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Exploring sequence space: harnessing chemical and biological diversity towards new peptide leads

Richard Obexer, Louise J Walport and Hiroaki Suga

From their early roots in natural products, peptides now represent an expanding class of novel drugs. Their modular structures make them ideal candidates for pooled library screening approaches. Key technologies for library generation and screening, such as SICLOPPS, phage display and mRNA display, give unparalleled access to tight binding peptides. Through combination with genetic code reprogramming and chemical modifications, access to more natural product-like libraries, spanning non-canonical peptide space, is readily achievable. Recent advances in these fields enable introduction of diverse non-standard motifs, such as cyclisation and backbone methylations. Peptide discovery platforms now allow robust access to potent, highly functionalised peptides against virtually any protein of interest, with typical binding constants in the nanomolar range. Application of these optimised platforms in a drug discovery setting has the potential to significantly accelerate identification of new leads.

Address
Department of Chemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Corresponding author: Suga, Hiroaki (hsuga@chem.s.u-tokyo.ac.jp)

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Introduction

Secondary metabolites of diverse organisms, including polyketides, alkaloids and peptides, have been a source of inspiration for drug development by chemists for many decades (Figure 1a). For example penicillins (**1**) are enzymatically modified tripeptides that represent one of the dominant classes of peptide-based drugs used today. Historically, synthetic exploitation of the chemical space of such naturally occurring peptides has provided a common route to novel therapeutics. To address the global need for new drugs, rather than relying on serendipitous discovery, *de novo* target-directed lead creation has now become essential.

Peptides represent ideal drug scaffolds due to their simple modular structures that nonetheless can adopt a wide variety of complex topologies. Their size and dense functionalisation make them particularly attractive for 'undruggable' targets [1,2], such as those involving protein–protein interactions (PPIs). Selective discovery of functionally tailored peptides is, however, experimentally challenging due to the sheer size of their sequence space. To date, rational approaches for tailoring peptide properties have had limited success. Instead, chemists and biologists have developed screening technologies to identify novel peptide leads for targets of therapeutic importance [3,4].

