Review

Pharmaceutical BIOPROCESSING

Pharm. Bioprocess.
(2013) 1(5), 467–485

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.

Stefano Menegatti[1], Amith D Naik[2] & Ruben G Carbonell*[1,2]

[1]Department of Chemical & Biomolecular Engineering, North Carolina State University, 911 Partners Way, Raleigh, NC 27695–7905, USA
[2]Biomanufacturing Training & Education Center (BTEC), 850 Oval Drive, Raleigh, NC 27695, USA
*Author for correspondence:
Tel.: +1 919 515 5118
Fax: +1 919 515 5831
E-mail: ruben@ncsu.edu

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

FUTURE SCIENCE

10.4155/PBP.13.54 ISSN 2048-9145

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 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 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 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-aminohexyl-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,

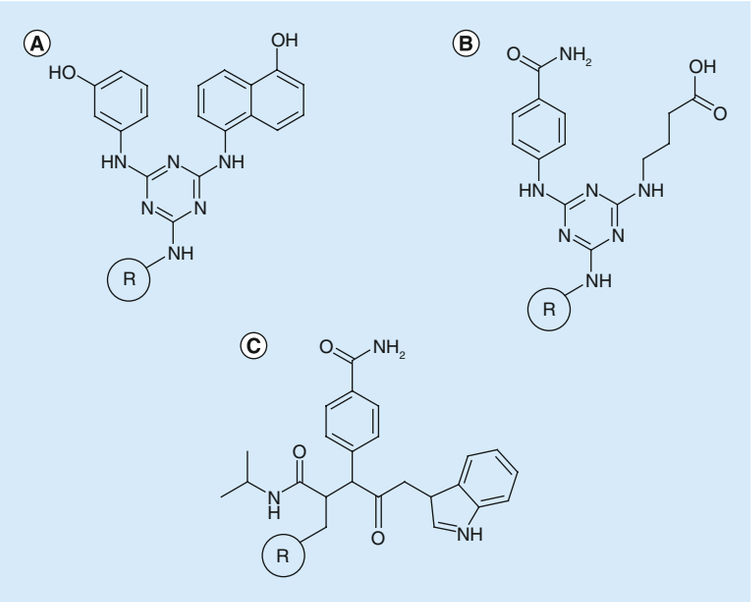

Figure 1. Triazinyl-based affinity ligands for the purification of immunoglobulins. (A) Ligand 22/8; **(B)** ligand 8/7; **(C)** ligand A2C 11/1. R: Sepharose resin.

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 preselected 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 IgG_3 subclass, shows weak binding to mouse IgG_1 and bovine IgG_1, 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 $(Arg\text{-}Thr\text{-}Tyr)_4$-$Lys_2$-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	$8.6\ 10^{-7}$	[52,110–112]
EPIHRSTLTALL	Anti-Tac IgG1 antibody (HAT)	N.P.	[113,114]
$(Arg\text{-}Thr\text{-}Tyr)_4$-$Lys_2$-Lys-Gly (TG19318 or PAM)	IgG-Fc of different animal species, and IgM, IgA, IgE, and IgY	$3.0\ 10^{-7}$ (for IgG)	[115–121]
$(DArg\text{-}DThr\text{-}DTyr)_4$-$DLys_2$-DLys-DGly (D-PAM)		N.P.	[122,123]
AEGEFINVPMMVDGITMGDPAK (M[46–2])	Murine anti-human tumor-associated tenascin-C (Tn-C) mAb	$1.7\ 10^{-9}$	[124]
PMMVDGITMG	Murine anti-human tumor-associated tenascin-C (Tn-C) Fab	N.P.	[124]
D_2AAG	IgG-Fc	$4.7\ 10^{-6}$	[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	$2\ 10^{-8}$	[135]
50-mer	IgA	N.P.	[136]
CCHQRLSQRK	IgM	N.P.	[137]

N.P.: Not provided.

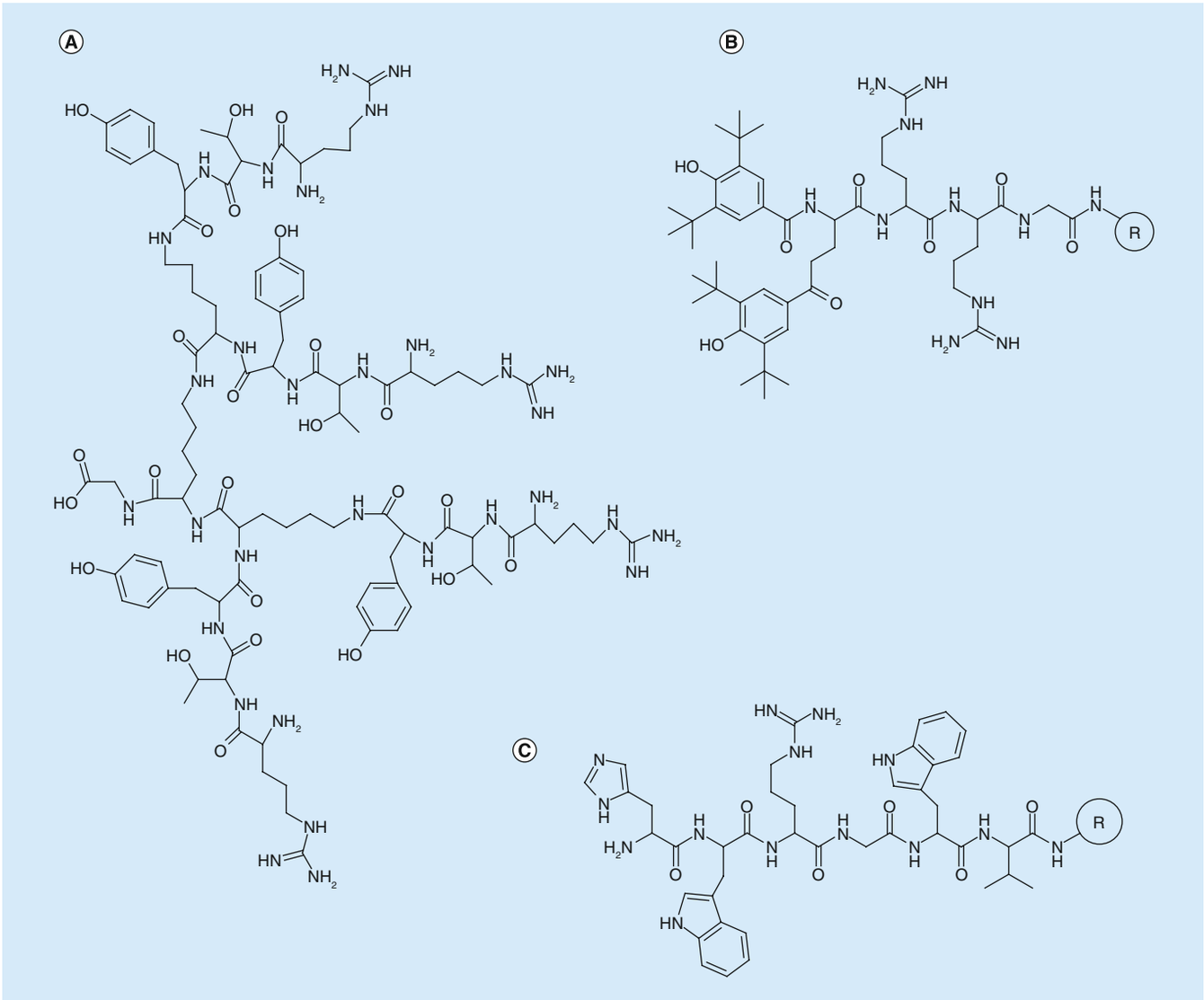

Figure 2. Protein A mimetic peptide ligands for the purification of immunoglobulins. (A) TG19318; **(B)** ligand D2AAG; **(C)** HWRGWV. R: Resin.

