Pharmaceutical BIOPROCESSING

Bespoke affinity ligands for the purification of therapeutic proteins

This article provides an overview of the current challenges and trends in bioprocessing, with a focus on recent advances in the affinity purification of the principal classes of biotherapeutics, including monoclonal antibodies, glycoproteins, vaccines and erythropoietin. Affinity chromatography is usually applied during large-scale protein purification; it involves affinity ligands of biological or synthetic origin. The high productivity that is currently achieved during upstream processing is placing an increasing burden on the downstream production phase which suffers from limited capacities. Consequently, while genetic engineering is helping to increase the stability and capacity of natural ligands, *in silico* approaches combined with combinatorial chemistry may be used to implement economical purification strategies based on synthetic ligands for the improvement of downstream processing and biomanufacturing.

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Therapeutic proteins currently constitute a group of blockbuster products of the pharmaceutical industry, with forecasted sales expected to reach US$165 billion by 2018 [1]. The principal classes of biopharmaceuticals include monoclonal antibodies (mAbs), growth hormones, insulin, vaccines, enzymes, cytokines, blood clotting factors and erythropoietin (EPO). Recombinant therapeutic proteins have been playing a prominent role in the treatment of several diseases, such as cancer and cardiovascular, inflammatory, autoimmune and neurological disorders. In addition, more than two-thirds of these biologics are glycoproteins [2]. However, some licensed therapeutic proteins are starting to lose their patent protection and will be replaced by biologic drugs known as biosimilars. These products resemble the original biotherapeutic drugs in terms of efficacy and safety, but they are developed by different manufacturers. Additionally, more than 700 biosimilar products are currently under development and this is also driving competitive companies to reduce manufacturing costs [3]. Moreover, continuous development of large-scale bioreactor production of recombinant proteins and a significant increase in upstream titers (approximately 10 g/l of mAbs) is raising the pressure on downstream processing and resulting in higher process-related costs [4].

Chromatography is the main unit operation in bioprocessing; however, it also constitutes the major downstream bottleneck due to the employment of costly chromatographic adsorbents. It consists of capture and polishing steps for the removal of aggregates and impurities arising from host cells. During the capture step, affinity chromatography is still the most commonly used method that can achieve high selectivity, purity and yield [5]. Specific biorecognition between affinity ligands and target proteins is driven by key functional groups and is based on multiple molecular interactions of hydrophobic and hydrogen bonds in addition to electrostatic and Van der Waals forces.

A wide range of affinity ligands is being used depending on the biophysical properties of the protein to be purified. Affinity adsorbents were first based on biological or biospecific ligands such as immunoglobulins, enzymes, bacterial proteins, lectins,

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Key terms

Biospecific adsorbents: Ligands of biological origin, derived from natural sources such as bacteria and yeast, able to bind specific target molecules. They include bacterial immunoglobulin-binding proteins, lectins, antibodies and nucleic acids.

Pseudobiospecific affinity ligands: They are fully synthetic ligands that display affinity to a specific target. They are classified as biomimetics (peptidic and nonpeptidic *de novo* ligands) and nonbiomimetics (metals and dye-ligands).

hormones and nucleic acids. More recently, camelid-derived single domain antibody fragments and engineered antibodies such as affibodies have been developed [6].

However, despite significant advancements in the development of **biospecific adsorbents**, some limitations are still being encountered in bioprocessing; they are mainly related to biomolecules production cost, their lot-to-lot variability, toxicity and low stability during cleaning-in-place and sterilization-in-place treatments [7]. This has contributed enormously to the emergence of inexpensive synthetic **pseudobiospecific affinity ligands** which include immobilized metals, hydrophobic ligands, dyes and biomimetic ligands [8–11]. Biomimetic ligands, in particular, have evolved from peptidic ligands to rationally designed *de novo* ligands, such as triazine and Ugi ligands, based on combinatorial chemistry and supported by *in silico* molecular modeling [12–16]. In addition, various affinity approaches such as expanded-bed chromatography, continuous chromatography and monolithic chromatography are also being applied in order to reduce bioprocessing cost and downstream steps [17].

This review tackles challenges currently encountered in affinity chromatography for the purification of therapeutic proteins, including mAbs and their fragments, glycoproteins, vaccines and EPO, with an emphasis on the development of *de novo* affinity ligands for the implementation of cost-effective downstream processing protocols.

Bioprocessing: challenges & future needs

Due to the burgeoning costs of healthcare expenditure and the emergence of biosimilars, there is increasing pressure on the biopharmaceutical industry to reduce manufacturing costs [18]. Biopharmaceutical production is currently facing many challenges in the upstream and downstream processing; in particular, large-scale purification of high-titer products that results from high-dose requirements and large volume demand.

Major manufacturing expenses include the use of large-scale stainless steel bioreactors (≥1000–2000 l) which contribute to approximately 90% of the upstream bioprocessing costs, and purification of high concentrations (approximately 5–10 g/l) of recombinant proteins from culture media (Chinese hamster ovary [CHO], yeast or *Escherichia coli* cell supernatants) [19,20], which may account for approximately 50–80% of the total production cost [21]. In addition, removal of contaminants and reduction of protein aggregates which display potential immunogenicity, also constitute critical prerequisite for effective bioprocessing.

Chromatographic separation techniques that consist of multistep downstream procedures are typically used to remove host-cell nucleic acids and proteins, aggregates, endotoxins and viruses from cell-culture harvest material. The process usually includes a capture step such as affinity chromatography, followed by intermediate purification with cation exchange (CEX) chromatography or hydrophobic interaction chromatography for the removal of aggregates, and a polishing step consisting of anion exchange (AEX) chromatography to remove host cell proteins and DNA, endotoxins and viruses [22]. In addition, other operational methods such as expanded-bed adsorption chromatography and mixed-mode chromatography are employed for the separation of biotherapeutics from aggregates and endotoxins [23–25].