Peptide discovery strategies

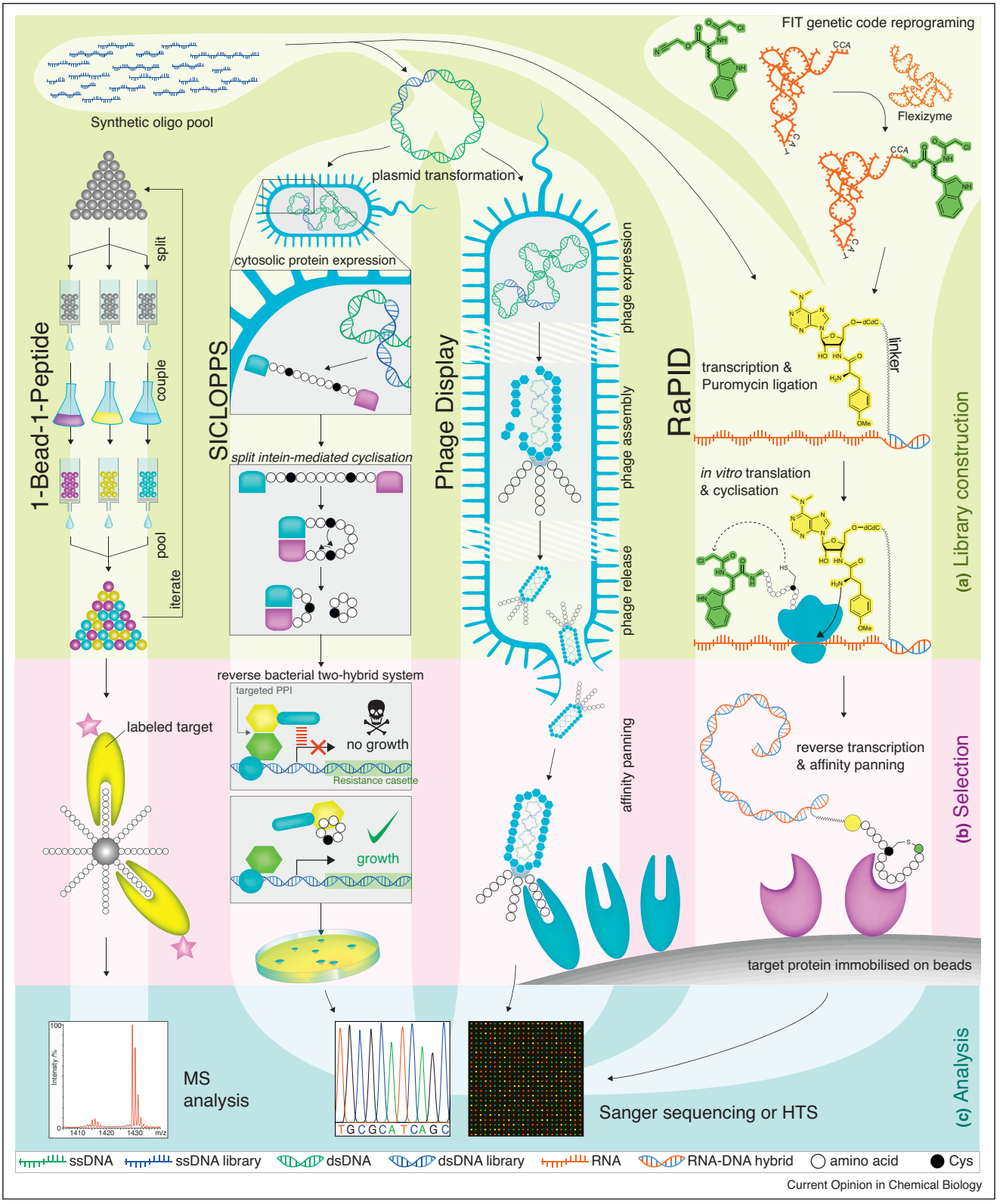
Conceptually, state-of-the-art peptide discovery strategies all follow the same scheme: (1) creation of a pooled peptide library, (2) isolation of peptides with desired properties, and (3) sequence determination of selected peptides (Figure 2). The increase in throughput was enabled by transition from an array format, where the size of the analysed library is limited by reagent quantities and workload, to pooled technologies in which all members are manipulated in parallel. Maintaining a one-to-one link between the peptide sequence and phenotype is the foundation of pooled selection strategies. Recent progress has been significantly enhanced by technical advances in DNA synthesis and sequencing. Taken together the advantages afforded by molecular biology screening techniques have led to their dominance over the past 25 years.

An overview of the most common technologies described in the literature is given in Table 1. Adhering to the principles outlined above, all techniques are somewhat related and share common steps. Requirements during the target selection step can inform the choice of technique. Here we focus on specific examples to highlight unique aspects of these technologies.

Combinatorial chemistry-based screening

The one-bead-one-peptide (1B1P) method, as pioneered by Lam et al. in 1991 [5], is among the oldest examples of pooled screening technologies. Split and pool synthesis techniques were developed to generate diverse libraries of beads, each coated with multiple copies of a unique peptide. Modern protocols allow routine generation of

Figure 1



Examples of natural and selected peptides. *N*-Methylations are highlighted in pink, non-canonical side chains are indicated in green, cyclisations are emphasised in blue, *D*-amino acids are indicated with an orange star and yellow highlights indicate cysteine alkylations for cyclisation. **(a)** Examples of natural cyclic peptides include penicillins (**1**), Cyclosporin A (**2**) and α-Amanitin (**3**). **(b)** Cyclic peptides selected against various

libraries of up to 10^7 compounds, exploiting the whole repertoire of resin-compatible chemistries. Thus diverse backbones, peptoids, D-amino acids and peptide cyclisations are readily accessible, giving the largest degree of freedom of all discussed methods. Fluorescent activated cell sorting (FACS) or magnetic bead sorting are now used for positive bead isolation followed by sequence analysis by partial Edman degradation and mass spectrometry (PED-MS) or MS alone [6,7].

