Affinity Chromatography — from Textile Dyes to Synthetic Ligands by Design

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Very significant progress has been made since the mid-1970s when dye ligands were first introduced.

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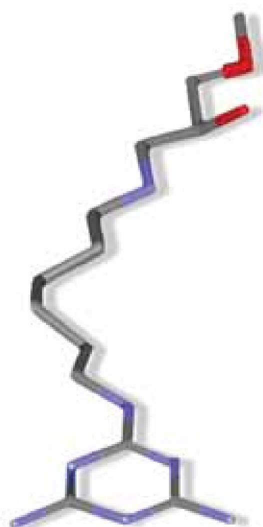
At its widest definition, affinity chromatography encompasses techniques from immobilized metal chelate affinity to molecular imprinting and technologies from affinity capillary electrophoresis to affinity precipitation, affinity partitioning, and affinity membranes.[1] A very narrow definition would focus on specific or selective, reversible interactions between the immobilized ligand and target, dependent on a unique topological relationship involving orientation and molecular reactions. These include biological as well as biological mimic ligands. This is an area that has seen very significant growth and research development in recent years. The advent of affinity chromatography is usually attributed to Cuatrecasas, Wilchek, and Anfinsen.[2] In 1968 — a year after the introduction of cyanogen bromide activation — they described the purification of certain enzymes



■ **Figure 1.** Triazine Structure with Two Substitution Positions and a Spacer Arm to the Matrix.

on "inhibitor gels" synthesized by coupling the inhibitor to CNBr-activated agarose. They introduced the notion of protein purification based on biologically functional pairs, the molecular recognition between a target protein and an immobilized partner. The technique was rapidly assimilated into the protein purification armory. Three years later, Cuatrecasas' review cited 100 papers and applications, ranging from immunoaffinity to the isolation of nucleic acids and the separation of complex cellular structures and cells.[3] By 1984, the body of literature had swollen to 1,800 papers, and a 2004 Medline search revealed 35,000 affinity chromatography citations.[4]

Perhaps affinity chromatography's greatest success at scale has been monoclonal antibody purification. The demand for Protein A resins is more than 10,000 liters annually, increasing at 50% per year and representing a market in excess of $50 million in 2002.[5] The use of immunoaffinity chromatography enables the production of both plasma-derived[6] and recombinant[7] coagulation factors VIII and IX, as well as other plasma proteins and biopharmaceuticals from natural and recombinant sources. Process development is constantly seeking high efficiency processing with the minimum number of steps and maximum output at the required purity. Larsson discussed customizing ligands to the separation task-at-hand instead of relying on "off-the-shelf," group-specific methodologies.[8] Indeed the concept of "designer" adsorbents was introduced ten years earlier at the peak of expectations in biopharmaceutical product development, but at a time (1992) when FDA had approved only 12 recombinant products.[9]

SERENDIPITOUS ORIGINS

Subramanian described the serendipitous origins of dye affinity chromatography.[10] Blue dextran, a 2,000 kDa soluble polymer with the covalently bound dye Cibacron Blue F3GA, was developed to measure the void volume in gel filtration columns introduced in the early 1960s. It had been noted that some proteins, when co-chromatographed with Blue dextran, interacted with the marker. It was established in 1968 that the dye chromophore was responsible for binding.[11,12] Thus, the evolution of dye affinity is concurrent with the general development of modern separation techniques. The introduction of the terms mimetic or biomimetic derives from the proposed (and now discarded) mechanism of dye affinity in which the structures of the chromophores mimic the naturally occurring heterocyclic nucleotides on which many proteins and enzymes depend.[13] The synthetic nature of the ligand also led to the introduction of the term pseudo-affinity, referring to the ligand's lack of biological function.

