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Affinity Chromatography

History, Perspectives, Limitations and Prospects

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Summary

Biomolecule separation and purification has until very recently steadfastly remained one of the more empirical aspects of modern biotechnology. Affinity chromatography, one of several types of adsorption chromatography, is particularly suited for the efficient isolation of biomolecules. This technique relies on the adsorbent bed material that has biological affinity for the substance to be isolated. This review is intended to place affinity chromatography in historical perspective and describe the current status, limitations and future prospects for the technique in modern biotechnology.

Key Words: Affinity; chromatography; biomimetic; ligands; synthetic; proteins; purification; design; combinatorial synthesis.

1. Introduction

Traditional techniques for biomolecule separation based on precipitation with pH, ionic strength, temperature, salts, solvents or polymers, ion exchange or hydrophobic chromatography are slowly being replaced by sophisticated chromatographic protocols based on biological specificity. Affinity techniques exploit highly specific biorecognition phenomena and are ideally suited to the purification of biomolecules. In affinity chromatography, the specific adsorption properties of the bed material are realized by covalently attaching the ligand complementary to the target biomolecule onto an insoluble matrix. If a crude
cell extract containing the biologically active target is passed through a column of such an immobilized ligand, then all compounds displaying affinity under the given experimental conditions will be retained by the column, whereas compounds showing no affinity will pass through unretarded. The retained target is then released from the complex with the immobilized ligand by changing operational parameters such as pH, ionic strength, buffer composition or temperature. Conceptually, the technique represents chromatographic nirvana: Exquisite selectivity combined with high yields and the unparalleled simplicity of a ‘load, wash, elute’ philosophy. However, experience over the last 3–4 decades has shown that there is a very high penalty to pay for the implicit specificity and simplicity of affinity chromatography, which has important ramifications for commercial use and process development.

2. Historical Perspective

Affinity chromatography is a particular variant of chromatography in which the unique biological specificity and reversibility of the target analyte and ligand interaction is utilized for the separation. It is possible to distinguish four phases in the development of the technique starting from the early
realization of the technique, through the research phase, the impact of the nascent biopharmaceutical industry to the likely effect of the ‘omics’ revolution.

2.1. Early Beginnings

The concept of resolving complex macromolecules by means of biospecific interactions with immobilized substrates has its antecedents reaching back to the beginning of the 20th century. The German pharmacologist Emil Starkenstein (1884–1942) in a paper published in 1910 on the influence of chloride on the enzymatic activity of liver α-amylase was generally considered to be responsible for the first experimental demonstration of the biospecific adsorption of an enzyme onto a solid substrate, in this case, starch. Not long after, Willstätter et al. appreciably enriched lipase by selective adsorption onto powdered stearic acid. It was not until 1951, however, that Campbell and co-workers first used the affinity principle to isolate rabbit anti-bovine serum albumin antibodies on a specific immunoadsorbent column comprising bovine serum albumin coupled to diazotised p-aminobenzyl-cellulose. This technique, now called immunoaffinity chromatography, became established before the development of small-ligand selective chromatography, where Lerman isolated mushroom tyrosinase on various p-azophenol-substituted cellulose columns, and Arsenis and McCormick purified liver flavokinase and several other FMN-dependent enzymes on flavin-substituted celluloses. Insoluble polymeric materials, especially the derivatives of cellulose, also found use in the purification of nucleotides, complementary strands of nucleic acids and certain species of transfer RNA.

2.2. Research Phase

The general notion of exploiting strong reversible associations with highly specific substrates or inhibitors to effect enzyme purification was evident in the literature in the mid-1960s, although the immense power of biospecificity as a purification tool was not generally appreciated until 1968 when the term ‘affinity chromatography’ was coined. It was recognized that the key development required for wider application of the technique was that the solid-phase adsorbent should have a number of desirable characteristics:

… the unsubstituted matrix or gel should show minimal interactions with proteins in general, both before and after coupling to the specific binding group. It must form a loose, porous network that permits easy entry and exit of macromolecules and which retains favourable flow properties during use. The chemical structure of the supporting material must permit the convenient and extensive attachment of the specific ligand under relatively mild conditions, and through chemical bonds that are stable to the conditions of adsorption and elution. Finally, the inhibitor
groups critical in the interaction must be sufficiently distant from the solid matrix to minimise steric interference with the binding process \(^{(12)}\).

