

The hidden potential of small synthetic molecules and peptides as affinity ligands for bioseparations

While extensively used as drugs, small synthetic molecules have not yet been widely applied in the industry as affinity ligands for the purification of biopharmaceuticals. Yet, a substantial amount of published research indicates that synthetic ligands, such as triazine scaffolds, amino acids, and peptides show a great deal of promise for becoming the next generation affinity ligands for bioseparations. In this review, we present a comprehensive account on small synthetic ligands, from triazine dyes to the most recent polycyclic peptide ligands, selected for targeting high-value biopharmaceuticals, such as immunoglobulins, blood factors and therapeutic enzymes. These ligands could play a significant role in improving downstream processing and helping the bioprocessing industry overcome the urgent issues of costs and availability posed by growing economies on the global pharmaceutical market.

The impact of biopharmaceuticals on human therapy has increased exponentially in the last three decades and a variety of products are now available for the treatment of severe diseases [1–5]. While the demand for biotherapeutics is growing rapidly, owing to higher life expectancy in advanced countries and the access of developing countries to the global drug market [6–11], their high costs still preclude the availability of these drugs for specific therapies to large segments of the world's population [12–14]. On the other hand, with the expiration of many patents in the near future and the introduction of biosimilars to the market, biomanufacturers will be exposed to a more globally competitive climate that is perhaps more receptive to technological innovation [15–18]. Meeting the large demand for products at lower prices while complying with increasingly stringent regulatory environments [19], requires a significant rethinking of manufacturing strategies, in particular the introduction of new technologies and the design of sustainable platform approaches to purification [20–22]. The most critical area

in the biomanufacturing scenario is downstream processing. In order to guarantee the high purity (>99.9%) standards required for human therapeutics [23,24], current processes comprise multiple purification steps, which can result in low yields and often account for a significant fraction of the overall manufacturing costs. To overcome the limitations of existing processes, it is essential to develop a cost-effective, robust, and compact platform technology that can easily respond to variations in product titer, impurity profile, and feed volume [22,25,26]. Among the available technologies, [affinity chromatography](#) is the most specific and effective technique for protein purification [27–29]. Affinity chromatography exploits the principle of biomolecular recognition, that is, the ability of biologically active molecules to form specific and reversible complexes with affinity ligands, and has been proposed as a capture step for high value therapeutics, such as blood factors, enzymes and antibodies [30–34]. Polyclonal and monoclonal antibodies (mAbs) in particular, with their high potential for immunotherapy and

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Key Terms**Affinity chromatography:**

Adsorption chromatographic technique based on biomolecular recognition between a ligand and a biological product, used as a capture step for high value biotherapeutics, such as blood factors, enzymes and antibodies.

Synthetic affinity ligand: Small molecule (MW ~0.1–2 kDa) that forms a noncovalent complex with a target biomolecule and that can be produced by chemical synthesis.

Triazine scaffold: Compound comprising a triazinyl based scaffold substituted with polyaromatic ring systems and electron exchanging groups that mimic the binding of natural substrates.

drug delivery, are currently the most heavily consumed protein therapeutics [35–38]. The industrial affinity purification of whole antibodies and Fab fragments is based on the use of biological ligands, such as Protein A, Protein G and Protein L [39–42]. These ligands are highly specific and result in >90% antibody purity in a single capture step. However, they suffer from several drawbacks, such as low chemical stability, weak or no binding to certain classes of mAbs, immunogenicity, with the risk associated with the leaching of ligand fragments in the product mainstream, and harsh elution conditions made necessary by the high binding affinity, which can cause product aggregation and compromise the biological activity of the eluted protein such as catalytic antibodies [43–45]. To address these issues, over the last two decades engineered versions of Protein A have been presented, which offer higher stability and binding capacity (40–60 mg/ml) [39,46]. These newer protein-based adsorbents, however, are very expensive (15,000–20,000 US\$/liter) and the immunogenicity of leachates is still a concern. To overcome these drawbacks, academia and industry have proposed **synthetic affinity ligands** as chemically robust, efficient, non-immunogenic alternatives to protein ligands [27,47]. The application of these compounds for affinity purification of biotherapeutics can help address the needs of developing countries for low-cost disposable process alternatives for new facilities producing follow-on biologics, as well as new protein capture steps for whole new classes of drugs that are not antibody based and for which there are no available protein ligands. Among the various classes of compounds introduced in the last three decades, the most promising are **triazine scaffolds** and peptides [28,48–50]. Owing to their synthetic nature and small size, these molecules have excellent chemical stability and can be produced under cGMP conditions at a lower cost. However, unlike protein ligands, synthetic ligands require correct orientation on the resin and high ligand density for large binding capacity, and each new ligand must be characterized in terms of toxicity and immunogenicity properties. However, the results summarized in this paper indicate that small synthetic affinity ligands can be highly competitive in terms of yield and purity for industrial applications and can offer other advantages such as stability, mild elution conditions, and potentially lower immunogenicity concerns.

Triazine ligands

This class of ligands comprises triazinyl scaffolds substituted with polyaromatic ring systems and electron exchanging groups that mimic the binding of natural substrates. Lowe and coworkers pioneered the discovery and application of these compounds for the affinity purification of proteins, and have discovered a number of specific ligands, particularly for immunoglobulins and enzymes [51–58]. The bifunctional ligand 22/8, for example, functionalized with anilino and tyramino substitutions (**Figure 1A**), mimics the IgG-binding dipeptide motif Phe132-Tyr133 of Protein A [57,59]. The ligand shows good affinity ($K_D = 9.6 \cdot 10^{-6}$ M) and broad specificity, as it can capture antibodies of different classes, such as IgA and IgM, and animal species, such as chicken, cow, rabbit, pig, horse, rat, goat, sheep and mouse. The agarose-based adsorbent shows binding capacity above 50 g/l and withstands the harsh conditions required for resin cleaning and sanitization. The ligand can be used to recover IgG from plasma with high purity (>90%), but relatively low yield (67–69%), and requires drastic elution conditions (0.1 M glycine-HCl at pH 2.9) that can cause product aggregation and denaturation. Based on ligand 22/8, two triazinic compounds have been designed and commercialized by ProMetic Biosciences Inc. under the name of MabSorbent A1P and A2P. The latter has an antibody binding capacity ranging from 30 to 40 g/l, but is affected by antifoam agents and its use is limited to polyclonal serum or cell cultures without Pluronic F68 [28]. A second triazinic compound, called ligand 8/7, functionalized with 4-aminobenzamide and aminobutanoic substitutions (**Figure 1B**), was selected for the purification of Fab, F(ab')₂ and scFv fragments [60,61]. Ligand 8/7 binds both the κ and λ light chain of IgG with good affinity ($K_D = 2.6 \cdot 10^{-6}$ M). The agarose-based adsorbent, however, shows low binding capacity (~2 g/l). Recently, the same group has developed a new synthetic, although non-triazinic, ligand that mimics Protein G for the purification of mammalian immunoglobulins, including camelid IgGs that only contain a heavy chain. The ligand, called as A2C 11/1, binds IgG with a K_D of $4.78 \cdot 10^{-6}$ M, and as indicated by *in silico* docking, targets the residues Asn35 and Trp43 of Protein G [62]. The adsorbent A2C-Sepharose binds antibodies from sera of different species, such as human, cow, goat, mouse, sheep, pig, rabbit, and rat, with a static capacity of about 25 g/l and returns a product purity of approximately 65%.

