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Beyond the Canonical 20: Peptide Discovery with Non-Canonical Amino Acids

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Abstract: Amino acids are central to biology as signaling molecules and as the building blocks of peptides and proteins, which represent an expanding class of drugs with vast therapeutic potential. The precise modulation of individual residues in therapeutic peptides and proteins is crucial to enhance their pharmacological properties. Expanding beyond the twenty proteinogenic amino acids to include non-canonical amino acids (ncAAs) offers powerful strategies to optimize the stability, selectivity, and potency of peptides. Including ncAAs in the early discovery phase can significantly accelerate lead development and clinical translation. This review examines how diverse platforms integrate ncAAs in early discovery and compares the capabilities and limitations of these discovery technologies. Finally, key challenges are outlined that must be addressed to drive future innovations and explore new therapeutic avenues. Together, these approaches mark a shift towards peptide drug discovery where non-canonical chemistry is not an exception but a necessity.

Keywords: Amino acids · Drug discovery · Peptides · Therapeutics

Nathalie M. Grob earned her BSc in Pharmaceutical Sciences and MSc in Pharmacy from the University of Basel in 2012 and 2014, respectively. She completed her doctoral studies on radiolabeled peptides from 2015–2019 at ETH Zürich with Profs. Schibli and Mindt. In 2020, she joined Prof. Pentelute in the Department of Chemistry at MIT for her postdoctoral training where she worked on methods for the discovery of peptides and peptide-encoded small molecules. Since April 2024, she has been an Assistant Professor in the Institute of Pharmaceutical Sciences at ETH Zürich supported by an SNSF Starting Grant and leads the research group for Peptide-Based Drug Discovery. Her team develops discovery platforms to address pharmaceutical challenges of peptide-based modalities to pursue both established and emerging target classes.

1. Introduction: Why Amino Acids Matter

Alpha-Amino acids are fundamental building blocks of life. Many amino acids (AAs) and their derivatives are endogenous signaling molecules including neurotransmitters and hormones. Beyond their critical roles in physiology, the 20 canonical amino acids (cAAs) encoded by the standard genetic code of eukaryotes constitute the primary building blocks of peptides and proteins. For pharmaceutical scientists and medicinal chemists, these biopolymers are of particular interest for two reasons: firstly, proteins of human or pathogenic origin account for more than 95% of the molecular targets of approved drugs.[1] Secondly, peptides and proteins themselves are a growing class of drugs in both established and emerging disease areas with immense therapeutic potential.[2] The later became apparent through the first medical use of insulin in 1922, which transformed the treatment of patients with type-1 diabetes mellitus and extended their lives by decades.[3] The research group for Peptide-Based Drug Discovery is particularly interested in advancing these concepts toward the development of next-generation peptide therapeutics.

Access to synthetic peptides was crucial to systematically explore the potential of this class of drugs. Innovations including solid-phase peptide synthesis (SPPS) in 1963[4] and recombinant DNA technology in 1977[5] enabled precise control over the sequences of synthetic peptides at the level of individual amino acids. This is a powerful capability as even single-residue changes can have profound impact on pharmacological properties including potency, selectivity, solubility, and metabolic stability. For instance, modern insulin analogs used in the treatment of type-1 diabetes mellitus differ from the human hormone by only a few mutations that modulate their pharmacokinetics, allowing for precise control of blood glucose levels.[6]

Advances in peptide drugs highlighted the need for greater chemical diversity to optimize therapeutic effects. Nature itself extends the repertoire of amino acids beyond the canonical 20. Modified or non-canonical amino acids (ncAAs) are key components of natural products and secondary metabolites across all kingdoms of life, being used as potent toxins, defense agents, and mediators of molecular communication.[7,8] In peptides and proteins, post-translational modifications (PTMs) such as phosphorylation, hydroxylation, or glycosylation play critical roles in fine-tuning activity, stability, and signaling cascades.[9] The broad variety of peptide natural products demonstrates how ncAAs can enrich structural and functional diversity.[10] These concepts have been successfully translated into medicinal chemistry, where ncAAs are core structures in numerous small-molecule and peptide drugs, underscoring their broad utility for modulating bioactivity and pharmacology.

