[61,62,156–158] Examples include phage display,[159] split-intein circular ligations of peptides and proteins (SICLOPPS),[160] and mRNA display.[161] In phage display, the cyclization is usually achieved by disulfide bond formation.[62] Here, we focus on the progress in the other two directions which utilizes more stable covalent linkages for cyclization.

SICLOPPS is a method for the high-throughput screening of macrocyclic peptide variants produced by intracellular expression and split intein mediated cyclization (Fig. 8A).[160,162] It facilitates the construction of libraries comprised of up to about 10^8 sequences and the screening process can be performed both in prokaryotes and in eukaryotes.[163] For example,

in order to evolve peptide macrocycles to suppress the toxicity of human α-synuclein, a presynaptic neuronal protein relevant to Parkinson's disease,[164,165] Lindquist and co-workers[26] constructed a library containing more than 30 million cyclic peptide octamers *via* intein-mediated protein trans-splicing. After selection on a series of filtering assays in a yeast synucleinopathy model, two peptide macrocycles were picked and confirmed to possess reproducible and specific activities with suppressed toxicity. These selected peptide macrocycles were introduced into *C. elegans* Parkinson's disease model and found to significantly reduce dopaminergic neurodegeneration. Another example aimed to engineer peptide macrocycles to regulate the interactions between the heterodimeric hypoxia-inducible factor (HIF)-1α and -1β, which is crucial for cancer therapy.[166,167] Tavassoli and co-workers[27] constructed a library containing more than 3.2 million cyclic hexapeptides by SICLOPPS and screened out active peptide

macrocycles for the inhibition of HIF-1 heterodimerization. One of the selected peptide macrocycles, named *cyclo*-CLLFVY, was observed as a good inhibitor to bind with the PAS-B domain (amino acid residues 235–350) of HIF-1α. These examples demonstrate the potential of SICLOPPS in developing highly active macrocyclic peptide drugs for disease treatments.

Unlike phage display or SICLOPPS, the *in vitro* transcription and translation process and the ability to conveniently incorporate noncanonical amino acids notably enlarge the library size of mRNA display to up to 10^{12}–10^{14}.[63,157,168] Moreover, with flexizymes,[66,169] a class of artificially evolved ribozymes capable of charging tRNA with various non-standard amino acids, Suga and co-workers[170,171] have developed an attractive mRNA display methodology, termed random nonstandard peptide integrated discovery (RaPID) platform. As shown in Fig. 8B, the RaPID system allows the synthesis of cyclized peptide libraries by genetic code reprogramming and spontaneous posttranslational cyclization. Iterative rounds of affinity-based selection and gradual enrichment of the active species give rise to the desired peptide macrocycles for targeted proteins. Using this technology, they successfully identified a series of peptide macrocycle-based inhibitors to factor XIIa (an initiator of the contact system),[172–174] calcium and integrin-binding protein 1 (an intracellular protein implicated in the survival and proliferation of triple-negative breast cancer),[175] influenza viral envelope protein hemagglutinin,[176] and human epidermal growth factor receptor (EGFR),[177,178] which provide potent drug candidates in antithrombotics, pneumonia prevention, and cancer therapy. Through effective cyclization and incorporating noncanonical amino acids, such as β-amino acids, D-amino acids, and L-carboranylalanine, these peptide macrocycles showed not only high inhibitory activity but also protease resistance and/or good cell permeability. Other exciting examples include the discovery of low nanomolar inhibitors of prolyl hydroxylase isoform 2,[179] isoform-selective Akt kinase[180] or NAD-dependent deacetylase sirtuin 2,[181] and the evolvement of cyclic peptides with the high binding ability to the interleukin-6 receptor.[182] The RaPID also exhibited its commercial promise to develop the potential macrocyclic inhibitor of programmed death 1/programmed death ligand 1(PD-1/PD-L1).[183]

With these advances, the identification of peptide macrocycles with target biological functions is no longer considered a major obstacle in drug discovery and development. More significant challenges now may lie in the poor membrane permeability, low oral bioavailability, and metabolic instability of these drug candidates.[157] Cyclization is expected to play vital roles in optimizing, at least partially, these crucial pharmacological properties for clinical translation.

Biomedical applications of protein macrocycles

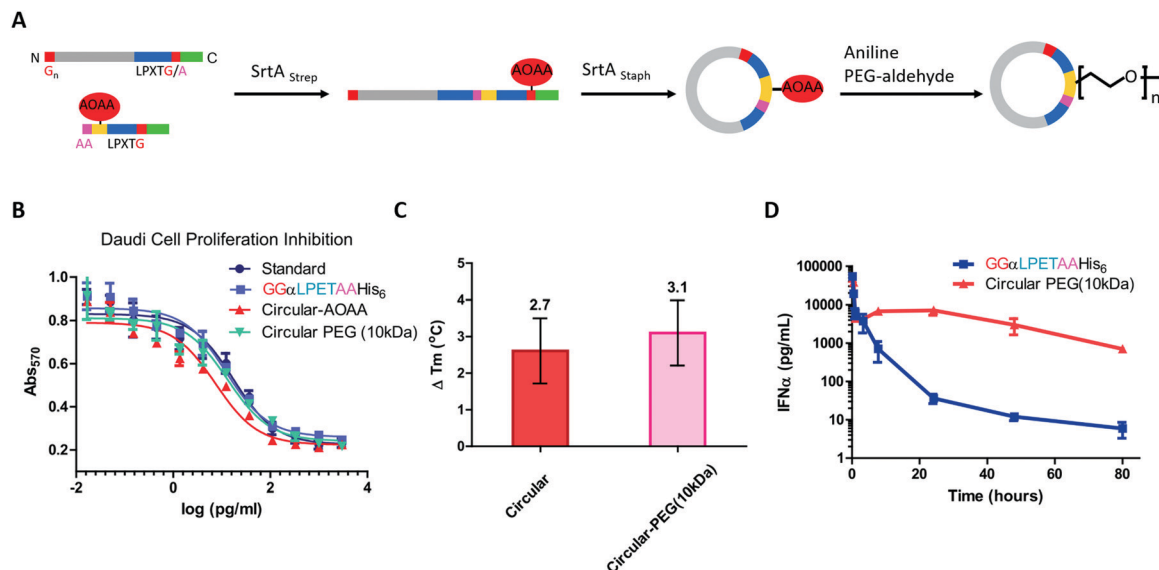Compared with small-molecule drugs, protein-based therapeutics exhibit unique functional benefits, such as high specificity and activity and fewer side effects. However, they also suffer some disadvantages, including inherent instability and poor pharmacokinetics.[184,185] To solve these problems, a lot of works on protein-macrocycle-based therapeutics have emerged, which can not only improve the stability of protein drugs but also lead to other functional benefits, such as optimized tissue penetration or binding affinity.[24,25,186] Moreover, these benefits could be further improved by combining with other strategies, such as PEGylation,[187] which remarkably optimize the pharmacokinetic behaviors of protein drugs. Herein, we would like to discuss the cyclization of important protein-based therapeutics, such as cytokines, enzymes, and single-chain variable fragment (scFv) antibodies, to illustrate how cyclization influences their behaviors.

