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Peptides and pseudopeptide ligands: a powerful toolbox for the affinity purification of current and next-generation biotherapeutics

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ABSTRACT

Following the consolidation of therapeutic proteins in the fight against cancer, autoimmune, and neurodegenerative diseases, recent advancements in biochemistry and biotechnology have introduced a host of next-generation biotherapeutics, such as CRISPR-Cas nucleases, stem and car-T cells, and viral vectors for gene therapy. With these drugs entering the clinical pipeline, a new challenge lies ahead: how to manufacture large quantities of high-purity biotherapeutics that meet the growing demand by clinics and biotech companies worldwide. The protein ligands employed by the industry are inadequate to confront this challenge: while featuring high binding affinity and selectivity, these ligands require laborious engineering and expensive manufacturing, are prone to biochemical degradation, and pose safety concerns related to their bacterial origin. Peptides and pseudopeptides make excellent candidates to form a new cohort of ligands for the purification of next-generation biotherapeutics. Peptide-based ligands feature excellent target biorecognition, low or no toxicity and immunogenicity, and can be manufactured affordably at large scale. This work presents a comprehensive and systematic review of the literature on peptide-based ligands and their use in the affinity purification of established and upcoming biological drugs. A comparative analysis is first presented on peptide engineering principles, the development of ligands targeting different biomolecular targets, and the promises and challenges connected to the industrial implementation of peptide ligands. The reviewed literature is organized in *(i)* conventional (α-)peptides targeting antibodies and other therapeutic proteins, gene therapy products, and therapeutic cells; *(ii)* cyclic peptides and pseudo-peptides for protein purification and capture of viral and bacterial pathogens; and *(iii)* the forefront of peptide mimetics, such as β-/γ-peptides, peptoids, foldamers, and stimuli-responsive peptides for advanced processing of biologics.

1. Introduction

The introduction of vaccines in the prophylaxis of infectious diseases [1,2] and monoclonal antibodies (mAbs) in the treatment of cancer and autoimmune disorders [3,4] are amongst the highest – if not the highest – achievement of 20th century medicine. By drastically cutting infant mortality and increasing the odds of the survival for patients affected by diseases until then deemed incurable, these molecules have demonstrated that biological drugs, or "biotherapeutics", hold true promise to improve the quality of life of humankind at large [5,6]. These efforts continue at a new level in the 21st century with the consolidation and expansion of the first-generation biotherapeutics, and the introduction of next-generation biological drugs. These include new protein species, such as bi-specific antibodies [7] and endonucleases (*e.g.*, CRISPR Cas) [8], and complex biologics, such as viral vectors for gene therapy (*e.g.*, adeno associated virus, AAV) [9], Car-T cells for cancer therapy [10], and stem and progenitor cells for tissue engineering and regenerative medicine [11].

The increased molecular complexity of these species, while improving their therapeutic efficacy, poses new challenges to their manufacturing. In particular, downstream processing represents

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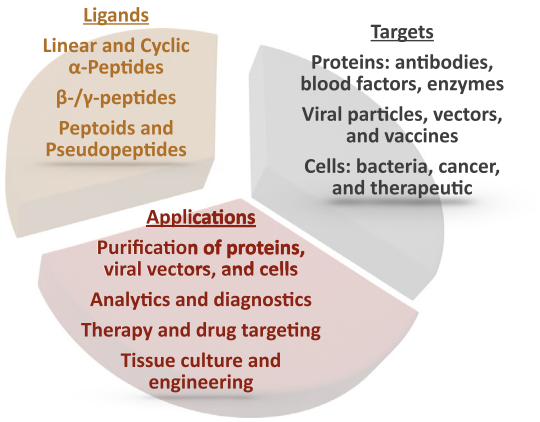

Fig. 1. Overview of the current scenario of peptide and pseudopeptide ligands, their biomolecular and biological targets, and main applications of peptide ligand-mediated adsorption of biotherapeutics and biologics.

one of the major voices of cost, as demonstrated by three decades of industrial mAb manufacturing [12]. The discovery and engineering of mAb-specific protein ligands (*e.g.*, Protein A and G) played a crucial role in enabling the manufacturing of large amounts of mAbs, but also impacted significantly on their price [13]. On the other hand, only few engineered ligands are available for the next-generation biotherapeutics. While some new ligands have recently been introduced, based on camelid antibody fragments targeting human Fab-κ or Fab-λ for the purification of bispecific mAbs [14], and different AAV serotypes [15], most of the above mentioned targets remain orphan of ligands.

The dearth of affinity ligands targeting the biotherapeutics of the future poses an urgent need as well as a major opportunity: to establish a new generation of ligands that possess the desirable qualities of protein ligands – namely, the high affinity and selectivity – without the downsides of high cost, biochemical lability, and toxicity and immunogenicity. In this context, synthetic peptide and pseudopeptides represent a promising family of scaffolds to develop cost effective affinity ligands (Fig. 1) [16]. Peptides, both natural and engineered, feature an excellent biorecognition activity towards proteins and other biomolecules [17], while also being amenable to affordable large-scale synthesis with minimal to no variability. Most notably, the advancements in peptide chemistry through the last decade have expanded this family well beyond the natural (α-)peptides, introducing β- and γ-peptides [18], peptoids [19], foldamers [20], as well as peptide hybrids integrating stimuli-responsive moieties for controlling the target capture and release using external signals [21]. The exploration in chemical diversity has been accompanied by a comparable effort in structural diversity, resulting in the development of cyclic and polycyclic ligands with superior binding strength and selectivity [22]. Completing this toolbox are the myriad methods for engineering protein-targeting peptides and pseudopeptides, grouped in combinatorial selection methods [23] and rational design [24]. The former include biological display methods, such as phage [25], mRNA [26], and yeast [27] display, and the screening of synthetic libraries in either solid or liquid phase [28,29].

The implementation of these chemistries and sequence engineering approaches has resulted in a plethora of peptide and pseudopeptide ligands for a wide variety of targets. Such is the variety of ligand properties and applications that not only the novice, but also the experienced reader may feel bewildered before the mole of literature. To provide guidance exploring this field, we endeavored to select and summarize the major contributions to the engineering of synthetic peptide-based ligands for the affinity purification of biotherapeutics. We have structured this work using the chemical classification of the ligands and the typology of biological targets as criteria to organize the reviewed material. In so doing, we trust that this contribution will prove useful to present the state-of-the-art of the field, demonstrate the value of its ancillary role to biotechnology and biomedicine at large, and propose directions that hold the most promise to advance the future of this technology.

2. Peptide design, discovery, and development: perspectives and challenges

Implementing peptide-based ligands in industrial biomolecular and biological separations stands at the crossroad between *(i)* the fundamental physicochemical features of these molecules, *(ii)* the technology of discovery and development of biospecific sequences, *(iii)* the scalability of synthesis and conjugation to chromatographic supports, and *(iv)* the industrial and regulatory requirements (Fig. 2).

2.1. Fundamental properties of peptide-based ligands

The realm of peptides comprises a large variety of aliphatic and aromatic foldamers [30], some of which – *e.g.*, linear and cyclic α-peptides and peptoids – have been widely utilized as ligands, whereas others – *e.g.*, β- and γ-peptides, and the so-called Class A and Class B peptide mimetics [31] – have been explored to a lesser degree. Together with the progress in the chemistry of polyamide backbones, a considerable effort has been devoted towards expanding the diversity of side chain functional groups beyond the natural ones – also known as “canonical” or “proteinogenic” – to include a broad variety of non-natural moieties [17].

The first element determining the biorecognition activity of peptide-based ligands is the balance between enthalpic and entropic contributions in the free energy of target:ligand interaction [32,33]. The enthalpic contribution is mostly determined by the physicochemical identity of the moieties displayed on the side chain or along the backbone of the monomers, and specifically their ability to form non-covalent interactions (*i.e.*, electrostatic, π-effects, van der Waals forces, and hydrophobic effects) with their partners on the binding epitope of the target [34-36]. The entropic contribution is determined by the disruption of the water shell on the binding site and the loss of conformational freedom that the ligand undergoes upon binding. The latter is always an unfavorable contribution, but it can be mitigated by increasing the rigidity of the ligand [37-40]. In proteins, the binding epitopes comprise amino acids optimally displayed within a structured framework that grants them a characteristically high binding affinity and selectivity [41].

Linear (α-)peptides displaying natural amino acids form the historical core – and currently the largest group – of peptide-based ligands for bioseparations [16,42,43]. Being affordable and scalable, linear canonical α-peptides have been developed for the purification of antibodies and other therapeutic proteins, gene therapy products, and therapeutic cells [44]. Among all peptides, however, they are the most prone to biochemical degradation and their high flexibility causes a substantial penalty to their binding energy [45]. Resistance to proteolytic enzymes can be achieved by replacing natural with non-natural amino acids [46]. These also improve the chemical diversity of α-peptides by introducing moieties that form stronger non-covalent interactions, thus boosting the enthalpic component of the binding energy. Modifying the backbone chemistry has also proved a valuable design parameter to increase biochemical stability and binding affinity: β- and γ-peptides [47,48] as well as N-substituted peptides – known as “peptoids” or poly N-substituted glycines [49] – are not prone to proteases and feature backbones with a different profile of hydrophobicity *vs.* hydrogen

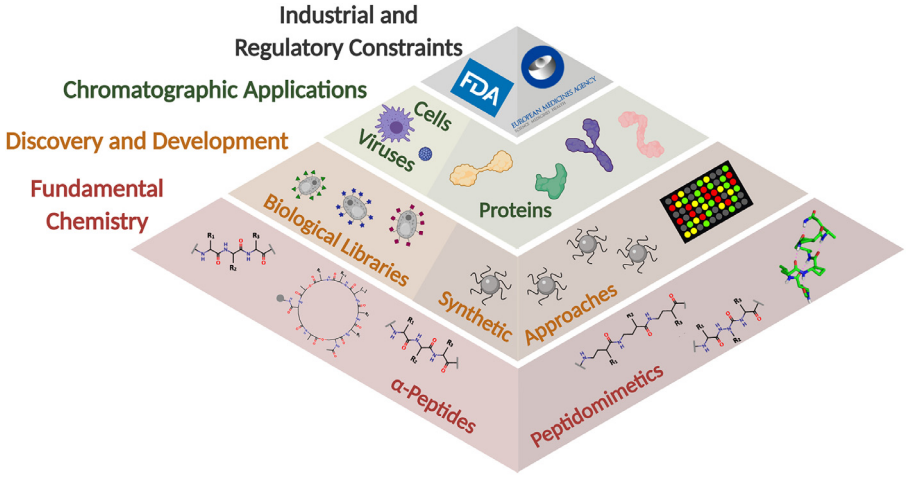

Fig. 2. Interplay between physicochemical features, technology of discovery and development, application towards different target, and industrial and regulatory requirements connected to biospecific peptide-based ligands.

bond-forming moieties; these have been shown to improve binding selectivity by privileging side chain group-based interactions with the target protein [19,50,51].

To mitigate the entropic penalty, constrained peptides with rigid backbone and orientation of the residues have been introduced: these include cyclic and polycyclic peptides with one or more intra-cycle covalent linkage [52-54], and peptide foldamers featuring a cyclic alkyl, alkenyl, or aryl backbone [55]. Foldamers are particularly interesting owing to their protein-like ability to fine-tune the presentation of the residues [56,57] and enhance their interaction with the target, thus optimizing both enthalpic and entropic components to the binding energy [58,59]. A rigid structure, on the other hand, is not necessarily an immovable one. Recently, linear and cyclic peptides as well as peptide mimetics integrating stimuli-responsive moieties have been introduced, whose binding activity for a target can be switched *on*-and-*off* reversibly and remotely via exposure to external fields [60,61]. These ligands feature efficient photo-isomerization of both free and protein-bound forms, substantial variation in protein binding energy upon photo-isomerization, and stability of the protein-bound isomer under physiological conditions [62,63].

The advancements in peptide synthesis technology are closing the "affinity gap" between peptide-based ligands and their protein counterparts. While small differences remain, the increasingly protein-like biorecognition mechanism of recently discovered peptides and pseudopeptides attests their potential as next-generation ligands for biomanufacturing.

2.2. Discovery and development technologies

The identification and optimization of biospecific peptide-based ligands relies on the high-throughput construction and interrogation of large ensembles of sequences – known as "libraries" – against the desired biomolecular or biological target. A myriad of *in vitro* and *in silico* tools are now available for building and screening libraries of peptides and pseudopeptides.

The *in vitro* technology for *de novo* discovery of peptide-based ligands relies on biological and synthetic libraries. Biological libraries are all liquid-phase and include mRNA-, yeast-, ribosomal-, and phage-display technologies [64]; the latter has been recognized with the 2018 Nobel Prize in Chemistry (to George Smith and Sir Gregory Winter) for its potential as a drug discovery tool [65,66]. These libraries are characterized by a large diversity ($>10^9$ variants), allow facile sequencing owing to the recent development in high-throughput gene sequencing [67,68], and enable the identification of ligands with high affinity and selectivity via directed evolution [69,70]. As a result, display technology is the tool of choice for the identification of ligands for difficult bioseparations, where the target is present at low titer (*e.g.*, therapeutic viral vectors and cells) in a highly complex medium. On the other hand, by relying on the cellular biosynthetic machinery, biological libraries limit the inclusion of amino acids with non-natural backbone and side chain groups, or complex structural modifications beyond simple cyclization [71-73]. Furthermore, while enabled by codon editing, the exclusion of specific amino acids from biological display peptide libraries - and therefore the resulting ligands - is challenging. This poses an issue in industrial bioseparations, where the use of alkaline conditions mandated for the cleaning-in-place of the adsorbent prevents the use of peptide ligands containing asparagine or glutamine [74].

The limitations inherent to biological libraries can be overcome by synthetic libraries, which are available in either liquid or – predominantly – solid phase. The latter are the offspring of combinatorial synthesis methods that enabled the construction of diverse library formats (*e.g.*, overlapping, alanine scanning, positional, truncation, random, and scrambled [75,76]) on a variety of substrates (*e.g.*, polymer beads and porous resins, membranes, microarrays, etc. [77-79]). The major advantages of synthetic libraries include *(i)* a virtually infinite variety of chemical and structural editing and *(ii)* the direct screening of ligand candidates on solid phase. The former includes the display of any non-natural residue, the hybridization of different backbones, cyclization and poly-cyclization, and the integration of stimuli-responsive moieties, all of which improve biorecognition strength, selectivity, and process control [62,80,81]. Among synthetic libraries on solid phase, the microarray [82,83] and the "one-bead-one-peptide" (OBOP) [84,85] have been applied countless times to the discovery of peptide and peptide mimetic ligands for protein targeting and purification as these methods are easy and affordable to apply and are well suited to high throughput screening. Critical to this end has been the introduction of equipment enabling rapid library screening, such as the machines for automated miniaturized synthesis on 2D substrates, MALDI-MS imaging for peptide microarrays [86], and microfluidic devices for automated screening and sorting of OBOP libraries [28,29,87,88].

