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Review

Design and selection of ligands for affinity chromatography

N.E. Labrou*

Laboratory of Enzyme Technology, Department of Agricultural Biotechnology, Agricultural University of Athens, 75 Iera Odos Street, GR-11855 Athens, Greece

Abstract

Affinity chromatography is potentially the most selective method for protein purification. The technique has the purification power to eliminate steps, increase yields and thereby improve process economics. However, it suffers from problems regarding ligand stability and cost. Some of the most recent advances in this area have explored the power of rational and combinatorial approaches for designing highly selective and stable synthetic affinity ligands. Rational molecular design techniques, which are based on the ability to combine knowledge of protein structures with defined chemical synthesis and advanced computational tools, have made rational ligand design feasible and faster. Combinatorial approaches based on peptide and nucleic acid libraries have permitted the rapid synthesis of new synthetic affinity ligands of potential use in affinity chromatography. The versatility of these approaches suggests that, in the near future, they will become the dominant methods for designing and selection of novel affinity ligands with scale-up potential.

Keywords: Reviews; Combinatorial design; Ligand design; Molecular docking; Phage display; Ribosome display; Systematic evolution of ligand by exponential enrichment; Affinity adsorbents; Proteins; Peptides

Contents

*Tel./fax: +30-210-529-4308.
E-mail address: lambrou@aua.gr (N.E. Labrou).

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1. Introduction

During the 20th Century there has been enormous progress in understanding the molecular basis of disease processes [1–3]. At the same time, technological advances in the genomics area and the efforts in proteomics research have increased the possibility of discovering many proteins with desirable therapeutic functions [4,5]. Some of them have been successfully applied to commercial production.

The biotechnology industry is suffering from a severe shortage of manufacturing capacity for recombinant protein therapeutics [6]. According to a recent survey by the Pharmaceutical Research and Manufactures of America, 122 biologics were either in phase III trials or awaiting US Food and Drug Administration (FDA) approval [6]. Some of them, such as therapeutic antibodies, will require enormous quantities (>1 g per patient per year) of protein to satisfy the market demand [7]. To overcome this request, technical improvements that will increase the production either by increasing expression level in bioreactors or improving the purification efficiency are considered [8].

Chromatography is a highly selective separation technique commonly used for the isolation and purification of biological macromolecules [9,10]. Chromatography is unique in achieving the high standards of product purity dictated by the regulatory authorities for commercial bioproducts [10]. A wide range of chromatographic techniques has been used for large-scale biopurifications: size-exclusion chromatography, ion-exchange, hydroxyapatite, hydrophobic interaction chromatography, reversed-phase chromatography, and affinity chromatography [8].

Affinity, in the chemical setting, is the attractive force of varying strength for individual elements or compounds that causes atoms and molecules to combine and stay combined [11]. These binding interactions are used by affinity chromatography for the purposes of purification. Exploitation of the principles of affinity to separate compounds is not a new concept. The idea was developed by Starkensten in 1910, who was able to bind α-amylase to insoluble starch. Affinity chromatography as it is known today was introduced in 1968 by Cuatrecasas et al. [12,13], and since then it has developed into a powerful tool in biomedical research and biotechnology [14]. Although affinity chromatography is used extensively on a laboratory scale, its widespread acceptance has been limited at the preparative scale because of the high cost of the affinity ligands and their biological and chemical instability [15]. Only recently, the development of new methods for screening, selection and design of stable synthetic ligands, has opened the opportunity of exploitation of such materials on a large-scale [16].

2. Design of an affinity chromatography process

Each protein separation process must be individually optimised and no general rules can predict the factors and parameters for a specific separation. However, some general factors that influence the design of an affinity chromatography process and its incorporation in a final purification protocol are well known [14,15]. These factors are mainly determined by the nature and quality of the desired final product and its intended use [14,15,17–21]. For example, proteins for industrial use need not always be absolutely pure whereas therapeutic proteins need to be extremely pure (>99.9%) to minimise the risk of side effects or immunogenic response [15,17,18]. Also, the desired amount of the final product must be defined as it may influence the process. For example, a final dose of pharmaceutical is not known until the end of the clinical trials [19,20]. When Genentech started its tissue plasminogen activator process in 1982, the expected dose was 1 mg, but after the progress of clinical trials its dosage increased to 100 mg. This necessitated changes in the process, such as switching host cells from *Escherichia coli* to Chinese hamster cells, and changing the downstream processing purification protocol [22].