Another triazine-based ligand, ligand 8–6, was identified for the affinity purification of IgY. The ligand, immobilized on Sepharose, was used to recover IgY from chicken, duck, and pigeon yolk, with resulting capacity of 74.8 g/l, and recovery and purity up to

75 and 90%, respectively [63]. Other similar synthetic affinity ligands have been developed for the purification of antibody therapeutics from plant extracts, such as Phe-Trz-Asp LAK-mimetic and 4E10lig ligands, identified for the purification of anti-HIV mAB 2F5 and 4.E10 from corn and tobacco extract, respectively [64,65].

Amino acids & peptides

The second major class of synthetic affinity ligands comprises amino acids and peptides. Single amino acids, such as tryptophan, phenylalanine and histidine have been promoted as 'mixed mode' affinity ligands [28,34,66]. These ligands bind the target protein through a combination of hydrophobic, π -stacking, hydrogen bonding and electrostatic interactions. Naik *et al.* have proposed the use of tryptophan immobilized on polymethacrylate matrix Sepabeads for the purification of polyclonal IgG from serum and monoclonal IgG from a cell culture supernatant, with purity and recovery of 90 and 85%, respectively [67]. Vijayalakshmi *et al.* have proposed the use of immobilized histidine for the purification of catalytic antibodies from the serum of lupus patients [68]. The histidyl-aminoethyl-Sepharose gel allowed a 60% recovery of the antibodies under gentle elution conditions and at high purity.

Single amino acids used as synthetic affinity ligands operate by a mechanism of mixed-mode binding, that is, a combination of hydrophobic and electrostatic interactions. Similar fully synthetic ligands have been proposed (Capto MMC, Nuvia cPrime, MEP Hypercel) that show good binding capacity. These ligands, while inexpensive and highly chemically robust, do not exhibit the necessary specificity for high purity product in the capture step as obtained using Protein A. For this reason, they are most frequently used in industry as an intermediate purification step for immunoglobulins after the Protein A capture step. It has been noted that the lack of specificity of these ligands could be overcome by screening libraries of synthetic compounds and by including a negative selection step to ensure that the ligands avoid interactions with proteins other than the target [69]. However, it is unlikely that high specificity can be achieved with such simple chemical functionalities even with additional screening steps.

Small peptides have been shown to overcome some of these specificity issues. Thanks to their rich functionality and wide diversity, peptides have become a class of molecules of great interest in biotechnology and medicine. These compounds, which can be considered as segments of proteins, are natural candidates for mimicking the fine mechanisms of biomolecular recognition that result in specific binding to target biomolecules. Owing to their high chemical and structural versatility,

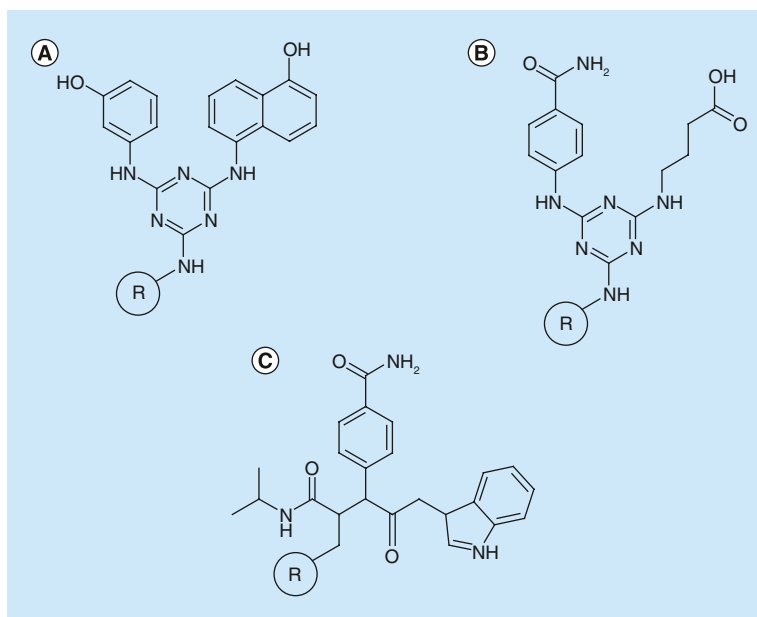


Figure 1. Triazinyl-based affinity ligands for the purification of immunoglobulins. (A) Ligand 22/8; (B) ligand 8/7; (C) ligand A2C 11/1. R: Sepharose resin.

as well as good chemical stability, peptides are attractive leads for the discovery of drugs, molecular probes, and affinity ligands for the purification of high-value biomolecules [24,28,33,70–75]. The value of the molecular recognition power of peptides is demonstrated by the vast literature published in the field of drug discovery and by the fact that more than 100 peptide-based drugs have already been put on the market. As drugs, peptides occupy a window of molecular weights (500–5000 Da) between small molecules and protein biologics, showing the size and manufacturing advantages of the former and some of the specificity and potency of the latter. Since the basic mechanisms of biomolecular recognition are the same, the technology employed for developing peptide drugs, such as the rational and combinatorial approaches, can be employed for the identification of peptide affinity ligands [76–78]. The rational approach aims to tailor a peptide sequence that fits into the pre-selected binding pocket of a protein target using data of the crystal structures available on databases and computational tools that simulate protein–peptide interactions *in silico* [32,49]. The combinatorial approach is based on the design of peptide libraries and screening processes to identify ligands that bind to a given target protein. Several library formats exist, either biological, such as phage-, yeast-, ribosome-, and mRNA-display libraries, or synthetic, in both solid and liquid phase [79–84]. The use of solid-phase combinatorial peptide libraries was introduced by Lam and co-workers [85–89]. The screening of these libraries, reported numerous times in the literature, comprises blocking steps before contacting

Table 1. Peptide ligands for the affinity purification of proteins.

Sequence	Target	Ref.
WQEHYN, WQETYQ and YENYGY	Fibrinogen	[90]
VIWLVR	α -1-proteinase inhibitor	[92]
WHWRKR	α -lactalbumin	[93,94]
FLLVPL	Fibrinogen	[91]
YYWLHH	Staphylococcal enterotoxin B	[95–97]
WRW	Porcine ParvoVirus	[98–100]
GVNFEVVG / GVNFTVVG, and GVFFELVG / GVFFEIVG	S-protein	[101]

with the target protein and subsequent steps of negative screening to ensure high target selectivity of the identified leads [90,91]. Our laboratory has extensively used these tools to discover peptide ligands for the affinity purification of a variety of target proteins from complex mixtures. **Table 1** presents a list of peptide ligands identified in our laboratory using solid-phase libraries built on chromatographic resins for affinity separations. While showing dissociation constants in the range of 10^{-5} – 10^{-6} M, these ligands have been found very effective for protein purification or capture of pathogens from complex mixtures [90–101].