Cytokines are a class of small-molecule proteins regulating cell growth, differentiation, and immune responses.[188] As an important section of protein-based therapeutics, many cytokines, such as IFN, granulocyte colony stimulating factor (GCSF), interleukins, and hGH, have been approved by FDA for the treatment of various diseases. As an elegant example, Ploegh and co-workers[22] constructed a cyclic-branched IFN-α coupled with 10 kDa PEG through two sequential transacylation procedures catalyzed by two Sortase A variants[189] recognizing different sequences (LPETA for SrtAstrep and LPETG for SrtAStaph) (Fig. 9A). The engineered cyclic-branched IFN-α showed not only enhanced *in vivo* stability and thermal stability over its linear counterpart (Fig. 9B and C), but also improved pharmacokinetics over its non-PEGylated counterpart (Fig. 9D). However, considering the multiple-step synthesis approach and the low efficiency of PEGylation, this strategy is complicated by a low overall yield (∼10%). Alternatively, Lu[24] and Gao[25] groups reported two methods to construct long-circulating cyclic IFN-α, respectively. Compared with cyclic-branched IFN-α, these cyclic IFN-α exhibited better *ex vivo* and *in vivo* tumor penetration, which should be explained by the compact structures. These results suggest that cyclization may be combined with other strategies, such as PEGylation[22,190] and ABD fusion,[25] to further improve its performance.

Apart from IFN-α, GCSF was also cyclized by Sortase A[22] or split-intein[191]-mediated ligation. Honda and co-workers[191] constructed a series of connecting loops to develop different cyclic variants, and one of them showed better thermal resistance with a 13 °C increase in T_m. hGH[192] and interleukin-1 receptor antagonist[102] were also cyclized to show the enhanced stability against thermal denaturation and comparable biological activities. However, it is difficult to evaluate their therapeutic potential due to the lack of *in vivo* data.

Enzymes are also a kind of potential therapeutics. Nine recombinant enzymes have been approved for clinical use from January 2014 to July 2018.[194] However, unlike the cyclization of cytokines, studies on the cyclization of enzymes have mainly focused on model proteins. As a classical model, BLA has been cyclized either by intein-mediated peptide ligation[79] or SpyTag/SpyCatcher chemistry[132] as mentioned above. All these cyclic BLA and cyclic-branched BLA showed better stability against thermal denaturation than their linear counterparts.

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Fig. 9   Synthesis of cyclic-branched IFN-α and PEG conjugate *via* two sequential transacylation procedures mediated by Sortase A (A). The cyclic-branched IFN-α and PEG conjugate shows optimized bioactivity (B), improved thermal stability (C), and prolonged circulation time (D).[22]

Monoclonal antibodies seem to be the most important section of protein-based therapeutics considering their dominance in terms of product approvals or market value.[194] They usually show high affinity and specificity but need to be produced in mammalian cells with a high cost. Thus, scFv antibodies, whose heavy and light variable fragments are usually connected with a short peptide linker, have emerged to be an alternative option. However, scFv antibodies suffer from their instability. The inter-domain interactions would lead to aggregate formation. To suppress scFv oligomerization,

Morioka and co-workers[193] constructed a kind of cyclic scFv antibody by Sortase A-mediated peptide ligation (Fig. 10A). By doing so, aggregation was markedly suppressed without disturbing the binding activities and thermal stability of the scFv antibody (Fig. 10B). This work is a useful attempt to produce cyclic scFv antibodies, holding great promise for further use in biomedical fields.