Certain factors determine the success of an affinity chromatographic step. Some of them are: (1) the ligand selectivity, (2) recovery, (3) throughput, (4) reproducibility, (5) stability and maintenance and (6) economy [18]. The selectivity of the ligand perhaps is the most important parameter and will be discussed in detail in the next paragraph. Recovery is determined by the amount of biological active protein that is eluted after the end of the process, and throughput is among the several factors that influence dramatically the cost effectiveness of the

process [19]. Reproducibility must be determined in order for the process get validation. Differences in purity should be taken into consideration before a purification method is incorporated in a large scale protocol [20]. Ligand stability is also an important factor which determines the durability of the column itself and the cost of the process [17].

Downstream processing costs can be minimal in the case of industrial enzymes, because the purity requirements are low. In contrast, the purity of pharmaceutical proteins, especially those to be administered should be very high (99.9%) [20]. In such cases, purification and recovery costs can account for as much as 80% of the total manufactured cost. Product cost increases with the number of steps involved in the process, not only because of the capital and operating costs but also because the overall yield decreases with the number of steps [19,21].

3. Ligands for affinity chromatography

The rapid growth of bioinformatics and molecular docking techniques and the introduction of combinatorial methods for systematic generation and screening of large numbers of novel compounds, has made feasible the rapid and efficient generation of ligands for affinity chromatography [22–25]. These techniques will be discussed in the following sections.

3.1. Protein-structure-based design

The strategy for the rational design of affinity ligands involves retrieving structural information about the target protein from suitable databases and identifying a potential binding site on the protein. The target site may be an active site, a solved-exposed region or a site involved in binding of a natural complementary ligand [26]. There are two important factors for successful structure-based ligand design: firstly, the ability to correctly predict the conformation of the designed ligand and secondly the ability to correctly predict the binding affinity of the designed ligand [15,17,25–27]. The theories behind protein–ligand binding are moving away from the historic lock-and-key and induced-fit theories. The current theory describes proteins in a pre-existing ensemble of conformation states, thus the flexibility of both ligand and protein must be taken into account for successful ligand design [26].

Some successful protein-structure ligand design approaches have been recently published [28,29]. For example, the anthraquinone biomimetic dye–ligand for L-lactate dehydrogenase (LDH), was designed by exploiting the three dimensional structure of the porcine heart LDH-(*S*)-lactate-NAD^+ complex (PDB code 5LDH) (Fig. 1) [28]. The ligand is an analogue of the textile dye Cibacron Blue 3GA and bore 2-(4-aminophenyl)ethyloxamic as a terminal biomimetic moiety. Ligand design was based on a match between the polar and hydrophobic regions of the enzyme binding site with those of the biomimetic moiety. The length of the ketoacid biomimetic moiety was determined to approach the enzyme catalytic site and to form charge–charge interactions

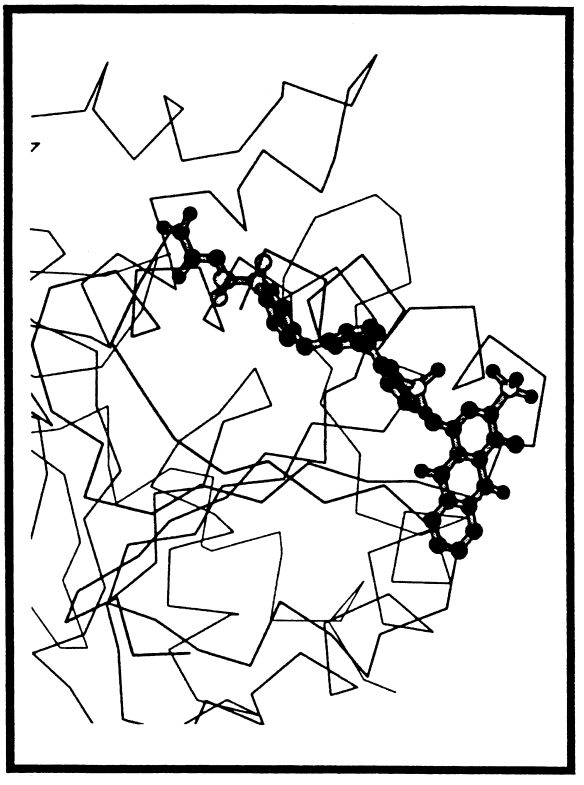


Fig. 1. (A) Diagram of the biomimetic dye bearing 2-(4-aminophenyl)ethyloxamic as terminal biomimetic moiety bound to the coenzyme-binding site of L-lactate dehydrogenase (LDH).

