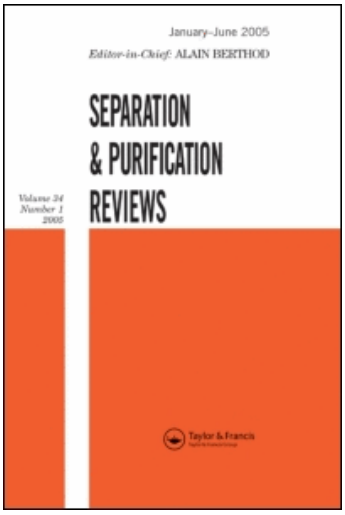

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Recent Developments in the Use of Group-Specific Ligands for Affinity Bioseparations

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RECENT DEVELOPMENTS IN THE USE OF GROUP-SPECIFIC LIGANDS FOR AFFINITY BIOSEPARATIONS

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INTRODUCTION

One of the major obstacles encountered in the commercialization of biochemical products is their separation and purification since it can represent up to 60% of the total production costs[1]. Using conventional methods such as filtration, centrifugation, extraction, precipitation, and chromatography usually requires many separation steps in order to isolate biomolecules with similar physico-chemical properties. Conventional techniques suffer from a lack of selectivity for a specific biomolecule in a complex mixture, especially when the biomolecules in the mixture have similar properties. A dramatic increase in selectivity is obtained when the method of

recovery is based on affinity interactions between the desired macromolecule and its complementary ligand.

Affinity interactions are most succinctly quantified in terms of the dissociation constant, K_D. Low values represent a very stable complex, while high values denote a weakly associated complex. Ligands can be classified as highly specific or group specific according to the strength of their interactions with the biomolecule of interest. Highly specific ligands have dissociation constants from $>10^{-3}$ M for enzyme substrates to up to 10^{-15} M for the avidin-biotin interaction[2]. Group specific ligands are less specific since they can bind to more than one particular molecule or biopolymer subgroup. However, the fact that highly specific ligands offer a very stable interaction results in one of its limitations - low ability to unbind ligate from ligand without irreversible damage to one or both. Another concern is the greater cost of highly specific ligands vs. group specific ligands. This can be of crucial importance if the purified metabolite is not a high value product.

The purpose of this review is to discuss how the more versatile and economical group specific ligands can be used in bioseparations. In order to be able to use group specific ligands for the separation of biomolecules they are often immobilized onto a support or carrier. Hydrophilic polymers such as polysaccharides, polyacrylamide, porous glass, and polyhydroxymethyl-acrylamide are currently popular for use as carriers. In order to immobilize ligands, these carriers have to be activated by covalently binding the following constructs in the order given: (1) an anchor group; (2) a spacer arm; and (3) a reactive terminal

group. This tripartite structure provides a stable anchor bond to the carrier, a spacer arm which gives flexibility to the ligand allowing it to interact with the biomolecule with minimal steric hindrance, and a reactive terminal group which allows immobilization of the ligand. Also, in order to prevent nonspecific binding it is important that after coupling of the ligand the remaining unreacted active groups should be neutralized. Different activation procedures are available[3] depending on the physico-chemical properties of the ligand and carrier. However, discussion of these procedures is beyond the scope of this review.

Based on the source from where group specific ligands are obtained, they can be subdivided into two categories: biological ligands or synthetic ligands. It is useful to briefly discuss in this article each ligand in terms of its binding preferences and strength. The rest of this article is then delegated to reviewing how some or all of these group specific ligands are used in precipitation; ultrafiltration; extraction using two-phase aqueous, reversed micelle, and perfluorocarbon systems; chromatography and membrane separations.

BRIEF DESCRIPTION OF GROUP SPECIFIC LIGANDS

Biological Ligands

Coenzymes

About two-thirds of known enzymes need nucleotide coenzymes to perform their catalytic activity. For this reason

nucleotide coenzymes such as AMP, ATP, NAD, and NADP can be used as group specific ligands. They have proven effective for more than two decades[4,5,6] in the purification of dehydrogenases, kinases, and transaminases.

Lectins

These are sugar-binding proteins that are usually of plant origin but which also occur in animals and bacteria. Among the best known lectins are jackbean concalavin A (Con A), which specifically binds α-D-glucose and α-D-mannose residues, and wheat germ agglutinin which specifically binds β-*N*-acetylmuramic acid and α-*N*-acetylneuraminic acid.

Protein A

This is one of the membrane proteins expressed by most strains of *Staphylococcus aureus* and shows special binding avidity to the Fc region of IgG[7]. The protein A-IgG complex can usually be dissociated at a pH of 4.0.

Protein G

Like protein A, protein G binds the Fc region of IgG; but unlike protein A, protein G binds a greater fraction of monoclonal and polyclonal IgG. Because of the greater avidity of protein G for IgG, the conditions required to dissociate the complex are harsher, often leading to loss of biological activity.

Synthetic Ligands

Reactive Dyes

Triazine dyes (e.g., Cibacron Blue F3G-A and Procion Red HE-3B) have been used extensively to mimic coenzymes that bind a number of dehydrogenases, hexokinases, alkaline phosphatases, carboxypeptidase G, and Ribonuclease A. These dyes are hydrophilic molecules consisting of a reactive group and a chromophore. The reactive group is a triazine ring containing one or more labile chlorine atoms. It is through the chlorine atom that the dye is coupled onto a support. The chromophores (e.g., azo, anthraquinone, and phtalocyanine) contain sulphonate groups which make them soluble in water, and an amino group to which the reactive group can be attached. The mechanism by which proteins adsorb onto reactive dyes is not well understood.