The search for peptide ligands has focused on three main targets, that is, immunoglobulins, blood factors, and therapeutic enzymes. mAbs are regarded as the most effective protein therapeutics for treating cancer and autoimmune diseases. More than 20 Mab-based drugs have received US FDA approval and are now on the market [102–104]. These products generated an annual revenue of approximately \$50 billion in 2012,

and throughout 2015 the estimated compound annualized growth rate is approximately 9.2% [105]. Currently, industry favors Protein A chromatography for the capture step in the antibody purification process [42]. This protein ligand, however, suffers from problems of high cost and low chemical stability, in particular towards the harsh alkaline conditions used for periodical cleaning and sanitization, and the associated risk of release of immunogenic fragments in the product mainstream [69]. Other technical concerns are related to the high binding strength and narrow binding specificity of Protein A. Due to the low dissociation of the Protein A–antibody complex ($K_D = 10^{-8}$ – 10^{-9} M), elution needs to be performed at very low pH (~ 2.5), with the risk of product denaturation and irreversible aggregation [106,107]. Furthermore, Protein A does not bind the human IgG₃ subclass, shows weak binding to mouse IgG₁ and bovine IgG₁, and does not bind goat and chicken antibodies [108]. Much research has been carried out to discover Protein A mimetic peptides that bind the constant portion (Fc) of antibodies with milder affinity and broader specificity, to enable the development of industrial processes of affinity purification of polyclonal antibodies from plasma fractions and more cost-effective purification of mAbs from recombinant sources. Several peptide sequences have been identified for affinity purification of antibodies from complex media and are reported in **Table 2** [39,52,109–137].

Among these antibody-binding peptides, three Protein A–mimetic sequences show particularly interesting characteristics. The first is the tripeptide tetramer (Arg-Thr-Tyr)₄-Lys₂-Lys-Gly (**Figure 2A**), identified

Table 2. Immunoglobulin-binding peptide ligands.

Sequence	Target	K_D (M)	Ref.
APAR	GM-CSF monoclonal antibody	N.P.	[109]
PDTRPAP	Carcinoma-associated anti-MUC1 mucin antibody C595, diabody fragment C595	$8.6 \cdot 10^{-7}$	[52,110–112]
EPIHRSTLTALL	Anti-Tac IgG1 antibody (HAT)	N.P.	[113,114]
(Arg-Thr-Tyr) ₄ -Lys ₂ -Lys-Gly (TG19318 or PAM)	IgG-Fc of different animal species, and IgM, IgA, IgE, and IgY	$3.0 \cdot 10^{-7}$ (for IgG)	[115–121]
(DArg-DThr-DTyr) ₄ -DLys ₂ -DLys-DGly (D-PAM)		N.P.	[122,123]
AEGEFINVPMMVDGITMGDPAK (M _[46–2])	Murine anti-human tumor-associated tenascin-C (Tn-C) mAb	$1.7 \cdot 10^{-9}$	[124]
PMMVDGITMG	Murine anti-human tumor-associated tenascin-C (Tn-C) Fab	N.P.	[124]
D ₂ AAG	IgG-Fc	$4.7 \cdot 10^{-6}$	[39,125]
HWRGWV, HYFKFD, and HFRRHL	IgG-Fc of different animal species, and IgM, IgA, and IgY	10^{-6} – 10^{-7}	[126–134]
DPQYRALMGENQDLRKREGQYQDKIEELE	IgA	$2 \cdot 10^{-8}$	[135]
50-mer	IgA	N.P.	[136]
CCHQRLSQRK	IgM	N.P.	[137]

N.P.: Not provided.

