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REVIEW PAPER

Cell-free Biosynthesis of Peptidomimetics

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Abstract A wide variety of peptidomimetics (peptide analogs) possessing innovative biological functions have been brought forth as therapeutic candidates through cell-free protein synthesis (CFPS) systems. A key feature of these peptidomimetic drugs is the use of non-canonical amino acid building blocks with diverse biochemical properties that expand functional diversity. Here, we summarize recent technologies leveraging CFPS platforms to expand the reach of peptidomimetic drugs. We also offer perspectives on engineering the translational machinery that may open new opportunities for expanding genetically encoded chemistry to transform drug discovery practice beyond traditional boundaries.

Keywords: peptidomimetics, cell-free protein synthesis systems, non-canonical amino acids, translational machinery

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1. Introduction

Peptides have been potent molecules in drug discovery due to their excellent tunability of biochemical properties at the atomic level using different combinations of amino acids [1-5]. Motivated by the efficacy of peptides in treating diseases, researchers have explored strategies for efficient drug discovery. Many innovative peptide drugs developed through such ways have been approved in the market [6,7]. For example, insulin, a peptide comprised of 51 amino acids, first obtained from the canine pancreas in the 1920s [8], is a critical component of treatment for patients with diabetes mellitus. Later in the 1970-80s, human insulin was first synthesized recombinantly [9] and commercialized [10]. It was the biotechnology that provided an alternative pipeline for drug manufacturing and thus enabled mass production that met large-scale market demands and societal needs (Fig. 1A) [11,12].

Despite these advantages, in general, peptides made of natural amino acids have seen less use as key pharmacological materials for the following reasons [13-15]. First, their linear architectures are too unstructured under physiological conditions, lowering the probability of effective interactions with target molecules. Second, they are often impermeable to cell membranes and vulnerable to proteolysis. Third, their biochemical space and functionality are limited to what can be achieved by the combination of the 20 natural amino acids. Thus, we posit that creating new platforms that enable the biosynthesis (polymerization), selection, and characterization of unique peptides bearing non-canonical entities would accelerate the discovery of novel peptides with therapeutically powerful functions. Further, such a platform may be a crucial tool to produce novel peptidomimetic materials as they enhance molecular rigidity [16], resistance to undesired enzymatic degradation reactions [17,18], and biological functions with enhanced target binding [19].

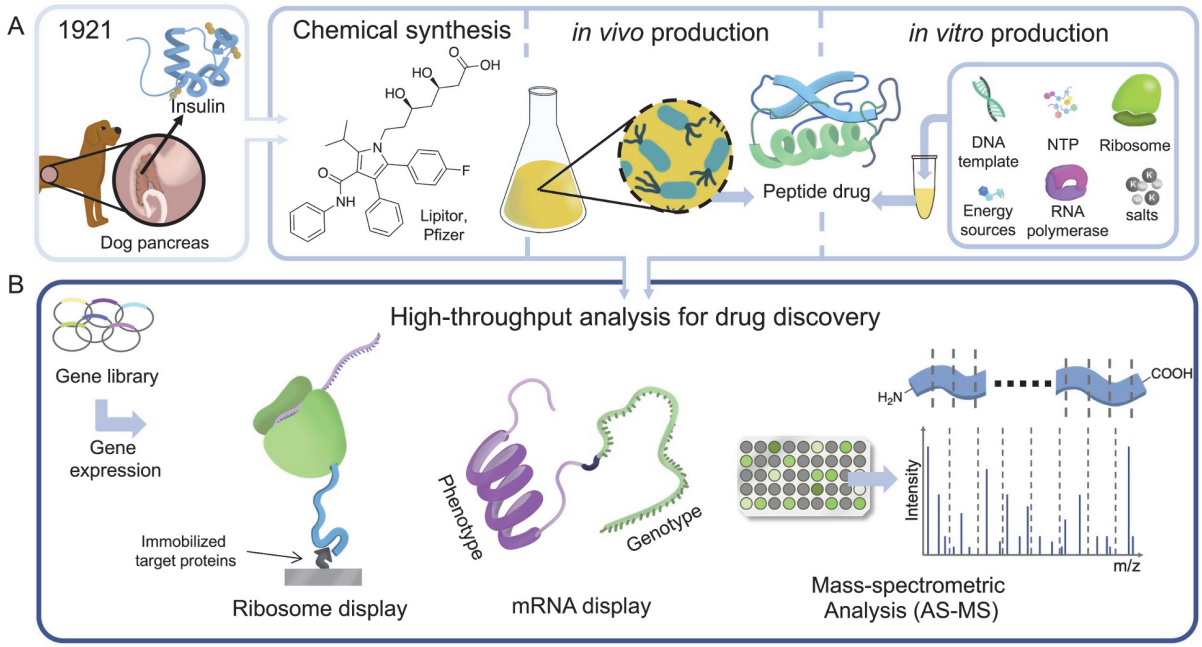

Fig. 1. An overview of peptidomimetics synthesis. (A) Biomanufacturing of peptide drugs. Initially, drugs were directly purified from animals, and later obtained using chemical and biological syntheses. Peptide drugs were commonly produced using either *in vivo* or *in vitro* biological systems. (B) Recently, the open environment of cell-free protein synthesis (CFPS) has been used for new drug discovery. CFPS integrated with high-throughput analysis techniques (display and mass spectrometry) enables screening of affinity peptidomimetic molecules that bind to a target protein from a vast peptide library.

In this review, we highlight recent advances in drug discovery platforms that have expanded the opportunity for efficient biosynthesis of proteins and peptides with the potential to unlock novel pharmacological properties. We will not present in detail the chronicled development of peptide drugs approved in the market and the bio-orthogonal synthetic handles technically required to cyclize or crosslink linear peptides, as these have been recently summarized in several prior and excellent reviews [20,21]. Rather, we focus on the use of cell-free protein synthesis (CFPS) and evolution strategies that would enable the novel peptidomimetics discovery. We first highlight the advantages of CFPS systems as a promising platform for the peptidomimetics production, and then discuss key display technologies crucial to linking the expressed products to their genotype, which allows the selection of peptidomimetics with the biological functions of interest from a library. We also describe recent technologies developed to characterize peptidomimetic molecule libraries using mass spectrometry [22]. Next, we present recent efforts made to incorporate non-canonical monomers into a peptide polymer backbone followed by the formation of non-peptide backbone *i.e.*, esters [23,24], thioamides [25], and thioesters [26] rather than polypeptides using a CFPS platform. Finally, we offer perspectives on the potential of peptidomimetics produced from CFPS platforms [27-30]. Importantly, CFPS containing engineered translational machinery would allow precise tuning of pharmacological properties taking advantage of the chemical moieties inaccessible via current peptide synthesis technologies (Fig. 1B).

2. CFPS Allows the Integration of Non-canonical Entities into Peptidomimetic Molecules

The discipline of synthetic chemistry has been prolific at enabling the synthesis [31-33] and discovery of small molecule drugs [34,35], improving human health worldwide [36-39]. The synergistic integration of cheminformatics with synthetic chemistry has further accelerated the pace of modern drug discovery efforts and has enhanced the efficacy of synthetic drugs [40]. Algorithm-guided rational design has successfully uncovered 'hits' from the vast chemical space for traditional drug target proteins such as receptors or membrane channels [41]. While their small size allows these molecules to penetrate cell membranes via passive diffusion [42], it also limits their interaction with target proteins at a restricted area. Hence both application and targets are limited, and the number of new small molecule drugs approved yearly has remained constant [43,44].

