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ARTICLE

Peptides as Programmable Molecular Scaffolds: From Chemical Synthesis and Engineering to Translational Medicine

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Peptides have evolved from naturally occurring ligands and classical hormones into a versatile and engineerable class of functional molecules. This review provides a comprehensive overview of the technological advances that collectively enable programmable peptide engineering across the entire discovery-to-development pipeline. We first discuss innovations in automated flow synthesis, chemoselective ligation, noncanonical residue incorporation, backbone editing, conformational constraint, and late-stage functionalization that have transformed peptide chemistry from linear sequence assembly into a modular engineering scaffold. We then examine modern discovery approaches including phage display, and mRNA display with the RaPID system, alongwith computational and AI-enabled design strategies that accelerate hit identification and multi-parameter optimization. Biophysical characterization techniques, cellular target engagement assays, and emerging delivery strategies are also reviewed as critical tools for bridging biochemical potency with intracellular activity. Finally, we discuss the translational barriers facing peptide therapeutics and the engineering strategies that have enabled successful clinical applications. Together, these advances establish a new era which peptides are no longer viewed as inherently labile biomolecules but as chemically programmable scaffolds whose structures and functions can be precisely engineered.

Introduction

Peptides have emerged as a compelling molecular modality at the interface of chemistry and biology, with recent breakthrough advances spanning diverse fields such as biopharmaceutical discovery, medical diagnostics, and therapeutic development. Positioned between small organic molecules and macromolecules, peptides combine several features that make them uniquely attractive for both chemical biology and therapeutic development. Compared with small molecules, peptides can more effectively engage larger, flatter, or more dynamic biomolecular surfaces, mimicking and including protein–protein interaction interfaces that are often difficult to modulate with conventional drug-like compounds. Compared with antibodies and other protein therapeutics, peptides are substantially smaller, more synthetically accessible, and generally more amenable to sequence- and structure-based optimization, while still retaining high target specificity and strong molecular recognition. Accordingly, peptides are increasingly viewed not simply as intermediates between established modalities, but as a versatile and engineerable class of functional tools. [1–5]

Despite these advantages, peptides were historically constrained by a set of well-recognized liabilities that limited their broader translational success. Peptides composed from natural amino acids—particularly linear peptides—or those bearing only minimal modification often suffer from rapid proteolytic degradation, short plasma half-lives, poor membrane permeability, and low oral bioavailability, which have traditionally restricted many peptide drugs to parenteral administration and biased their use toward extracellular targets.[1–3] Earlier peptide discovery and optimization pipelines were also comparatively narrow, relying heavily on endogenous ligand sequences and structures, classical medicinal chemistry optimization, or relatively low-throughput screening paradigms. In parallel, peptide manufacturing has long faced substantial practical burdens in solvent usage, purification, and process intensity, underscoring that the field's limitations were not solely biological but also synthetic and operational. [5–8]

However, the current field is markedly different compared to decades ago. Recent years have seen a convergence of technological advances that are reshaping what peptides can be and how they can be developed. Improvements in synthetic chemistry and manufacturing have made increasingly complex peptide structures more accessible, while modern peptide engineering and chemical methodology tool strategically allow researchers to tune their stability, affinity, conformation, permeability, and pharmacological behavior more deliberately[3–5]. These advances include automated and rapid flow-based synthesis, the efficient preparation of cyclic scaffolds, expanded use of noncanonical building blocks, peptide stapling and macrocyclization strategies, and a broader conceptual shift from sequence assembly to programmable molecular engineering.[9–13]

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At the same time, the accessible chemical and functional space of peptides is expanding through innovations that bridge chemistry, screening, and computation. Random, nonstandard peptide discovery approaches, RNA/mRNA display and phage display platforms have greatly broadened the diversity of accessible bioactive ligands, particularly in the macrocyclic peptide space, while enabling downstream affinity maturation and chemical tailoring and modifications.[13,14] More recently, AI and deep-learning–enabled macrocycle design has begun to demonstrate that peptide binders can be generated with increasing precision using data-driven and structure-guided approaches, further accelerating the transition from empirical discovery to more predictive design.[15] Together, these developments support the view that peptide science is no longer centered only on naturally occurring ligands or classical peptide hormones, but is instead entering a broader "chemical multiverse" in which synthesis, discovery, and design are becoming further matured and refined.[4,13–15]

Accordingly, this review focuses on the advances in technologies spanning the peptide upstream-to-downstream pipeline, rather than around individual achievements focused on specific target proteins. Our central propose is that peptide chemistry and biology have entered a new phase of opportunity because modern enabling technologies are increasingly transforming peptides from inherently labile biomolecules under physiological conditions into stable, programmable, optimizable, and translatable molecular platforms. [1–5] We therefore focus on the tools and strategies that are driving this transformation, including innovations in peptide synthesis and chemical engineering, discovery and medicinal optimization, biophysical characterization, cellular target engagement, and downstream, late-stage translational development. By emphasizing these interconnected advances, we aim to highlight not only where the field currently stands, but also how emerging technologies are redefining the future trajectory of peptide science across discovery and application.[9–15]

Importantly, these advances are not independent developments, but mutually reinforcing innovations that collectively improve peptide discovery, synthesis and chemical modification, performance, and translational feasibility.

Advances in Peptide Synthesis and Chemical Engineering

Modern peptide chemistry is no longer defined solely by the efficient assembly of linear amino acid sequences, but increasingly by the programmable engineering and modification of peptide structure, stability, and function. This transition is important because linear peptides, although now rapidly accessible through solid-phase and automated synthesis, often remain limited by conformational flexibility, short biological half-lives, and developable potentials. Thus, rapid synthesis provides the necessary starting point, but it does not by itself convert a peptide sequence into a stable biological probe or therapeutic candidate. In this section, we organize recent advances as a workflow that begins with rapid access to linear peptide scaffolds and then proceeds through conformational control, convergent assembly, building units expansion, and late-stage functional diversification.

In earlier generations of peptide science, synthetic effort was often dedicated to overcoming practical limitations in sequence elongation, coupling efficiency, and purification. The initial breakthrough in this area can be traced back to 1963, when Merrifield designed and successfully synthesized bradykinin using solid-phase peptide synthesis.[16] From that point onward, the rate of synthesizing linear peptides composed of natural amino acids has drastically increased. In recent years, many automated peptide synthesizers can even complete the synthesis of a 20-mer peptide within two hours. This principle enabled the efficient preparation of linear peptides and later made automated peptide synthesis possible and allows rapid preparation of analogues for sequence–activity relationship studies, metabolic stability evaluation, and medicinal chemistry optimization. Also, in the drug discovery field, this ability to generate many related sequences rapidly is particularly valuable because it provides the upstream material required for systematic downstream engineering (**Figure 1A**).[9,10,17,18] Within them, flow-based SPPS represents an important extension of conventional and automated SPPS by improving reagent delivery, mixing, temperature control, cycle time, and reproducibility compared with batch synthesis. These advantages make flow platforms particularly useful for difficult sequences, rapid analogue preparation, and optimization campaigns.[10,17]

However, rapid access to linear peptide precursors is only the first step in peptide engineering, because many linear peptides remain conformationally flexible and metabolically labile due to their free termini and solvent-exposed amide bonds. Head-to-tail cyclization provides one of the most established strategies to address these limitations by removing terminal degradation sites and constraining the peptide backbone into a closed topology, which can improve proteolytic resistance, reduce conformational entropy, and enhance target recognition in favourable cases. Recent automated flow-enabled methods for preparing head-to-tail cyclic peptides therefore represent an important bridge between rapid precursor synthesis and topology-driven scaffold engineering, allowing constrained peptide architectures to be accessed more efficiently from linear precursors.[9] Beyond head-to-tail cyclization, broader macrocyclization and stapling strategies have expanded the ways in which peptide conformation can be controlled. Side-chain-to-side-chain cyclization, disulfide formation, thioether formation, biaryl linkages, and hydrocarbon stapling each impose distinct geometric constraints and chemical properties.[24–29] These approaches can reduce conformational entropy, stabilize bioactive conformations, increase resistance to proteolysis, and, in selected cases, improve cell permeability or intracellular activity. Hydrocarbon stapling, for example, has been widely used to stabilize α-helical peptides that target protein–protein interactions, whereas newer chemoselective stapling methods allow unprotected or minimally protected peptides to be constrained under milder conditions.[29] However, conformational constraint is not universally beneficial.