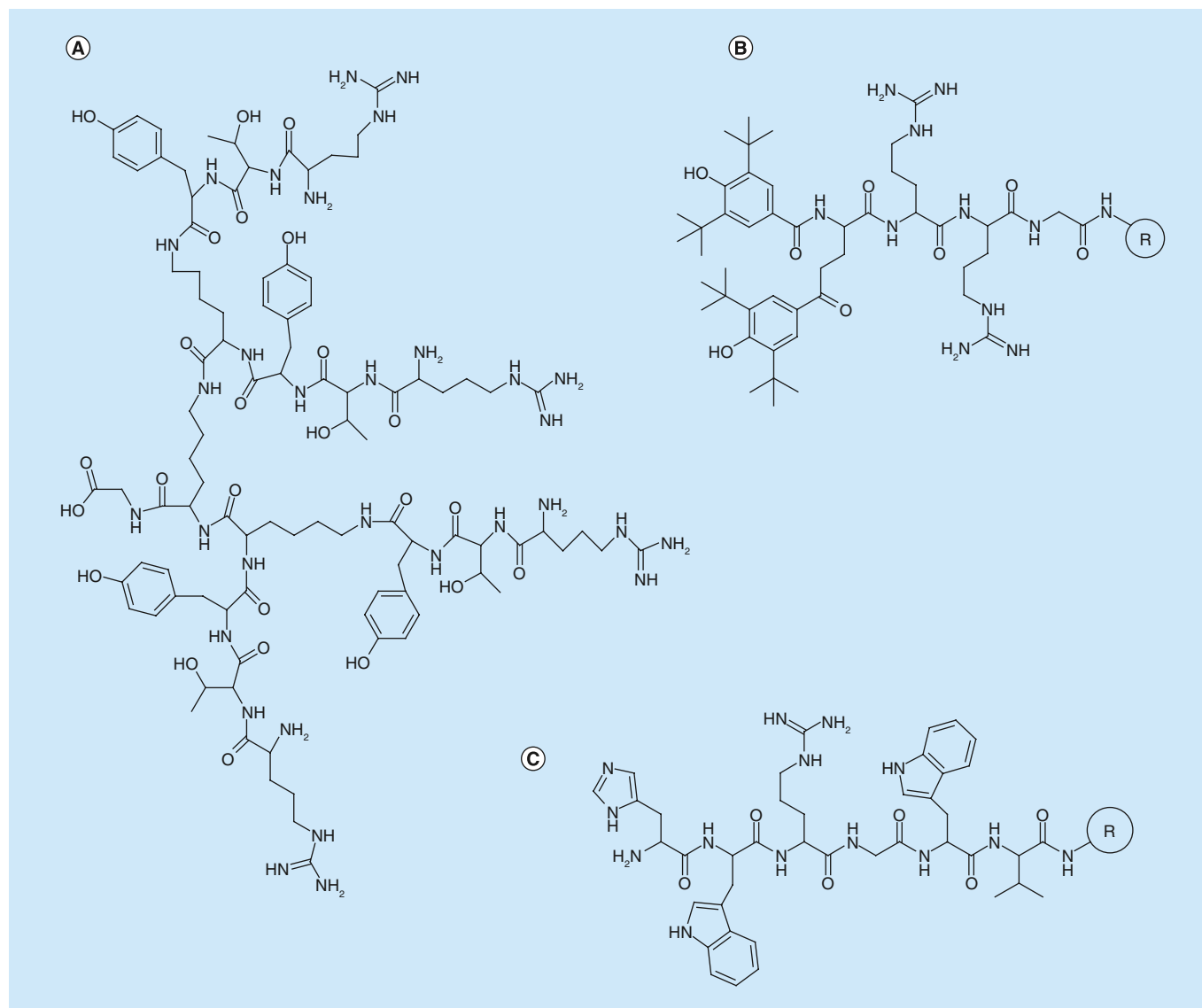


Figure 2. Protein A mimetic peptide ligands for the purification of immunoglobulins. (A) TG19318; (B) ligand D2AAG; (C) HWRGWV. R: Resin.

by Fassina and co-workers and known as TG19318 or PAM (Protein A mimetic), which binds the Fc portion of IgG [115,116,121]. PAM has been reported to show a wide range of specificity in terms of animal source (human, cow, horse, pig, mouse, rat, rabbit, goat, sheep and chicken) and antibody classes (IgG, IgY, IgM, IgA and IgE), with resulting protein purity in the range of 90–95% [117–120]. The ligand is also highly stable to harsh sanitization conditions, such as long incubation times with 1 M NaOH. By replacing all amino acids with D stereoisomers, a protease-resistant version of the peptide (called D-PAM) has been developed [122,123].

Recently, Lund *et al.* have identified a peptide ligand, D₂AAG (Figure 2B), which specifically binds the Fc fragment of IgG with $K_D = 10^{-5}$ M and is stable to contact with 0.1 M NaOH [125]. The adsorbent prepared

by coupling this peptide on agarose-based WorkBeads resin (Bio-Works) shows a dynamic binding capacity of up to 48 g/l and purifies IgG from harvest cell culture fluid with purity and recovery above 90%. The adsorbent was also shown to separate aggregated IgG from non-aggregated IgG, indicating that the ligand could be used both as a primary capture step for IgG purification as well as a subsequent polishing step.

Finally, our group has developed three hexapeptide sequences, HWRGWV (Figure 2C), HYFKFD, and HFRRHL that bind the Fc region of human IgG, with a K_D in the range of 10^{-5} – 10^{-6} M [132–134]. These three ligands identified by combinatorial screening showed a common motif comprising histidine, aromatic amino acids (W, Y, F) and basic amino acids (R, K). The relatively low binding strength allows the elution of

Key Term**Linear and structured peptides:**

Highly specific sequences of amino acids that can exhibit either a linear or a non-linear structure, the latter being either non-covalently (e.g., α -helices or β -sheets) or covalently (cyclic and polycyclic) constrained.

the antibody at milder conditions (pH 4.0–5.0) than those required by Protein A (pH 2.5), with reduced risk of aggregation and loss of product bioactivity [133]. Characterization of HWRGWV-hIgG interaction by mass spectrometry and docking simulations indicate that the ligand-binding site can be found in the loop Ser383-Asn389 (SNGQPEN), located in the CH₃ domain of the pFc portion of hIgG [134]. This HWRGWV sequence has been studied in great detail for its ability to isolate IgG and it has been tested against a variety of complex sources, including cell culture media, commercial CHO cell culture supernatants, Cohn II and III fraction of human plasma, transgenic milk and whey, and transgenic plant extract. In all these cases the adsorbent afforded product yields and purities up to 90 and 95%, respectively [128,130,131]. Further studies have been performed to improve the chemical stability and binding capacity of peptide-based resins. A method of surface resin modification was developed to enable the production of an alkaline-stable peptide-based adsorbent. The adsorbent HWRGWV-Toyopearl produced by peptide synthesis on the modified chromatographic resin was used over 200 cycles of IgG purification, each followed by alkaline cleaning and sanitization with aqueous 0.1 M NaOH, without significant loss of capacity and selectivity [129]. Further, a highly efficient protocol of ligand coupling was developed in order to optimize ligand distribution and accessibility on the resin pore surface. The adsorbents HWRGWVC-Toyopearl (peptide density of 60 μ moles/ml) and HWRGWVC-WorkBeads (peptide density of 50 μ moles/ml) gave dynamic binding capacities of 40 g/l and 60 mg/ml (residence time 5 min), respectively [138]. The ligand was also found to bind IgG from different species, for example, mouse, llama, bovine, goat, rabbit, and chicken, and purify them from crude sera [TAKAAKI T *ET AL.* SILICA GEL BASED PEPTIDE RESINS FOR AFFINITY PURIFICATION OF IMMUNOGLOBULINS (2013), MANUSCRIPT IN PREPARATION]. This indicates that the peptide could be used for purifying mAbs from hybridoma cell culture and ascites fluids. To prevent the proteolytic degradation of the peptide ligand by the enzymes present in these fluids, in particular trypsin and chymotrypsin, variants of the peptide ligands have been developed by replacing natural amino acids with non-natural residues [MENEGATTI S *ET AL.* PROTEASE-RESISTANT HEXAPEPTIDE LIGANDS FOR THE PURIFICATION OF ANTIBODIES FROM ANIMAL PLASMA (2013), MANUSCRIPT IN PREPARATION]. The variant HW_MCitGW_MV (W_M: N_{in}-methyl-tryptophan, Cit: citrulline) shows resistance to both proteases over a high number of injections.

The ligand HWRGWV was also used to bind human immunoglobulins of different classes, such as IgA and IgM [126,127]. Chromatographic tests with HWRGWV-Toyopearl resin at varying concentration of peptide ligand were performed to separate IgG, IgA and IgM from a cell culture supernatant and Cohn II and III fraction. After pretreatment with caprylic acid precipitation or combination of caprylic acid and polyethylene glycol precipitation, three elution fractions were obtained. The first fraction contained pure IgG (>95%), while the two subsequent fractions were respectively enriched in IgA (42% hIgA and 56% hIgG) and IgM (46% hIgM, 28% hIgA and 24% hIgG).