In silico tools have acquired an increasingly higher status in the field of peptide discovery and development [89]. Recent advancements in hardware technology, in fact, have boosted the power and

accessibility of computing resources, enabling faster simulations of more complex systems [90,91]. This has morphed the *in silico* evaluation of protein:peptide interactions from the validation to the discovery phase, where virtual libraries of peptides and peptide mimetics can be screened in a high-throughput fashion against putative binding sites on target biomolecules [19,92]. A myriad of proprietary as well as open access algorithms are available for performing "druggability" (*i.e.*, identify putative binding sites on the surface of the target biomolecule) [93,94], "docking" (*i.e.*, identify binding poses of a candidate ligand on the target biomolecule) [95], and molecular dynamics (*i.e.*, refine the target:ligand complex and evaluate the binding energy and affinity) simulations [96]. While the current landscape of *in silico* tools is mostly focused on the design of α-peptides ligands, recent advancements in force field development are opening avenues towards the screening of virtual ensembles of peptoids, foldamers, and other peptide mimetics [97]. Alongside discovery, computational analysis maintains its role in ligand evaluation and validation, as it provides a molecular-level decomposition of the binding energy at the level of either the single residue or the single energetic contribution (*e.g.*, electrostatic, hydrogen bonding, or hydrophobic interactions) [98,99]. This information is critical to carry out a rational optimization of the ligand sequence and operation [100,101]. Of particular relevance in this regard is the ability of current tools to simulate the immobilization of the ligand on a solid phase [102-104], as well as the ionic strength and pH of the environment in which the target:ligand complex is formed [105,106], to evaluate realistically chromatographic process steps (binding, washing, and elution).

2.3. Scalability and conjugation to chromatographic substrates

One of the major advantages that synthetic peptides and peptide mimetics hold over their protein counterpart is the ability to be mass manufactured affordably and with no batch-to-batch variability [107]. Liquid phase Fmoc/tBu synthesis [108], in particular, affords large amounts (>10 kg) of highly pure peptide at prices as low as $ 10 – 15 per gram, enabling inexpensive manufacturing of peptide-based affinity adsorbents. Reducing the cost of bioprocessing – especially the downstream segment – remains a key objective, and an increasing number of companies are directing their attention to synthetic ligands, including peptides and peptide mimetics [42], as a means to make the manufacturing of biologics more affordable. It must be noted, however, that industrial peptide manufacturers can currently synthesize only linear α-peptides confidently. Advanced compounds, like α-peptides integrating non-natural amino acids, peptoids and foldamers, and cyclic and polycyclic structures, on the other hand, are subjected to a steep price increase due to the limited availability of the necessary building blocks and special reagents for difficult couplings or orthogonal deprotection steps. Nonetheless, it is foreseen that the manufacturing of advanced peptides and peptide mimetics will progress, just like the synthesis of α-peptides which has evolved from a niche technology to a mainstream commodity.

Furthermore, chemical synthesis allows appending residues dedicated to the conjugation of the ligand onto the chromatographic substrate, either via traditional (*e.g.*, the N-terminal or lysine's side chain amino group to an NSH ester- or epoxide-activated substrate; or the thiol group of a cysteine to a maleimide-, epoxide-, or alkylhalide-activated substrate [109]) or orthogonal conjugation strategies (*e.g.*, thiol-ene or alkine-azide "click" chemistry employing amino acids displaying "clickable" groups such as allylglycine, propargylglycine, or azidolysine [110]). A multitude of chromatographic substrates have been demonstrated for the conjugation of peptide and pseudopeptide ligands, including agarose and sepharose, polymethacrylates, and silica resins [111,112], or polysulfone, polyester, and polyamide membranes [113,114].

The ligand density of peptides on substrates can be accurately controlled, which is critical to minimize variability among lots of peptide-based affinity adsorbents, due to small size of peptide ligands and the use of precise conjugation chemistry.

A critical aspect still in need of improvement is represented by the values of binding capacity. The contending protein-based affinity adsorbents feature values > 40 mg of target protein per mL of adsorbent [115,116]. While scant, the available literature on optimizing the capacity of peptide-based adsorbents has demonstrated that, whilst high values are reachable, they require a laborious *ad hoc* optimization, which is inherently connected to the character of peptides as small-molecule ligands. Their size is in fact comparable to the nanoscopic features of the chromatographic support surface, which imposes the use of spacer arms and careful optimization of their surface density to ensure effective display to capture the target biomolecule. Nonetheless, a large degree of variability is unavoidable and peptide and pseudopeptide ligands that provide high binding capacity on silica resin, afford rather different capacities on Sepharose or polymethacrylate resins [111]. Growing efforts towards the purification of complex targets, such as viruses and cells, pose new challenges to the design of peptide-based adsorbents. Critical in this regard is the formation of multipoint interactions between the biological targets and the ligands, which increases dramatically the binding strength – a phenomenon known as "avidity" – and makes the elution of the target challenging [44,117,118]. Overcoming these issues requires a holistic rethinking of peptide-based ligands and the adsorbent, where the ligand surface density, the topology of the pore surface, and the pore size are adjusted concurrently to afford an optimal balance between capacity and selectivity of capture, and effectiveness of release and bioactivity of the recovered product.

2.4. Industrial applications and regulatory concerns

The growing success of companies such as LigaTrap Technologies [119], Avitide [120], and Astrea Bioseparations [121] demonstrates that peptides and peptide mimetics are on course to become the next-generation ligands for bioseparations. Biological therapeutics are rapidly expanding beyond mAbs and a multitude of new affinity ligands and adsorbents will be soon needed to meet the demand of clinics and biotech companies worldwide. Two decades of Protein A-based technology has taught invaluable lessons about the ideal properties of affinity ligands and associated regulatory requirements.

In this regard, several key properties of peptide and peptide mimetic ligands are only superficially known, such as their *(i)* ability to effectively clear the myriad of host cell proteins (HCPs) and nucleic acids, especially those that pose safety concerns to patients [122,123], to the levels mandated by regulatory bodies (< 100 ppm), *(ii)* chemical and biochemical stability – and resulting binding capacity and selectivity – across the intended lifetime of the adsorbent (up to 200 cycles), and *(iii)* safety and biocompatibility. To date, only a few peptide and peptoid ligands have undergone in-depth scrutiny of these properties, whereas the vastest majority of published ligands have been investigated only regarding their binding affinity and capacity as well as the product recovery and purity they afford.

Reassurance that these challenges will be met successfully is given by the outstanding chemical and biochemical stability of peptide mimetics. While in fact the secondary amide bonds in the backbone of α-peptides can be hydrolyzed by the acidic buffers used for product elution and the alkaline solutions used for cleaning-in-place, the tertiary amide bonds forming the backbone of peptoids and foldamers feature a substantially higher stability

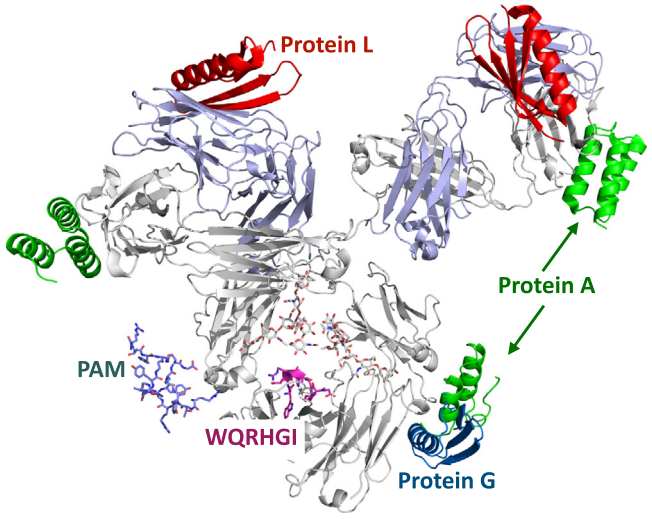

Fig. 3. IgG binding sites of the earliest (PAM, violet, PDB: 3D6G) and latest (WQRHGI, magenta, [38]) published IgG-binding peptide ligands in comparison with protein ligands Protein A (green, PDB: 1FC2), Protein G (blue, PDB: 1FCC), and Protein L (red, PDB: 4HKZ).

[124]. The superior strength and durability of peptide mimetics, combined with their excellent biorecognition properties and ease of conjugation, suggests that these molecules will soon occupy a role of preeminence in the proscenium of peptide-based ligands.

3. Linear α-peptide and small protein ligands

3.1. Purification of therapeutic antibodies

Monoclonal and polyclonal antibodies represent the largest class of biotherapeutics currently on the market, with more than 60 products approved in the US alone [125]. Monoclonal antibodies (mAbs) form the current arsenal in the fight against cancer, autoimmune and neurodegenerative diseases, and viral diseases [126]. Polyclonal antibodies (pAbs) are supplied to patients affected by autoimmune, infectious, and idiopathic diseases [127,128], and have garnered interest as potential treatments in the recent viral pandemic [129,130]. The chromatographic purification of antibodies, while established, suffers from cost and footprint overrun [131,132]. A key role in this context is played by Protein A media, currently the industrial standard for mAb capture via affinity chromatography, due to their high selectivity and binding capacity. Protein A adsorbents, however, are expensive (up to $15,000 per liter), require rather harsh elution conditions, and are prone to leaching toxic and immunogenic ligand fragments upon repeated use [92,133]. To address the growing demand for therapeutic mAbs while also containing the costs and improving the safety of the purification process, short peptides have emerged as alternative ligands (Fig. 3) [134].

Linear α-peptides in particular can be manufactured affordably on the large scale, and are inherently safer and more robust than bacterial protein ligands. They exhibit good binding affinity and selectivity, and enable product elution under milder conditions, thus reducing the risk of product denaturation and aggregation [19,135]. The first antibody-binding peptides date back to the work of Fassina *et al.* on the "Protein A-mimetic" (PAM) peptide [136] and its *inverso* form D-PAM [137]. Following these pioneering studies, remarkable advances have been made in the field of small linear peptide ligands for IgG purification (Table 1).

Our group has produced substantial work on peptide ligands for antibody purification, beginning with a series of studies on HWRGWV, HFRRHL, and HYFKFD targeting the Fc region of IgG (IgG-Fc) by screening solid-phase synthetic hexapeptide libraries [138]. Peptide HWRGWV was evaluated in depth by purifying therapeutic mAb from commercial Chinese Hamster Ovary (CHO) cell culture fluids [112,139] and a *Lemma* plant extract [140], performing comparably with Protein A and Protein G control resins. The ligand was also utilized for purifying human polyclonal antibodies from an immunoglobulin-rich paste of human plasma derived from the Cohn-Oncely [141,142], as well as silk milk and whey returning high IgG yield and purity. Billakanti *et al.* utilized HWRGWV for the extraction of polyclonal bovine IgG from milk with purity above 85%, demonstrating the usefulness of the ligand for the production of nutraceuticals [143]. Of interest is also the ability of this ligand to target all four subclasses of human IgG [138], human IgA and IgM [144-146], as well as animal (*e.g.*, rabbit and llama [111,138]) antibodies with high selectivity, affording high product quality from diverse sources. This is particularly relevant in demonstrating the effectiveness of IgG-binding peptide ligands over traditional bacterial ligands Protein A, Protein G, and Protein L, whose targeting ability is rather narrow [147-149]. The work on HWRGWV also demonstrated the flexibility of synthetic peptide ligands towards conjugation on different substrates. The peptide has in fact been conjugated to polymethacrylate (Toyopearl) and agarose (Sepharose and WorkBeads) resins [112,139], a ceramic fluorapatite (CFT) matrix [150], a megaporous cryogen [151], and disposable silica resins with optimized functional density, and particle and pore size [111]. The latter study is particularly interesting, since it demonstrates the amenability of peptide ligands towards the development of single-use, disposable and recyclable adsorbents.

Combinatorial strategies for ligand discovery alternative to screening synthetic solid-phase libraries rely on biopanning of liquid-phase biological libraries [149,159]. In recent work, Kruljec *et al.* screened three phage-display libraries of linear heptameric, linear dodecameric peptides, and cyclic nonameric peptides against IgG-Fc. AGNGSYWYGVWF was selected among the identified sequences as model to develop variants via N- and C- terminal trimming, alanine scanning, and sequence mutagenesis. The optimized peptide GSYWYDVWF was utilized to recover human IgG from spiked growth medium and crude human serum with 95% purity, and sustained functional performance over 25 chromatographic runs [126]. Following a similar approach, Sun *et al.* identified candidate ligands by panning a heptapeptide phage-display library against IgG-Fc. The sequences were evaluated via molecular docking and molecular dynamics (MD) simulations to estimate the free energy of binding and the contribution of each amino acid in the sequence. All seven candidates showed affinity towards human, canine, and murine IgG as well as human IgM [160].

The use of integrated computational-experimental approaches to discover and refine IgG-targeting peptides has become mainstream in recent years. Huang *et al.* utilized the IgG:Protein A complex as a starting model for the biomimetic design of affinity peptide ligands [161]. Based on the *in silico* study of the IgG:Protein A interaction, the authors constructed an *in silico* ensemble of 8-mer peptides and screened it against IgG-Fc using the docking software Autodock Vina. Based on the preliminary docked structure, a round of sequence refinement followed via flexible docking in Rosetta FlexPrepDock, which returned 15 candidate peptides. Upon refinement via MD simulations, the sequence FYWHCLDE was selected for experimental evaluation via IgG purification from serum and cell culture supernatants, affording purity and yield $\sim$ 90% [162-164]; in line with the initial simulations, FYWHCLDE and its variants FYCHWALE and FYCHTIDE were found to target the Protein A binding site on the Fc region of IgG. In subsequent work, the group developed the dual-peptide affinity system FYWHCLDE-FYCHTIDE, which showed a synergistic effect in IgG binding, substantially increasing product yield and purity compared to single-ligand adsor-

Table 1
Overview of published results on antibody-binding peptide ligands.

Peptide	Target	Antibody Source	Purity (%)	Yield (%)	REF
HWRGWV	hIgG	cMEM	95.8	89.6	144, 152
HWRGWV	hIgA	cMEM	91.6	83.8	144
HWRGWV	hIgA	CHO CCCF	96	90.3	146
HWRGWV	SIgA	CHO CCCF	94.3	91.73	146
HWRGWV	hIgM	cMEM	75.7	86.0	144
HWRGWV	hIgM	Human B lymphocyte cells	> 95	>95	146
HWRGWV	MAb2	CHO CCCF	> 94	>84	[112,139]
HWRGWV	MAb1	CHO CCCF	> 94	>84	[112,139]
HYFKFD	MAb1	CHO CCCF	93	86	139
HFRRHL	MAb1	CHO CCCF	95	84	139
HWRGWV	mAb	Lemma plant extract	90	96	140
HWRGWV	pAbs	Cohn fraction II+III of human plasma	95	84	153
HWRGWV	pAbs	Bovine skim milk	92	74	[143,153]
HWRGWV	pAbs	Bovine whey	93	85	153
HWRGWV	mAb	CHO CCCF	> 90	> 85	111
HWRGWV-KPRSVSG	hIgG	E.coli lysate	94	87	150
CEWW	IgG	cMEM	95.2	35.8	154
CEWW	IgG	CHO CCCF	95.8	53.3	154
HEYW	IgG	cMEM	98.7	61.1	154
HEYW	IgG	CHO CCCF	98.7	65.8	154
DWHW	IgG	cMEM	95.6	79.6	154
DWHW	IgG	CHO CCCF	98.1	85.4	154
Ac-YFRH	mAb	CHO CCCF	99	89	155
W-ABI	mAb	CHO CCCF	98.9	88.6	156
FYE-ABI	hIgG	Human serum	93.9	88.9	157
GSYWYDVWF	hIgG	Spiked growth medium and human serum	95	N/A	126
GVKCTWSSIVDWVCVDM	IgY	Chicken egg yolk	> 90	~70	158

bents [164]. The same group also developed variant FYCHWQDE, which was utilized to purify IgG from diluted clarified human serum at 80% yield and 90% purity [165,166]. In a similar manner, Wei *et al.* utilized Autodock Vina to identify tetrameric peptides with high IgG-Fc affinity and low human serum albumin affinity. The lead candidate peptides DWHW, CEWW, and HEYW were evaluated experimentally by studying the effect of salt, pH, and flow rate on binding performance. In particular, DWHW was able to purify IgG from CHO cell culture supernatants with 95% purity and 85% yield [154]. Based on previous IgG-binding peptide ligands found in literature, Wang *et al.* designed a focused tetrameric peptide library comprising two aromatic residues followed by an arginine and an aromatic/aliphatic residue, and used the Flexible Docking module of Discovery Studio to screen the library against the protein A binding site of IgG-Fc. Upon MD refinement of the docked structures, the lead candidate ligand Ac-YFRH was selected for experimental studies, affording a remarkable IgG purification from a clarified CHO cell culture supernatants with 99% purity and 89% yield [155]. Tong *et al.* utilized alanine scanning and MD simulations to evaluate the binding to a highly conserved consensus binding site and several Fc-binding ligands including protein A, protein G, and Fc receptors for IgG (FcγRs) [167]. These studies demonstrated the importance of hydrophobic interactions and aromatic residues in Fc targeting. Accordingly, tryptophan-mimetic 5-amino-benzimidazole (ABI) was adopted as a ligand for mAb purification via multimodal charge induction chromatography. The ligand was tested by isolate mAb from cell culture fluids, affording 88.6% yield and 98.9% purity [156]. In an effort to further develop this ligand, MD simulations were used to guide the design of complementary tripeptide ligands to balance the hydrophobic and electrostatic contributions to Fc binding. The selected tripeptide FYE was conjugated to the ABI moiety and the resulting ligand was utilized to isolate human IgG from human serum with 88.9% yield and 93.9% purity [157].