The removal of impurities arising from host cells is an essential step in purification. Current good manufacturing practice (cGMP) regulations specify that the content of host cell proteins should be less than 100 ppm and aggregates less than 1%, whereas host cell DNA ≤100 pg [26,27]; consequently, highly sensitive quantitative PCR-based systems have been developed [28]. In addition, various analytical methods are applied in order to detect and quantify different contaminants and leachable components in crude systems. These include high-pressure liquid chromatography–mass spectrometry, UV-visible spectroscopy, gas chromatography and Fourier-transform infrared spectroscopy [29,30]. Moreover, high-throughput approaches are being employed in order to improve screening and process optimization, reduce costs and save processing time.

Disposable bioreactors, usually made of plastic, are now being considered as alternative systems for upstream processing. However, due to aspects like leachables and extractables, the utilization of single-use technologies in commercial applications is still limited. They are being tested at small scale in research and development and clinical assays [31], and they have been employed in few facilities at some pharmaceutical companies (e.g., Shire Plc). On the other hand, during the affinity chromatography step of downstream pro-

cessing, biological adsorbents are still being extensively used despite their high cost; in particular, antibody-binding ligands, such as protein A, which are also being improved to achieve high capacity and stability and lower ligand leaching. Additionally, single-use membrane absorbents [32] and monoliths [33,34], as well as continuous chromatography systems, are being considered for the reduction of purification costs. In fact, although single-use membrane adsorber technologies are still a very small segment, they are also one of the fastest growing downstream processing technologies in terms of evaluation and adoption. Moreover, synthetic affinity ligands, particularly nonpeptidic adsorbents, in other words, biomimetics, have also shown to be efficient for the implementation of economical and 'greener' alternative approaches, but they are still at a very early stage of development [7]. In addition to innovations in single-use bioreactors and purification platforms, further improvements in different areas of bioprocessing are being achieved, with advanced statistical and engineering approaches that are helping to speed process optimization [35].

Affinity chromatography: current approaches

During the past few decades, an array of ligands were developed and studied to improve protein purification. The affinity ligand, a stationary phase that selectively retains a target of interest, is an important factor that determines the success of affinity chromatography [36]. Several parameters determine the success of an affinity ligand; these include the ligand's affinity to its target, its specificity, feasibility of immobilization, stability in harsh washing and elution conditions and retention of target-binding capacity after attachment to the matrix [37]. Most immobilized ligands that are being used currently are biospecific ligands; a group of naturally derived substances such as antibody-binding proteins, antigens, bacterial receptors, enzymes, transport proteins and carbohydrate-binding proteins such as lectins.

Anti-antibodies offer an attractive approach with relatively large binding constants. They also provide high specificity for antibody purification with the heavy- and light-chain-antibody constant domains (C_H and C_L) as potential targets. These can be exemplified by **single-domain camelid antibodies** which offer the advantages of small size, high affinity, stability and a 3D structure that enables binding to novel epitopes [38]. Antibodies or related agents can be immobilized on traditional affinity supports such as agarose or attached to alternative supports including silica or monolithic materials. They can be used to purify hormones, enzymes, peptides, viruses and other biologically relevant substances [39]. Antigen-specific antibodies can be isolated by immobilizing antigens, either whole or specific peptides representing antigenic epitopes, onto chromatographic matrices [40]. However, nonuniformity of the antibody ligand immobilized to the column can reduce the capture efficiency of the target therapeutic protein. Additionally, the large molecular size of antibodies often limits their coupling efficiency to the stationary phase and its resultant binding capacity.

The most commonly used ligands in the downstream processing of mAbs and Fc-fusion proteins are Staphylococcal protein A (from *Staphylococcus aureus*) and Streptococcal protein G (from group C and G streptococci) isolated from bacterial cell walls. They allow binding of antibodies from various species, although, their affinities vary according to antibody subclasses [41,42]. Compared with protein A and G, protein L, isolated from *Peptostreptococcus magnus*, has the ability to bind immunoglobulins from different classes (IgG, IgM, IgY, IgD and IgE). However, the application of this method requires the production and purification of the pure natural ligands for immobilization to the affinity matrix. Harsh elution conditions, such as extreme pH, detergents or organic solvents can easily denature biomolecules coupled to affinity columns [37]. Immunoaffinity and other biological media, in general, cannot readily withstand strong cleaning and sanitization conditions, and often leakage of biomolecules takes place during purification.

As an alternative, aptamers, also termed 'chemical antibodies', have been proposed as substitute antibodies. Aptamers are single stranded DNAs, RNAs or a combination of nucleic acids with nonnatural nucleotides capable of adopting 3D structures that interact specifically with a target molecule [43]. Aptamers were developed as affinity ligands for a large number of small molecules, soluble proteins, membrane-bound receptors, cell surface epitopes, entire cells and even whole organisms [44–47]. Aptamers have a higher affinity to large size targets, with dissociation constants (K_d) within picomolar or nanomolar concentrations [48]. However, the identification of aptamers capable of binding to a specific target site is not rational. Instead, it involves random screening from vast combinatorial libraries that comprise 10^{13}–10^{15} different sequence motifs by numerous cycles of *in vitro* selection [49]. Moreover, being biologically oriented, chemical

Key term

Single-domain camelid antibodies: They constitute the variable region or antigen-binding domain (VHH) of heavy-chain antibodies found in the Camelidae family (camels and llamas). The single VHH domain (~12 kDa) is derived from a fully functional immunoglobulin.

modifications are needed to enhance their nuclease resistance, binding affinity or structural stability [50].

