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Review

Preparative affinity chromatography of proteins

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ABSTRACT

This review describes the role of affinity chromatography in large-scale protein purification. The currently available affinity supports are examined with respect to their use in the preparative scale. Factors that play a major role in successful large-scale affinity purification are discussed. The use of stable synthetic, group-specific ligands, is advocated in the place of fragile, biological counterparts. The use of these affinity media and the validation of affinity chromatography in the process-scale purification of therapeutics are addressed.

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1. INTRODUCTION

Of all the separation mechanisms currently used in macromolecular separation sciences, affinity chromatography is considered to be the most specific, since it is based on the unique specificity inherent in a ligand–biomacromolecule interaction [1,2]. The absolute dependence of affinity interaction on biological recognition rather than physico-chemical properties implies that the technique is suitable for preserving the biological and immunological activity of the isolated protein or antibody. Affinity chromatography is also suitable in cases where the protein to be isolated is (1) of extreme value and (2) present in very dilute solutions, as low as a few micrograms per milliliter which makes them unstable during the isolation process. Affinity-based techniques are able to concentrate dilute amounts of the expensive molecule and stabilize the protein when adsorbed onto a ligand for which it has a natural affinity.

For these reasons, the use of affinity chromatography is on the rise in laboratories. Although it is widely accepted as a powerful technique capable of purifying a solution sometimes as much as 3000 fold in a single step, its use has been limited at the preparative scale because of two main problems: the high cost of the affinity ligand (media) and the instability of such media in a multicycle, pyrogen-free, hard to validate environment [3].

In this review, an attempt is made at possibly overcoming the limitations of this technique and put together the commercial availability of preparative affinity-based chromatographic supports.

1.1. Factors important in the design of large-scale chromatography of proteins

The requirements of a large-scale purification protocol are mainly determined by the nature and quality of the desired final product and its intended use. For example, proteins for therapeutic use need to be extremely pure to minimize the risk of unwanted side effects or immunogenic response. Conversely, materials for use in industrial processes need not always be absolutely pure. So, it is important to define if, for example, any one impurity is more important than the others, or is there any acceptable contamination (of less than 1%) of any known impurity. Four factors dictate at what stage affinity chromatography can best be exploited. These are (1) the concentration of the desired product in the starting material, (2) the composition of other components in the starting material along with its physical and chemical properties, (3) the desired product purity and (4) the volume of material to be processed. However, there are some common parameters which dictate the success of a preparative chromatographic process, whether it is based on affinity, ion-exchange or hydrophobic mode [4]. Some of these pertinent factors are:

Resolution (selectivity)
Recovery
Throughput
Reproducibility
Stability
Maintenance
Economy
Convenience

Each stage of an affinity chromatography process: adsorption, washing, elution and regeneration needs to be optimized before the process is scaled up [5,6]. In the adsorption stage, the molecule of interest is brought into contact with the affinity matrix and the interactions are allowed to occur. In the wash cycle, loosely or non-specifically bound molecules are removed. During elution, the molecule to be purified is released from the affinity ligand. The regeneration stage prepares the matrix for the next cycle.

Resolution of an affinity chromatographic

separation depends on the specificity and selectivity of the interaction between the immobilized ligand and the molecule to be separated. In an ideal separation process, the association constant of the complex should be neither too low nor too high, in the range of 10^3–10^8 *M*. The efficiency of the chromatographic system is also important in determining resolution.

Recovery or the amount of active protein that is recovered at the end of the process and throughput are among the several factors that determine its cost effectiveness. While producers of high-value therapeutics, for example, interferon, remain unconcerned about process economics, others dealing with products of biotechnological origin no longer have that option. Separation costs account for 50–80% of total production costs and chromatography is often the most expensive unit process in a separation protocol. More economical chromatographic systems, therefore, will become increasingly important. According to one survey, in a typical large-scale process, labor costs emerge as the most significant contributor in the overall cost of the protein or drug [7].

Moreover, in a multistep purification process of a biomolecule, each step needs to be consistent and reproducible. The stability of the chromatographic media is an important criterion in the success of affinity chromatography since the ligands are often fragile biological molecules. Affinity media can lose its effectiveness because of unstable ligands, growth of bacteria and mould leading to microbial contamination, clogging due to the presence of insoluble matter in the sample and in the eluting buffers. Accumulation of denatured protein, lipids, nucleic acids etc., that are not eluted during the regeneration process can also limit the lifetime of the column. A preparative column sees more protein in three or four preparative cycles than an analytical column sees in two or three hundred cycles. Under these conditions, maintenance of the affinity media becomes very important. Stringent cleaning-in-place procedures are recommended by the manufacturers of the media to prolong its lifetime. The feasibility of such measures should be taken into consideration before a purification method is scaled up [8].

1.2. Implication of affinity chromatography theory on scaling up

An important requirement for a successful scale-up of affinity chromatography is a thorough understanding of the fundamental mechanisms involved in the separation mechanism and this is provided by several reviews [6,9,10]. In addition, these authors have addressed the predications, implications and limitations of affinity chromatography models. Geometric parameters to consider while scaling up a purification protocol are length and diameter of the column, and particle size and pore size of the affinity support. Key physical parameters are injection volume, flow-rates, temperature and pH. Chemical parameters to be considered are solvent composition, nature of the stationary phase and operating parameters like backflushing and mobile phase recycling [6].

Although each protein separation process must be individually optimized and no general equation can indicate the best set of parameters for a specific separation problem, several groups are developing algorithms to predict optimum flow-rates, gradients, buffer compositions and temperatures that will be best for a particular purification process [11,12]. Yamamoto and Sano [13] have reported a short-cut method for predicting the productivity of affinity chromatography. Productivity is described as the amount of the target protein recovered per unit volume per unit time. The experimental breakthrough curves for several different packing media (40- and 10-μm porous particles, 2.5-μm non-porous particles) are determined by this method.

2. THE AFFINITY SUPPORT

As in the case of supports used in other modes of chromatography, affinity matrices need to satisfy certain criteria [14,15]. The desirable properties of affinity supports are:

Inert
Hydrophilic
Non-biodegradable
Chemically and physically resistant
Easily derivatized
Easily packed in large columns
Macroporous

The choice in affinity matrices and the requirements of an ideal matrix have been the subject of several reviews. In process-scale operations, the three most important characteristics are high adsorption capacity, high mass transfer coefficient and high liquid flow-rate through adsorbent bed [16]. In process-scale operations, in addition to the above three characteristics, the cost of the packing material is also an important consideration. Physical characterization of the support material in terms of its pore size, particle size, pore volume and pore surface area will help in determining the mass transfer rate of solutes within the chromatographic bed and its effective ligand binding capacity [17]. The reader is referred to an excellent review on optimization and scale-up of affinity chromatography [9].

Based on the specific pore size of support materials, affinity supports can be classified into four main categories [18].

(1) Non-rigid or soft support materials like agarose which are macroporous with a molecular size exclusion limit of upto $40 \cdot 10^6$ daltons.

(2) Rigid support materials like silicas, aluminas or zerolites which exhibit a permanent porosity. These materials maintain their porosity when exposed to vapors or immersed in liquids and are available in pore sizes ranging from 60–5000 Å [19].

(3) Non-porous microparticulate materials which are made of either polymer or silica [20].