Undoubtedly the earliest (1973) and most studied ligand is Cibacron Blue F3GA (Ciba-Geigy), which is also available as Procion Blue H-B (Imperial Chemical Industries) and C.I. Reactive Blue 2 (the Color Index name). These textile dyes from different manufacturers are no longer available, but single synthetic analogue adsorbents are manufactured by at least two suppliers of chromatographic media. The first reported separations predate plasma proteome studies by 30 years and concern the depletion of serum albumin from human plasma to enable identification and purification of low

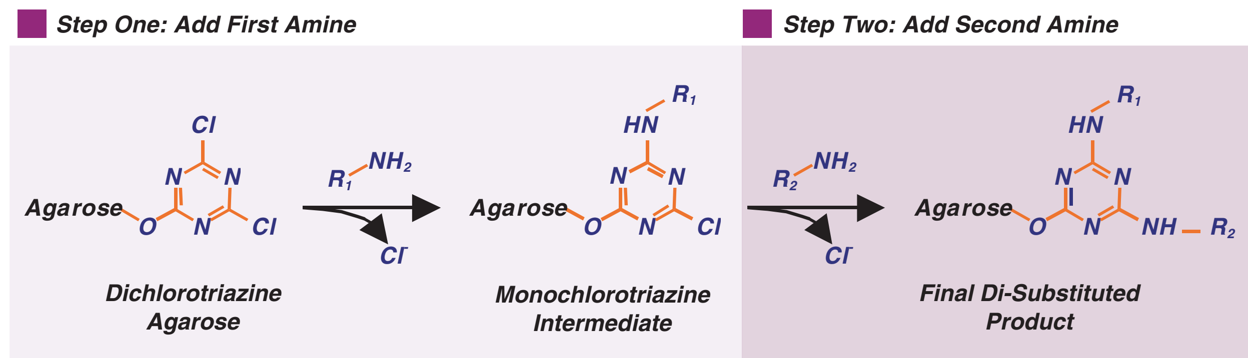


Figure 2. Basic Chemistry of Ligand Synthesis from Dichlorotriazine-Agarose

concentration proteins.[14] This was carried out using a Procion Blue dextran-Sepharose conjugate. At the time, Travis and Pannell were interested in α_1-antitrypsin and described the difficult separation of this protein from albumin at high ionic strength where any non-specific ion exchange binding is at a minimum.[15] This paper identified an initial problem with dye affinity chromatography, namely the leakage of the dye into the eluates. In later studies, the authors used the dye directly conjugated with agarose, thus reducing the leakage problem, improving the binding capacity to 40 mg/mL gel, and enabling elution of the albumin under non-denaturing conditions.[16]

The 1970s academic focus on protein purification is particularly evident in a 1979 review listing over 100 different proteins and enzymes purified using immobilized triazine dyes.[17] This also heralds a migration from a dye descriptor to a chemical identification of the core triazine. Additionally, it is notable that the dyes under investigation were triazine, substituted at two positions and coupled to the matrix through the third.

At this time it became evident that it was necessary to screen dye-ligand columns to find the most selective adsorbent.[18,19] Atkinson *et al*. noted that "this dye (C.I. Reactive Blue 2), however, is only one of a large family of triazine dyes, most of which bind proteins," indicating the need for a systematic approach to selection.[20] It was also believed at this time that these ligand adsorbents would be highly suited to downstream processing.[21]

Stellwagen (1990) noted that "since the color, and hence the structure, of each reactive dye is different, each reactive dye will have a somewhat different affinity for a bifunctional site on a given protein. Unfortunately, the affinity of a particular reactive dye for a bifunctional site cannot be predicted with any confidence, necessitating an empirical screening procedure to optimize chromatography."[22] Only two years later, Lowe *et al*. wrote, "A fundamental advantage of affinity techniques is their predictive and rational character, since the ligand selected is designed to interact specifically with the protein to be purified."[23]

At this pivotal point, dye affinity technology began to shift to *de novo* synthesis and rational ligand design.[9] However, because some triazinyl derivatives are colored — although most are not — the concept of dye ligand chromatography, in which the ligand is chosen from a random battery of commercial dyestuffs, has lived on past its expiration date. Two reviews have summarized the field in the context of bioprocessing applications.[24, 25]