Despite the extensive use of biospecific ligands in protein purification, this class of ligand presents various drawbacks. The majority of these ligands are produced in bacteria, which also requires their purification and this increases manufacturing and bioprocessing costs, in addition to the risk of contamination by viruses, pyrogens and DNA. Further disadvantages include instability, potential ligand leakage, storage, labor, sterilization and low binding capacities. These shortcomings have led to an ongoing pursuit for alternative ligands with improved characteristics to replace biospecific ligands [51].

Pseudobiospecifc ligands exploit intrinsic properties of the protein at the molecular level. Commonly used pseudobiospecific ligands include hydrophobic, thiophilic, mixed-mode affinity ligands, metals, dyes and biomimetic ligands [7]. They are promising candidates in comparison to biospecific ligands with the advantages of being cheaper, more robust, structurally simple, less toxic and highly stable with resistance to harsh sterilization conditions. Although the affinity of such ligands is generally lower than that of biospecific ligands, it is sufficient to ensure selectivity. However, pseudobiospecific ligands are not as specific as biological ligands and thus, significant process optimization is required for each individual protein to attain high selectivity. As a result, pseudobiospecific ligands have yet to achieve widespread acceptability as sole ligands in downstream protocols [37].

In order to overcome these issues, great effort has been put into the discovery and validation of more efficient and less expensive purification methods. Synthetic ligands are a new genre of compounds that circumvent most of the shortcomings associated with traditional biospecific ligands. Synthetic ligands allow for easy chemical modifications, high-density stationary phase immobilization and, additionally, they are often resistant toward degradation. Synthetic ligands include metal-ion, dye-based, peptidic and biomimetic peptoidal ligands. Dye-based ligands rely on the binding of reactive dyes to proteins selectively and reversibly; examples include Cibacron-Blue F-G3A (GE Healthcare Life Sciences, NJ, USA), which has been used for decades for protein purification [52]. Metal ions as immobilized ligands have seen growing use in analyzing membrane proteins, histidine-tagged proteins and phosphorylated proteins [53]. Metal ions such as Ni^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+} are often used and have specific interactions with targets like amino acids, peptides, proteins and nucleic acids. Peptidic ligands are short linear peptides composed of amino acid residues of different physicochemical properties; the most common example is the multimeric peptide, protein A mimetic, specific for the Fc fragment of immunoglobulins. Nevertheless, synthetic ligands expand the capacity of purification strategies and offer a reliable, cost-effective, scalable and stable means of purification. Rational design, combinatorial synthesis and parallel screening of several biomimetic triazine and Ugi ligands, known as *de novo* ligands, have resulted in the successful purification of various proteins at small scale [7,12–16].

Design, synthesis & evaluation of *de novo* affinity ligands

The development of potential low-cost affinity ligands for target therapeutic proteins involves a general strategy that includes computer-aided design based on the analyses of x-ray crystallographic structures and NMR data of biomolecules and combinatorial chemistry. The design of *de novo* synthetic ligands relies on the fundamental understanding of specific molecular interactions of the complexes between the target protein and its natural ligand if they are available, or through structural studies of the protein of interest, in order to identify potential binding sites (Figure 1). Each ligand of the designed library consists of different components that mimic key amino acid residues of the natural ligand's binding site, or is potentially based on complementarity of target residues; thus, the ligand functional groups are designed to interact specifically with the target-binding site. The ligands are synthesized on chromatographic resins such as chemically modified agarose beads; the synthetic route first involved triazine chemistry and was based on the substitution of 1,3,5-*sym*-trichlorotriazine with two amine compounds that allow different protein interaction mechanisms. This synthetic approach has since evolved to generate greater ligand variety by using the four-component Ugi reaction in which an oxo-component (i.e., aldehyde-activated agarose beads), a primary or secondary amine, a carboxylic acid and an isonitrile component are condensed, in a one-pot reaction, to yield a peptoidal scaffold product (Figure 2A) [12,54]. In comparison with triazine chemistry, the Ugi reaction allows greater scaffold diversity (Figure 2B). Potential Ugi ligands have been developed recently for the purification of various proteins of interest, including immunoglobulins and their fragments, recombinant human EPO (rHuEPO) and glycoproteins [12–16]. For example, a protein G mimetic was developed for antibody purification by energy minimizing the configuration of an ideal synthetic ligand to resemble the wild-type residues in bacterial protein G in terms functional groups, solvent accessibility and hydrophobicity. It has been shown previously that harsh elution conditions, such as a very low pH and high concentrations of