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, D_2AAG (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 CH_3 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 µmoles/ml) and HWRGWVC-WorkBeads (peptide density of 50 µmoles/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].

Table 3. Peptide affinity ligands for blood coagulation factors.

Sequence	Target	K_D (M)	Ref.
Ac-RVRSFY	Von Willebrand Factor	$5\ 10^{-7}$	[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_2NH)EYC (3-IAA)-EYΨ(CH_2NH)C	Factor VIII	N.P.	[143–145]
YANKGY	Factor IX	N.P.	[146]

ΨNH indicates that the peptide bond CONH has been replaced by CH_2NH.
IAA: Indolacetic acid; N.P.: Not provided.

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-AFHNFAHAK, 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 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.
EIFPGNSKTYYAE (fragment of anti-lysozyme antibody) AVLERAARSVLLNAP, STLLPPELSETPNAT, and ELAPEDPEDSALLED (analogous to the amino acid sequence of herpes simplex glycoprotein D)	Lysozyme	$1.7\ 10^{-10}$	[157,158]
YDLRYDRERA, GQGWYDLRYDRERA, KLASNYDTNGDGWI(Nle), LASNYDTNGDGWI(Nle), KLASNYDTNGDGWI(Nle)GLWELGKGPPQRVWNVGYGTLS	Lysozyme	$8\ 10^{-8}$	[159]
YNFEVL	Ribonuclease S	$5.5\ 10^{-6}$	[147,160]
YIYGSFK	p60(c-src) protein tyrosine kinase	$5.5\ 10^{-5}$	[161]
NWMMF	Glycosomal gPGK	$8\ 10^{-5}$	[162]
VAR, TAR, VPR, and TPR	Trypsin	$3.8\ 10^{-5}$	[163]
D-His-Pro-Phe-His-Leu-Ψ(CH_2NH)-Leu-Val-Tyr	Renin	N.P.	[164]
TRWLVYFSRPYLVAT	α-amylase	$4.4\ 10^{-9}$	[165]
FHENWS	α-amylase	$4\ 10^{-6}$	[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 alpha-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 Gαi1 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 byproducts 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 IgG_1 and IgG_4 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 × 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 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 5. Cyclic peptides (by disulfide bond) selected from phage-display libraries.

Sequence	Target	K_D (M)	Ref.
Cyclo[**C**HPQF**C**], cyclo[**C**HPQFP**C**], and cyclo[**C**HPQGPP**C**]	Streptavidin	6.6 10^{-7}	[179]
Cyclo[**C**D(R/L)A(S/T)P(Y/W)**C**]	Avidin and neutravidin	1–5 10^{-5}	[180]
Cyclo [**C**CFSWRCR**C**]	Chymotrypsin	10^{-6}	[181]
AEGTGDHL-cyclo[**C**GAWFRP**C**-]-DAEPGEGGGGK	Factor VIII	7.2 10^{-7}	[182]
H2N-cyclo[**C**FHH**C**]$_2$-KG	IgG (Fc and Fab fragments)	2 10^{-5}	[183]
HMVcyclo[**C**LAYRGRPV**C**]FAL, HMVcyclo[**C**LSYRGRPV**C**]FSL	IgA	1.6–3.3 10^{-8}	[184]

C indicates cysteine residues employed for peptide cyclization.

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[AFCpYCNVLNE] and cyclo[AIKpYQNYLNE]	SH2 domains of Grb2	[212]
Cyclo[ANIpYDNVLNE]	Tensin	[212]
Cyclo[YHIGIGPVIVITAAE] and others	Calcineurin/NFAT interaction complex	[213]
Cyclo[aWYQ-Fpa-Nle-aaaEK]-K and cyclo[aI-Fpa-RYWaaE]K	HIV-1 capsid/human Lysyl-tRNA synthetase complex	[214]

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

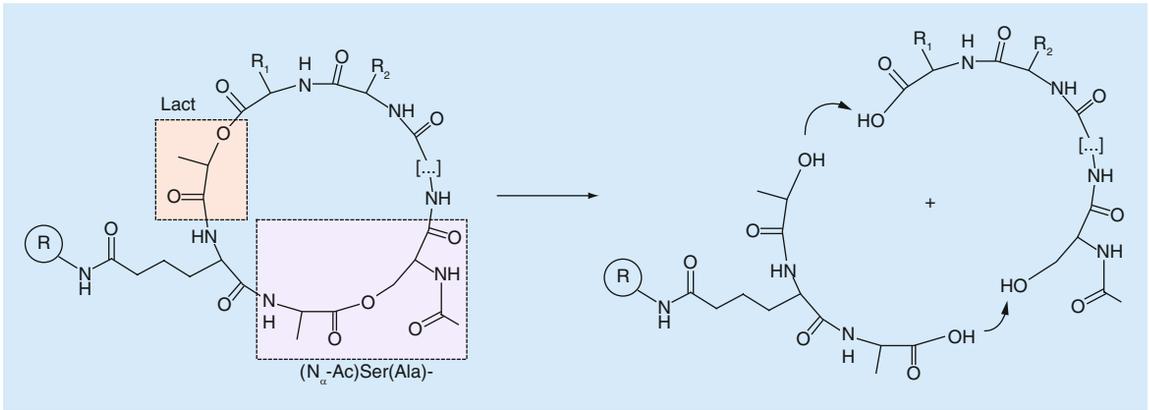

Figure 3. Model structures of the cyclic dilactone cyclo[(Na-Ac)S(A)-X1X2X3X4X5X6-Lact-E] and linearized structure Ac-S-X1X2X3X4X5X6.
Reproduced with permission from [216] © American Chemical Society (2013).

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$_\alpha$-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 cyclization 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 × 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.

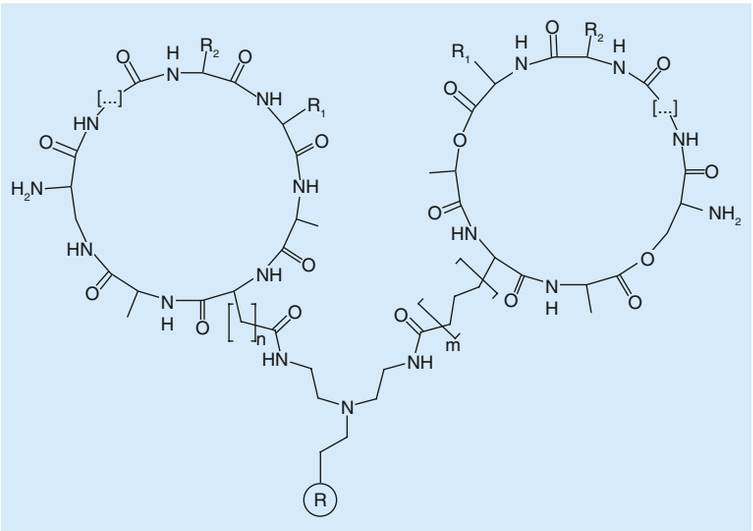

Figure 4. Structure of bicyclic peptide.

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 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 biopharmaceutics 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.

Financial & competing interests disclosure

The WR Kenan Jr Institute for Engineering, Technology and Science and the North Carolian Biotechnology Center, provided financial support this work. NC State University owns intellectual property created by all three authors of this paper, who also could benefit financially from licensing and other commercial opportunities. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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.