In this seminal paper, the general principles and potential application of affinity chromatography were enunciated and have largely remained unchanged until the present date. The paper contained several important contributions. First, it generalized the technique to all potential enzyme purifications via immobilized substrates and inhibitors and exemplified the approach by application to staphylococcal nuclease, \(\alpha\)-chymotrypsin and carboxypeptidase A. Second, it introduced for the first time a new highly porous commercially available ‘beaded’ matrix of agarose, Sepharose, which displayed virtually all of the desirable features listed above \(^{(13)}\) and circumvented many of the issues associated with conventional cellulosic matrices available at that time. Agarose is a linear polysaccharide consisting of alternating 1,3-linked \(\beta\)-D-galactose and 1,4-linked 3,6-anhydro-\(\alpha\)-L-galactose units \(^{(13)}\). Third, the report exploited the activation of Sepharose by treatment with cyanogen bromide (CNBr) to result in a derivative that could be readily coupled to unprotonated amino groups of an inhibitory analogue to generate a highly stable Sepharose-inhibitor gel with nearly ideal properties for selective column chromatography \(^{(14,15)}\). The use of CNBr activation chemistry was a milestone in the development of the technique, because the complex organic chemistry required for the synthesis of reliable immobilized ligand matrices had previously prevented this technique from becoming generally established in biological laboratories. Fourth, the report introduces the notion of spacer arms to alleviate steric interference and exemplifies the concept by showing the dramatically stronger adsorption of \(\alpha\)-chymotrypsin to the immobilized inhibitor D-tryptophan methyl ester when a 6-carbon chain, \(\varepsilon\)-amino caproic acid, was interposed between the Sepharose matrix and the inhibitor. When the inhibitor was coupled directly to the matrix, incomplete and unsatisfactory resolution of the enzyme was observed. Fifth, the report emphasizes the importance of selective affinity for the immobilized inhibitor by demonstrating the absence of adsorption of chemically inhibited enzymes such as DFP-treated \(\alpha\)-chymotrypsin or CNBr-treated nuclease to their respective adsorbents \(^{(12)}\). Finally, this paper emphasizes the efficacy of relatively low-affinity inhibitors and suggests that unusually strong affinity constants are not an essential requirement for utilization of these techniques for the rapid single-step purification of proteins.

Affinity chromatography caught the eye of many researchers worldwide and there followed a spate of publications purporting to purify proteins and other biomolecules by every conceivable class of immobilized ligand. However, troubling issues relating to the chemistry of the ligand attachment still remained. For example, there was much debate on how adsorbents should be synthesized \(^{(16)}\); the ‘solid-phase assembly’ approach was more facile and advocated the
attachment of ligands to spacer arms already present on the pre-activated affinity matrix, whereas the ‘pre-assembly’ approach uses conventional organic chemistry to modify the ligand with a suitably derivatized spacer arm, after which the whole assembly is coupled to the matrix. The solid-phase assembly approach lead to inhomogeneity problems where there were multiple sites on the target ligand or the coupling chemistries were incomplete, whereas the pre-assembled ligand spacer arm unit could be pre-characterized by conventional chemical techniques and studies in solution to yield useful advance information on binding specificity and kinetic constants. The present authors believe that a combination of both strategies represents an effective means of developing new and well-characterized affinity adsorbents for the purification of target proteins.