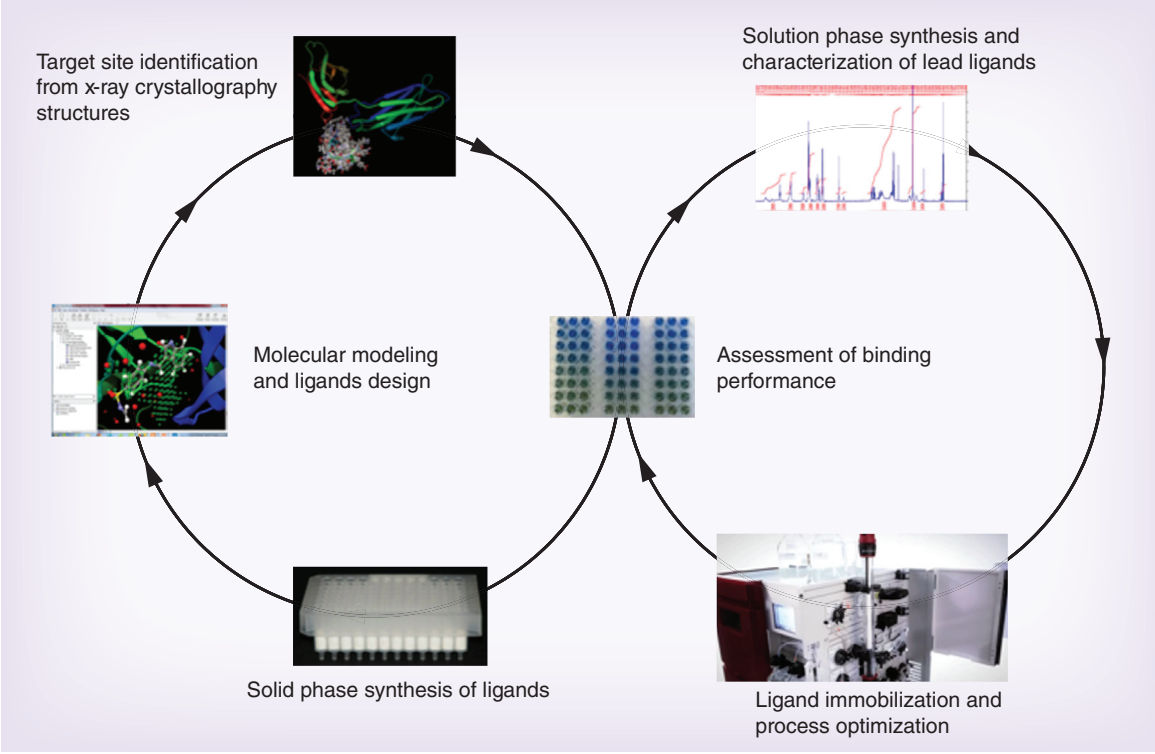

Figure 1. Development of *de novo* affinity ligands. The research strategy includes *in silico* molecular modeling and design of ligand libraries based on structural analyses of biomolecule complexes. The library is synthesized by combinatorial approaches, screened and chromatographic performance of the ligands is assessed. Lead ligands are then synthesized in solution and characterized before immobilization on the chromatographic matrix for process optimization.

hydrophobic bond disruptors, such as ethylene glycol or propylene glycol are needed to release the captured product [13,17]. However, these conditions can damage some the eluted proteins and reduce the overall purification yield. Hence, the ideal ligands will have an acceptable selectivity and a moderate affinity toward the target product ($K_d \sim 10^{-7}$–10^{-5} M).

To date, the efficiency of Ugi ligands has been evaluated mainly with small-scale affinity chromatography using pure and crude biological samples, in other words, pure immunoglobulins, EPO and glycoproteins, CHO and yeast cell supernatants; the purity of the isolated proteins was estimated with SDS-PAGE lane densitometry and values in the range of 80–99% purity were achieved in a single step. In addition, the affinity of different Ugi ligands was evaluated with partition equilibrium studies and the ligands displayed affinity constants (K_d) in the range 10^{-7}–10^{-5} M toward their target proteins [12–16]. However, it is difficult to analyze and characterize the ligands synthesized directly on a solid phase consisting of 6% cross-linked agarose beads, and in order to provide supporting evidence for the synthesis of the ligands on the resin, they have been evaluated through control experiments by testing the chromatographic performance of the Ugi components at each stage of the organic synthesis and solution-phase synthesis of ligands similar to those immobilized on the matrix and analysis with liquid chromatography–mass spectrometry and NMR spectroscopy [12–16]. In addition, the affinity adsorbents *per se* are being currently investigated, and new techniques such as solid state NMR will be used to analyze the molecular structure of the ligands synthesized *in situ* on the solid matrix.

Affinity purification of therapeutic proteins

The affinity technique applied during the capture step of the purification process depends mainly on the biochemical and physical properties of the protein to be purified, the expression system used and the production scale. A wide variety of natural and synthetic affinity adsorbents are currently available; however, due to downstream bottlenecks, and in an attempt to reduce the number of purification steps, enormous effort is being devoted to develop potential and cost-effective affinity ligands, with respect to their safety, efficacy, stability and compliance with cGMP. Advances and challenges in the affinity purification of different proteins of interest in the biopharmaceutical sector are addressed below; these include mAbs and their fragments, glycoproteins, vaccines and rHuEPO.

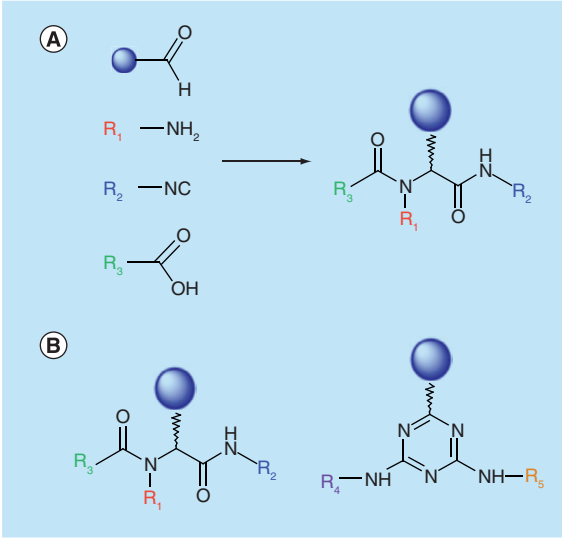

Figure 2. *De novo* affinity ligands immobilized on solid support. (A) Ugi ligand formation: Ugi reaction involves an oxo-component (e.g., aldehyde-activated agarose beads; blue sphere), a primary or secondary amine (R_1), an isonitrile group (R_2) and a carboxylic acid (R_3) which are condensed to generate a single scaffold. **(B)** Comparison of Ugi and triazine ligands immobilized on agarose-functionalized matrices (immobilized triazine ligand is formed by substitution of 1,3,5-*sym*-trichlorotriazine with two primary amine components R_4 and R_5). Only one ligand is displayed per bead for simplicity. Chemical structures were drawn using MarvinSketch (v4.1.13; ChemAxon Ltd, Hungary). Adapted with permission from [12].