Biopolymers such as peptides, glycans, and nucleic acids

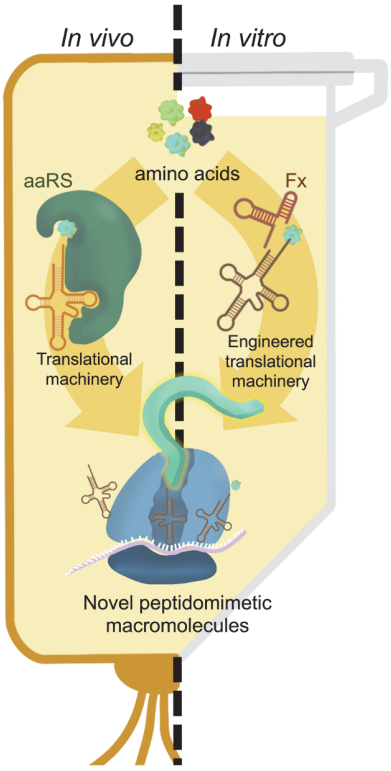

Fig. 2. Fx-mediated acylation produces a tRNA:substrate complex like those made in cells by aaRSs. For the site-specific incorporation of non-canonical substrate into a polymeric chain, a tRNA:substrate complex must be formed before it is accepted by the ribosome. Fx catalyzes the acylation of non-canonical substrates activated with esters to the 3'-OH of a tRNA. Fx: flexizyme, tRNA: transfer RNA, aaRS: aminoacyl-tRNA synthetases.

are uniquely suited as novel therapeutics [45-48] due to their outstanding specificity in intricate reaction conditions. These polymeric molecules provide a larger contact area with target proteins, and the scaffolds of the polymers can be effectively decorated with various functional groups by specifying the non-canonical entities that are compatible with the biological machinery. One powerful emerging technology for this type of biopolymer synthesis is the CFPS platform, which allows one to manipulate the cell's machinery *in vitro* using purified enzymes or crude extracts, enabling control of a wide variety of biosynthetic reactions with non-biological chemical analogs. The fundamental principle of CFPS is to set up *in vitro* transcription and translation reactions containing endogenous biological machinery extracted from the cell and to supplement them with reaction components (energy sources, co-factors, salts, amino acids, and nucleic acids) required for biological reactions of interest [49-54].

The unique characteristic of the CFPS platform is that the open reaction conditions enable the incorporation of a wide variety of non-canonical entities into the protein synthesis reaction, without being hindered by cellular uptake and toxicity that can be problematic *in vivo*. Notably, attempts to incorporate non-canonical amino acids (ncAAs) into proteins have yielded new molecular scaffolds and structural diversity [55-58]. In order to integrate the ncAA into the protein synthesis, it must be attached covalently to a transfer RNA (tRNA), forming an aminoacyl-tRNA or mis-acylated tRNAs. Multiple methods have been developed to charge ncAAs to the 3'-OH of tRNA. The conventional strategy for the acylation reaction of such ncAAs to tRNAs is to engineer aminoacyl-tRNA synthetases (aaRS) by directed evolution strategies so the new enzymes can charge cognate ncAAs to tRNA pairs. Unfortunately, aaRSs have a narrow range of promiscuity toward ncAAs, and the scope of ncAAs charged by engineered aaRSs has been mostly confined to lysine derivatives. Another route to form acylated tRNAs is via a semisynthetic approach, including chemical synthesis of dinucleotide pdCpA (5'-phospho-2'-deoxyribocytidylylriboadenosine) or pCpA, ester coupling reaction with ncAA substrates, and enzymatic ligation (T4 ligase) with a truncated tRNA lacking the two nucleotides at the 3'-end. However, the chemical acylation steps are technically challenging and demand laborious jobs. Recently, an alternative way to synthesize mis-acylated tRNAs that uses an RNA catalyst was devised (Fig. 2). This RNA catalyst, called flexizyme (Fx), was initially evolved using SELEX (systematic evolution of ligands by exponential enrichment) [59], and has since been refined to efficiently acylate amino acid substrates to the 3'-end of tRNA [60-66]. Recent structural studies on Fx [67] and molecular modeling simulations [68] suggest that the terminal J1a/3 base pair of its recognition site strongly interacts with an aromatic group (specifically a benzyl group) of substrates and thereby catalyzes the acylation of chemical substrates to the 3'-hydroxyl group of tRNA.

The key advantage of Fx is its ability to flexibly charge onto the terminal 3'-hydorxyl group of tRNAs virtually any amino acid containing a benzyl group with an activated ester (leaving group) [59,66,69]. This promiscuity toward non-canonical substrates significantly expands the reach of chemical building blocks compatible with endogenous biological machinery. This accommodating feature of Fx toward non-canonical chemical substrates enables formation of tRNA:amino acid complexes that are typically produced by aaRS in cells.

Since its development, Fxs have been used to site-specifically incorporate a wide variety of non-canonical chemical substrates into a peptide or protein. More than 200 non-canonical chemical substrates have been site-specifically incorporated into a peptide or protein using the Fx system. These include α-amino acids bearing a non-canonical side chain (*e.g.*, *p*AzF - *p*-azidophenylalanine)

[70,71], backbone-extended amino acids (β-, γ-, δ-, *etc.*) [72-76], N-alkylated amino acids [77-79], D-amino acids [80,81], oligomeric amino acids (peptides and sidechain-functionalized) [80], thio acids [26], and carboxylic acids containing a moiety of aromatic [82], heteroaromatic [83], hydroxy acid [23], aminooxy [58], and hydrazine [58]. Recently, we have developed substrate design rules and significantly expanded the scope of non-canonical substrates accepted by the protein translation machinery [68]. Briefly, the design rules were empirically determined through efforts to interpret the variations in acylation yields across different substrates using Fxs, when the authors observed that structural and electronic factors determine the yield of Fx-mediated tRNA-charging reaction. The design rules generally follow these observations. i) Substrates with structural similarity to phenylalanine give high tRNA acylation yield, ii) Substrates decreasing the electron density around the carbonyl carbon accelerate the acylation, iii) Substrates bearing a bulky moiety that can sterically block the nucleophilic attack of tRNA to the carbonyl slow down the acylation reaction, giving a low yield. Guided by these design rules, one can use the molecular structures of the substrate to generally predict the acylation efficiency, which significantly reduces the random trial-and-error stage of experiments.