This review examines how ncAAs are incorporated in widely used peptide discovery platforms, how they expand the structural landscape, and how they enable the translation of peptide binders into tailored therapeutics.

2. The Benefits of ncAAs in Peptide Drugs

Peptides are valuable therapeutics due to their high efficacy and target selectivity combined with generally low toxicity and non-concerning, readily cleared metabolites.[11] However, their

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inherently low proteolytic stability, rapid elimination, and limited membrane permeability can represent major obstacles for therapeutic success.[11] To address these challenges, chemical modifications and ncAAs are frequently incorporated into lead structures.[12] Backbone modifications, bioisosteres, and D-amino acids are widely used to protect protease cleavage sites and thereby enhance metabolic stability. Moreover, backbone modifications can modulate structural conformation and enhance cell permeability (Fig. 1).[13,14] Macrocyclization, achieved through linkages of the termini or *via* side chains, provides an additional strategy for tailoring the overall peptide structure with potential benefits on target binding, metabolic stability, and cell permeability.[15] Chemically-modified side chains can further expand functional diversity as ncAAs that feature halogenated, hydroxylated, or extended aromatic functional groups can fine-tune target interactions and enhance the selectivity and potency of the resulting candidates. Both cAAs and ncAAs may also serve as handles for bioconjugation by site-selective functionalization with lipids, polyethylene glycol, small-molecule pharmacophores, or electrophilic groups to grant further control over pharmacokinetics and the mechanism of action.[16,17]

Collectively, ncAAs expand the structural landscape to generate viable peptide therapeutics. Yet, ncAAs are often introduced only during optimization of structure–activity relationships (SAR) at the hit-to-lead development stage in labor-intensive and time-consuming cycles of synthesis and bioactivity profiling. Therefore, current efforts aim to incorporate ncAAs directly into the early discovery process.