In a recent effort, our group developed an integrated computational-experimental approach for the discovery of linear peptide ligands with high purification performance [92]. An ensemble of 60,000 linear hexametric variants of HWRGWV were initially generated and screened *in silico* using a high-throughput search algorithm to identify sequences targeting IgG-Fc. The selected sequences were then negatively screened *in silico* against a panel of 24 model host cell proteins (HCPs) to down-select candidate ligands with high affinity and selectivity. Sequences WQRHGI and MWRGWQ were conjugated to Toyopearl resins and evaluated by measuring by static binding capacity (52.6 and 57.5 mg of IgG per mL of adsorbent, respectively) and dynamic binding capacity ($DBC_{10\%}$ of 30.1 and 36.4 mg/mL, respectively, at 2 minutes residence time). Measurements of binding affinity via isothermal titration calorimetry (ITC) confirmed the *in silico* values of binding energy and the affinity-like binding activity of both peptides. WQRHGI-WorkBeads resin was utilized to purify a therapeutic mAb from an industrial CHO cell culture harvest, affording a remarkable 500-fold reduction in HCP titer and performing consistently over 100 chromatographic cycles.

While most antibody therapeutics on the market or in clinical trials are IgG-based, increasing attention has been given to Immunoglobulin A (IgA) and M (IgM). IgA has value in the treatment of infectious and malignant diseases [146], while monoclonal IgM offers promising anticancer and antitumor activity in melanoma patients [168]. Compared to the mole of studies on IgG purification, much less work has been conducted on the purification of IgA and IgM. Our group has demonstrated the utility of ligand HWRGWV towards IgA and IgM recovery from recombinant sources: artificial cell culture fluid mimetic, with 80% yield and 90% purity for hIgA, and 75.7% yield and 86.0% purity for hIgM [144,152]; a CHO cell culture supernatant and a human B lymphocyte cell culture supernatant with both purity and yield above 90% [145]. Notably, the dynamic binding capacity of HWRGWV-Toyopearl resin for IgM is much greater than protein-based affinity ligands and competitive with commercial adsorbents KAPTIVE-M, CaptureSelect IgM, and Ultralink Immobilized Mannan Binding Protein [145].

Recently, avian immunoglobulins (IgY) have gained increasing attention for their therapeutic potential as they represent a read-

Table 2
Overview of published results on non-antibody protein-binding peptide ligands.

Peptide	Target	Antibody Source	Purity (%)	Yield (%)	REF
W-W	HSA	N/A	N/A	N/A	[187]
DWDLRLLY	Caps	Cell lysate	70.1	N/A	[188]
DYWWQSWE	PCV2 Cap	N/A	98	90	[189]
KVPLITVSKAK	rhFSH	CHO CCCF	94	41	[190]
SMWRTYIGSGSG	hGH	Pichia pastoris CCCF	91	20	[191]
SMWRTYHGSGSG	hGH	Pichia pastoris CCCF	95	80	[191]
HRCGSWLHPCLA	BDDrFVIII	CHO CCCF	99.9	85	[192,193]
EYKSWEYC	BDDrFVIII	Diluted plasma	N/A	N/A	[194,195]
(3-IAA)Eψ[CH2NH]YC	FVIII	FBS-containing cell culture medium	N/A	N/A	[196]

ily available and economical substitute to IgG [169-172]. Protein A and G, however, do not bind IgY, and conventional IgY purification procedures achieve low yield and purity. In the effort to develop effective ligands for IgY purification, Khan *et al.* screened a T7 phage display library of decameric disulfide-cyclic peptides. Of the 30 sequences identified, 3 showed particularly high binding affinity and specificity, and were further characterized via enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), immunoprecipitation, and affinity chromatography. The selected peptide GVKCTWSSIVDWVCVDM was biotinylated and immobilized onto a HiTrap Streptavidin HP column, and used to extract IgY from chicken egg yolk, affording $\sim$ 70% yield and $>$90% purity [80].

Recent advances in antibody engineering have led to the development of recombinant fragments for use as functional alternatives to whole mAbs. While offering advantages in terms of therapeutic efficacy and cost effectiveness, these antibody derivatives introduce new challenges to downstream processing. Protein A chromatography, in fact, cannot capture non-Fc fragments or selectively purify target heterodimer bispecific antibodies from the homodimers. To address these issues, Nascimiento *et al.* screened phage display libraries to identify the first peptide ligands targeting Fab-κ [149]. Of three selected sequences WHYNWQDVSDRQ (A5), WIPNSEFEHERT (B1), and HQNHHSTFWEIY (C7) found to target Fab with micromolar affinity, B1 was selected for its binding consistency across the panel of target Fabs and immobilized onto NHS activated Sepharose Fast Flow for evaluation via affinity chromatography. The peptide managed to recover Fab from a crude *E. coli* cell lysate with 84% yield and 90% purity, enabling gentle elution conditions (pH 5) and reproducible results across multiple chromatographic cycles. In a similar manner, Akiyama *et al.* screened a phage-display library of linear peptides against consensus sequences comprising heavy and light-chain variable-regions of human antibodies, and designed by aligning the Fv region of human IgG [173]. Library screening returned several candidate ligands, a subset of which were characterized in combination by purifying trastuzumab-derived single chain variable fragments from Rosetta2 *E. coli*, affording 60% yield.

3.2. Purification of other therapeutic proteins

With regards to downstream processing, mAbs have the advantage of a conserved structural framework that can targeted by a single ligand irrespective of the antigen specificity. Non-antibody protein therapeutics, on the other hand, rarely share conserved structural regions, and affinity ligands must therefore be tailored to every target (Fig. 4). Antibody substitutes, blood factors, therapeutic enzymes, and hormones are the most targeted species (Table 2).

Small-molecular-weight scaffolds, including adnectins [174], anticalins [175] DARPins [176], knottins [177], and affibodies [178] hold true promise as alternatives to antibodies in human therapy and biotechnology [179,180]. These species feature customizable binding affinity and can be expressed inexpensively and at high titer in bacteria, and [180]. Among them, affibodies hold a prominent position, with more than a dozen products on the market, regulatory approval for human therapy [181,182], and a growing body of literature demonstrating their value in basic research [183-185]. Yet, the purification of affibodies does not benefit from an established platform technology, limiting their availability and increasing their price. In a recent study, Barozzi *et al.* presented the discovery of peptides that target the constant regions of affibodies by screening a solid-phase synthetic peptide library simultaneously against multiple model affibodies [186]. The selected sequences were evaluated via chromatographic studies and *in silico* docking to demonstrate targeting of the conserved affibody domain. Peptide IGKQRI was further validated through purification of an anti-ErbB2 affibody from an *Escherichia coli* lysate affording in 71% yield and 91% purity.

A prominent role among blood factors is played by Factor VIII (FVIII), which is utilized in the treatment of Hemophilia A [197]. Intravenous supplementation of FVIII requires the production and purification of massive amounts of FVIII worldwide, which traditionally relied on immunoaffinity chromatography followed by ion exchange-based product polishing [198,199]. To replace expensive antibody ligands, Jungbauer *et al.* reported a series of octameric peptides with high affinity to FVIII identified by screening a combinatorial spot library. The peptide EYKSWEYC showed the best performance for FVIII purification by affinity chromatography from diluted plasma, although the values of purity and yield were not reported [194,195]. In later work, Kelly *et al.* screened phage-display libraries to identify peptides targeting the recombinant B-Domain Deleted Factor VIII (BDDrFVIII). The identified sequence HRCGSWLHPCLA was used to purify BDDrFVIII from CHO fluids affording 85% recovery and 99.9% purity [192,193]; this peptide provides a 10,000-fold reduction in host cell proteins and host cell-derived DNA, comparable in performance with immunoaffinity purification techniques.

Another family of major interest among protein therapeutics is that of hormones, whose best-known members are insulin, erythropoietin, and gonadotropic hormones [200-202]. Among the latter, follicle stimulating hormone (FSH) is clinically used to assist reproduction technologies of in-vitro fertilization (IVF) and intracytoplasmic sperm injection [203,204]. In a recent study, Messina *et al.* designed a short peptide ligand KVPLITVSKAK for the purification of rhFSH via affinity chromatography. The peptide sequence was selected by generating and screening variants of the FSH receptor exoloop 3 [205]. As the variant with the highest affinity for FSH, KVPLITVSKAK was conjugated to the chromatography resin SulfoLink agarose and utilized to purify rhFSH from a crude sample of CHO cell supernatant, returning 94% purity and 41% yield [190]. In another study, Chandra *et al.* developed a peptide affinity ligand for the human growth hormone (hGH) by screening a small peptide library using the hGH-binding protein and human prolactin receptor as reference binders [191]. The peptides in the library were designed via receptor epitope mapping, identification of interacting regions and adjacent loops, and mutated variants of

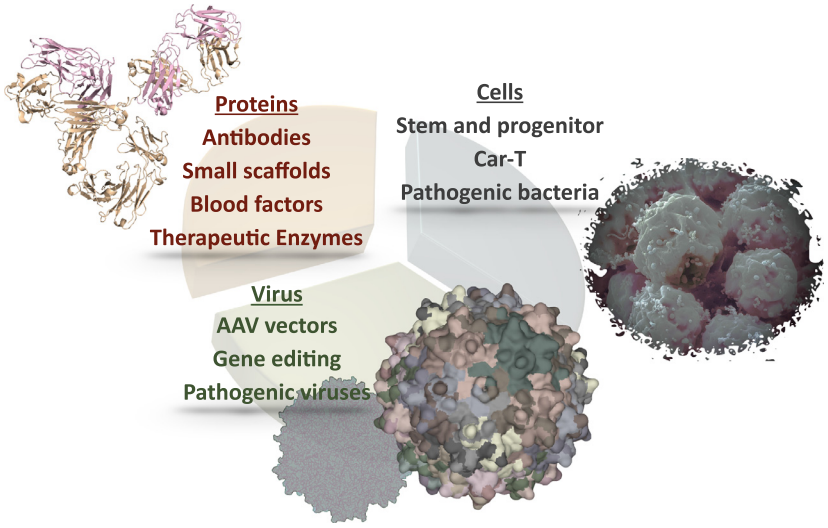

Fig. 4. Overview of the current scenario of biotherapeutics: protein-, virus-, and cell-based targets.

these sequences. Using a microarray screening method, the candidate ligand SMWRTYIGSGSG was selected to purify hGH from a *Pichia pastoris* cell culture fluid, affording 91% purity but only 20% yield. Histidine mutagenesis of the ligand resulted in the peptide variant SMWRTYHGSGSG, which was found able of capturing hGH from the same *Pichia pastoris* fluid with 95% purity and 80% yield.

Among the emerging enzyme therapeutics, CRISPR-Cas nucleases hold a prominent place owing to the ubiquitous application of CRISPR in biotechnology, medicine, and bioprocessing [8,206]. Despite the incipient clinical trials and the growing use of Cas nucleases in organism engineering, affinity ligands targeting these proteins are missing, posing a major roadblock to the long-term success of this technology. To address this challenge, Day *et al.* implemented an orthogonal dual fluorescence method to screen a solid-phase peptide library against a model mixture of *Streptococcus pyogenes* Cas9 spiked in *E.coli* cell lysate and identify Cas9-binding peptides. Selected octameric sequences GYYRYSEY and YYHRHGLQ were shown to target the RecII domain of Cas9, and utilized to purify Cas9 from a *E.coli* cell lysate with recovery of 86∼89% and purity of 91∼93% [207].

In a recent study, Trasatti *et al.* presented a method for the rational design of peptide affinity ligands for purifying a recombinant human therapeutic enzyme [24]. The putative binding domains on the surface of the enzyme were initially identified *in silico* and utilized as target sites to screen a virtual library of peptides constructed by mutating helical peptides derived from zipper-like proteins. The peptide variants with predicted complementarity to the target were evaluated experimentally via microarray screening to identify candidate ligands with high binding affinity and gentle elution conditions. Selected peptides were further evaluated in batch chromatography studies, where peptide-based resins captured and eluted the target with substantially higher purity compared to commercial mixed-mode resin material.

In recent work, Martinez-Ceron *et al.* developed a peptide against the Phospholipase A2 (PLA2) subunit of Crotoxin, a toxin in the venom of the rattlesnake species *Crotalus durissus terrificus* [208]. PLA2 has demonstrated antiviral activity against yellow fever and dengue viruses [209], although no protein ligands are available for its large-scale purification. The authors synthesized and screened a solid-phase combinatorial library of decameric peptides against biotinylated PLA2, using Streptavidin-Peroxidase and α-Chloronapthol/H_2O_2 to evaluate binding. The selected peptides were sequenced with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Among the ligands that were evaluated experimentally, the leading candidate peptide was shown to capture up to 97% of PLA2 in the source venom and remove most of the endogenous proteins present therein.

3.3. Peptide ligands for protein purification in flow-through mode

In traditional biomanufacturing practice, the validation of a batch of therapeutic mAb only required the certification of residual impurities (HCP and DNA) to be below the FDA-imposed limits. Today, the introduction of advanced analytical techniques for protein identification and quantification has shown that mAb batches with acceptable global level of impurities can contain amounts of single high-risk HCP impurities (HR-HCPs) that either are a threat to patient health (*e.g.*, are toxic or immunogenic) or can degrade the product or its excipients during storage resulting in harmful products. A growing body of literature is also documenting that commercial Protein A and polishing adsorbents struggle to remove particular HR-HCPs [210-212]. Different HR-HCPs have been highlighted both on a process-basis and a product-basis [212,213]. In some instances, HR-HCPs can be effectively cleared, but this requires implementing additional steps in the purification process [214,215]. “Hard-to-remove” HR-HCPs, however, have been reported to cause delays in FDA clinical trials of mAbs [216,217] and process approval [218,219], and recall of mAb batches [220,221]. To address this challenge, Lavoie *et al.* developed a set of peptide ligands that target the HCPs produced by CHO cells, with minimal binding of the mAb product [222] by screening combinatorial libraries of linear tetrameric and hexameric peptides [28,223]. The selected sequences were conjugated onto Toyopearl resins and utilized to purify therapeutic mAbs by capturing CHO HCPs in flow through mode (Fig. 5). Optimization of the loading conditions (buffer composition and pH, and loading ratio) afforded a >90% purity of the mAb product and a >80% yield. Importantly, the proteomics analysis of the effluent via mass spectrometry demonstrated that hard-to-remove and high-risk HCPs were effectively retained [222], demonstrating the potential of this adsorbent for scrubbing HCPs prior to Protein A capture step in a mAb purification platform [224]. Further improvement of this technology may require targeted screening of peptide libraries against individual HCPs that are escaping capture by the current ensemble of peptide ligands.