3. CFPS Can Be Integrated with Display Technologies for Discovery of Molecules with Therapeutic Potential

Display techniques (Fig. 3) are a powerful strategy to discover candidate molecules with high affinity and specificity against immobilized target proteins [84-86]. Display technologies using a wide variety of cellular machinery, such as phage [87], cell-surface [88], mRNA [89], and ribosome [90] have enabled the production of and subsequent selection from myriad numbers of peptide molecules, typically reaching 10^{12}-10^{13} molecules at a time [84,91-95]. In selection, vast numbers of variants are subjected to the same chemical and biological conditions simultaneously to yield the 'best' molecules in a designed reaction environment. The power of selection stems from the capability to propagate genetic information through multiple rounds of selection and funnel them into a set of candidates under the selection

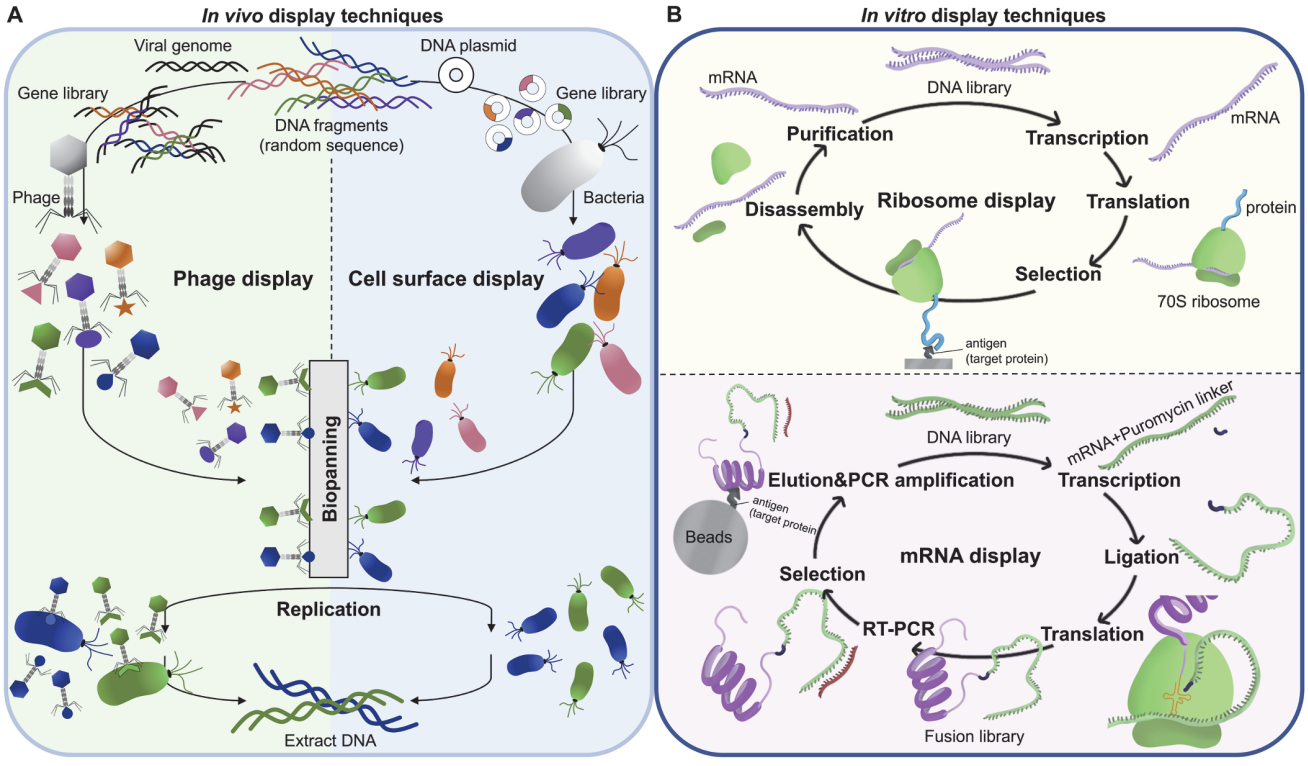

Fig. 3. An overview of display technologies used to select peptide-based molecules binding to target proteins with high affinity. (A) Selection platforms that display peptides on the surface of living systems (phage display and cell-surface display). (B) A schema of *in vitro* selection platforms widely used for drug discovery. A large number of macrocyclic peptidomimetics has been discovered using the RaPID system, which integrate the CFPS platform, Fx technology, and mRNA display. The selected phenotypes are identified through the genetic template that are covalently linked to the phenotype. RaPID: Random non-canonical Peptide Integrated Discovery, CFPS: cell-free protein synthesis, Fx: flexizyme.

pressure introduced at each round (*i.e.*, directed evolution). Although *in vivo* display methods (Fig. 3A), such as phage display and cell-surface display, have been widely used to select peptides or proteins of interest, they are typically limited by the cellular environment. Specifically, disadvantages are i) restriction to canonical building blocks (as discussed above), ii) the limited transformation efficiency, typically capping library size at 10^9, iii) bacterial cells lacking many post-translational modifications, and iv) peptide synthesis that must rely on ribosome-mediated polymerization limiting scaffold diversity (*i.e.*, mainly peptide bonds). The advantage of the *in vitro* display systems described below is their ability to scale to 10^{12}-10^{14} unique library candidates, as no transformation process is required, and easy integration of non-canonical or modified building blocks.

Ribosome display [96-98] (Fig. 3B) is a selection strategy for discovering peptides or proteins that bind to desired targets. It relies on formation of a ternary complex (peptide chain-ribosome-mRNA). When the ribosome reaches a stop codon on mRNA or RNA-DNA junction in the absence release factors, the ribosome stalls while still bound to the mRNA and the nascent peptide chain. Thus, the genotype (mRNA) of each variant can be enriched alongside the phenotype (peptide) bound to the desired target. The selection pipeline includes an iterative cycle of library production, peptide capture, reverse transcription polymerase chain reaction (RT-PCR) for cDNA production, and DNA amplification (Fig. 3B). Ribosome display has been widely applied to peptidomimetics discovery as it enables the production of large libraries (~10^{12}) through PCR, which generates random sequence variants at each selection round. In past years, several studies have demonstrated the importance of ribosome display for the selection of peptidomimetics [99-101].

mRNA display (Fig. 3B) is a widely used technique that relies on covalently binding the ribosomally-produced peptides or proteins (phenotype) to their coding genes (genotype). In the system, a library of DNA is first transcribed to mRNA, which is then ligated to single-stranded DNA containing a puromycin molecule at the 3'-end. As described in the ribosome display technique, the ribosome halts the translation reaction at a stop codon, for example, when no release factors are involved in translation termination. The puromycin at the end of mRNA enters the A site of the peptidyl-transferase center (PTC), where it forms a covalent bond with the growing peptide at the P site, thus coupling the peptide and mRNA. Next, the mRNA-peptide hybrid is pulled down through target proteins immobilized on magnetic beads and reverse transcribed to generate a cDNA library. The cDNA is then enriched by PCR amplification, sequenced, and cast into another round of selection (Fig. 3B). While many of new peptidomimetic drugs have been discovered using this technique [19,56,102-105], unmodified natural peptides generally have poor resistance to proteasomal degradation and poor cellular uptake.

The RaPID (Random non-canonical Peptide Integrated Discovery) system was built on this mRNA display technique, but designed for peptidomimetics selection more specifically. By integrating the Fx system into the mRNA display platform, RaPID enables generic recoding and the integration of ncAAs into the resulting peptidomimetic library [19]. Fx can integrate not only post-transcriptional modification usually lacking in bacteria, but also include residues that allow for the formation of cyclic peptides, or macrocycles. A library of randomized cyclic peptides bearing ncAA is then added to a target protein immobilized on beads. Cyclic peptides with binding affinity to the target proteins are amplified by PCR using the attached mRNA and identified by further rounds of selection, amplification, and high-throughput sequencing. The selected macrocyclic peptides offer an intermediate size between small molecule drugs and protein biologics, two popular representative classes of pharmaceuticals.