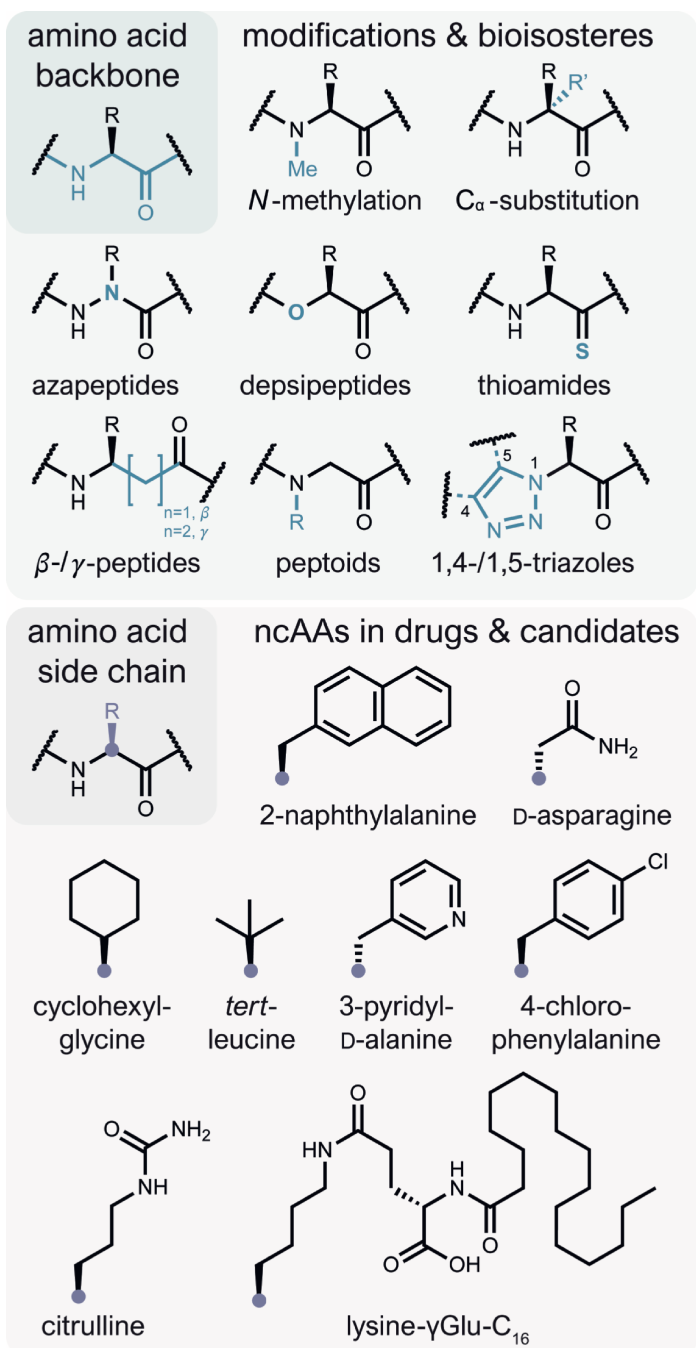

Fig. 1. Common backbone modifications (top) and selected ncAAs used in approved peptide drugs and candidates under clinical evaluation (bottom).

3. Discovery Technologies and Their Ability to Go Beyond 20

Bioactive peptides from nature remain a rich source of inspiration for structure-based development of new drugs,[18] but structure-agnostic, *de novo* approaches are equally critical to advance therapeutic strategies in areas with limited or no treatment options. The following technologies have been most impactful for the *de novo* discovery of peptide binders which serve as crucial starting points for the development of therapeutic drug candidates (Fig. 2).

3.1 *Display Technologies*

Display technologies physically connect peptides expressed *via* a translational machinery to their encoding oligonucleotide sequence, enabling the selection of *de novo* binders from large, combinatorial libraries of 10^9–10^{14} members.[19] Today, the two most applied technologies are phage display and mRNA display, and peptides with potent bioactivity have been discovered *via* both platforms using affinity-based selections. However, the display technologies differ in their ability to explore chemical space with ncAAs.

Phage display, first described in 1985, enables the expression (display) of peptides on the surface of bacteriophages by recombinantly linking their encoding DNA to the gene of a phage coat protein (Fig. 2A).[20] This technology has been successfully applied in affinity-based selections (biopanning) to identify peptide binders to various targets of interest. Several hits proceeded to clinical evaluation after extensive optimization, ultimately resulting in four approved peptide drugs.[21] The introduction of ncAAs in phage libraries can be achieved either by enabling their expression during phage production *via* genetic code expansion (GCE)[22] or by chemical, post-translational modification of phage-displayed peptides.[23] However, *in vivo* incorporation of ncAAs by GCE is technically difficult as the ncAAs need to be transported into the host harboring the respective tRNA–tRNA synthetase pair that is orthogonal to the host system to avoid incorporation at undesired locations. Still, numerous derivatives of phenylalanine, lysine, and tyrosine were successfully expressed by phage-displayed libraries, but only few examples reported designs with more than one ncAA per library.[23,24] Alternatively, phage-compatible chemistry has proven effective for introducing PTMs to achieve residue-specific alterations, particularly for the generation of macrocyclic peptides through disulfide bonds or cysteine alkylation with unnatural linkers.[26,27] Such chemical strategies help expand the use of ncAAs in phage systems

In contrast, mRNA display is more permissive to incorporate ncAAs by GCE and genetic code reprogramming as the translation occurs *in vitro* using purified components and not in live host cells.[28–30] The display method emerged in 1997 as a peptide discovery platform by enabling the direct linkage of translated peptides to their encoding mRNA *via* puromycin at the 3' end (Fig. 2B).[31] The later development of flexizymes as catalytic RNAs with the ability to charge ncAAs onto tRNAs for ribosomal translation and application within the Random nonstandard Peptides Integrated