Reactive dyes offer many attractive features. They are available in large quantities and low cost and they offer a variety of chemically distinct chromophores displaying a wide spectral range, and high molar extinction coefficients[8]. A major drawback for their industrial application is the possibility that leakage might contaminate purified biochemicals and render the product toxic. Regnault and coworkers[9] studied the dye Remazol Yellow GGL bound to Sepharose using human plasma transthyretin as a model system. They measured dye leakage under different elution conditions, tested different storage solutions, and suggested various forms of virus inactivation. In a recent series of articles[10,11,12] in vitro toxicity assays for Cibacron Blue F3G-A and Procion Red HE-3B were provided. These assays monitored the behavior of HeLa cells, human fibroblasts, and *E. coli* as a function

of dye concentration. It was reported that neither dye influenced HeLa cell viability at concentrations up to 62.5 μg/ml (normal leakage is around 2 μg/ml). A review of the data yields the conclusion that a 20% drop in cell growth is evident while in the presence of Cibacron Blue F3G-A in its native form at concentrations as low as 0.244 μg/ml. It was also reported that there was no change in fibroblast cell growth or morphology for up to 21 days at dye concentrations of 10 μg/ml. One criticism of this reported result is that twenty one days is too short a span of time to ensure that the dyes have no toxic effect on cells. Also, with regards to the genotoxic studies, there was no secretion of β-galactosidase by *E. coli* at dye concentrations from 3-39 μg/ml. More extensive toxicity studies are needed on the effect of these and other dyes on cells.

Metal Ions

The interaction of metal ions with specific amino acids on the surface of proteins was first used by Porath in chromatography so as to separate serum proteins using metal ions immobilized by iminodiacetate[13]. In comparison to other affinity based separations, immobilized metal affinity separations offer high ligand stability and protein loading, mild elution conditions, and complete ligand recovery at relatively low cost[14].

Until now most of the purifications studies using metal ions have relied on transition metal ions (e.g., Cu(II), Ni(II), Fe(III), and Zn(II)) which interact with indole and imidazole groups (e.g., try and his). Based on Pearson's hard-soft acid base theory[15,16,17] these are

moderately hard acids and bases, respectively. While some metal ions which are hard acids have been used, such as Ca(II), metal ions exhibiting soft acid properties have not been widely investigated because of concerns over their stability and toxicity. Recent work[18] has shown that Ag(I) and Pt(II) ions can be used in bioseparations. Soft acids (e.g., Ag(I), Cu(I), Au(I), Hg(II), Pb(II), Pd(II), Pt(II), and Cd(II)) interact with soft bases (i.e., sulfur-containing functional groups in chemicals and biomolecules), thus offering a more extensive library of functional group targets for metal affinity chromatography.

PRECIPITATION

Precipitation has been a technique widely used for many years to recover proteins[19]. When affinity ligands are introduced to increase selectivity it is known as affinity precipitation. The physicochemical aspects of this technique as well as practical applications have been extensively developed in recent reviews[20,21]. This article will provide a general view of the effect of different process variables as well as give an update on the current applications of the technique to the selective recovery of biomolecules.

In this technique a phase change is induced whereby proteins precipitate. Non-affinity means of achieving supersaturation include: (1) addition of a salt; (2) a change in pH; (3) addition of a polar organic solvent; (4) addition of a nonionic polymer. The addition of salts such as ammonium sulfate precipitates proteins because the protein

solubility is markedly reduced by an increase in the salt concentration in solution. This technique is inexpensive but causes some denaturation since most low molecular weight salts are difficult to remove from precipitated protein[21]. Adjusting the pH to the isoelectric point will bring the protein to its minimum solubility, thus inducing precipitation. Reducing the medium dielectric constant enhances electrostatic interactions precipitating the protein. A way to achieve this is by adding organic solvents[22] miscible in water, such as acetone, ethanol, methanol, and isopropanol. Adding nonionic polymers can also induce proteins to precipitate by reducing the amount of water available for solvation[23]. Proteins which denature at a different temperature than the protein of interest can be removed first by achieving their denaturation temperature[22] which lowers their solubility since hydrophobic groups come in contact with the aqueous phase making it precipitate.

Use of Group-Specific Ligands

Group specific ligands can be used in affinity precipitation by forming either homobifunctional or heterobifunctional ligands[21]. Homobifunctional ligands are two identical ligands attached by a spacer arm. They crosslink proteins until the network formed precipitates. Heterobifunctional ligands serve as affinity ligands for the macromolecule to be separated while functioning as a ligand carrier whose solubility properties can be altered in order to make it precipitate. Some of the most recent specific applications of these types of ligand formulations are discussed below starting with

Table 1. Group-Specific affinity precipitation systems.

Homobifunctional

Biomolecule	Ligand	Carrier (type§)	Reference
lactate dehydrogenase	NAD	N.A.*	24
glutactate dehydrogenase	NAD	N.A.	25
yeast alcohol dehydrogenase	NAD	N.A.	25
lactate dehydrogenase	o-Cibacron Blue F3G-A	N.A.	27
myoglobin	Cu(II)	N.A.	29
hemoglobin	Cu(II)	N.A.	29
concavalin A	Cu(II)	N.A.	32
proinsulin	Ni(II), Mn(II)	N.A.	34

Heterobifunctional

trypsin	trypsin inhibitor	chitosan (pH)	36
β-glucosidase	none	chitosan (pH)	37
D-lactate dehydrogenase	Cibacron Blue	Eudragit 100 (pH)	38.39
trypsin	trypsin inhibitor	alginate (X)	41
protein A	IgG	PIPA¶ (T)	42
C-reactive protein	hapten	PIPA (T)	43
lactate dehydrogenase	Cibacron Blue	Eudragit 100 (T)	44
Pyruvate kinase	Cibacron Blue	Eudragit 100 (T)	44
lactate dehydrogenase	Cu(II)	PVCL (T)	45
avidin	biotin	DMPEA (S)	47

§ pH:pH dependent, X:ionic cross-linking, T:temperature dependent, S:surfactant.
¶ PIPA:poly(N-isopropylacrylamide), PVCL:poly(N-vinylcaprolactam), DMPEA:dymiristoylphatidylethanolamine. * N.A. indicates not applicable.

homobifunctional ligands which have been more widely used as group specific ligands. The use of homobifunctional and heterobifunctional ligands in the affinity precipitation of specific biomolecules is referenced in Table 1.

Homobifunctional Ligands

NAD

Nicotinamide adenine dinucleotide (NAD) in its oxidized form NAD^+ was one of the first homobifunctional ligands used in affinity

precipitation of dehydrogenases[24,25]. NAD^+ has been used to purify several NAD^+-dependent dehydrogenases, including pig heart lactate dehydrogenase[24], bovine liver glutactate dehydrogenase and yeast alcohol dehydrogenase[25]. Even at yields greater than 90% this type of ligand is problematic since it is very expensive and more susceptible to biological degradation than other ligands. These issues have likely contributed to the lack of mention of the use of this ligand in the technical literature during the past ten years.