A further key development introduced in the early 1970s was that of ‘group-specific’ (17) or ‘general ligand’ (18) adsorbents. An important advantage of ligands with a broad bioaffinity spectrum, such as the coenzymes, lectins, nucleic acids, metal chelates, Protein A, gelatine and heparin, is that it was not obligatory to devise a new organic synthetic strategy for every projected biospecific purification. However, a possible disadvantage of the group-specific approach is that the broad specificity of the adsorption stage required a compensatory specific elution step to restore the overall biospecificity of the chromatographic system. Nevertheless, of the thousands of enzymes that have been assigned a specific Enzyme Commission number, approximately one-third involve one of the four adenine coenzymes (NAD+, NADP+, CoA and ATP), and not surprisingly, these classes of enzymes were the first to be targeted by this approach (17–19) and subsequently extensively exploited in the purification of oxido-reductases by affinity chromatography and in enzyme technology (20–22).

Until this point in time, most of the studies had generated rules-of-thumb on how to apply the technique of affinity chromatography to selected purifications. However, it became apparent on even a rudimentary examination of the theoretical basis of the technique (23) that the implicit assumption that the observed chromatographic adsorption of the target protein to the immobilized ligand was due exclusively to biospecific enzyme–ligand interactions was misguided. The large discrepancies observed between what was anticipated on the basis of the biological affinity for the immobilized ligand and what was observed experimentally to be the case were found to be due to the largely unsuspected interference by non-biospecific adsorption, which, in many cases, completely eclipsed the biospecific adsorption (24–25). O’Carra and co-workers (24–25) demonstrated that spacer arms do not always act simply as passive links between biospecific ligands and the polymer matrix and described methods for the control of interfering non-specific adsorption effects and for the optimization of affinity chromatography performance by a logical and systematic appraisal.
of reinforcement effects and, where applicable, kinetic and mechanistic factors. Whilst the necessity for spacer arms interposed between the ligand and matrix was recognized very early in order to alleviate steric interference, it was not until later that it was realized that the aliphatic hydrocarbons commonly employed as spacers could act as hydrophobic ligands in their own right. In a study with pre-assembled AMP ligands containing spacer arms of varying degrees of hydrophilicity and hydrophobicity, it was found that enzymes bound preferentially to ligands tethered via hydrophobic spacer arms and that the notion of constructing adsorbents comprising a ligand attached to a matrix via a hydrophilic arm in order to ameliorate non-specific hydrophobic interactions may not be a viable proposition. Alternative strategies of combating these undesirable effects, such as inclusion of low concentrations of water-miscible organic solvents in the buffers (e.g., ethylene glycol, glycerol or dioxane), were adopted as they resulted in dramatically improved recoveries of the released enzyme.

Several other advances in ligand selection also had a dramatic effect on the development of the technique of affinity chromatography. Originally, selective adsorbents were fabricated with natural biological ligands as the exquisite selectivity of enzymes, antibodies, receptor and binding proteins and oligonucleotides for their complementary ligands was rational and easily justified on economic grounds. However, experience has shown that the majority of biological ligands are difficult to immobilize with retention of activity and often lead to prohibitively expensive adsorbents that have limited stability in a multi-cycle sterile environment. Paradoxically, the key feature of affinity chromatography, exquisite selectivity, is also its biggest weakness, because off-the-shelf adsorbents other than those with group specificity are often commercially unavailable. Ideal adsorbents for large-scale applications should combine features of selective and non-selective adsorbents, be inexpensive, have general applicability and be stable to a variety of adsorption, elution and sterilization conditions, with specially synthesized quasi-biological ligands offering the best hope of finding general purpose, inexpensive and stable adsorbents.