(4) A novel polymeric support which is made of some large pores that allow solutes to penetrate the diameter of the particle by a convective

TABLE 1

SUPPORT MATERIALS USED IN PREPARATIVE AFFINITY CHROMATOGRAPHY

Trade name	Particle size (μm)	Support material	Supplier[a]
Trisacryl	40–80 60–180	Acrylic polymer	IBF
Ultrogel	60–140	Polyacrylamide or agarose	IBF
Sepharose 4B	60–140	Agarose	PLKB
6B	45–165		
Sephadex	10–300	Cross-linked dextran	PLKB
Superose	10–12	Agarose	PLKB
Macrosorb		Various organic powders	SO
Eupergit C	30	Methacrylamide–methylbisacrylamide	RP
Affigel	80–150	Agarose	B
Matrex cellufine	45–105	Cellulose	A
Bakerbond wide-pore	40	Polymer clad silica	JTB
AvidGel P	40–120	Polymer	BP
HiTrap	34	Agarose	PLKB
Poros	15–25	Polymer	PBS
Fractogel TSK	32–63	Vinyl polymer	M
Protein pak	37–55	Silica–cellulose	W/M
Cross-linked agarose	45–165	Agarose	P
Controlled pore glass	74–125 or 125–177	Glass	P
HiPac	30	Silica	C
Preflex	–	Fluoropolymer	D
Magnogel	60–140	Polyacrylamide agarose with 7% Fe_3O_4	IBF

[a] A = Amicon; B = Bio-Rad; BP = Bioprobe international; C = Chromatochem; D = DuPont; P = Pierce; PBS = Perceptive Biosystems; PLKB = Pharmacia LKB; M = Merck; RP = Rohm Pharma; SO = Sterling Organics; W/M = Waters Millipore.

flow and some small diffusive pores that provide the large surface area [21]. This new support operates under the term "perfusion chromatography".

A mathematical model for perfusion chromatography is reported by Liapis and McCoy [22]. The values for several variables which can be used to evaluate column performance and also the breakthrough curves are compared for perfusive particles and purely diffusive adsorbent particles. The results suggest that for adsorption systems composed of relatively fast or infinitely fast interaction kinetics between the adsorbate molecules and the active sites, the use of perfusive particles can have the potential to provide improved column performance [22].

The binding capacity of the support material is directly proportional to its surface area [17,23]. Foster and Anderson [23] have shown that capacity is related to surface area and the ability of the solute to difffuse in and out of the porous silica. This observation is based on a binding study of immunoglobulin G (IgG) to Protein A. In the case of the binding of proteins to immobilized Cibacron Blue F3G-A, Horstmann *et al.* [24] conclude that the maximum capacities obtained increase with decreasing particle size in Sepharose-based affinity supports. Rounds *et al.* [25] demonstrate that in anion-exchange chromatography, the binding of proteins is dependent on accessible surface area (that is the total pore surface area of the support excluding the external particle surface) rather than the total surface area of the support, indicating that wide pores in addition to high surface area provide maximum capacity.

Table 1 lists the commercial availability of supports composed of medium-sized particles that can withstand higher flow-rates than the conventional rigid supports used in HPLC. The main support materials are cellulose, silica, porous glass, synthetic organic polymers such as polyacrylamide and other polymers such as methacrylate. Quartz fibers have recently been reported as an alternative to spherical packing since they are non-porous and can be derivatized in several different ways [26]. The choice of separation media for preparative chromatography is also addressed by Low [27]. Please read Janson and Kristiansen [15] for a detailed survey of affinity packings and their commercial availability.

3. AFFINITY LIGANDS

3.1. Choice of chemistry in coupling ligands to support materials

In order for the ligand to be stable, it needs to be covalently coupled to the support by some functional groups that can be easily and conveniently derivatized. Several reviews deal with the choices available in the coupling chemistry [28,29]. A wide range of ready-to-use or preactivated support materials are commercially available [15].

Fig. 1 depicts some of the commonly used coupling strategies for supports with hydroxyl or amide groups. Although there is a wide choice in activation procedures, their use is limited in process-scale applications. The time required to immobilize the affinity ligand to a preactivated support is an important consideration in large-scale operations especially when the ligand happens to be a fragile biological molecule which may not be stable over long periods of immobilization time. Contamination of the final product with the ligand due to leakage is yet another deciding factor while choosing the coupling chemistry. Stability of the affinity support is hence always questioned and verified.

Stability of the affinity ligand is important while purifying therapeutic grade products. For example, in the purification of interferon α-2a by immunoffinity chromatography, the contamination of the purified product by IgG was evaluated [30]. About 60 ng mouse IgG per ml is found in the eluate after several hundred cycles although the capacity of the immunosorbent for interferon has not decreased. Contamination of copper in the range of 30–50 ng/ml was also reported from a copper chelate column. A new immobilization technology based on adsorption of biomolecules onto fluorocarbon surfaces is recently reported [31]. Proteins are perfluoroalkylated before being introduced to the fluorocarbon matrix. The authors claim enhancement of avidity of proteins for the support and mainte-

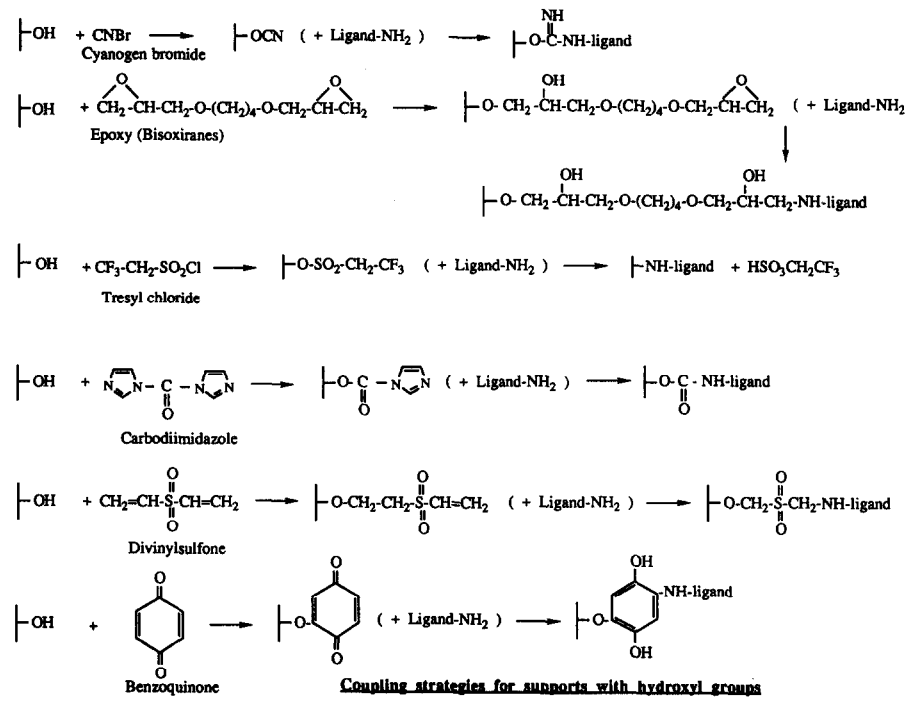

Fig. 1. Coupling strategies for supports with hydroxyl and amide groups.

nance of biological activity and biospecificity [31,32]. McCreath *et al.* [33] report the use of a perfluorocarbon emulsion in a fluidized bed for the purification of human serum albumin using affinity chromatography. The liquid affinity support is reported to be stable under operational conditions with no coalescence for at least a year. Jervis [34] outlines desirable characteristics of an ideal activation/coupling chemistry as (1) rapid, efficient formation of a stable, uncharged, covalent bond under mild conditions with no side reactions, (2) easy blocking of residual activated groups with simple, hydrophilic, uncharged groups and (3) use of inexpensive, non-toxic reagents in procedures which can be scaled up.