Reactive Dyes

In affinity precipitation reactive dyes form homobifunctional ligands separated by a spacer arm. Bifunctional ligands must bind at least two protein units simultaneously, and the protein should contain three or four ligand binding sites. This is necessary for the formation of an insoluble protein network consisting of proteins cross-linked with the bis-ligands, which results in the selective precipitation of that protein from solution[26].

Pearson et al.[27] using a *o*-isomer of Cibacron Blue F3G-A was able to precipitate rabbit muscle lactate dehydrogenase. The bound protein dissociated with the competitive ligand NADH, resulting in a 97% yield. This compares favorably with the 90% yield of lactate dehydrogenase using bis-NAD[7]. Some years later Morris and Fisher[28] failed to precipitate lactate dehydrogenase using Cibacron F3G-A in an attempt to reproduce previous findings by Pearson et al.[27]. These authors failed to notice that in Pearson et al. the dye that was able to

precipitate the protein was not Cibacron F3G-A but an isomer, since the former failed to make a precipitate.

Metal Ions

The application of metal affinity interactions to affinity precipitation is in the complexation of bis-metal chelates with strong electron donor groups of proteins (i.e., histidine) eventually forming an insoluble protein network equivalent to that using reactive dyes[29]. Van Dam et al.[29] used bis-Cu(II) chelate to precipitate sperm whale myoglobin and hemoglobin, both of which contained several histidine residues. In contrast, horse heart cytochrome c which contains only one histidine residue failed to precipitate. The interactions between histidine and transition metal ions such as Cu(II), Ni(II), and Zn(II); and soft metal ions such as Ag(I)[18] have been correlated using amino acids[18], peptides[30], and proteins of known histidine content[31]. A point of concern with the method of Van Dam et al.[29] is the long time (i.e., 48 hrs) that the precipitation was allowed to continue before recovering the protein. This does not compare favorably with precipitation times using other ligands. In a more recent study Agarwal and Gupta[32] were able to obtain a 4.5 fold purification of Con A by performing an ammonium sulphate precipitation prior to precipitation with $CuSO_4$.

Capitalizing on metal-histidine affinity, recombinant DNA methods have been used to create histidine-containing cleavable affinity tails which can be engineered into proteins in order to increase selectivity[33]. This was recently achieved by Ko et al.[34] for the purification of proinsulin containing a $(His)_{10}$-X-Met affinity tail. Ko

and colleagues used Cu(II), Mg(II), Ni(II), and Mn(II), bis-metal chelates with the latter two ions giving better selectivity.

Heterobifunctional Ligands

The use of heterobifunctional ligands seem to have become the norm in affinity precipitation in general. Specific applications of these ligands will be presented in terms of the precipitant (i.e., polymer) since it is still not widely used with group specific ligands.

pH dependent

These type of polymers contain both hydrophobic and ionic groups making it possible to induce hydrophobic aggregation by altering the pH of the solvent. In a recent review[35] the use of these polymers and their respective solubility ranges were discussed. One of these, chitosan, which precipitates at pH values greater than 6.5 has been used in affinity precipitation. Chitosan has been used with soy bean trypsin inhibitor as an affinity ligand in the purification of trypsin[36]. The complex was precipitated at a pH of 8.5 and the trypsin was dissociated by lowering the pH to 2.5. In a recent study Homma et al.[37] used a different strategy for the purification of β-glucosidase from cellulase. It was found that chitosan without any affinity ligand was able to precipitate all the components of cellulase except for β-glucosidase which remained in solution after increasing the pH to 8.0.

A more recent application involved using Eudragit S 100, a copolymer of methacrylic acid and methylmethacrylate which

precipitates at a pH lower than 4.5[38]. This polymer was used to purify D-lactate dehydrogenase from the cell homogenate of *Leuconostoc mesenteroides*. Eudragit S 100 was first modified with ethanolamine in order to precipitate contaminating proteins and cell debris. After removal of the precipitate, Eudragit S 100 was activated using the reactive dye Cibacron Blue which selectively precipitated D-lactate dehydrogenase by lowering the pH to 2.5. Guoqianang et al.[39] where able to integrate this system with extraction using an aqueous two-phase system containing polyethylene glycol (PEG) which gave a lactate dehydrogenase yield of 54%.

Ionic Cross linking

These type of polymers precipitate when cross-linked forming a large complex by the addition of a specific polyvalent counterions. An example is alginate, which is a natural polymer from algae cross-linked by Ca(II) ions to form an insoluble precipitate[40]. Alginate activated with soybean trypsin inhibitor was used in the purification of trypsin[41] with a yield of about 50%. Trypsin was dissociated from the complex by either adding arginine or lowering the pH. Ligand recovery was only 60%.

Temperature dependent

Temperature dependent polymers precipitate with small changes in temperature. Temperature changes necessary have to be small enough to insure functional stability of the macromolecule. An

example of these types of polymers is poly(N-isopropylacrylamide) which precipitates at 32°C in aqueous solution. Poly(N-isopropylacrylamide) was recently used to bind protein A using IgG as the affinity ligand[42]. This polymer was also activated with the hapten p-aminophenyl-phosphorylcholine in order to purify rabbit C-reactive protein from acute phase serum[43]. Precipitation was achieved by increasing the temperature to 32°C. After removing the liquid phase, the precipitate was redissolved and the protein was dissociated by buffer addition at 4°C. This resulted in a yield of 80% .

The use of thermosensitive polymers with group specific ligands was also demonstrated in the purification of lactate dehydrogenase and pyruvate kinase from porcine muscle[44]. Eudragit S 100 activated with Cibacron Blue precipitated both proteins by increasing the temperature to 40°C and addition of calcium ions. The precipitate was redissolved in calcium free buffer at room temperature, and the proteins where dissociated by increasing the salt concentration. Of the two enzymes, lactate dehydrogenase gave the highest yield. Another application of group specific ligands involved using poly(N-vinylcaprolactam) activated with chelated copper to purify lactate dehydrogenase[45]. Of the 35-45% lactate dehydrogenase recovered, only 3-5% retained activity. The authors concluded that protein molecules as well as copper ions were stripped off the polymer during precipitation.