GENESIS OF SYNTHETIC LIGAND ALTERNATIVES

The shortcomings of dyes as affinity ligands has been recognized for some time. They are rarely single chemical entities and — produced as bulk chemicals for a consumer product industry — they do not fulfill the rigorous requirements of biopharmaceutical development and manufacturing. It had been noted that textile dyes contain isomers

Library XY

Unbound (Flow through): HSA mg/mL

Second Amine	First Amine							
	A	B	C	D	E	F	G	H
1	0.915	0.954	0.805	0.907	1.009	1.194	0.279	0.718
2	1.056	0.113	0.894	0.907	0.947	0.998	0.915	0.915
3	0.977	0.779	0.773	0.788	1.071	0.990	0.345	0.630
4	0.835	0.701	0.694	0.692	0.960	0.869	0.301	0.586
5	1.066	0.918	1.073	1.043	0.945	1.069	0.856	0.869
6	0.960	1.041	0.884	0.864	0.990	1.154	0.490	0.847
7	0.788	0.290	0.684	0.728	0.732	1.024	0.326	0.567
8	0.669	0.624	0.849	0.592	0.875	0.888	0.237	0.647

Elution: HSA mg/mL

Second Amine	First Amine							
	A	B	C	D	E	F	G	H
1	-0.096	-0.002	-0.062	-0.057	-0.040	-0.106	0.533	-0.062
2	-0.047	0.864	-0.074	-0.059	-0.077	-0.123	0.015	-0.077
3	-0.049	-0.222	-0.025	-0.064	-0.217	-0.074	0.469	0.059
4	-0.062	-0.052	-0.042	-0.037	-0.081	-0.131	0.373	-0.049
5	0.037	0.062	-0.040	-0.027	-0.040	-0.069	0.054	-0.007
6	-0.084	-0.153	-0.099	0.017	-0.084	-0.101	0.309	-0.040
7	0.151	0.521	0.111	0.069	-0.062	-0.042	0.570	0.299
8	-0.030	0.002	-0.079	-0.067	-0.062	-0.057	0.635	0.047

Figure 3. Secondary Screen of a Symmetrical Library (# XY) (8 x 8 array) for a Recombinant Albumin. The figures represent protein concentration using a simple BCP assay. The ligand B2 demonstrate a development opportunity, since near neighbors do not exhibit similar binding and elution properties.

of the main product, stabilizing and diluent agents, as well as anti-dusting agents such as dodecylbenzene. Of course, these impurities and contaminants must be removed.[26] In one study of Cibacron Blue F3GA, up to 15 different colored components were identified. Furthermore, different preparations did not necessarily contain the same components.[27] In 1988, Burton et al. reported, for the first time, the synthesis of single isomer variants of C.I. Reactive Blue 2, thus marking the link between dye structure and protein binding.[28]

Additionally, the interactions between target proteins and dye structures present many different alternatives for highly specific binding of proteins at their active sites and less specific interactions at other sites. These reactions may include a complex combination of electrostatic, hydrophobic, hydrogen bonding, and charge transfer reactions. Thus, ligand engineering or design is a way of targeting specific binding.

Work on biomimetic ligands, as defined by Clonis, was initiated in 1984 and is exemplified by two development projects.[29] The first biomimetic dye ligand was developed for trypsin by linking benzamidine to the reactive chlorotriazine through a diaminomethylbenzene group.[29] The ligand was designed on the basis of the unusual cationic substrate preference of trypsin-like enzymes. In a second example, Reactive Blue 2 was specifically redesigned to confer specificity for calf intestinal alkaline phosphatase.[30]

From there, it was a short step to generate well-defined dye adsorbents in order to develop a series of adsorbents for which the ligand structures and the ligand density were known and could be controlled.[31] However, there are many more variables in the performance of affinity chromatography. Greater degrees of sophistication are required, both in the design of the ligand and the ligand-adsorbent conjugate and in the execution of the technique. In a second step toward designed ligands (but still restricted to the concept of “designer dyes”), the interaction between the “parent” dye (Cibacron Blue) and analogs with horse liver alcohol dehydrogenase was established using X-ray crystallography.[32] A new set of terminal-ring (opposite to the anthraquinone) analogs were synthesized with favorable affinity for ADH. These ligands belong to a second generation of adsorbents, the results of rational molecular design techniques.

