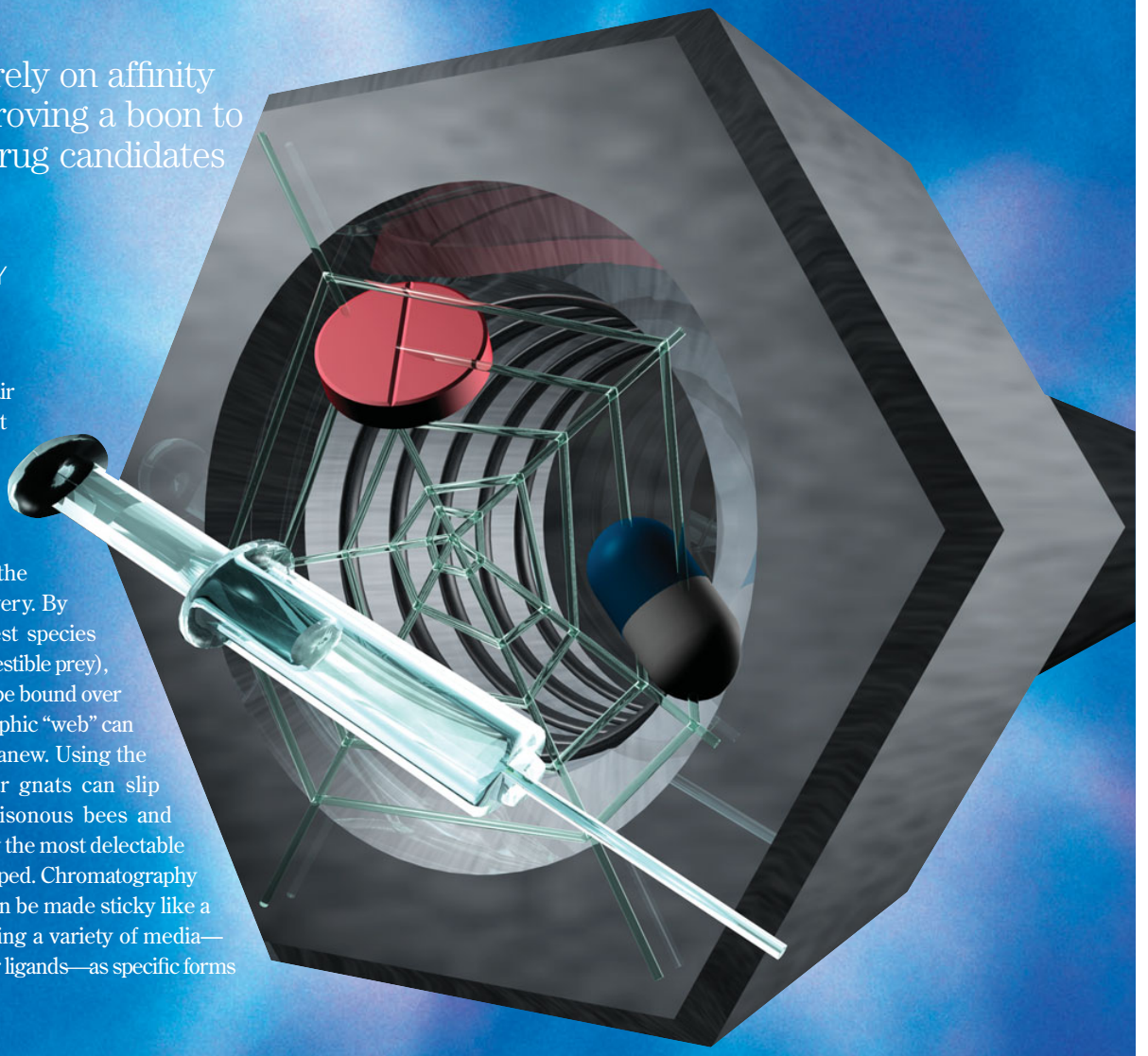
Sticking with affinity chromatography

LC methods that rely on affinity interactions are proving a boon to the discovery of drug candidates and targets.