Surfactants

When using surfactants, ligands are immobilized on phospholipids which are then solubilized in aqueous solution using nonionic surfactant[46]. As macromolecules bind the complex its

solubility decreases until it precipitates. Powers et al.[47] were able to precipitate avidin by adding dymiristoylphosphatidylethanolamine activated with biotin. A model which considered the four binding sites on avidin was proposed to analyze the data. Avidin was never eluted, and possible ways to achieve this dissociation were not given.

ULTRAFILTRATION

Ultrafiltration is a technique by which molecules or particles are separated according to size. The method is very simple and attractive since it does not require a change of phase, addition of chemicals, or large energy demands, and is capable of processing large volumes at a high rate. Even with these features this method has a major drawback, a lack of chemical selectivity. This lack of selectivity extends not only to chemically similar molecules but even to physically different ones since there needs to be a difference in molecular weight of at least ten-fold for proper resolution of two solutes.

There are two general methods by which affinity ligands are utilized in ultrafiltration. Affinity ligands are used in either water-insoluble or water-soluble polymeric ligand carriers. The use of insoluble and insoluble ligand-carriers in the affinity ultrafiltration of specific biomolecules is referenced in Table 2.

Water-Insoluble Polymeric Carriers

These polymers are cross-linked in order to form a porous membrane to which ligands are attached on its surface and in the pore

Table 2. Group-Specific affinity ultrafiltration systems.

Insoluble Carriers

Biomolecule	Ligand	Carrier	Reference
IgG	protein A	PEUU	49,50
IgG	protein A	MPHF-chitosan	50
IgG	protein G	sepharose	51
IgG_1	protein G	Amicon's MAC	52
malate dehydrogenase	Cibacron Blue F3G-A	nylon	53
human serum albumin	Cibacron Blue F3G-A	PEI	54
bovine serum albumin	Cibacron Blue F3G-A	latex	55
alkaline phosphatase	Active Red K2BP	cellulose	56
histidine	Cu(II)	polysulfone	57
lysozyme	Cu(II)	MPGHF	58

Soluble Carriers

trypsin	trypsin inhibitor	AABA¶	59,60
urokinase	trypsin inhibitor	AABA	61
vancomycin	D-Alanyl-D-alanine	dextran	62

¶ MPH:microporous hollow fiber, PEI:poly(etylene imine), MPGHF:microporous glass hollow fiber, AABA:N-acryoyl-m-aminobenzamide.

walls. When a mixture is passed through the membrane, the macromolecule to be purified will be retained by the affinity ligands coupled onto the membrane. Some of the most common carriers include polysulfone, cellulose, and porous glass. These are chosen based on their chemical and mechanical stability, and their capacity to bind the affinity ligand. Some of the most recent specific applications of these types of carriers will be discussed. When using water-insoluble carriers, spacer arms are usually used in order to make the ligand more accessible to the protein to be purified. The type and length of spacer arm to be used depends on the type of carrier and ligand as well as the

protein-ligand affinity[2]. A more specific description of the use of spacer arms is beyond the scope of this review.

Protein A

Langlotz and Kroner[48] coupled protein A, as an affinity ligand, to a polymeric composite membrane in order to separate IgG. The binding capacity was determined by measuring the amount of IgG eluted. After repeating this sequence 30 times there was a 30% loss in binding capacity, of which 20% occurred after the first run. This initial loss in activity was attributed to noncovalently bound protein. In a different approach Bamford and coworkers[49] used poly(ether-urethane-urea), a polymer used in prosthetic vascular grafts, as a carrier for protein A. This polymer when electrostatically-spun produces a fibrous network with a high internal surface area. When the IgG binding was tested they found the nonspecific binding to be low, and a binding capacity of 152-290 $\mu g/cm^2$ using three disks was slightly higher when compared with about 90 $\mu g/cm^2$ obtained by Langlotz and Kroner[48] for a single membrane.

Recently Klein and coworkers[50] modified microporous hollow fiber membranes using chitosan in order to couple protein A. The concentration of chitosan was found to be an important variable in determining the binding capacity of the membrane. Too much chitosan would increase the hydraulic resistance while too little chitosan would have low ligand capacity.

Protein G

One of the first applications of protein G as an affinity ligand in

ultrafiltration was performed by Chen[51] for the purification of bovine IgG, resulting in purification of about 90% of the protein. The protein G immobilized onto Sepharose was used in a method called affinity-recycle extraction. In this method the protein G-Sepharose beads were maintained in the outer section of a hollowfiber filtration unit with a 20 μm filter. This section was divided into two compartments, one for the adsorption step, and the other for the elution step. In a recent study[52] Malakian characterized Amicon's MAC affinity membrane, using protein G as the ligand, for the separation of IgG1. The binding capacity of 63 $\mu g/cm^2$ remained relatively constant after 100 runs.

Reactive Dyes

Champluvier and Kula[53] modified several nylon-based ultrafiltration membranes to couple the dye Cibacron Blue F3G-A for the isolation of the enzyme malate dehydrogenase. The resulting membranes adsorbed 200-400 $\mu g/cm^2$ of the enzyme, and they remained within that range after six runs. The same dye was coupled to a poly(ethylene imine) membrane to isolate human serum albumin from plasma[54] with a binding capacity of 40 $\mu g/cm^2$ as well as submicron latex particles[55] for the recovery of bovine serum albumin. Guo et al.[56] obtained a 40-fold purification of alkaline phosphatase when using the dye Active Red K2BP on a microporous cellulose membrane.

Metal Ions

Rodemann and Staude[57] used polysulfone membranes with copper metal chelates in order to separate histidine from

phenylalanine, threonine, and alanine. Serafica and coworkers[58] used the same metal chelate to separate lysozyme from α chymotrypsinogen using modified microporous glass hollow fiber membranes with a capacity of 12.2 μg/ml of membrane. Surface modifications to the membrane resulted in an increase in the surface concentration of ligand.

Water-Soluble Polymeric Carriers

These are high molecular weight polymers which are soluble in water (e.g., dextrans, polyacrylamide) to which the ligand is attached. The biomolecule of interest binds in batch to the ligand-activated polymer from a mixed population. This is followed by conventional filtration, which retains the soluble biomolecule-bound polymer based on the great difference in size from the unbound biomolecules. The bound biomolecule is then eluted in batch using appropriate dissociating agent. Finally both the biomolecule of interest and the soluble polymer are separated by conventional filtration. The water-soluble polymeric carrier offers a high binding capacity since all possible ligand-binding sites are accessible. Also, because of the flexible nature of the polymer in solution, the ligand is more accessible to its complementary macromolecule.