Lowe *et al.* reviewed the advent of computer-aided ligand design, which has subsequently developed to include design based on X-ray crystallographic data, NMR protein structures, and homology data from suitable databases.[33] This has been made possible by concomitant software developments.[33] In addition, design may start from peptide ligand models as mimics of protein-protein binding interactions. Peptides themselves can be used as ligands, but present an expensive and less stable choice for bioprocessing. Instead, the peptide template can be used to model a synthetic alternative.

There are several different approaches to ligand design and synthesis that form the basis of the technology used today for commercial development of third-generation

adsorbents, including:

- optimization of existing ligands (analog synthesis);
- rational design (computer modeling of ligand structures);
- systematic screening of ligand arrays;
- rational design combined with library construction and screening.

Often, insufficient structural information is available for a target protein, and, in some cases, even the target may be unknown. If a biological ligand is known or a peptide ligand has already been developed, those models may be used. It is not surprising, therefore, that most commercially developed ligands and adsorbents have been derived from targeted construction and screening of solid phase libraries. Lowe recently reviewed combinatorial approaches to affinity chromatography.[34] Although virtual libraries can be constructed from, for example, the Available Chemicals Directory's 2.5 million entities, and these libraries can be reduced to real and manageable sub-libraries, this route has not been commercially successful for bioprocessing.[35] It is important to note that earlier screening of solution phase libraries has also failed to produce any commercial adsorbents, since the orientation of the ligand to its target in solution and as an immobilized entity may present different aspects to the protein. In this article, only solid phase libraries and screening will be discussed.

It is possible to identify two major steps in the development of "customized" ligands: 1) construction and screening of suitable libraries, known as intelligent combinatorial chemistry and 2) development of the ligand adsorbent conjugate.

LIBRARY CONSTRUCTION

Customized robotics systems are available (for example, see www.chemspeed.com) that enable the parallel synthesis of reasonably large libraries. A 96-well plate format is convenient. A successful approach employed by ProMetic BioSciences is to construct libraries in micro-column arrays in which each well contains 250µL of packed adsorbent (PuraBead). Each unique adsorbent is based on a 100µm agarose bead media, epoxy-activated and coupled to a triazine, with or without a spacer of one to six carbons (Figure 1). Triazine is selected because of its stability, ease of derivation, and safety. The resultant dichlorotriazine can be derived either symmetrically (with the same ligand at each of the two positions) or with different moieties at each of the substitution sites.[36] The reaction scheme, where R1 and R2 are amine substituents, is shown in Figure 2. One thousand amines provide a diversity of 500,000 different ligands based on a single triazine scaffold.

$$v = \frac{n}{2}(n+1) = \frac{n^2}{2}$$

where v is the total number of possible combinations and n is the number of amines.

In addition, triazine lends itself to dendrimetic expansion, and therefore larger, branched ligands may be developed with even greater diversity than those available from single triazine derivation.[37] Such ligands offer the possibility of improved specificity and selectivity for "difficult-to-isolate" molecular species.

Pre-Project Stage
• Target product profile
• Milestone definition
Stage 1: Ligand Discovery (2 - 6 Months)
• Target protein purity (selectivity)
• Impurity profile
Stage 2: Adsorbent/Chromatography Development (2 - 6 Months)
• Capacity
• Recovery
• Alkali resistance
• Binding and elution conditions
Stage 3: Commercialization (6 -12 Months)
• Adsorbent scale-up
• Stability
• Ligand loss (leakage)
• Safety (toxicity)
• Drug master file

Figure 4. Development Timeline and Major Activities for Customized, Synthetic Ligand Adsorbents. The commercialization stage entails preparation for routine manufacturing, consistency lot production, and initiation of long-term studies. Drug master file information also is generated at this stage.