Enhancing diversity through molecular biology

The challenge for molecular biology selection systems, with their unparalleled library sizes, is to generate as chemically creative peptides as nature. To identify macrocylic peptides as potent as naturally occurring ones it is desirable to closely mimic nature by including artificial building blocks and post-translational modifications (PTMs) (Figure 1a) [8]. All molecular biology approaches mentioned in this review rely on the ribosome and associated translation machinery. Thus incorporation of non-canonical building blocks is non-trivial, and represents the focus of much current research.

Split-intein circular ligation of peptides and proteins (SICLOPPS) is an *in vivo* method for discovering head-to-tail cyclised peptides that requires no genetic code reprogramming. Applying amber stop codon suppression, incorporation of unnatural amino acids has been demonstrated [9]. Peptides are typically six amino acids long, including a cysteine residue for splicing [10]. Linkage between genotype and phenotype is achieved through plasmid propagation within cells. The main bottleneck is the achievable library size of 10^{7-9}, which is limited by transformation efficiency. Unlike most other approaches discussed, this technology allows for functional selection (not only binding) through implementation of a reverse two-hybrid system. Whilst originally implemented in *Escherichia coli*, SICLOPPS has since been extended to eukaryotic cells, allowing use of more disease-relevant models [11,12].

Instead of assaying peptides *in cellulo*, peptides can be displayed on a surface through membrane/envelope protein fusion and manipulated *in vitro*. The most commonly used display technology, phage display, relies on filamentous phages, such as M13 [13]. Initial plasmidic libraries are transformed into *E. coli* for expression, assembly and release of peptide-tagged phages. Subsequently, phages are affinity panned to immobilised target protein, and binders are isolated. Enrichment is achieved through iterative selection cycles with reamplified phages. Since this system combines *in vivo* and *in vitro* steps, the library size remains limited by transformation efficiency, resulting in a library diversity of $\sim 10^9$. Insertion of the *in vitro* selection step is advantageous, allowing ready combination with other *in vitro* techniques; peptides can be chemically or enzymatically post-translationally modified increasing chemical diversity and allowing for cyclisation, and, most importantly, selection against virtually any protein target is possible [4,14,15].