Using an affinity polymer synthesized by copolymerizing the trypsin inhibitor *N*-acryoyl-*m*-aminobenzamidine with acrylamide, Luong et al.[59] isolated trypsin from chymotrypsin. They obtained a yield of 90% with product purity of 98% using cross flow filtration. The same group recovered trypsin from porcine pancreatic extract[60] obtaining a yield of 77% with a product purity of 97% using the same

ligand. This ligand was used again[61] to recover urokinase from a mixture containing peroxidase giving a yield of 86% and from crude urine giving a yield of 49%. More recently[62] D-alanyl-D-alanine bound to dextran was used as a ligand in order to recover the antibiotic vancomycin at a yield of 57% from whole broth using centrifugal ultrafiltration.

EXTRACTION

This section deals with extraction processes where group specific affinity interactions are exploited. Biphase separations can be accomplished using aqueous polymer systems, reversed miscelles, and more recently using perfluorocarbon emulsions. They are all based on the partitioning of one or more biomolecules between two immiscible phases in order to isolate a product from a complex mixture.

Aqueous Two-Phase Extraction

Aqueous two-phase systems are formed when two aqueous solutions of hydrophilic polymers or a polymer and a salt are mixed at a high water concentration (i.e., >80%). This system is very attractive for the isolation of biomolecules since the isolation occurs under very mild conditions[63]. The system most widely studied is composed of polyethylene glycol (PEG) and dextran (Dx), since systems using PEG and salt are of high ionic strength which might influence the interactions between biomolecule and ligand.

The distribution of biomolecules between phases is measured in terms of the partition coefficient which is the ratio of the concentration of the biomolecule in the upper phase over that on the lower phase. This distribution is affected by surface properties of the biomolecule as well as by the composition of the two-phase system. The biomolecule will have affinity for the phase which offers most electrical, hydrophobic, hydrophilic, or conformational interactions. The degree of these interactions is in turn affected by polymer concentration and molecular weight, salt type and concentration, temperature, and pH[63].

An important aspect of partitioning is that when the protein is at its isoelectric point (pI), salt effects are negligible. When proteins have a net negative charge their partition coefficients increase following the Hoffmeister series:

$$ClO_4^- < SCN^- < I^- < Br^- < CL^- < CH_3COO^- < F^- < SO_4^{2-} < HPO_4^{2-} < PO_4^{3-}$$

and

$$K^+ < Na^+ < NH_4^+ < Li^+ < (C_4H_9)_4N^+$$

When the biomolecules are positively charge the opposite is true[63,64]. Thus a combination of the proper salt and the proper pH, which affect the net charge of the protein, can steer the preferential partitioning to either phase.

Temperature influences phase formation of polymer/polymer systems by requiring lower polymer concentrations at lower

Table 3. Group-Specific affinity aqueous two-phase systems.

Biomolecule§	Carrier-Ligand¶	System	Reference
G6PDH	PEG-Cibacron Blue	PEG-dextran	70
G6PDH	PEG-Procion Yellow HE-3G	PEG-dextran	70
lactate dehydrogenase	PEG-Procion Yellow HE-3G	PEG-dextran	71
lactate dehydrogenase	Eu-Cibacron Blue 3GA	PEG-dextran	72
phosphofructokinase	PEG-Cibacron Blue F3G-A	PEG-dextran	73
lactate dehydrogenase	Dx-Procion Yellow HE-3G	PEG-dextran	69
G6PDH	Dx-Procion Yellow HE-3G	PEG-dextran	69
phosphoglycerate kinase	Dx-Procion Yellow HE-3G	PEG-dextran	69
hemoglobin	PEG-Cu(II)	PEG-dextran	74
phosphorylated proteins	PEG-Fe(III)	PEG-dextran	75
hydroxyisocaproate	PEG-Cu(II)	PEG-dextran	76
lactate dehydrogenase	PEG-Cu(II)	PEG-dextran	77
alcohol dehydrogenase	PEG-Cu(II)	PEG-dextran	78
lactate dehydrogenase	PEG-Cu(II)	PEG-dextran	78
malate dehydrogenase	PEG-Cu(II)	PEG-dextran	78

§ G6PDH:glucose-6-phosphate dehydrogenase. ¶ PEG:polyethylene glycol, Eu:Eudragit S 100, Dx:dextran.

temperatures. However, for salt polymer systems, higher concentrations are required for phase partitioning[63]. Various mathematical models have been proposed to predict the behavior of aqueous two-phase systems as a function of these and other variables[65,66,67].

The most common ligand carrier is PEG, although ligands have also being coupled to Dx[68,69] and Ficoll[69]. Many recent articles involving the use of group specific ligands are presented, in which either reactive dyes or metal ions are used. The use of different carrier-ligands systems in the affinity aqueous two-phase extraction of specific biomolecules is referenced in Table 3.

Reactive Dyes

Cheng and coworkers[70] studied the extraction of glucose-6-phosphate dehydrogenase (G6PDH) in a PEG-Dx aqueous two-phase system with the addition of polymers carrying charged groups and the ligands Cibacron Blue F3G-A and Procion Yellow HE-3G. It was found that when polyelectrolytes are forced into a phase with salts, they have a very strong effect on the partitioning of the enzyme, and can enhance or decrease the partition when reactive dyes are employed. Joelsson and Tjerneld[71] extracted lactate dehydrogenase (LDH) from pig muscle homogenate using Procion Yellow HE-3G bound to PEG in a PEG-Dx aqueous two-phase system. After affinity partitioning and back-extracting twice with fresh lower phase, a 21-fold purification with a 79% yield was obtained. Alternatively, Guoaqiang and coworkers[72] recovered LDH by combining two extractions with a precipitation step. The first step extracted LDH nonspecifically into the lower phase (i.e., Dx) in a PEG-Dx system. For the second extraction Cibacron Blue 3GA was linked to a polymer, Eudragit S 100, whose solubility is pH-dependent. This polymer partitions almost completely to the top, PEG-rich phase making it possible to partition LDH to the top phase. The LDH-ligand complex was then precipitated by lowering the pH to 5.1. The enzyme was recovered with a 54% yield and a 12-fold purification after eluting with 0.5 N NaCl. Kopperschläger[73] recovered phosphofructokinase (PFK) from baker's yeast homogenate combining the PEG-Dx aqueous two-phase system with other nonspecific techniques. First, the beaker's yeast homogenate was fractionally precipitated with PEG, followed by affinity partitioning using Cibacron Blue F3G-A bound to PEG as a ligand. This resulted in a 58-fold

purification and 67% yield of PFK. The enzyme was further purified by a DEAE-cellulose batch treatment of the top phase, followed by gel filtration. A 142-fold purification and a 30% yield was obtained after the gel filtration.