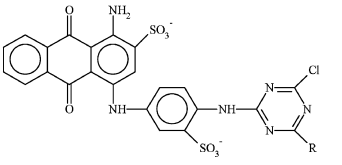
with Arg-171. This biomimetic dye in its immobilised form exhibited improved purifying ability for LDH from bovine heart and when it was integrated in a two-step purification protocol, produced pure enzyme of specific activity equal to 600 U/mg and 56% recovery [28]. The same adsorbent was proved to be effective in the purification of LDH from other than bovine heart sources, such as chicken liver and muscle, pig muscle, bovine pancreas and pea seeds [28].

3.2. Protein-function-based design

This approach is applied where the three dimensional structure of the target protein is not available and is relies on the incorporation of certain structural features on the ligand which can be:

1. a certain required molecular shape. For example linear anionic molecules, such as heparin, have been of general value for the purification of DNA-binding enzymes [30]. Other examples involve the application of triazine dye Cibacron Blue 3GA for the purification of nucleotide binding enzymes [27,31]. Cibacron Blue 3GA is believed to adopt a three-dimensional structure which resembles that of nucleotide and also possesses the required geometry and combination of ionic and hydrophobic groups capable of forming adequate interactions with the nucleotide binding site of certain enzymes [31];
2. a specific functional group. For example, benzamidine group as ligand for trypsin-like proteases, hydroxamic acid and iminodiacetic acid as ligands for metal-binding proteins and metalloproteases [32–34]. Also, analogues of phenylboronic acid, as ligands for glycoproteins [14], immobilised wheat germ agglutinin (WGA-agarose) and serotonin as ligands for the purification of sialoglycoproteins [35,36];
3. a structural model derived from the combination of structural moieties which are known substrates, inhibitors, effectors or cofactors. For example the keto-carboxyl biomimetic chimeric dye–ligands which were designed for the purification of (keto)carboxyl-recognising enzymes [27]. These ligands comprise two enzyme-recognition moieties: the anthraquinone chromophore (a strong competitive inhibitor for dehydrogenases) and the terminal substrate–mimetic moiety linked to the triazine ring. The substrate–mimetic moieties have carboxyl or ketoacid structures, analogous to those of the natural ligands (substrates, inhibitors) of carboxyl recognising enzymes (Fig. 2, CK-BM1, CK-BM2, CK-BM3). These biomimetic-dyes, in their immobilised form, were used to purify several (keto)carboxyl-group recognising enzymes, such as formate dehydrogenase from *Candida boidinii* (on CK-BM1, in a single chromatographic step, leading to SA >7 U/mg and recovery >60%) [37], L-malate dehydrogenase from bovine herd (on CK-BM2, in two chromatographic steps, leading to SA >30 U/mg and recovery >40%) [38], oxaloacetate decarboxylase from *Pseudomonas stutzeri* (on CK-BM3, in three chromatographic steps, leading to SA >300 U/mg and recovery >30%) [39].

Melissis et al. have reported the design of glutathionyl-biomimetic dye–ligands (GSH-BM) for glutathione-recognising enzymes [40]. These ligands comprise two enzyme-recognition moieties: the anthraquinone chromophore and the terminal glutathionyl-moiety linked to the triazine ring (Fig.



Dye-ligand	(-R)
CK-BM1	-SCH_2COCOO^-
CK-BM2	-*p*- $HN\text{-}C_6H_4\text{-}NHCOCOO^-$
CK-BM3	-*m*-$HN\text{-}C_6H_4\text{-}COO^-$
GSH-BM1	-$HNC_{10}H_{15}N_2O_6SO_3^-$
GSH-BM2	-$HNC_{10}H_{14}N_2O_6SCH_3$
GSH-BM3	-$SC_{10}H_{14}N_2O_6NH_2$

Fig. 2. Structures of the anthraquinone (keto)carboxyl-biomimetic dyes (KC-BM1-3) and of the anthraquinone glutathionyl–biomimetic dyes (GSH-BM1-3).

2). These biomimetic-dyes, in their immobilised form, were used to purify several glutathione recognising enzymes, such as glutathione *S*-transferase, glutathione reductase, and formaldehyde dehydrogenase [40].

3.3. Combinatorial approaches

Over the past 10–15 years, several methods have emerged that allow the generation of up to 10^{15} different proteins, peptides or nucleic acids [41]. The availability of such large libraries allows the selection of molecules with high affinity and specificity. Binding molecules from first generation libraries can be used as parental scaffolds to produce additional libraries from which higher affinity binders can then be selected [42]. Several laboratories have recently reported the application of combinatorial methods to the selection of affinity ligands from libraries which were based on (poly)peptides, polynucleotides and substituted triazines [41–46].