Besides simple cyclization, a more complicated, higher order protein [n]catenane based artificial antibody has been developed by Zhang and co-workers.[149] Based on the synthetic
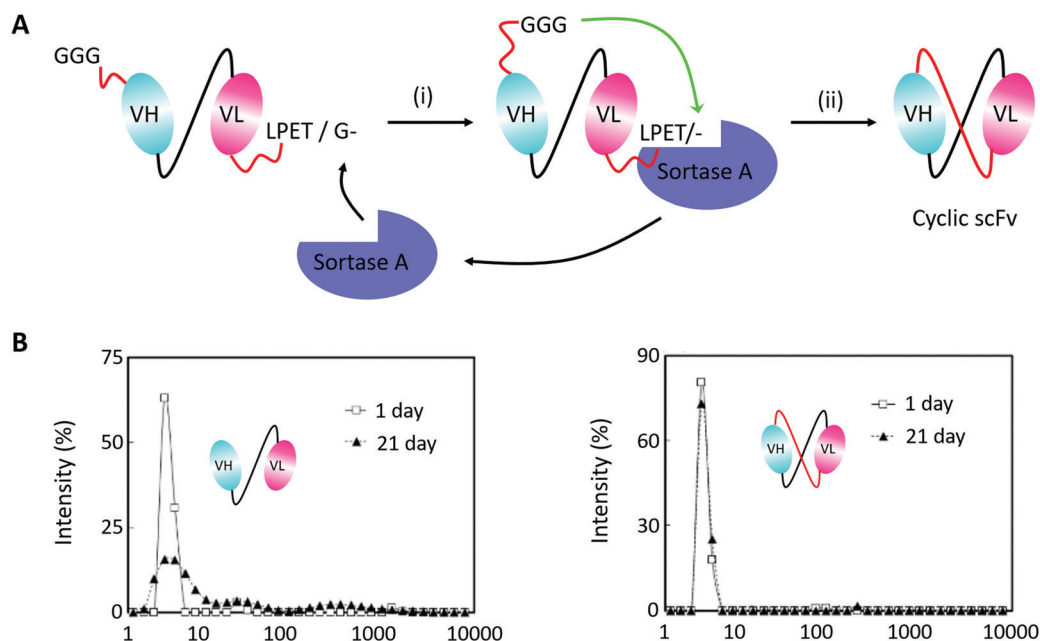
Fig. 10   (A) Scheme for Sortase A-mediated scFv cyclization and (B) analysis of molecular size distributions by dynamic light scattering: linear scFv (left) and cyclic scFv (right) (B).[193]

strategy mentioned above, a human epidermal growth factor receptor 2 (HER2)-specific affibody (AffiHER2 that targets the HER2 receptor specifically) has been genetically encoded into the scaffolds of protein [3]catenane or [4]catenane, giving rise to bivalent and trivalent artificial antibodies termed [3]catbody and [4]catbody. Due to the multivalent effect and stability enhancement of the [n]catenane scaffold, both [3]catbody and [4]catbody exhibit increased binding affinities to HER2 receptors, significantly prolonged circulation time, and optimized tumor accumulation. This indicates that protein macrocycles and their variants can not only increase the stability of target protein drugs but also bring in other functional benefits, such as multi-valent effects.

Conclusions

Inspired by naturally occurring peptide/protein macrocycles and their variants with enhanced stability and biological activity, extensive efforts have been directed to explore the biological syntheses and biomedical applications of such macrocycles over the past two decades. There are many advantages in biological synthesis, including high efficiency and selectivity, fully genetically programmable, and compatible with various functional proteins, making it a convenient way to produce peptide/protein-based macrocycles with tailor-designed topologies and functional benefits. In particular, significant advances have been gained in discovering new cyclic peptide-based drugs and in improving the pharmacokinetic properties of protein drugs.

Despite these progresses, much work is still urgently needed. The extensive presence of peptide/protein-based macrocycles in nature should be understood in detail, particularly their formation mechanism and structural diversity. The detailed study on the structure–property relationship requests a moderate diversity of artificial peptide/protein-based macrocycles. Although the "assembly-reaction" synergy is powerful, there is still limited toolkits of genetically encoded protein entangling and ligation motifs. This toolbox should be largely expanded and optimized in terms of their reactivity and specificity to facilitate the synthesis of diverse macrocycles. Computer-aided methodology and direct evolution have been considered powerful to optimize these protein toolkits. In addition, it is also important to consider the scale-up synthesis of these macrocycles in industry. With their rapid development, we believe that more and more peptides/protein-based macrocycles will be successfully developed with increasing structural complexity, evolving from simple circle to multicycles (Type I), cyclic-branch (Type II), and further to knots and links (Type III and IV). Functional benefits will be gained in this endeavor, which shall open a new avenue for peptide/protein-based drug discovery and enable advanced therapeutics for biomedical applications.

Author contributions

The review was written, and the literature analyzed, through contributions by all authors. Each of the authors gave approval to the final version of the manuscript. W. W. H. and J. G. contributed equally.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1 T. Yamamoto and Y. Tezuka, *Polym. Chem.*, 2011, **2**, 1930–1941.

2 F. M. Haque and S. M. Grayson, *Nat. Chem.*, 2020, **12**, 433–444.

3 C. D. Roland, H. Li, K. A. Abboud, K. B. Wagener and A. S. Veige, *Nat. Chem.*, 2016, **8**, 791–796.

4 Y. Tezuka, *Acc. Chem. Res.*, 2017, **50**, 2661–2672.

5 H. L. Frisch and E. Wasserman, *J. Am. Chem. Soc.*, 1961, **83**, 3789–3795.

6 Y. Tezuka, *Topological polymer chemistry: progress of cyclic polymers in syntheses, properties, and functions*, World Scientific, 2013.

7 E. Flapan, *When topology meets chemistry: a topological look at molecular chirality*, Cambridge University Press, 2000.

8 K. Shimokawa, K. Ishihara and Y. Tezuka, *Topology of Polymers*, Springer Nature, 2019.

9 Y. Tezuka, *Isr. J. Chem.*, 2020, **60**, 67–74.

10 X.-W. Wang and W.-B. Zhang, *Trends Biochem. Sci.*, 2018, **43**, 806–817.

11 Z. Qu, S. Z. Cheng and W.-B. Zhang, *Trends Chem.*, 2021, **3**, 402–415.

12 W.-B. Zhang and S. Z. Cheng, *Giant*, 2020, **1**, 100011.

13 Y. Tezuka and T. Deguchi, *Topological Polymer Chemistry*, Springer Nature, 2022.

14 H.-X. Zhou, *Acc. Chem. Res.*, 2004, **35**, 123–130.

15 H.-X. Zhou, *J. Mol. Biol.*, 2003, **332**, 257–264.

16 H.-X. Zhou, *J. Am. Chem. Soc.*, 2003, **125**, 9280–9281.

17 T. L. Aboye and J. A. Camarero, *J. Biol. Chem.*, 2012, **287**, 27026–27032.

18 R. J. Clark, M. Akcan, Q. Kaas, N. L. Daly and D. J. Craik, *Toxicon*, 2012, **59**, 446–455.

19 N. K. Williams, E. Liepinsh, S. J. Watt, P. Prosselkov, J. M. Matthews, P. Attard, J. L. Beck, N. E. Dixon and G. Otting, *J. Mol. Biol.*, 2005, **346**, 1095–1108.