In order to study the effect of ligand carrier on biomolecule partitioning, Johansson and Joelsson[68] tried using Dx as a ligand carrier for Procion Yellow HE-3G instead of PEG. Dextran has the advantage of containing more reactive groups than PEG for ligand binding. The enzymes LDH, G6PDH, and 3-phosphoglycerate kinase preferentially partitioned into the Dx phase. The dye-Dx phase could become either the top or bottom phase by the addition of salts. More recently Birkenmeier[69] studied the partitioning of human serum albumin (HSA) and prealbumin in the presence of two different immobilized dyes. They tested all possible combinations of two dyes (i.e., Cibacron Blue F3G-A and Remazol Yellow GGL) and three polymers (i.e., PEG, Ficoll, and Dx) in a two phase aqueous system.

Metal Ions

Wuenschell and coworkers[74] were one of the first to apply metal ions as affinity ligands in aqueous two-phase systems. An aqueous two-phase system of PEG-Dx consisted of an iminodiacetic acid (IDA) derivative of PEG in order to chelate Cu(II). This system was used to study the extraction of heme proteins containing different numbers of exposed histidine residues. The results showed that protein partitioning was strongly dependent on the number, as well as on the state of ionization of the residues, since partitioning was pH

dependent. Cytochrome c which has only two histidine residues showed almost no increase in the partition coefficient using chelated Cu(II), while hemoglobin with 20-24 residues showed 24 to 37-fold increases in the partition coefficient. Recently the same group[75] studied the partitioning of several phosphorylated and nonphosphorylated proteins in a Fe(III)IDA-PEG-Dx two-phase system, since Fe(III) has a high affinity for phosphoryl groups. They found almost no increase in partition coefficient in the unphosphorylated proteins, while phosphorylated proteins showed a 3 to 15-fold increase, with phosvitin (40% phosphate) having more than a 1000-fold increase.

Schustolla and coworkers[76] found a 1000-fold increase in the partition coefficient of D-2-hydroxyisocaproate in a Cu(II)IDA-PEG-Dx two-phase system. Direct extraction from the cell homogenate resulted in a sharp decrease in efficiency with an 11-fold purification and a 90% yield of the protein. Using the same affinity two-phase system, Otto and Birkenmeier[77] studied the differences in partition of different LDH isoenzymes from different species. There was a large difference in the partition coefficient in the presence of the Cu(II) chelate between the isoenzymes ranging from a 2.5-fold to a 30,000-fold increase, making this a viable method for the separation of LDH isoenzymes. However this procedure was repeated with Ni(II) and Zn(II) yielding almost no difference in partitioning between isoenzymes. In a similar study, Pesliakas and coworkers[78] used Cu(II), Ni(II), Zn(II), and Cd(II) for the affinity extraction of yeast alcohol dehydrogenase (YADH), two different lactate dehydrogenases (HLDH and MLDH), and malate

dehydrogenase (HMDH). Cu(II) was the only metal chelate that showed a marked difference between the dehydrogenases with affinity increasing in the order MLDH>YADH>HMDH >>HLDH.

Reversed Micelle Extraction

The distribution of a biomolecule between reversed micellar and aqueous phase seems to be affected by different electrostatic interactions such as pH, ionic strength, temperature, and type of surfactant[79]. The biomolecule is extracted into the reversed micellar phase when its net charge opposes that of the polar head of the reversed micelle since the net charge of the biomolecule is pH dependent. Thus a modification of the pH above or below the pI of this biomolecule will give the proper net charge so as to oppose that of the polar head and hence become extracted into the reversed micellar phase. Many enzymes are not stable below their pIs, and careful selection of the surfactant is required. It has also been noted that the pH required for extraction increases with ionic strength[79]. Moreover, an increase in ionic strength decreases electrostatic interactions between the charged biomolecule and the polar head of the surfactant making it harder to extract the biomolecule into the reversed micellar phase[79]. The type of salt used affects the biomolecule partition in the same way as in the aqueous two-phase system since the core of the reversed micelle is also aqueous. As the temperature increases, the partition of the biomolecule into the reversed micellar phase is less favorable and subsequently decreases. This property can be used as a recovery procedure during back-extraction[80]. Affinity surfactants are surfactants with an affinity ligand

as the polar head, affecting the partition of the complementary biomolecule into the reversed micellar phase.

Woll and coworkers[81] were one of the first to use affinity surfactants to isolate biomolecules. They used bis(2-ethylhexyl)sodium sulfosuccinate (AOT) reversed micelles with the glucoside *n*-octyl-β-D-glucopyranoside as the affinity ligand for Con A. Using this ligand they found close to 100% partitioning of Con A at a pH close to its pI, while no partitioning was observed without the ligand. Confirmation that an affinity interaction was the important component in this system was proven when partitioning was inhibited using the free ligand. Using the findings by Woll and coworkers[81], that Con A could be extracted using AOT reversed micelles, Paradakar and Dordick[82] used Con A as a carrier for the glycoprotein horseradish peroxidase in order to facilitate the extraction. A separation factor of 16 was obtained using horseradish peroxidase and the unglucosylated alkaline phosphatase. More recently Chen and Jen[83] used a similar system to extract Con A. Using the AOT reversed micelles with β-glucosides of different alkyl lengths, they found octyl-glucoside to give the best extraction. This was a confirmation of the work of Kelley and coworkers[84] who found the same results not only with β-glucosides, but also with amino acids and carboxylates. Chen and Jen[83] also found that back-extraction of Con A showed a significant loss of β-glucosides, limiting the reusability of the reversed micellar phase. An alternative back-extraction method was proposed by Leser and coworkers[85] in which the biomolecules in the reversed micellar phase are adsorbed onto silica on the aqueous phase. The organic solvent was removed and the biomolecules eluted by

optimizing pH and ionic strength. Reversed micellar extractions have also been obtained using not only octyl-glucoside but also natural amphiphiles (such as lecithin) and alkyl boric acids to extract Con A, myelin basic protein, and α chymotrypsin, respectively[86]. A 8-, 3-, and 20-fold increase in extraction was found in the presence of the affinity ligands of Con A, α chymotrypsin, and myelin basic protein, respectively.