3.3.1. Synthetic peptide libraries

Synthetic peptide libraries are collections of random peptide molecules, encompassing all the possible sequences for a given peptide length [47–50]. Peptides are synthesised chemically using solid-phase peptide synthesis methods. The synthesised peptides are either cleaved from a soluble peptide library or remain on the resin in the one-bead-one-structure peptide library [51]. A basic in vitro screen for a combinatorial library is to pass the library mixture over a surface on which the protein of interest has been immobilised [52]. The ligands that are retarded in their passage are those that exhibit affinity with the immobilised protein molecules, and so these compounds are usually good candidates as affinity chromatography ligands [41,52].

Using this approach a peptide ligand with the sequence F–L–L–V–P–L has been developed for the purification of fibrinogen [41]. The ligand immobilised and the corresponding adsorbent were used to purify human fibrinogen. A low ionic strength buffer at pH 4 was used successfully to elute adsorbed fibrinogen from the column with high purity, and minimal loss of biological activity. The authors suggest that this general approach can be used to develop peptide ligands for the affinity purification of diverse proteins on a large scale [41].

Nord et al. describe the use of two small protein ligands which were selected from libraries based on an M_r 7000 folding domain derived from *Staphylococcal* protein A [53]. The two ligands, anti-apo and anti-*Taq*, bind to their respective targets, apolipoprotein $A\text{-}1_M$ and *Taq* DNA polymerase, with dissociation constants in a micromolar range and were used, in their immobilised form, to purify their respective proteins from clarified *E. coli* cell lysate [53].

While only 10 monoclonal antibodies have been approved so far, there are more than 100 antibodies in clinical trials and hundreds more in preclinical development [54]. This underlines the requirements of the study and develops high affinity ligands and methods for efficient purification of immunoglobulins [55–61]. The search for synthetic ligands able to bind to immunoglobulins has been undertaken mainly through the synthesis and screening of combinatorial peptide libraries [55,56]. So far, two successful applications have been published. The first approach has yielded a protein A mimetic peptide using three screening cycles [57]. In the first screening cycle a sublibrary with arginine at the *N*-terminus was identified (60% inhibition). Subsequently, a second multimeric sublibrary was synthesised, with arginine at the *N*-terminus, the amino acid in position 2 defined, and the third unknown. This second sublibrary was screened and the best inhibitory activity was found in the sublibrary with threonine in the second position. A third sublibrary, composed of 18 tripeptide tetramers of the general formula $(R–T–X)_4–K_2–K–G$, allowed the final identification of the most active multimer as $(R–T–Y)_4–K_2–K–G$ (TG19318). Adsorption of antibodies on TG19318 affinity columns occurs with neutral buffers at low ionic strength, conditions fully compatible with the use of crude feedstock derived from cell culture supernatants. Elution of adsorbed immunoglobulins were achieved simply by changing the buffer to acid or weakly alkaline conditions, with acetic acid at pH 3.0 or sodium bicarbonate at pH 9.0 [57]. The ligand has a much broader specificity than protein A, since not only does it purify IgG from different sources, but also IgY from egg yolk [58], IgM from ascetic fluid, sera, or cell culture super-

natants [57], IgA from cell culture supernatants [60] and IgE from cell culture supernatants [61].

In another approach, the screening strategy led to the identification of peptide H, a cyclic dimeric peptide of formula $(C–F–H–H)_2K–G$, where the two cysteine residues at the *N*-terminus are covalently linked by a disulfide bridge [62]. When tested in affinity chromatography experiments, this ligand proved useful for mouse and rat IgG purification, but less selective for human and rabbit IgG. The capacity for mouse IgG was close to 1 mg IgG/ml of derivatised CH-Sepharose 4B [62].

3.3.2. Affinity selection by biopanning: phage display

Phage display has rapidly matured and evolved as a tool for discovering high affinity ligands for affinity chromatography [46,63]. This technology which is based on the utilisation of phage display libraries in a screening process known as biopanning has accelerated identification of highly selective ligands. The versatility of phage display has become apparent through discovery of peptide ligands that bind to targets whose natural ligands or substrates are not only proteins or peptides, but also nonpeptides [64].