4. Cyclic Structures and Motifs in Therapeutic Reagents

Macrocyclic peptides have been long-desired architectures for next-generation therapeutics as they overcome some of the disadvantages of linear peptides. Importantly, cyclization of a molecule provides resistance against undesired enzymatic or extracellular degradation [17], which can enable oral delivery [18,42]. Macrocyclic conformations often stabilize a floppy architecture, which decreases the entropic penalty upon binding to target proteins. The intermediate size of cyclic peptides imparts target selectivity without losing the ability to penetrate cell membranes. The appropriate size (4-20 monomers) of cyclic peptides does not rouse the risk of immunogenicity [106,107]. There are numerous chemical and biological strategies to form cyclized or constrained structures with higher specificity and affinity for a protein of interest than their linearly structured counterparts (Fig. 4). The simplest cyclic conformation of peptides is a single loop made by a selective chemical reaction between the two residues at the N- and C-terminus. Another structure used to present bioactivities is a stapled form in which the dihedral rotation of the peptide backbone is locked with various covalent linkers, resulting in a stable secondary structure. A further improvement in the cyclic architecture has produced two loops which contains a three-way junction at the center of a peptide molecule. This bicyclic (or bridged) structure [108] has shown greater conformational rigidity and proteolytic stability than the monocyclic structure,

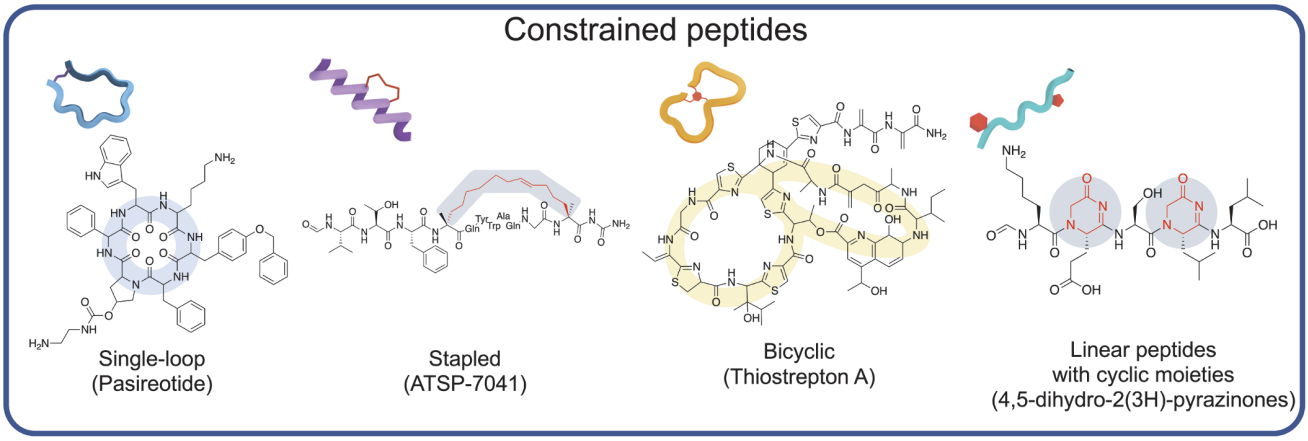

Fig. 4. Formats of constrained peptidomimetic molecules. Peptidomimetics molecules are produced in many different structures and sizes. The simplest cyclic structure can consist of a single-loop of an amino acid chain. Stapled peptides also form a single-loop architecture using a covalent linker between two residues in a peptide with a rigid secondary structure. Bicyclic frames feature a three-way junction, which can produce more rigid structure, giving a larger contact area with target proteins.

which can increase binding affinity and half-life of peptides. Cyclization adds another dimension to peptidomimetic design, because the drugs structural motifs can be tuned by altering the position of amino acid residues [109-111] or chemical linkers connecting the bridges in a peptide [112].

Although macrocyclic structures are of great interest, smaller cyclic motifs found in natural products have also drawn interest as a promising synthetic target for their biological activity. More recently, naturally occurring peptides bearing a backbone-modified scaffold have shown high proteolytic stability and binding affinity to their target proteins [19,113]. A representative example of natural products with such architectures and bioactivities is the class of ribosomally synthesized and post-translationally modified peptides (RiPPs) [114]. The precursor of RiPPs is initially produced to contain a peptidic backbone with elongated motifs at the N-and C-terminus. These leader or recognition sequences recruit the post-transcriptional modification machinery to both cleave themselves off and introduce cyclic motifs between monomers of the peptide, which results in characteristic pharmacological activity (Fig. 5A) [115,116]. Significant progress has been made in the realm of *in vivo* systems for the biomanufacturing of RiPPs [117-119], however, reducing the cost and timing required to engineer host cells remains challenging. Recent studies have shown that the limits of living cells could be overcome by integrating CFPS platforms with the Fx system to produce RiPPs [19,57,120]. The ribosome-mediated polymerization in CFPS provides an ideal template backbone (peptide) that can be further modified with non-canonical motifs. The open environment allows for easy supplementation of non-canonical substrates beyond naturally occurring

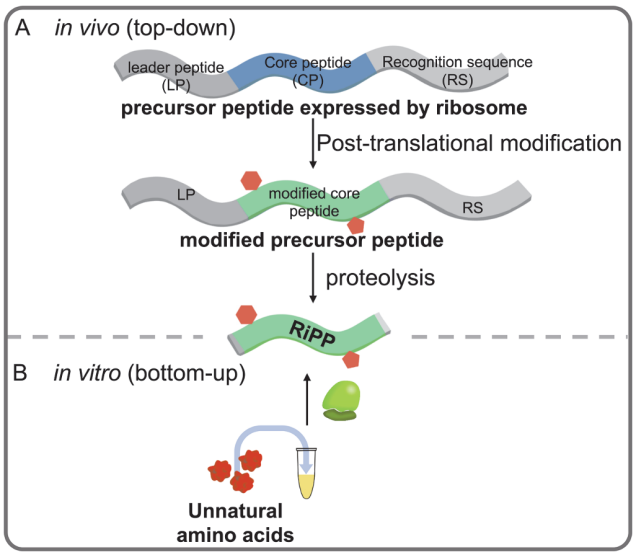

Fig. 5. Biosynthetic pathway for RiPPs. (A) In nature, RiPPs are produced through a top-down strategy; an extensive precursor molecule is synthesized then processed into a final mature peptide. (B) RiPPs, however, can be synthesized through a bottom-up strategy using rationally designed non-canonical chemical substrates on a CFPS platform. RiPPs: ribosomally synthesized and post-translationally modified peptides, CFPS: cell-free protein synthesis.

post-translational modifications, thus unlocking novel chemistries and allowing assembly of new types of RiPP analogs with a bottom-up approach that would not be possible in living cells (Fig. 5B). By combining production of libraries containing cyclic frameworks with the selection of target molecules using mRNA display, research now possesses a powerful discovery pipeline for biopolymers

with therapeutic properties. In the following section, we will discuss a few representative strategies developed to produce novel heterocyclic backbone motifs.