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Cyclization or stapling can reduce activity if the imposed geometry is incompatible with the binding conformation, and improved stability does not necessarily guarantee improved permeability or in vivo exposure. Therefore, these strategies are most powerful when integrated with structural, biophysical, and cellular evaluation rather than treated as generic solutions to peptide liabilities (**Figure 1B**).

In the parallel of the cyclization and stapling directly address conformational and stability limitations, chemoselective ligation strategies solve a different problem: the difficulty of accessing long, aggregation-prone, or architecturally complex peptides by direct chain elongation. Traditional SPPS becomes increasingly challenging as sequence length increases, because incomplete couplings, deletion products, aggregation, and purification problems can accumulate. Chemoselective ligation provides a convergent solution by allowing shorter peptide fragments to be synthesized separately and then joined through selective bond-forming reactions. Native chemical ligation (NCL) remains the foundational example, enabling peptide thioesters and N-terminal cysteine-containing fragments to react under aqueous conditions to form native amide bonds. More recent advances, including enhanced NCL conditions and KAHA-based ligation strategies, have expanded the practicality of fragment coupling and enabled access to challenging peptide and protein-like targets.[19,20] The conceptual importance of ligation is that it shifts peptide synthesis from a strictly linear process to a modular assembly strategy. Unlike cyclization or stapling, ligation does not necessarily improve metabolic stability or conformational control by itself. Instead, it expands what can be built, including longer peptides, segmentally modified constructs, cyclic or branched architectures, and multifunctional peptide conjugates that may be difficult to obtain by direct SPPS alone. Water-compatible ligation and native peptide cyclization methods further illustrate how convergent assembly can intersect with topology control.[21] At the same time, ligation methods have important limitations, including sequence-dependent reactivity, junction requirements, functional-group compatibility, and protecting-group considerations. These trade-offs make direct comparison among ligation strategies important, as summarized in **Figure 1C**. Overall, ligation complements topology-focused methods by increasing the accessible size, modularity, and architectural complexity of peptide structures.

In parallel with advances in synthesis and assembly, peptide chemists have increasingly expanded peptide composition beyond the twenty canonical amino acids. Noncanonical amino acids, D-amino acids, N-methylated residues, β-amino acids, aza-peptide motifs, depsipeptide linkages, peptoid-like structures, and other backbone modifications provide ways to tune properties that cannot be achieved through natural sequence variation alone.[22,23] These modifications can improve proteolytic stability, alter hydrogen-bonding patterns, modulate conformational preferences, increase target affinity, introduce reactive handles, and adjust physicochemical properties associated with permeability and solubility. Backbone editing further extends this concept by changing the connectivity and chemical identity of the peptide backbone itself. Recent strategies such as post-translational O-to-N acyl shift chemistry show that peptide-like molecules can be diversified after initial assembly to generate backbone-embedded motifs and heterocycle-containing architectures.[23] However, noncanonical residues or backbone modifications may also reduce binding affinity, disrupt secondary structure, complicate synthesis, or introduce new developability challenges. Their value therefore lies in enabling systematic property tuning rather than providing a universal solution to peptide limitations (**Figure 1D**).

Late-stage functionalization provides a final layer of peptide engineering-boarding the toolbox for applications. Whereas noncanonical residues and backbone modifications are often introduced during sequence assembly, late-stage functionalization allows advanced peptide scaffolds to be diversified after synthesis (**Figure 1E**). This is strategically valuable because an optimized peptide sequence or constrained scaffold does not need to be rebuilt from the beginning each time a new functional group is required. Instead, site-selective, or residue-selective chemistry can be used to install fluorophores, affinity tags, imaging groups, delivery motifs, bio-orthogonal handles, linkers, or payloads directly onto a prepared peptide. Recent examples include tryptophan-selective functionalization for tetrazine-compatible bio-orthogonal ligation, palladium-catalyzed modification of phenylalanine-containing peptides, photocatalytic functionalization at selenocysteine, and cysteine-selective dual functionalization using multifunctional reagents.[30–33] Other approaches combine macrocyclization with functional installation, illustrating how structural constraint and functional diversification can be integrated within a single synthetic strategy.[34] However, late-stage modification can alter binding, solubility, aggregation behaviours, permeability, or metabolic stability, and site selectivity, reaction compatibility, and purification may become practical challenges for longer or highly functionalized peptides

Taken together, these advances define a coherent progression in modern peptide chemistry. SPPS and flow synthesis provide rapid access to linear peptide scaffolds; cyclization and stapling address conformational flexibility and proteolytic susceptibility; ligation expands the size and modularity of accessible architectures; noncanonical residues and backbone editing broaden the chemical space available for property optimization as well as increasing of inherent stability; and late-stage functionalization enables advanced scaffolds to be converted into probes, conjugates, or therapeutic candidates. The central challenge is therefore no longer simply whether a peptide sequence can be synthesized, but how efficiently that sequence can be converted into a stable, functional, and developable molecular scaffold.