Monoclonal antibodies & antibody fragments

Therapeutic mAbs constitute one of the best-selling biopharmaceuticals, with a global market expected to reach US$58 billion by 2016 [55]. They have been successfully applied in the treatment of various diseases, including cancer, arthritis and other inflammatory disorders [56]. The current gold standard in the industry for the purification of mAbs and antibody-based products is the use of protein A affinity resins [57]. Protein A is able to bind specifically to immunoglobulins by interacting primarily with the hinge region between the CH_2 and CH_3 domains of the Fc fragment. However, despite its superior performance in purifying antibody products, protein A has several disadvantages: affinity columns are very expensive (i.e., US$15,000 per l of resin) [58], the biological nature of the ligand makes it susceptible to proteolytic degradation and harsh conditions are used for protein elution and column regeneration (i.e., acidic pH). Purification of Fab fragments and other isotypes of immunoglobulin involves other immobilized bacterial proteins such as protein G and protein L, which mainly bind to the Fab region of antibodies [59]; they are often used to purify immunoglobulin subclasses which are not able to bind to protein A, such as IgG_3 [7].

Nevertheless, all biological ligands share similar drawbacks. However, recombinant protein A affinity resins are continuously undergoing significant improvement, particularly in terms of stability and binding capacity. Examples include, Toyopearl AF-rProtein A-650F, a recombinant protein A that has been produced recently and optimized in order to increase the stability of the ligand toward alkaline solutions used during cleaning and sanitization procedures. In addition, protein A resins can now display high binding capacities reaching 50–70 mg/ml, with eluted antibody purities of approximately 99% [60].

While biological ligands are being continually improved, synthetic ligands are also being developed. Biomimetic peptidic ligands based on peptide libraries have started to be used (e.g., protein A mimetic and cyclic peptides) [61,62]. However, although peptides are relatively stable to cleaning-in-place and sterilization-in-place treatments, problems have been encountered regarding their resistance to enzymatic degradation due to the fragility of the fissile peptide bond [61,62]. Therefore, in an attempt to overcome some limitations of biological and synthetic peptidic ligands, a new approach has been devised involving biomimetic nonpeptoidal ligands, also called *de novo* ligands. They are purely synthetic, proven to be stable, and are developed using low-cost combinatorial approaches [7,63]. For example, triazine ligands such as Mabsorbent A1P and A2P are robust to the harsh cleaning conditions and much more affordable than protein A columns. However, such ligands have lower specificity and selectivity and can bind to other impurities in the feed stream [64,65]. Nevertheless, the washing and elution conditions can be optimized in order to elute a pure product in high yield (approximately 95% pure IgG) [66]. On the other hand, Ugi ligands mimicking protein L and protein G have been developed and shown specificity to IgG from different species including heavy-chain only camelid IgGs, in addition to Fab and Fc fragments, all isolated from mammalian or yeast crude samples, with purity reaching 99% at small scale [12–14]. Moreover, an Ugi ligand mimicking protein G in particular, was able to bind and purify IgG and Fab fragments from crude samples under near physiological ionic strength and pH [14].

It should be noted that several factors still need to be optimized and must be taken into consideration regarding *de novo* affinity ligands. To date, the ligands have been tested only at small scale and assessed directly on the solid phase; it has been challenging to characterize them in definitive chemical criteria. Currently, they are being further investigated through solution-phase syn-

thesis and analytical studies, in order to confirm their chemical structure and integrity. Furthermore, safety analyses will be performed and purification protocols optimized at large scale to meet regulatory requirements. Therefore, the availability and application of inexpensive affinity approaches coupled to single-use technologies could be more affordable than currently used affinity adsorbents for biopharmaceutical production. However, it should be noted that most biomanufacturers are still reluctant to adopt new purification processes at the industrial scale, mainly due to the lack of proven cost-effective protein A alternatives which would be acceptable to the regulatory authorities.

Glycoproteins

Glycosylation has a significant impact on the biological activity and pharmacological and clinical properties of recombinant proteins. About 70% of pipeline products are glycosylated; therapeutic glycoproteins include antibodies, clotting factors, colony stimulating factors, EPO, interferon and hepatitis B and C vaccines [67–69].

Production of therapeutic recombinant glycoproteins, usually expressed in mammalian systems, leads to mixtures of **glycoforms** with potentially different biological properties, because glycan synthesis is not template-mediated. The heterogeneous mixture presents not only regulatory difficulties but also challenges in determining exact structure–activity relationships [70]. The ability to isolate and purify individual recombinant glycoforms with defined properties is therefore crucial, as the potential consequences and side effects after administration can be detrimental to the health of the patient [71]. Manufacturing defined glycoforms offers a substantial opportunity to improve product consistency.