References

Papers of special note have been highlighted as:
- ■ of interest
- ■■ of considerable interest

1 Gill DS, Damie NK. Biopharmaceutical drug discovery using novel protein scaffolds. *Curr. Opin. Biotechnol.* 17(6), 656–658 (2006).

2 Strohl WR, Knight DM. Discovery and development of biopharmaceuticals: current issues. *Curr. Opin. Biotechnol.* 20(6), 668–672 (2009).

3 Szlachcic A, Zakrewska M, Otlewski J. Longer action means better drug: tuning up protein therapeutics. *Biotechnol. Adv.* 29(4), 436–441 (2011).

4 Walsh G. Second-generation biopharmaceuticals. *Eur. J. Pharm. Biopharm.* 58(2), 185–196 (2004).

5 Werner RG. Identification and development of new biopharmaceuticals. *Arzeinmittelforschung* 48(5), 535–530 (1998).

6 Dickson M, Gagnon JP. The cost of new drug discovery and development. *Discov. Med.* 4(22), 172–179 (2004).

7 Grootendorst P, Pierard E, Shim M. Life-expectancy gains from pharmaceutical drugs: a critical appraisal of the literature. *Expert. Rev. Pharmacoecon. Outcomes Res.* 9(4), 353–364 (2009).

8 Kremer M. Pharmaceuticals and the developing world. *J. Econ. Perspect.* 16(4), 67–90 (2002).

9 Lage A. Global pharmaceutical development and access: critical issues of ethics and equity. *MEDICC Rev.* 13(3), 16–22 (2011).

10 Pecoul B, Chirac P, Trouiller P, Pinel J. Access to essential drugs in poor countries: a lost battle? *JAMA* 281(4), 361–367 (1999).

11 Short RV. Population growth in retrospect and prospect. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 364(1532), 2971–2974 (2009).

12 Dranitsaris G, Truter I, Lubbe MS, Amir E, Evans W. Advances in cancer therapeutics and patient access to new drugs. *Pharmacoeconomics* 29(3), 213–224 (2011).

13 Miller FG, Joffe S. Balancing access and evaluation in the approval of new cancer drugs. *JAMA* 305(22), 2345–2346 (2011).

14 Tangka FK, Trogdon JG, Richardson LC, Howard D, Sabatino SA, Finkelstein EA. Cancer treatment cost in the United States: has the burden shifted over time? *Cancer* 116(14), 3477–3484 (2010).

15 Cornes P. The economic pressures for biosimilar drug use in cancer medicine. *Target Oncol.* 7(Suppl. 1), S57–S67 (2012).

16 Dranitsaris G, Amir E, Dorward K. Biosimilars of biologic drug therapies: regulatory, clinical and commercial considerations. *Drugs* 71(12), 1527–1536 (2011).

17 Hou JJC, Codamo J, Pilborough W, Hughes B, Gray PP, Munro TR. New frontiers in cell line development: challenges for biosimilars. *J. Chem. Technol. Biotechnol.* 86(7), 895–904 (2011).

18 Roger SD. Biosimilars: current status and future directions. *Expert. Opin. Biol. Ther.* 10(7), 1011–1018 (2010).

19 Woollett GR. Innovation in biotechnology: current and future states. *Clin. Pharmacol. Ther.* 91(1), 17–20 (2012).

20 Bhambure R, Kumar K, Rathore AS. High-throughput process development for biopharmaceutical drug substances. *Trends Biotechnol.* 29(3), 127–135 (2011).

21 Nfor BK, Verhaert PD, van der Wielen LA, Hubbuch J, Ottens M. Rational and systematic protein purification process development: the next generation. *Trends Biotechnol.* 27(12), 673–369 (2009).

22 Shukla AA, Thömmes J. Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol.* 28(5), 253–261 (2010).

23 Kelley BD. Biochemical engineering: bioprocessing of therapeutic proteins. *Curr. Opin. Biotechnol.* 12(2), 173–174 (2001).

24 Lowe CR. Combinatorial approaches to affinity chromatography. *Curr. Opin. Chem. Biol.* 5(3), 248–256 (2001).

25 Gottschalk U, Brorson K, Shukla AA. The need for innovation in biomanufacturing. *Nat. Biotechnol.* 30(6), 489–492 (2012).

26 Kelley BD. Industrialization of mAb production technology: the bioprocessing industry at a crossroads. *MAbs* 1(5), 443–452 (2009).

27 Cramer SM, Holstein MA. Downstream bioprocessing: recent advances and future promise. *Curr. Opin. Chem. Eng.* 1(1), 27–37 (2011).

28 Gagnon P. Technology trends in antibody purification. *J. Chromatogr. A* 1221, 57–70 (2012).

29 Low D, O'Leary R, Pujar NS. Future of antibody purification. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 848(1), 48–63 (2007).

30 Ayyar BV, Arora S, Murphy C, O'kennedy R. Affinity chromatography as a tool for antibody purification. *Methods* 56(2), 116–129 (2012).

31 Chon JH, Zarbis-Papastoitsis G. Advances in the production and downstream processing of antibodies. *N. Biotechnol.* 28(5), 458–463 (2011).

32 Clonis YD. Affinity chromatography matures as bioinformatic and combinatorial tools develop. *J. Chromatogr. A* 1101(1–2), 1–24 (2006).

33 Lowe CR, Lowe AR, Gupta G. New developments in affinity chromatography with potential application in the production of biopharmaceuticals. *J. Biochem. Biophys. Methods* 49(1–3), 561–574 (2001).

34 Smith C. Striving for purity: advances in protein purification. *Nat. Method* 2, 71–77 (2005).

35 Chester K, Pedley B, Tolner B. Engineering antibodies for clinical applications in cancer. *Tumour Biol.* 25(1–2), 91–98 (2004).

36 de Oliveira JR, Nunes FB, Pires MGS, da Silva Melo DA, Donadio MVF. Antibodies as anti-infective agents in medicinal chemistry. *Anti Infect. Agents Med. Chem.* 7, 249–257 (2008).

37 Kontermann RE. Alternative antibody formats. *Curr. Opin. Mol. Ther.* 12(2), 176–183 (2010).

38 Kuroki M. Possible applications of antibodies or their genes in cancer therapy. *Anticancer Res.* 26(6A), 4019–4025 (2006).

39 Darcy E, Leonard P, Fitzgerald J, Danaher M, O'Kennedy R. Purification of antibodies using affinity chromatography. *Methods Mol. Biol.* 681, 369–382 (2011).

40 Griep R, Mcdougall G. Analysis and purification of antibody fragments using Protein A, Protein G, and Protein L. In: *Antibody Engineering*. Kontermann RE, Dübel S (Eds). Springer Berlin Heidelberg, Berlin, Germany, 301–315 (2010).

41 Grodzki AC, Berenstein E. Antibody purification: affinity chromatography – Protein A and Protein G Sepharose. *Methods Mol. Biol.* 588, 33–41 (2010).

42 Hober S, Nord K, Linhult M. Protein A chromatography for antibody purification. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 848(1), 40–47 (2007).

43 Bloom JW, Wong MF, Mitra G. Detection and reduction of Protein A contamination in immobilized Protein A purified monoclonal antibody preparations. *J. Immunol. Methods* 117(1), 83–89 (1989).

44 Follman DK, Fahrner RL. Factorial screening of antibody purification processes using three chromatography steps without Protein A. *J. Chromatogr. A* 1024(1–2), 79–85 (2004).

45 Scriba GKE. Affinity Chromatography. In: *Molecular biology in medicinal chemistry*, Dingermann T, Steinhilber D, Folkers G (Eds). John Wiley and Sons, NJ, USA, 211–241 (2006).