Protein A mimetic peptides, however, also come with some disadvantages. First, due to the lower affinity constant, small peptides require a higher ligand density than that needed for protein ligands. Also, being small molecules for which the display is important, peptide ligands require specific orientation on the chromatographic resin. The higher amount of peptide required to functionalize the resin, however, is balanced by the much lower cost per gram of peptides as compared with Protein A. The cost of peptide synthesis is highly dependent on the length of the sequence and the volume of production and, for hexapeptides made on a large scale, is likely below \$25/g at 95% purity. While we cannot propose a detailed analysis of the overall resin costs, including materials and labor costs, we deem it safe to state that the manufacturing costs of peptide-based adsorbents would be significantly lower than that of protein ligand based affinity supports. Additionally, the methods developed for coupling protein ligands can be applied for the immobilization of peptides in order to attain optimal ligand density and the required orientation. We have explored several of these strategies to fine tune ligand density, obtaining very good results in terms of binding capacity and product purity [138]. One final, yet crucial, issue is the determination of the immunogenicity and toxicity of peptide ligands. So far there are no reports in the literature for any of the sequences listed above that address these issues. Specific assays need to be developed for the sequences reported above to ensure compliance of these ligands with regards to safety and regulatory requirements.

Besides antibodies, peptide ligands have been identified for numerous other biotherapeutic proteins, among them, blood coagulation factors, such as Factor VII, VIII, IX and von Willebrand factor. The deficiency of these factors causes hemophilia (Factors VII, VIII and IX) and bleeding diathesis (von Willebrand factor) of the skin and mucous membranes. To restore correct hemostasis, patients are treated with injection of purified factors obtained either from human blood plasma

or recombinant sources. Due to the requirements of high purity (>99.9%) as well as immunological and virus safety, the cost of coagulation factor concentrates is very high and limits the implementation of prophylactic therapy as well as extended therapeutic and surgical use in patients suffering with bleeding disorders. Currently, these biomolecules are purified through a sequence of chromatographic steps, including immunoaffinity chromatography, that have a significant impact on the product costs. In order to reduce the number of purification steps and hence reduce the costs, several research teams have sought small peptide ligands for the affinity purification of blood coagulation factors. A list of these sequences is reported in **Table 3** [139–146].

Several studies have been performed to increase the resistance of peptide ligands to proteolysis and the binding capacity of peptide-based affinity adsorbents. A **linear peptide** ligand has also been found for another blood protein of high value, erythropoietin [147]. The two best sequences, Ac-FHHFAHAK and Ac-AFHNEFAHAK, were coupled on Sepharose resins and used for the purification of recombinant erythropoietin from a CHO cell culture supernatant. The ligands showed K_D values of 1.8 μM and 2.7 μM , respectively and afforded a protein purity of 95 and 97%, respectively, along with yields above 90%. On average, all these peptides offer very good product recovery and purity from plasma or recombinant sources and represent a highly promising class of ligands for affinity purification of blood proteins.

Finally, several peptide ligands have also been identified for the purification of therapeutic enzymes. The application of enzymes in pharmaceutical and food industry, scientific research, and human therapy has

Table 3. Peptide affinity ligands for blood coagulation factors.

Sequence	Target	K_D (M)	Ref.
Ac-RVRSFY	Von Willebrand Factor	$5 \cdot 10^{-7}$	[139]
RDRKWNCTDHVC	Factor VIII (light chain)	N.P.	[140]
EYKSWEYC EYHSWEYC EYISWEYC	Factor VIII	N.P.	[141,142]
WEYC and WDYC (3-IAA)EYC (3-IAA)DYC (3-IAA) Ψ (CH ₂ NH)EYC (3-IAA)-EY Ψ (CH ₂ NH)C	Factor VIII	N.P.	[143–145]
YANKGY	Factor IX	N.P.	[146]

Ψ (CH₂NH) indicates that the peptide bond CONH has been replaced by CH₂NH.
IAA: Indolacetic acid; N.P.: Not provided.

increased considerably in the last decade and a growing number of products are awaiting for FDA approval or are entering Phase III of clinical evaluation [148–152]. Enzymes are used in clinical settings for either analytical or therapeutic purposes. Therapeutic enzymes are currently used to treat a broad spectrum of diseases, from food intolerance and allergies to leukemia, ulcers, fibrosis, and inflammations [153,154]. These products generate substantial revenues worldwide and their market in the USA alone was estimated at \$1.15–1.17 billion in 2011 [155]. Currently, after extraction and concentration, enzymes are purified via chromatographic processes comprising up to five steps [156]. Combinatorial libraries of peptides have been used for the identification of sequences with affinity for enzymes. Several examples are listed in **Table 4** [157–166].

As shown in **Tables 2–4**, small linear peptide ligands discovered for the purification of immunoglobulins, blood factors, and enzymes show average dissociation

Table 4. Peptide ligands for the affinity purification of enzymes.

Sequence	Target	K_D (M)	Ref.
EIFPGNSKTYAE (fragment of anti-lysozyme antibody) AVLERAARSVLLNAP, STLLPELSETPNAT, and ELAPEDPEDSALLED (analogous to the amino acid sequence of herpes simplex glycoprotein D)	Lysozyme	$1.7 \cdot 10^{-10}$	[157,158]
YDLRYDRERA, GQGWYDLRYDRERA, KLASNYDTNGDGWI(Nle), LASNYDTNGDGWI(Nle), KLASNYDTNGDGWI(Nle)GLWELGKGPPQRVWNVGYGTLS	Lysozyme	$8 \cdot 10^{-8}$	[159]
YNFEVL	Ribonuclease S	$5.5 \cdot 10^{-6}$	[147,160]
YIYGSFK	p60(c-src) protein tyrosine kinase	$5.5 \cdot 10^{-5}$	[161]
NWMMF	Glycosomal gPGK	$8 \cdot 10^{-5}$	[162]
VAR, TAR, VPR, and TPR	Trypsin	$3.8 \cdot 10^{-5}$	[163]
D-His-Pro-Phe-His-Leu- Ψ (CH ₂ NH)-Leu-Val-Tyr	Renin	N.P.	[164]
TRWLVIYFSRPLYVAT	α -amylase	$4.4 \cdot 10^{-9}$	[165]
FHENWS	α -amylase	$4 \cdot 10^{-6}$	[166]

N.P.: Not provided.

constants of 10^{-5} – 10^{-7} M. Longer linear sequences can reach affinity values closer to those characteristic of protein ligands (10^{-8} – 10^{-9} M). Yet, while having a lower affinity, all the peptide sequences herein reported have been shown to afford binding capacity and product purities comparable to those offered by protein adsorbents or via immunoaffinity chromatography.

Several other peptide ligands have been identified for protein affinity purification. The sequence IQHPQ was identified by Lam *et al.* to bind Streptavidin [167]. Two other sequences, LNIVSVNGRHX and DNRIRLQAKXX (where X represents an undetermined residue), were identified by Pennington for α -6-b-1-integrin [168]; the heptamer HWWWPAS was found by Dong *et al.* for insulin [169]; the peptide EFDWNH was discovered by Lehman for anti-insulin antibodies [170]; GLERPE for prion protein by Lathrop [171]; four hexapeptides, APRQPP, DQDQDT, EGKQRR and HQHRQR, were found by Noppe for Lactoferrin [172]; several peptides for β -actin were discovered by Miyamoto [173]; and finally, a peptide for α -n-b-3 integrin, RGD, was found by Xiao *et al.* [174].