2.2.1. Synthetic Ligands

The reactive textile dyes are a group of synthetic ligands that have been widely exploited to purify an astounding array of individual proteins. The archetypal dye, Cibacron blue F3G-A, contains a triazine scaffold substituted with polyaromatic ring systems solubilized with sulphonate or carboxylate functions and decorated with electron withdrawing or donating groups. It has been the subject of intensive research ever since it was found serendipitously to bind to yeast pyruvate kinase when co-chromatographed with blue
dextran on a Sephadex G-200 gel filtration column. Subsequent studies demonstrated that it was the reactive chromophore of blue dextran, Cibacron blue F3G-A, that was responsible for binding and not the dextran carrier itself. Sepharose-immobilized Cibacron blue F3G-A is advantageous for large-scale affinity chromatography as it is low cost, generally available, easily coupled to a matrix and exhibits protein-binding capacities that exceed those of natural ligand media by factors of 10–100. Furthermore, synthetic dyes are almost completely resistant to chemical and enzymatic attack and are hence readily cleaned and sterilized in situ, are less prone to leakage than other ligands and yield high capacity, easily identified adsorbents.

It is believed that these dyes mimic the binding of natural anionic heterocyclic substrates such as nucleic acids, nucleotides, coenzymes and vitamins. However, concerns over the selectivity, purity, leakage and toxicity of the commercial dyes limited their use and led to the search for improved “biomimetic” dyes and the adoption of rational molecular design techniques. For example, inspection of the interaction of Cibacron blue F3G-A with horse liver alcohol dehydrogenase provided a sound basis for rational ligand design. X-ray crystallography and affinity labelling studies showed that the dye binds to the coenzyme-binding domain of the enzyme with the anthraquinone, diaminobenzene sulphonate and triazine rings adopting similar positions as the adenine, adenosine ribose and pyrophosphate groups respectively of NAD$^+$. It appeared that the terminal aminobenzene sulphonate ring of the dye was bound to the side of the main NAD$^+$-binding site in a crevice bounded by the side chains of cationic (Arg/His) residues. Thus, the synthesis, characterization and assessment of a number of terminal ring analogues of the dye confirmed the preference for a small, anionic o- or m-substituted group and substantially improved the affinity and selectivity of the dye for the protein. These conclusions have been confirmed with more recent studies with a range of new analogues and demonstrate how the use of modern design techniques can greatly improve the selectivity of biomimetic ligands.

2.2.2. De Novo Ligand Design

The recently acquired ability to combine knowledge of X-ray crystallographic, nuclear magnetic resonance (NMR) or homology structures with defined or combinatorial chemical synthesis and advanced computational tools has made the rational design of affinity ligands even more feasible, powerful, logical and faster. The target site on the protein may be a known active site, a solvent-exposed region or motif on the protein surface or a site involved in binding a natural or complementary ligand. However, the design of a complementary affinity ligand is at best only a semi-rational process, as numerous
unknown factors are introduced during immobilization of the ligand. The affinity of the immobilized ligand for the complementary protein is determined partly by the characteristics of the ligand per se and partly by the matrix, activation, spacer and coupling chemistry. Studies in free solution with soluble ligands do not fairly reflect the chemical, geometrical and steric constraints imposed by the complex three-dimensional matrix environment. Nevertheless, three distinct approaches to ligand design can be distinguished: first, investigation of the structure of a natural protein–ligand interaction and the use of the partner as a template on which to model a biomimetic ligand [40]; second, construction of a molecule which displays complementarity to exposed residues in the target site [41–44]; and third, direct mimicking of natural biological recognition interactions [45].

Peptidal templates comprising two or three amino acids have been used to design highly selective affinity ligands for IgG [40–42], kallikrein [46] and elastase [44] and were synthesized by combinatorial substitution of a triazine scaffold with appropriate analogues of the amino acids.