The principle underlying the selection of phage displayed peptides is schematically depicted in Fig. 3. The affinity selection of the phage displayed peptides can be performed in two different ways. Either the library can be incubated directly with an immobilised target or preincubated with a target, prior to capture on a solid support. As in affinity chromatography, noninteracting peptides are washed

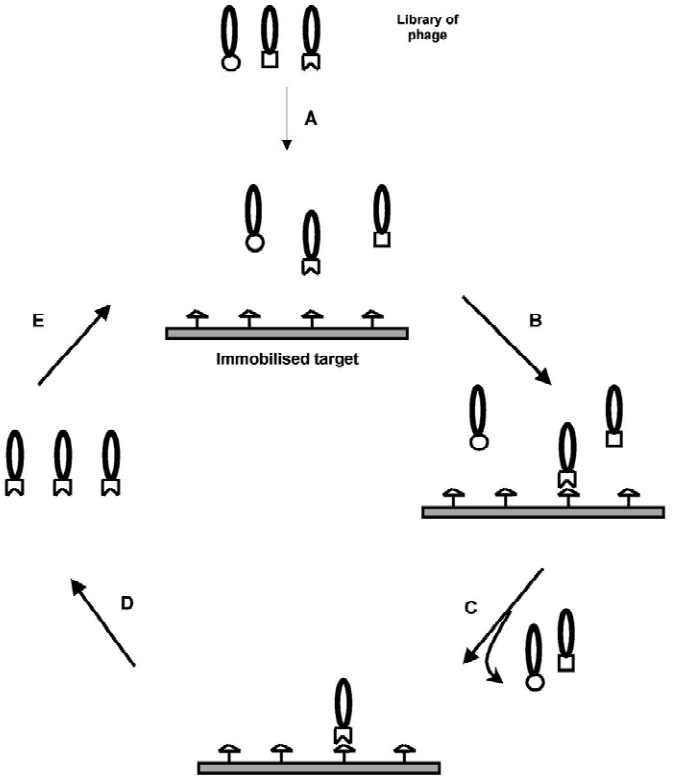


Fig. 3. General scheme of selecting phage from a phage-displayed combinatorial peptide library. In step A the displaying peptides are introduced into microtiter plate wells with immobilized target. After incubation (step B), the nonbinding phage are washed away. The bound phage are then recovered (step C). Phage particles are transferred to another tube and the bacteria are infected to produce more phage particles (step D). The amplified phage are then rescreened (step E), to complete one cycle of affinity selection. After three or more rounds of screening, the phages are plated out and individual plaques (clones) are further analysed by DNA sequencing.

away and then interacting peptides/proteins are eluted specifically or nonspecifically. These interacting phage display peptides can be amplified by bacterial infection to increase their copy number. This screening/amplification process can be repeated as necessary to obtain higher-affinity phage display peptides. The desired sequences are obtained by DNA sequencing of isolated phage DNA.

A successful application of this technology for selection of peptide ligands for affinity chromatography has been reported [42,65,66]. For example, the immunoglobulin-binding domain of protein A, mentioned earlier, has been displayed on the surface of phage, raising the potential for screening mutant forms of protein A with improved specificity or milder elution properties [63,65]. In another example, phage peptide library screening yielded a lead peptide (R–L–R–S–F–Y) that interacts with von Willebrand Factor (vWE) [66]. Conservative substitutions of terminal residues of the lead peptide led to a second peptide, R–V–R–S–F–Y, which was more efficient in the affinity purification of vWF from protein mixtures. The purification of vWF from human plasma was demonstrated using affinity chromatography on immobilised *N*-acetyl–R–V–R–S–F–Y–K. Divalent salts, such as calcium and magnesium chloride, were used to elute the retained vWF with 82.5% recovery [66].

Gaskin et al. described the use of a library of heptapeptides displayed on the surface of filamentous phage M13 as a potential source of affinity ligands for the purification of *Rhizomucor miehei* lipase [42]. The authors demonstrate that the interaction of the best ligand with lipase was due to both the heptapeptide sequence and the presence of a part of the phage coat protein. This conclusion was further verified by immobilising the whole phage on the surface of magnetic beads and using the resulting conjugate as an affinity adsorbent. This work presents the possibility of preparing phage-display affinity materials [42].

3.3.3. Affinity selection by ribosome display and SELEX

These technologies have not been applied so far for the discovery and selection of ligands for large scale affinity chromatography. However, the versatility of these approaches for rapid generation of high affinity ligands from very large initial libraries (10^{15} to 10^{16} sequences), suggests that, in the near future, they will become the dominant methods for designing and selection of novel affinity ligands with scale up potential [67–70].

The principle underlying ribosome display is schematically depicted in Fig. 4 and summarised as follows [71,72]. A DNA library, encoding a peptide of interest fused to a carboxyterminal spacer region, which allows the peptide product to be displayed clear on the ribosome. A strong transcriptional promoter is introduced upstream of the gene and in vitro transcription and translation is carried out. The absence of a stop codon prevents the release of the mRNA and peptide from the ribosome and, under the appropriate conditions, a stable ternary complex is formed. This complex is then exposed to the molecular target, which is often immobilised on a surface. If peptides bind to the target with sufficient affinity, they are retained. Peptides with low affinity are washed away. The mRNAs from the bound complexes are recovered by dissociating the complex, usually by EDTA. cDNA is produced by reverse transcription, and amplified by polymerase chain reaction (PCR). The recovered sequences may be subjected to a subsequent selective round of enrichment [71,72].