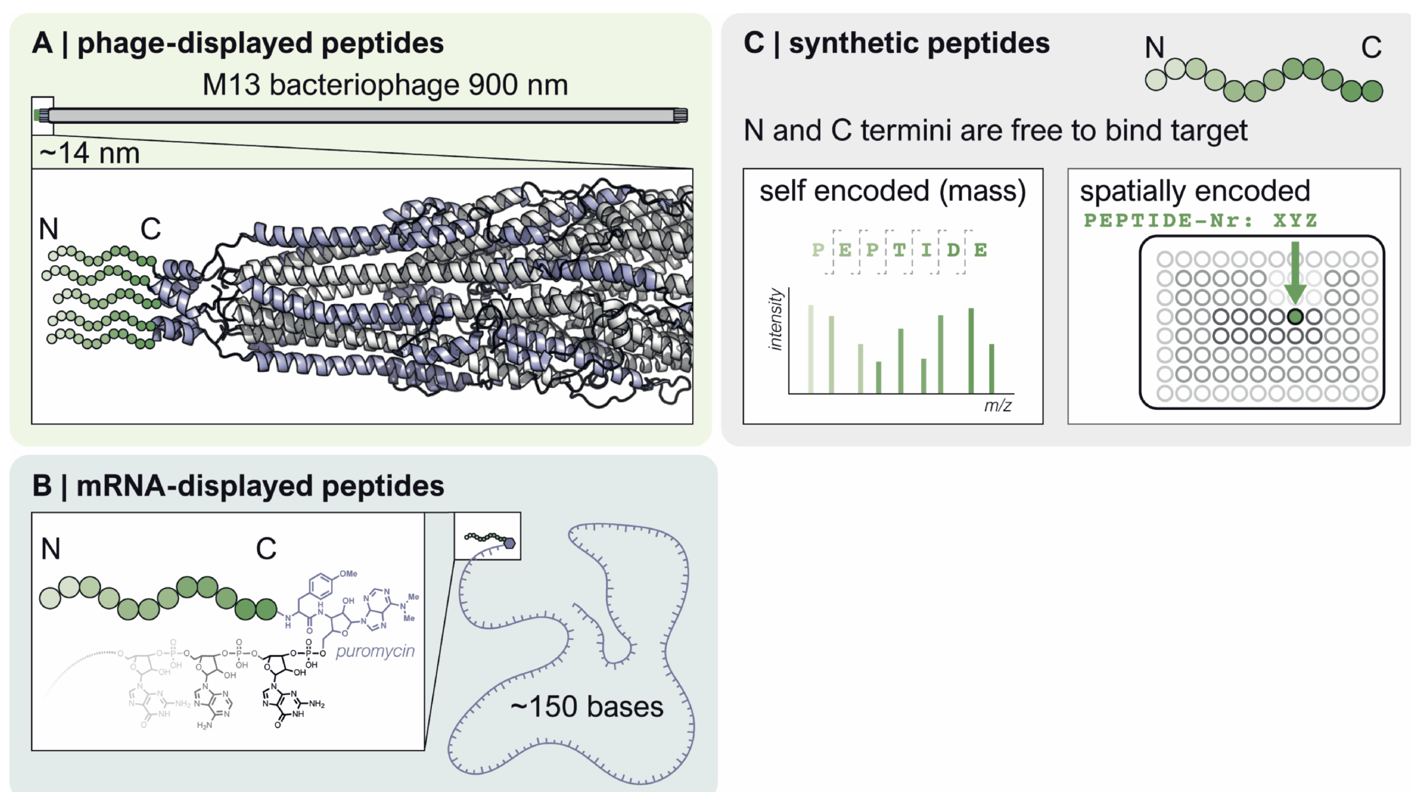

Fig. 2. General scaffolds in technologies for the *de novo* discovery of peptide binders. A) Peptides (4–5 per phage) are displayed on M13 bacteriophages (relative proportions), magnification of the pIII coat protein with N-terminally fused peptides adapted from PDB 8ixk.[25] B) Peptides linked to their encoding mRNA via puromycin in proportion to complete mRNA code. C) Synthetic peptides are self encoded through their mass or position in an array (spatial).

Discovery (RaPID) system allowed the ribosomal incorporation of diverse ncAAs, including D-residues, *N*-methylated AAs, β- and γ-AAs, and backbone-modified residues to generate both linear and macrocyclic peptide scaffolds.[32,33] Reprogramming of the genetic code even enabled the design of libraries with multiple ncAAs.[34] mRNA display has been used to generate immense libraries of over 10^{14} members and has identified macrocyclic peptide hits with remarkable stability and potency yielding several lead structures currently in advanced clinical evaluation and one approved drug.[19] These successful representatives demonstrate that including ncAAs directly in the early discovery phase not only expands structural diversity but can also accelerate the path from discovery of a binder to development of a therapeutic lead.