46 Hahn R, Shimahara K, Steindl F, Jungbauer A. Comparison of Protein A affinity sorbents III. Life time study. *J. Chromatogr. A* 1102(1–2), 224–231 (2006).

47 Denizlia A, Pişkin E. Dye-ligand affinity systems. *J. Biochem. Biophys. Methods* 49(1–3), 391–416 (2001).

48 Huse K, Böhme HJ, Scholz GH. Purification of antibodies by affinity chromatography. *J. Biochem. Biophys. Methods* 51(3), 217–231 (2002).

49 Labrou NE. Design and selection of ligands for affinity chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 790(1–2), 67–78 (2003).

50 Roque AC, Silva CS, Taipa MA. Affinity-based methodologies and ligands for antibody purification: advances and perspectives. *J. Chromatogr. A* 1160(1–2), 44–55 (2007).

51 Burton NP, Lowe CR. Design of novel affinity adsorbents for the purification of trypsin-like proteases. *J. Mol. Recognit.* 5(2), 55–68 (1992).

52 Clonis YD, Labrou NE, Kotsira VP, Mazitsos C, Melissis S, Gogolas G. Biomimetic dyes as affinity chromatography tools in enzyme purification. *J. Chromatogr. A* 891(1), 33–44 (2000).

53 Clonis YD, Lowe CR. Triazine dyes, a new class of affinity labels for nucleotide-dependent enzymes. *Biochem J.* 191(1), 247–251 (1980).

54 Clonis YD, Lowe CR. Affinity chromatography on immobilised triazine dyes. Studies on the interaction with multinucleotide-dependent enzymes. *Biochim. Biophys. Acta* 659(1), 86–98 (1981).

55 Clonis YD, Stead CV, Lowe CR. Novel cationic triazine dyes for protein purification. *Biotechnol. Bioeng.* 30(5), 621–627 (1987).

56 Filippusson H, Erlendsson LS, Lowe CR. Design, synthesis and evaluation of biomimetic affinity ligands for elastases. *J. Mol. Recognit.* 13(6), 370–381 (2000).

57 Li R, Dowd V, Stewart DJ, Burton SJ, Lowe CR. Design, synthesis, and application of a protein a mimetic. *Nat. Biotechnol.* 16(2), 190–195 (1998).

▪ **First paper on triazine scaffold ligands for antibody purification.**

58 Palanisamy UD, Winzor DJ, Lowe CR. Synthesis and evaluation of affinity adsorbents for glycoproteins: an artificial lectin. *J. Chromatogr. B Biomed. Sci. Appl.* 746(2), 265–281 (2000).

59 Teng SF, Sproule K, Husain A, Lowe CR. Affinity chromatography on immobilized 'biomimetic' ligands: synthesis, immobilization and chromatographic assessment of an immunoglobulin G-binding ligand. *J. Chromatogr. B Biomed. Sci. Appl.* 740(1), 1–15 (2000).

60 Roque ACA, Taipa MA, Lowe CR. An artificial Protein L for the purification of immunoglobulins and Fab fragments by affinity chromatography. *J. Chromatogr. A* 1064(2), 157–167 (2005).

61 Roque ACA, Taipa MA, Lowe CR. Synthesis and screening of a rationally designed combinatorial library of affinity ligands mimicking Protein L from *Peptostreptococcus magnus*. *J. Mol. Recognit.* 18(3), 213–224 (2005).

62 Qian J, El Khoury G, Issa H, Al-Qaoud K, Shihab P, Lowe CR. A synthetic Protein G adsorbent based on the multi-component Ugi reaction for the purification of mammalian immunoglobulins. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 898, 15–23 (2012).

63 Dong D, Liu H, Xiao Q, Li R. Affinity purification of egg yolk immunoglobulins (IgY) with a stable synthetic ligand. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 870(1), 51–54 (2008).

64 Platis D, Maltezos A, Ma JK-C, Labrou NE. Combinatorial *de novo* design and application of a biomimetic affinity ligand for the purification of human anti-HIV mAb 4E10 from transgenic tobacco. *J. Mol. Recognit.* 22(6), 415–424 (2009).

65 Platis D, Sotriffer CA, Clonis YD, Labrou NE. Lock-and-key motif as a concept for designing affinity adsorbents for protein purification. *J. Chromatogr. A* 1128(1–2), 138–151 (2006).

66 Zhao G, Dong XY, Sun Y. Ligands for mixed-mode protein chromatography: principles, characteristics and design. *J. Biotechnol.* 144(1), 3–11 (2009).

67 Naik AD, Raina M, Lali AM. AbSep - an amino acid based pseudobioaffinity adsorbent for the purification of immunoglobulin G. *J. Chromatogr. A* 1218(13), 1756–1766 (2011).

68 Vijayalakshmi MA. Pseudobiospecific ligand affinity chromatography. *Trends Biotechnol.* 7(3), 71–76 (1989).

69 Ghose S, Hubbard B, Cramer SM. Evaluation and comparison of alternative to Protein A chromatography: mimetic and hydrophobic charge induction chromatographic stationary phases. *J. Chromatogr. A* 1122(1–2), 144–152 (2006).

▪ **Comprehensive paper on small molecule ligands for protein purification.**

70 Cha J, Lim J, Zheng Y *et al.* Process automation toward ultra-high-throughput screening of combinatorial one-bead-one-compound (OBOC) peptide libraries. *J. Lab. Autom.* 17(3), 186–200 (2012).

71 Dettner F, Hänchen A, Schols D, Toti L, Nusser A, Süssmuth RD. Total synthesis of the antiviral peptide antibiotic feglymycin. *Angew. Chem. Int. Ed. Engl.* 48(10), 1856–1861 (2009).

72 Perez SA, von Hofe E, Kallinteris NL *et al.* A new era in anticancer peptide vaccines. *Cancer* 116(9), 2071–2080 (2010).

73 Uttamchandani M, Yao SQ. Peptide microarrays: next generation biochips for detection, diagnostics and high-throughput screening. *Curr. Pharm. Des.* 14(24), 2428–2438 (2008).

74 Westerlind U, Kunz H. Synthetic vaccines from tumor-associated glycopeptide antigens. *Chimia (Aarau)* 65(1–2), 30–34 (2011).

75 Wraith DC. Therapeutic peptide vaccines for treatment of autoimmune diseases. *Immunol. Lett.* 122(2), 134–136 (2009).

76 Ahrens VM, Bellmann-Sickert K, Beck-Sickinger AG. Peptides and peptide conjugates: therapeutics on the upward path. *Future Med. Chem.* 4(12), 1567–1586 (2012).

77 Albericio F, Kruger HG. Therapeutic peptides. *Future Med. Chem.* 4(12), 1527–1531 (2012).

78 Carredano E, Baumann H. Affinity ligands from chemical combinatorial libraries. *Methods Biochem. Anal.* 54, 259–267 (2011).

79 Bratkovic T. Progress in phage display: evolution of the technique and its application. *Cell. Mol. Life Sci.* 67(5), 749–767 (2010).

80 Casey JL, Coley AM, Foley M. Phage display of peptides in ligand selection for use in affinity chromatography. *Methods Mol. Biol.* 421, 111–124 (2008).

81 Lowman HB. Bacteriophage display and discovery of peptide leads for drug development. *Annu. Rev. Biophys. Biomol. Struct.* 26, 401–424 (1997).

82 Nakajima E, Goto Y, Sako Y, Murakami H, Suga H. Ribosomal synthesis of peptides with C-terminal lactams, thiolactones, and alkylamides. *Chembiochem* 10(7), 1186–1192 (2009).