The enrichment of low-abundance proteins in complex fluids using ensembles of peptide ligands immobilized on chromato-

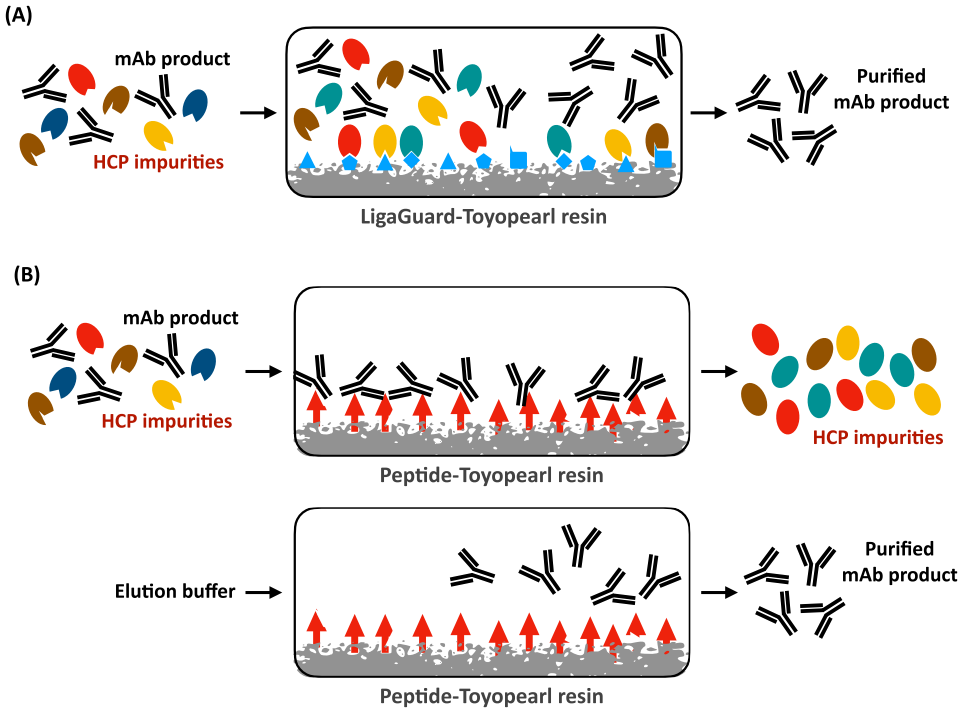

Fig. 5. Comparison of **(A)** flow-through mode vs. **(B)** bind-and-elute mAb purification using peptide ligands.

graphic substrates has also been investigated as a means to improve the outcome of proteomic studies. This technology, now known as mixed-bed affinity chromatography [225], was introduced by Thulasiraman *et al.*, who employed full combinatorial peptide libraries to capture proteins via broad-spectrum intermolecular interactions in flow through mode [226]: as a complex protein mixture flows through the adsorbent, the less abundant proteins are captured and gradually build up into the column, while high-titer proteins rapidly saturate their binding sites and the excess flow through; upon saturation, all proteins are desorbed from the adsorbent, yielding a solution enriched in low-abundant proteins by up to 3 – 4 orders of magnitude [227]. The equalization of protein titers improves dramatically the detection of the species in solution. Contrary to mixed-mode resins where one ligand captures multiple proteins [228], mixed-bed affinity chromatography utilizes a pooled resin where each bead is functionalized with different ligands. This technique is particularly attractive for its ability to magnify diluted species, and has great potential for preparative and diagnostic applications, and proteomic studies on biological fluids [229-231] and plant extracts [232,233]. Combinatorial peptide libraries for low-abundance protein enrichment are now commercially available under the name ProteoMiner (Bio-Rad, CA, USA). When tested against seven other protein depletion strategies, ProteoMiner provided the best protein enrichment, affording a 1.5-fold increase in protein detection [234]. D'Ambrosio *et al.* [235], Ma *et al.* [236], and Liu *et al.* [237] used ProteoMiner and its carboxylated version to remove highly abundant proteins from chicken egg white or blood sample, increasing the number of identified proteins compared to the previously available list. Simo *et al.* also used the ProteoMiner kit to enrich and study the low-abundance proteome of red blood cells by removing high-abundance species such as albumin and immunoglobulins [238]. Restuccia *et al.* used the kit to detect novel protein species in human serum [239]. Fasoli *et al.* also reported the proteomic characterization of African Puff Adder snake venom after pre-fractionation using ProteoMiner beads to amplify low-abundance proteins, thus contributing to a more comprehensive description of the venom proteome [240]. In another study, Li *et al.* solubilized proteins from human tissue in Triton X-100 prior to loading onto ProteoMiner beads, and eluted the enriched proteins with a urea solution. The detergent's solubilization of the high-titer hydrophobic proteins present in tissues and cells enabled the capture and identification of new proteins from the four human tissues including eight membrane/secreted proteins and five nucleus proteins [241]. This approach has spurred a number of recent advances in analytical protein enrichment using peptide libraries on chromatographic substrates. In a recent study, Candiano *et al.* developed a peptide library modified with Alcian Blue 8GX, a cationic dye targeting protein glycosylation groups, to improve the detection of glycoproteins and glycopeptides in the human urinary proteome [242].

3.4. Purification of gene therapy products

3.4.1. Purification of DNA.

Plasmid DNA has recently emerged as a promising biotherapeutic class for vaccination and gene therapy applications [243,244]. Among the many forms of plasmid DNA - open circular, relaxed circular, linear, supercoiled, and supercoiled denatured conformations - the supercoiled isoform is considered to be the most effective, owing to its stability and antigenicity. As with protein therapeutics, this growth poses the need of affinity ligands for purifying not just plasmid DNA, but specific isoforms of plasmid DNA as well [245].

In early studies, chromatographic substrates functionalized with arginine-rich peptide ligands were shown to bind specifically and separate the supercoiled and open circular isoforms of plasmid DNA by interacting with the DNA backbone and specific nucleotide bases. These ligands have since been characterized and incorporated into numerous platforms for the purification of plasmid DNA [246,247]. Bai *et al.* have studied the effect of ligand density and spacer length on the binding efficiency of arginine towards supercoiled plasmid DNA and enable large scale purification. Longer spacer arms and increased ligand density improved binding capacity of the ligand, providing a more feasible chromatography

method for large scale supercoiled plasmid DNA purification from clarified cell lysate [248]. In addition, Cardoso *et al*. utilized di- and tri- arginine decorated monoliths as a plasmid DNA purification platform. Compared to a single arginine monolith, epoxy monolithic columns functionalized with di- and tri-arginine homopeptides showed higher dynamic binding capacity of plasmid DNA, but also caused longer retention times, and reduced elution efficiency and yield [249]. These authors have also employed arginine-based ligands to develop a negative-mode chromatography platform to remove host impurities from plasmid DNA. The preferential binding of RNA, proteins, genomic DNA, and endotoxin impurities, in fact, inhibited plasmid DNA capture, allowing up to 99% of plasmid DNA recovery from an *E. Coli* lysate [250].

In another study, Ferreira *et al.* compared the performance of L-tyrosine and oligo-L-tyrosine peptides for the affinity purification of supercoiled plasmid DNA. The study of binding conditions of pVAX1-*LacZ* and pcDNA3-FLAG-p53 plasmids to oligo-L-tyrosine peptides via SPR demonstrated the ability of these ligands to separate the supercoiled isoform from the open circular and linear isoforms of plasmid DNA [251]. In a similar study, Santos *el al.* used L-tyrosine and L-tryptophan as ligands for purifying the supercoiled isoform of pPH600, a plasmid forming the G-quadruplex secondary structure. L-tyrosine in particular was able to recover supercoiled pPH600 with high purity and yield [252]. SPR and chromatographic analysis of single L-amino acid ligands demonstrated that these ligands have specific biorecognition of supercoiled pVAX-*LacZ* and pPH600 plasmids [253].

Minicircle DNA (mcDNA) vectors are similar to plasmid DNA vectors, but are depleted of the bacterial sequences [254]. These minimal plasmids avoid the transgene silencing and immunologic responses caused by the bacterial elements, resulting in improved biologic efficacy [255]. Despite their therapeutic promise, no downstream process is available to meet the growing demand for mcDNA. In an effort to develop a minicircle DNA affinity ligand, Gaspar *et al.* evaluated the binding of novel arginine-based peptides to mcDNA via SPR, and found that binding is improved by lowering the ionic strength of the binding environment, indicating that electrostatic interactions play a central role in peptide-target binding [256]. These researchers utilized a zinc-binding HHHHHHCC peptide to mimic a DNA-binding zinc-finger motif, which, when complexed with the Zn^{+2} ion, exhibited significant DNA binding and was used to purify mcDNA with comparable performance with RR dipeptide ligands [256,257].

3.4.2. Purification of viral therapeutics

Viral products play an increasingly important role in therapy and prophylaxis [258-260]. Recent advancements in genetic engineering and recombinant virus production have improved the efficacy and titer of vaccines and vectors for gene therapy [261,262]. On the other hand, major challenges remain in their downstream processing, where critical quality attributes such as capsid protein makeup and full/empty capsid ratio remain unmet [260]. As the current purification methods are expensive, time-consuming, and difficult to scale-up, researchers have explored the use of synthetic ligands to improve chromatographic purification platforms.

Among the viral vectors for gene therapy, a prominent role is held by Adeno-Associated Virus (AAV) [258,263]. Commercial chromatographic adsorbents for AAV purification employ ligands derived from engineered single-chain (*e.g.*, camelid) antibodies and antibody fragments, such as the POROS™ CaptureSelect™ AAVX Affinity Resin or AVB Sepharose HP resin [264-266]. Together with high binding capacity and selectivity, however, these adsorbents suffer from the shortcomings inherent to protein ligands, such as limited targeting range and biochemical stability. The development of robust and cost-effective peptide ligands is highly sought after to improve AAV biomanufacturing, especially owing to their potential to enable single-use purification tools.

Pulichela and Asokan pioneered the discovery of AAV-binding peptides by screening a phage display library of heptameric peptides against AAV serotype 8 (AAV8) capsids [267]. The selected sequence GYVSRHP, named Pep8, was characterized for AAV binding on solid-phase, demonstrating the ability to bind not only AAV8, but also AAV1, AAV2, AAV5, AAV6, and AAV9 capsids. Pep8-agarose resin was therefore evaluated by purifying AAV8 from clarified HEK293 cell lysates. Gel electrophoresis and qPCR analyses demonstrated the ability of Pep8 to capture and enrich AAV8 vectors from dilute sources.

Virus-like particles (VLPs) are regarded as the next frontier of vaccines, owing to the ease of recombinant expression, high titer, and optimal presentation of the antigen [268,269]. Fernandes *et al.* utilized phage display to discover peptide ligands targeting retrovirus-like particles expressing the envelope protein Ampho4070A (VLPs-AMPHO) [270]. The lead peptide candidate CAAALAKPHTENHLLT was immobilized on agarose resin and evaluated against null virus-like particles (null-VLPs) and VLPs-AMPHO. The resulting adsorbent demonstrated selective VLPs-AMPHO capture (~16 μg/g resin) and virtually no null-VLP binding. While the VLPs recovered in elution were not assessed for biological activity, the recovery yields of the viral vectors reached 90-100% under mild elution using 12 mM imidazole in physiological buffer (PBS, pH 7.4) [270]. In a similar study Zheng *et al.* screened a phage display peptide library to discover peptide ligands targeting recombinant Porcine circovirus type II (PCV2) Cap protein [271]. PCV2 is a circular, single-stranded DNA virus with porcine pathogenicity and is responsible for significant economic losses to the swine industry [272]. The PCV2 Cap protein is the main antigenic determinant of PCV2 [273] and its recombinant form is utilized to prepare vaccines against PCV-associated diseases. The selected sequence YHDCFSAGFCIG, characterized via SPR, exhibited a remarkable binding affinity for PCV2 Cap protein, with a dissociation constant (K_D) in the nanomolar range. Owing to its high affinity, the peptide was utilized as primary capture agent to develop an ELISA kit with high sensitivity for the detection of PCV2 Cap protein [271]. Finally, YHDCFSAGFCIG was conjugated to magnetic beads and characterized for the affinity purification of PCV2 Cap protein from recombinant *E. coli* BL21 (DE3) cell culture, affording high yield and purity. In a more recent study, Hao *et al.* utilized molecular docking to identify peptide sequences targeting the PCV2 Cap protein [189]. The leading ligand candidate DYWWQSWE was characterized via SPR, demonstrating high PCV2 Cap protein binding affinity, and utilized to purify the protein from recombinant BL21 cell culture fluids with 98% purity and 90% yield.

Building on the work of *in silico* development of single protein-binding peptides, MD simulations have been applied to identify peptide ligands for viral particles. In one example, Li *et al.* adopted a computational approach to identify putative binding sites on murine polyomavirus virus-like particles and develop targeted peptide ligands. Murine polyomavirus virus-like particles are capsids formed by self-assembled capsomeres and have demonstrated interesting potential towards multiple therapeutic applications including vaccination, gene therapy, drug delivery, and materials science [188]. The authors initially studied the interaction between the capsomeres and a minor coat protein on the capsid of murine polyomavirus and identified “hot spots”, namely putative binding sites on the surface of the capsomeres. A virtual library of peptide ligands was docked *in silico* on the binding sites using Autodock Vina, and selected complexes were refined and validated via MD simulations. The leading candidate DWDLRLLY was finally validated experimentally, affording capsomere enrichment from a 15.6% purity feedstock to 70.1% purity elution fraction [188].

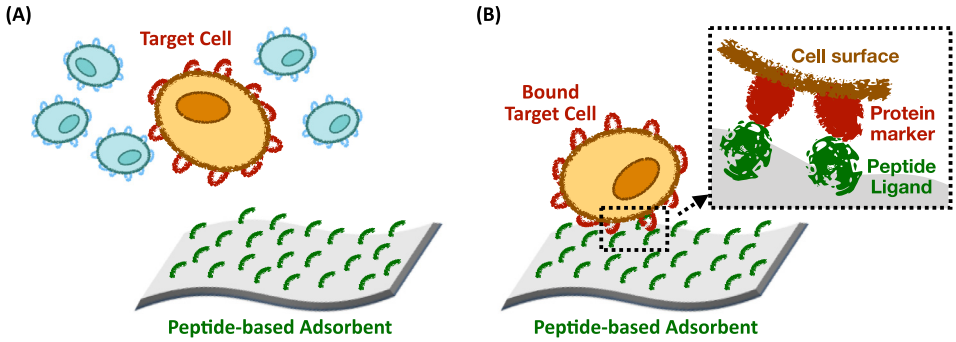

Fig. 6. **(A)** Adsorption and **(B)** cell binding via multi-point protein:peptide interactions (avidity).