Innovations in display technologies have enabled the use of diverse ncAAs but variations in translation and sequencing efficiencies may introduce biases in selection results. Moreover, the C-terminal linkage of the peptide to its encoding phage or mRNA restricts conformational freedom and may cause steric hindrance, representing important limitations.

3.2 *Chemically Synthesized Libraries*

SPPS has been transformative for accessing peptides that incorporate amino acids beyond the 20 cAAs. Robust and well-established synthesis protocols based on AAs featuring the base-labile fluorenylmethoxycarbonyl (Fmoc) protecting group and orthogonal, acid-labile protecting groups including *tert*-butyl (*t*Bu), *tert*-butyloxycarbonyl (Boc), trityl (Trt), and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) can efficiently incorporate hundreds of commercially available, chemically-diverse ncAAs, thereby facilitating the production of highly tailored peptide sequences.[35] Therein, modifications of the peptide backbone, alternative linkages between amino acids, incorporation of ncAAs with uncommon functional groups, and site-specific functionalization of side chains are readily accessible by established reactions, which routinely yield high-quality products. The use of excess reagents drives reactions to completion, and the immobilization of the growing peptide on a solid phase minimizes workup and purification. Different solid supports and resin-compatible chemistry can be employed to accommodate various cleavage conditions and expand the scope of accessible transformations that provide a high level of control, structural diversity, and compatibility with late-stage functionalization strategies.[16]

These robust SPPS protocols have been leveraged for the generation of peptide libraries, which have become powerful tools in peptide drug discovery (Fig. 2C). The first combinatorial peptide libraries prepared by SPPS and used for screening were reported in 1991, establishing the foundation for systematic exploration of chemical space beyond the canonical 20.[36–38] One-bead-one-compound (OBOC) libraries (Fig. 3A) can be generated by combinatorial split-and-pool synthesis in the timeframe of a few days and contain multiple copies of a unique peptide per bead with typical library sizes of 10^6–10^9 members.[39] Unlike display technologies discussed in section 3.1, these synthetic libraries are not encoded by an appendant genetic tag and thus require alternative methods for hit identification. For the discovery of novel peptide binders, screening has typically been pursued by on-bead screening or solution-phase screening.

In on-bead screening, peptides remain covalently attached to the solid support, and target binding or bioactivity can be detected visually by equipping the target with dyes, radionuclides, fluorophores, or through enzyme-linked colorimetric indicators.[39] Beads that retain the labeled target are isolated manually or with techniques such as fluorescence-assisted particle sorting, and the peptide sequence is determined either by Edman degradation sequencing[40] or by tandem mass spectrometry, which has become the method of choice in recent years.

For the solution-phase approach, peptides are cleaved from the solid support prior to evaluation in binding assays or functional