3.2. Ligand density and binding capacity of affinity media

The degree of substitution or the ligand density of the support material is one of the several factors that affect the adsorption characteristics of affinity supports [17]. The binding capacity of the affinity matrix usually increases in proportion to the amount of ligand immobilized. However, in a study were several immunosorbents are compared, it is noted that the antibody binds more efficiently to low substituted immunosorbents and the processing time can be decreased by using higher flow-rates with low substituted sorbents [35]. Low substitution also helps in optimizing elution conditions since elution is often a problem when high-affinity antibodies are used. The disadvantage in using a larger column packed with a low substituted sorbent is that (1) there is more washing and eluting buffer consumption and (2) the final product is collected in a larger volume, thereby diluting the molecule of interest [35,36].

In large-scale applications, it may be necessary to sacrifice high specific activity or rates of adsorption of the more porous supports in favor of increased stability and lifetime associated with the more rigid supports. In one study, silica is used as the base support and proteins are immobilized through diol groups. Although the total amounts of activity and protein immobilized decreased at low ligand densities, the specific activity of two model proteins increased at low ligand (carboxylate spacer arm) densities [37]. From a practical point of view, large-scale operators typically aim for high ligand density. Hearn and Davies [38] report that in the case of immunoaffinity supports, at high antibody densities, the accessibility of the immobilized antibody decreases due to steric resistance as the antigen size increases.

3.3. Choice of ligands

In affinity-based interaction, the ligand plays a very significant role in the success of the purification protocol. In addition to specificity, the affinity ligand has to be stable and economical. If the affinity support is specific for one particular sample, it is frequently not economical and its use is limited as a chromatographic tool.

3.3.1. Natural versus synthetic ligands

The concept of general ligand chromatography was introduced to overcome the "one protein–one ligand" practice [39]. General ligand chromatography is best used when the protein of interest is present in reasonable concentration. Tables 2 and 3 describe some of the more popular group-specific affinity ligands and their properties and commercial availability. General ligands can be classified as biological (examples are nucleotides, lectins, Protein A and Protein G (Table 2) or non-biological (pseudospecific or synthetic), such as dyes, metals and amino acids (Table 3).

While natural ligands have an inherent biological specificity for a molecule, synthetic or pseudobiospecific ligands are often made specific by varying and optimizing the binding and elution conditions. Natural ligands are often unstable and so its use is limited at ambient or elevated temperatures or under other harsh conditions. Stringent cleaning-in-place procedures are also difficult to perform on these affinity media. Often, the high specificity means low dissociation constants and stronger elution buffers. The synthetic ligands are robust and not susceptible to denaturation. They are also more stable under strong elution conditions and more cost effective than their biological counterparts.

TABLE 2

READY TO USE GROUP SPECIFIC NATURAL LIGANDS

Ligand	Trade name	Support	Supplier[a]
Concanavalin A	Con A Sepharose	Agarose (45–165 μm)	PLKB
Wheat germ Lectin	Lentil lectin Sepharose 4B	Agarose (45–165 μm)	PLKB
Helix pomatia Lectin	*Helix pomatia* Lectin Sepharose 4MB	Agarose (200–300 μm)	PLKB
Heparin	Heparin Avidgel P	Polymer (40–120 μm)	BP
	HiTrap Heparin	Agarose (34 μm)	PLKB
	Heparin Sepharose CL-6B	Agarose (45–165 μm)	PLKB
IgG	IgG Sepharose 6 FF	Agarose (45–165 μm)	PLKB
Protein A	HiPac Protein A	Silica (30 μm)	C
	AffiPrep Protein A	Polymer (40–60 μm)	B
	HiTrap Protein A	Agarose (34 μm)	PLKB
	Protein A AvidGel F	Polymer (40–120 μm)	BP
	POROS A	PSDVB (15–25 μm)	PBS
Protein G	Gammabind Protein G	Silica (30 μm)	G
	HiTrap Protein G	Agarose (34 μm)	PLKB
	Protein G Sepharose 4 Fast flow	Agarose (45–165 μm)	PLKB
	POROS G	SVDB (15–25 μm)	PBS
Benzamidine	Benzamidine Sepharose 6B	Agarose (45–165 μm)	PLKB
5′ AMP	5′ AMP Sepharose	Agarose (45–165 μm)	PLKB
2′5′ ADP	2′5′ ADP Sepharose 4B	Agarose (45–165 μm)	PLKB
7-Methyl-GTP	7-Methyl-GTP Sepharose 4B	Agarose (45–165 μm)	PLKB

[a] For abbreviations of supplier's names see Table 1.

TABLE 3

READY TO USE GROUP SPECIFIC SYNTHETIC LIGANDS

Ligand	Trade name	Support	Supplier[a]
Cibacron Blue F3G-A	HiTrap Blue	Agarose (34 μm)	PLKB
	Blue Sepharose 6 fast flow	Agarose (45–165 μm) 6% cross-linked	PLKB
	Blue Sepharose CL-6B	Agarose (45–165 μm) 6% cross-linked	PLKB
Procion Red HE-3B	Red Sepharose CL-6B	Agarose (45–165 μm) cross-linked	PLKB
Lysine	Lysine AvidGel P	Polymer (40–120 μm)	BP
	Lysine Sepharose	Agarose (40–165 μm)	PLKB
Arginine	Arginine Sepharose	Agarose (40–165 μm)	PLKB
IgG binding	Avid AL	Polymer (40–120 μm)	BP
Chelating support for IMAC	Chelating Sepharose FF	Agarose (45–165 μm)	PLKB
	HiTrap chelating Sepharose	Agarose (34 μm)	PLKB

[a] For abbreviations of supplier's names see Table 1.

3.3.2. Specific natural ligands

3.3.2.1. Immunoaffinity ligands. The most popular among the specific natural ligands is the antigen–antibody interaction that is consolidated under "immunoaffinity chromatography". Immunopurification has been reported as the ultimate affinity-based method where the immobilized antibody raised against a particular protein interacts with a single surface feature of the protein, not necessarily a ligand binding site (in this way it differs from a true affinity adsorbent) [40]. Antigen–antibody interaction being very specific has a high binding constant (a low dissociation constant (K_d), 10^{-8} to 10^{-6} *M* for polyclonal antibodies and 10^{-12} to 10^{-8} *M* for monoclonal antibodies). Too low a K_d is often a major problem in this case.

Both monoclonal and polyclonal antibodies have been used and literature abounds with purification methods based on this concept. Elution is usually effective at a low pH of 2–3 (typically a glycine–HCl buffer is used). High salt can alternatively be used if the interaction happens to be largely electrostatic. Chaotropic salts such as thiocyanate or lithium bromide have also been infrequently used. In all these cases, the antigen is exposed to harsh conditions and its stability is threatened. Elution is milder in the case of a monoclonal antibody column since the dissociation constant is at least an order of magnitude higher than with polyclonals. Since clones of monoclonal antibodies can be stored in liquid nitrogen, an indefinite supply of a particular antibody is possible. All the coupled IgGs on the affinity media are specific for the desired enzyme, so binding capacity of the adsorbent is generally at least ten fold higher than with a polyclonal antibody immobilized column [40].

An example of a typical positive immunopurification is described for the purification of interleukin-2 (IL-2). A monoclonal antibody to interleukin-2 is immobilized on cyanogen bromide-activated Sepharose at a concentration of 8 mg IgG/ml gel. Equilibration buffer is 0.1 *M* phosphate, pH 7.5 containing 0.5 *M* NaCl. After loading the column with crude IL-2-derived from *E. coli* lysate, the unbound protein is washed with the equilibration buffer containing 1.0 *M* potassium chloride. IL-2 is consequently eluted by lowering the pH to 4.0. The eluent is directly neutralized to pH 7.5 by adding 2 M Tris–HCl, pH 7.5. A purification of about 1000 fold is achieved by positive immunopurification [40].