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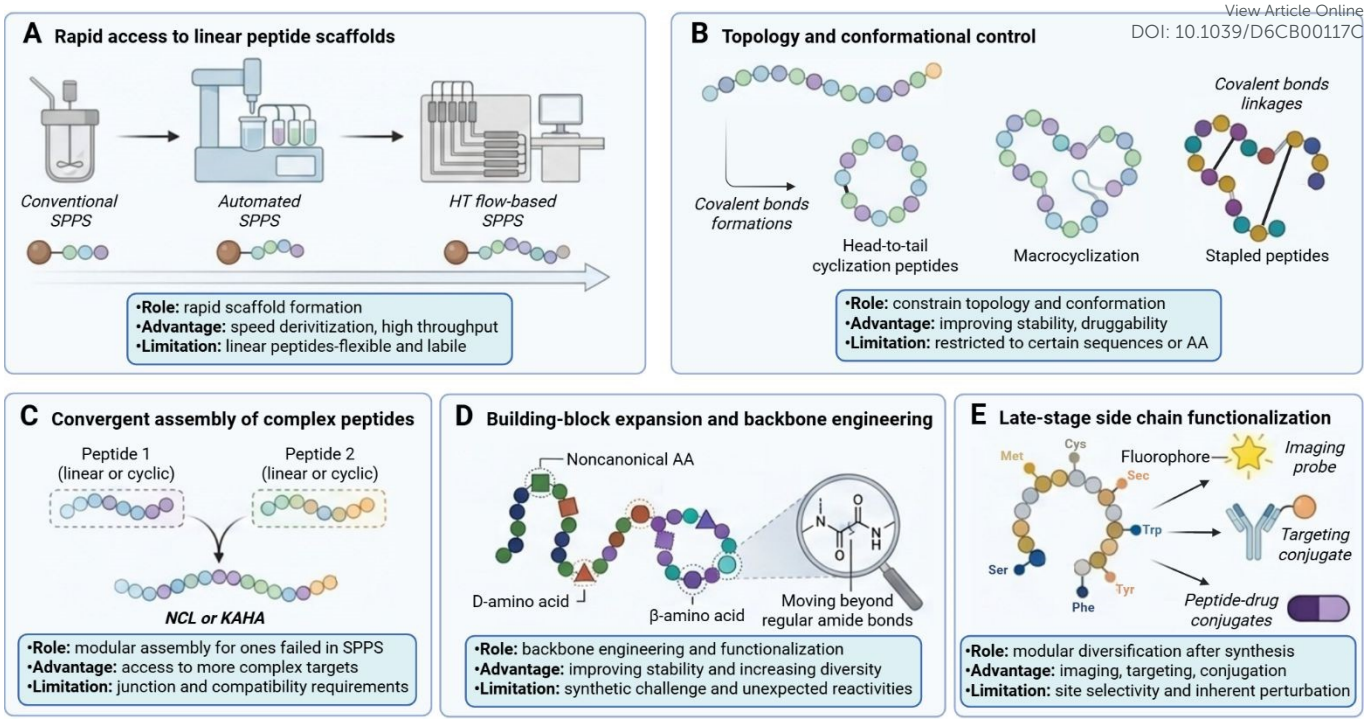

Figure. 1 Representative advances in modern peptide engineering, from rapid solid-phase synthesis to post-assembly structural and functional diversification. **(A)** Conventional, automated, and flow-based SPPS enable rapid access to linear peptide precursors for analogue synthesis and downstream engineering. **(B)** Conformational constraint through macrocyclization and stapling reduces peptide flexibility, preorganizes bioactive conformations, and can enhance stability and molecular recognition. **(C)** Chemo selective ligation methods, including native chemical ligation (NCL) and α-ketoacid–hydroxylamine (KAHA) ligation, support convergent assembly from short peptide fragments and facilitate access to longer, more complex, and more highly functionalized peptide architectures. **(D)** Incorporation of noncanonical amino acids expands peptide chemical space beyond the 20 canonical residues and enables backbone-level editing, thereby improving metabolic stability, broadening accessible design space, and introducing functional handles. **(E)** Representative residue-selective late-stage functionalization strategies enable modular diversification of peptides for imaging, targeting, and conjugation, including Cys-selective dual functionalization, Trp chemoenzymatic handle installation, Phe C(sp^2)–H annulation, and Sec-selective photocatalytic functionalization as well as other possible amino acids side chains.

Peptide Discovery, Medicinal Optimization, and Assay-Guided Design

For most of the twentieth century, therapeutic peptides were discovered by rational design - mimicking endogenous ligands or truncating natural hormones, exemplified by the systematic modification of oxytocin (**Figure 2A**) that yielded carbetocin (A medication used to reduce or prevent excessive bleeding following childbirth),[35] and the iterative structural refinement of native glucagon-like peptide-1 that produced semaglutide which is used to treat type 2 diabetes.[36,37] The transformation of peptide drug discovery has been both conceptual and technological: modern library-based platforms now interrogate 10^8-10^{13} sequences in a single experiment,[38,39] chemical biology has extended accessible sequence space far beyond the 20 canonical amino acids,[40] and computational and AI tools now accelerate every step of the pipeline. The central question has shifted from *which sequence should we design?* to *which platform will most efficiently reveal the optimal sequence?* The bottleneck is no longer generating peptide diversity, it is identifying and optimizing the most translatable diversity. To clarify the distinct roles of these approaches, Table 1 summarizes representative peptide discovery and optimization strategies discussed in this section, highlighting their major advantages and limitations.

From Rational Design to Library-Enabled Peptide Discovery

Despite the broader shift toward library-based discovery, rational design has achieved notable success in specific cases. A prominent example is the development of macrocyclic inhibitors of the hepatitis C virus NS3/4A protease through rational design based on the natural substrate of the viral protease, using macrocyclisation to rigidify the peptide scaffold. This strategy ultimately produced several approved drugs, including Paritaprevir, Grazoprevir, Voxilaprevir, and Glecaprevir (**Figure 2B**).[41] However, this success has largely been limited to this target class, highlighting the challenges of generalizing rational macrocycle design and motivating the shift toward library-based discovery platforms. The defining advantage of library-based

discovery over rational design is epistemic: rather than exploring a scientist's hypothesis space, selection platforms use the target itself to identify productive sequences by direct competitive binding, routinely uncovering active sequences with unusual residue combinations and backbone modifications that no hypothesis-driven approach would have generated.[39,42] Two platform technologies have driven this shift, each accessing a distinct region of chemical and structural space as discussed below.

Phage Display Phage display presents peptide libraries of 10^8-10^{10} sequences on bacteriophage coat proteins; iterative affinity panning against an immobilized target,[43] followed by next-generation sequencing (NGS), identifies enriched binders (**Figure 3A**).[44] Heinis and coworkers extended this approach by crosslinking displayed peptides with tris-(bromomethyl)benzene (TBMB), generating constrained bicyclic scaffolds with superior proteolytic stability and binding surface complementarity.[45] This approach discovered BT8009 (Bicycle Therapeutics), a Nectin-4-targeting bicyclic peptide-toxin conjugate now in Phase I/II, subsequently optimized via non-canonical amino acid substitution to achieve K_d 2.5 nM and high degree of target selectivity.[46,47]

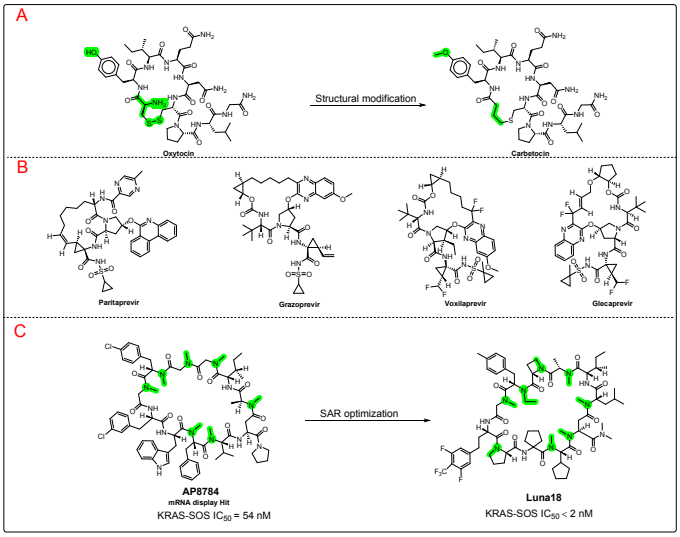

Figure 2. (A and B) Chemical structures of carbetocin, paritaprevir, grazoprevir, voxilaprevir, and glecaprevir. **(C)** chemical optimization of AP8747 into Luna18 as a KRAS-targeting cyclic peptide inhibitor.