3.4.3. Removal of pathogenic contaminants from biological fluids.

Bacterial and viral contamination in human therapeutics is a major concern in the biomanufacturing industry, given the large amount of therapeutic drugs derived from mammalian cell cultures or plasma fractionation [274]. The downstream processing of therapeutic products is required to comprise at least two distinct clearance steps and clear 99.99% of contaminating viral particles and bacterial toxins (*i.e.*, 4 log reduction). In this context, the use of peptide ligands to capture virus particles and bacterial toxins in bioprocess streams has proven its potential to ensure product safety and patient's health.

The group led by Carbonell has pioneered the discovery of peptide ligands for contaminant removal from biological fluids [275-278]. In an early study, the peptide YYWLHH was selected by screening a solid-phase combinatorial library of hexametric peptides against staphylococcal enterotoxin B. The peptide, which features antibody-like affinity for the capsid, was conjugated to Toyopearl resin and utilized to remove enterotoxin B from a mixture of SEB in either *E. coli* lysate or *S. aureus* fermentation broth in a single step, achieving more than 70% reduction [279]. In another study, the team screened a solid-phase library for trimer peptides to identify ligands that capture porcine parvovirus (PPV). The selected peptides WRW, KYY, and RAA provided high clearance (4.5–5.5 log) of PPV particles from buffer, although only WRW was able to remove all detectable PPV at relatively low injection volumes when challenged against diluted human blood plasma. This work demonstrated that small peptide ligands hold promise to replace nanofiltration or anion exchange chromatography in removing viruses from biomanufacturing fluid streams [280]. In a subsequent study, in the attempt to improve the capture of PPV by reducing its non-specific binding to plasma proteins, the authors examined the influence of peptide density and the use of hydrophilic spacer arms on the efficiency of virus removal. Their results demonstrated that low WRW density enhanced binding selectivity, facilitating specific peptide-virus capture in the presence of plasma proteins [275].

Rogers *et al.* utilized phage display screening to identify dodecameric peptides targeting Norwalk virus-like particles (NV VLPs). Norovirus (NoV) causes an estimated 21 million cases of gastroenteritis in the US annually, resulting in high hospitalization rates and loss of life in severe cases [281]. Immunosorbent diagnostics (*e.g.*, ELISA kits and lateral flow assays) are currently utilized for NoV diagnosis detection. However, the development, production, and quality control of anti-NoV antibodies are laborious and expensive, and pose a defined need for robust and affordable detection tools. Peptide NV-N-R5-1, isolated by the team, targets the protruding domain of the VP1 capsid protein and was utilized in lieu of antibodies to develop an ELISA test providing a limit of detection of 1.56 ng of NV VLP [282].

In another study, Memczak *et al.* developed three peptides derived from complementarity determining regions of anti-hemagglutinin antibody heavy chain against influenza A spike glycoprotein. The peptides were identified as substitutes to antibodies to detect and characterize influenza viruses and aid in the development of vaccines and therapeutics. In particular, sequence ARDFYDYDVFYYAMD (PeB) and its mutant variant ARDFYGYDVFFYAMD (PeB^{GE}) showed strong binding of influenza A/Aichi/2/68 H3N2 and other medically relevant influenza strains, demonstrating potential for application in viral detection and treatment [283].

3.5. Purification of Therapeutic Cells

Cell purification technology plays a key role in advanced therapeutic and diagnostic applications [284], including personalized cell therapy [285], tissue engineering [286], disease monitoring [287], as well as drug discovery [288] and fundamental cell biology [289]. Numerous cell isolation techniques have therefore been proposed to meet the growing demand for phenotypically pure cell populations. While several of these methods rely on the size and the superficial physicochemical properties of cells [290], affinity-based separations have become an established mainstream in cell purification [291]. Affinity-based cell separations have traditionally relied on antibodies targeting surface proteins that are unique or over-expressed on the target cells. Despite their significant cost, antibodies are attractive owing to their high affinity and selectivity [292]. When immobilized on a solid surface, antibodies drive cell capture by "avidity", which refers to the multivalent, also known as multi-point, interaction between the antibodies and multiple copies of the target protein on the cell's surface [293] (Fig. 6). Due to the high inherent binding strength of antibodies, avidity results in very strong cell capture [294], which imposes harsh elution conditions that can be detrimental to cell functionality and viability [295]; furthermore, the high binding strength of antibodies can also trigger undesired intracellular signaling cascades as well as cell death [296]. To overcome these limitations, peptides have emerged as alternative affinity ligands for cell separations. Peptides typically exhibit milder affinity compared to antibodies, which enables using gentler elution conditions that can preserve cell viability and functionality [297]. The advances in peptide selection via screening of phage display [298], mRNA display [299], and synthetic [300] peptide libraries as well *in silico* peptide ensembles [301-303] have spurred a remarkable growth in peptide-based tools for cell purification.

Cell-targeting peptides are commonly immobilized onto solid substrates to capture cells via adhesion, and are often used in diagnostic applications, where a particular cell phenotype is indicative of a disease. For example, circulating tumor cells (CTCs) shed from primary tumor tissue are typically indicative of metastasis [304]. Accordingly, the isolation of CTCs from peripheral blood plays a significant role in monitoring disease progression and evaluating therapeutic efficacy. Bai *et al.* discovered the peptide VRRDAPRFSMQGLDACGGNNCNN specific to the EpCAM biomarker, which is

over-expressed on CTCs but not on other blood cells [305]. This anti-EpCAM peptide ($K_D \sim 1.98$ nM) showed comparable binding affinity to that of an anti-EpCAM antibody ($K_D \sim 0.269$ nM), and was conjugated to magnetic nanoparticles to isolate breast, prostate, and liver cancer cells from spiked human blood, affording high capture efficiency (>90%) and selectivity (>93%). Other EpCAM-binding peptides have been identified for use in capturing CTCs for diagnostic applications [306]. Peptides specific to other biomarkers have also been used to isolate cancer cells and CTCs. One example is represented by the RGD peptide, which targets the $\alpha v\beta 3$ integrins overexpressed on many cancer cell types [307]. Xiao *et al*. functionalized nanofibers integrated into a microfluidic device with RGD peptides to capture cancer cells and CTCs [308]. A549 cancer cells expressing high levels of $\alpha v\beta 3$ integrin were captured with high efficiency (91.8%), but only moderate purity (33.1%), from a spiked suspension of white blood cells. Notably, the RGD peptide was conjugated to the nanofiber using an acid-labile linker, which enabled nondestructive recovery of the captured cells.

Cell-binding peptides have also been applied to the rapid detection of low concentration pathogenic bacteria in food sources or bodily fluids. The early detection of pathogens is often difficult due to their low concentrations, and most samples must be concentrated to increase the bacterial concentration above the limit of detection (10^3 to 10^6 CFU/mL) to enable analysis [309]. Sample concentration, however, is laborious and can result in a loss of sample. To overcome this issue, immobilized anti-microbial peptides (AMPs) are frequently employed as ligands to develop bioassays where enrichment and detection of bacteria is achieved on the same device [310-314]. In one example, Qiao *et al.* developed a high-sensitivity electrochemical biosensor for quantifying *E.coli* O157:H7 cells by combining the capture of bacteria using the antimicrobial peptide Magainin I (GIGKFLHSAGKGKAFVGEIMK) with enzymatic signal amplification [315]. The assay could detect *E.coli* cells spiked into apple juice and ground beef samples for concentrations as low as 84 and 233 CFU/mL, respectively. As the identity of pathogens in a sample is often unknown, an ideal detection platform must be able to detect a variety of pathogens. The use of peptide arrays allows for multiplexed detection of pathogens as demonstrated by Paradoux *et al.* [309]. In this work, six different AMPs were spotted onto a chip for surface plasmon resonance imaging. Each AMP exhibited a distinct binding with the pathogens tested and allowed for the unequivocal identification of unknown pathogens within a sample by comparing the binding pattern on the array to the binding profile of known pathogens.

Beyond diagnostics and pathogen detection, adhesion-based cell separation is also advantageous in tissue engineering applications. Prior to culturing on scaffolds, in fact, cell populations must frequently be enriched from a heterogeneous suspension obtained from a tissue sample. Ploufee *et al*. developed microfluidic devices functionalized with peptide REDV or VAPG to enrich endothelial or smooth muscle cells, respectively [316]. These peptides are derived from cell-binding proteins: REDV is found in the type III connecting region of fibronectin [317], while VAPG is derived from elastin [318]. Using these peptide ligands, endothelial and smooth cells were recovered at 86% and 83% purity, respectively, corresponding to a 3-fold enrichment compared to the inlet concentration. To improve the performance of the device, the phage-derived peptide HGGVRLY was utilized in lieu of REDV to improve the capture endothelial cells [319].

A significant challenge when separating cells for tissue engineering is posed by the low abundance of target cell types in pathological tissues [320]. Cells derived from primary tissues are generally anchorage-dependent and will not perform further functions, like proliferation, unless adsorbed onto a scaffold that mimics the extracellular matrix of the native tissue [321]. Peptide ligands are ideal binding moieties for recruiting particular cell types to adhere to substrate biomaterials. The RGD peptide has been extensively utilized for broad-spectrum cell adhesion on tissue culture substrates [322,323], although more cell-specific ligands are known. In one study, Shao *et al*. enhanced the adhesion of synovium-derived mesenchymal stem cells by conjugating the heptapeptide LTHPRWP on polycaprolactone electrospun meshes and human decalcified bone scaffolds [324]. Peptides targeting mesenchymal stem cells (DPIYALSWSGMA [320], as well as disulfide cyclic peptides CDNVAQSVC [325] and CTTNPFSLC [326]) and human dermal fibroblasts (GTPGPQGIAGQRQVV [327]) have also been used for cell recruitment to scaffolds.

As shown above, affinity-based cell separations have relied heavily on the use of peptide ligands derived from nature rather than engineered synthetic peptides. Given the growing demand for pure cell populations and the broadening diversity of cell targets, synthetic peptide ligands are destined to play a leading role in this field. Screening combinatorial libraries is a key technology to develop cell-targeting peptides: Liu *et al.* have utilized it to identify peptides specific to cancer cells and tumors [328]. Andrieu *et al.* performed *in vivo* phage display to discover peptides docking on target tissues and organs [329]. Similarly, library screening has served to identify peptides targeting embryonic stem cells [330], hematopoietic stem cells [331], neural cells [332], dendritic cells [333], tumor-associated macrophages [334], erythrocytes [335], chondrocytes [336,337], and osteoblasts [176]. The application of combinatorial selection technologies towards the discovery of cell-binding peptides is likely to increase as novel biomarkers are identified that are unique to target cell populations. In this context, peptide ligands binding EGFR [338], VEGF [339], VCAM-1 [340,341], VPAC-1 [342], FLT3 [343], E-selectin [344], TfR [345], CXCR4 [346], CD4 [347], CD81 [348], CD133 [349], and CD206 [350] have been discovered.

4. Cyclic peptide and pseudopeptides

Cyclic peptides and pseudopeptides such as α-peptides comprising non-natural amino acids, β- and γ-peptides, azapeptides and peptoids (N-substituted peptides), and peptide foldamers represent a growing realm of ligands with superior biorecognition properties as well as higher chemical and biochemical stability [134,351-354] (Fig. 7). The wide chemical diversity of pseudopeptides featuring residues with synergistic combinations of charged, hydrogen-bonding, and hydrophobic moieties, and optimal presentation on β- and γ- backbones enhance the enthalpic component of the free energy of binding; the rigid structure of cyclic and polycyclic peptides, and foldamers removes detrimental entropic components, thus equally contributing to stronger binding. Inspired by natural compounds, cyclic peptide ligands have emerged rather early in the literature as superior ligands [355-357], whereas pseudopeptides are a rather recent addition to the field [19,50,135,354,358-360].

4.1. Cyclic peptide ligands

Cyclic peptides have long been recognized as excellent scaffolds to develop synthetic affinity ligands targeting protein and cell therapeutics given their superior binding affinity and selectivity, and resistance to proteolysis compared to their linear counterparts.

The formation of a disulfide bond between cysteine residues flanking a protein-targeting peptide segment represents the simplest way to achieve peptide cyclization; furthermore, because it avoids the need for chemical ligation, it was the earliest strategy used to construct biological display libraries of cyclic peptides [355-357]. In an early study, Delano *et al.* screened a M13 phage display library of disulfide cyclic peptides against the Fc region of

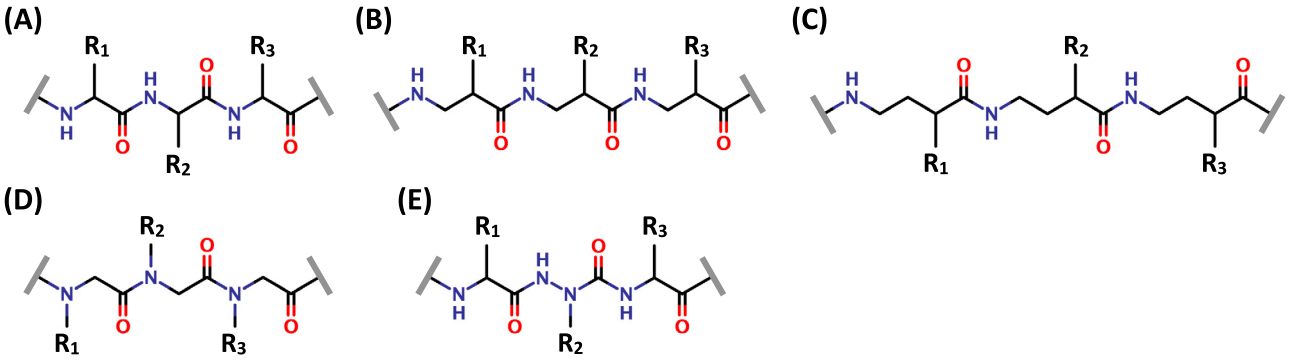

Fig. 7. Structure of **(A)** α-peptides, **(B)** β-peptides, **(C)** γ-peptides, **(D)** azapeptides, and **(E)** peptoids (N-substituted peptides).

IgG. The selected 13-mer sequence DCAWHLGELVWCT, named Fc-III, was found to bind the Fc region of IgG with affinity comparable to that of Protein A and Protein G [355]. In a later study, the hairpin-inducing D-Pro-L-Pro template was introduced as a N- to C- link of Fc-III to form a bicyclic structure, which improved its IgG binding affinity 80-fold [196]. In a recent study aimed at reproducing the increased affinity without the synthetic effort associated with D-Pro and N- to C- cyclization, Gong *et al.* replaced the D-Pro-L-Pro linker with two cysteine residues to mimic the bicyclic structure with a second disulfide bond. This variant, denoted as Fc-III-4C, featured a 30-fold increase in IgG binding affinity compared with the Fc-III cognate [361]. In a separate study to determine the effect of cyclization on binding performance, derivatives of PEG-conjugated Fc-III peptide, referred to as Fc-binding peptide (FcBP) in this paper, were prepared and tested [353]: FcBP-Ser was prepared by substituting both cysteine residues with serine, while FcBP-Red was prepared by reducing the disulfide bond of FcBP with DTT prior to use. NHS-activated Sepharose^{TM} 4 Fast Flow resins functionalized with FcBP, FcBP-Ser, FcBP-Red, Protein A, and Protein G ligands were evaluated for IgG binding and purification. As anticipated, FcBP-Ser-resin showed a significant decrease in IgG binding capacity; on the other hand, FcBP-Red showed considerable IgG binding capacity despite the absence of a rigid cyclic structure, and enabled IgG elution at pH 4.8, providing much gentler elution conditions compared to protein A and FcBP resins. Finally, the FcBP resin was utilized to isolate antibodies from porcine and human sera, affording 95% product purity and long lifecycle. Screening a synthetic library of disulfide-cyclic peptides, Verdoliva *et al.* selected the sequence $(CFHH)_2$-KG, denoted as "FcRM", as a ligand for IgG purification. The cyclic peptide proved useful for the purification of mouse monoclonal antibodies from crude hybridoma supernatants and polyclonal antibodies from human serum with both yield and purity higher than 90%. Further investigation demonstrated that FcRM can bind both Fab and Fc fragments of different isotypes, suggesting the presence of multiple peptide binding sites on the antibody. This makes FcRM useful for both the purification of antibodies and appealing for the generation of Fc-receptor antagonists [356].