5. Non-canonical Chemical Substrates Enable Formation of Novel Polymeric Backbones

Several studies have explored cyclic structure formations that are inaccessible in conventional small-molecule drugs. For example, site-specifically incorporated ncAAs bearing either an electrophile (*e.g.*, chloroacetyl moiety) or a nucleophile (*e.g.*, cysteine-containing) on their side chains were used to form a covalent thioether bond, linking the N- and C-termini of the linearly expressed peptides in an intramolecular fashion (Fig. 6A) [65,121-127]. Building upon this advancement, a non-canonical motif that can be self-cyclized non-enzymatically to form a bicyclic backbone was developed. A cysteine-proline-glycolic acid motif was incorporated in the middle of a peptide, which subsequently formed a diketopiperadine (dkp)-thioester backbone through a self-rearrangement reaction (Fig. 6B) [128].

As an advanced study, a more complicated cyclic structure was shown to form *in vitro*. For example, azolines, 5-membered heterocycles, are the alternative backbone of many natural products [129,130]. Cyanobactins containing four azoline-based motifs on a peptide backbone display anti-tumor and antiviral activities (Fig. 6C). In cells, cyanobactins are ribosomally synthesized as a long precursor peptide bearing cysteine, serine, and threonine which is then modified by the azoline-producing enzyme PatD. PatD catalyzes the cyclodehydration reaction, yielding azolines with the nucleophile (thiol and hydroxyl group) on the side chain of the three natural amino acids. In a recent study, a CFPS platform for the *in vitro* biosynthesis of azoline-containing peptides was established [131], and later refined to enable the direct synthesis of azoline groups on a peptide in 'one-pot' in the presence of the precursor and the

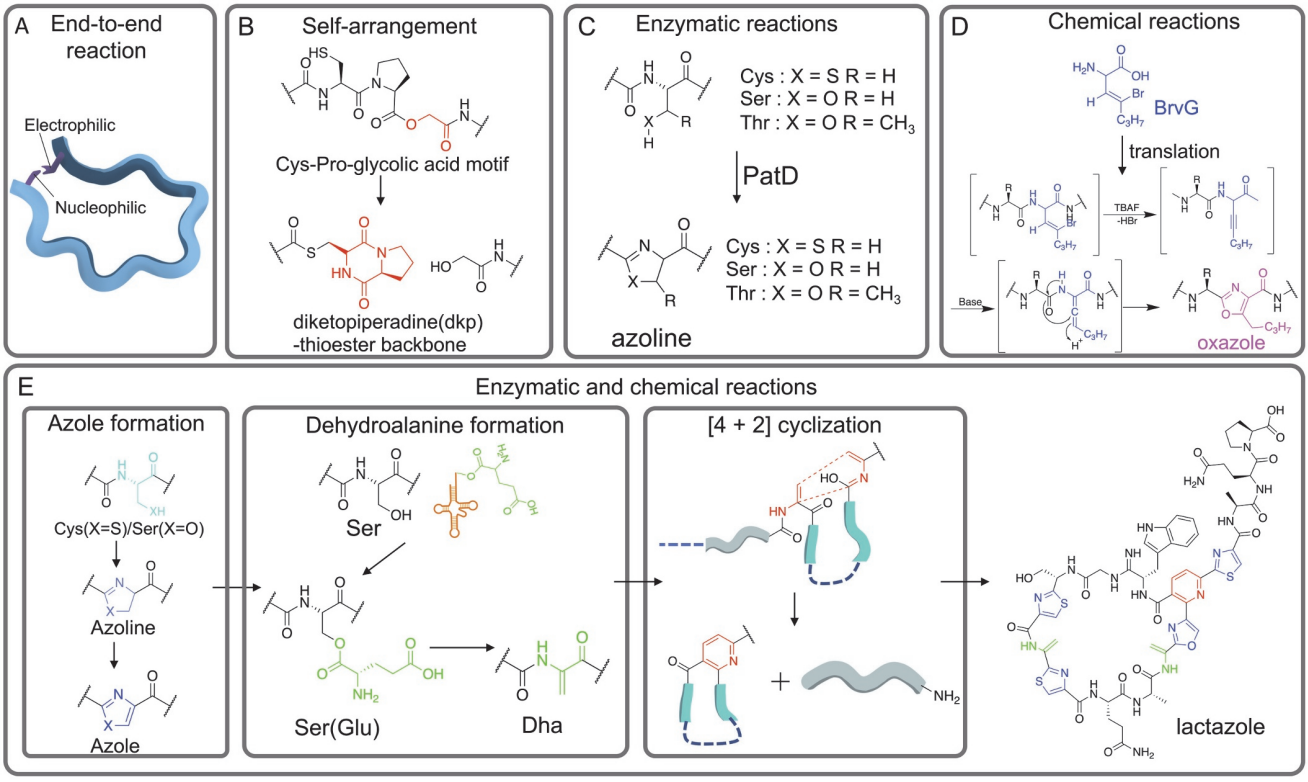

Fig. 6. Building cyclic structures. Chemical and enzymatic reactions generate a variety of cyclic motifs. (A) The most straightforward way to build a macrocyclic structure is an end-to-end intramolecular reaction between a nucleophile and an electrophile present on a peptide chain. (B) Rational design of specific residues in a peptide provides smaller cyclic motifs, an essential motif for a pharmaceutical property of a biopolymer. The Cys-Pro-glycolic acid motif on a peptide undergoes an intramolecular aminolysis reaction, resulting in a cyclic structure, diketopiperadine. (C) Enzymes produce small cyclic motifs into a peptide. PatD gives an azoline ring when specific natural residues (Cys, Ser, or Thr) are present in a peptide chain. (D) Chemical reactions also provide a cyclic motif with pharmaceutical properties into a peptide. A ncAA designed to contain a reactive functional group on the sidechain forms an oxazole by a set of chemical reactions. (E) A series of enzymatic and chemical reactions on rationally designed ncAAs provide a more sophisticated cyclic architecture. ncAAs: non-canonical amino acids.

recombinant PatD enzyme [132]. However, this system is limited by the need to add auxiliary enzymes for the azoline ring production. This was overcome by directly introducing an azole ring into the peptide chain via incorporation of the ncAA carrying a 4-bromovinylglycine group, which is subsequently transformed by dehydrobromination into azoline (Fig. 6D). Significant in this work compared to the previous works is that the incorporation of ncAA into a peptide eliminates the need for a precursor design and an addition of enzymes that transforms the precursor to an azole ring motif on a peptide-based polymer. Moreover, the extra sequences (leader or recognition sequences) that will be removed through the post-translational modification are not required for the creation of a cyclic motif. This work demonstrates that the rational design of ncAA can directly produce a target motif site-specifically through via translation, facilitating biomanufacturing and drug discovery. Furthermore, these cyclization strategies utilizing enzymatic and chemical reactions can be combined to form two different heterocyclic motifs (azole and pyridine) on a peptide backbone, as recently demonstrated in the biosynthesis of a thiopeptide (lactazole) (Fig. 6E) [57]. The ability of CFPS to site-specifically incorporate cyclization sites of various chemistries expands the scope of peptidomimetics that can be synthesized in this way.