2.2.3. Combinatorial Ligand Synthesis

However, in many cases, there is inadequate or insufficient structural data on the formation of complexes between the target protein and a substrate, inhibitor or binding protein, to design molecules de novo to interact with the exposed residues of a specified site and ensure that the ligand has complementary functionality to the target residues. A good example of this approach is the design, synthesis and evaluation of an affinity ligand for a recombinant insulin precursor (MI3) expressed in Saccharomyces cerevisiae [43]. Preliminary molecular modelling showed that a lead ligand comprising a triazine scaffold substituted with aniline and tyramine, showed significant π-π overlap with the aromatic side chains of B:16-Tyr and B:24-Phe from the biomolecule, and was thus used as a guide to the type of directed solid-phase combinatorial library that might be synthesized. A library of 64 members was synthesized from 26 amino derivatives of bicyclic, tricyclic and heterocyclic aromatics, aliphatic alcohols, fluorenes and acridines substituted with various functionalities. The solid-phase library was screened for MI3 binding and elution, and fractions from each column were analyzed by reversed-phase high performance liquid chromatography by reference to the known elution behaviour of authentic MI3. Under the specified conditions, the most effective ligands appeared to be bisymmetrical ligands substituted with aminonaphthols or aminonaphthoic acids, with very high levels of discrimination being noted with various ring substituents. Modelling studies showed that bisymmetrical bicyclic-ring ligands displayed more complete π-π overlap with the side chain of residues B:16-Tyr and
B:24-Phe, than the single-ring substituents of the original lead compound used to direct library synthesis. However, despite the value of computer modelling in visualizing putative interactions, the complexity of the three-dimensional matrix environment, with largely unknown ligand–matrix, coupling, activation and spacer molecule chemistry interactions, suggests that rational design and combinatorial chemistry together should be evoked to develop effective affinity ligands. Nevertheless, despite these reservations, the symmetrical ligand 23/23 was synthesized de novo in solution, characterized and immobilized to agarose beads, whence affinity chromatography of a crude clarified yeast expression system revealed that MI3 was purified on this adsorbent with a purity of >95% and a yield of 90% (43). This study showed that a defined structural template is not required and that a limited combinatorial library of ligands together with the use of parallel screening protocols allows selective affinity ligands to be obtained for target proteins.

One of the most widely used combinatorial technologies is based on biological vehicles as platforms for the presentation of random linear or constrained peptides, gene fragments, cDNA and antibodies. The non-lytic filamentous bacteriophage, M13, and the closely related phages, fd and f1, are the most commonly exploited vectors with random peptides displayed on the surface of the phage by fusion of the desired DNA sequence with the genes encoding coat proteins (47, 48). Combinatorial libraries containing up to $10^9$ peptides can be generated and selected for the desired activity by ‘biopanning’ of the phage pool on a solid-phase immobilized target receptor. Bound phage particles are eluted, amplified by propagation in Escherichia coli and the process repeated several times to enrich iteratively for the peptide with the desired binding properties, and whose sequence is determined from the coding region of the viral DNA. Phage display libraries have been successfully applied to epitope mapping, vaccine development, the identification of protein kinase substrates, bioactive peptides and peptide mimics of non-peptide ligands and are eminently suitable as a source of affinity ligands for chromatography or analysis (49). However, a limitation of the phage display approach is that peptides may only function when the peptide is an integral part of the phage-coat protein and not when isolated in free solution (50). These limitations can be circumvented to some extent by using conformationally constrained peptides (51), although issues relating to retention of their function on optimization, scale-up and use on various solid-phase matrices still remain (52). An alternative approach based on ribosome display for the evolution of very large protein libraries differs from other selection techniques in that the entire procedure is conducted in vitro and is particularly appropriate for the screening and selection of folded proteins (53). Other scaffolds exploiting domains from proteins such as fibronectin (“monobodies”), V domains (“minibodies”) or α-helical bacterial
receptor domains ("affibodies") have been shown to yield specific binders, with usually mM affinities, from libraries of up to $10^7$ clones \(^{(54)}\).

Recently, it has been shown that peptides of a modest size isolated from a combinatorial library using a simple genetic assay can act as specific receptors for other peptides \(^{(55)}\). However, peptide arrays are known to offer advantages, particularly in signal-to-noise ratio and in the chromatographic optimization steps \(^{(56)}\). A good example of the use of randomized synthetic peptidomers for the affinity purification of antibodies has been reported \(^{(57)}\). The lead peptide mimics *Staphylococcus aureus* protein A in its ability to recognize the Fc fragment of IgG and offers a one-step isolation of 95% pure antibody from crude human serum. Panels of peptides derived from a combinatorial library were also shown to bind human blood coagulation factor VIII \(^{(58)}\).