While easy to screen and construct, disulfide-cyclic peptides suffer from the chemical lability of the disulfide bond, which can be easily hydrolyzed in mild reducing conditions. The resulting free cysteines can form disulfide bonds with the both the protein target and impurities in the feed, or become oxidized thus altering the binding activity of the ligand. Alternative cyclization strategies relying on chemical ligation have therefore been explored. Following the work by Robert and coworkers [362,363], Menegatti *et al.* constructed and screened a mRNA display libraries of chemically cyclized peptides to discover cyclic peptide ligands targeting IgG-Fc (Fig. 8). The cyclization of library peptides was achieved via solid-phase crosslinking using the bis-amine-reactive disuccinimidyl glutarate (DSG) [364,365]. Among the selected ligand candidates evaluated by IgG binding emerged the cyclic peptide cyclo[*DSG*-M-WFRHY-K], which was ultimately used to purify therapeutic mAbs from clarified CHO cell culture fluids with 96% yield and 93% purity [364]. Using this method, Bowen *et al.* identified cyclic peptides targeting the WW domains of Yes-Associated Protein 1 (WW-YAP) [117]. The mRNA display library was screened against yeast cells displaying WW-YAP and magnetized with iron oxide nanoparticles to isolate of WW-binding mRNA-peptide fusions. The selected peptides cyclo[*DSG*-M-AFRLC-K] and cyclo[*DSG*-M-LDFVNHRSRG-K] featured high affinity for WW ($K_D \sim \mu$M) and were evaluated via chromatographic binding in competitive conditions. In a recent study, Bacon *et al.* utilized a yeast-display library of DSG-cyclized peptides to discover ligands binding interleukin 17 (IL17). The selected peptide cyclo-[*DSG*-RMRWLRGRR-K] featured high IL17 affinity ($K_D \sim 0.3$ μM), supported by *in silico*-calculated $K_D \sim 0.1$ μM [27]. Besides chemical ligation, enzymatic routes have been explored for the cyclization of peptides displayed on biological libraries. In particular, several groups have demonstrated the use of transglutaminase to form peptide-protein conjugates or peptide macrocycles [366-369]. In recent work, Bowen *et al.* utilized this approach to construct yeast display libraries of cyclic peptides, whose construct was optimized to evaluate the efficiency of cyclization via dual fluorescence flow cytometry [370]. Library screening against YAP and its WW domain returned the cyclic peptide cyclo[*E*-LYLAYPAH-*K*], which featured high binding affinity ($K_D \sim 0.84$ and 1.67 µM for WW and YAP, respectively) and selectivity.

An inherent advantage of biological libraries as tools for ligand discovery is the presence of a polynucleotide encoding for the displayed peptide, which can be easily expanded and sequenced to identify the candidate peptide ligand. Sequencing cyclic peptide leads selected from synthetic libraries, on the other hand, is laborious and involves significant guesswork. In an effort to improve sequencing throughput and confidence, Pei and coworkers developed the "one-bead-two-peptide" method, wherein every library bead is segregated into an outer layer, which displays cyclic peptides and is accessible to the target protein, and an inner core, which is impervious to proteins and contains the linear peptide precursor. The selected beads are analyzed via Edman degradation to determine the sequence of the linear peptides in the core [360]. This method has been successfully applied to identify cyclic peptides [371-373] and peptide/peptoid hybrids [374-377] with protein-binding activity. In later work, our group developed an alternative sequencing strategy that employs libraries of cyclic depsipeptides, where the protein-binding sequence is framed in its cyclic form by two ester bonds [371]. Following screening, the selected beads are treated with an aque-

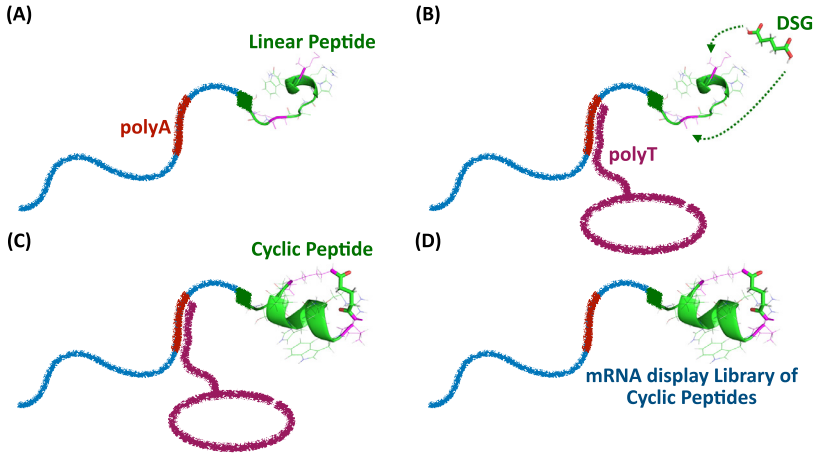

Fig. 8. Production of a mRNA display library of cyclic peptides: **(A)** preparation of a precursor mRNA-display library of linear peptides, **(B)** adsorption of the mRNA-display library on a polyT solid phase and reaction with DSG, **(C)** mRNA-display library of cyclic peptides, and **(D)** desorption of the library.

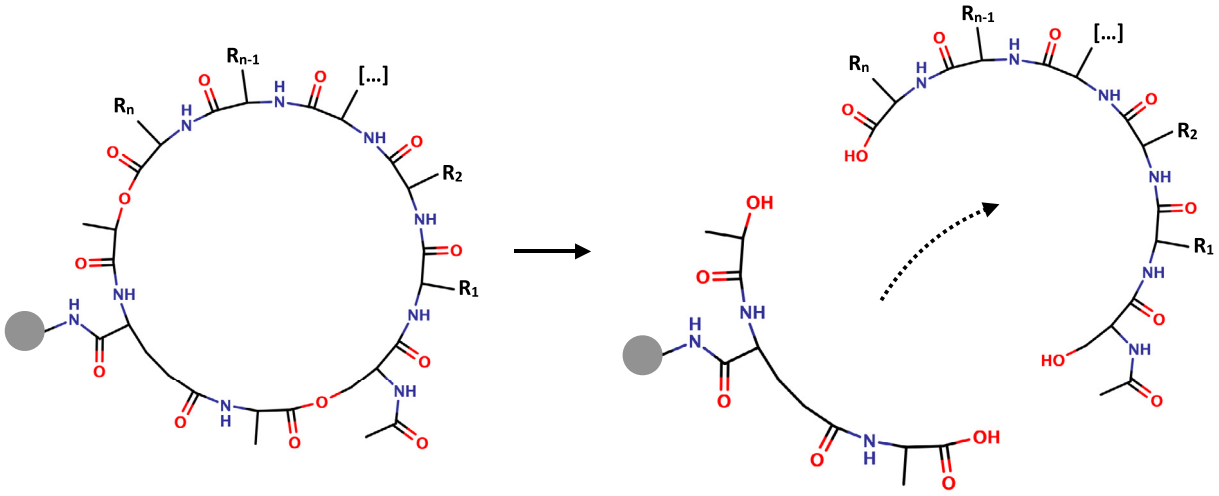

Fig. 9. Model structure of the cyclic depsipeptide and its linearized structure upon alkaline treatment.

ous alkaline solution that cleaves the framed peptide as a linear sequence and releases it in solution, enabling *de novo* sequencing via mass spectrometry (Fig. 9). This method has been demonstrated via the discovery of cyclic peptides targeting IgG-Fc and recombinant human erythropoietin (rHuEpo). The Fc-binding peptide cyclo[(Nα-Ac)-S(G)-RWHYFK-Gly-E] was conjugated to Toyopearl resins and demonstrated IgG binding on par with control ligands [371]. The rHuEpo-binding cyclic peptides cyclo[(Nα-Ac)Dap(A)-FSLLSH-AE] and cyclo[(Nα-Ac)Dap(A)-VVFFVH-AE] were selected from a library designed after the Epo-binding site of the Epo receptor [352]. The peptides were analyzed by molecular docking and sequence mutagenesis using non-natural amino acids to gain molecular-level understanding of the rHuEpo:peptide interaction, improve binding selectivity, and enable elution under mild conditions. The sequence FSLLSH was selected for its high affinity ($K_D = 0.46\mu M$) and utilized to purify rHuEpo from a CHO cell culture fluid, affording yield and purity above 90%, and a *Pichia pastoris* cell culture fluid, resulting in product yield and purity of 96% and 84%, respectively [378].

Cyclic peptides are also finding a role in cell purification, a field until recently dominated by linear peptides. In particular, peptides with multiple randomized cycles are most attractive as each cycle will bind to a unique epitope on the cell, thereby increasing specificity [379]. Phage-derived bicyclic peptides have been identified for several cell receptors, including the human epidermal growth factor receptor 2 (HER2) [380], NOTCH1 [381], various members of the integrins family [382], and the Platelet-Derived Growth Factor Receptor-beta (PDGFR-β) [383]. These peptides have been shown to mediate the adhesion of cells onto solid substrates [384], although true chromatographic application have not yet been demonstrated.

4.2. α-peptide ligands incorporating non-natural amino acids

Next to the proteinogenic amino acids, which comprise the 20 canonical and several natural non-canonical amino acids (*e.g.*, ornithine, citrulline, pyrrolysine, selenocysteine, etc.) [385,386], a myriad of synthetic non-canonical amino acids (sncAAs) have been developed for integration in α-peptide ligands either by engineering the translation machinery of cells [387] or traditional peptide synthesis via Fmoc/tBu chemistry [135]. The amino acids in this family display chemophore groups that provide synergistic combinations of binding modes (*i.e.*, electrostatic, hydrophobic, hydrogen bonding, etc.) [135] or the D-enantiomeric form of the proteinogenic residues that occur in nature as L enantiomers [137]. The outstanding chemical diversity of sncAAs increases dramatically the target binding affinity and selectivity of peptide ligands as well as their chemical and biochemical stability (*e.g.*, resistance to proteolysis) [135].

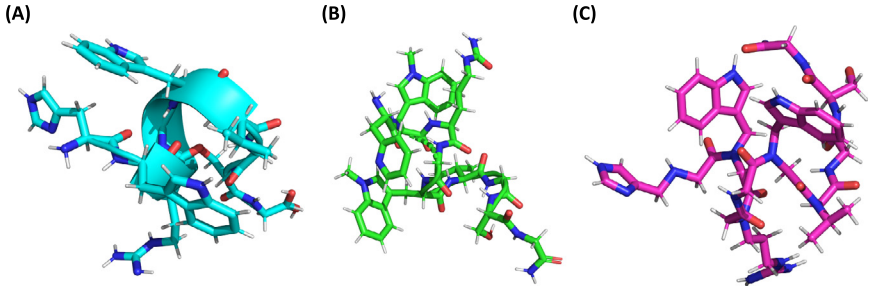

Fig. 10. Structural comparison of IgG-binding ligands **(A)** peptide HWRGWV, **(B)** peptide Ac-HW$_{Met}$CitGW$_{Met}$V, and (C) peptoid PL16.

Several researchers have explored the use of single ncAAs as advanced mixed-mode ligands. In one example, Bresolin *et al.* utilized *ortho*-phospho-L-serine (opSer) immobilized on agarose gel to capture IgG via immobilized metal ion affinity chromatography (IMAC) [388]. Upon optimization of the binding conditions (sodium phosphate at pH 6.5) and target properties (high isoelectric point ranging between 8.15 and 8.45), the opSer-agarose resin demonstrated a binding capacity of 24.2 mg/mL and extracted IgG from human serum with purity of 89% and yield of 58%. The binding capacity of opSer for cationic proteins is higher than that of other singular amino acids owing to the electrostatic interactions facilitated by the combination of phosphate and carboxyl groups. In analogous work, Afonso *et al.* demonstrated the use of *ortho*-phospho-L-tyrosine (opTyr) as a ligand for the purification of micro interfering RNA (miRNA), achieving 52% purity and 71% yield from a marine photosynthetic bacterium *R. sulfidophilum* DSM 1374 cell culture fluid [389]. The work on opTyr was extended in two studies by Pavan *et al.*, who showed that the amino acid can serve as affinity ligand for IgG purification [390,391]. The optimization of the binding conditions to a low conductivity zwitterionic buffer improved the capacity and selectivity of IgG adsorption, resulting in ~91% purity and 48% yield of IgG from human plasma; furthermore, by targeting the Fab region of IgG, P-Tyr enables fractionating a mixture of Fab and Fc fragments, affording Fab recovery and purity of 86.2% and 98%, respectively.

The application of ncAAs to the purification of IgG has been extended to peptide sequences. In one example, our group replaced cationic and aromatic residues in IgG-binding peptides HWRGWV, HFRRHL, and HYFKFD with non-natural analogs to develop protease-resistant variants [135]. A virtual library of non-natural peptide variants was screened *in silico* via molecular docking against the crystal structure of human IgG. Selected variants were conjugated to Toyopearl resin and evaluated via IgG purification upon intermediate exposure to strong proteases. In particular, Ac-HW$_{Met}$CitGW$_{Met}$V (Fig. 10), comprising methylated tryptophan (W$_{Met}$) and citrulline (Cit) in lieu of tryptophan and arginine, showed a strong resistance against trypsin and α-chymotrypsin, and extracted polyclonal IgG from Cohn II+III fraction of human plasma with 94% yield and 92% purity. Notably, the N-terminal acetylation and the ncAAs reduced the positive charge of the peptide, thus increasing its binding selectivity by suppressing non-specific binding of anionic proteins (*e.g.*, albumin). In a later study, Islam *et al.* demonstrated that variant Ac-HW$_{Met}$CitGW$_{Met}$V, when conjugated to WorkBeads resin via a tris(2-aminoethyl)amine (TREN) spacer, provides superior HCP clearance (2.15 log$_{10}$) from a mAb expressing industrial CHO cell culture supernatant [133]. In a similar work, Saavedra *et al.* developed the peptide affinity ligand Ac-FFVRp-opSer-EVFFK to purify phospholipase A$_2$ (PLA2), a toxin with pharmacological applications and found in the venom of the *Crotalus durissus terrificus* snake [392]. The peptide, designed to mimic a phospholipid, folds in a two tailed β-sheet and isolated PLA2 from the venom with 90% purity. In another study, Collinsova *et al.* screened an ensemble of pseudopeptides with general formula Ac-Xaa-DL-Ala-ψ[PO$_2^-$-CH$_2$]-DL-Leu-Xaa′-(βAla)$_2$ immobilized on amino-PEGA resin [393]. The selected ligand Val-Phe-ψ[PO$_2^-$-CH$_2$]-Leu-His-NH$_2$ was found able to purify the rat liver betaine homocysteine S-methyltransferase (BHMT) with 80% yield and > 90% purity.