mRNA Display and the RaPID System mRNA display scales libraries to 10^{13} unique sequences, with each peptide covalently attached to its encoding mRNA enabling direct sequencing-based hit identification without deconvolution.[48] The RaPID (random nonstandard peptides integrated discovery) system integrates mRNA display with genetic code reprogramming: engineered flexizymes (flexible tRNA aminoacylating ribozymes) charge non *N*-methyl, D-configured, and β-amino acids onto transfer RNA (tRNA) for ribosomal incorporation,[49] while a chloroacetyl initiator spontaneously macrocyclizes with a downstream cysteine - meaning every library member is a drug-like macrocycle before selection begins (**Figure 3B**).[50,51] The LUNA18 program exemplifies the full discovery-to-clinic arc: a RaPID screen against $KRAS^{G12D}$ yielded hit AP8747; alanine scanning mapped the pharmacophore; iterative non-canonical amino acid substitution produced an 11-mer cyclic peptide with cellular IC_{50} 1.4 nM (AsPC-1) and oral bioavailability 21–47%, now in Phase I (**Figure 2C**).[52]

Hit-to-Lead Optimization in Peptide Medicinal Chemistry

Library selection produces hits with measurable affinity but insufficient drug-like properties. Alanine scanning maps the pharmacophore: positions where substitution costs >2 kcal/mol are critical contacts to retain; permissive positions become ADME handles.[53] Metabolic stability can be improved through backbone N-methylation (eliminating H-bond donors and sterically blocking protease active sites), incorporation of D-amino acids at protease-sensitive positions, and macrocyclization to remove free termini, together extending plasma half-life from minutes to hours.[54] Additional localized backbone modifications including stereochemical inversion, atom replacement (e.g., azapeptides[55] or depsipeptides[56]), side-chain repositioning from the α-carbon atom to the amide nitrogen atom as in peptoids,[57] or backbone extension with β-amino acids can further expand structural diversity and improve drug-like properties. Cell permeability is optimized by modulating cLogP within a 2–5 windows and exploiting conformational chameleonic behavior, wherein polar NH groups are buried in membrane-compatible conformations while exposed in aqueous environments.[58] Multi-parameter optimization (MPO) tracks potency, selectivity, stability, and permeability simultaneously.[59] Our research group has demonstrated this logic across immune checkpoint targets: structure-based design of constrained cyclic peptide as TIM-3 inhibitors yielded candidates validated by SPR and cell-based assays,[60] while iterative design of second-generation cyclic LAG-3 inhibitors improved potency and selectivity through systematic backbone modification and biophysical characterization.[61]

Assay-Guided Iterative Design

Hit-to-lead optimization is a self-correcting feedback cycle in which biological data from each round directly dictates the next synthesis iteration. Tier 1 biochemical assays - SPR, BLI, ITC-measure binding affinity and kinetics, rapidly ranking analog series. Tier 2 cell-based assays confirm cellular activity: failure redirects chemistry toward permeability or efflux rather than further affinity gains. Tier 3 mechanistic assays, particularly CETSA, confirm direct target engagement inside intact cells. This tiered framework has been widely applied in peptide drug discovery. Cyclic peptide inhibitors of protein-protein interactions, such as ICOS/ICOSL[62,63] and CD28,[27] are refined through iterative synthesis guided by orthogonal biophysical and cellular assays, enabling high potency and selectivity.

Table 1. Comparison of major peptide discovery and optimization approaches.

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Approach	Core principle	Main advantages	Key limitations	Representative use
Rational design	• Known ligand/substrate • Structure-guided analogues	• Mechanistically clear • Focused optimization	• Prior knowledge required • Limited generalizability	Optimization of endogenous peptide hormones; substrate-inspired macrocyclic inhibitors
Phage display	• Phage-displayed libraries • Iterative panning	• Robust selection • Large libraries • Constrained scaffolds	• Genetically encoded bias • Display-format effects • Target immobilization artifacts	Discovery of peptide binders and bicyclic peptide scaffolds
mRNA display / RaPID	• mRNA-linked peptides • Genetic-code reprogramming • Macrocyclization	• Very large libraries • Direct sequencing • Macrocycle access	• Specialized expertise • Selection bias • Optimization still required	Discovery of nonstandard macrocyclic peptides such as KRAS-targeting cyclic peptide leads
Hit-to-lead optimization	• Alanine scanning • Backbone modification • Macrocyclization	• Improved stability • Better potency/selectivity • Multiparameter tuning	• Property trade-offs • Repeated synthesis/testing	Optimization of cyclic peptide inhibitors and peptide leads for improved ADME properties
Assay-guided design	• Binding assays • Cellular assays • Target-engagement assays	• Orthogonal validation • Cellular relevance • Avoids affinity-only optimization	• Assay artifacts • Permeability barriers • Context dependence	SPR/BLI/ITC for binding, cell assays for activity, CETSA for intracellular engagement
Computational and AI-enabled design	• Structure prediction • Generative models • Sequence design • Closed-loop learning	• Faster prioritization • Large design space • Pre-synthesis filtering	• Experimental validation needed • Model/data dependence • Structural assumptions	AlphaFold/RFdiffusion/CycleDesigner-guided peptide binder discovery

Computational and AI-Enabled Peptide Discovery

Computational tools complement experimental assays by accelerating sequence generation, prioritization, and optimization. AlphaFold3 predicts peptide-protein complex structures including non-canonical residues, providing binding hypotheses before synthesis.[64,65] RFdiffusion (RFdiffusion extension) generates de novo peptide binders with picomolar affinity,[15] while CycleDesigner combines CyclicMPNN sequence optimization,[66] RFdiffusion-generated macrocyclic scaffolds, and advanced structure prediction to yield cyclic peptides that fold accurately (RMSD <1.5 Å) and validate experimentally.[67]

AI and machine learning models further guide sequence prioritization and optimization. Models trained on SAR data reduce synthesis burden approximately tenfold, and FEP calculations quantitatively pre-screen residue substitutions. Computational pipelines have successfully designed macrocyclic inhibitors for targets such as TREM2 and SLIT2/ROBO1, [39, 68] demonstrating where peptide-based approaches outperform small molecules. AI-guided design of the cyclic peptide CIP-3 targeting the immune checkpoint receptor CD28, highlighting the potential of computational approaches to generate functional peptide modulators of T-cell co-stimulation.[34] Closed-loop predict-synthesize-test-retrain cycles can compress discovery timelines from years to months, underscoring that the modern bottleneck in peptide drug discovery is no longer generating diversity but efficiently identifying and optimizing the most translatable sequences.

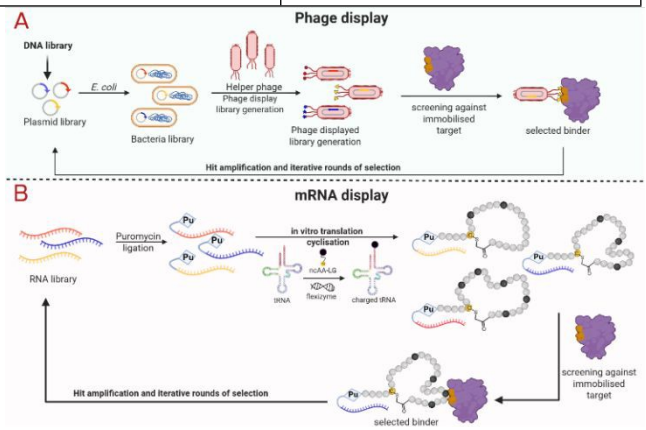

Figure 3. Library-Enabled Peptide Discovery. **(A)** Phage display: A plasmid library is first transformed into E. coli, which are subsequently infected with helper phages to generate a library of peptides displayed on the phage coat protein. The phage library is screened against an immobilized target, and enriched sequences are amplified and subjected to additional selection rounds before final sequencing. **(B)** mRNA display: A randomized mRNA library is linked to a puromycin molecule, translated into peptides, and typically cyclized via a thioether bond. Non-canonical amino acids (ncAAs) are incorporated using the FiT system, in which an artificial flexizyme charges the ncAAs onto tRNAs for ribosomal incorporation during translation. Peptides are then subjected to affinity selection, and enriched sequences are amplified across successive rounds. This combination of mRNA display with ncAA incorporation is referred to as the RaPID method.