83 Rothe A, Hosse RJ, Power BE. *In vitro* display technologies reveal novel biopharmaceutics. *FASEB J.* 20(10), 1599–1610 (2006).

84 Takahashi TT, Austin RJ, Roberts RW. mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.* 28(3), 159–165 (2003).

85 Lam KS, Lebl M, Krchnák V. The 'one-bead-one-compound' combinatorial library method. *Chem. Rev.* 97(2), 411–448 (1997).

■■ **Very important paper on one-bead-one-compound libraries.**

86 Lam KS, Renil M. From combinatorial chemistry to chemical microarray. *Curr. Opin. Chem. Biol.* 6(3), 353–358 (2002).

87 Liu R, Lam KS. Peptide and non-peptide combinatorial libraries. In: *Encyclopedia of Molecular Cell Biology and Molecular Medicine*. John Wiley and Sons, Inc., NJ, USA (2006).

88 Liu R, Marik J, Lam KS. A novel peptide-based encoding system for 'one-bead one-compound' peptidomimetic and small molecule combinatorial libraries. *J. Am. Chem. Soc.* 124(26), 7678–7680 (2002).

89 Yao N, Wu CY, Xiao W, Lam KS. Discovery of high-affinity peptide ligands for vancomycin. *Biopolymers* 90(3), 421–432 (2008).

90 Mondorf K, Kaufman DB, Carbonell RG. Screening of combinatorial peptide libraries: identification of ligands for affinity purification of proteins using a radiological approach. *J. Pept. Res.* 52(6), 526–536 (1998).

91 Kaufman DB, Hentsch ME, Baumbach GA *et al.* Affinity purification of fibrinogen using a ligand from a peptide library. *BIotechnol. Bioeng.* 77(3), 278–289 (2002).

92 Bastek PD, Land JM, Baumbach GA, Hammond DH, Carbonell RG. Discovery of alpha-1-proteinase inhibitor binding peptides from the screening of a solid phase combinatorial peptide library. *Separ. Sci. Technol.* 35(11), 1681–1706 (2000).

93 Gurgel PV, Carbonell RG, Swaisgood HE. Fractionation of whey proteins with a hexapeptide ligand affinity resin. *Bioseparation* 9(6), 385–392 (2000).

94 Gurgel PV, Carbonell RG, Swaisgood HE. Studies of the binding of alpha-lactalbumin to immobilized peptide ligands. *J. Agric. Food Chem.* 49(12), 5765–5770 (2001).

95 Wang G, Carbonell RG. Characterization of a peptide affinity support that binds selectively to staphylococcal enterotoxin B. *J. Chromatogr. A* 1078(1–2), 98–112 (2005).

96 Wang G, Carbonell RG. Design of adsorptive columns for specific pathogen removal: application to staphylococcal enterotoxin B. *Biotechnol. Prog.* 22(5), 1358–1367 (2006).

97 Wang G, De J, Schoeniger JS, Roe DC, Carbonell RG. A hexamer peptide ligand that binds selectively to staphylococcal enterotoxin B: isolation from a solid phase combinatorial library. *J. Pept. Res.* 64(2), 51–64 (2004).

98 Heldt CL, Gurgel PV, Jaykus LA, Carbonell RG. Identification of trimeric peptides that bind porcine parvovirus from mixtures containing human blood plasma. *Biotechnol. Prog.* 24(3), 554–560 (2008).

99 Heldt CL, Gurgel PV, Jaykus LA, Carbonell RG. Influence of peptide ligand surface density and ethylene oxide spacer arm on the capture of porcine parvovirus. *Biotechnol. Prog.* 25(5), 1411–1418 (2009).

100 Heldt CL, Hernandez R, Mudiganti U, Gurgel PV, Brown DT, Carbonell RG. A colorimetric assay for viral agents that produce cytopathic effects. *J. Virol. Methods* 135(1), 56–65 (2006).

101 Huang PY, Carbonell RG. Affinity chromatographic screening of soluble combinatorial peptide libraries. *Biotechnol. Bioeng.* 63(6), 633–641 (1999).

102 Breedveld FC. Therapeutic monoclonal antibodies. *Lancet* 355(9205), 735–740 (2000).

103 Brekke OH, Sandlie I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat. Rev. Drug. Discov.* 2(1), 52–62 (2003).

104 Melero I, Hervas-Stubbs S, Glennie M, Pardoll DM, Chen L. Immunostimulatory monoclonal antibodies for cancer therapy. *Nat. Rev. Cancer* 7(2), 95–106 (2007).

105 Elvin JG, Couston RG, van der Walle CF. Therapeutic antibodies: market considerations, disease targets and bioprocessing. *Int. J. Pharm.* 440(1), 83–98 (2013).

106 Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. *AAPS J.* 8(3), E572-E579 (2006).

107 Shukla AA, Gupta P, Han X. Protein aggregation kinetics during Protein A chromatography. Case study for an Fc fusion protein. *J. Chromatogr. A* 1171(1–2), 22–28 (2007).

108 GE Healthcare. HiTrap rProtein A FF, HiTrap Protein A HP, HiTrap Protein G HP. Uppsala, Sweden (2005).

109 Camperi SA, Iannucci NB, Albanesi GJ *et al.* Monoclonal antibody purification by affinity chromatography with ligands derived from the screening of peptide combinatory libraries. *Biotechnol. Lett.* 25(18), 1545–1548 (2003).

110 Lan JCW, Ling TC, O'sullivan DA, Lyddiatt A. Comparison of two matrices for selective recovery of C595 diabody fragment (dbFv) from *Escherichia coli* lysates. *Proc. Biochem.* 42(3), 335–343 (2007).

111 Murray A, Sekowski M, Spencer DI, Denton G, Price MR. Purification of monoclonal antibodies by epitope and

mimotope affinity chromatography. *J. Chromatogr. A* 782(1), 49–54 (1997).

112 Price MR, Hudecz F, O'Sullivan C, Baldwin RW, Edwards PM, Tendler SJ. Immunological and structural features of the protein core of human polymorphic epithelial mucin. *Mol. Immunol.* 27(8), 795–802 (1990).

113 Ehrlich GK, Bailon P. Identification of model peptides as affinity ligands for the purification of humanized monoclonal antibodies by means of phage display. *J. Biochem. Biophys. Methods* 49(1–3), 443–454 (2001).

114 Ehrlich GK, Bailon P, Berthold W. Phage display technology. Identification of peptides as model ligands for affinity chromatography. *Methods Mol. Biol.* 147, 209–220 (2000).

115 Fassina G, Ruvo M, Palombo G, Verdoliva A, Marino M. Novel ligands for the affinity-chromatographic purification of antibodies. *J. Biochem. Biophys. Methods.* 49(1–3), 481–490 (2001).

116 Fassina G, Verdoliva A, Palombo G, Ruvo M, Cassani G. Immunoglobulin specificity of TG19318: a novel synthetic ligand for antibody affinity purification. *J. Mol. Recognit.* 11(1–6), 128–133 (1998).

117 Palombo G, De Falco S, Tortora M, Cassani G, Fassina G. A synthetic ligand for IgA affinity purification. *J. Mol. Recognit.* 11(1–6), 243–246 (1998).

118 Palombo G, Rossi M, Cassani G, Fassina G. Affinity purification of mouse monoclonal IgE using a Protein A mimetic ligand (TG19318) immobilized on solid supports. *J. Mol. Recognit.* 11(1–6), 247–249 (1998).

119 Palombo G, Verdoliva A, Fassina G. Affinity purification of immunoglobulin M using a novel synthetic ligand. *J. Chromatogr. B Biomed. Sci. Appl.* 715(1), 137–145 (1998).