Diversity-optimized combinatorial libraries can be constructed *in silico* using Cerius2 (www.accelrys.com) software. Diverse "R" groups are used as input, "combichem" molecular descriptors are calculated for all fragments, and a principal component analysis (PCA) is performed. A coverage-based diversity selection is used to produce a subset of the PCA space, and an analogue builder tool is used to combine R groups with a triazine core. In this way, a diversity-optimized virtual library is constructed. R group subsetting and proportional sampling functions are used to select sets of R groups to generate a 16 x 16 combinatorial library with maximum diversity.[38] These libraries, or subsets of them, may then be synthesized to contain anionic, neutral, cationic, hydrophilic, and hydrophobic R groups in a 96-well format.

LIBRARY SCREENING

General libraries are screened — the primary screen — under the desired separation conditions (conditions of the feed stream, stability of the protein, etc.) of defined pH, ionic strength, and buffer composition. The screen is carried out in a straightforward load, wash, elute, sanitize sequence using, for example, a Tecan Genesis (www.tecan.com) robotic liquid handing system. Eluates are assayed for the target protein. The initial screen allows the identification of lead ligands with promising binding and dissociation characteristics. These ligands are selected, and sublibraries are constructed using analogues of the general library's primary amines. The sublibrary is screened — the secondary screen — using the conditions of the primary screen. Further iterations may be necessary to home in on the target ligand. A typical screening result is shown in Figure 3.

A lead ligand is selected for development. The development program involves three stages, starting with discovery, in which the selectivity of the target ligand is established to meet a desired purity (activity) specification. In the second stage, adsorbent and chromatography development lead to the establishment and optimization of capacity, recovery, binding, and elution conditions and the alkali resistance (stability) of the adsorbent. In the third stage, the adsorbent is scaled up for manufacturing, stability and leakage studies are initiated, and safety (toxicity) data are generated in preparation for submitting a Drug Master File to FDA. This development process is summarized in Figure 4 for a typical customized ligand and adsorbent development program from a client-oriented vendor.

PROPERTIES

Initially, dye ligands were pursued not only for their biomimetic function but also because they were readily available from major chemical companies; they were cheap and they were stable when attached to a support matrix. Very significant progress has been made since the mid-1970s when dye ligands were introduced, and the major differences and improvements are given in Table 1.

The advantages of synthetic ligands compared to their biological counterparts have been summarized by Lowe *et al.*[39] and Boyer and Tsu.[40] Table 2 is an adaptation of their view, modified to encompass specifically designed ligands.

Because they are based on triazine chemistry, the synthetic ligands under discussion have some similarities with their dye predecessors. These ligands, therefore, display significant stability and resistance to both chemical and biological degradation. This property also confers long-term stability and re-usability, and synthetic ligand adsorbents can be used for at least 100 cycles.[41] In this context it may be remembered that the colorfast, reactive (triazine) dyes developed during the 1950s were designed for covalent bonding to cellulosic fabrics (in contrast to earlier animal and synthetic fibers) under mild conditions with minimum leaching.

These affinity adsorbents display dynamic protein-binding capacities in the range 5 up to 40 mg/mL. Most absorbents used in bioprocessing have a capacity of 15 to 20

mg/mL at a linear flow rate of 100 cm/hour. They are comparable with Protein A adsorbents and most ion exchangers. They may be regarded as a particular compound family similar to the families of ion exchangers, and they certainly show similar characteristics in bioprocessing.

LEACHABLES

As has been previously mentioned, ligand leakage was noted when Blue dextran was coupled to agarose and significantly reduced when the ligand was coupled directly. Eketorp[42] discussed the leakage of ligand from cyanogen bromide-coupled products, which have now generally been discarded in favor of oxirane coupling through a far more stable epoxy linkage. However, as with all adsorbents used in chromatography, leaching with or without degradation of the matrix, is an important consideration.