The technology termed systematic evolution of ligands by exponential enrichment (SELEX) was introduced in 1990 by Tuerk and Gold [67]. Currently, this technology is widely employed for screening nucleic acid ligands (called ‘‘aptamers’’) with potential applications in affinity chromatography [70]. The principle underlying SELEX is depicted in Fig. 5 and summarised as follows. A DNA pool is chemically synthesised with a region of random mutagenised sequence flanked on each end by constant sequence and with T7 RNA polymerase promoter at the 5′ end. This DNA is amplified by a few cycles of PCR and subsequently transcribed in vitro to make an RNA pool. The RNA molecules are then selected based on whether they bind to the chosen target protein, for example, by passing them through an affinity column derivatised with the target. The retained RNAs are eluted, reverse transcribed, amplified by PCR, transcribed, and then the entire cycle is repeated. In vitro selection has been used to identify aptamers to targets covering a wide range of sizes, including simple ions, peptides, proteins, or-

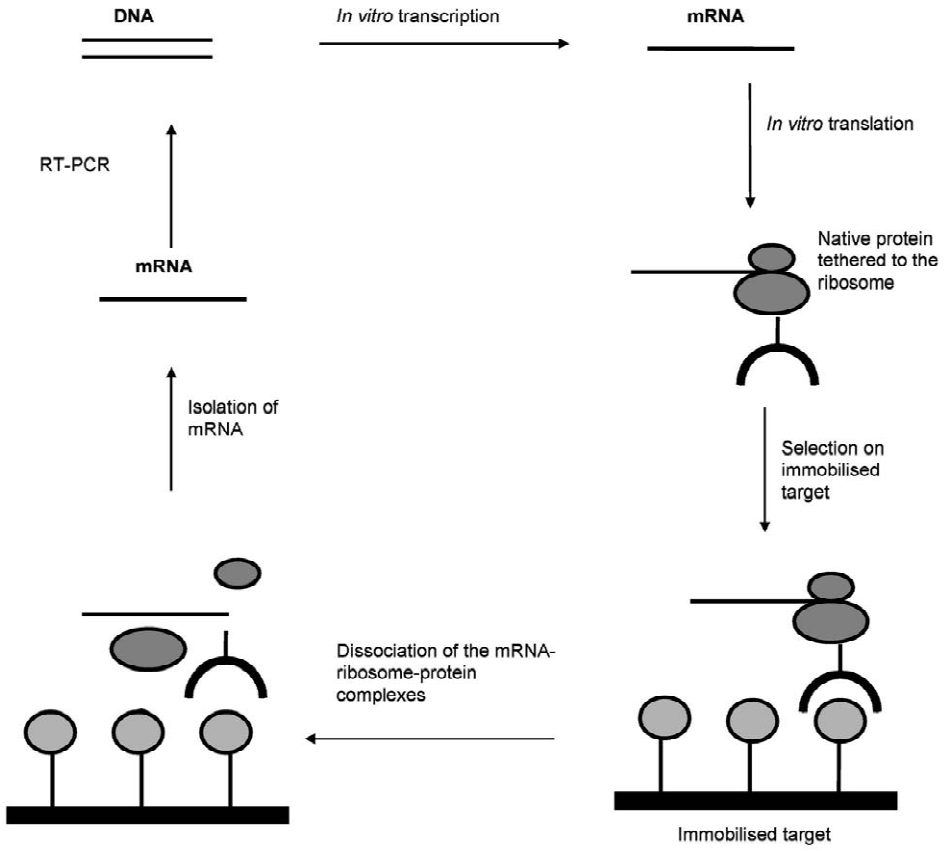


Fig. 4. Schematic diagram of the ribosome display. A DNA library is first transcribed to mRNA. The mRNA is translated in vitro. Translation is stopped and the ribosome complexes are stabilised by increasing the magnesium concentration. The desired ribosome complexes are affinity purified from the translation mixture by binding of the protein to the immobilised target. Unspecific ribosome complexes are removed by intensive washing. The bound ribosome complexes can then be dissociated by EDTA, and mRNA can be reverse transcribed to cDNA. This DNA is then used for the next cycle of enrichment, and a portion can be analyzed by cloning and sequencing.

ganelles, viruses, and even entire cells (reviewed in Ref. [68]).

Roming et al. have recently reported a successful application of an immobilised DNA aptamer for the purification of a recombinant human L-selectin-immunoglobulin fusion protein from Chinese hamster ovary cell. An impressive 1500-fold purification with 83% recovery was achieved [70].