Biophysical Screening and Mechanistic Characterization of Peptide–Target Interactions

Challenges of biophysical peptide binding characterization

Biophysical characterization of peptide binding presents several experimental challenges due to the intrinsic physicochemical properties of many peptides.[1] Peptides are often flexible and highly charged, which can complicate the determination of well-defined conformations and binding modes. In addition, many peptides are surface-active, leading to nonspecific adsorption to experimental surfaces (e.g., capillaries, plates) and potential artifacts in assays.[2] Additionally, they are also frequently prone to weak and transient interactions, resulting in low-affinity binding that can be difficult to detect or quantify with standard techniques. Furthermore, potentially low stability, including susceptibility to aggregation, degradation, or conformational changes, can further complicate reproducible measurements and interpretation of binding data.[69] Together, these factors make rigorous biophysical analysis of peptide-target interactions particularly demanding and require thorough orthogonal validation of the interaction.

Binding affinity and kinetics

Common methods for determining binding affinities include surface plasmon resonance (SPR),[60] biolayer interferometry (BLI),[70] microscale thermophoresis (MST) and spectral shift (SpS),[67] among others. SPR and BLI are particularly well-suited to determine kinetic parameters (e.g., k_{on}, k_{off}, residence time) while MST and SpS allow for a rapid assessment of binding. Isothermal titration calorimetry (ITC) provides additional thermodynamic insights such as binding enthalpy and entropy as well as the stoichiometry of the interaction.[71] However, due to the potential weak interaction between the target and the peptide, characterization using these methods alone can be challenging. Therefore, combining various techniques is key for a successful peptide binding characterization.

Current and emerging high-throughput biophysical approaches

The establishment of high-throughput screening (HTS) platforms requires screening methods that are robust and scalable to process large peptide libraries. A classic approach are solution-phase HTS assays such as fluorescence polarization (FP) or FRET-based assays (TR-FRET, AlphaScreen).[72,73] FRET or AlphaScreen assays generate a proximity-based signal when a peptide and the protein of interest interact (**Figure 4B**). They can be upscaled to 384- or 1536-well plates and provide robust results due to the high sensitivity of the assay. For example, Wilson and co-workers have described a screening method that uses AlphaScreen technology to identify small peptides that disrupt the interaction between the OX40 receptor and its ligand.[74] Microarray-based platforms (e.g., pHLA microarray) use solid surfaces (e.g., cavity chips) to immobilize thousands of peptides and probe them with a protein of interest.[75] Binding is then detected via fluorescence or label-free imaging. Peptide microarrays are especially useful in T cell receptor (TCR)-pHLA screenings as demonstrated by Kramer and co-workers.[75] They created an ultra-HTS platform using microcavity chips to store the peptide library (**Figure 4B**). The library can be activated for the screening with biotinylated HLA that is added to the cavities. Wherever a peptide binds, it forms a stable complex that is transferred to a streptavidin (SA)-coated substrate and subsequently analyzed by a method of choice (e.g., highSCORE to assess TCR binding).[75] Mass spectrometry-based platforms like LiF-MS and affinity-selection mass spectrometry (AS-MS) are useful methods for identifying binding peptides in a high-throughput setting.[76, 77] By combining various techniques, AS-MS demonstrates how this interactive approach can improve results. As an example, Zhang and co-workers have developed a workflow in which BLI is used as a primary screening method to capture binders, which are then eluted and sequenced using tandem MS (**Figure 4B**).[76] This technique enables the screening of large libraries with real-time monitoring, low background signal, and high specificity.[76]

Binding-site mapping

NMR spectroscopy can be used as a powerful tool to map binding sites in peptide-protein interactions, especially for flexible ligands and weak complexes.[78] In typical experiments, an isotopically labeled protein (e.g., ^{15}N) is titrated with the peptide and chemical shift perturbations are monitored in two-dimensional spectra such as HSQC (**Figure 4D**).[78] Residues showing significant shifts upon peptide addition identify regions at or near the binding interface. Since NMR provides residue-specific information, it enables precise localization of the interaction surface and can also yield insights into binding affinity and exchange dynamics.[78] One major caveat is the high sample and time consumption of protein-NMR experiments. However, there have been efforts to fully automate the binding characterization via microfluidic 2D NMR as published by Plata and co-workers, making the technique more suitable for high-throughput screening.[78] Another method for binding site mapping is ligand-footprinting mass spectrometry (LiF-MS).[77] This technique maps peptide-protein binding surfaces by tethering a photoactivatable, cleavable crosslinker to a peptide ligand, which transiently crosslinks to nearby protein residues during binding.[79] After cleavage, small covalent marks remain on the protein and are identified by tandem MS, providing spatial constraints that define where the ligand footprinted the protein surface (**Figure 4D**).[77, 79]

Orthogonal workflows for peptide validation

In peptide drug discovery, a typical workflow begins by establishing proof-of-binding with techniques such as MST/SpS or BLI/SPR through single-dose (medium or high throughput) and then dose-response experiments (Figure 4A,B). Once binding is confirmed, further biophysical studies (e.g., with BLI, SPR, etc.) should characterize binding kinetics and, where possible, thermodynamic parameters (e.g., with ITC, Figure 4C). Finally, binding site mapping with NMR or MS can provide additional insight into the location and nature of the binding interface (Figure 4D).[78] Ultimately, for peptide leads, a combination of orthogonal biophysical methods is indispensable. Binding mechanisms can only be defined in a reliable way through complementary approaches to establish confidence in the interaction and successfully support downstream translation.