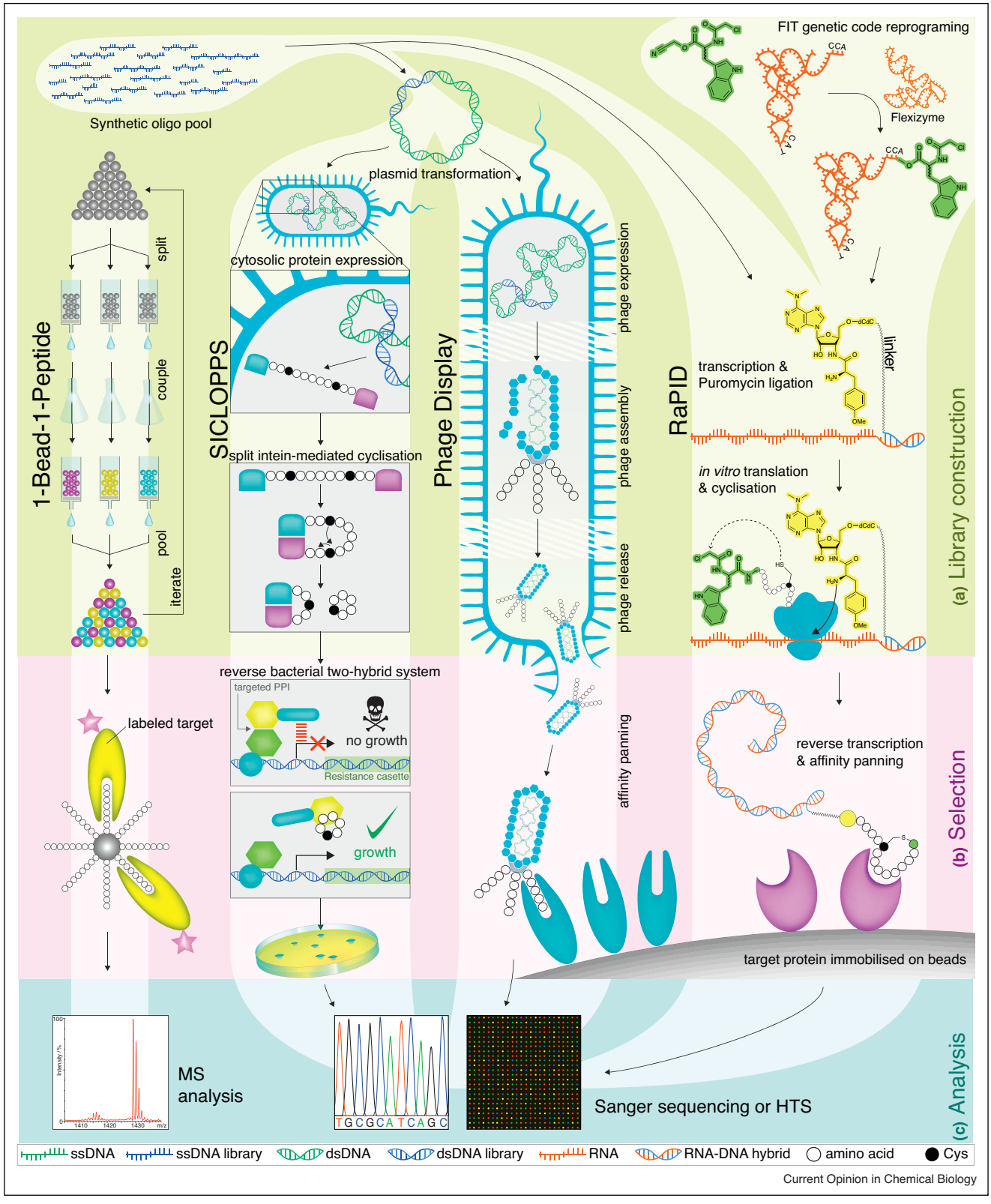
Approaching maximal diversity through *in vitro* technologies

Translation of the libraries *in vitro* alleviates the need for transformation, increasing the accessible library sizes by five orders of magnitude up to 10^{13-14}. Without the compartmentalisation provided by a cell, the peptide-nucleic acid linkage must occur concomitant with translation to maintain unambiguous linkage. A prototypic example is mRNA display, where prior to translation, an antibiotic (puromycin) that normally leads to peptide termination is covalently appended to each RNA strand [16]. Upon peptide translation, puromycin reacts with the peptide at the stop codon position, resulting in a covalent linkage between peptide and RNA. As for other display technologies affinity panning with an immobilised target is the method of choice for identifying binders. To propagate the sequence, RNA is reverse transcribed and the whole procedure is repeated multiple times. Upon enrichment, sequences are elucidated through DNA sequencing.

Reprogramming and 'post synthesis modifications' are the primary means of modulating physicochemical properties of peptides, essential for developing peptides with drug-like properties. In particular, peptide backbone modifications, including azolines, β-amino acids, backbone *N*-methylation and cyclisation (Figure 3), can lead to increased potency through enhanced structural rigidity, proteolytic stability and cell permeability (c.f. cyclosporine (**2**), Figure 1) [17]. Recently, through guided sequence optimisation, Wilbs et al. decreased the K_i of a phage selected peptide fivefold by a single α- to β-amino acid substitution [18]. Rather than inclusion through reverse engineering, *a priori* introduction of artificial amino acids in screening technologies will give rise to new generations of potent peptide drugs.