120 Verdoliva A, Basile G, Fassina G. Affinity purification of immunoglobulins from chicken egg yolk using a new synthetic ligand. *J. Chromatogr. B Biomed. Sci. Appl.* 749(2), 233–242 (2000).

121 Verdoliva A, Cassani G, Fassina G. Affinity purification of polyclonal antibodies using immobilized multimeric peptides. *J. Chromatogr. B Biomed. Appl.* 664(1), 175–183 (1995).

122 D'Agostino B, Bellofiore P, De Martino T, Punzo C, Rivieccio V, Verdoliva A. Affinity purification of IgG monoclonal antibodies using the D-PAM synthetic ligand: chromatographic comparison with Protein A and thermodynamic investigation of the D-PAM/IgG interaction. *J. Immunol. Methods* 333(1–2), 126–138 (2008).

123 Verdoliva A, Pannone F, Rossi M, Catello S, Manfredi V. Affinity purification of polyclonal antibodies using a new all-D synthetic peptide ligand: comparison with Protein A and Protein G. *J. Immunol. Methods* 271(1–2), 77–88 (2002).

124 Bellofiore P, Petronzelli F, De Martino T *et al.* Identification and refinement of a peptide affinity ligand with unique specificity for a monoclonal anti-tenascin-C antibody by screening of a phage display library. *J. Chromatogr. A* 1107(1–2), 182–191 (2006).

125 Lund LN, Gustavsson PE, Michael R *et al.* Novel peptide ligand with high binding capacity for antibody purification. *J. Chromatogr. A* 1225, 158–167 (2012).

126 Liu Z, Gurgel PV, Carbonell RG. Effects of peptide density and elution pH on affinity chromatographic purification of human immunoglobulins A and M. *J. Chromatogr. A* 1218(46), 8344–8352 (2011).

127 Liu Z, Gurgel PV, Carbonell RG. Purification of human immunoglobulins A, G and M from Cohn fraction II/III by small peptide affinity chromatography. *J. Chromatogr. A* 1262, 169–179 (2012).

128 Menegatti S, Naik AD, Gurgel PV, Carbonell RG. Purification of polyclonal antibodies from Cohn fraction II + III, skim milk, and whey by affinity chromatography using a hexamer peptide ligand. *J. Pept. Sci.* 35(22), 3139–3148 (2012).

129 Menegatti S, Naik AD, Gurgel PV, Carbonell RG. Alkaline-stable peptide ligand affinity adsorbents for the purification of biomolecules. *J. Chromatogr. A* 1245, 55–64 (2012).

130 Naik AD, Menegatti S, Gurgel PV, Carbonell RG. Performance of hexamer peptide ligands for affinity purification of immunoglobulin G from commercial cell culture media. *J. Chromatogr. A* 1218(13), 1691–1700 (2011).

131 Naik AD, Menegatti S, Reese HR, Gurgel PV, Carbonell RG. Process for purification of monoclonal antibody expressed in transgenic Lemna plant extract using dextran-coated charcoal and hexamer peptide affinity resin. *J. Chromatogr. A* 1260, 61–66 (2012).

132 Yang H, Gurgel PV, Carbonell RG. Hexamer peptide affinity resins that bind the Fc region of human immunoglobulin G. *J. Pept. Res.* 66(Suppl. 1), 120–137 (2005).

▪ **First publication on the authors' hexapeptide ligands for antibody purification.**

133 Yang H, Gurgel PV, Carbonell RG. Purification of human immunoglobulin G via Fc-specific small peptide ligand affinity chromatography. *J. Chromatogr. A* 1216(6), 910–918 (2009).

134 Yang H, Gurgel PV, Williams DK Jr *et al.* Binding site on human immunoglobulin G for the affinity ligand HWRGWV. *J. Mol. Recognit.* 23(3), 271–282 (2010).

135 Johnsson E, Andersson G, Lindahl G, Hedén LO. Identification of the IgA-binding region in streptococcal protein Arp. *J. Immunol.* 153(8), 3557–3564 (1994).

136 Sandin C, Linse S, Areschoug T, Woof JM, Reinholdt J, Lindahl G. Isolation and detection of human IgA using a streptococcal IgA-binding peptide. *J. Immunol.* 169(3), 1357–1364 (2002).

137 Neff S, Jungbauer A. Monolith peptide affinity chromatography for quantification of immunoglobulin M. *J. Chromatogr. A* 1218(17), 2374–2380 (2011).

138 Kish WS, Naik AD, Menegatti S, Carbonell RG. Peptide-based affinity adsorbents with high binding capacity for the purification of monoclonal antibodies. *Ind. Eng. Chem. Res.*, 52(26), 8800–8811 (2012).

139 Huang PY, Baumbach GA, Dadd CA *et al.* Affinity purification of von Willebrand factor using ligands derived from peptide libraries. *Bioorg. Med. Chem.* 4(5), 699–708 (1996).

140 Necina R, Amatschek K, Schallaun E, Schwinn H, Josic D, Jungbauer A. Peptide affinity chromatography of human clotting factor VIII. Screening of the vWF-binding

domain. *J. Chromatogr. B Biomed. Sci. Appl.* 715(1), 191–201 (1998).

▪ **Details peptide ligands for the purification of human erythropoietin, a very important biopharmaceutic.**

141 Pflegerl K, Hahn R, Berger E, Jungbauer A. Affinity chromatography of human blood coagulation factor VIII on monoliths with peptides from a combinatorial library. *J. Sep. Sci.* 23(1), 47–58 (2000).

142 Pflegerl K, Hahn R, Berger E, Jungbauer A. Mutational analysis of a blood coagulation factor VIII-binding peptide. *J. Pept. Res.* 59(4), 174–182 (2002).

143 Knör S, Khrenov A, Laufer B *et al.* Efficient factor VIII affinity purification using a small synthetic ligand. *J. Thromb. Haemost.* 6(3), 470–477 (2008).

144 Knör S, Khrenov AV, Laufer B, Saenko EL, Hauser CA, Kessler H. Development of a peptidomimetic ligand for efficient isolation and purification of factor VIII via affinity chromatography. *J. Med. Chem.* 50(18), 4329–4339 (2007).

145 Knör S, Laufer B, Khrenov A *et al.* Development of a small molecule peptidomimetic affinity ligand for efficient purification of the large protein factor VIII. *Adv. Exp. Med. Biol.* 611, 151–152 (2009).

146 Buettner JA, Dadd CA, Baumbach GA, Masecar BL, Hammond DJ. Chemically derived peptide libraries: a new resin and methodology for lead identification. *Int. J. Pept. Protein Res.* 47(1–2), 70–83 (1996).

147 Martínez-Ceron MC, Marani MM, Taules M *et al.* Affinity chromatography based on a combinatorial strategy for erythropoietin purification. *ACS Comb. Sci.* 13, 251–258 (2011).

148 Kang TS, Stevens RC. Structural aspects of therapeutic enzymes to treat metabolic disorders. *Hum. Mutat.* 30(12), 1591–1610 (2009).

149 Lequette Y, Boels G, Clarisse M, Faille C. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. *Biofouling* 26(4), 421–431 (2010).

150 McGrath BM, Walsh G. Directory of therapeutic enzymes. *Biochemistry (Moscow)* 71(11), 1289–1290 (2006).

151 Murray BA, Fitzgerald RJ. Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Curr. Pharm. Des.* 13(8), 773–791 (2007).

152 Vellard M. The enzyme as drug: application of enzymes as pharmaceuticals. *Curr. Opin. Biotechnol.* 14(4), 444–450 (2003).