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RSC Chem. Biol., 2022, **3**, 815–829 | 825

20 D. R. Boutz, D. Cascio, J. Whitelegge, L. J. Perry and T. O. Yeates, *J. Mol. Biol.*, 2007, **368**, 1332–1344.

21 C. Zong, M. J. Wu, J. Z. Qin and A. J. Link, *J. Am. Chem. Soc.*, 2017, **139**, 10403–10409.

22 M. W. Popp, S. K. Dougan, T. Y. Chuang, E. Spooner and H. L. Ploegh, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 3169–3174.

23 J. Hu, W. Zhao, Y. Gao, M. Sun, Y. Wei, H. Deng and W. Gao, *Biomaterials*, 2015, **47**, 13–19.

24 Y. Hou, Y. Zhou, H. Wang, R. Wang, J. Yuan, Y. Hu, K. Sheng, J. Feng, S. Yang and H. Lu, *J. Am. Chem. Soc.*, 2018, **140**, 1170–1178.

25 J. Guo, J. Sun, X. Liu, Z. Wang and W. Gao, *Biomaterials*, 2020, **250**, 120073.

26 J. A. Kritzer, S. Hamamichi, J. M. Mccaffery, S. Santagata, T. A. Naumann, K. A. Caldwell, G. A. Caldwell and S. Lindquist, *Nat. Chem. Biol.*, 2009, **5**, 655–663.

27 E. Miranda, I. K. Norderen, A. L. Male, C. E. Lawrence, F. Hoakwie, F. Cuda, W. Court, K. R. Fox, P. A. Townsend and G. K. P. Ac Kham, *J. Am. Chem. Soc.*, 2013, **135**, 10418–10425.

28 A. Zorzi, K. Deyle and C. Heinis, *Curr. Opin. Chem. Biol.*, 2017, **38**, 24–29.

29 E. M. Driggers, S. P. Hale, J. Lee and N. K. Terrett, *Nat. Rev. Drug Discovery*, 2008, **7**, 608–624.

30 E. Marsault and M. L. Peterson, *J. Med. Chem.*, 2011, **54**, 1961–2004.

31 D. Whitford, *Proteins: Structure and Function*, J. Wiley & Sons, Hoboken, NJ, 2005.

32 G. A. Brar and J. S. Weissman, *Nat. Rev. Mol. Cell Biol.*, 2015, **16**, 651–664.

33 M. Trabi and D. J. Craik, *Trends Biochem. Sci.*, 2002, **27**, 132–138.

34 C. K. Wang, Q. Kaas, L. Chiche and D. J. Craik, *Nucleic Acids Res.*, 2008, **36**, D206–D210.

35 L. Cascales and D. J. Craik, *Org. Biomol. Chem.*, 2010, **8**, 5035–5047.

36 D. J. Craik, N. L. Daly, T. Bond and C. Waine, *J. Mol. Biol.*, 1999, **294**, 1327–1336.

37 D. C. Ireland, R. J. Clark, N. L. Daly and D. J. Craik, *J. Nat. Prod.*, 2010, **73**, 1610–1622.

38 R. Burman, S. Gunasekera, A. A. Strömstedt and U. Göransson, *J. Nat. Prod.*, 2014, **77**, 724–736.

39 J. D. Hegemann, M. Zimmermann, X. Xie and M. A. Marahiel, *Acc. Chem. Res.*, 2015, **48**, 1909–1919.

40 M. O. Maksimov, S. J. Pan and L. A. James, *Nat. Prod. Rep.*, 2012, **29**, 996–1006.

41 N. L. Daly and D. J. Craik, *Curr. Opin. Chem. Biol.*, 2011, **15**, 362–368.

42 R. L. Duda, *Cell*, 1998, **94**, 55–60.

43 W. R. Wikoff, L. Liljas, R. L. Duda, H. Tsuruta, R. W. Hendrix and J. E. Johnson, *Science*, 2000, **289**, 2129–2133.

44 C. Helgstrand, W. R. Wikoff, R. L. Duda, R. W. Hendrix, J. E. Johnson and L. Liljas, *J. Mol. Biol.*, 2003, **334**, 885–899.

45 A. D. Gillon, I. Saska, C. V. Jennings, R. F. Guarino, D. J. Craik and M. A. Anderson, *Plant J.*, 2008, **53**, 505–515.

46 I. Saska, A. D. Gillon, N. Hatsugai, R. G. Dietzgen, I. Hara-Nishimura, M. A. Anderson and D. J. Craik, *J. Biol. Chem.*, 2007, **282**, 29721–29728.

47 O. Saether, D. J. Craik, I. D. Campbell, K. Sletten, J. Juul and D. G. Norman, *Biochemistry*, 1995, **34**, 4147–4158.

48 N. L. Daly and D. J. Craik, *J. Biol. Chem.*, 2000, **275**, 19068–19075.

49 M. Martínez-Bueno, M. Maqueda, A. Gálvez, B. Samyn, J. V. Beeumen, J. Coyette and E. Valdivia, *J. Bacteriol.*, 1994, **176**, 6334–6339.

50 C. González, G. M. Langdon, M. Bruix, A. Gálvez and M. Rico, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 11221–11226.

51 P. Tongaonkar, P. Tran, K. Roberts, J. Schaal, G. Ösapay, D. Tran, A. J. Ouellette and M. Selsted, *J. Leukocyte Biol.*, 2011, **89**, 283–290.