As use was increased in the early '90s, particularly of Cibacron Blue/CI Reactive Blue adsorbents, leachates and potential toxicity came under scrutiny. Initial studies focused on the development of immunochemical quantification, since the sensitivity of a spectrophotometric determination at 608 nm (for Cibacron Blue) was only about 1µg/mL dye.[43] The free dye is non-antigenic, and antibodies can only be raised when the dye is conjugated with, for example, mannosylated BSA or Keyhole Limpet Hemocyanin (KLH).[44]Using this latter method, a 1,000-fold sensitivity increase was attained in a competitive inhibition assay. Even further improvement was achieved in an enzyme-linked immunosorbent assay (ELISA) capable of measuring CI Reactive Blue at concentrations down to 10 ppm (10 nM), representing a 3,000-fold higher sensitivity over direct spectrophotometric analysis.[45] This assay formed the basis for a commercial product, and similar assays for other ligands have subsequently been developed. For example, Novo Nordisk has reported the stability of a symmetrical synthetic ligand developed for the purification of coagulation Factor VIIa (NovoSeven).[46] In this study, a reverse phase HPLC assay was developed with a sensitivity corresponding to a quantification limit of 6.2 ng/mL with 100µL injections. The response was linear over a 0 to 500 ng range for leachates, both in the presence and in the absence of the protein. Leakage during storage in 20% ethanol for 24 hours at 23°C was <0.002%, and leakage significantly declined at +5°C. Cleaved ligand adsorbent conjugate, after regeneration with 0.5 M sodium hydroxide, could be washed out with 2.5 column volumes, and it was estimated that 3,000 batch runs could be performed if a 1% ligand density decrease (caused by alkaline regeneration) was accepted.

Table 1. Comparison of Synthetic Biomimetic Ligands with Earlier Dye Ligands.

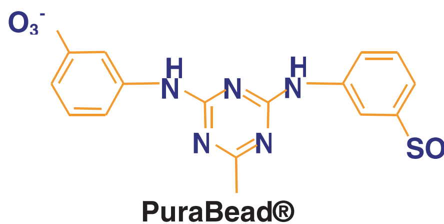
Criterion	Synthetic/biomimetic ligand	Dye ligand
Design	Discovery by modeling and combinatorial chemistry	As dyestuff for dyeing cellulosic materials
Immobilization	Highly stable epoxide-derived linkage	CNBr-linkage etc.
Structure	Synthesis of symmetrical or asymmetrical, single or branched triazines	Usually triazine with chromophores
Synthesis	Purpose generated for bioseparations	Not controlled for biotechnology applications
Specificity Capacity	Designed to target Optimized: high—up to 40 mg protein/mL adsorbent	Not easily predicted Variable
Alkali resistance	Withstand normal alkali regeneration and sanitization procedures	Variable
Leakage	Very low. Measured using ELISA Storage studies performed	Reported in literature
Safety	No active chlorines. No potentially toxic substituents used. Safety documented through toxicity studies, etc.	High