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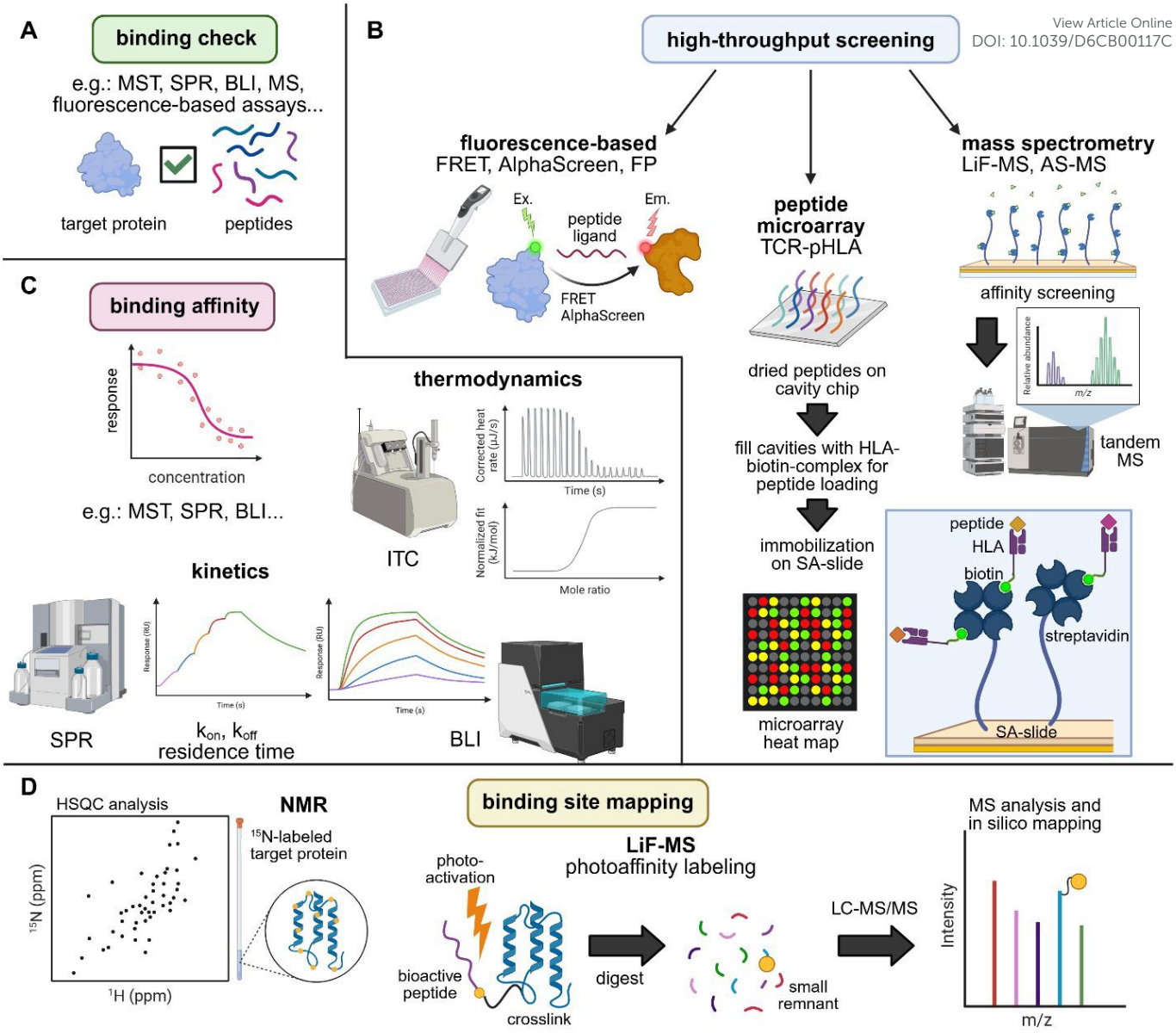

Figure 4. Biophysical characterization of peptide binding. (**A**) First step in most evaluations is a single-dose binding check which can be achieved with various methods (e.g., MST, SPR, BLI, etc.). (**B**) Literature-known high-throughput screening (HTS) methods to evaluate peptide binding. Includes fluorescence-based screenings in solution (e.g., FRET, AlphaScreen, FP), peptide microarray (e.g., TCR-pHLA microarray), and MS-based screening (e.g., AS-MS). (**C**) Binding affinity can be determined using multiple methods. Techniques like SPR and BLI provide additional kinetic information whereas ITC delivers thermodynamic parameters. (**D**) NMR and MS techniques establish binding site mapping.

Cellular Technologies for Peptide Biology and Intracellular Target Engagement

Why Biochemical Potency Is Not Enough

A peptide that binds its target with nanomolar affinity in biophysical assays may fail to produce a biological response in cells. Peptides face a cascade of barriers largely absent for small molecules, including poor passive membrane permeability, entrapment within endosomal compartments, and rapid proteolytic degradation **(Figure 5)**.[80] Stapled peptides targeting MDM2–p53 exemplify this disconnect, early leads bound MDM2 potently yet showed negligible cellular p53 activation, primarily because they could not access the cytosol at effective concentrations.[81] These failures motivated the development of cellular technologies that address three questions biochemical data alone cannot answer: does the peptide bind its target inside cells, can it overcome delivery and stability barriers to reach the correct compartment, and does that engagement ultimately produce a physiological outcome?

Measuring Intracellular Peptide-Target Binding

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The first requirement for an intracellular peptide therapeutic is evidence that it physically engages its target within the cellular milieu. The cellular thermal shift assay (CETSA) detects such engagement by measuring ligand-induced changes in protein thermal stability inside intact cells.[82] When a peptide binds its target, the complex resists thermal denaturation, producing a measurable shift in aggregation temperature. Verma and colleagues applied CETSA directly to peptide drug discovery, confirming that stapled peptides engage both MDM2 and MDM4 intracellularly and comparing uptake and binding efficiency across analogs.[83] The assay has evolved from Western blot detection to SplitLuc CETSA, using a HiBiT tag for luminescence readouts in 1536-well format, and to real-time CETSA (RT-CETSA), which captures complete melt profiles from single samples.[84,85] The NanoBRET target engagement assay provides a complementary approach using energy transfer between a NanoLuc-tagged target and a cell-permeable fluorescent tracer to quantify intracellular affinity and residence time, and is beginning to find application with cell-permeable constrained peptides.[86] However, even when binding is confirmed, a key question remains: did the peptide reach the cytosol efficiently, or does limited delivery restrict the observed engagement?

Evaluating and Overcoming Peptide Delivery and Stability Barriers

A peptide may show target engagement in permeabilized cells yet fail in intact cells simply because it never reaches the cytosol. The chloroalkane penetration assay (CAPA) quantifies compartment-specific cytosolic access: cells expressing cytosolic HaloTag react covalently with chloroalkane-tagged peptides, and residual HaloTag labeling measured by flow cytometry yields a CP50 value that distinguishes true cytosolic delivery from endosomal entrapment.[87] CAPA has been applied to profile stapled peptides, cyclic cell-penetrating peptides, and miniature proteins, and has been extended to compare penetration across multiple human cell lines, revealing cell-type-dependent uptake efficiency.[88] The NanoClick assay offers a complementary high-throughput format, combining in-cell click chemistry with NanoBRET detection to measure cumulative cytosolic exposure of azide-functionalized macrocyclic peptides in 96- and 384-well plates.[89]

Beyond measurement, several strategies address delivery barriers directly. Cell-penetrating peptides (CPPs) derived from viral sequences, most notably the TAT peptide from HIV-1 and penetratin from Drosophila Antennapedia, have been widely conjugated to peptide cargoes to enhance cellular uptake.[90] However, quantitative assays such as CAPA have revealed that a significant fraction of CPP-delivered material remains trapped in endosomes, and cytosolic access is frequently lower than total uptake suggests.[87] For peptides that remain impermeable even with CPP conjugation, microfluidic mechanoporation platforms such as DµVS transiently permeabilize cell membranes, enabling direct cytosolic delivery and coupling with functional assays to deconvolute penetration from engagement.[91] With delivery confirmed and binding established, the final question is whether engagement translates into a biological outcome.