In negative immunopurification, the immobilized antibody is specific for a particular contaminant. Equilibration and elution conditions are based on the same concept as for the positive immunopurification. If the antigen (contaminant) is not required for further use, its stability is not a major problem during the regeneration process of the column. A very commonly practiced example of negative immunopurification is the removal of bovine serum albumin (BSA) from serum samples prior to analysis. BSA is bound to the anti BSA antibody while the rest of the sample flows through. The binding capacity of the column for BSA has to be carefully studied in this case. In one case, BSA content of 10% (w/w) was reduced to less than 0.05% (w/w) demonstrating at least a 200-fold reduction of BSA in a single step [40].

It is important to understand the immobilization process of the antibody to the solid support especially when it is to be used in large-scale operations. Immunosorbents prepared through the carbohydrate moiety of the IgG molecule (oriented coupling) show dramatic increases in antigen capacity over those prepared by the random coupling through primary amino groups of the IgG [41]. The theoretical antigen-binding capacity of the immunosorbent is decreased when the immobilization chemistry relies primarily on the reactivity of the IgG's free lysine residues with an activated ester or other reactive groups on the support.

3.3.2.2. Oriented versus random coupling. Coupling the antibody to a solid-phase support which has been activated with a terminal hydrazide group is an example of oriented coupling [42]. The vicinal hydroxyl groups of the antibodies' carbohydrate moieties is oxidized to aldehydes by sodium periodate. These aldehyde groups react with the matrix hydrazide. Since the reactive carbohydrates are usually located outside the antigen binding domain and no other groups within the protein will react with the matrix hydrazide, an oriented coupling is fa-

vored. By carefully adjusting the pH and reaction times, the tendency for random coupling of amino groups that results in a sterically hindered immunoligand can be controlled [42]. Although hydrazide chemistry through the Fc region of the antibody is reported to be successful in increasing the binding capacity, recent evidence suggests that these carbohydrate moieties can be found on the Fab region of antibodies as well. In addition, the distribution of these carbohydrate moieties are reported to be highly antibody specific leading to variability with the hydrazide immobilization technique [43].

The use of immunoaffinity chromatography on a preparative scale is well cited in the literature. In one study, a 200-ml preparative column is reported to be capable of generating greater than 0.4 g of antibody in a single run [44]. IgG from mouse and human sources are coupled to a commercial polymeric support derivatized with N-hydroxysuccinimide. Coupling efficiency varies from 42 to 97% depending on the ligand type and concentration [44]. Highsmith *et al.* [45] evaluated three different coupling strategies for the purification of factor IX by immunoaffinity chromatography. Three different resins namely Sepharose CL2B activated with cyanogen bromide a synthetic polymer bead activated with 2-fluoro-1-methyl pyridinium toluene-4-sulfonate (FMP) and a cross-linked 2% agarose with free hydrazide groups are evaluated for their binding capacity to bind factor IX. The purity and recovery of factor IX compared. The FMP resin exhibited the lowest capacity, binding only 2% of the factor IX feed, cyanogen bromide-Sepharose bound 87% while the hydrazide resin bound 43%. The results suggest that hydrazide activation may be insufficient to orient monoclonal antibody and also other factors such as steric hindrances and diffusional resistances during immobilization may be important. The authors emphasize that these results are specific for factor IX and the antibody used in their study.

3.3.3. Group-specific natural ligands

3.3.3.1. Nucleotides. Nucleotides such as NAD and ATP have been used to purify nucleotide binding proteins but the cost of the nucleotide-immobilized media and the instability of the ligand are major drawbacks in adapting these methodologies on a large scale [46].

3.3.3.2. Lectins. Immobilized lectins constitute an important part of group-specific ligands [47]. They are extensively used as affinity supports for selectively binding carbohydrates and glycoproteins. While selecting a lectin to purify a glycoprotein, the nature of the oligosaccharide linked to the protein of interest has to be evaluated. A detailed account of the variety of lectins and their commercial availability is documented [47].

The most popular among the lectins is concanavalin A (Con A). Con A is coupled to a variety of supports; one of which is a widepore, polymer-coated, silica-based support which has aldehyde functional groups for coupling [48]. These widepore matrices are unique in that they can withstand a much higher pressure than the normal silica-based supports since they are made of 30–70 μm particles. Hence, these are particularly useful for preparative chromatography of proteins. Con A exhibits specificity towards α-D-mannosyl or glucosyl moieties of proteins and has been used in the purification of several glycoproteins, notably hormonal proteins, human angiotensinogen, the prohormone of the angiotensin peptides, human prostatic acid phosphatase (a diagnostic indicator in the detection of prostatic cancer), α-fetoprotein, membrane-bound glycosyltransferases and other glycoconjugates containing mannose or glucose [49–53].

Lectin affinity chromatography is carried out under very mild conditions, elution being affected by low concentrations of the appropriate saccharide. Lectin affinity chromatography tolerates the presence of high salt concentrations in the binding buffer since interactions between the sugar residue on the glycoprotein and the lectin are not ionic in nature. This is advantagenous since salt prevents non-specific binding of proteins with the matrix. Binding of glycoproteins to lectin columns is pH dependent and samples are required to be buffered in the pH range of 6.8–7.5. Free sugars which may be frequently present in the cytoplasm or culture medium should be removed to avoid competition with the glycoproteins for lectin binding sites. The pres-

ence of detergents (more than 1%, w/v or v/v) will decrease the binding efficiency of lectin affinity columns. For example, sodium dodecyl sulphate (SDS) will reduce binding probably by unfolding the immobilized lectin.

Since the usefulness of lectin affinity chromatography is sometimes impaired by ligand release, detection and quantitation of ligand leakage from lectin columns have been studied [54]. In the case of mistletoe lectin-1 (ML-1) lectin release is dependent on the concentration of sugar in the eluting buffer. When Con A is immobilized on an aldehyde-activated support, leaching is reported to be minimal when monitored by radiolabelled Con A [48]. In another study, leaching of Con A during affinity chromatographic isolation of cell surface glycoproteins is outlined [55,56]. The authors recommend (1) using non-ionic/zwitterionic detergents and (2) executing the chromatography at lower temperatures to minimize the leaching of the ligand [56].

In a study dealing with the fractionation of N-linked oligosaccharides found in glycoproteins, several different combinations of serial lectin columns are employed [57]. Lectin affinity chromatography is also used in conjunction with other modes of chromatography to purify tumor necrosis factors (TNFs) [58]. TNF-α is a non-glycoprotein whereas TNF-β is a glycoprotein. TNF-β binds to lentil lectin and the binding can be reversed by α-methyl mannoside. Based on this interaction, TNF-β is purified several hundred fold [58].

3.3.3.3. Protein A and Protein G. Protein A and Protein G constitute a special class of ligands which is highly specific for immunoglobulins, immune complexes and monoclonal antibodies [59,60]. Protein A binds specifically to the Fc region of immunoglobulins from various species. It binds weakly to murine IgG1, horse IgG, chicken IgG, most IgA and IgMs. Protein A is not known to bind to human IgG3 or rat IgG2a and 2b. Protein G, on the other hand binds to human IgG3, rat IgG2a and 2b but does not bind to chicken IgG.

Binding to Protein A and Protein G is usually enhanced in the presence of high salt concentration like 3 *M* NaCl and at a high pH of 8–9. Elution is affected by a decreasing pH gradient using 0.1 *M* citric acid or 1 *M* acetic acid. The ligand is stable in the presence of 6 *M* guanidine–HCl which is used to regenerate the column. Binding is optimum in physiological buffers and elution requires 0.1 *M* glycine–HCl, pH 2.5–3.0. Eluted proteins are neutralized to avoid denaturation. Protein A and Protein G supports are available commercially, coupled to agarose, silica and other rigid matrices (Table 2).