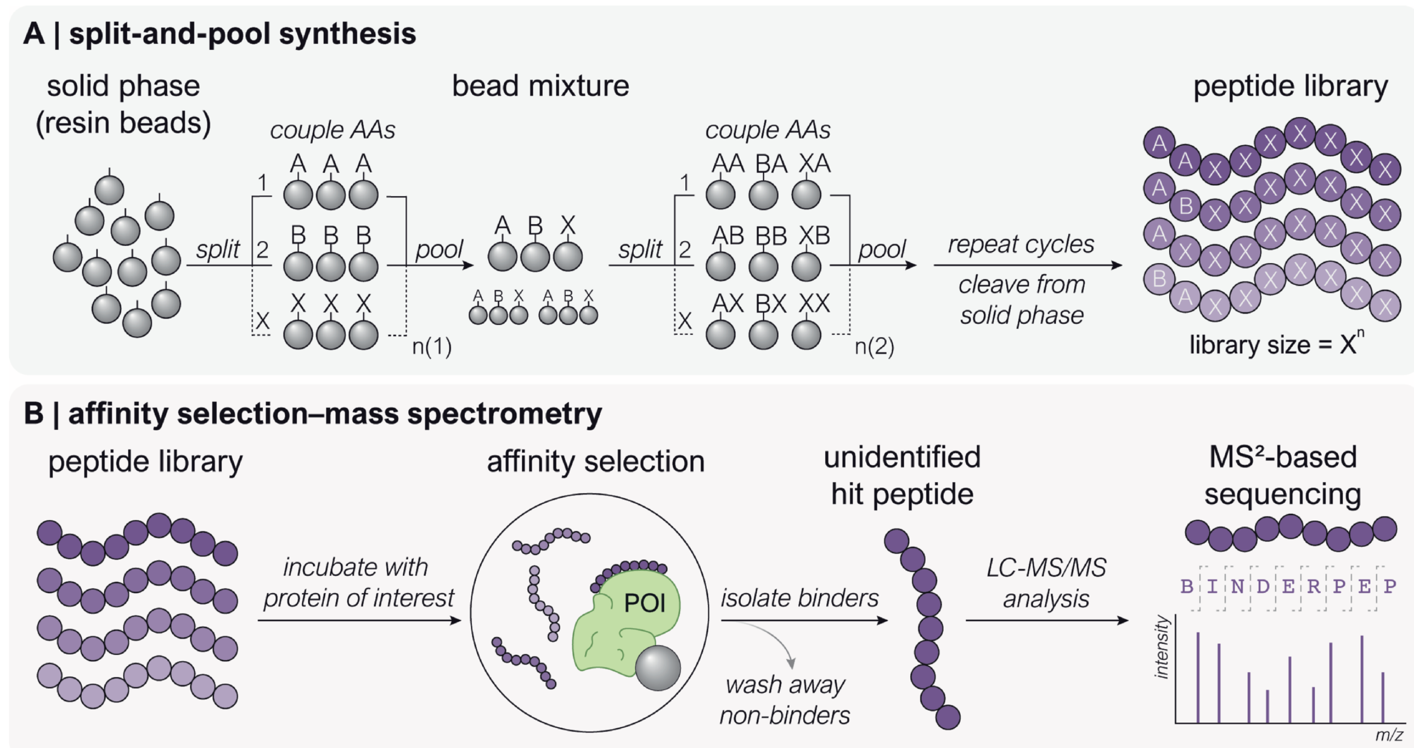

Fig. 3. Synthetic libraries for drug discovery. A) Split-and-pool synthesis on a solid support generates libraries with X^n diverse peptides, wherein X = number of AAs per split and n = number of split-and-pool cycles. B) Peptide libraries are used in affinity selections with a protein of interest (POI). Binders are isolated, and their sequence is determined by mass spectrometry.

screens. This approach benefits from the absence of constraints imposed by beads or oligonucleotide tags and allows the peptides to bind potential targets in any orientation. Early implementations generated large OBOC libraries to test cleaved peptides as isolated compounds or simple mixtures in spatially separated arrays.[38,39] Expanding these arrays to practical formats using 96-, 384-, and most recently 1536-well plates for high-throughput screening (HTS)[41] have since advanced this approach by generating large, information-rich datasets with valuable insights for SAR optimization and machine learning. Logistically, an appropriate infrastructure for automated liquid handling and storage is required for efficient library synthesis and screening.

An alternative approach for peptide identification in solution-phase screening is based on peptide sequencing by tandem mass spectrometry (MS/MS or MS^2). Therein, peptide ions are subjected to fragmentation along their backbone resulting in characteristic ion patterns recorded in secondary mass spectra that allow elucidation of their sequence.[42] Peptides are therefore self-encoded by their mass and fragmentation pattern. Early applications showed successful enrichment of peptides from mixtures of 19 members by affinity-based selection with a target antibody followed by sequencing using MS/MS.[43] Owing to significant advances in instrumentation and sequencing algorithms driven by the field of proteomics, affinity selection–mass spectrometry (AS-MS, Fig. 3B) can identify binders from self-encoded combinatorial libraries with 10^6–10^9 members through MS/MS-based sequencing after selection in solution using free or immobilized targets.[44–46] In a single assay, millions of synthetic peptides containing cAAs and ncAAs can be simultaneously evaluated for target binding, which supports both *de novo* discovery campaigns and structure-guided, focused designs.[47] The concept has also been applied for the discovery of small-molecule binders covalently attached to encoding peptide tags.[48–51] To ensure confident sequence determination, high-resolution and high-throughput mass spectrometers coupled to liquid chromatography (LC-MS/MS) are needed to deconvolute the complex mixtures after affinity selections. Unlike oligonucleotide codes in display methods, peptides cannot be amplified, which requires robust optimization of workflows for successful enrichment and confident, unbiased hit identification.