Cyclic peptide ligands

Small linear peptides show a great deal of promise, owing from their ability for specific biorecognition, chemical stability, low immunogenicity and toxicity, and ease of synthesis. Yet, a new generation of ligands, cyclic peptides, is gaining interest in the wider scenario of biotechnological applications. Due to their conformational rigidity, structured (cyclic) peptides possess higher affinity, specificity and enzymatic stability compared with their linear counterparts, and they are therefore promising candidates in the areas of drug discovery and identification of ligands for bioseparations [175–178]. The first libraries of cyclic peptides used for ligand discovery were prepared by a phage-display technique, where a disulfide bond between two cysteines framed the peptide sequence to attain cyclization. A list of disulfide-bridged cyclic peptides that bind specifically to target proteins is presented in Table 5 [179–184]. In all these studies, the affinity of the selected cyclic peptides was found to be higher than those of the corresponding linear sequences.

Since the pioneering work of McLafferty on phage-display libraries [185], many disulfide-bridged cyclic peptides with high affinity for protein targets have been identified. However, the liability of the disulfide bond limits the application of these cyclic peptides to research and medicinal environments. Under reducing conditions, such as high pH, the -S-S- bond hydrolyses and the cyclic peptide loses its structure. Hence, disulfide-bridged cyclic peptides cannot find application as affinity ligands for purification of protein therapeutics

in the industry, where alkaline conditions are regularly employed for resin cleaning and regeneration [186–188].

Biological peptide libraries, such as phage display, bacterial display, yeast display, and so forth, remain very powerful tools for identifying high-affinity ligands, owing to the mechanism of sequence selection by directed evolution. This process consists in iterating cycles of library generation and selection, which gradually defines homology patterns and enables the identification of a final candidate ligand with very high affinity and selectivity. Methods for attaining peptide cyclization on biological libraries other than the formation of disulfide bonds have been developed to discover cyclic peptide ligands. Roberts and co-workers, for example, have published a method for peptide cyclization on mRNA-display libraries, which comprises a liquid-phase crosslinking reaction between two primary amino groups using the homobifunctional linker disuccinimidyl glutarate [189]. Using this technology, a library was prepared and screened against the G α i1 protein to identify a cyclic peptide that binds the target with ‘antibody-like affinity’ ($K_D = 2.1$ nM). The cyclic peptide was found to bind the target protein 15-fold more tightly than its linear counterpart and to possess enhanced proteolytic stability [190]. The cyclization method as described by these researchers, however, suffers from moderate yields, confined in the range of 30–50%, and is open to formation of undesired by-products resulting from poly-modification and intermolecular crosslinking. To avoid these side reactions, we have recently proposed a variant of this peptide cyclization on a ‘reversible solid-phase format’ [191]. After translation, the liquid-phase library of mRNA-peptide hybrids is adsorbed on solid phase, reacted with disuccinimidyl glutarate, washed and eluted. The spatial segregation of the peptides on a solid phase and the optimized crosslinking conditions avoid the formation of unwanted byproducts and afford high peptide cyclization yield and library purity (>90%). Library screening against IgG-Fc was performed under very stringent conditions to ensure the identification of candidate binders in a single step. The selected sequences showed a marked similarity in amino acid composition, with a predominance of histidine, aromatic (W, F and Y) and basic (R and K) residues, similarly to the above mentioned linear hexapeptides selected from a solid-phase synthetic library screened against the same target. The selected sequence cyclo[Link-MWFRHY-K] (Link: glutarate) was shown to bind IgG from different mammalian species, namely human, mouse, rabbit, chicken, goat and cow, and was used to purify therapeutic mAbs of IgG₁ and IgG₄ subclasses from two commercial cell culture supernatants. The product yield and purity (both at 95%) as well as the binding

strength ($K_D = 7.6 \times 10^{-6}$ M) were slightly higher than those given by the linear hexapeptides.

Interestingly, biological display libraries have been recently enriched with the inclusion of non-natural (also called 'non proteinogenic') amino acids. These expand significantly the chemical and structural diversity by enabling post-translational modifications that are not possible with natural amino acids. mRNA-display libraries of cyclic peptides have been created using non-natural amino acids to enable macrocyclization and screened for the isolation of high affinity peptide ligands against the protease thrombin. These ligands have been found to possess dissociation constants in the low nanomolar range, thereby reinstating the potential of these compounds for the discovery of highly selective drugs, inhibitors and affinity ligands [192].

While having a track record of success, biological libraries suffer from some limitations. The variety of non-natural amino acids and other synthetic groups that they allow is still quite limited and their screening process is fairly laborious. Solid-phase synthetic libraries, on the other hand, enable a wide range of chemical and structural variations. Several strategies of peptide cyclization have been proposed, which employ both natural and non-natural amino acids. The former include end-to-tail, end- or tail-to-side chain, and side chain-to-side chain cyclization by reaction between glutamic or aspartic acid and lysine residues [193,194]. Among the latter, ring closing metathesis and Click chemistry are notable techniques. Ring closing metathesis refers to the intramolecular olefin metathesis catalyzed by a Ruthenium-based Grubbs' reagent between two allylglycines located at the ends of the peptide [195–200]. Click chemistry consists of a Huisgen cycloaddition between the alkyne and the azide residues of non-natural amino acids, such as propargylglycine and an azido amino acid, leading to a triazole link [201–205]. The major issue related to these methods is the difficulty of sequencing the cyclic peptides carried by the beads selected from library screening. The techniques routinely used for sequencing linear peptides, Edman degradation and single stage MS/MS, cannot be employed for cyclic peptides. Some MS-based methods have been reported for sequencing cyclic peptides, although they entail considerable effort and high level of uncertainty. In a mass spectrometer, in fact, the cyclic peptide undergoes ring opening at multiple positions to produce a complex mixture of fragments, making spectral interpretation difficult and highly uncertain [206–211].

A few strategies have been proposed to circumvent the difficulties involved in the post-screening hit identification. Pei and co-workers have developed a method for the synthesis of a one-bead-two-peptide

Table 5. Cyclic peptides (by disulfide bond) selected from phage-display libraries.

Sequence	Target	K_D (M)	Ref.
Cyclo[CHPQFC], cyclo[CHPQFPC], and cyclo[CHPQGPPC]	Streptavidin	6.6×10^{-7}	[179]
Cyclo[CD(R/L)A(S/T)P(Y/W)C]	Avidin and neutravidin	$1-5 \times 10^{-5}$	[180]
Cyclo [CCFSWRCRC]	Chymotrypsin	10^{-6}	[181]
AEGTGDHL-cyclo[CGAWFRPC]- DAEPGEGGGGK	Factor VIII	7.2×10^{-7}	[182]
H2N-cyclo[CFHHC] ₂ -KG	IgG (Fc and Fab fragments)	2×10^{-5}	[183]
HMVcyclo[CLAYRGRPVC]FAL, HMVcyclo[CLSYRGRPVC]FSL	IgA	$1.6-3.3 \times 10^{-8}$	[184]

C indicates cysteine residues employed for peptide cyclization.

library [207]. This procedure comprises the segregation of each library bead into an outer layer, which displays cyclic peptides and is accessible to the target protein, and an inner core, which contains the peptide linear precursors and is impervious to proteins. After selecting the beads that bind the target protein through the cyclic ligands located in the outer layer, the linear peptides in the inner core are sequenced by partial Edman degradation [207]. This method has been successfully applied to the identification of cyclic peptides ligand with micromolar or submicromolar affinity against a variety of targets, reported in Table 6 [177,212–214]. Recently, the same method has been extended to the identification of peptoids and peptide/peptoid hybrids [215].