In order to circumvent the problems and limitations of the hydrazide chemistry in the immobilization of antibodies, Gersten and Marchalonis [61] linked antibody to Protein A-Sepharose [43]. The antigen-binding site of the antibody is left intact since Protein A binds to the Fc region. Immunoaffinity columns based on this concept were thus introduced by Schneider *et al.* [62] and Sisson and Castor [63] on matrices cross-linked with dimethylpimelimidate. These columns are very stable and no leakage of the antibody can be detected at low pH of the eluent. Some monoclonal antibodies of murine and rat origin bind weakly to Protein A and, therefore, it may not be possible to couple a high concentration of it.

However, Protein A can be replaced by Protein G and the complementary nature of its binding properties can be exploited [64]. Recombinant Protein G with its serum albumin binding domain deleted is reported to retain antibodies which results in higher binding capacity compared to those immobilized directly on activated agarose. Protein G-Sepharose is used to immobilize anti-IL-12 monoclonal antibody, 20C2 for the large scale purification of recombinant IL-12 [65]. The binding capacity of the affinity support and the recovery of IL-12 bound to the support are 79% and 100%, respectively, for Protein G-anti IL-12 support compared to 75% and 60%, respectively, for the hydrazide support. When the antibody is immobilized on a N-hydroxysuccinimide activated support, the binding capacity and recovery are 58% and and 48%, respectively. Similar increase in binding capacity and recovery are found in the case of IL-2-specific monoclonal antibody.

3.3.3.4. Benzamidine. Synthetic inhibitors such as *m*- and *p*-aminobenzamidine have been

used as affinity ligands for the removal of proteases, purification of plasmin, plasminogen, trypsin, thrombin, urokinase, enterokinase etc. [66,67]. *p*-Aminobenzamidine which binds to the catalytic site of trypsin family proteases is used as an affinity ligand in its purification. Elution is effectively carried out by lowering the pH from 8.0 to 2.8. A high-performance affinity adsorent is reported using *p*-aminobenzamidine coupled to a hydrophilic vinylpolymer gel. Rapid separation of numerous proteases like prekallikrein, kallikrein and collagenase has been reported using benzamidine [66]. Ready to use *p*-aminobenzamidine supports are commercially available (Table 2).

3.3.3.5. Heparin. Heparin is a linear, highly sulfated glycosaminoglycn composed of repeating disaccharide units with anticoagulant properties [66,68]. Heparin is used in the purification of blood coagulation factors and lipoprotein lipases. Heparin is effective in the fractionation of bile salt stimulated lipase from human milk whey and elution is successful using a linear NaCl gradient [66]. Heparin immobilized on a polymer bonded phase is used to purify antithrombin III and thrombin [69]. Since heparin is polyanionic in nature, it interacts with many basic proteins like a cation exchanger and this has been demonstrated using a standard protein mixture containing trypsinogen, ribonuclease, α-chymotrypsinogen A, cytochrome *c* and lysozyme [66]. An improved method for the immobilization of heparin is reported by reductive amination between aminohexyl bonded phase and the terminal formyl group of heparin [70]. This chemistry is reported on supports for both low- and high-pressure chromatography.

Recombinant HIV-1 reverse transcriptase is purified to homogeneity using heparin Sepharose in combination with other modes of chromatography [71]. Reverse transcriptase is purified by step elution from 0.15 *M* NaCl to 0.35 *M* NaCl in 20 m*M* Tris–HCl containing 2% glycerol, 1 m*M* EDTA and 1 m*M* dithiothreitol (DTT). There are several growth factors that bind to heparin. These are fibroblast growth factor (FGF), endothelial cell growth factor (ECGF), cartilage-derived growth factor (CDGF) etc. These growth factors are purified using a combination of cation-exchange and heparin-affinity-based separations. Bound proteins are eluted from the immobilized heparin column with a salt gradient of 0.1–3.0 *M* NaCl. Immobilized heparin is commercially available (Table 2).

3.3.3.6. Boronate. Phenyl boronate (PBA) is yet another affinity ligand with wide applicability [66] (Table 2). It has high selectivity for vicinal *cis*-diols and α-hydroxy carboxy acids and can be used for purifying carbohydrates and biomolecules containing carbohydrate moieties. Binding is effectively carried out in low ionic strength buffer such as 50 m*M* Tris–HCl, pH 8.0 containing 20 m*M* $MgCl_2$. Elution can be carried out using sorbitol (0–5 m*M* gradient) in the same buffer. A low pH of about 5.0 is recommended for the regeneration of this sorbent. However, there is hardly any reference of the use of this ligand in the preparative mode.

3.3.4. Specific and group-specific synthetic ligands

The concept of pseudobiospecific affinity chromatography was recently reviewed by Vijayalakshmi [72]. Pseudobiospecific affinity chromatography is based on the use of inexpensive, non-fragile ligands such as dyes, metals, amino acids and other synthetic molecules.

3.3.4.1. Synthetic dyes. Dye ligand affinity chromatography has been extremely popular in the eighties and several of these dye sorbents are now routinely used [73]. Among them, by far the most utilized is Cibacron Blue F3G-A. These dyes, belonging to the triazine class are thought to mimic the nucleotide binding sites of enzymes and thus exhibit affinity for nucleotide-dependent enzymes. Dyes have often replaced nucleotides as affinity ligands in large-scale purification of biomolecules. Affinity chromatography supports based on immobilized dyes are of low cost, exhibit high binding capacity, offer a wide binding selectivity and are very stable and easy to regenerate [73]. Chemical modification of the aromatic ring of the dye has made it possible to alter the binding specificity of the dye and also increase the affinity of the dye for proteins [74].

Triazine dyes have been immobilized on a variety of supports and are commercially available (Table 3). Numerous examples of purifica-

tion of proteins are reported using triazine dyes. However, it is not always possible to predict the binding interactions between proteins and dyes. It is therefore often necessary to screen the dyes to arrive at a suitable ligand [75].

There are several examples of large-scale purification of proteins using dye ligand affinity chromatography. In the case of yeast ATP:AMP phosphotransferase, a remarkable 120-fold purification is achieved with elution by a salt gradient from a 40-ml column to which 2.5 kg of yeast extract is applied, indicating an extraordinary specificity of binding [76]. In the case of glycerokinase from *B. stearothermophilus*, a 3.5-l column of immobilised Procion Blue MX-3G is used to purify 10 g of impure enzyme. The enzyme is eluted biospecifically by low concentration of Mg-ATP in phosphate buffer with a recovery of 78% [77].

In the case of carboxypeptidase G_2 from Pseudomonas, it is reported that elution is affected by the presence of EDTA in the buffer [78]. The binding of this Zn-dependent enzyme is enhanced by the presence of 0.2 m*M* Zn^{2+}. Prior to elution, excess Zn^{2+} is removed from the column with EDTA at pH 5.8 and the enzyme is eluted when the pH is raised to 7.3 by 100 m*M* Tris–HCl. In these examples, the advantages of using dye ligand chromatography are (1) increased yield of the enzyme, (2) high capacity of the dyes which means smaller columns and (3) the use of rather inexpensive dye ligands when compared to nucleotides.

Immobilised Cibacron Blue F3G-A is also widely used in the large- to semi-preparative purification of plasma proteins. A semi-preparative system for the recovery of albumin from Cohn Fraction IV is outlined by Harvey [79]. A 1-l column of immobilized Cibacron Blue F3G-A is used to bind albumin which is then recovered in the range of 98% by elution with 0.2 *M* KSCN in 0.15 *M* NaCl. Twenty-seven other plasma proteins have also been separated by Cibacron Blue F3G-A [79]. Complete separation of antithrombin III and antitrypsin is achieved with 10- and 30-fold purification, respectively. Plasminogen has also been isolated from Cohn fraction III using dye ligand affinity chromatography [80].