52 G. C. Paesen, C. Siebold, K. Harlos, M. F. Peacey, P. A. Nuttall and D. Stuart, *J. Mol. Biol.*, 2007, **368**, 1172–1186.

53 L. S. Zhang and J. P. Tam, *J. Am. Chem. Soc.*, 1997, **119**, 2363–2370.

54 Y.-Q. Chen, C. Chen, Y. He, M. Yu, L. Xu, C.-L. Tian, Q.-X. Guo, J. Shi, M. Zhang and Y. M. Li, *Tetrahedron Lett.*, 2014, **55**, 2883–2886.

55 M. Chen, S. Wang and X. Yu, *Chem. Commun.*, 2019, **55**, 3323–3326.

56 L. Z. Yan and P. E. Dawson, *Angew. Chem., Int. Ed.*, 2001, **40**, 3625–3627.

57 J. W. Blankenship and P. E. Dawson, *J. Mol. Biol.*, 2003, **327**, 537–548.

58 J. W. Blankenship and P. E. Dawson, *Protein Sci.*, 2010, **16**, 1249–1256.

59 H. C. Hayes, L. Y. Luk and Y.-H. Tsai, *Org. Biomol. Chem.*, 2021, **19**, 3983–4001.

60 H. Y. Chow, Y. Zhang, E. Matheson and X. Li, *Chem. Rev.*, 2019, **119**, 9971–10001.

61 A. D. Foster, J. D. Ingram, E. K. Leitch, K. R. Lennard, E. L. Osher and A. Tavassoli, *J. Biomol. Screening*, 2015, **20**, 563–576.

62 C. Sohrabi, A. Foster and A. Tavassoli, *Nat. Rev. Chem.*, 2020, **4**, 90–101.

63 Y. Huang, M. M. Wiedmann and H. Suga, *Chem. Rev.*, 2019, **119**, 10360–10391.

64 K. Josephson, M. C. Hartman and J. W. Szostak, *J. Am. Chem. Soc.*, 2005, **127**, 11727–11735.

65 T. G. Heckler, L. H. Chang, Y. Zama, T. Naka, M. S. Chorghade and S. M. Hecht, *Biochemistry*, 1984, **23**, 1468–1473.

66 M. Ohuchi, H. Murakami and H. Suga, *Curr. Opin. Chem. Biol.*, 2007, **11**, 537–542.

67 H. J. Kang, F. Coulibaly, F. Clow, T. Proft and E. N. Baker, *Science*, 2007, **318**, 1625–1628.

68 S. J. de Veer, A. M. White and D. Craik, *Angew. Chem., Int. Ed.*, 2021, **60**, 8050–8071.

69 C. K. Wang, S. H. Hu, J. L. Martin, T. Sjogren, J. Hajdu, L. Bohlin, P. Claeson, U. Goransson, K. J. Rosengren and J. Tang, *J. Biol. Chem.*, 2009, **284**, 10672–10683.

70 P. Dabrowski-Tumanski and J. I. Sulkowska, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 3415–3420.

71 J. I. Sulkowska, *Curr. Opin. Struct. Biol.*, 2020, **60**, 131–141.

72 N. H. Shah and T. W. Muir, *Chem. Sci.*, 2014, **5**, 446–461.

73 T. W. Muir, D. Sondhi and P. A. Cole, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, 6075–6710.

74 T. C. Evans, *J. Biol. Chem.*, 1999, **274**, 3923–3926.

75 R. E. Thompson and T. W. Muir, *Chem. Rev.*, 2019, **120**, 3051–3126.

76 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. Kent, *Science*, 1994, **266**, 776–779.

77 J. A. Camarero and T. W. Muir, *J. Am. Chem. Soc.*, 1999, **121**, 5597–5598.

78 M. Q. Xu and T. C. Evans, Jr., *Methods*, 2001, **24**, 257–277.

79 H. Iwai and A. Plückthun, *FEBS Lett.*, 1999, **459**, 166–172.

80 J. A. Camarero, D. Fushman, D. Cowburn and T. W. Muir, *Biorg. Med. Chem.*, 2001, **9**, 2479–2484.

81 G. Volkmann and H. Iwai, *Mol. BioSyst.*, 2010, **6**, 2110–2121.

82 Y. Li, *Biotechnol. Lett.*, 2015, **37**, 2121–2137.

83 C. P. Scott, E. Abel-Santos, M. Wall, D. C. Wahnon and S. J. Benkovic, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 13638–13643.

84 H. Iwai, A. Lingel and A. Pluckthun, *J. Biol. Chem.*, 2001, **276**, 16548–16554.

85 M. C. Waldhauer, S. N. Schmitz, C. Ahlmanneltze, J. G. Gleixner, C. C. Schmelas, A. G. Huhn, C. Bunne, M. Buscher, M. Horn, N. Klughammer, J. Kreft, E. Schäfer, P. A. Bayer, S. G. Krämer, J. Neugebauer, P. Wehler, M. P. Mayer, R. Eils and B. D. Ventura, *Mol. BioSyst.*, 2015, **11**, 3231–3243.

86 T. S. Young, D. D. Young, I. Ahmad, J. M. Louis, S. J. Benkovic and P. G. Schultz, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 11052–11056.

87 K. Jagadish, R. Borra, V. Lacey, S. Majumder, A. Shekhtman, L. Wang and J. A. Camarero, *Angew. Chem., Int. Ed.*, 2013, **52**, 3126–3131.

88 H. Iwai, S. Züger, J. Jin and P. H. Tam, *FEBS Lett.*, 2006, **580**, 1853–1858.

89 J. Zettler, V. Schütz and H. D. Mootz, *FEBS Lett.*, 2009, **583**, 909–914.

90 A. A. Sesilja, Z. Sara, B. Edith, I. Hideo and L. Hilal, *PLoS One*, 2009, **4**, e5185.

91 S. K. Mazmanian, G. Liu and H. Ton-That, *Science*, 1999, **285**, 760.

92 H. Y. Chow, Y. Zhang, E. Matheson and X. Li, *Chem. Rev.*, 2019, **119**, 9971–10001.

93 R. Parthasarathy, S. Subramanian and E. T. Boder, *Bioconjugate Chem.*, 2007, **18**, 469–476.

94 J. M. Antos, W. L. Popp, R. Ernst, G. L. Chew, E. Spooner and H. L. Ploegh, *J. Biol. Chem.*, 2009, **284**, 16028–16036.