TOXICITY

As shown in Figure 1, the triazines are symmetrical hexameric rings of alternat-

ing carbons and nitrogens. Chlorotriazine derivatives such as atrazine are common herbicides and have been used since 1959 to control the growth of annual grasses and broadleaf weeds. In 1990, over 64 million acres of cropland were treated in the US. Triazines inhibit photosynthesis in plants and only show low-level toxicity in non-photosynthetic organisms. In a 1999 review, Eldridge *et al.* concluded that "triazines pose no reproductive or developmental hazard in animals. Triazines have also been assessed in more than 40 mutagenicity-genotoxicity tests using *in vivo* markers as well as prokaryotic and eukaryotic cells, and a complete weight of evidence analysis concluded that the herbicides are neither genotoxic nor mutagenic."[47] The same review concluded that "triazines have no intrinsic hormone activity and cannot support carcinogenesis on their own." Other studies report that "carcinogenic effects [of triazines] in rodents have no relevance for humans,"[48] and that they are not causally linked to mortality among triazine herbicide manufacturing workers.[49] Safety and toxicological information on triazine herbicides is available from the CDC Agency for Toxic Substance and Disease Registry (ATSDR) (www.atsdr.cdc.gov) and the Environmental Protection Agency (www.epa.gov). After a re-registration review, ATSDR concluded that "atrazine is not a likely human carcinogen." It should also be noted that typical leachate levels under operational conditions are at picogram to nanogram levels — if leaching occurs at all. These levels are between six to nine orders of magnitude lower than the levels at which triazine compounds are known to be safe.

Figure 5. Ligand Structure for Capture of Amediplase, a tPA-urokinase Fusion Protein. PuraBead® 6HF is a new "High Flow" agarose intermediate.



Clearly, information on herbicides is not a substitute for toxicology testing of leached ligands. However, the large body of information supporting the safety of triazine derivatives is a reasonable starting point, which, together with completed studies, indicates an adequate safety profile for the triazine-derived ligands tested to date. Such studies are normally carried out by adsorbent manufacturers at the request of clients and form part of a regulatory support file package supplied confidentially to the client. Santambien et al. looked at the *in vitro* toxicity of Reactive Blue 2 and Reactive Red 120,[50] but later, confidential toxicity studies were conducted. It should be noted that during ligand synthesis, the active chlorines of tri- and di-chlorotriazines are eliminated, and R-groups that could contribute to toxicity are discarded as candidate substituents.

SAFETY

Chromatographic adsorbents, eluents, and cleaning solutions that contact or potentially contact target biopharmaceutical products can potentially contaminate the final product. Synthetic ligand adsorbents can eliminate the potential hazards of animal- and microorganism-sourced ligands,[51] minimizing running, cleaning, and validation costs. However, the same care must be exercised with potential contaminants from eluent media and the risks from adventitious organisms. A significant advantage of synthetic ligand adsorbents is their tolerance to strong alkaline solutions, normally to 0.5 M, but for short periods to 1 M sodium hydroxide, as well as isopropanol alkalis.

APPLICATIONS

Dye ligands such as Cibacron Blue quickly found application in investigations of plasma fractionation and the recovery of albumin, the industry driver at the time, from Cohn Fraction IV.[52] Subsequently, a synthetic analogue adsorbent was developed, and has been applied to the commercial production of yeast recombinant albumin, Recombumin, now used as an excipient.[53,54] Procion Blue and Red triazine derivatives have been compared in the purification of α1-proteinase inhibitor with the finding that a Procion Red Fractogel was more effective.[55] Lysine-agarose was used to purify plasminogen from plasma, and the ligand was later adapted (L-lysine linked through 6-aminohexanoic acid to agarose) to the large-scale

recovery of the zymogen plasminogen, leading to an active plasmin product, from Fraction II + III of the Cohn process.[56-58] A case study by Datar *et al.* reports on extension of lysine-agarose use to the commercial recovery of tissue plasminogen activators (tPA) expressed in *E. coli*.[59] tPA has also been fractionated on a lysine derivative.[60]

A mimic of Protein A has been constructed through rational design and subsequent synthesis on a triazine backbone.[61-63] Two variants have been commercialized as MAbsorbent A1P and A2P, and A2P has been applied to the isolation of polyclonal IgG from ethanol precipitates of the Cohn and Kistler-Nitschmann processes.[64-66]

Following the rapid introduction of recombinant DNA technology into biopharmaceutical development, affinity chromatography was applied to cytokine purification, tissue necrosis factors, and, as mentioned previously, tPA purification.[67,68] Interferon-α was purified on Blue-agarose, and later work describes the application of a "Mimetic Green" adsorbent to interferon-α.[69] A simpler synthetic ligand, phenylboronate, has been used as a capture step for erythropoeitin.[70] Phenylboronate adsorbents are otherwise used for purification of glycoproteins containing *cis*-diols, as reviewed by Liu and Scouten.[71]