The integration of ncAAs in peptide ligands for improved affinity and stability was ~~also~~ extended to biological libraries. Screening a phage display library of branched peptides containing β-cyclohexyl-L-alanine (Cha), D-Ser, D-Asp, D-Arg, and β-Ala, Jacobsen *et al.* identify a peptide that isolates intact urokinase-type plasminogen activator receptor (uPAR) to a greater than 95% purity [394]. The receptor serves as a target for cancer treatment and the current purification strategy relies on costly and laborious immunoaffinity chromatography. The selected peptide ligand, denoted as AE152, was coupled to activated Sepharose and the resulting adsorbent featured a uPAR-binding capacity of 0.21 mg/mL, double that of the immunoaffinity resin R2 (0.103 mg/mL); furthermore, while achieving comparable purity levels, AE152 only captured intact uPAR, whereas R2 would target degraded uPAR as well.

Pseudopeptides have also been successfully applied to the purification of viral proteins. In one example, Martin *et al.* developed a CD4-mimetic peptide ligand for purifying the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, which is highly targeted in HIV vaccine development [395]. The ligand Tpa-NLHFCQLRC(K-Biot)SLGLLGRCApTFCACV, wherein Tpa is thiopropionyl and K-Biot is biotinyl-lysine, was immobilized on Streptavidin Sepharose resin and used to purify the glycoprotein gp120(SF162) from a HEK 293 cell culture supernatant to a purity greater than 90% and yield of 56%.

Transforming a peptide to its *inverso* and *retroinverso* forms has also proved effective in improving peptide stability without sacrificing affinity and selectivity. The application of this approach towards ligand development was pioneered by Verdoliva and coworkers, who developed D-PAM as the *inverso* form of the IgG-targeting tetrameric tripeptide (Arg-Thr-Tyr)$_4$-Ly$_2$-Lys-Gly, known as Protein A mimetic (PAM, TG19318) [136]. The authors demonstrated the ability of D-PAM to capture IgG of different animal species (*i.e.*, bovine, horse, human, goat, mouse, rabbit, and sheep) with high binding capacity (36-66 mg/mL) and purify it from plasma sources with purity (>95%) and recovery (60-91%) comparable to those offered by PAM [137,396]. In a later study, Dinon *et al.* further refined D-PAM by adding a phenylacetyl group to form D-PAM-Φ [397]. The new ligand achieved a 10-fold increase in dynamic IgG binding capacity compared to D-PAM and recovered a highly pure (> 90%) IgG product from several plasma sources. It was suggested that the superior affinity of D-PAM-Φ is due to the phenylacetyl group encouraging the display of the peptide's arginine side chain, which results in stronger IgG binding. Analogous studies were conducted by Giudicessi *et al.*, who developed D-variants of the previously discovered rHuEpo-binding peptide Ac-FHHFAHAK [398]. The resin-bound peptides were incu-

bated with trypsin, chymotrypsin, and CHO extract before being cleaved from the solid phase using ammonia vapor and analyzed via mass spectrometry. In particular, the retroinverse D-form of the peptide was shown to possesses a rHuEpo binding affinity comparable to that of its L-cognate, while also featuring a much greater resistance to proteolytic degradation.

Rajčanová *et al.* developed heptameric D/L-peptides VlPYFVl, VlPYYVl, and VlPFYVl as ligands for the affinity purification of porcine pepsin A and rat pepsin C [399]. The adsorbents obtained by conjugating the peptides to Sepharose featured a binding capacity of 35-44 mg of pepsin A per mL resin and negligible binding of pepsin C. In a similar study, Kučerová *et al.* utilized VfPFFVl to separate human pepsin from gastricsin with high yield and purity [400]. Agnew *et al.* employed click chemistry to promote the identification of high-affinity protein-binding peptides via library screening [401]. The peptide lklwfk-(D-Pra), initially selected as a weak ligand for bovine carbonic anhydrase II (bCAII, $K_D \sim 500$ μM), was bookended with two azide-displaying amino acids and conjugated to an anchor ligand via click chemistry. Upon a subsequent round of screening, (D-Pra)-kwlwGl-TzI-kfwlkl was selected, which bound bCBAII with higher affinity ($K_D \sim 3$ μM). An additional conjugation and screening round were conducted to identify a final ligand, rfviln-Tz_2-kwlwGl-Tzl-kfwlkl, which bound bCAII and its human analogue (hCAII) with antibody-like affinity ($K_D = 64$ and 45 nM, respectively).

4.3. Peptoid, azapeptide, and other peptide-mimetic ligands

N-substituted oligoglycines, known as peptoids, and azapeptides are gaining momentum as the next generation of peptide-mimetic compounds [402]. While sharing the same polyamide backbone of peptides, peptoids carry the side chain functional groups on the amide nitrogen, whereas azapeptides feature a nitrogen in lieu of the α-carbon. This endows them with a different space of conformations as well as a higher chemical and biochemical stability compared to their peptide counterparts [403-405]. Peptoids are traditionally synthesized via the sub-monomer approach [406,407], which employs functional primary amines to append the side chain group to the backbone, thus expanding dramatically the chemical diversity of this class of peptide-mimetics [19,406]. A wide variety of bioactive peptoids have been identified from combinatorial libraries, including antimicrobials [408-410], vectors for nucleic acid delivery [411], lung surfactant mimetics [412], analgesics [413], protein inhibitors [414,415], and agents for multidrug resistance reversal [416]. These applications show the potential of peptoids as ligands for biomolecular purification from complex mixtures. A wide number of methods have been developed to facilitate the use of peptoids in research, most of which are centered around the screening of libraries to identify affinity ligands [19,417-419]. This results in a diverse discovery environment, spanning from unbiased and fully combinatorial – as in the work by Wrenn *et al.*, who screened a library of 100 million peptoids to identify ligands which bind to the N-terminal SH3 domain of the proto-oncogene Crk [420] - to fully rational – as in the study by Nguyen *et al.*, who screened a library of only 12 peptide-peptoid hybrids to identify a high affinity ligand for the same protein [421].

Among the early methods of peptoid ligand development is the array screening technology. Alluri *et al.* reported the screening of large arrays of protein-detecting peptoids ($\sim 10^6$ compounds) on chips or encoded beads. The peptoids selected to target the mouse double minute 2 homolog (Nlys-Nbsa-Nlys-Nser-Nbsa-Npip-Nbsa-Npip) and glutathione S-transferase (Nbsa-Nlys-Nbsa-Npip-Nlys) showed good affinity to their targets ($K_D \sim 37$ - 62 μM) [417]. Following on this work, Reddy *et al.* modified the protein-detecting arrays into small-molecule microarrays (SMMs) using peptoids and introduced an *ad hoc* "fingerprinting" approach to identify protein-targeted ligands. In this approach, the majority of the peptoids in the library exhibit only above-background binding, whereas the few that possess high affinity for the target protein provide unique protein fingerprinting. Testing with several model proteins demonstrated that peptoid SMMs can detect proteins robustly and reproducibly in complex mixtures without sample labeling, thus proving applicable to bioassay development [422]. In a parallel study, Heine *et al.* reported the synthesis and screening of peptoid arrays on cellulose membranes for the *de novo* discovery of ligands or drugs, and to study protein:peptoid interactions. The submonomer synthesis was successfully adapted to SPOT technique on cellulose membranes [406,423] by replacing bromoacetic acid with its 2,4-dinitrophenylester. An array library of 8,000 peptoids and peptomers was screened against the anti-transforming growth factor α monoclonal antibody Tab-2, resulting in the identification of affinity ligands ($K_D \sim \mu$M) [418].

A series of studies demonstrated that libraries of hybrid peptoids comprising randomized sequences fused to a pre-ligand with moderate affinity for the target protein are powerful tools for the identification of ligands with high binding affinity. The group led by Kodadek reviewed the peptoid array technology and developed a high-throughput method to isolate synthetic protein-binding ligands with antibody-like affinity [377,419,422,424]. To this end, the authors constructed a large library (~78,000) of chalcone-terminated octameric peptoids displayed on Tentagel resin. The peptoid-chalcone hybridization was meant to provide bivalent interaction with the target protein and lead to the identification of high-affinity ligands. The library was screened using the automated bead-sorting machine COPAS (Union Biometrica, Holliston MA) against fluorescein-labeled Mdm2, and the isolated leads were sequenced by Edman degradation. The selected ligand featured a K_D of 1.3 μM for Mdm2, corresponding to a 170-fold increase in affinity compared to chalcone alone ($K_D \sim 220$ μM) [419]. The Kodadek group further explored this method by constructing a library of peptoids comprising the N-terminal hexameric ligand BKH2, which targets the KIX domain of the CREB-binding protein ($K_D \sim$ 195 µM), fused to downstream (C-terminal) hexameric randomized peptoids. Library screening against the KIX domain returned two ligands, which bound KIX with K_Ds of 4.6 µM and 8.3 µM [425]. In subsequent work, the group further implemented this method to identify ligands that selectively bind phosphorylated proteins [50]. In that study, a peptoid library was N-terminally fused with biotin and 3,4-dihydroxylphenylalanine (DOPA) and screened against the phosphorylated form of the phosphorylation-dependent interaction domain (PDID) of the Brd4 transcriptional coactivator. The N-terminal biotin served as a tag, while the DOPA group assisted in binding of the target via nucleophilic attack. The lead peptoid Biotin-DOPA-Nlys-Nlys-Nmea-Nlys-Ntyr-Nleu targeted the phosphorylated form of the target protein selectively, yet with weak affinity ($K_D \sim$ 50-100 µM).

The combination of modeling tools with experimental characterization of focused ensembles of ligand candidates is at the heart of the rational design of protein-binding peptoids for use in bioseparations. This field has been recently reviewed by Matos *et al.* [42], and it suffices here to report only a few cases studies. Following upon the development of peptide ligands for Factor VIII (FVIII), Knör *et al.* presented a procedure for designing minimal FVIII-targeting peptoids starting from known peptide ligands [196,426]. The authors demonstrated their method by downsizing the lead octapeptide EYHSWEYC into the mimetic (3-IAA)Eψ[CH_2NH]YC, which was used for purifying FVIII from FBS-containing medium with high yield and purity, and demonstrated resistance against serum proteases. The peptoid variant was proven to bind all isoforms of FVIII, demonstrating potential to reduce manufacturing costs compared to current immunoaffinity adsorbents. Following a similar approach, our group recently published

a series of studies on the discovery and characterization of peptoid ligands. We initially presented a rational-combinatorial method for translating protein-binding peptides into peptoids, using the IgG-binding peptide HWRGWV as reference ligand [19]. An ensemble of 60 variants generated by exploring the effect of physicochemical identity of the residues, their display and sequence, and the inclusion of glycine spacers, was screened against IgG, leading to the identification of peptoids PL-02, PL-16, and PL-22, which demonstrated superior binding activity than HWRGWV. Variants PL-16 and PL-22, in particular, featured high affinity ($K_D \sim 0.78$ μM and 0.54 μM) and binding capacity (48 and 57 mg of IgG per mL of adsorbent), and recovered human IgG from a CHO cell culture supernatant added with 1% fetal bovine serum (FBS) with 85% and 98% purity, respectively. The peptoids also demonstrated high resistance to strong alkali and proteolytic enzymes, and the ability to specifically capture monomeric IgG without binding aggregated species. A secondary result of this study was that peptoid PL-01, designed as the formal translation of HWRGWV, possessed a lower affinity ($K_D \sim 17$ μM) and capacity (21.6 mg/mL), indicating that structural optimization is a key aspect of peptide-to-peptoid ligand translation. We utilized PL-16 immobilized on sepharose resin (now LigaTrap® IgG Resin) to purify *(i)* monoclonal IgG_2 (93% purity and 67% yield) and IgG_3 (92% purity and 53% yield) antibodies from a CHO cell culture supernatant added with 1% fetal bovine serum (FBS), as well as IgG from a diluted HEK 293 cell culture supernatant (0.1 mg of IgG per mL of fluid) added with 0.1% fetal bovine serum (FBS) obtaining a remarkable 10-fold product enrichment; *(ii)* polyclonal IgG from serum with 95% purity and 63% yield; and *(iii)* IgM from a CHO cell culture fluid added with 5% FBS with 93% purity and 66% yield [359]. The latter result is particularly remarkable since the commercial adsorbents for IgM purification, HiTrap IgM HP resin and POROS CaptureSelect IgM matrix, afforded lower purity (76% and 92%) and much lower yield (14% and 11%). To further demonstrate the broad targeting range of PL-16 over protein ligands Protein A/G, we utilized PL16-sepharose resin to purify a panel of animal IgGs from the corresponding sera: mouse (yield of 47% and purity of 94%), rabbit (66.5% and 91.7%), goat (63% and 91-95%), donkey, llama (93% and 97%), and chicken IgY (42% and 92%).

As noted with peptides, cyclization of peptoids increases binding affinity. Gao *et al.* were first to complete a comparison of linear *vs.* macrocyclic peptoid libraries for discovering affinity ligands, and developed a method that could be extended to other types of oligomers [427]. The authors discovered that a library of macrocyles comprising 17-atom rings returned a higher number of stronger leads compared to three libraries of linear compounds. However, as the ring size increased to 20 and 23 atoms the difference between cyclic and linear libraries narrows, and it was concluded that steric constraints of the 17-membered cyclic peptoids is conducive to higher affinity compared to linear or flexible cyclic peptoids.

A number of studies by the Liang group recently reviewed by Knight *et al.* have also demonstrated that peptoid ligands possess chirality recognition power to fractionate mixtures of enantiomers [428]. Specifically, oligopeptoids constructed with S-N-(1-phenylethyl) (S-Nspe) glycine immobilized on silica resins for analytical chromatography were utilized to fractionate a mixture of 1,1′-bi-2-naph-thol (BINOL) derivatives. The enantioselectivity of the peptide ligands increased with the number of S-Nspe, reaching its maximum at 6 monomers. Short peptoids built with R-Nspe and capped with alkyl chains, or cyclic chiral and aromatic monomers (*e.g.*, N′-phenyl-L-proline and N′-phenyl-L-leucine) were also evaluated, demonstrating that N-fusions of achiral monomers improved the fractionation ability of R-Nspe peptoid trimers.

Introduced as affinity ligands by Barker *et al.* who employed them for the purification of chymotrypsin and trypsin [429], α-aza-peptides have demonstrated promising biorecognition activity. Most research on azapeptides, however, has focused on drug development, in particular inhibitors of protein targets like HIV protease [430], human neutrophil proteinase 3 [431], and the cluster of differentiation 36 class B scavenger receptor [432], and their use as affinity ligands is still in its infancy. This body of literature, however, demonstrates that azapeptides hold promise as synthetic ligands for affinity chromatography [405,433].

A number of other peptide-mimetics that have demonstrated potential as affinity ligands, though not in an affinity chromatography context, include ones for binding human chromodomain-containing proteins CBX7 and CBX8 [434], anti-AQP4 antibodies [424], the KIX domain of the CREB-binding protein [435], chemokine interleukin-8 [436], c-Jun N-terminal kinase 3 (JNK3) [437], polycomb repressive complex 2 (PRC2) [438], the SH2 (Src Homology 2) domain of oncoproteins and signal-transducing proteins [439], the SH3 domain of Src-family PTKs [421], and the evolutionary-conserved EVH1 protein domain [440]. Lund *et al.* developed the peptide-mimetics DAAG and D_2AAG as ligands for IgG purification from cell culture fluids. The ligands were immobilized on sepharose resin and featured a binding capacity of 24 and 48 mg/mL, respectively, and afforded product purities over 93% and recovery over 85% [441].