(Figure 1 Legend Continued) protein targets using peptide discovery platforms. Cyclo-CLLFVY (**4**) disrupts the HIF-1α/HIF-1β PPI and was identified using SICLOPPS [53]. Compound **5** is a sortase A inhibitor and was identified using phage dispay [54]. PB1m6 (**6**) is an allosteric inhibitor of the PPI between Semaphorin and Plexin B1 and was discovered using the RaPID platform. The co-crystal structure of Plexin B1 (grey) and PB1m6 (cyan) and a close up view thereof is shown below (PDB ID: 5B4W), indicating that the peptide-binding site is distant from the PPI surface. The peptide itself adopts a β-sheet like structure. Dashed yellow lines indicate intra and inter-molecular polar contacts [55•]. Peptide piHA-Dm (**7**) against HPA was identified through the RaPID system. The co-crystal structure (PDB ID: 5KEZ) of the peptide (cyan) and HPA (grey) shows an unusually compact conformation of the peptide [56•].

Figure 2



Overview of the main screening technologies used for cyclic peptide discovery. **(a)** All procedures require initial library generation (green background). In the case of the 1B1P method peptide libraries are synthesised directly on the beads by split-and-pool chemistry. Beads are divided and subjected to a single peptide coupling step, then repooled. This procedure is iterated until peptides of the desired length are

Alternative routes to diversity: discovery beyond the standard amino acid alphabet

Traditionally, reprogramming has been done via stop codon suppression or removal of canonical amino acids. More extensive genetic code reprogramming is particularly facile when carrying out *in vitro* display techniques [19–21]. To this end, enhanced library diversity can be achieved through use of a reconstituted translation systems (IVT, *e.g.* the PURE system) giving compositional freedom, for example through omission of aminoacyl tRNA synthetases (ARSs). An advantage of the IVT systems is that pre-aminoacylated tRNA can be directly supplied, providing the most direct method for reprogramming. Aminoacylated tRNAs can be chemically synthesised, though this is technically demanding [22]. Instead, artificial amino acids can be charged onto designated tRNAs using natural or modified ARSs [23]. As an alternative to enzymes, Suga created promiscuous aminoacylating ribozymes, so-called flexizymes, which essentially recognise activated carboxylates. Flexizymes thus allow extensive genetic code reprogramming with non-canonical amino acids (Figure 3) [24]. Overall the flexible *in vitro* translation (FIT) method remains the most versatile and least labour intensive procedure for genetic code reprogramming *in vitro*. In tandem with FIT, Iwane et al. recently exploited the PURE system to the extreme by removing all 20 ARSs and replacing them with charged tRNAs, elegantly demonstrating that the codon boxes can be split to allow maximum diversity [25••]. Using an orthogonal approach, instead of artificially transcribing all tRNAs, Lee et al. selectively depleted all *E. coli* $tRNA^{Arg}$ with colicin D to divide the arginine codon box [26•]. Integrating the FIT system with mRNA display gave rise to the random non-standard peptide integrated discovery (RaPID) system [27]. The versatility of this system is reflected in its broad use in the pharmaceutical industry today.

The translational tolerance of the ribosome has been extensively explored [17,28••,29,30]. Whilst side chain modifications are typically well accepted, backbone modifications present a greater challenge due to the strict contraints of the ribosomal machinery [31,32]. In a comprehensive study Fujino et al. demonstrated that 13 different β-amino acids can be successfully translated in multiple non-consecutive positions within a peptide [28••]. Likewise, incorporation of multiple *N*-alkylated amino acids can be performed using the FIT system [33]. Since 2008 peptide initiation with D-amino acids has been known to be well tolerated, producing largely non-formylated peptides [34]. Moreover, despite the traditional belief that elongation with D-amino acids is highly disfavoured, by use of a variety of precharged D-aminoacyl-tRNAs, increasing numbers of D-amino acids have been translated into internal positions [29,35]. Most recently, Katoh et al. demonstrated the ability to incorporate 10 consecutive D-serine residues by use of an optimised translation system in combination with a modified $tRNA^{Glu}$ [36••]. In a complementary approach, exploiting enzymes involved in lanthipeptide biosynthesis, Yang and Van Der Donk post-translationally introduced D-alanine from serine [37•]. Enzymatic epimerisation is a phenomenon also seen in PTM of ribosomal peptides as catalysed by PoyD during polytheonamide biosynthesis [38•]. Introduction of the PTM enzyme PatD to a modified version of the RaPID system led to cyclodehydration of cysteine, serine and threonine residues to form the corresponding azoline peptides [39•]. More recently, the requirements for the downstream regulatory sequence of PatD were revealed to be flexible, allowing production of a wide range of modified peptides with variable C-termini including peptide tags [40].