The biopharmaceutical industry, an adsorbent vendor, and academia collaborated in designing a novel synthetic ligand to recover the recombinant human insulin precursor MI3 from the crude fermentation broth of *S. cerevisiæ*.[72] In similar work, a novel, synthetic ligand was designed to replace a monoclonal antibody immunoaffinity step in the process for Factor VIIa (NovoSeven).[73,74]

Like tPA, urokinase has been purified using lysine derivatives. In addition, isolation of urokinases from urine has been reported using *p*-aminobenzamidine derivatives.[75,76] This and earlier work on tPA and plasminogen suggest that ligand structures containing positively charged groups, for example lysine and benzamidine, are candidates for improved downstream processing of a tPA-urokinase fusion protein — Amediplase. However, and surprisingly, screening general synthetic ligand libraries revealed ligand candidates (and a lead ligand through secondary screening), which contained strongly negatively charged groups, as shown in Figure 5. This led to the development of an entirely new synthetic ligand adsorbent for the tPA-urokinase fusion.[77]

Table 2. Comparison of Biomimetic Ligands with Conventional (Biological) Ligands.

Criterion	Synthetic/Biomimetic Ligands	Biological/Specific Ligands
Cost	Inexpensive	Usually expensive, e.g. monoclonal antibodies
Availability	Synthesized by adsorbent manufacturer	Biological origin, e.g. ascites, fermentation, etc.
Synthesis	Facile	Often long synthetic route and purification needed
Specificity	Moderate to high	Moderate to high
Capacity	High (up to 40 mg protein/mL adsorbent). >10% ligand utilization	Low. Typically 0.01-0.1% ligand utilization
Scale-up	Large scale use: columns at >100 liter scale	Limited application
Sterilizability	High	Mostly low or not sterilizable

The synthetic adsorbent developed for this product showed a capacity of 11.4 mg/mL, with ≥ 99% purity at 92% recovery from cell culture supernatant (5 mg/mL target protein) when operated at a column flow rate of 300 cm/hr. The adsorbent can be sanitized with 0.5 M sodium hydroxide and shows excellent stability in 1 M sodium hydroxide, 30% propan-2-ol/0.2 M sodium hydroxide, and in 20% ethanol. This demonstrates the benefits of developing synthetic ligands by screening combinatorial libraries and the major advance from the existing lysine-adsorbent (1 mg/mL capacity) chromatography, which produced a < 80% pure protein and could only be used for < 50 cycles.

FUTURE PERSPECTIVES

It is clear that what was originally referred to as dye-affinity or pseudo-affinity chromatography has been superseded by the far more

exact science of synthetic ligand chromatography. Even "biomimetic" may be a misleading term if it refers to a mimic of a dyestuff. The later ligands have all been developed from combinatorial libraries, frequently after

Synthetic ligand adsorbents can eliminate the potential hazards of animal- and microorganism-sourced ligands, minimizing running, cleaning, and validation costs.

computational chemistry study of the protein's target binding epitopes. The triazine backbone with diverse amine substitution has been a versatile and successful chemistry for synthetic ligand design and attachment through stable epoxy linkages to agarose and other matrices, such as those based on methacrylates.

This article has described the move towards dedicated, custom design of synthetic ligand adsorbents that are specifically designed and manufactured for the protein purification task at hand. General bioseparation unit operations of ion exchange chromatography and membrane steps will always retain their workhorse position in the biopharmaceutical industry. However, with ever increasing demands on purity, yield, and reliability — repeatability and consistency — synthetic ligand chromatography is set to assume a significant position in the bioseparations armory

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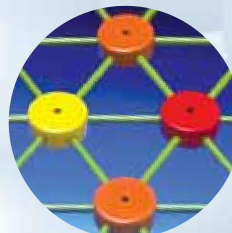

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