3.3.4. Structure-based combinatorial approaches

The combination of structure-based design and combinatorial chemistry has emerged as a new and promising approach to ligand design. The method relies on the use of structural information of the target protein to focus and limit the synthetic efforts onto those molecules that experimentally have been evaluated to bind to the target or predicted to bind by a computational approach [73].

A structure-based combinatorial approach for the design and selection of ligands was developed by Lowe and co-workers [15,17,74–77]. This approach involves the following steps: selection of an appropriate site on the target protein, design of a complementary ligand compatible with the three-dimensional structure of the site, synthesis of a solid-phase combinatorial library of structurally relative ligands, and screening of the library against the target protein [75]. These libraries were based on 1,3,5-triazine scaffold. 1,3,5-Triazine is of special interest due to its synthetic accessibility, by taking advantage of the

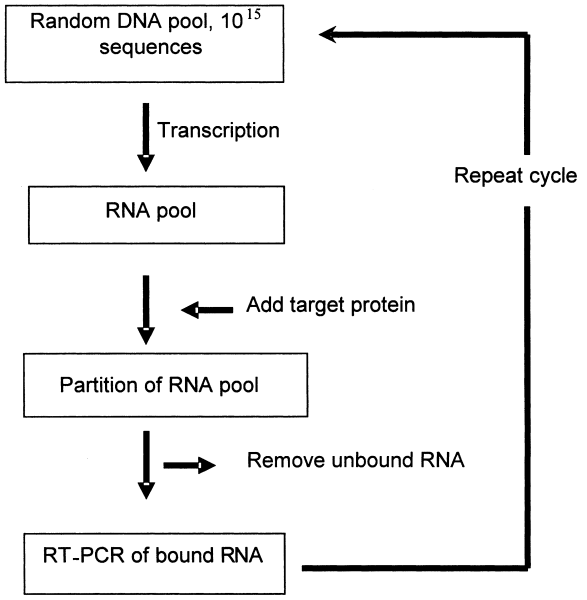


Fig. 5. In vitro selection of nucleic acid aptamers. The starting material for a SELEX experiment is typically a library of synthetic random-sequence DNA oligonucleotides containing different sequences. Each molecule contains a segment of random sequence flanked by primer-binding sequences at each end to facilitate conversion to double-stranded DNA by PCR. The library is converted to RNA in vitro using T7 RNA polymerase, which recognises a promoter sequence in the 5′ constant region. The target protein is immobilised on a solid support and mixed with the RNA library. The bound RNA is eluted from the target protein and converted to cDNA by reverse transcription and then amplified by PCR to produce a new DNA library enriched for the subset of sequences that encode ligands for the target protein. The cycle is repeated until a substantial fraction of the RNA mixture is found to bind to the target protein.

temperature-dependent successive displacement of the chloride atoms by different nucleophiles [15]. Other advantages of synthesis of triazine-based libraries for application in affinity chromatography is its direct immobilisation on amino- or hydroxyl-derivatised solid support, its high stability against biological and chemical degradation and its low cost [74]. Furthermore, the presence of electron-rich nitrogen sites increases its capability of forming hydrogen bonds with amino acid residues within the binding site, thus allowing stronger interaction with the protein [31].

Nonpeptidic biomimetic ligands for IgG have been developed based on the X-ray crystallographic structure of the complex between the B domain of protein A, the F_c fragment of IgG [76,77]. By using computer-aided molecular modelling, a series of biomimetic molecules around the protein A dipeptide Phe132–Tyr133 were designed. One of these ligands, which bore 3-aminophenol and 4-amino-1-naphthol moieties oriented on a triazine ring were found to bind human IgG. This biomimetic ligand when immobilised on agarose was successfully used for the purification of IgG from human plasma and ascetic fluid, with a purity of eluted IgG close to 98%. Both pH and ionic strength have a direct effect on binding, which is stronger near pH 7 and weaker at extremes of pH [76,77].

Similar approaches were also developed for the design of biomimetic ligands for the recombinant insulin precursor MI3 [75], and glycoproteins [74] and more recently to recombinant human clotting factor VIIa [44]. In the latter case, the rational design was based on the X-ray crystallographic structure of the complex of tissue factor and factor VIIa. The selected and optimised ligand comprises a triazine scaffold bis-substituted with 3-aminobenzoic acid and was shown to bind selectively to factor VIIa in a Ca^{2+}-dependent manner. The adsorbent purifies factor VIIa to almost identical purity (>99%), yield (99%), activation/degradation profile and impurity content (~1000 ppm) as the current immunoadsorption process, while displaying a 10-fold higher static capacity and substantially higher reusability and durability.