153 Shanley N, Walsh G. Applied enzymology: an overview. In: *Directory of therapeutic enzymes*, Mcgrath BM,Walsh G (Eds). Taylor and Francis, London, UK, 1–16 (2006).

154 O'Connel S. Additional therapeutic enzymes. In: *Directory of therapeutic enzymes*. Mcgrath BM, Walsh G (Eds). Taylor and Francis, London, UK, 261–290 (2006).

155 Aggarwal S. What's fueling the biotech engine – 2010 to 2011. *Nat. Biotechnol.* 29(12), 1083–1089 (2011).

156 McGrath BM. Enzyme engineering. In: *Directory of therapeutic enzymes*, Mcgrath BM, Walsh G (Eds). Taylor and Francis, London, UK, 17–54 (2006).

157 Welling GW, Geurts T, Van Gorkum J, Damhof RA, Drijfhout JW. Synthetic antibody fragment as ligand in immunoaffinity chromatography. *J. Chromatogr.* 512, 337–343 (1990).

158 Welling GW, van Gorkum J, Damhof RA, Drijfhout JW, Bloemhoff W, Welling-Wester S. A ten-residue fragment of an antibody (mini-antibody) directed against lysozyme as ligand in immunoaffinity chromatography. *J. Chromatogr.* 548(1–2), 235–242 (1991).

159 Lasonder E, Bloemhoff W, Welling GW. Interaction of lysozyme with synthetic anti-lysozyme D1.3 antibody fragments studied by affinity chromatography and surface plasmon resonance. *J. Chromatogr. A* 676(1), 91–98 (1994).

160 Smith GP, Schultz DA, Ladbury JE. A ribonuclease S-peptide antagonist discovered with a bacteriophage display library. *Gene* 128(1), 37–42 (1993).

161 Lam KS, Wu J, Lou Q. Identification and characterization of a novel synthetic peptide substrate specific for Src-family protein tyrosine kinases. *Int. J. Pept. Protein Res.* 45(6), 587–592 (1995).

162 Samson I, Kerremans L, Rozenski J *et al.* Identification of a peptide inhibitor against glycosomal phosphoglycerate kinase of *Trypanosoma brucei* by a synthetic peptide library approach. *Bioorg. Med. Chem.* 3(3), 257–265 (1995).

163 Makriyannis T, Clonis YD. Design and study of peptide-ligand affinity chromatography adsorbents: application to the case of trypsin purification from bovine pancreas. *Biotechnol. Bioeng.* 53(1), 49–57 (1997).

164 Mcintyre GD, Leckie B, Hallett A, Szelke M. Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-LeuR-Leu-Val-Tyr). *Biochem J.* 211(2), 519–522 (1983).

165 Wong DWS, Robertson GH, Tillin SJ, Wong C. identifying peptide ligands for barley α-amylase 1 using combinatorial phage display libraries. *J. Agric. Food Chem.* 46(9), 3852–3857 (1998).

166 Liu FF, Wang T, Dong XY, Sun Y. Rational design of affinity peptide ligand by flexible docking simulation. *J. Chromatogr. A* 1146(1), 41–50 (2007).

167 Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* 354(6348), 82–84 (1991).

168 Pennington ME, Lam KS, Cress AE. The use of a combinatorial library method to isolate human tumor cell adhesion peptides. *Mol. Divers.* 2(1–2), 19–28 (1996).

169 Dong XY, Fu L, Yu H. Affinity purification of insulin by peptide-ligand affinity chromatography. *Transactions of Tianjin University* 5, 313–317 (2007).

170 Lehman A, Gholami S, Hahn M, Lam KS. Image subtraction approach to screening one-bead-one-compound combinatorial libraries with complex protein mixtures. *J. Comb. Chem.* 8(4), 562–570 (2006).

171 Lathrop JT, Fijalkowska I, Hammond D. The Bead blot: a method for identifying ligand-protein and protein-protein

interactions using combinatorial libraries of peptide ligands. *Anal. Biochem.* 361(1), 65–76 (2007).

172 Noppe W, Plieva FM, Galaev IY, Vanhoorelbeke K, Mattiasson B, Deckmyn H. Immobilised peptide displaying phages as affinity ligands. Purification of lactoferrin from defatted milk. *J. Chromatogr. A* 1101(1–2), 79–85 (2006).

173 Miyamoto S, Liu R, Hung S, Wang X, Lam KS. Screening of a one bead-one compound combinatorial library for beta-actin identifies molecules active toward Ramos B-lymphoma cells. *Anal. Biochem.* 374(1), 112–120 (2008).

174 Xiao W, Wang Y, Lau EY *et al.* The use of one-bead one-compound combinatorial library technology to discover high-affinity αvβ3 integrin and cancer targeting arginine-glycine-aspartic acid ligands with a built-in handle. *Mol. Cancer Ther.* 9(10), 2714–2723 (2010).

175 Katsara M, Tselios T, Deraos S *et al.* Round and round we go: cyclic peptides in disease. *Curr. Med. Chem.* 13(19), 2221–2232 (2006).

176 Khan SN, Kim A, Grubbs RH, Kwon YU. Ring-closing metathesis approaches for the solid-phase synthesis of cyclic peptoids. *Org. Lett.* 13(7), 1582–1585 (2011).

177 Liu T, Joo SH, Voorhees JL, Brooks CL, Pei D. Synthesis and screening of a cyclic peptide library: discovery of small-molecule ligands against human prolactin receptor. *Bioorg. Med. Chem.* 17(3), 1026–1033 (2009).

178 Young TS, Young DD, Ahmad I, Louis JM, Benkovic SJ, Schultz PG. Evolution of cyclic peptide protease inhibitors. *Proc. Natl. Acad. Sci. USA* 108(27), 11052–11056 (2011).

179 Giebel LB, Cass RT, Milligan DL, Young DC, Arze R, Johnson CR. Screening of cyclic peptide phage libraries identifies ligands that bind streptavidin with high affinities. *Biochemistry* 34(47), 15430–15435 (1995).

180 Meyer SC, Gaj T, Ghosh I. Highly selective cyclic peptide ligands for NeutrAvidin and avidin identified by phage display. *Chem. Biol. Drug. Des.* 68(1), 3–10 (2006).

181 Krook M, Lindbladh C, Eriksen JA, Mosbach K. Selection of a cyclic nonapeptide inhibitor to alpha-chymotrypsin using a phage display peptide library. *Mol. Divers.* 3(3), 149–159 (1997–1998).

182 Kelley BD, Tannatt M, Magnusson R, Hagelberg S, Booth J. Development and validation of an affinity chromatography step using a peptide ligand for cGMP production of factor VIII. *Biotechnol. Bioeng.* 87(3), 400–412 (2004).

183 Verdoliva A, Marasco D, De Capua A *et al.* A new ligand for immunoglobulin g subdomains by screening of a synthetic peptide library. *Chembiochem* 6(7), 1242–1253 (2005).

184 Hatanaka T, Ohzono S, Park M *et al.* Human IgA-binding peptides selected from random peptide libraries: affinity maturation and application in IgA purification. *J. Biol. Chem.* 287(51), 43126 (2012).

185 Mclafferty MA, Kent RB, Ladner RC, Markland W. M13 bacteriophage displaying disulfide-constrained microproteins. *Gene* 128(1), 29–36 (1993).

▪ **Pioneering work on phage-display libraries.**

186 Andersson LO. Hydrolysis of disulfide bonds in weakly alkaline media. II. Bovine serum albumin dimer. *Biochim. Biophys. Acta* 200(2), 363–369 (1970).