Together, HTS and AS-MS highlight how synthetic libraries complement display technologies by offering direct access to chemically diverse peptides enriched with ncAAs.

4. Challenges and Opportunities for AS-MS

For practical reasons, synthetic OBOC libraries rarely exceed 10^9 members, whereas libraries with $2x10^{10}$ members and libraries exceeding 10^{14} members have been reported for phage and mRNA display, respectively.[52] Considering the size difference of several orders of magnitude between these approaches, one may ask: is bigger always better? Insights from mRNA display suggest that larger libraries indeed lead to the identification of binders with higher affinities.[53] Whether this correlation holds true for AS-MS remains to be confirmed. Although a synthetic, one-bead-multiple-compounds library with $2x10^{18}$ members was generated by flow synthesis,[54] its application in discovery campaigns using AS-MS will need to address limits in sequencing depth, binding equilibria, and solubility constraints. Advances in MS instrumentation, alternative sequencing methods,[55] and integration of machine learning for data deconvolution will further push these boundaries. Computational approaches are expected to work hand in hand with experimental methods to accelerate peptide discovery and optimization.[56,57]

Beyond library size, architectural diversity in AS-MS remains an important aspect. To date, most applications with self-encoded peptides have relied on linear peptides, as the complex fragmentation patterns of macrocyclic and stapled peptides pose additional challenges for *de novo* sequencing.[58] Yet, these topologies with conformational preorganization are highly attractive for drug development.[59] Only a few studies have explored small macrocyclic libraries or disulfide-linked peptides in AS-MS campaigns,[60–63] highlighting both the feasibility and potential

for future discovery of diverse scaffolds with drug-like properties.

The development of covalent peptide binders emerged as another strategy to address pharmacological shortcomings.[64,65] A previous effort by AS-MS focused on cysteine-reactive electrophiles,[66] but the scarcity of cysteine in the human proteome limits its potential applications.[67] Expanding the repertoire to include electrophiles with selective reactivity for lysine, tyrosine, or histidine would grant access to a broader range of protein targets.[68] Embedding such electrophiles directly into AS-MS libraries rather than by rational design during SAR optimization may significantly accelerate the development of peptide therapeutics with covalent modes of action. Key challenges remain in the analysis of crosslinked peptides and assessment of nonspecific reactivity.

Although incorporation of ncAAs into peptides offers clear pharmacological advantages, it may present a challenge for large-scale manufacturing. Peptides with cAAs can often be produced recombinantly, providing an efficient and sustainable 'green' route.[69] While minor non-natural elements may still be introduced by semi-recombinant approaches, peptides with multiple ncAAs will likely require fully synthetic routes, which are associated with high process mass intensity and a greater environmental footprint.[70] Future advances in biosynthetic engineering are crucial to ensure sustainable manufacturing of ncAA-containing peptides.

5. Concluding Remarks

ncAAs have evolved from niche modifications to essential tools in peptide drug discovery. Incorporating ncAAs at the discovery stage has the potential to reduce labor-intensive cycles of SAR and accelerate the development of screening hits into viable candidates for clinical translation. Beyond addressing inherent limitations of peptide drugs, ncAAs also enable access to challenging targets such as protein–protein interactions, offering new therapeutic avenues in areas of high unmet need. Looking ahead, the continued integration of non-canonical chemistry into discovery platforms will be key to shaping the next generation of peptide therapeutics. The research group for Peptide-Based Drug Discovery is committed to driving these efforts by developing and applying innovative, non-canonical chemistry to peptide design and discovery.

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