Although immobilized dyes have a great potential for the large-scale purification of a variety of biomolecules, the potential toxicity of ligand which leaches from the support remains a major problem.

3.3.4.2. Metals. Immobilized metal affinity chromatography (IMAC) was introduced in the mid 1970s and since then it has been extensively applied in the purification of proteins containing tryptophan or histidine residues [81]. The most commonly used metal ions in IMAC belong to the first transition series [82]. Examples are Cu^{2+}, Zn^{2}, Co^{2+} and Ni^{2+}. Metal affinity chromatography has been successfully used in the purification of serum proteins, human interferon and recombinant tissue plasminogen activator [83,84].

The different forms of interferon α-2a are separated by copper affinity chromatography on a preparative scale using a 4-l copper column [33]. Elution is carried out using dilute acetic acid containing 0.1 *M* NaCl and 0.1% Tween-20. Purification of transferrin on a Zn^{2+} chelate column is accomplished using a linear imidazole gradient. The imidazole gradient elution can be repeated several times without loss of column performance [33].

IMAC on Cu(II)-chelating Sepharose is found to be very effective in the purification of catalase from *Penicillium chrysogenum* [85]. Catalase is desorbed by lowering the ionic strength and pH simultaneously and the Cu^{2+} support is regenerated by washing with 50 m*M* sodium phosphate, pH 7.3 containing 1.0 *M* NaCl and 0.2 *M* imidazole followed by 0.2 *M* EDTA to strip off remaining Cu^{2+}. Catalase recovery is in the order of 85%. IMAC is found to be superior to ion-exchange, hydrophobic interaction, size-exclusion and Con A affinity chromatography in this case.

Ni^{2+} immobilized on a nitrilotriacetatic acid (NTA) has a high affinity for proteins and peptides containing adjacent histidines [86]. Several recombinant fusion proteins and protein fragments having a polyhistidine affinity peptide at the C and/or N-terminus are purified using Ni^{2+}-NTA support [87,88].

High resolution of peptides by IMAC has been reported [89]. The effect of solute structure,

ligand density and salt concentration on the retention of a variety of synthetic peptide hormones on a column of chelating copper bonded phase is recently published [90]. The results suggest that peptides containing aromatic and hydroxy amino acids are retarded more than the others. These amino acids, in addition, strengthen the existing strong binding of peptides containing histidine, tryptophan or cyteine to Cu^{2+}. Pilot-scale processing of *E. coli* cells was reported to yield about 70 mg of a homogeneous preparation of human interferon r (rhIFN-r) by using an immobilized Ni^{2+} column in conjunction with ion-exchange and size-exclusion chromatography [91]. About 65% average recovery of activity is reported in the IMAC step using imidazole as the eluent. The levels of DNA, bacterial endotoxins and Ni^{2+} ions are determined in the final purified product. IMAC has a distinct advantage over reversed-phase chromatography for the large-scale purification of recombinant proteins since aqueous buffer is used in the former. A few chelating supports for IMAC are commercially available (Table 3).

When a polyhistidine peptide is fused into a recombinant protein such as mouse dihydrofolate reductase, the complex can be purified by IMAC on a Ni^{2+} column followed by removal of the histidine affinity peptide with carboxypeptidase A [88]. The concept of fusion for the purification of proteins is again used for separating proinsulin [92]. A histidine-tryptophan peptide is fused to proinsulin and then purified on a Ni^{2+}-iminodiacetic acid support. This method can be exploited in the preparative purification of a variety of heterologously produced proteins.

In one study, a monoclonal antibody is covalently modified with a chelating peptide, lysine-glycine-$(histidine)_6$ and retained on a nickel-IMAC support [93]. The molar antigen-binding ratio is 1.4 indicating an oriented immobilization which results in greater accessibility for the antigen. Some difficulty in eluting the bound antigen by acid elution is observed although this approach results in immunosorbents with high binding capacity.

3.3.4.3. Amino acids. The use of amino acids as affinity ligands is relatively less exploited. Amino acids such as lysine, arginine, tryptophan and histidine have been used as affinity ligands for the purification of proteins [72]. The versatile nature of histidine as a general ligand for the purification of chymosin, IgG and carboxypeptidase Y has been demonstrated [94]. Charge transfer and ionic interactions are attributed for the retention of these very different kinds of proteins on immobilized histidine [94]. Immobilized histidine has proved to be very efficient in removing pyrogen from therapeutic grade protein products [95]. Lysine has been reported to have affinity for plasmin, plasminogen and plasminogen activator [96]. A large-scale purification of recombinant tissue-type plasminogen activator is accomplished using a continuous chromatography system comprising of a Zn^{2+}-chelate column coupled to a lysin column [97]. Lysine coupled to agarose is commercially available (Table 3). An oxirane-activated agarose gel substituted with histidine is shown to bind serum proteins including IgG at low ionic strength. IgG can be eluted with Tris buffer containing 0.2 *M* NaCl [98].

3.3.4.4. Affinity-tag ligands. A number of purifications based on "affinity tag procedures" have been reported for proteins which have lost their original biological activities [99,100]. When biological activity such as catalytic activity, binding activity to some substances or antigenicity as a result of mutagenesis is lost, the protein can be fused to another well characterized protein and its affinity can be exploited. Examples of these are the binding specificity of β-galactosidase, protein A, glutathione-S-transferase and maltose-binding protein to *p*-aminophenyl-β-thiogalactoside, IgG, glutathione and amylose, respectively [101–103]. In some cases, fusion to such large proteins can impair the structure or function of a target protein and so some proteins have been fused to smaller peptides. Examples of such coupling strategy are oligocysteine for the use of a thiopropyl column, oligohistidine for IMAC, oligophenylalanine for hydrophobic chromatography, oligoarginine for cation-exchange chromatography and a specific haptenic peptide for a Ca^{2+}-dependent antibody [104–107].

An enzymatically inactive derivative of trypsin, anhydrotrypsin is immobilized for use in

affinity chromatography since it has high specificity for C-terminal tryptic peptides which lack arginine and lysine [108]. The outcome of this observation is a method called arginine-tail method which consists of the following (1) introduction of an arginine residue at the C-terminal by mutagenesis, (2) adsorption of the arginine-tailed protein on immobilized anhydrotrypsin and (3) elution of the arginine-tailed protein with a specific inhibitor of anhydrotrypsin which is benzoylglycylarginine [109]. The added arginine residue is removed by carboxypeptidase B. This method has been reported to have advantages over other affinity-tag procedures since the addition of a simple arginine residue at the C-terminal will have only minimum effect on the structure of the target protein. Human haemoglobin α-chain, which originally has a C-terminal arginine is separated based on this concept by Ishii *et al.* [110]. However, contamination of the final product with host proteins is a major issue and needs to be closely addressed.

In a unique example of purification of connective tissue metalloproteinases, several different forms of affinity chromatography are exploited [111]. The metalloproteinase, namely collagenase, stromelysin and gelatinase have a general strategy for their isolation and purification. After initial concentration and removal of interfering culture medium compounds, they are subjected to ion-exchange chromatography followed by heparin-affinity chromatography. Stromelysin is purified on immobilized red dye ligand chromatography, collagenase on Zn^{2+} iminodiacetic acid-Sepharose and gelatinase on gelatin-Sepharose. All three proteins are finally chromatographed on a size-exclusion column. Recently a new affinity-based support consisting of hydroxamic acid is reported for the purification of collagenase [112]. However, its affinity for the other metalloproteinases has not been clarified.