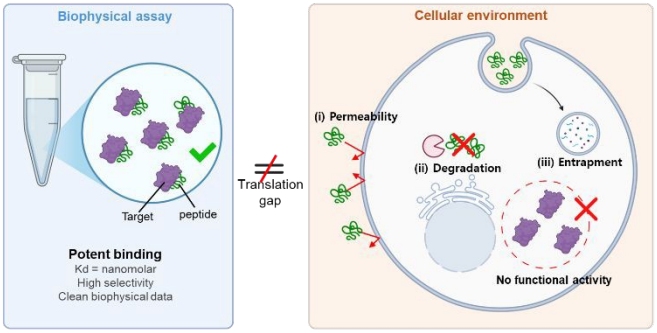

Figure 5. The translation gap between biochemical peptide binding and intracellular activity. (Left) In biochemical assays, peptides bind their target proteins with high affinity (nanomolar Kd), suggesting therapeutic potential. **(Right)** In the cellular environment, multiple barriers prevent effective target engagement: **(i)** poor membrane permeability blocks cytosolic entry, **(ii)** cytosolic proteases degrade peptides that reach the intracellular space, and **(iii)** endosomal entrapment following energy-dependent uptake diverts peptides to lysosomal degradation.

Assessing Functional Modulation of Intracellular Targets

The ultimate validation of a peptide drug candidate is evidence that it modulates target protein function inside living cells. Transcriptional reporter assays provide a straightforward readout of downstream biological consequences. For example, p53 reporter cell lines confirm that stapled-peptide-mediated dissociation of the MDM2 complex reactivates p53-dependent transcription.[81,92] Counter-screens for membrane disruption (LDH release) and luciferase interference remain essential to distinguish genuine target modulation from off-target cytotoxicity that amphipathic or cationic peptides can produce.[92] To move beyond downstream readouts and directly monitor the protein–protein interaction itself, NanoBiT (NanoLuc Binary Technology) splits NanoLuc into LgBiT and SmBiT subunits that reconstitute a luminescent signal only when fused partner proteins interact.[93] Peptide-mediated PPI disruption extinguishes this signal, enabling quantitative monitoring of intracellular PPI inhibition. Stapled peptides have been shown to dissociate p53-MDM2 and KRAS-effector complexes inside cells using this approach.[92] We recently developed a NanoBiT-based high-throughput screening platform for the NOS1–NOS1AP interaction, a PPI implicated in cardiovascular and neuropsychiatric disorders.[94] Stable CHO-K1 cell lines engineered with NOS1-HiBiT and NOS1AP-LgBiT fusions achieved a signal-to-background ratio exceeding 240-fold and were validated using the peptide inhibitor TAT-GESV. A pilot screen of 10,240 compounds identified 19 validated hits with IC_{50} values as low as 2.54 µM.[94] Importantly, this platform architecture, stable cell lines with engineered split-luciferase PPI reporters, is readily adaptable to other intracellular PPI

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targets, providing a general screening framework applicable to both small-molecule and peptide drug discovery.

Peptides as Tools for Intracellular Biology

When delivery, target engagement, and functional modulation are rigorously established, peptides become uniquely precise tools for interrogating intracellular biology. Stapled peptides have elucidated the relative contributions of MDM2 versus MDMX to p53 regulation, while cell-permeable cyclic peptides have served as probes to disrupt KRAS nanoclustering and downstream oncogenic signaling.[92,95] The convergence of binding assays, delivery evaluation, and functional reporters into an integrated workflow is transforming peptides from promising biochemical binders into biologically validated agents. As these cellular technologies mature, they will increasingly define the translational path for peptide therapeutics targeting intracellular protein-protein interactions.

From Cell-Based Activity to Translation: Advancing Peptide Therapeutics Toward Clinical Application

Peptides have emerged as an attractive class of therapeutic molecules because of their ability to combine high specificity with flexible design. Advances over the past twenty years in technologies for identifying and screening peptides have allowed researchers to find many potential peptide-based drugs that exhibit good activity in both biochemical and cellular assays. However, translating these potential drugs into clinically efficacious therapies has proven difficult. Many peptides that demonstrate excellent in vitro efficacy do not progress through the preclinical or clinical phases of development because they cannot withstand the rigors of preclinical testing due to their short half-lives, rapid clearance from the body, poor pharmacokinetic profiles, and barriers to delivery.[96] Consequently, overcoming these challenges has become one of the key issues surrounding the development of peptide-based therapeutics. The major translational barriers and representative engineering strategies discussed in this section are summarized in **Figure 6**.

Pharmacokinetic and pharmacodynamic barriers to translation

One of the main challenges associated with peptide translation is the instability due to enzymatic breakdown via proteolytic enzymes. Because peptides naturally exist in the body, they are extremely susceptible to degradation by these enzymes leading to very short in vivo half-lives.[97,98] To overcome this issue, several chemical strategies have been developed to enhance peptide stability. Cyclization which is a method often used in the creation of stable peptides by limiting their degree of flexibility and shielding them from exposure to proteolytic cleavage by altering their structure. Another technique is hydrocarbon stapling. It has been used stabilize α-helical peptides to increase their resistance to proteolytic cleavage and improve their ability to bind to their target molecule. There are other methods employed to increase the stability of peptides against proteolysis, such as using D-amino acids or N-methylated amino acids, which also not only provide protection against degradation but retain their bioactivity.[98]

Pharmacokinetic limitations pose a second challenge when developing peptide-based therapies. The small molecular weight of peptides has resulted in rapid (renal) clearance, causing peptides to have very short systemic exposure time. Thus, researchers have developed numerous chemical strategies to modify peptides in order to extend the peptide half-life in the circulation.[99] The most frequently used chemical modification method is PEGylation, where polyethylene glycol (PEG) is covalently bonded to the peptide to increase the hydrodynamic size (thereby reducing the renal clearance) of the peptide.[100] Another well-accepted modification method employed to enhance the peptide half-life is through lipidation, which relies on the reversible interaction between the peptide and serum albumin to prolong the circulation time and improve pharmacokinetic (PK) characteristics.[101] Additionally, some peptides have been developed with engineered albumin-binding motifs, providing further enhancement of systemic stability and persistence.

Delivery poses a third key obstacle for peptide drugs, especially when the target protein is inside cells. While peptides can readily bind to and interact with extracellular protein receptors, delivery into cells is much more difficult because the plasma membrane is not permeable to most peptides.[102] Cell-penetrating peptides (CPPs) have been explored as potential delivery vehicles to facilitate cellular uptake of peptide drugs. Additionally, nanoparticle-based delivery systems are being developed as potential solutions for both improving stability and targeting of peptides along with their delivery to the inside of cells.[102] Nanoparticles, such as lipid nanoparticles, polymeric carriers and other nanomaterials can protect the peptide from breakdown while allowing controlled release at the desired site of action.[103]

To successfully translate peptide therapeutics into the clinic, rigorous preclinical evaluation is critical. Animal studies are important tools for assessing the pharmacology of peptide drugs, including the pharmacokinetics, biodistribution and pharmacodynamic characteristics of the peptide. These studies help determine if the peptide can be delivered at a pharmacologically relevant (therapeutic) concentration to the target site of action, as well as if the peptide has an acceptable safety profile.[104] Toxicity and immunogenicity of peptide drugs will also need to be evaluated carefully, as the use of peptide drugs can sometimes lead to an immune response due to the use of repeating doses. The development of imaging technologies and analytical tools has enhanced the ability to evaluate the distribution of peptides and target engagement in vivo.