6. Mass Spectrometry Elucidates Peptidomimetics with High Affinity to Target Proteins

As discussed above, display techniques isolate peptidomimetics bound to specific protein targets and enrich them through iterative selection cycles. Ideally, a platform for accelerating drug discovery should combine high chemical diversity with the rapid identification of lead compounds selected from libraries. Recent strategies combining display techniques based on affinity selections and mass spectrometry techniques have enabled direct structural analysis of selected candidates [22,133,134]. The great advantage of this system is that no additional reaction for phenotype-genotype linkage is required, as the information of selected compounds can be directly deduced from the phenotypic sequence. This reduces the selection procedure required for mRNA capture, reverse transcription, and DNA amplification to enrich the genotype output, therefore accelerating the identification process. Also, peptide library synthesis is not limited by the ability of enzymes to synthesize biopolymers, but rather made possible through chemical synthesis. ncAAs, in general, are not efficiently incorporated into a peptide by the translational machinery, because the ribosome and other enzymes have been evolved to utilize the 20 natural amino acids. However, the polymerization efficiency of amino acid monomers through solid-phase chemical synthesis is almost identical [135], therefore, both canonical and non-canonical libraries can be produced with virtually unlimited structural diversities through chemical synthesis approaches [136]. Building upon these advantages, the production of libraries using chemical synthesis is also poised to significantly impact the rate of drug discovery and grow the realm of therapeutics from small molecules to biological polymers. Recently, Zhang *et al.* [22] developed the affinity selection-mass spectrometry (AS-MS) technique and discovered a set of peptidomimetic molecules that specifically bind to angiotensin converting enzyme 2 (ACE2) [22]. ACE2 is an important biomarker for cardiovascular disease and a receptor for endocytosis for SARS-Cov-2 infection [137]. In this study, the peptide library containing ~10^6 different synthetic peptides were synthesized on solid-phase using both canonical and ncAA building blocks. Through rapid single tandem mass analysis, the authors discovered non-canonical peptidomimetics (Fig. 7) with high affinity (K_D = 19-123 nM) and enhanced serum stability, showing the great potential of a drug discovery platform integrated with mass spectrometry as a useful tool to select novel peptidomimetic drugs bearing a mixture of canonical and ncAAs.

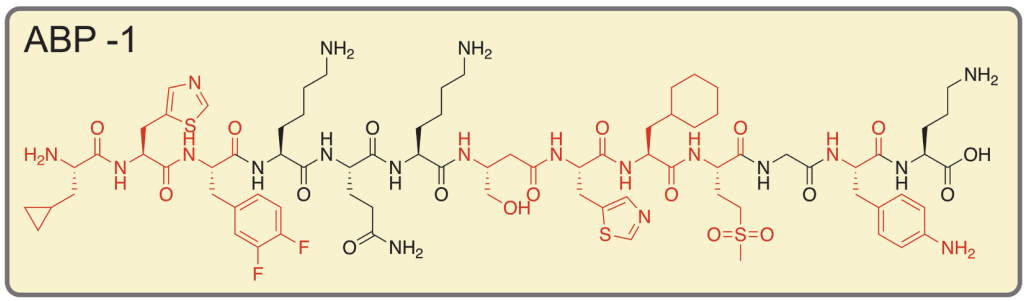

Fig. 7. A peptidomimetic binder molecule discovered by AS-MS integrating mass-spectrometric analysis with high-throughput technology. From a random peptide library, ABP-1 was found to bind to ACE2 proteins with nanomolar affinity (K_D = 19 nM). The residues in red are non-canonical building blocks. AS-MS: affinity selection-mass spectrometry, ACE2: angiotensin converting enzyme 2.

7. Towards Ribosome-mediated Synthesis of Backbone-modified Biopolymers

The cell-free system offers an exciting platform for the direct production of a large variety of backbone-modified biopolymers with novel bioactivities, if the catalytic power of the translation machinery and other enzymes can be leveraged towards non-canonical monomers. A key focus for the synthesis of non-canonical backbone polymers is engineering of the translation apparatus (tRNAs, aaRSs, elongation factors, and ribosome) that has evolved to support life by synthesizing proteins made of α-amino acids. Leveraging its ability to extend natural amino acids into peptides or proteins towards polymerization of ncAA would change the paradigm of peptidomimetics production. In cell-free systems, variations on mRNA input (or synthetic DNA) and non-canonical monomers acylated to tRNA by the Fx allows production of a variant biopolymer bearing a non-amide backbone [138]. Towards this vision, hydroxy acids have been used [23,24] as monomers for the translation apparatus to synthesize polyesters containing an ester as a backbone. Fx was used to reprogram the genetic code and generate new tRNA:hydroxy acid pairs (so-called 'mis-acylated tRNA') and the hydroxyacids on the mis-acylated tRNAs were polymerized to an ester backbone [23]. This study demonstrated consecutive incorporations of up to 12 α-hydroxy acids in a row, effectively using the ribosome for polyester synthesis (Fig. 8). This proof-of-concept illustrates that the genetic code reprogramming approach may enable the production of various block-co-polymers that cannot be synthesized with the current polymerization chemistry. Furthermore, ribosome-catalyzed polymerization, if scalable, could provide a green chemistry platform that is desirable for large-volume biosynthesis at a lower environmental impact.

Building upon this work, recent studies have shown the ability to produce thioester [26,139]-containing peptide *in vitro*. Specifically, synthetic thio acid monomers (Fig. 8) were charged onto tRNA and incorporated into a peptide by standard ribosome-mediated polymerization mechanism. Additionally, aminocarbothio-acid substrates was designed (Fig. 8) for the production of an even more exotic backbone, thioamides [25] *in vitro*. While the incorporation of the aforementioned monomers into a peptide backbone was inefficient and the yield was relatively low (5-250 pmol of products / 5 μL of translation reaction) [124], these works are profound in that the ribosome-mediated polymerization enables the production of altered backbones using rationally designed substrates. Ribosomal synthesis offers a more straightforward way of creating exotic backbones, which were only accessible through hard-to-engineer polyketide synthases and similarly complex multi-step reactions [140,141].

8. Engineering the Protein Translation Systems

Although a combination of the 20 canonical amino acids can yield a remarkable diversity of chemical properties, their potential for novel biological function is limited. The role of selenocysteine [142] and pyrrolysine [143] found in redox reactions and methanogenesis has showcased the

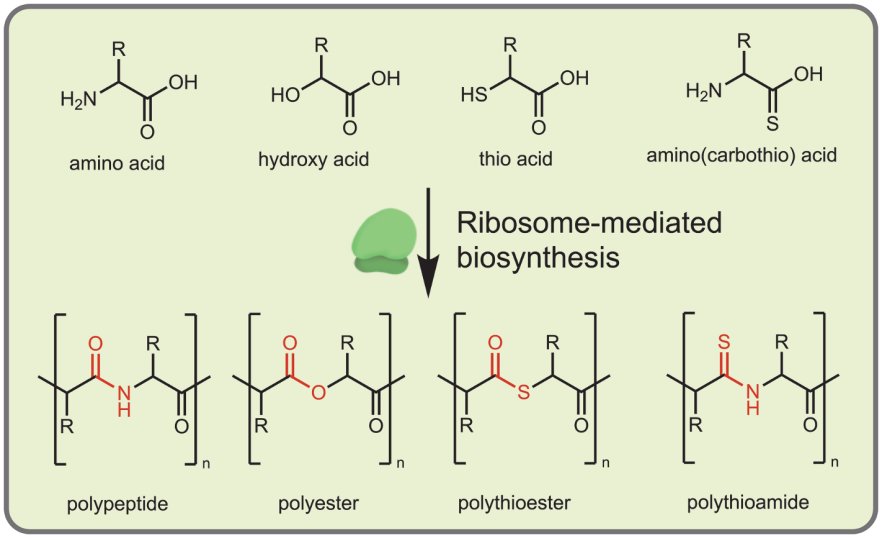

Fig. 8. Biopolymers produced by ribosome-catalyzed polymerization. Non-canonical monomers charged to tRNA by Fx form non-peptide backbone when they are polymerized by the ribosome, giving opportunity to produce a wide variety of block-co-polymers using CFPS. tRNA: transfer RNA, Fx: flexizyme, CFPS: cell-free protein synthesis.