4. Large-scale dye–ligand affinity chromatography

Dye–ligand adsorbents represent one of the most important developments in protein purification over the past 30 years [14,27,31]. The range of dyes available, their low cost, and ease of immobilisation allows a rapid screening of a variety of adsorbents for both positive and negative selectivity, thereby enabling the very effective technique [31]. Dye–ligand adsorbents are available for analytical, preparative and large-scale work [24].

Wide application of dye–ligand affinity chromatography has been found in the purification of pharmaceutical proteins [78–83]. Some recent exam-

Table 1
Examples of the application of dye–ligand affinity chromatography in large scale protein and enzyme purification

Protein/enzyme purified	Source (with amount of starting material)	Ligand	Ref.
Formate dehydrogenase	*C. boidinii*, 50 l	Procion Red HE3b	[84]
Enterotoxins A, B, C2	*Staphylococcus*	Red A	[85]
α-Antitripsin	Human serum, 0.5 l	Cibacron Blue F3G-A	[86]
Carboxypeptidase G2	*Pseudomonas* spp., 5 kg paste	Procion Red H-8BN	[87]
Glucokinase	*B. stearothermophilus*, 28 kg paste	Procion Brown H-3R	[88]
Interferon	Human fibroblasts, 10–15 l	Cibacron Blue F3G-A	[89]
Phosphotransferase ATP:AMP	Beef heart, 26 kg	Cibacron Blue F3G-A	[90]

ples involve the purification of human complement factor B, factor C2, factor II, factor IX, trypsin, chymotrypsin and proteinase 3 [83]. Also, human recombinant α-interferon was purified using a single step procedure on a mimetic dye–ligand adsorbent, yielded monomeric α-interferon with specific activity of 2.8×10^8 IU/mg [80]. Other examples involve the purification of follicle-stimulating hormone [79], pituitary gonadotropins [80], ricin A chain [81] and human serum albumin [82].

Despite the wide and successful application of dye–ligands in affinity chromatography of pharmaceuticals, concerns over their leakage and toxicity have limited their use in biopharmaceutical manufacturing. Proteins purified using these methods are suited for analytical or technical uses [82]. Some examples of the application of dye–ligand affinity chromatography in large scale protein and enzyme purification are shown in Table 1.

5. Validation aspects of affinity chromatography

In the industrial context, the affinity procedures should be well validated and documented [20]. Depending on the source of protein (animal tissue, human serum, recombinant microorganism, transgenic animals or plants) the specific contaminants that are likely to be encountered depend, to a large extent, on which of these systems is used for the production of the desired protein. A number of possible contaminants of potential concern include: viruses and DNA, foreign proteins, pyrogens (bacterial endotoxins and lipopolysaccharides), protein aggregated, misfolded, or degraded. The commonly encountered degraded variants are deamidated forms, oxidized forms, products of proteolysis and forms with variations in carbohydrates [20].

Another contaminant of potential concern includes ligand leakage from the matrices [17]. Immobilised ligand may leak from the column and contaminate the product. In other instances after repeated use, the affinity adsorbent may become fouled with contaminants which are not washed and eventually start eluting in the eluate. For example, Cibacron Blue 3GA-Sepharose adsorbent was used on a pilot scale to produce 250 g albumin on a 50 l column with high purity (98–100%) and yield (82%) [91]. The long life of the adsorbent renders this approach very attractive and economically acceptable. However, safety and toxicity issues associated with the leakage of the dye from the column and the ability of the dye column to adsorb virus particles from human plasma [92,93] the albumin purified by this method has been rejected for clinical applications.

6. Conclusions

The unique specificity and reversibility of biological interactions have opened a new horizon for the development of purification technologies. Considering the resolution, recovery, yield and capacity, affinity chromatography has the potential to replace existing process technologies. As we enter the 21st century, we have begun to realise the enormous potential of the role of proteins in reducing the severity and incidence of many diseases. As the number of protein therapeutics in clinical evaluation continues to rise, more efficient production methodologies must be developed. In addition, with the

recognition of the growing shortfall in worldwide manufacturing capacity for biopharmaceuticals, such considerations are a significant driving force for the development of intensified and integrated downstream processes, principally through the use of affinity chromatography. However, the success of any affinity purification protocol is ultimately dependent on the availability of suitable ligands. Hence it is the design and synthesis of appropriate ligands which is often the rate limiting step in introducing affinity-based purification protocols. The use of rational and combinatorial approaches for designing highly selective and stable synthetic affinity ligands with scale-up potential will have a significant impact on future applications of affinity chromatography.

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