Our group also has proposed a strategy for easy and high throughput sequencing of cyclic peptides leads, based on the use of a solid-phase library of reversible cyclic peptides [216]. The cyclic structure comprises two ester bonds that frame the functional sequence, that is, the portion of the peptide that directly interacts with the target protein, as shown in Figure 3.

Table 6. Cyclic peptide ligands identified from screening one-bead-one-peptide libraries.

Sequence	Target	Ref.
Cyclo[ARYQSRVE] and cyclo[AIYQSRVE]	Human prolactin receptor	[177]
Cyclo[AFCpYCNVLE] and cyclo[AIKpYQNYLNE]	SH2 domains of Grb2	[212]
Cyclo[ANIpYDNVLE]	Tensin	[212]
Cyclo[YHIGIGPVIVITAAE] and others	Calcineurin/NFAT interaction complex	[213]
Cyclo[aWYQ-Fpa-NIe-aaaEK]-K and cyclo[al-Fpa-RYWaaE]K	HIV-1 capsid/human Lysyl- tRNA synthetase complex	[214]

Uppercase lettering indicates L-amino acids, lowercase indicate D-amino acids.

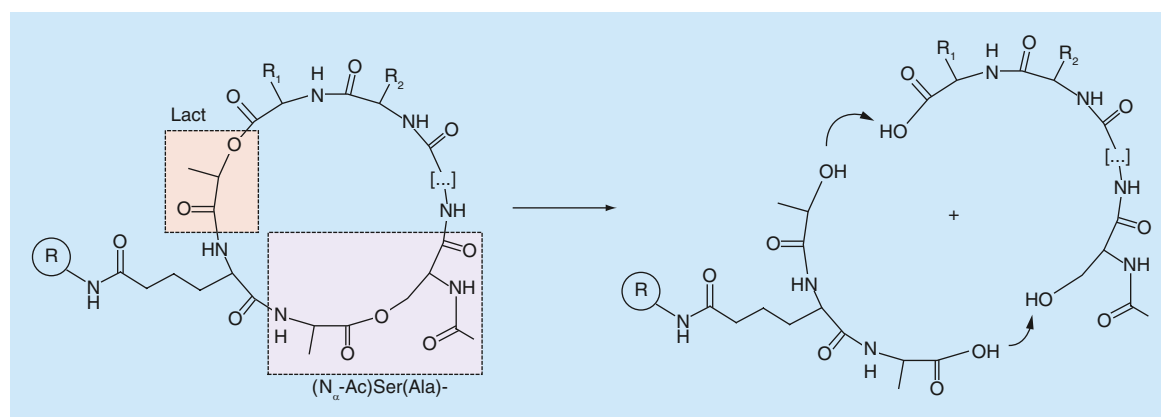


Figure 3. Model structures of the cyclic dilactone cyclo[(Na-Ac)S(A)-X1X2X3X4X5X6-Lact-E] and linearized structure Ac-S-X1X2X3X4X5X6.

Reproduced with permission from [216] © American Chemical Society (2013).

Ester bonds are sufficiently stable at neutral pH to allow screening the library with the peptides retaining their cyclic structure, whereas they are rapidly hydrolyzed in alkaline conditions. Beads selected from library screening are treated with an alkaline aqueous-organic solution, which cleaves the functional sequence from the resin surface and releases it in liquid phase. The linearized sequences are then analyzed by single step ESI-MS/MS. This method has been demonstrated through the selection of an IgG-binding cyclic peptide from a small model library. The sequence cyclo[(N_α-Ac)-S(G)-RWHYFK-Lact-E] was selected, synthesized on a chromatographic resin, and tested for antibody binding. To avoid ligand loss due to hydrolysis during the elution step (acid buffer) and resin sanitization (alkaline buffer) and to maintain the ligand cyclic structure, the ester bonds were replaced with amide bonds. This modification has no consequence on the structure of the ligand, which retains its binding capacity as demonstrated by chromatographic binding tests.

While on average cyclic peptides show decidedly improved selectivity and binding strength when compared with linear peptides, they are also more difficult and costly to produce. Peptide cyclization, especially when not based on disulfide bridges, requires specially protected amino acids and additional reaction steps for cyclization that are sequence-dependent and require careful optimization. This makes cyclic peptide ligands more expensive than their linear counterparts and could limit their application to specific cases where the target biologic is extremely dilute and very high affinity is required – in the nanomolar range. It is difficult to provide an estimate of the cost of large-scale production of cyclic peptide ligands since this would be highly dependent on the length and flexibility of the peptide sequence, as well as on the particular cycliza-

tion method. However, it is reasonable to assume that large-scale synthesis of a synthetic cyclic ligand would result in significant reductions in cost.

Polycyclic peptides

Recent advances in chemical and biochemical synthesis have opened the way to a new generation of highly complex peptide architectures. Multi-domain peptides, that is, ligands capable of binding the target molecule via multisite interactions, are very promising candidates for attaining the high levels of affinity required in difficult bioseparations [217]. Bicyclic and tricyclic peptides are regarded as promising structures for creating ligands that mimic the hand-in-glove binding mechanism characteristic of natural protein-protein biorecognition. Recently, Heinis *et al.* have presented a method for producing phage-display libraries of bicyclic peptides. The formation of bicyclic peptides is achieved by reacting three cysteine residues located on the peptide with *tris*-(bromomethyl)benzene linker. The library was screened against human kallikrein and a ligand was identified that binds the protein target with $K_d = 1.5 \times 10^{-9}$ M [218–220]. A study was also presented on the effect of peptide length on the efficiency of the *bis*-cyclization reaction and the affinity of the resulting ligands [221]. In a recent study, the same team has presented a method for the synthesis of tricyclic peptides that comprises the use of the above mentioned linker to form the first two cycles, followed by the enzyme transglutaminase that bridges between lysine and asparagine thereby forming the third loop [222,223]. Our group has developed a method for the synthesis of bicyclic and tricyclic peptides on solid phases. These structures comprise independent peptide cycles connected by a spacer arm (Figure 4). This conformation is expected to allow better access of the cyclic binding units into neighbor binding sites, thus promoting

an 'avidity effect' within the polycyclic ligand for the target biomolecule.