The generic bioseparation process used for the purification of therapeutic glycoproteins currently consists of a sequential purification procedure, usually including a capture step consisting of large-scale affinity chromatography based on immobilized lectins or boronates. Lectins have seen growing interest over the past few years; they are found in plants, animals, as well as in microorganisms and display specificity for certain types of carbohydrate residues [72]. Lectins have been widely used to isolate and identify glycoproteins, glycopeptides, glycolipids and oligosaccharides. The most commonly used proteins are concanavalin A (Con A), wheat germ agglutinin and jacalin. Con A is isolated from *Canavalia ensiformis* and displays specificity to targets containing α-D-mannose or α-D-glucose residues. Wheat germ agglutinin, isolated from *Triticum vulgaris*, binds to D-*N*-acetyl-D-glucosamine (GlcNAc) residues, and the plant lectin, jacalin, isolated from jackfruit seeds *Artocarpus integrifolia*, binds

Key term

Glycoforms: Mixture of glycoproteins of variable glycosylation patterns usually produced by post-translational modification in an eukaryotic expression system.

to galactose or mannose residues [73]. Other lectins also used in affinity chromatography include mannose-binding proteins, which have affinity toward mannose and GlcNAc residues and *Sambucus nigra* agglutinin that binds sialylated glycopeptides. Elution of glycoproteins from lectin affinity resins can be achieved at neutral pH, using competitive eluents such as glucose, mannose and methyl-α-D-glucoside. Alternatively, buffers containing sodium borate, ethylene glycol or urea, changes in temperature or ionic strength can be also considered. However, as a class of biological ligands, lectins share the same disadvantages mentioned above; in addition, Con A, is very toxic to mammalian cells in culture and can cause agglutination of erythrocytes [74].

Boronate columns have been used for the separation of sugars and nucleic acid components since 1970 [75]. These ligands have been employed as less expensive and more stable alternatives to lectins to separate a wide range of *cis*-diol-containing compounds, including nucleosides, nucleotides, nucleic acids, carbohydrates, glycoproteins and enzymes [76]. The most commonly used boronic acid ligand is 3-aminophenylboronic acid immobilized on agarose beads. However, 3-aminophenylboronic acid interacts optimally with glycoproteins at pH values above 8.5, and this basic pH may affect the biological activity of the target protein [76]. Moreover, boronic acid ligands are not specifically selective to *cis*-diol-containing glycoproteins [77]. With the development of biomimetic ligands, alternative glycoprotein-binding adsorbents, such as synthetic triazine and Ugi ligands have been designed and assessed at small scale, based on natural carbohydrate-binding ligands [16,78]. They have been tested with glucose oxidase and kallikrein as model glycoproteins and were able to achieve 92–99% glycoprotein purity, with affinity dissociation constants K_d in the range of 10^{-6}–10^{-5} M [16,78]. Glycoengineering of biotherapeutic proteins is likely to present future opportunities for employment in the biopharmaceutical sector. In order to improve efficacy, functionality, cost–effectiveness and patentability, it would be desirable to produce homogeneous glycoform products in high yield. Improvements in the development of economical affinity ligands for large-scale purification will have an important impact on downstream processing of therapeutic glycoproteins and the resolution of glycoforms.

Key term

Virus-like particle: Noninfectious, immunogenic macromolecular structures of viral proteins; they lack the viral genome and can be used to develop vaccines.

Vaccines

Since the first discovery of vaccination by British physician Edward Jenner in 1803 for smallpox [79], there has been a surge to increase vaccine production for different viruses. Influenza viruses, in particular, pose a great risk to society; influenza viral strains change yearly leading to epidemics and, in certain instances, pandemics such as the 'greatest medical holocaust in history' of 1918 H1N1 Spanish Flu strain [80].

The cGMP manufacturing procedure for influenza vaccines uses fertilized chicken eggs in the upstream processing to grow the virus. In addition, for efficient production mammalian cell platforms are used to manufacture vaccines and **virus-like particle** vaccines. In 2012, the US FDA approved the first cell culture influenza virus vaccine, Flucelvax® (Novartis, MA, USA), which uses Madin-Darby Canine Kidney cells in culture as the host to grow the influenza vaccine [81]. The cell-based platform ensures readiness in the event of a pandemic, as the cells can be banked and available upon request. Furthermore, cell-based technology is flexible because it does not depend on egg supply and is suitable for patients with egg-based allergies [82].

The cGMP egg-based downstream process, it consists of virus inactivation, followed by extensive filtration and concentration steps to yield a concentrate before centrifugation. Finally, an ultrafiltration step is followed by terminal sterile filtration to generate the monovalent vaccine [83,84]. In general, the unit operations do not vary significantly for the cell-based vaccines. Therefore, depending on the chemistry of the potential contaminants, most vaccine chromatography processes use a purification chain that encompasses negative chromatography that binds to impurities rather than the product; the charged contaminants bind monolithic AEX columns or CEX resins. The final steps are usually size exclusion chromatography or tangential flow filtration to get the highest purity to satisfy safety requirements and comply with regulatory agencies [84].

In an attempt to improve multistep purification protocols and reduce process time, affinity chromatography has been applied for the purification of vaccines and virus-like particle vaccines containing hemagglutinin glycoprotein; *Erythrina cristagalli* agglutinin (from coral tree lectin) was used to target the variable domain of hemagglutinin [85]. However, this method lacks selective purification of different strains, and may lead to the contamination of the purified product by toxic lectins in case of leaching. Therefore, there is a palpable need to devise an economical approach to purify vaccines focusing on their constant domains to selectively isolate various strains. Thus, alternative affinity approaches and single-use systems are currently being considered in the vaccine manufacturing industry.