More commonly, modifications are introduced chemically. Kitov et al. have pioneered the use of phage-compatible aldehyde-oxime chemistry to modify aldehyde-terminated peptides, for introduction of wide-ranging modifications including sulfonamides, biotin and mannose [41]. They used this chemistry to identify potent chemically-conjugated warhead peptides in functional selections [42]. Similar modifications have been introduced via cysteine disulphide formation and alkylation [43,44]. This chemistry has also proved extremely versatile for peptide cyclisations. Mild alkylation conditions compatible with display technologies are used to

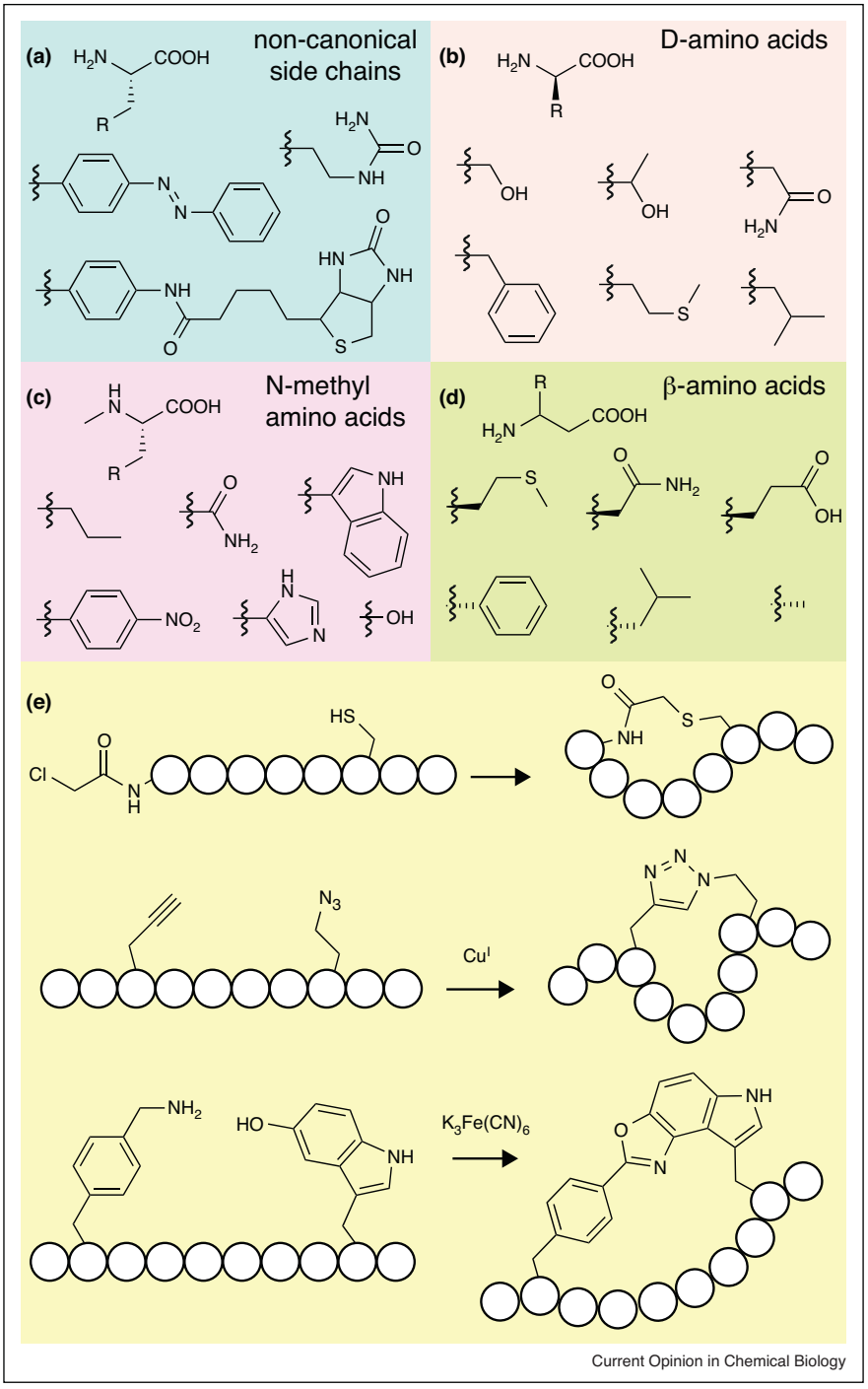
(Figure 2 Legend Continued) achieved. In all other methods library diversity is created by DNA synthesis and subsequent transcription and translation. For SICLOPPS the DNA library is transformed into cells as plasmids. The expressed pre-peptides undergo self-splicing, yielding the final cyclic peptides. In phage display the DNA library is transformed into bacteria as plasmids to allow phage expression, assembly, with individual members of the peptide library expressed on their surface, and subsequent release. In mRNA display the DNA library is transcribed and translated using an *in vitro* translation system. Each mRNA molecule is covalently linked to its encoded peptide by puromycin during translation. This procedure can be combined with the FIT system to give the RaPID system. In the FIT system ribozymes known as flexizymes are used to charge tRNA with any unnatural amino acids of choice. **(b)** Following library synthesis, sequences with desired properties are isolated (pink background). For 1B1P approaches, peptide bearing beads are incubated with, for example fluorescently labeled target for FACS. In the SICLOPPS system peptides are selected using a functional assay. Targets are fused to a transcription factor allowing implementation of a reverse bacterial 2-hybrid system. If peptides disrupt the desired PPI cells survive. In all other cases the cells die. *In vitro* technologies (*e.g.* phage display and RaPID) peptides are selected by affinity panning with bead-immobilised target. The recovered sequences serve as input for repetition of the selection process to strongly enrich binding sequences. **(c)** Finally, sequences of isolated/enriched peptides are determined (blue background). For 1B1P positive clones are analysed by MS. For molecular biology-based methods top hits are identified by DNA sequencing, either through standard Sanger sequencing or high throughput sequencing.