Perfluorocarbon Emulsions

Perfluorocarbon emulsions form in an aqueous solution using perfluoroalkylated surfactants which orient themselves on the surface of fluorocarbon liquid droplets. These surfactants can be modified in order to include affinity ligands[87,88] for isolating specific biomolecules. Perfluorocarbons are an attractive affinity support because they are: (1) inert; (2) hydrophobic in nature; and (3) about three times heavier than water, thus making it easy to separate the phases by sedimentation in a contacting vessel.

Boedeker and Lenhoff[88] where among the first to use liquid perfluorocarbons as affinity supports. Monoclonal antibodies specific for the marker dansyl (5-di-ethylaminonaphtalene-1-suphonyl) where coupled onto the perfluorocarbon supports to bind dansylated lysine and bovine serum albumin (BSA). Using a continuous stirred tank reactor as a contacting vessel about 7 and 15 minutes were necessary to saturate 0.1 μM of ligand with 50-times excess lysine and an unknown amount of BSA, respectively. One of the main problems encountered was dissociating the dansylated proteins from the highly specific ligand.

More recently McCreath and coworkers[89] used the reactive dye CI Reactive Blue 4 coupled to a polymeric fluorosurfactant based on poly(vinyl alcohol) in order to isolate human serum albumin from blood plasma in a fluidized bed. An average separation factor of 1.4 was obtained with an average purity of 90% HSA. The binding capacity of the affinity emulsion was found using frontal analysis to be 0.59 mg/ml for HSA, with a 13.5% mol/mol ligand usage. The major drawback of this method was that the emulsion droplets became unstable after long operations. In trying to create a milder contacting environment, a new type of reactor was designed[90]. This reactor consisted of four continuous mixer-settler stages: the first one for adsorption, the second for washing, the third for elution, and the fourth to regenerate the affinity emulsion, which is then recycled to the first stage. This resulted in a 1.52-fold purification with a 70% HSA yield after elution. The same contacting vessel was tested more recently[91] for the purification of glucose-6-phosphate (G6PDH) dehydrogenase from yeast homogenate using Procion Red H-E7B. This resulted in a 18-fold purification with 61% yield of G6PDH after elution.

AFFINITY CHROMATOGRAPHY

Affinity chromatography is a highly selective purification technique which employs affinity ligands specific for one or more biomolecules. The ligands are coupled to a solid support in such a way

so as to form a stable but reversible complex with its complementary biomolecule. There are many types of supports, organic or inorganic, and they vary according to their bulk and surface chemical structure, as well as in their pore structure. In affinity chromatography the choice of support depends on the type of ligand to be used. Conventional chromatography uses a few different types of supports such as: dextrans with varying degrees of cross-linking, molecular sieves, or ion exchangers.

The process of affinity chromatography for the isolation of biomolecules is composed of the sequence: adsorption, washing, elution, and regeneration. The first step is adsorption of the specific biomolecule to the ligand from a multicomponent feed mixture. Then the column is washed to remove anything that was not specifically bound to the ligand. Elution of the biomolecule is accomplished by decreasing the binding strength of the ligand and its corresponding biomolecule. This can be done by altering the pH or the ionic strength, or by using denaturing agents or competing ligands. The last step is regeneration of the ligand to be used again for another purification cycle. While the binding step is critical for the success of the technique, the elution and regeneration steps are also of great importance for commercial viability. The choice of elution method will determine, not only, the amount of product recovered, but also its integrity. Regeneration is extremely important when expensive ligands (e.g., antibodies) are used, but less critical when group-specific ligands are involved.

This section focuses on new applications of reactive dyes and metal ions as affinity ligands in chromatography. Both techniques

Table 4. Group-Specific affinity chromatography systems.

Biomolecule	Ligand	Support	Reference
lactate dehydrogenase	Cibacron Blue 3G-A	HEMA¶	94
lactate dehydrogenase	Cibacron Blue	Sepharose	95
alcohol dehydrogenase	Scarlet	Sepharose	95
phosphofructokinase	Cibacron Blue F3G-A	Sepharose	96
IgG_2	Rubine R/K-5BL	Sepharose	98
S-oxynitriliase	Cu(II)	Sepharose	99
Igs	Cu(II)	Novarose	103
VP2	Ni(II)	Sepharose	104
amino acids	Ag(I), Pt(II)	polyacrylamide	105
b-BSA	Pt(II)	polyacrylamide	106

¶ HEMA:polyhydroxyethyl methacrylate.

have been extensively reviewed in previous publications[92,93]. In order to complement these reviews, the following discussion describes some limitations when using reactive dyes and metal ions.

The use of different ligands in the affinity chromatography of specific biomolecules is referenced in Table 4.

Reactive Dyes

Much of the work that is being done in affinity chromatography today involves the shielding of conventional chromatography supports in order to minimize nonspecific binding. This is of great importance in affinity chromatography since the biomolecule of interest is usually present at very low concentrations and any nonspecific binding would result in very low recovery. This is because

nonspecific elution would recover all biomolecules bound, while specific elution would only elute the affinity ligand-bound biomolecules, leaving behind the biomolecules of interest that were nonspecifically-bound. Mislovicová and coworkers[94] reduced nonspecific binding associated with the support polyhydroxyethyl methacrylate (HEMA) by filling macropores with a dextran gel to obtain the composite D-HEMA. The behavior of this support was tested using the reactive dye Cibacron Blue 3G-A to recover LDH. There was more than a 3-fold increase in recovery of LDH after elution with the composite D-HEMA at a yield of 82%. In another study Galaev and Mattiasson[95] used poly(N-vinylpyrrolidone) (PVP) to reduce nonspecific binding on Blue Sepharose and Scarlet Sepharose using LDH and secondary alcohol dehydrogenase, respectively, as model systems. This resulted in nearly 100% recovery for both systems using either specific or nonspecific elution of the enzymes. In another study[96] the same group studied the interactions of poly(vinyl pyridine) (PVP) and poly(vinyl alcohol) (PVA) with Cibacron Blue F3G-A in order to minimize the nonspecific interactions with the dye. PVA demonstrated a hydrophobic interaction with the dye while PVP showed an electrostatic interaction, making it a better choice for use in charge shielding. The shielded dye was coupled onto Sepharose CL 4B and used for affinity chromatography of PFK from baker's yeast homogenate. A 56% yield was obtained after specific elution of PFK with 27-fold purification.