A similar approach to peptide phage display involves the use of oligonucleotide-based combinatorial biochemistry, in which the nucleotides on the DNA polymerase-encoding gene 43 regulatory loop of bacteriophage T4 are randomized \(^{(59,60)}\). The so-called systematic evolution of ligands by exponential enrichment (SELEX) technology can yield high affinity/high specificity ligands for virtually any molecular target. Several of the ligands, aptamers, that emerge from this method, where starting libraries may contain up to $10^{14}$–$10^{15}$ sequences, have been shown to have pM-nM affinities for their binding partners. DNA-aptamer affinity chromatography has recently been applied to the purification of human L-selectin from Chinese hamster ovary cell-conditioned medium \(^{(61)}\). The aptamer column resulted in a 1500-fold single-step purification of an L-selectin fusion protein with an 83% recovery. Figure 2 summarizes the various types of affinity ligand and the stages in their development.

### 2.3. Impact of the Biopharmaceutical Industry

The development of novel therapeutic proteins must rank amongst the most laborious and capital intensive of all industrial activities. The nascent biotechnology industry faces two principal challenges in fulfilling this promise to deliver new therapeutics. The first relates to the production of specified therapeutic proteins at an appropriate price, scale and quality. Many of the potential customers, particularly health service providers, are struggling to contain rising costs and are thus cautious about using high-cost therapies based on biopharmaceuticals. As much as 50–80% of the total cost of biomanufacturing is incurred during downstream processing, purification and polishing. Thus, the need to revise existing production processes to improve efficiency and yields is high on the agenda of many manufacturers. Furthermore, changes in the regulatory climate have shifted the focus of regulation from defining production processes
Fig. 2. Types of affinity ligands utilized in the separation of biomolecules.

By itself to the concept of the “well-characterized biologic.” Under this regime, the final protein will be required to have defined purity, efficacy, potency, stability, pharmacokinetics, pharmacodynamics, toxicity and immunogenicity. The product should also be analyzed, not only for contaminants such as nucleic acids, viruses, pyrogens, residual host cell proteins, cell culture media, leachates from the separation media and unspecified impurities, but also for the presence of various isoforms, originating from post-translational modifications in the host cell expression system, such as glycosylation, sulphation, oxidation, misfolding, aggregation, misalignment of disulphide bridges and nicking or truncation. A thorough characterization of the potency, purity and safety of proteinaceous drugs using high performance hyphenated techniques is now required.

This new challenge has necessitated a radical re-think of the design and operation of purification processes, with the options being largely dictated by their speed of introduction, effectiveness, robustness and economics. Conventional purification protocols are now being substituted with highly selective and sophisticated strategies based on affinity chromatography. This technique provides a rational basis for purification and simulates and exploits natural biological processes such as molecular recognition for the selective purification of the target protein. Affinity chromatography is probably the only technique currently able to address key issues in high-throughput proteomics and scale-up. The principal issue is to devise new techniques to identify highly selective affinity ligands, which bind to the putative target biopharmaceuticals. Not surprisingly, the value of computer-aided design and combinatorial strategies for the design of ultra stable synthetic ligands has been appreciated.
A further issue of concern to the FDA and relating to both biological and synthetic ligands is that of leakage. The regulatory authorities insist that any biological ligand used in the manufacture of a therapeutic product meet the same requirements as the end product itself. This notion extends even to how the affinity ligand is produced and purified. A good example of this strategy lies in the design, synthesis and chromatographic evaluation of an affinity adsorbent for human recombinant Factor VIIa. The requirement for a metal ion-dependent immuno-adsorbent step in the purification of the recombinant human clotting factor, FVIIa, and hence scrutiny by the FDA, has been obviated by using X-ray crystallography, computer-aided molecular modelling and directed combinatorial chemistry to design, synthesize and evaluate a stable, sterilizable and inexpensive “biomimetic” affinity adsorbent. The ligand comprises a triazine scaffold bis-substituted with 3-aminobenzoic acid and was shown to bind selectively to FVIIa in a Ca\(^{2+}\)-dependent manner. The adsorbent purifies FVIIa to almost identical purity (>99%), yield (99%), activation/degradation profile and impurity content (~1000 ppm) as the current immuno-adsorption process, while displaying a 10-fold higher capacity and substantially higher reusability and durability. A similar philosophy was used to develop synthetic equivalents to Protein A and Protein L.