importance of expanding chemical diversity of the monomer building blocks. aaRS/tRNA reassignment has been a crucial strategy to transmit the genetic information from mRNA to a tRNA [144-148]. This strategy may be essential for the industrial-scale synthesis of new biopolymers. However, to create a new aaRS/tRNA pair with ncAA, aaRS has to be modified to orthogonally accept the target ncAA. As aaRSs have evolved to possess high fidelity against canonical amino acids, engineered orthogonal aaRS/tRNA pairs are often outcompeted by the endogenous aaRS/tRNA pairs in cells, resulting in low yields of ncAA incorporation. In addition, aaRS engineering via directed evolution requires complex design methods, and the library size is often limited to few residues in the active site (*e.g.*, for six degenerate sequences, $20^6 = \sim 10^7$) other library sizes used for selection [149-152]. Compared to the drawbacks of the aaRS engineering approach, CFPS offers the excellent flexibility to use a broad selection of ncAAs, and therefore, it has been extensively used to introduce unnatural backbones [153] for peptidomimetic molecules [154,155] and polymeric materials [138,156]. The direct addition of the tRNA complex charged with non-canonical substrates avoids the laborious and hard-to-predict successes of engineering a naturally occurring aaRS towards new substrates.

The ribosome, the workhorse of peptide synthesis, has been a focus for repurposing to accept new substrates (Fig. 8) containing a nucleophile in their molecular structure. The catalytic core (*i.e.*, PTC) of the ribosome has already been shown that it can be mutated on the ribosomal RNA (rRNA) level and that such engineered ribosomes [73,157,158] can enhance the incorporation efficiency of β-, D-amino acids, and dipeptides into a polypeptide chain [159-164]. Building upon this advancement, we have also demonstrated the incorporation of cyclic γ-amino acids [75] and fluorescent amino acids [83,165] into a peptide using engineered ribosomes. These results suggest that the ribosome's preference toward L-α-amino acids evolutionarily optimized to support life are not critically conserved and the ribosome can be engineered to have new catalytic activity beyond nature's limit.

The rational design of backbone-altered chemical substrates can introduce novel pharmaceutical activities when it is incorporated into a peptide *in vitro*. However, synthesizing the same products *in vivo* faces a major challenge. Production of exogenous molecules *in vivo* entails the engineering of cellular machinery, so the complex biosynthetic reaction networks do not affect cell viability. To this effect, *in vivo* biosynthesis of exogenous peptides requires the creation of two separate, orthogonal translation systems in the cell: one to support life, and one to interface with the ncAA and produce the desired product (Fig. 9A).

One key to this 'division of labor' is the orthogonal ribosome (Fig. 9), which minimizes translation of the endogenous genetic messages, while decoding orthogonal mRNA encoding specific biopolymers. This orthogonality is achieved by modified Shine Dalgarno (SD) and anti-SD

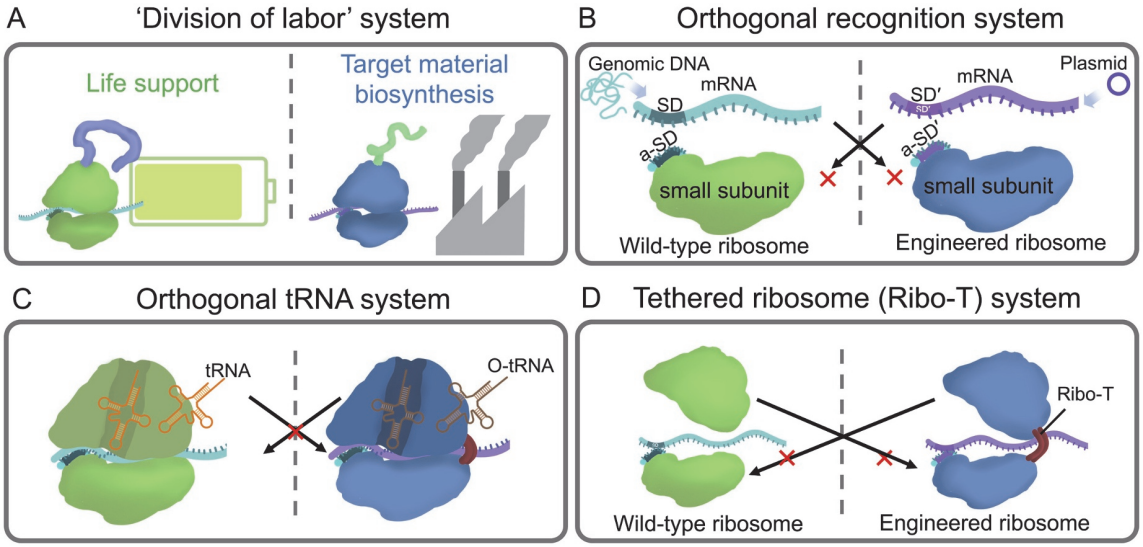

Fig. 9. Overview of orthogonal translation systems. (A) The goal of an orthogonal system is to embed an additional system enabling the biosynthesis of desired materials separately from the central system supporting life in the cell. (B) To introduce orthogonality in the cell, the small subunit of the ribosome has been engineered to have a different SD sequence so that it reads a corresponding engineered mRNA input in high efficiency rather than the endogenous transcriptome. (C) Introducing non-canonical substrates in the cell has been made possible using orthogonal tRNAs that do not cross-interact with the endogenous aaRSs and natural amino acids. (D) A recent study attaching the two subunits covalently has demonstrated orthogonality, showing the incorporation of ncAAs into a peptide polymer. SD: Shine Dalgarno, tRNA: transfer RNA, aaRS: aminoacyl-tRNA synthetases, ncAAs: non-canonical amino acids.