Kirchberger and coworkers[97] studied the interactions between NADH oxidase from *Thermus thermophilus* HB8 and twenty eight triazine dyes. This choice of enzyme is a result of its capacity to bind

the coenzymes NADH and FAB. It was found that all triazine dyes bound the FAD binding site of the enzyme, while Procion Red H-8BN also bound, to some extent, the NADH binding site. In another study[98], human immunoglobulin G 2 (IgG_2) was isolated by employing the strong affinity for the copper-containing dye Rubine R/K-5BL from a solution containing other subclasses of IgG. It seems that the group-specific interactions of dye and metal ion is synergistic and yields highly-specific ligands. Because of this strong association between the dye and IgG_2 a systematic screening of eluents was necessary to recover IgG_2 in its native state. Many mobile phase conditions allowed recovery of the bound IgG_2.

Metal Ions

Woker and coworkers[99] isolated the hydroxynitrile lyase S-oxynitrilase from the homogenate of *Sorghum bicolor* through a series of steps, some involving chelated Cu(II). One of the steps consisted of extraction using an aqueous two-phase system, followed by affinity extraction using Cu(II)-IDA-PEG and Dx. This was followed by immobilized metal ion affinity chromatography (IMAC) using Cu(II)-IDA, and subsequently followed by gel permeation. This process resulted in a 60% yield and a 150-fold purification. Other strategies were explored, but there was no appreciable increase in yield or purification. In order to elucidate some aspects of the molecular basis of IMAC retention, Todd and coworkers[100] varied the number and position of histidine residues in iso-1-cytochrome c and compared the adsorption isotherms of different variants. Proteins with multiple histidine residues did not follow a Langmuir-type adsorption,

suggesting that more than one metal ligand is interacting with it. Hansen and coworkers[101] were able to purify synthetic peptides lacking histidine by binding their α-amino group to either Cu(II) or Ni(II) using IMAC. In a more recent paper the same group[102] was able to selectively purify histidine containing peptides using IMAC. The found that the histidine-containing peptides would always bind Cu(II), with or without the α-amino group blocked. On the other hand both histidine and α-amino groups were necessary for optimal retention on Ni(II), suggesting two distinct types of interactions that can be used to selectively purify histidine-containing peptides. Boden and coworkers[103] used the high capacity gel Novarose coupled to tris(2-aminoethyl)amine with chelated Cu(II) to purify goat immunoglobulins (Igs) in one step. The adsorption of Igs from goat serum was performed at high flow rate, pH 6.0 and at low flow rate, pH 7.0, obtaining a binding capacity of 17 and 15 mg Igs/ml, respectively. The proteins were eluted by linearly decreasing the pH to 5.5. Another one-step procedure was achieved by Wang and coworkers[104] in the purification of the chimeric counterpart of viral VP2 from the infectious bursal disease virus using IMAC with Ni(II). The difference between the two proteins, which allowed for the separation, was five histidine residues.

Until recently most purification studies using IMAC have relied on using transition metal ions (e.g., Cu(II), Ni(II), and Zn(II)) which interact with indole and imidazole groups (e.g., try and his). Based on Pearson's hard-soft acid base theory[15,16,17] these are moderately hard acids and bases, respectively. An alternative is to use soft acids (e.g.,

Ag(I), Cu(I), Au(I), Hg(II), Pb(II), Pd(II), Pt(II), and Cd(II)) to interact with soft bases (i.e., sulfur-containing functional groups in chemicals and biomolecules). With this in mind, García and coworkers[105] immobilized the soft metal ions Ag(I) and Pt(II) onto a polyacrylamide support activated with glutaraldehyde and thiourea to study the retention of different amino acids. Their results at pH 7.0 showed the following retention order for Ag(I): His > Met >> Trp >> Tyr > Phe > Asx > Pro, while for Pt(II) the order was: Met >> Trp > Tyr > His = Phe. At pH 4.7, methionine is retained longer than histidine on the silver ion resin. IMAC using Pt(II) was used by the same group[106], to purify biotinylated BSA (b-BSA) from unlabeled BSA at pH 4.8. After elution with imidazole/HCl buffer at pH 7.0 60% of the bound b-BSA was recovered.

The union of membrane technology and chromatography appropriately called membrane chromatography has also seen the use of group specific ligands. An excellent source of information on the types of group specific systems that have been employed in membrane chromatography can be found in a recent review of the field by Thömmes and Kula[107]. They report the use of dyes such as Cibacron Blue, Procion Red and Yellow, amino acid such as phenylalanine and tryptophan, as well as metal ions immobilized by IDA.

CONCLUSIONS

Group specific ligands have been effectively introduced into a number of bioseparation processes. However, more work is needed in

some of these applications in order to overcome some challenges such as the toxic effect of dyes and the stability of metal ions. The overall approach of targeting biological products based on a specific aspect of their interaction chemistry is attractive since the use of simple chemicals, biochemicals, or metal ions as ligands is much less expensive and usually less susceptible to degradation than protein or antibody ligands.

In order to further this approach, a better understanding of group specific ligand design is needed. Design issues that could be addressed include: (1) more ligand types in order to target the large array of biochemical functional groups; (2) cost effective "templating" of ligand designs so that a specific sequence presented by a biopolymer can be targeted; (3) improvements in ligand toxicity testing; and (4) improvements in immobilization procedures and ligand leakage testing.

The industrial future of bioseparations will include a greater emphasis on cost-effective techniques with the entrance into the public domain of the first generation of biotechnology products. A judicious use of group specific ligands will certainly play a role in many of these and in future products of the biotechnology industry.

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