These molecules are very interesting and promising leads for the development of ligands and drug, due to their level of binding strength and stability which are almost unmatched by any other synthetic molecule of similar size. While only a few such compounds are under clinical trials, recent research indicates that polycyclic peptide therapeutics could potentially be developed for a broad range of diseases [223]. Simple forms of bicyclic peptides (cyclic peptides having an inner tether) are already being proposed as scaffolds for pharmaceutical research. While many groups are contemplating the possibility of using synthetic ligands, primarily linear peptides, in place of protein ligands for the purification of IgGs and other protein pharmaceuticals, the use of cyclic and polycyclic peptides is a more distant goal.

Conclusion

This literature survey indicates that small, easy to synthesize, non-immunogenic, and cost-effective synthetic molecules offer promising alternatives to protein ligands for bioseparations. Of special interest is the class of peptide ligands, which, in the past three decades, has shown a tremendous improvement in terms of both numbers and performance for a wide variety of high-value biological targets. Peptides combine the advantages of small synthetic molecules, such as biochemical stability and ease of synthesis, with levels of affinity and specificity sufficient to afford values of dynamic binding capacity and product purity similar to those obtained using biological protein ligands. The potential of peptides is further increased by the constant development of novel synthetic and computational tools. The introduction of non-natural amino acids and of new reaction mechanisms open the way to higher levels of molecular complexity, and thus affinity, specificity, and stability, at lower synthesis costs. New refined computational tools for molecular design and simulation of protein–peptide interactions offer an invaluable help to chemists and engineers for better understanding the phenomena of molecular biorecognition and for the design of more effective ligands and drugs. With all these resources, peptides offer a great deal of promise for the identification of novel ligands for developing new diagnostics, therapeutics and bioseparation technologies. While the peptide sequences discovered in the past showed K_D values in the range of 10^{-6} – 10^{-7} M, sterically constrained ligands are now being discovered with affinity in the nanomolar ('antibody-like') range. While peptide drugs have found numerous applications, the potential of peptides as affinity ligands for downstream bioprocessing has been but minimally utilized. Two

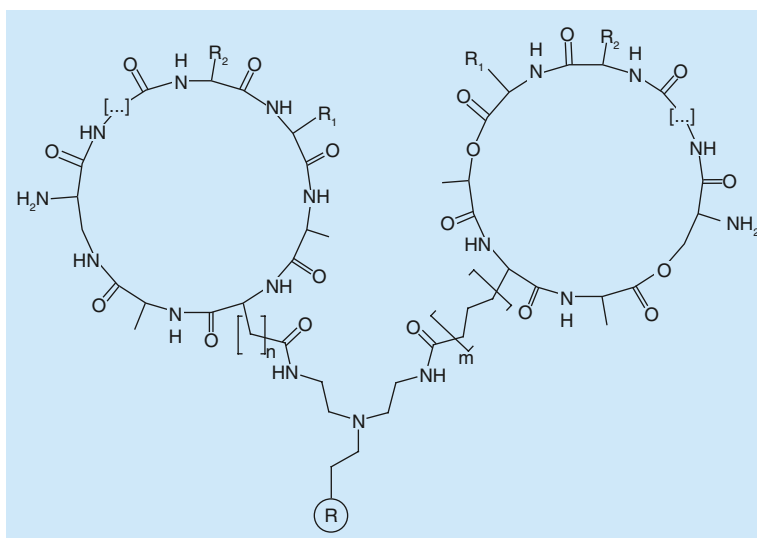


Figure 4. Structure of bicyclic peptide.

major barriers hinder the development of small peptide ligands for industrial affinity separations:

- » The difficulty in implementing process changes in highly regulated existing processes;
- » The lack of financial motivation for major resin manufacturers to develop real low-cost alternatives to highly profitable affinity resins.

So far, resin manufacturers have instead focused on providing less specific alternatives such as high capacity ion exchangers and mixed-mode adsorption resins, that might supplant Protein A, but are even less specific than peptides and require additional chromatography polishing steps to achieve the required product purity. Yet, it is conceivable that under the increasing pressure for radical changes in downstream processing technologies peptides may play a strong role in future, low cost, sustainable bioprocessing technologies.

Future perspective

The increasing demand for affordable biopharmaceuticals from growing economies and an aging world population is forcing a comprehensive rethinking of bioprocess technologies. For downstream processing in particular, several measures are on the horizon, such as the development of compact, disposable, modular units, continuous processes, measures to reduce solvent usage, and the adoption of novel ligands for the purification of a variety of biotherapeutics currently isolated by less efficient techniques. Although a number of protein affinity ligands are known in the art for affinity purifications, small, easy to synthesize, non-immunogenic, and cost-effective synthetic molecules hold the highest promise for major advances in future

downstream bioprocessing. Peptides in particular are expected to play a crucial role in providing new solutions for the purification of protein pharmaceuticals. The rise in the use of peptide ligands is being spurred by the ongoing development of novel synthetic and computational tools. The recent introductions of non-natural amino acids and of new reaction synthesis mechanisms are opening the way to higher levels of molecular complexity, and thus increased ligand affinity, specificity, and stability, at lower synthesis costs. Furthermore, advanced computational tools for molecular design and simulation of protein–peptide interactions will provide chemists and engineers a better understanding of the phenomena of molecular biorecognition for the design of novel ligands. With these resources at hand, peptides are indeed the leading

candidates for the identification of a new generation of ligands for diagnostics, therapeutics and bioseparation technologies.

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Executive summary

Background

» There is a growing worldwide demand for low cost, more widely accessible biopharmaceuticals and biosimilars. Downstream processes are major contributors to manufacturing costs of biologics. Affinity chromatography has the potential for reducing costs by reducing processing steps. Large protein ligands used in affinity chromatography (Protein A, Protein G and Protein L) are costly, lack stability, and require harsh elution conditions that can reduce yield and purity. Small synthetic affinity ligands can help overcome these issues.

Triazine ligands

» This general class of small ligands has been used for the purification of human antibodies and their fragments as well as other proteins. They are less specific than protein ligands and require manipulation of the binding buffers to achieve desired purity, but they are much less costly than protein ligands.

Amino acids & linear peptide ligands

» Amino acids have been used as mixed-mode pseudo-affinity ligands. Peptide-based ligands for a wide variety of target proteins can be discovered via high-throughput screening techniques. Linear peptides have been used for the purification of human and animal antibodies, blood factors, enzymes and other applications. Peptide ligands can be cost effective and more selective than triazine ligands.

Cyclic & polycyclic peptide ligands

» Sterically constrained peptides, such as cyclic peptides, can exhibit greater specificity and stronger avidity than linear peptides. Disulfide-linked cyclic peptides have been identified but they tend to be unstable under downstream process conditions. Chemically stable cyclic peptides can be identified using liquid-phase mRNA-display libraries as well as synthetic solid-phase libraries. Cyclic and polycyclic peptides show great promise as ligands for affinity purification as well as diagnostics.

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