4.4. The latest frontier: stimuli-responsive peptides

The latest frontier in protein purification is represented by the use of remote physical stimuli, in lieu of pH and ionic strength, to control binding and release of target biologics to and from the chromatographic substrate [442]. This technology is ideal for biochemically labile products, such as physical dimer proteins and cells, whose growing demand in research and clinical settings makes their isolation from complex sources an emerging issue in bioseparation research. Outside a narrow range of solution conditions (pH and composition), in fact, labile biologics undergo rapid denaturation and loss of bioactivity. The integration of stimuli-responsive molecular switches into protein-binding peptides enables the design of ligands that capture the target protein via native affinity and release it when exposed to an external stimulus (*e.g.*, light) of appropriate intensity. Stimuli-responsive switches, in fact, feature a reversible and controlled isomerization. Hence, exposure to stimuli triggers a reversible rearrangement of the ligand structure, thus acting as ON/OFF switch of protein binding/elution (Fig. 11). This enables performing the entire chromatographic process on a single optimal aqueous solvent, thus combining high product bioactivity with process intensification. A number of stimuli-responsive switches and peptide geometries have been proposed mostly revolving around the use of near UV light excitation. Linkers that have been used include azobenzenes, diarylethenes, acylhydrazones, hemithioindigos, stilbenes, and rhodopsin-like molecules, as reviewed below.

4.4.1. Azobenzene-peptide hybrids

A prominent role among stimuli-responsive linkers for peptide modification is held by azobenzenes [22,443]. Native azobenzene undergoes a trans-to-cis isomerization upon irradiation with 320 nm light, and a reverse cis-to-trans isomerization when exposed to 450 nm light. The values of wavelength can be either red- or blue-shifted respectively by adding electron-donating or withdrawing groups in *ortho* position to the azo group [443]. The integration of azobenzene in peptides and proteins to endow the host with light-controlled conformation has been studied extensively and optimized using both *in silico* and experimental approaches [444-452]. Lesser effort, however has been dedicated to the engineering of azobenzene-peptide hybrids with light-controlled biorecognition activity [450,453-456]. Typically, two are the approaches towards

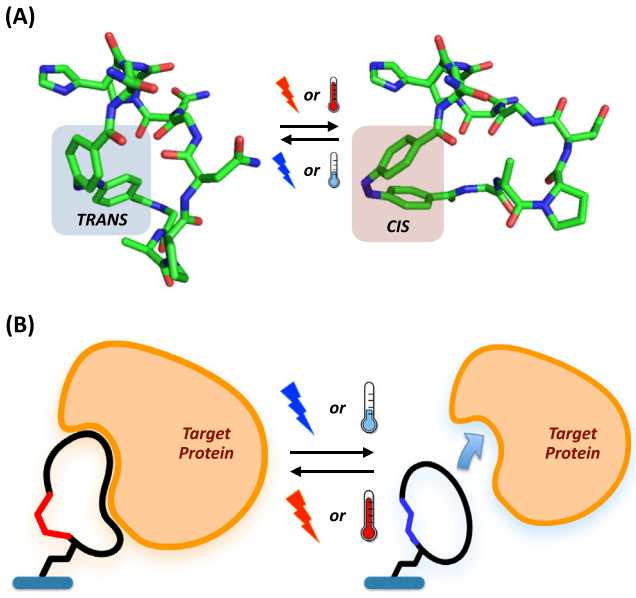

Fig. 11. Mechanism and function of stimuli-controlled peptide ligands: (A) stimuli-induced conformational switch and resulting (B) stimuli-controlled adsorption and release of a biomolecular target.

integrating native and modified azobenzenes into peptide and proteins, namely *(i)* hybridization with the combinatorial sequences of the libraries utilized for *de novo* discovery of photo-switchable ligands or *(ii)* conjugation to scaffolds and ligands with known structure. Myrhammar *et al.*, for example, incorporated an azobenzene linker into affibodies and utilized them to build an adsorbent enabling light-controlled binding and elution of IgG [457]. The authors reported that, while elution was triggered upon exposure of the column to UV light, a substantial amount of protein remained adsorbed and was later eluted upon acidic regeneration of the resin. Other groups hybridized azobenzenes with peptides at the stage of library screening. Jafari *et al.* constructed a phage display library of cysteine-bookened peptides cyclized using a bis-thiol-reactive azobenzene linker, and screened it against streptavidin. The selected ligand ACGFERERTCG featured a 4.5-fold difference in binding strength (K_D) between its cis and trans isomers [458]. In a follow-up study, Bellotto *et al.* created a combinatorial phage display library of cysteine-bookended heptapeptides cyclized with 3,3'-bis(sulfanato)-4,4'-bis(bromoacetamido) azobenzene and screened it against streptavidin; prior to screening, the library was exposed to UV light to isomerize the ligands to the cis configuration and bias the selection towards cis-binders and trans-eluters. The selected peptides exhibited good binding selectivity, and a 3-fold shift in binding strength (K_D) upon light exposure [450]. Liu *et al.* extended the selection of azobenzene-hybrid peptides to ribosome display using an azobenzene-modified lysine that was inserted into each peptide of the library [459]. The peptides were collectively converted into their *trans* isomers and screened against streptavidin microbeads. Following library adsorption, the beads were irradiated with UV light to induce a trans-to-cis photo-switching and induce loss of streptavidin binding. The selected peptides LA37(QAVLIMVAVXASSGQLGQFEGSDYKDDDDK), LA40(QAHSCXVTIDVFFGQLGQFEGSDYKDDDDK), and LA81 (QAGVTXRRFIXYVGQLGQFEGSDYKDDDDK) were evaluated on solid phase and demonstrated increasing degrees of binding affinity with LA81 exhibiting the greatest binding indicating a dissociation constant of 6.31 μM. The conformational structure and binding of the LA81 peptides were shown to be reversibly photo-controlled upon UV/Vis excitation. [459].

The incorporation of azobenzene into known sequences has also been explored. For example, Vaselli *et al.* conjugated the sequence of GRGDS to a glass substrate using an azobenzene linker via "click chemistry" [460], and characterized both the surface distribution of GRGDS-Azo peptides via atomic force microscopy (AFM) and Raman spectroscopy, and the isomerization of the azobenzene-peptide via contact angle and through UV-Vis spectroscopy. The authors showed spatial control of binding and elution of human umbilical vein endothelial cells using light patterns demonstrating applicability to cell purification. In this context, Parisot *et al.* [452] developed hybrids of the FLAG-tag peptide DYKDDDDK with an azobenzene-displaying amino acid and evaluated their light-controlled binding of FLAG antibodies; the location of the photo-responsive amino acid was systematically varied to attain light-controlled biorecognition.

Our group recently developed a hybrid computational-combinatorial approach based on a novel azobenzene-cyclized peptide structure where the length of the protein-binding peptide segment and the cyclization geometry are optimized to achieve a balance between chain flexibility and rigidity, which ultimately affords efficient photo-isomerization and a strong shift in binding affinity. To demonstrate our design, we developed photo-switchable peptides targeting vascular cell adhesion marker 1 (VCAM1) starting from a known VCAM1-binding linear peptide. An ensemble of azobenzene-cyclized variants was initially designed and screened *in silico*, and the selected variants evaluated via surface plasmon resonance (SPR). In particular, $cyclo_{AZOB}$[G-VHAKQHRN-K] featured efficient light-controlled binding of VCAM1 with $K_{D,Trans}/K_{D,Cis} \sim 130$. Finally, the biotin-$cyclo_{AZOB}$[G-VHAKQHRN-K] was used to fluorescently label brain microvascular endothelial cells (BMECs), showing co-localization with anti-VCAM1 antibodies in *cis* configuration and negligible binding in *trans* configuration (Fig. 12).

4.4.2. Hemithioindigo-peptide hybrids

Like azobenzene, hemithioindigos have been widely investigated for their photo-switching activity and regulation of peptide structure and function [461-465]. These photochromic switches comprise two structural groups, a hemistilbene and a hemithioindigo, and undergo reversible isomerization from a stable ground-state E isomer to a photo-stationary state with high conversion to a metastable Z isomer in response to visible wavelengths of light (> 400 nm) [466]. The photochromic behavior of *hemithioindigo-peptide hybrids* and its impact on bioactivity has been recently reviewed by Kitzig *et al.* [463]. Batjargal *et al.* evaluated the impact of photo-isomerization of hemithioindigos in the context of protein structure and function, including *(i)* the use of hemithioindigos as ligands to induce/inhibit protein activity, *(ii)* the incorporation of hemithioindigos into amino acid side-chains to alter protein structure and activity, and *(iii)* the incorporation of hemithioindigos as linkers in the protein primary structure and bioactivity [467]. Füllbeck *et al.* incorporated different meta- and para-substituted hemithioindigo photo-switches into linear and cyclic peptides whose structures are relevant in cell signaling [462]. The authors evaluated the photo-switching kinetics of the hemithioindigo-peptide hybrids via UV-Vis and IR spectroscopy, and found that the hemithioindigo reverts to the ground-state more slowly when incorporated into peptides and can therefore induce long-term changes in the secondary structure of peptide hosts.

4.4.3. Diarylethenes-peptide hybrids

Diarylethenes are a class of photochromic molecules that undergo reversible cyclization reactions between a ring-open and a ring-closed form in response to visible and UV light, respectively. The incorporation of diarylethenes into linear and cyclic

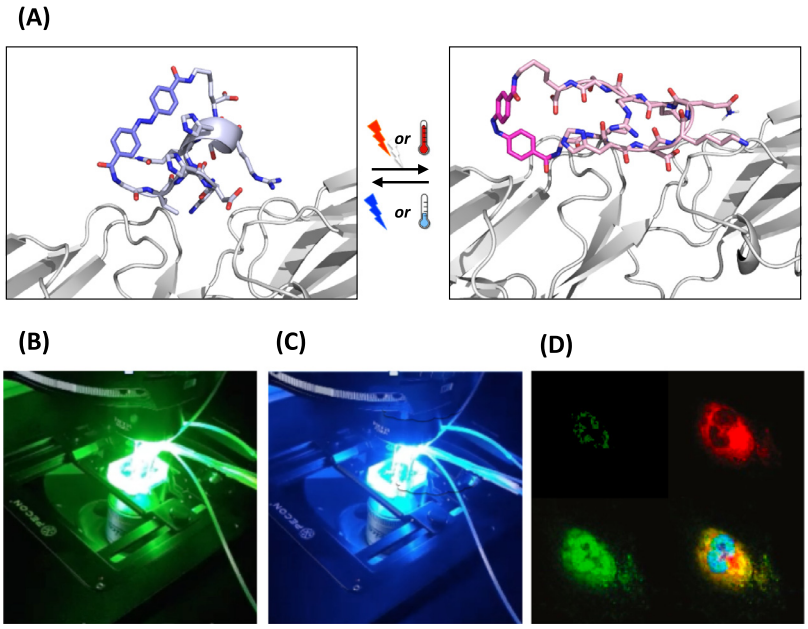

Fig. 12. Structure of the VCAM1:peptide complex formed by docking azobenzene-cyclic peptide cyclo$_{AZOB}$[G-VHAKQHRN-K*] in **(A)** trans and cis configurations on the crystal structure of VCAM1 (PDB ID: 1VCA); selection of protein-binding peptides using **(B)** green light for protein binding and **(C)** blue light for protein elution; **(D)** imaging of brain microvascular endothelial cells (BMECs) with rhodamine-labeled anti-VCAM1 antibody, fluorescein-labeled cyclo$_{AZOB}$[G-VHAKQHRN-K*], and overlay.

peptides as well as protein structures has deepened the understanding of the interconnected nature of photo-switching and peptide behavior. By analyzing the kinetics of diarylethene photoisomerization in different cyclic peptidomimetics, Schweigh *et al.* found that specific cyclic peptide frameworks greatly impact photo-switching ability [468]. Several studies have been conducted to demonstrate the value of diarylethene-linked peptides as photoswitchable ligands. Fujimoto *et al.*, for example, incorporated diarylethenes into several linear DNA-binding peptides with uniform α-helical structure, and showed that the photoisomerization of diarylethene drastically altered the secondary structure of the peptides and consequently their DNA binding affinity [469]. In another study, diarylethene residues were incorporated in cyclic peptide mimetics of gramicidin S, endowing them with light-controlled secondary structure as well as antimicrobial activity and hemolytic activity [470]. The incorporation of diarylethenes into cyclic peptides forming β-hairpin showed similar results on their secondary structure and biological activity (*i.e.*, cytotoxicity) towards eukaryotic cells [471]; while not applied to purification, the effectiveness of diarylethenes in regulating peptide structure and bioactivity has been demonstrated.

4.4.4. Stilbene-peptide hybrids

Stilbenes are a class of molecules with a similar structure to azobenzenes [472,473]. Waldeck *et al.* characterized the photoswitching performance of stilbene derivatives when incorporated into linear trimer peptidomimetics to evaluate their use in lieu of azobenzene in photo-switching applications [474]. The stilbene derivatives isomerize in response to 280 nm and 300 nm light, with half lives of several hours and negligible thermal isomerization. Erdélyi *et al.* incorporated stilbene into two sets of peptide sequences of differing lengths to achieve light-controlled inhibition of the R1 and R2 subunits of the *M. tuberculosis* ribonucleotide reductase [472]. The linear peptides were either modeled after the R1 subunit binding region or comprised only of select residues expected to endow binding activity. Photoisomerization resulted in differential binding of the inhibitory peptide sequence to the R2 subunit, with the *E* isomers demonstrating a stronger binding than the *Z* isomers. The opposite trend in light-controlled binding was observed with shorter peptide sequences, although the influence of photo-isomerization on bioactivity remained. In later work, Karlsson *et al.* inserted a stilbene derivative into a peptide sequence that acted as an artificial hydrolase [475]. The enzymatic activity relied on both secondary helical motifs as well as tertiary aggregation of dimers. By inserting the stilbene into the backbone to replace the turn in a helix-turn-helix motif, the tertiary structure of the enzyme was made light-modulated, resulting in controllable enzyme activity. Specifically, a 42% increase in the observed rate constant of the catalytic reaction was observed upon photoisomerization [473].

5. Conclusions

The growing demand for affordable biopharmaceutics led by the ageing world population and the increasing exposure to risk factors (*e.g.*, air and water pollution) poses an urgent need to rethink the manufacturing of biological therapeutics. Innovating process design and tools employed in the downstream segment will be critical to grant access to large amounts of highly pure and bioactive biotherapeutics, while containing cost to patients. A key role in particular will be played by synthetic ligands that are selective, robust, safe, and easily and reproducibly manufactured. Peptides are at the front line of this initiative, owing to the large variety of formats, physicochemical properties, and modes of operation, which makes them suitable as affinity ligands for the purification of a wide range of biotherapeutics, from proteins, to viral vectors, and cells. It comes as no surprise that peptide-based affinity tools are now on the market and gathering significant attention from multiple end users, from research labs to biopharmaceutical companies. Recent innovations in peptide chemistry, such as the introduction of non-natural amino acids and the hybridization of peptides with stimuli-responsive moieties, are taking peptide ligands a step further, enabling efficient purification of biological targets that traditional chromatographic approaches were inadequate to target. In parallel, powerful computational tools are being introduced to aid the design of peptide and pseudopeptide ligands with optimal target affinity and selectivity, and provide chemists and engineers with a molecular-level understanding of biorecognition phenomena on solid phase. With these resources at hand, peptides

are indeed the leading candidates for engineering the next generations of ligands for diagnostic bioseparation technologies.

Declaration of Competing Interest

No personal or financial interests exist.

CRediT authorship contribution statement

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