2.4. The “Omics” Revolution

The “omics” technologies of genomics, proteomics and metabolomics collectively have the capacity to revolutionize the discovery and development of drugs. Genomics specifies the patterns of gene expression associated with particular cellular states, whereas proteomics describes the corresponding protein expression profiles. However, many key aspects of proteomics, such as the concentration, transcriptional alteration, post-translational modification, intermittent or permanent formation of complexes with other proteins or cellular components, compartmentation within the cell, and the modulation of biological activity with a plethora of small effector molecules, are not encoded at the genetic level but influence the function of the protein and can only be clarified by analysis at the protein level. These modulations often play a crucial role in the activity, localization and turnover of individual proteins. The inability of classical genomics to address issues at the protein level in sufficient detail is a crucial shortcoming, as most disease processes develop at this level. Thus, the field of proteomics will require the development of a new toolbox of analytical and preparative techniques that allow the resolution and characterization of complex sets of protein mixtures and the subsequent purification of individual target therapeutic proteins.
Affinity Chromatography is regarded as an indispensable tool in proteomics allowing the discrimination of proteins by diverse principles based on reverse-phase, ion exchange, size-exclusion, hydrophobic and affinity interactions. The technique is potentially useful not only for the separation of specific groups of proteins, but also for the exploration of post-translational modifications and the study of protein–protein and protein–ligand interactions.

Furthermore, the use of affinity chromatography to enrich scarce proteins or deplete over-abundant proteins is a powerful means of enhancing the resolution and sensitivity in two-dimensional electrophoresis (2D-PAGE) or mass spectrometry (MS) analysis. Isotope-encoded affinity tags may represent a new tool for the analysis of complex mixtures of proteins in living systems. Alternatively, element-encoded metal chelates may also prove helpful for affinity chromatography, quantification and identification of tagged peptides from complex mixtures by LC-MS/MS.

A significant development in affinity techniques for proteomics is the use of fusion tags or proteins for expression and purification. A large choice of systems is available for expression in bacterial hosts, with a further selection amenable for eukaryotic cells. Amongst the most popular fusion partners for molecular, structural and bioprocessing applications are the polyArg, hexaHis-tag, glutathione-S-transferase and maltose-binding protein. Other less commonly employed expression tags include thioredoxin, the Z-domain from Protein A, NusA, GB1 domain from Protein G and others. A recent comparison of the efficiency of eight elutable affinity tags for the purification of proteins from E. coli, yeast, Drosophila and HeLa extracts shows that none of these tags is universally superior for a particular system because proteins do not naturally lend themselves to high throughput analysis and they display diverse and individualistic physicochemical properties. It was found that the His-tag provided good yields of tagged protein from inexpensive, high capacity resins but with only moderate purity from E. coli extracts and poor purification from the other extracts. Cellulose-binding protein provided good purification from HeLa extracts. Consequently, affinity tags are invaluable tools for structural and functional proteomics as well as being used extensively in the expression and purification of proteins. Affinity tags can have a positive impact on the yield, solubility and folding of their complementary fusion partners. Combinatorial tagging might be the solution to choosing the most appropriate partner in high throughput scenarios.