Peptide therapeutics success story

Many peptide therapeutics have advanced to clinical use despite the numerous difficulties encountered in their translation to this level. The high-profile example of the success of this class of compounds is represented by the analogs of the glucagon-like peptide-1 (GLP-1), with now many GLP-1 analogs available for the treatment of type 2 diabetes and obesity.[105] The GLP-1 analogs contain chemical modifications to increase their stability and extend their time in circulation, thus maintaining their efficacy in

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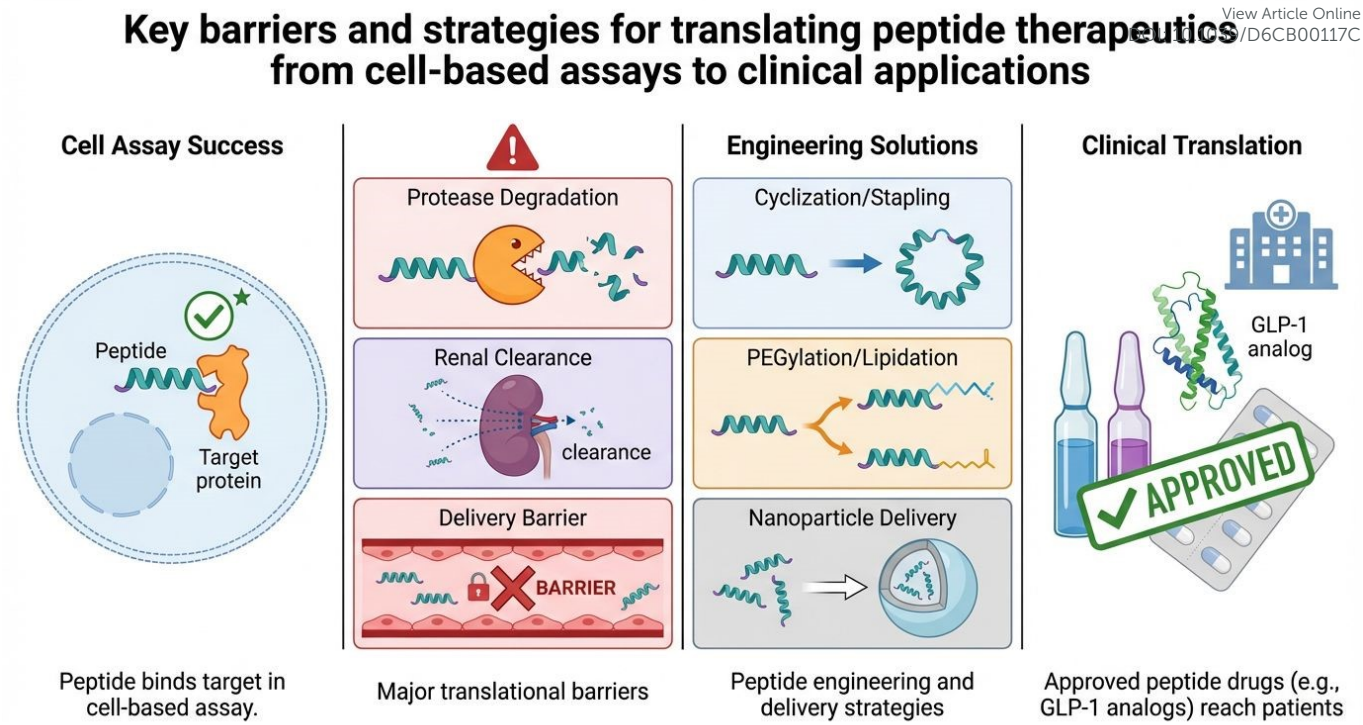

Figure 6. Translational barriers and engineering strategies for advancing peptide therapeutics from cell-based studies to clinical applications.

Peptides often demonstrate strong binding and functional activity against their target proteins in cell-based assays. However, successful translation to in vivo and clinical settings is frequently limited by several biological barriers. Major challenges include rapid proteolytic degradation, which reduces peptide stability in biological fluids; rapid renal clearance due to the small size of many peptides; and limited cellular or tissue delivery caused by membrane permeability barriers. To overcome these limitations, multiple engineering strategies have been developed. Structural stabilization approaches such as peptide cyclization or hydrocarbon stapling can enhance conformational stability and protease resistance. Pharmacokinetic optimization strategies, including PEGylation or lipidation, increase molecular size and promote interactions with serum proteins, thereby prolonging circulation time. In addition, nanoparticle-based delivery systems can improve peptide stability and facilitate targeted delivery. Together, these advances help enable the successful translation of peptide candidates into clinically approved therapeutics, such as glucagon-like peptide-1 (GLP-1) analogs used in the treatment of metabolic diseases.

vivo. In addition, improvements in the engineering and delivery of peptide hormones and agonists to receptors have benefited from the advancement of peptide engineering and delivery technologies.

Emerging technologies will continue to expand the ability to utilize peptides therapeutics. The advances in computational modeling and artificial intelligence are allowing for the efficient identification of peptide sequences with optimal structural and pharmacological characteristics.[106] New therapeutic approaches, such as peptide-drug conjugates and using targeted degradation methods to modulate difficult-to-achieve biological targets, are presenting additional opportunities for the development of clinically relevant peptide therapeutics and support the connection of potential cell-based discoveries to viable peptide therapeutics.[103]

In summary, however, significant hurdles exist, but the ongoing advancement in peptide chemistry techniques, delivery systems, and preclinical validation methods continues to provide an enhanced probability that peptides found during early phases of research will successfully move into clinical studies. With continued development in these areas, peptide medications are projected to take on increasing significance within the field of drug discovery today.

Conclusions

Peptide chemistry and biology are no longer defined primarily by the efficient preparation of linear sequences or by the rediscovery of endogenous ligands. Instead, the field is increasingly organized around enabling technologies that allow peptides to be discovered, engineered, validated, and translated in a far more programmable manner. Across the sections of this review, a consistent theme appears: progress is greatest when synthesis, discovery, mechanistic characterization, and translational design are treated not as separate stages, but as an integrated workflow. Rapid and automated synthesis now shortens the path from concept to molecule; display technologies, medicinal optimization, and computational design expand the space of accessible binders; and orthogonal biophysical and cellular assays make it possible to distinguish simple biochemical potency from genuine intracellular target engagement and therapeutic potential.[5,103,110] At the same time, recent advances in

developability-focused chemistry—including stabilization against metabolism, half-life extension, and more sophisticated delivery strategies—are helping shift peptide research away from "bind first, fix later" toward earlier multiparameter optimization.[106,107]

Looking forward, the next major advances in peptide science will likely come from tighter coupling of these capabilities. Rather than optimizing affinity, permeability, stability, manufacturability, and in vivo exposure sequentially, future workflows will increasingly need to address them in parallel. This will favor closed-loop platforms in which synthesis, screening, structural analysis, AI-guided design, and developability assessment iteratively inform one another.[5,110] Such integration should be especially important for complex peptide that sit beyond traditional linear therapeutics, including highly constrained macrocycles, orally enabled peptide drug candidates, targeted delivery systems, and peptide-based chimeras for induced proximity or targeted protein degradation.[15,108,109] The field should also continue to expand beyond potency-driven discovery toward context-aware design, where tissue access, intracellular trafficking, dosing route, and mechanism of action are considered intrinsic parts of molecular design rather than downstream obstacles.[103,107,109]

More broadly, the future of peptide therapeutics will depend not only on making better binders, but on making a whole better system: molecules that are chemically accessible, biologically credible, clinically practical, and scalable to manufacture. In that sense, the maturation of peptide science is not simply producing more peptide candidates; it is redefining peptides as a versatile chemical-biological platform capable of addressing targets and mechanisms that remain difficult to reach with conventional small molecules or larger biologics. If the current convergence of molecular engineering, predictive design, and translational technologies continues, peptides are prepared to progress to an even more central position in next-generation chemical biology and therapeutic discovery.[4,107–110]

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.