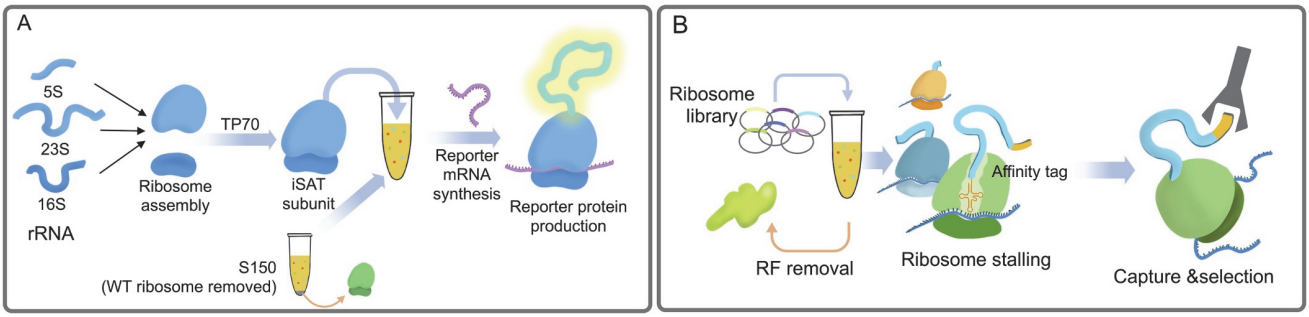

Fig. 10. Ribosome evolution platforms. (A) iSAT. T7-transcripts of 23S, 16S, and 5S rRNA are produced from DNA templates and assembled into active ribosomes with TP70 r-proteins. (B) RISE selects mutant ribosomes from a random DNA library by capturing mutants capable of synthesizing desired polymers. iSAT: *in vitro* synthesis, assembly, and translation, RISE: *in vitro* methodology for ribosome synthesis and evolution.

sequence pair [166] on the small subunit of the ribosome and target mRNA, respectively [165,167-169]. This allows the orthogonal ribosome to only recruit custom messages designed for target material productions, while wild-type ribosomes carry on supporting the cell's life, without being affected by the orthogonal ribosome's activity. A key constraint of the orthogonal translation system is that the small subunit giving orthogonal property with an anti-SD sequence is not permanently paired with the large subunit that is engineered for an altered ability to polymerize at the catalytic core (*i.e.*, PTC [170,171]). This so-called two-body system can yield an association of undesired hybrid ribosome pair, *i.e.*, natural large-subunit + anti-SD-modified small-subunit pairing. Since ribosome subunits are freely interchangeable, orthogonality of the small subunit is not transferred onto the large subunit, and any significant engineering of the large subunits PTC is prohibited since it may be disruptive to natural translation. To address this problem, a tethered ribosome was developed by linking the two 23S and 16S rRNA strands into a transcript [165,167,172, 173], so that the two subunits are conjoined and exclusively directed towards synthesizing novel peptidomimetics through new chemistries [165,167-169,173-177].

Although introducing an orthogonal translation system *in vivo* provides excellent potential for new peptidomimetics synthesis, challenges related to the specificity of aaRS for tRNA charging remain an issue. As an alternative approach, *in vitro* ribosome engineering has emerged as an exciting opportunity. For ribosome synthesis *in vitro*, rRNAs are produced by *in vitro* transcription from a DNA template and assembled into functional ribosomes by supplementing ribosomal proteins. This approach is integrated into a one-pot system called iSAT (*in vitro* synthesis, assembly, and translation) (Fig. 10A), which enables the direct synthesis of rRNA, as well as functional testing of resulting ribosomes in *E. coli* cell extract [92,178-180]. The extract used in iSAT (*E. coli* S150) lacks natural ribosomes due to an ultracentrifugation step during extract preparation, thereby allowing for the assembly of modified ribosomes and subsequent protein synthesis by the new ribosome. For example, Liu *et al*. [181] have demonstrated the multi-site incorporations of *p*-acetyl-phenylalanines (*p*AcFs) into proteins by the ribosome assembled through the iSAT system. A key feature of iSAT is that it can be used to make mutant ribosomes that are functionally active [171,182,183]. More recently, an *in vitro* ribosome evolution and selection platform called RISE has been developed (Fig. 10B) [95]. By integrating iSAT and ribosome display, they demonstrated *in vitro* synthesis of a pool of ribosome variants. Only active ribosomes initiate translation and stalls on mRNA after synthesizing target peptides, forming a ternary complex. The nascent peptides protruding from the exit tunnel can be used to pull down translationally active rRNA sequences. The captured ribosomes are then identified through RT-PCR, DNA-amplification, and high-throughput sequencing. This platform enables the discovery of new ribosomes variants capable of polymerizing non-canonical monomers to backbone-modified polymers more efficiently. We anticipate these new *in vitro* ribosome engineering platforms will play a pivotal role in providing new synthetic tools that accelerate the rate of peptidomimetic drug discovery.

9. Summary and Perspectives

Biosynthetic approaches for peptidomimetics production have made up for the shortcomings of synthetic methods (Table 1) for drug discovery. However, many challenges remain on the way toward the goal of fast, specific peptidomimetic compound discovery. First, final peptido-mimetic products selected *in vitro* for target proteins may present lower resistance against proteolysis, possess toxicity to the host cells, or disrupt protein folding, even if they are

Table 1. Comparison of chemical synthesis-based and biosynthesis-based approach for the synthesis and screening of peptidomimetics

Features	Synthetic approach	Biosynthetic approach
Length of produced peptide	Allows a wide range of peptides up to ~50 aa in length	Allows longer peptides, but usually produces peptides composed of < 15 aa in length
Freedom of ncAA usage	High Virtually any ncAAs can be used, provided the sidechain is stable for solid-phase peptide synthesis reaction.	Low to moderate The incorporation efficiency of ncAAs is highly dependent on the preference of the ribosome.
Yields	Moderate (mg) to high (g)	Low (ng-μg)
Purity of product	Low to moderate	High
Library size (scalability)	Moderate (~10^6)	High (10^{12}-10^{14})
Rate of synthesis	20 aa/day	20 aa/sec
Optimal platform	Suitable for mass production	Ideal for screening or selection

ncAAs: non-canonical amino acids.

complete in theory. Second, the selection approach used to discover peptidomimetics from a library mainly relies on the solid binding affinity toward target proteins by protein-protein interaction [4,184-187], which may not necessarily yield an efficient inhibitor of bioactivities. Third, the library size is somewhat limited to 10^{12}-10^{14} [188], but increasing the size of a library does not proportionally increase the chance of drug discovery, because randomizing more diverse positions for non-canonical substrate incorporations will produce more incomplete libraries that may not include the most efficient candidates for the target proteins. Finally, CFPS typically yields low amounts of peptides, ranging from mg·mL^{-1} [189] to ~1 mg·L^{-1} [190]. This low production output might not suffice the market's demand if newly discovered peptidomimetic prove to be successful. Perhaps the development of chemical synthesis will be needed for mass production.

Looking forward, we believe the CFPS platform combined with ribosomal synthesis and engineering the molecular translation systems would allow the discovery and production of more attractive peptidomimetic molecules that are pharmacologically relevant. The ability to polymerize amino acid monomers using an iterative cycle will yield molecules bearing non-canonical backbones more efficiently than nature's biosynthetic pathway. Integration of computational modeling with vast quantities of peptidomimetics bound to target proteins will refine the structure of new candidates [69,191-195]. Along the way, using CFPS platforms for peptidomimetic drugs will shed light on the evolution of molecular translation machines and serve as a cornerstone to open a new chapter of new peptidomimetic macromolecules production beyond natural limits.

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Author's Contributions

All the authors contributed to all aspects of the article.

Ethical Statements

M.C.J. is a cofounder of SwiftScale Biologics, Stemloop, Inc., Design Pharmaceuticals, and Pearl Bio. The interests of M.C.J. are reviewed and managed by Northwestern University in accordance with their competing interest policies. All other authors declare no competing interests. Neither ethical approval nor informed consent was required for this study.

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