187 Federici G, Duprè S, Matarese RM, Solinas SP, Cavallini D. Is the alkaline cleavage of disulfide bonds in peptides an alpha-beta elimination reaction or a hydrolysis? *Int. J. Pept. Protein Res.* 10(3), 185–189 (1977).

188 Gilbert HF. Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol.* 251, 8–28 (1995).

189 Millward SW, Takahashi TT, Roberts RW. A general route for post-translational cyclization of mRNA display libraries. *J. Am. Chem. Soc.* 127(41), 14142–14143 (2005).

190 Millward SW, Fiacco S, Austin RJ, Roberts RW. Design of cyclic peptides that bind protein surfaces with antibody-like affinity. *ACS Chem. Biol.* 2(9), 625–634 (2007).

191 Menegatti S, Hussain M, Naik AD, Carbonell RG, Rao BM. mRNA display selection and solid-phase synthesis of Fc-binding cyclic peptide affinity ligands. *Biotechnol. Bioeng.* 110(3), 857–870 (2013).

192 Schlippe YVG, Hartman MC, Josephson K, Szostak JW. *In vitro* selection of highly modified cyclic peptides that act as tight binding inhibitors. *J. Am. Chem. Soc.* 134(25), 10469–10477 (2012).

193 Blackburn C, Kates SA. Solid-phase synthesis of cyclic homodetic peptides. *Methods Enzymol.* 289, 175–198 (1997).

194 Romanovskis P, Spatola AF. Preparation of head-to-tail cyclic peptides via side-chain attachment: implications for library synthesis. *J. Pept. Res.* 52(5), 356–374 (1998).

195 Grubbs RH, Miller SJ, Fu GC. Ring-closing metathesis and related processes in organic synthesis. *Acc. Chem. Res.* 28(11), 446–452 (1995).

196 Kazmaier U, Hebach C, Watzke A, Maier S, Mues H, Huch V. A straightforward approach towards cyclic peptides via ring-closing metathesis – scope and limitations. *Org. Biomol. Chem.* 3(1), 136–145 (2005).

197 Miller SJ, Blackwell HE, Grubbs RH. Application of ring-closing metathesis to the synthesis of rigidified amino acids and peptides. *J. Am. Chem. Soc.* 118(40), 9606–9614 (1996).

198 Reichwein JF, Versluis C, Liskamp RM. Synthesis of cyclic peptides by ring-closing metathesis. *J. Org. Chem.* 65(19), 6187–6195 (2000).

199 Scholl M, Trnka TM, Morgan JP, Grubbs RH. ChemInform abstract: increased ring closing metathesis activity of ruthenium-based olefin. metathesis catalysts coordinated with imidazolin-2-ylidene ligands. *ChemInform* 30(25), (2010).

200 Robinson AJ, Elaridi J, Van Lierop BJ, Mujcinovic S, Jackson WR. Microwave-assisted RCM for the synthesis of carbocyclic peptides. *J. Pept. Sci.* 13(4), 280–285 (2007).

201 Jagasia R, Holub JM, Bollinger M, Kirshenbaum K, Finn MG. Peptide cyclization and cyclodimerization by cui-mediated azide-alkyne cycloaddition. *J. Org. Chem.* 74(8), 2964–2974 (2009).

202 Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem. Int. Ed. Engl.* 40(11), 2004–2021 (2001).

203 Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. *Drug Discov. Today* 8(24), 1128–1137 (2003).

204 Moses JE, Moorhouse AD. The growing applications of click chemistry. *Chem. Soc. Rev.* 36(8), 1249–1262 (2007).

205 Turner RA, Oliver AG, Lokey RS. Click chemistry as a macrocyclization tool in the solid-phase synthesis of small cyclic peptides. *Org. Lett.* 9(24), 5011–5014 (2007).

206 Eckart K, Schwarz H, Tomer KB, Gross ML. Tandem mass spectrometry methodology for the sequence determination of cyclic peptides. *J. Am. Chem. Soc.* 107(24), 6765–6769 (1985).

207 Joo SH, Xiao Q, Ling Y, Gopishetty B, Pei D. High-throughput sequence determination of cyclic peptide library members by partial Edman degradation/mass spectrometry. *J. Am. Chem. Soc.* 128(39), 13000–13009 (2006).

208 Lin S, Liehr S, Cooperman BS, Cotter RJ. Sequencing cyclic peptide inhibitors of mammalian ribonucleotide reductase by electrospray ionization mass spectrometry. *J. Mass Spectrom.* 36(6), 658–663 (2001).

209 Ngoka LC, Gross ML. Multistep tandem mass spectrometry for sequencing cyclic peptides in an ion-trap mass spectrometer. *J. Am. Soc. Mass Spectrom.* 10(8), 732–746 (1999).

210 Redman JE, Wilcoxen KM, Ghadiri MR. Automated mass spectrometric sequence determination of cyclic peptide library members. *J. Comb. Chem.* 5(1), 33–40 (2003).

211 Schilling B, Wang W, McMurray JS, Medzihradszky KF. Fragmentation and sequencing of cyclic peptides by matrix-assisted laser desorption/ionization post-source decay mass spectrometry. *Rapid Commun. Mass Spectrom.* 13(21), 2174–2179 (1999).

212 Zhang Y, Zhou S, Wavreille AS, DeWille J, Pei D. Cyclic peptidyl inhibitors of Grb2 and tensin SH2 domains identified from combinatorial libraries. *J. Comb. Chem.* 10(2), 247–255 (2008).

213 Liu T, Qian Z, Xiao Q, Pei D. High-throughput screening of one-bead-one-compound libraries: identification of cyclic peptidyl inhibitors against calcineurin/NFAT interaction. *ACS Comb. Sci* 13(5), 537–546 (2011).

214 Dewan V, Liu T, Chen KM *et al.* Cyclic peptide inhibitors of HIV-1 capsid-human lysyl-tRNA synthetase interaction. *ACS Chem. Biol.* 7(4), 761–769 (2012).

215 Thakkar A, Cohen AS, Connolly MD, Zuckermann RN, Pei D. High-throughput sequencing of peptoids and peptide-peptoid hybrids by partial edman degradation and mass spectrometry. *J. Comb. Chem.* 11(2), 294–302 (2009).

216 Menegatti S, Ward KL, Naik AD, Kish WS, Blackburn RK, Carbonell RG. Reversible cyclic peptide libraries for the discovery of novel affinity ligands. *Anal. Chem.* 85(19), 9229–9237 (2013).

217 Krishnamurty VM, Estroff LA, Whitesides GM. Multivalency in ligand design. In: *Fragment-based approaches in drug discovery*, Jahnke W, Erlanson DA (Eds). John Wiley and Sons, Inc., NJ, USA (2006).

218 Angelini A, Heinis C. Post-translational modification of genetically encoded polypeptide libraries. *Curr. Opin. Chem. Biol.* 15(3), 355–361 (2011).

▪ **Pioneering work on polycyclic peptides.**

219 Heinis C. Bicyclic peptide antagonists derived from genetically encoded combinatorial libraries. *Chimia (Aarau)* 65(9), 677–679 (2011).

220 Heinis C, Rutherford T, Freund S, Winter G. Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nat. Chem. Biol.* 5(7), 502–507 (2009).

221 Rentero Rebollo I, Angelini A, Heinis C. Phage display libraries of differently sized bicyclic peptides. 4, 145–150 *Med. Chem. Comm*. (2012).

222 Touati J, Angelini A, Hinner MJ, Heinis C. Enzymatic Cyclisation of Peptides with a Transglutaminase. *ChemBioChem* 12(1), 38–42 (2011).

223 Baeriswyl V, Heinis C. Polycyclic peptide therapeutics. *ChemMedChem* 8, 377–384 (2013).