Table 1

Summary of key features of screening technologies commonly used for cyclic peptide discovery

		Library size	DNA form	Screening host	Peptide copy number	Library size restriction	Peptide/nucleic acid linkage	Cyclic	Non natural amino acids/ PTMs/backbone modification	Screening method
	One-bead-one-peptide [7]	10^6	N/A	*In vitro*	Multivalent beads	Library construction	N/A (sequence determination by MS)	Various chemistries	Yes—all resin compatible chemistries	Binding assays
	SICLOPPS [10]	10^9	Plasmid DNA	*In cellulo*	Multicopy	Transformation efficiency	*In cellulo*	Head-to-tail cyclisation via split intein chemistry	Possible with amber codon suppression	Functional assays (*e.g.* bacterial reverse 2 hybrid system)
	Peptide on plasmid [62]	10^9	Plasmid DNA	Bacteria	Multicopy	Transformation efficiency	Peptide fusion to LacI	Possible	Possible with amber codon suppression	Binding assays
Surface display	Prokaryotic [63]	10^9	Plasmid DNA	Bacteria	Multivalent	Transformation efficiency	*In cellulo*	Yes—commonly by post translational cysteine alkylation	Possible with amber codon suppression	Binding assays
	Eukaryotic [64]	10^7		Yeast						
	Phage [13,65]	10^9		Phage						
In vitro display	CIS [66]	10^{14}	DNA	*In vitro*	Single copy	Translation scale	Peptide fusion to DNA replication initiator protein (RepA)	Yes—commonly	Yes—possible	Binding assays
	Ribosome [67]		mRNA				Stable protein–ribo some–mRNA complex			
	mRNA [16]						Covalent puromycin linkage			
	RaPID [68] /TRAP [69]							Yes—commonly *N*-acetyl chloride chemistry	Extensive reprogramming using the FIT system	