2.5. Resolution of Isoforms

Heterogeneity in proteins may arise due to variations in post-translational modifications during the synthesis of a protein in native, recombinant or
transgenic systems. These variations may include altered glycosylation, unnatural or incomplete disulphide bond formation, partial proteolysis, amino- and carboxy-terminal sequence alterations and oxidation or deamidation of amino acids, unnatural phosphorylation or dephosphorylation, myristoylation or sulphation of amino acids. The expressed proteins may then differ in function, kinetics, structure, stability and other properties affecting their biological role. Most proteins produced by recombinant DNA technology for in vivo administration are glycosylated and may have glycoform heterogeneity due to variable site occupancy of the sugar moieties on the protein or due to variations in the carbohydrate sequence. Consequently, in the future, it may be important to be able to isolate and purify recombinant glycoforms with defined glycosylation and biological properties prior to administration because mixtures of isoforms could have serious side effects on human health. The concepts of rational design and solid-phase combinatorial chemistry have been used to develop affinity adsorbents for glycoproteins \(^{(81, 82)}\). The strategy for the resolution of glycoforms involves generation of synthetic ligands that display affinity and selectivity for the sugar moieties on glycoproteins but which have no interaction with the protein per se. A detailed assessment of protein–carbohydrate interactions from a number of known X-ray crystallographic structures was used to identify key residues that determine monosaccharide specificity and which were subsequently exploited as the basis for the synthesis of a library of glycoprotein-binding ligands \(^{(82, 83)}\). The ligands were synthesized using solid-phase combinatorial chemistry and were assessed for their sugar-binding ability with several glycoproteins. Partial and completely deglycosylated proteins were used as controls. A triazine-based ligand, bis-substituted with 5-aminoindan, was identified as a putative glycoprotein-binding ligand, because it displayed particular affinity for mannose moieties. These findings were substantiated by interaction analysis between the ligand and mannose moieties through NMR experiments \(^{(83)}\). \(^{\text{1}}\)H-NMR studies and molecular modelling suggested involvement of the hydroxyls on the mannose moiety at C-2, C-3 and C-4 positions. Small peptides selected from a library of 62,000 chemically synthesized peptides have also been shown to display some selectivity for binding monosaccharides, although their application in the chromatographic resolution of glycoproteins was not established \(^{(84)}\).

3. Conclusions

This review has looked at the history, current status and prospects for affinity chromatography and identified techniques that are able to rationalize the design and selection of affinity ligands for the purification of pharmaceutical proteins.
Two strategies are evident: first, screening for target binding to large combinatorial libraries of peptides, oligonucleotides, antibodies, various natural binding motifs and synthetic ligands and, secondly, the introduction of a design step to reduce the size of the directed libraries. The approach adopted depends to a large extent on what information is available at the outset; if structural data is at hand, the design approach is possible, whilst in the absence of such information, which may be the case in many proteomics applications, a combinatorial screen would be the only route available. The present author prefers the ‘intelligent’ approach, because it drastically reduces the chemistry and screening necessary to identify a lead ligand. Nevertheless, combinatorial screening is still required to obviate many of the unknowns involved in the interaction of protein with solid-phase immobilized ligands. A key aspect of this system is that the chromatographic adsorption and elution protocols can be in-built into the total package at the screening stage and therefore lead to very rapid conversion of a hit ligand into a working adsorbent. Rapid screening techniques based on fluorescently labelled proteins (85), ELISA (64), surface plasmon resonance (86) and the quartz crystal microbalance (87) are now available.

The use of synthetic ligands offers a number of advantages for the purification of pharmaceutical proteins. First, the adsorbents are inexpensive, scaleable, durable and reusable over multiple cycles. Secondly, the provision of a ligand with defined chemistry and toxicity satisfies the regulatory authorities. Finally, the exceptional stability of synthetic adsorbents allows harsh elution and cleaning-in-place and sterilization-in-place protocols to be used. These considerations remove the potential risk of prion or virus contamination, which may arise when immunoadsorbents originating from animal sources are used. Other types of affinity ligand based on peptide, oligonucleotide or small protein libraries are likely to be less durable under operating conditions, which employ harsh sterilization, and cleaning protocols.

References