95 J. G. Bolscher, M. J. Oudhoff, K. Nazmi, J. M. Antos, C. P. Guimaraes, E. Spooner, E. F. Haney, J. J. Garcia Vallejo, H. J. Vogel, W. van't Hof, H. L. Ploegh and E. C. Veerman, *FASEB J.*, 2011, **25**, 2650–2658.

96 K. Strijbis, E. Spooner and H. L. Ploegh, *Traffic*, 2012, **13**, 780–789.

97 J. Zhang, S. Yamaguchi, H. Hirakawa and T. Nagamune, *J. Biosci. Bioeng.*, 2013, **116**, 298–301.

98 J. J. Bellucci, J. Bhattacharyya and A. Chilkoti, *Angew. Chem., Int. Ed.*, 2015, **54**, 441–445.

99 M. A. Jackson, E. K. Gilding, T. Shafee, K. S. Harris and D. J. Craik, *Nat. Commun.*, 2018, **9**, 2411.

100 N. L. Daly, K. J. Rosengren and D. J. Craik, *Adv. Drug Delivery Rev.*, 2009, **61**, 918–930.

101 G. K. T. Nguyen, W. H. Lim, P. Q. T. Nguyen and J. P. Tam, *J. Biol. Chem.*, 2012, **287**, 17598–17607.

102 G. K. Nguyen, A. Kam, S. Loo, A. E. Jansson, L. X. Pan and J. P. Tam, *J. Am. Chem. Soc.*, 2015, **137**, 15398–15401.

103 G. K. Nguyen, X. Hemu, J. P. Quek and J. P. Tam, *Angew. Chem., Int. Ed.*, 2016, **55**, 12802–12806.

104 M. Schmidt, A. Toplak, P. Quaedflieg, J. Maarseveen and T. Nuijens, *Drug Discovery Today*, 2017, **26**, 11–16.

105 K. S. Harris, T. Durek, Q. Kaas, A. G. Poth, E. K. Gilding, B. F. Conlan, I. Saska, N. L. Daly, D. Van and D. J. Craik, *Nat. Commun.*, 2015, **6**, 10199.

106 Q. Wu, H. L. Ploegh and M. C. Truttmann, *ACS Chem. Biol.*, 2017, **12**, 664–673.

107 R. Yang, Y. H. Wong, G. Nguyen, J. P. Tam, J. Lescar and B. Wu, *J. Am. Chem. Soc.*, 2017, **139**, 5351–5358.

108 K. M. Mikula, I. Tascón, J. J. Tommila and H. Iwa, *FEBS Lett.*, 2017, **591**, 1285–1294.

109 K. S. Harris, R. F. Guarino, R. S. Dissanayake, P. Quimbar, O. C. McCorkelle, S. Poon, Q. Kaas, T. Durek, E. K. Gilding, M. A. Jackson, D. J. Craik, N. L. van der Weerden, R. F. Anders and M. A. Anderson, *Sci. Rep.*, 2019, **9**, 10820.

110 B. J. Smithies, Y.-H. Huang, M. A. Jackson, K. Yap, E. K. Gilding, K. S. Harris, M. A. Anderson and D. J. Craik, *ACS Chem. Biol.*, 2020, **15**, 962–969.

111 K. Yap, J. Du, F. B. H. Rehm, S. R. Tang, Y. Zhou, J. Xie, C. K. Wang, S. J. de Veer, L. H. L. Lua, T. Durek and D. J. Craik, *Nat. Protoc.*, 2021, **16**, 1740–1760.

112 T. Passioura and H. Suga, *Trends Biochem. Sci.*, 2014, **39**, 400–408.

113 T. Kawakami, A. Ohta, M. Ohuchi, H. Ashigai, H. Murakami and H. Suga, *Nat. Chem. Biol.*, 2009, **5**, 888–890.

114 T. Passioura and H. Suga, *Chem. – Eur. J.*, 2013, **19**, 6530–6536.

115 H. Kwon, C. J. Squire, P. G. Young and E. N. Baker, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 1367–1372.

116 M. Walden, J. M. Edwards, A. M. Dziewulska, R. Bergmann, G. Saalbach, S. Y. Kan, O. K. Miller, M. Weckener, R. J. Jackson and S. L. Shirran, *eLife*, 2015, **4**, e06638.

117 J. A. Pointon, W. D. Smith, G. Saalbach, A. Crow, M. A. Kehoe and M. J. Banfield, *J. Biol. Chem.*, 2010, **285**, 33858–33866.

118 F. Zhang and W. B. Zhang, *Chin. J. Chem.*, 2020, **38**, 864–878.

119 R. M. Hagan, R. Björnsson, S. A. Mcmahon, B. Schomburg and U. Schwarz-Linek, *Angew. Chem., Int. Ed.*, 2010, **49**, 8421–8425.

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RSC Chem. Biol., 2022, **3**, 815–829 | **827**

120 G. Yin, J. Wei, Y. Shao, W.-H. Wu, L. Xu and W.-B. Zhang, *Chin. Chem. Lett.*, 2021, **32**, 353–356.

121 F. Sun and W. B. Zhang, *Chin. J. Chem.*, 2020, **38**, 894–896.

122 L. Xu and W.-B. Zhang, *Polymer*, 2018, **155**, 235–247.

123 J. Fang and W.-b Zhang, *Acta Polym. Sin.*, 2018, 429–444.

124 J. Wei, L. Xu, W.-H. Wu, F. Sun and W.-B. Zhang, *Sci. China: Chem.*, 2022, 1–11.

125 B. Zakeri and M. Howarth, *J. Am. Chem. Soc.*, 2010, **132**, 4526–4537.

126 B. Zakeri, J. O. Fierer, E. Celik, E. C. Chittock, U. Schwarz-Linek, V. T. Moy and M. Howarth, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, E690–E697.