Figure 3



Examples of non-canonical amino acids and chemistries accessible by genetic code reprogramming or post-translational modifications. **(a–d)** A selection of non-natural amino acids that can be incorporated using the FIT system **(e)** Cyclisation methods using one (top) or multiple (middle, lower) non-natural amino acids.

create both cyclic and bicyclic libraries [45–47], and to chemically staple short alpha-helical peptides [48]. Combining this bicycle bridging moiety with the classic *N*-acetyl chloride cyclisation of the RaPID system, Bashiruddin et al. even created tricycles [49]. Alternatives for cyclisations include enzymatic lanthipeptide formation [50], and crosslinking of two artificial amino acids [51,52].

Recent applications in drug discovery

A number of recent examples highlight the success of peptide-based selection techniques for targeting medicinally relevant protein targets. Using the SICLOPPS system, Miranda et al. identified inhibitors for the HIF-1α/HIF-1β PPI (**4**, K_D = 124 nM) [53]. Using phage display, Rebollo et al. selected bicyclic inhibitors of Sortase A (**5**, K_D = 1.5 μM), the first inhibitor for this target [54]. Through use of the RaPID system Matsunaga et al. elicited a 16 amino acid peptide (**6**, K_D = 3.5 nM) against Plexin B1, a Semaphorin receptor implicated in osteoporosis [55•]. Intriguingly the best peptide binds to a crevice distant from the Semaphorin binding site, yet still allosterically inhibits the PPI (IC_{50} = 1.5 μM) (Figure 1b). Likewise, Jongkees et al. selected a potent nonapeptide inhibitor of human pancreatic α-amylase (HPA, **7**, K_I = 7 nM) exhibiting exemplary selectivity over related amylases [56•]. The identified peptide is composed of a small cycle with a long tail that binds to HPA in an unusually compacted conformation (Figure 1b).

Outlook

Platforms for peptide discovery have matured beyond academia and are now widely adopted by the pharmaceutical industry. Nevertheless new avenues remain to be explored, especially to reduce labour and artifact accumulation. The advent of readily available, affordable next-generation sequencing technologies is revolutionising library screening approaches by facilitating early identification of enriching sequences. For example, its application to display has allowed isolation of the best peptide sequences within a single selection round [57,58]. Jalali-Yazdi et al. recently developed a method for identifying the binding constants of over 20 000 peptides within their library directly through population sequencing at various times [59••]. They identified peptides with picomolar affinities, notably finding that enrichment rank did not directly correlate with binding affinity. In a further innovative use of deep sequencing, Svensen et al. recently adapted the Illumina sequencing chip to allow ribosomal peptide synthesis of a DNA library directly as clusters on the chip [60••]. By combining this with sequencing of the immobilised DNA library it should soon be possible to combine the large diversity of screening-based methods with the relative speed of array-type systems. The full potential of these technologies is only now beginning to emerge.

The results outlined above have showcased our ability to identify peptides that interact and manipulate a wide-range of medicinally relevant proteins, many of which are challenging to target with small molecules. Peptide leads can act as models for small molecule drug design. Direct applications of peptide therapeutics, especially for intracellular targets, are yet hampered by bioavailability and cell permeability [61]. Whilst advances in genetic code reprogramming and post synthesis peptide modifications have gone some way towards addressing these pharmacokinetic hurdles, a convincing general solution has not yet emerged to address this shortcoming. Efforts towards engineering ever less peptide-like, but nonetheless ribosomally expressed molecules provide an interesting avenue to access unexplored chemical spaces.

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