3.3.5. Paralog ligands, antisense ligands and other synthetic ligands

One of the ways of surmounting the instability of expensive affinity ligands is to use synthetic or pseudospecific ligands as described above. Yet another solution is the use of small peptides or similar polymers that mimic the binding dynamics of a protein with the whole ligand. The concept of weak affinity interactions between the ligand and the molecule to be separated was first introduced in the eighties [113,114]. In weak affinity, the dissociation constant is higher than in conventional affinity interaction which means that dissociation can actually occur under mild isocratic conditions without adverse pH changes or the presence of chaotropic agents. The major limitation of weak affinity chromatography is an efficient and logical method to produce weak ligands to the target of interest. In spite of this limitation, this approach has been effectively used in immunoassays and has been the basis of the concept of paralog chromatography [115]. Paralogs are short peptides that simulate the binding site of the antigen in terms of the overall bulk, hydrophobicity, charge etc. Paralogs provide some of the specificity of the more rigid antibodies while retaining the ease of use and broad applicability of ion-exchange chromatography. With the advent of new technology by which peptides can be easily synthesized, the practice of paralog chromatography has a very promising future.

The affinity of peptides encoded by the antisense strands of DNA, known as antisense (AS) peptides and those coded by the corresponding sense strands has been exploited in the purification of native popypeptides such as Arg^8-vasopressin-bovine neurophisin II biosynthetic precursor, recombinant c-raf protein and the Arg^8-vasopressin-receptor complex [116]. The purification of human interferon β from a recombinant Chinese hamster ovary cell line is recently reported using As peptides [117]. The AS-IFN peptides are synthesized by the solid-phase method on a resin which is then used as the affinity support. The results obtained are comparable to those obtained with a monoclonal anti-hIFN-β column. The possibility of deducing the correct AS peptide sequences on the basis of the sequence of proteins to be purified makes this approach general and practical.

3.3.5.1. Other synthetic ligands. A number of supports for purifying IgG using synthetic affinity chromatography has gained widespread attention. Porath and collaborators used a thiophillic support to purify IgG in the presence of high salt

and purified IgG can be eluted by lowering the salt concentration [118]. The advantage of totally synthetic low-molecular-mass affinity ligands is again seen in the use of Avid AL, a synthetic affinity ligand for IgG that mimics immobilized bacterial antibody receptor [119]. Avid AL is commercially available (Table 3). In contrast to Protein A or Protein G, Avid AL can withstand acid, base, organic solvent, proteolytic enzymes and autoclaving treatments. Rapid preparative purification of goat IgG up to 12 g from phosphate-buffered saline (PBS)-diluted serum in a single run is discussed by Ngo and Katter [119]. A 1.5-l Avid AL radial flow column was used at a flow-rate of 75–150 ml/min. Binding of IgG does not require a high concentration of salt and elution is carried out at neutral pH. The antibodies purified with neutral buffer consistently show higher binding activity which is yet another advantage of this type of purification protocol. Avid AL is reported to be stable under conditions used to depyrogenate affinity supports.

The cyclic oligopeptide, bacitracin A is a well known synthetic affinity ligand for the purification of proteinases [120]. Silica-sorbents with bacitracin attached through its two amino groups is reported to be specific for proteinases. Human thrombin, pig pepsin, serine proteinase from *Thermoactinomyces vulgaris* and bovine trypsin are reported to bind to this support. Elution is effective in the presence of 1 *M* NaCl and 25% isopropanol.

4. NON-SPECIFIC INTERACTIONS IN AFFINITY CHROMATOGRAPHY

The basis of affinity chromatography is the specific interaction between the immobilized ligand and the molecule to be isolated. So, in principle any other non-covalent interactions such as hydrogen bonding and hydrophobic interactions that may arise between any molecule in solution and any part of the derivatized affinity support should be minimized [121]. Non-specific adsorption are generally characterized as either hydrophobic or ionic. Hydrophobic adsorption results from interactions between non-polar side chains of proteins and the support material, the spacer arms between support and ligand or from the ligand itself. The indiscriminate use of long spacer arms frequently leads to hydrophobic interference. Incomplete attachment of ligands, leaving free functional groups is another source of hydrophobic interaction. During ligand coupling, the use of excess ligand is prudent as well as longer reaction times which would assure that the reaction has gone to completion. Free functional groups can alternatively be blocked with hydrophilic molecules. Using hydrophilic spacer arms will also reduce hydrophobic interactions.

Ionic interactions are also of some concern since proteins are polyelectrolytic in nature. Ionic interactions can arise from the matrix, the spacer arm, the ligand or the coupling agent. CNBr-activated Sepharose was used, in one study to assess the degree of non-specific adsorption of IgG. The adsorption study suggested residual cationic charges on the support [122]. Supports made of polyacrylamide may contain carboxyl groups, due to hydrolysis of amide groups, especially at alkaline pH. Glass beads contain negative charges and are anionic at neutral pH, promoting non-specific protein binding.

Ionic interactions can be controlled to a certain degree by adjusting the ionic strength of the equilibration buffer. Salt concentration in the range of 0.25–0.5 *M* NaCl/KCl can suppress ionic effects, although high concentration of salt will promote hydrophobic effects. If charged groups are not involved in the binding mechanism, slight variation in the ionic composition of the buffer will not affect the binding mechanism between the protein and the ligand.

Non-specific interaction is difficult to be ruled out even in carefully prepared affinity supports. It is prudent to elute the protein using selective desorbing agents, such as low concentration of substrate, competitive inhibitors or soluble forms of the ligand instead of lowering the pH or adding salt in the eluting buffer.

5. VALIDATION OF AFFINITY CHROMATOGRAPHY IN THERAPEUTIC-GRADE PROTEIN PURIFICATION

Apart from the quality of the final product, written standard operating procedures for operation and maintenance of the equipment are

essential. In order for routine production of a product, approval is required with respect to the equipment and utilities used in the process and the use of hardware and software.

Therapeutically useful proteins are required to follow a range of therapeutic regimens which include the types of contamination that may occur and the possible clinical consequences of the various types of contaminants [113]. Therapeutically important proteins are divided into four groups: (1) regulatory factors such as hormones, cytokines, lymphokines and other factors of cellular growth and metabolism, (2) blood products such as serum-derived blood factors and fibrinogen activators, (3) vaccines and (4) monoclonal antibodies. The degree of purity and contamination varies in these four groups of proteins since they are often derived from different sources. Sources of these proteins can vary from extracted animal tissue, human serum, cultured microorganisms, mammalian cells infected with various viruses, recombinant host-vector expression systems and hybridomas of murine origin. Level of purity also depends on the dosage level of therapeutics. For example, insulin is given two or three times daily for a lifetime, interferon may be administered for a limited time at an intense rate while plasminogen activator or monoclonal bodies are given as a single-shot therapy at a high dose. The immunogenic contaminants present in a protein that is given in a daily therapy is of more concern that the same contaminant present in a single dose-therapy. The nature of the illness that is being treated is also a deciding factor in the acceptable levels of contamination. Despite these differences, it has been possible to adhere to a common guideline while preparing a therapeutically useful protein product [123]. Table 4 gives a guideline of the acceptable levels of contamination of the four main potential contaminants in therapeutic grade protein products. These figures, although a useful place to start, are by no means absolute for any therapeutic preparation.

There are four main concerns when a foreign protein is injected into man. These are (1) antigenicity, (2) transformation of contaminating DNA, (3) transmissible diseases and (4) pyrogenicity. There are two possible routes by which impure preparations may give rise to immunity.