Erythropoietin

Human EPO is a hematopoietic cytokine and is the prime regulator of erythropoiesis [76]. Following the identification of the gene encoding EPO in 1985 [86], rHuEPO has become a major therapeutic agent to treat anemia associated with chronic renal disease, Zidovudine-induced anemia of HIV infection and AIDS, the treatment of cancer patients on chemotherapy and for surgical patients to prevent the need for red blood cell transfusion.

Currently, there are two Erythropoietin Stimulating Agents approved by the FDA: Epoetin alpha (Epogen®, Procrit®/Eprex®; Amgen, CA, USA), and darbepoetin (Aranesp®; Amgen), an EPO analog. The market value of EPO-based products is approximately US$9.5 billion per annum [87]. However, downward price pressures, increasing demand on healthcare budgets and rising competition from biosimilars, are driving action to maintain its market share and prevent a further decline in sales [88]. Developments in upstream and downstream manufacturing have focused on reducing the overall manufacturing costs without compromising the purity, efficacy and biological activity of the product [87].

EPO is a heavily glycosylated single-chain protein with carbohydrate components constituting approximately 35–40% of its molecular mass. The degree of glycosylation affects efficiency of protein production, receptor affinity, plasma half-life, stability and excretion [67]. The carbohydrate chains of EPO are mainly responsible for its integrity and stability, being the starting point of new rHuEPO developments of hyperglycosylated analogs like darbepoetin alpha with longer serum half-lives [89,90]. rHuEPO produced in mammalian cells is fully glycosylated. In the case of EPO production, the sequence of amino acids, as well as the amount of glycosylation, must be correct in order to achieve the desired efficacy *in vivo*. Mammalian cell lines are thus used for their ability to produce *O*-glycosylation and *N*-glycosylation similar to human EPO. Epoetin alpha was the first rHuEPO; it has been commercially produced since 1989 in a CHO cell line which was transfected with the human EPO cDNA gene. More recently, lymphoblastoid cells have also been used to express rHuEPO [91,92].

The conventional purification process for rHuEPO involves multistep downstream procedures; several patents exist for the purification of rHuEPO that comprise different chromatographic steps [93]. One protocol involves consecutive AEX, reverse-phase and size-exclu-

sion chromatography steps, while others include several chromatographic methods such as hydroxyapatite, hydrophobic interactions and affinity chromatography. Concentration, diafiltration, ultrafiltration and dialysis are also commonly used as intermediate steps [93–97]. Several ligands have been used for the affinity purification of EPO including boronates, dyes and mAbs [94,98]. One protocol describes a purification process comprising dye affinity chromatography, where Cibacron®-Blue F-3GA or other triazine dyes are covalently bound to a polysaccharide matrix. EPO binds at low ionic strength and slightly acidic pH values, while the elution takes place by increasing pH and salt concentration [99]. A more recent protocol also utilizes the commercially available Blue-Sepharose affinity ligand used during the capture step, followed by AEX, reverse phase and CEX chromatography steps [98]. However, concerns over the selectivity, purity, leakage and toxicity of commercially available dyes have limited their industrial use for therapeutic applications and has steered research into alternative biomimetic ligands and affinity adsorbents.

Immunoaffinity columns are often used for the purification of EPO from urine and for the detection of EPO levels and isoforms, with an application in sports doping [100–102]. The use of immunoaffinity columns for the purification of rHuEPO, on the other hand, started decades ago, along with its use as a purification step in the separation of EPO isoforms based on different isoelectric points [103,104]. However, immunoaffinity media, in general, cannot withstand strong cleaning and sanitization conditions, and often leakage of antibodies takes place during purification, hindering its application in therapeutic protein production [105].

Research by Martínez-Ceron *et al.* (2011) has led to the synthesis of a peptide ligand that successfully binds to rHuEPO [106]. This approach was based on random screening of a peptide library for rHuEPO binding. Although peptide ligands are more commercially available and FDA compliant, they do suffer from the limitations cited previously.

More recently, a synthetic ligand was designed utilizing combinatorial chemistry based on the four-component Ugi reaction. The ligand, containing histamine, succinic acid and benzyl isocyanide components was designed to mimic functional groups found on EPO receptor residues (Figure 3). This rational approach