127 A. H. Keeble, A. Banerjee, M. P. Ferla, S. C. Reddington, I. Anuar and M. Howarth, *Angew. Chem., Int. Ed.*, 2017, **56**, 16521–16525.

128 A. H. Keeble, P. Turkki, S. Stokes, I. N. A. Khairil Anuar, R. Rahikainen, V. P. Hytonen and M. Howarth, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 26523–26533.

129 G. Veggiani, T. Nakamura, M. D. Brenner, R. V. Gayet, J. Yan, C. V. Robinson and M. Howarth, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 1202–1207.

130 W.-B. Zhang, F. Sun, D. A. Tirrell and F. H. Arnold, *J. Am. Chem. Soc.*, 2013, **135**, 13988–13997.

131 C. Schoene, S. P. Bennett and M. Howarth, *Sci. Rep.*, 2016, **6**, 21151.

132 C. Schoene, J. O. Fierer, S. P. Bennett and M. Howarth, *Angew. Chem., Int. Ed.*, 2014, **53**, 6101–6104.

133 M. Si, Q. Xu, L. Jiang and H. Huang, *PLoS One*, 2016, **11**, e0162318.

134 J. Wang, Y. Wang, X. Wang, D. Zhang, S. Wu and G. Zhang, *Biotechnol. Biofuels*, 2016, **9**, 79.

135 J. O. Fierer, G. Veggiani and M. Howarth, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, E1176–E1181.

136 C. M. Buldun, J. X. Jean, M. R. Bedford and M. Howarth, *J. Am. Chem. Soc.*, 2018, **140**, 3008–3018.

137 X. L. Wu, Y. Liu, D. Liu, F. Sun and W.-B. Zhang, *J. Am. Chem. Soc.*, 2018, **140**, 17474–17483.

138 X.-D. Da and W.-B. Zhang, *Angew. Chem., Int. Ed.*, 2019, **58**, 11097–11104.

139 X. W. Wang and W.-B. Zhang, *Trends Biochem. Sci.*, 2018, **43**, 806–817.

140 T.-t Yang, X.-D. Da and W.-B. Zhang, *Acta Polym. Sin.*, 2020, **51**, 804–816.

141 L. Xu and W.-B. Zhang, *Sci. China: Chem.*, 2018, **61**, 3–16.

142 S. T. D. Hsu, Y. T. C. Lee, K. M. Mikula, S. M. Backlund and H. Iwa, *Front. Chem.*, 2021, **9**, 663241.

143 X. W. Wang and W.-B. Zhang, *Angew. Chem., Int. Ed.*, 2016, **55**, 3442–3446.

144 X. W. Wang and W.-B. Zhang, *Angew. Chem., Int. Ed.*, 2017, **56**, 13985–13989.

145 D. Liu, W.-H. Wu, Y. J. Liu, X. L. Wu, Y. Cao, B. Song, X. Li and W.-B. Zhang, *ACS Cent. Sci.*, 2017, **3**, 473–481.

146 X. Bai, Y. Liu, J. Lee, J. Fang, W.-H. Wu, J. Seo and W.-B. Zhang, *Giant*, 2022, **10**, 100092.

147 Y. Liu, W.-H. Wu, S. Hong, J. Fang, F. Zhang, G. X. Liu, J. Seo and W. B. Zhang, *Angew. Chem., Int. Ed.*, 2020, **59**, 19153–19161.

148 Y. Liu, Z. Duan, J. Fang, F. Zhang, J. Xiao and W.-B. Zhang, *Angew. Chem., Int. Ed.*, 2020, **59**, 16122–16127.

149 W.-H. Wu, X. Bai, Y. Shao, C. Yang, J. J. Wei, W. Wei and W.-B. Zhang, *J. Am. Chem. Soc.*, 2021, **143**, 18029–18040.

150 Q.-H. Guo, Y. Jiao, Y. Feng and J. F. Stoddart, *CCS Chem.*, 2021, **3**, 1542–1572.

151 R. M. Freidinger, D. F. Veber, D. S. Perlow, J. R. Brooks and R. Saperstein, *Science*, 1980, **210**, 656–658.

152 D. F. Veber, R. G. Strachan, S. J. Bergstrand, F. W. Holly, C. F. Homnick, R. Hirschmann, M. L. Torchiana and R. Saperstein, *J. Am. Chem. Soc.*, 1976, **98**, 2367–2369.

153 E. Valeur, S. M. Guéret, H. Adihou, R. Gopalakrishnan, M. Lemurell, H. Waldmann, T. N. Grossmann and A. T. Plowright, *Angew. Chem., Int. Ed.*, 2017, **56**, 10294–10323.

154 P. Edman, *Annu. Rev. Biochem.*, 1959, **28**, 69–96.

155 D. A. Horton, G. T. Bourne and M. L. Smythe, *J. Comput.-Aided Mol. Des.*, 2002, **16**, 415–431.

156 K. Jin, *Future Med. Chem.*, 2020, **12**, 1687–1690.

157 A. A. Vinogradov, Y. Yin and H. Suga, *J. Am. Chem. Soc.*, 2019, **141**, 4167–4181.

158 N. K. Bashiruddin and H. Suga, *Curr. Opin. Chem. Biol.*, 2015, **24**, 131–138.

159 G. P. Smith, *Science*, 1985, **228**, 1315–1317.

160 C. P. Scott, E. Abel-Santos, M. Wall, D. C. Wahnon and S. J. Benkovic, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 13638–13643.