TABLE 4

ACCEPTABLE LEVELS OF COMMON CONTAMINANTS IN THERAPEUTICS: USEFUL GUIDE

Compound	Level
Proteins	10 ppm
DNA	10 pg/dose
Bacterial endotoxins	35 ng/dose
Viruses	None should be present

Degraded or aggregated forms of the product may be more immunogenic than the native form. High levels of contaminating proteins can act as an adjuvant, thereby rendering the therapeutic protein more immunogenic than before. Immunity to the therapeutic protein or to the contaminants can have adverse clinical consequences. For patients who are immunodeficient, transformation of DNA or active oncogenes that may induce or promote tumors have to be avoided at all costs although there is hardly any evidence that this can happen. Contamination of agents known to be pathogenic in man especially in immunodeficient patients are a real threat during treatment. Proteins isolated from human tissue or serum can potentially be contaminated with the viruses causing AIDS, hepatitis or Creutzfeldt–Jacob disease. Human cell culture may have Epstein–Barr virus or other transforming viruses [124]. Hybridoma cell lines may have murine viruses. Proteins from these sources have to be devoid of such contamination. Bacterial endotoxins are yet another concern when the protein is cloned in Gram-negative bacteria. The liposaccharide components of the cell walls of these bacteria can cause fever or pyrexia if administered even in small quantities [125].

In the case of disease-causing viruses, even a single virus can transmit the disease. Evidence of complete absence of the virus is required. The levels of contamination indicated in Table 4 can be proved only if the analytical detection methods are in place. Several refined analytical protocols are being developed for the purpose. These include, immunoblotting for the detection of *E. coli* proteins, peroxidase–antiperoxidase (PAP) detection of bound rabbit antibodies,

radioimmunoassay of *E. coli* proteins, ultrasensitive silver staining, dot-blot hybridization analysis of *E. coli* DNA, the Limulus test for bacterial endotoxin and others [123].

Process validation should take into account the removal of all the above mentioned contaminants from a therapeutic grade protein. The purification protocol should take measures to eliminate these concerns during its operation. DNA being strongly acidic is usually best removed during ion-exchange chromatography, but is considerably reduced during other modes of chromatography, but is considerably reduced during other modes of chromatography. Proteins are difficult to remove and the best recommendation is to introduce affinity chromatography at a later stage in the purification protocol to remove such contaminants. Modified proteins in its deamidated form, aggregated form, oxidized form, incorrectly folded form or partially hydrolysed are some of the proteinaceous contaminants.

Bacterial endotoxins are removed by autoclaving or sterilizing reagents, glassware an plasticware. All operations are carried out under aseptic conditions. In the case of affinity media, it is extensively washed with pyrogen-free buffers until the eluate is free of pyrogen. Viruses are a potential contaminant in the preparation of serum proteins. Heat treatment (at 60°C for 10 h) has been used to inactivate viruses in serum protein preparations. Potential modification or inactivation of the protein of interest is to be guarded against during the process of heating, UV radiation or ionizing radiation.

In the case of affinity supports, especially with antibody ligands, validation involves extra steps in quality control and assurance. The Food and Drug Administration (FDA) wants proof that affinity ligands, which often leak off the column are fully characterized and removed from the final product. The product and the ligand both need to be validated. The FDA also demands extensive documentation of chromatographic materials used in the purification process. Several manufacturers therefore have drug master files on chromatographic supports.

Consideration on the technical and legal requirements involved in the use of affinity chromatography for clinical and biological applications recently gained attention since it is particularly suitable for removing toxic substances from patients, plasma *in vivo* and the separation of molecules intended for clinical or biological use [126]. Stoltz *et al.* [127] outlines the following technical and legal problems that must be taken into account while setting up affinity-based procedures.

(1) The choice of the affinity support, ligand and ligand linkage.

(2) The need to preserve the integrity of the molecule.

(3) The specificity of the procedure and the specific activity of the final product.

(4) The possible introduction of toxicity, bacterial or viral contamination, residual DNA and ligand leakage.

(5) The regeneration and reusability of the chromatographic support.

All these points have to be validated for the FDA. If the affinity ligand is a murine antibody, further validation such as the origin and characteristics of the myeloma, immunogen and immune parental cells have to be provided. Details of the cloning and fusion procedure, identification and characterization of the hybridoma cell line are also required. The monoclonal antibody has to be carefully characterized and its production details validated [126].

Although affinity and immunoaffinity-based techniques are undoubtedly very suitable for preparative purification, the regulatory requirements connected with these procedures must be carefully studied before a process is actually scaled-up.

6. CONCLUSIONS AND PROSPECTS

Affinity chromatography has a secure future since it is being increasingly used in large-scale purification of therapeutic products. Several references have been made to the problems associated with large scale affinity chromatography and how some of these can be overcome. An impressive example to cite, however, is the immunopurification of interferon which resulted in a 5000-fold increase in specific activity.

The progress of affinity-based processes is

hampered by the cost of affinity supports and the instability of such supports in large-scale operations. The cost of the support stems from the use of conventional biological ligands which are not easily available in a homogeneous form suitable for these as an affinity ligand. The instability of the affinity support is due to weak linkages between the ligand and the support and the fragility of the ligand itself. The use of synthetic ligands and chemistries that enhance stability of ligand to support linkage can to a certain extent solve these drawbacks of affinity chromatography.

With the stiff competition faced by biotechnology enterprises, controlling costs has become an important factor in their overall success. The many possible variations in the number and mode of chromatography in the purification protocol of a higher value therapeutic product allow a lot of flexibility to change and refine the process which ultimately dictates the associated costs of the final product. Fewer chromatographic steps means fewer steps to validate. While the average number of steps in a purification scheme is five or more, the use of affinity chromatography has the potential of bringing the number of steps down to two or three.

In the late eighties, the chromatography critics objected to preparative affinity chromatography on the basis that it is expensive, easy to ruin and hard to validate [3]. But the use of innovative ligands and better coupling strategies to improve matrix to ligand linkages have changed the status of affinity chromatography in the purification arena.

According to Knight [3] getting the FDA approval of a validation procedure without undue delay can contribute significantly to the success of the product and the company. Knight [3] reports that the cost of product quality control can vary between 15–50% of total chromatographic cost. So it is prudent to strike a balance between the number of fractions in which the product of interest elutes from a column and fraction volume. Fewer fractions mean less cost since less quality control is required while bigger fractions mean bigger losses of valuable product if something goes wrong.

As per Jones [128], the 21st century may yet be the golden age of chromatography. Jones also points out the clear distinction between scaling up macromolecules such as proteins and small synthetic molecules such as drugs. While the latter can be carried out using high-performance supports, the former is usually accomplished with low- to medium-performance chromatography supports primarily because of lack of suitable media. A range of new biomimetic ligands based on reactive triazine dyes has been introduced recently which show a remarkable ability to separate a wide range of proteins. These synthetic ligands demonstrate greater stability compared to the severe leakage experienced with the original textile dyes [128].

The past ten years in chromatography was reviewed with emphasis on the state of the art of the different modes of chromatography [3]. According to Hupe *et al.* [129], the success of today's biotechnology revolution depends heavily on the separation power of affinity chromatography. They have also discussed the progress in three areas pertaining to affinity chromatography namely affinity ligands, supports and coupling chemistry.

As improvements in the technology of chromatographic support materials are developed, the combination of the unique selectivity of an affinity interaction along with the improved performance of modern supports and the increased use of synthetic ligands assures affinity chromatography a commanding position in the future of preparative scale protein purification. In addition to the technological and economical constraints, it is also important that the regulatory restrictions imposed on therapeutic products be closely addressed and resolved.

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