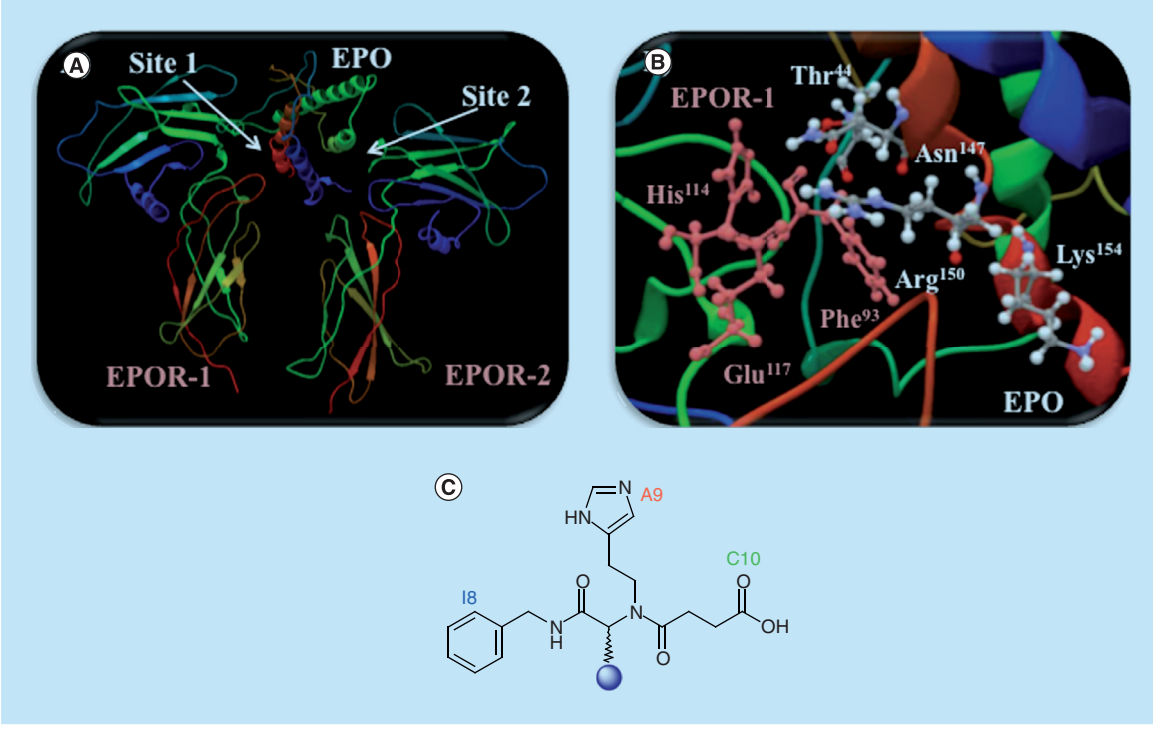

Figure 3. Design of an Ugi-scaffolded affinity ligand for erythropoietin. **(A)** X-ray crystallographic structure of EPO and EPOR complex (PDB accession code: 1CN4). EPO has two nonidentical binding sites; site 1 and site 2, each of which binds to one EPOR molecule. **(B)** Key receptor residues (Phe^{93}, His^{114} and Glu^{117}) to be mimicked at binding site 1 (EPOR-1, in red). **(C)** Ugi ligand A9C10I8 attached to an agarose-functionalized matrix (blue sphere). Only one ligand is displayed per bead for simplicity. Ugi reaction components: histamine (A9), succinic acid (C10) and benzyl isocyanide (I8). **(A & B)** were created using CLC Drug Discovery Workbench (v1.0; CLC bio, Denmark). The chemical structure was drawn using MarvinSketch (v4.1.13; ChemAxon Ltd).
EPO: Erythropoietin; EPOR: EPO-receptor.
Adapted with permission from [15].

allowed for a smaller library of ligands to be screened for binding. It was found that the lead ligand was able to purify rHuEPO from a spiked mammalian supernatant at small scale [15]. These initial steps suggest that a synthetic affinity ligand specific for rHuEPO could potentially reduce the overall number of purification steps and provide a more economic purification procedure.

Conclusion & future perspective

This review has addressed some aspects of therapeutic protein production, highlighting downstream bottlenecks resulting from constant improvements in expression titers and batch volumes. Conventional protocols applied for the affinity purification of prominent classes of biotherapeutics have been tackled, involving time-consuming processes and costly biological ligands used during the capture step. However, advances in affinity chromatography including engineering of recombinant biomolecules with enhanced chromatographic performance, development of *de novo* biomimetic synthetic ligands based on bioinformatics tools and combinatorial chemistry, in addition to innovations in bioprocess modeling and high-throughput screening methods will all contribute to facing the challenges created by improvements in upstream events. Furthermore, pressure and competition resulting from the emergence of biosimilars and the increasing number of approved biopharmaceuticals are also driving industries to devise more cost-effective technologies, including single-use systems (e.g., bioreactors and membrane adsorbers) that have started to be adopted at clinical scale and will be used for commercial applications. Continuous advancement in protein purification approaches and employment of expanded-bed affinity chromatography and monoliths will also contribute to the implementation of inexpensive manufacturing processes and the development of economical purification platforms.

Financial & competing interests disclosure

The authors have no 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. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

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

Executive summary

Background

- Affinity chromatography is one of the main downstream steps that incurs major expenses during bioprocessing, primarily due to the use of costly biological ligands.
- Implementation of economical purification platforms could be achieved through the development of synthetic affinity ligands and employment of single-use technologies.

Bioprocessing: challenges & future needs

- High productivity in upstream processing and emergence of biosimilars are placing pressure on the downstream production phase for reducing manufacturing costs.
- Main challenges in bioprocessing include achievement of high-product yields and high purity with respect to the safety, efficiency, stability, regulatory requirements and production cost of biotherapeutics.

Affinity chromatography: current approaches

- Despite their high cost, biospecific ligands are still being widely used in affinity chromatography, with continuous efforts to improve their performance, in an attempt to establish economical platforms.
- In parallel, alternative approaches are being developed including inexpensive synthetic ligands.

Design, synthesis & evaluation of *de novo* affinity ligands

- The global strategy is based on combinatorial chemistry, recently involving four-component Ugi reaction, and chromatographic assessment of ligands libraries supported by *in silico* molecular modeling.
- Lead ligands are subject to extensive analytical characterization for the optimization of chromatographic conditions and potential purification processes.

Affinity purification of therapeutic proteins

- Principal classes of biopharmaceuticals include monoclonal antibodies and their fragments, glycoproteins, vaccines and recombinant human erythropoietin.
- Downstream processing of different biotherapeutics tends to share similar limitations with the use of biological ligands as primary affinity adsorbents during the capture step.
- Synthetic affinity ligands have been developed at small scale for most proteins of interest. However, lead ligands need to be more characterized before scale-up of protein purification.

Conclusion & future perspective

- Advances in the development of various affinity approaches combined with innovations in process engineering, automation and single-use technologies will all contribute to the improvement of biomanufacturing.

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