161 R. W. Roberts and J. W. Szostak, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 12297–12302.

162 A. Tavassoli, *Curr. Opin. Chem. Biol.*, 2017, **38**, 30–35.

163 A. Tavassoli and S. Benkovic, *Nat. Protoc.*, 2007, **2**, 1126–1133.

164 T. F. Outeiro and S. Lindquist, *Science*, 2003, **302**, 1772–1775.

165 A. A. Cooper, A. D. Gitler, A. Cashikar, C. M. Haynes, K. J. Hill, B. Bhullar, K. Liu, K. Xu, K. E. Strathearn and F. Liu, *Science*, 2006, **313**, 324–328.

166 B. Keith, R. S. Johnson and M. C. Simon, *Nat. Rev. Cancer*, 2012, **12**, 9–22.

167 A. Giaccia, B. G. Siim and R. S. Johnson, *Nat. Rev. Drug Discovery*, 2003, **2**, 803–811.

168 K. Josephson, A. Ricardo and J. W. Szostak, *Drug Discovery Today*, 2014, **19**, 388–399.

169 T. Passioura and H. Suga, *Chem. – Eur. J.*, 2013, **19**, 6530–6536.

170 T. Passioura, T. Katoh, Y. Goto and H. Suga, *Annu. Rev. Biochem.*, 2014, **83**, 727–752.

171 Y. Goto and H. Suga, *Acc. Chem. Res.*, 2021, **54**, 3604–3617.

172 T. Katoh and H. Suga, *J. Am. Chem. Soc.*, 2022, **144**, 2069–2072.

173 W. Liu, S. J. de Veer, Y.-H. Huang, T. Sengoku, C. Okada, K. Ogata, C. N. Zdenek, B. G. Fry, J. E. Swedberg and T. Passioura, *J. Am. Chem. Soc.*, 2021, **143**, 18481–18489.

174 D. J. Ford, N. M. Duggan, S. E. Fry, J. Ripoll-Rozada, S. M. Agten, W. Liu, L. Corcilius, T. M. Hackeng, R. van Oerle and H. M. Spronk, *J. Med. Chem.*, 2021, **64**, 7853–7876.

175 V. A. Haberman, S. R. Fleming, T. M. Leisner, A. C. Puhl, E. Feng, L. Xie, X. Chen, Y. Goto, H. Suga, L. V. Parise, D. Kireev, K. H. Pearce and A. A. Bowers, *ACS Med. Chem. Lett.*, 2021, **12**, 1832–1839.

176 M. Saito, Y. Itoh, F. Yasui, T. Munakata, D. Yamane, M. Ozawa, R. Ito, T. Katoh, H. Ishigaki and M. Nakayama, *Nat. Commun.*, 2021, **12**, 1–11.

177 S. Imanishi, T. Katoh, Y. Yin, M. Yamada, M. Kawai and H. Suga, *J. Am. Chem. Soc.*, 2021, **143**, 5680–5684.

178 Y. Yin, N. Ochi, T. W. Craven, D. Baker, N. Takigawa and H. Suga, *J. Am. Chem. Soc.*, 2019, **141**, 19193–19197.

179 T. McAllister, T.-L. Yeh, M. Abboud, I. Leung, E. Hookway, O. King, B. Bhushan, S. Williams, R. Hopkinson and M. Münzel, *Chem. Sci.*, 2018, **9**, 4569–4578.

180 Y. Hayashi, J. Morimoto and H. Suga, *ACS Chem. Biol.*, 2012, **7**, 607–613.

181 J. Morimoto, Y. Hayashi and H. Suga, *Angew. Chem., Int. Ed.*, 2012, **124**, 3479–3483.

182 T. Passioura, W. Liu, D. Dunkelmann, T. Higuchi and H. Suga, *J. Am. Chem. Soc.*, 2018, **140**, 11551–11555.

183 T. Zarganes-Tzitzikas, M. Konstantinidou, Y. Gao, D. Krzemien, K. Zak, G. Dubin, T. A. Holak and A. Dömling, *Expert Opin. Ther. Pat.*, 2016, **26**, 973–977.

184 B. Leader, Q. J. Baca and D. E. Golan, *Nat. Rev. Drug Discovery*, 2008, **7**, 21–39.

185 S. Frokjaer and D. E. Otzen, *Nat. Rev. Drug Discovery*, 2005, **4**, 298–306.

186 A. Purkayastha and T. J. Kang, *Biotechnol. Bioprocess Eng.*, 2019, **24**, 702–712.

187 F. M. Veronese and G. Pasut, *Drug Discovery Today*, 2005, **10**, 1451–1458.

188 J. Oppenheim, *Int. J. Hematol.*, 2001, **74**, 3–8.

189 J. M. Antos, G. L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, W. L. Popp and H. L. Ploegh, *J. Am. Chem. Soc.*, 2009, **131**, 10800–10801.

190 J. Zhou, X.-Y. Zhang and Z.-Q. Su, *Chin. J. Polym. Sci.*, 2021, **39**, 1093–1109.

191 T. Miyafusa, R. Shibuya, W. Nishima, R. Ohara, C. Yoshida and S. Honda, *ACS Chem. Biol.*, 2017, **12**, 2690–2696.

192 N. Rasche, J. Tonillo, M. Rieker, S. Becker, B. Dorr, D. Ter-Ovanesyan, U. A. Betz, B. r Hock and H. Kolmar, *Bioconjugate Chem.*, 2016, **27**, 1341–1347.

193 S. Yamauchi, Y. Kobashigawa, N. Fukuda, M. Teramoto, Y. Toyota, C. Liu, Y. Ikeguchi, T. Sato, Y. Sato and H. Kimura, *Molecules*, 2019, **24**, 2620.

194 G. Walsh, *Nat. Biotechnol.*, 2018, **36**, 1136–1145.

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RSC Chem. Biol., 2022, **3**, 815–829 | **829**