BY MARK S. LESNEY



Like a spider in its silken lair seeking to capture insect morsels, researchers must often spin an affinity web in hopes of capturing new molecules and new targets to feed the insatiable maw of drug discovery. By selectively entrapping the best species (and releasing unwanted, indigestible prey), the most likely candidates can be bound over for digestion, the chromatographic "web" can be reset, and the hunt begun anew. Using the right affinity web, molecular gnats can slip through and be ignored, poisonous bees and wasps can break free, and only the most delectable of pharmaceutical flies are trapped. Chromatography beads or membrane sheets can be made sticky like a web for molecular capture using a variety of media—metals or enzymes, receptors or ligands—as specific forms of ionic glue.

Antibody angling

One of the conceptually easiest forms of affinity chromatography to understand is the classic antibody affinity column, generally known as immunoaffinity chromatography. Fittingly, IgG antibodies are normally first purified using their own form of affinity chromatography. They bind naturally to immobilized protein A—a wall component of *Staphylococcus aureus* that binds the Fc portion of IgG specifically (*1*). Subsequently, the purified antibodies (generally monoclonal—directed toward a specific antigen of interest) are attached to a chromatographic support for use in HPLC or capillary electrophoresis (CE) systems. A key factor is tightly binding the antibody to the support medium without interfering with its antigen-binding site. This requires the use of a properly sized spacer arm to attach the antibody to the support bead. Protein A, or a similarly functioned protein known as G, can be of service here as well. Because IgG binds to these proteins on the opposite side from the antigen-binding site, and far more strongly than does the antigen to the antibody, these molecules make ideal spacers. In principle, any antigen to which specific antibodies can be produced is capable of being highly purified in a one-step process using such a column—even to the level of bulk industrial processing.

Metal "bonding"

A less specific, but no less useful, form of affinity chromatography is known as immobilized metal affinity chromatography (IMAC).

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This form of chromatography, generally performed as HPLC, takes advantage of the fact that proteins have a differential ability to form metal ion complexes based on their amino acid sequences (primarily surface histidines) and phosphate attachments; this leads to a differential binding during chromatographic separation that can be exploited. The method is not specifically applicable to proteins that require metal cofactors, as these sites on the metalloproteins are generally unavailable and are swamped by the surface effects of the amino acids. In practice, chelating resins are generally used, most often with Cu^{2+} as the metal of choice (*1*). IMAC is a particularly valuable technique for purifying recombinant proteins that have been genetically engineered to have polyhistidine tags attached specifically for this purpose. The binding of surface histidine residues in proteins to IMAC columns can be enhanced by the use of high-molarity sodium chloride, as shown by Yi Li and Robert Beitle of the University of Arkansas (Fayetteville) in their recent purification scheme for recombinant green fluorescent protein from extracts of *E. coli* (*2*).

IMAC is also useful for isolating phosphorylated proteins, as the chelated metal selectively retards the phosphate groups. Since IMAC is not as specific as immunoaffinity chromatography, it is critical to be able to analyze and identify the proteins thus isolated. One way is the subsequent use of MS. This can be a problem, as samples must be eluted and the high-phosphate salts in the eluent removed before analysis can be performed. However, as shown in a recent paper by Christina Raska and colleagues at the University of North Carolina at Chapel Hill (*3*), IMAC can be used to isolate and enrich phosphopeptides from a peptide mixture immediately prior to MALDI-MS/MS analysis. They showed that the IMAC beads with bound phosphopeptide ligands could be placed directly on the MALDI target without prior purification for MS analysis.

Substrate stickers

One of the most powerful forms of modern affinity chromatography for use in drug discovery relies not on specific antibody binding or nonspecific metal attraction, but rather on the sensitive nature of the interaction in standard biological "lock and key" systems—either enzymes with substrates or receptors with ligands. It is obvious why such a form of chromatography would be of value to modern biomedicine—these are the very targets and the small molecules that bind to them that form the basis of nearly all drug interactions. Choose the right receptor target to bind to your beads, and you can easily sort through a combinatorial library for potential new drugs; choose the right drug ligand or mimetic, and you can easily sort through cell lysates for potential new targets.

The more selective the affinity web, the greater the efficiency of the entire enterprise.

Recent work in a number of laboratories has shown the power of this technique as a research tool.

Takenori Tomohiro and colleagues at several Japanese institutions, for example, developed a chromatographic method for linking cisplatin-damaged DNA to Sephadex G-50 beads for the isolation of binding proteins from HeLa cells that would specifically attach to these damaged sequences (*4*). Cisplatin is a platinum-containing chemotherapeutic agent used to attack a variety of cancers including those of the ovary, testes, and bladder. Cisplatin acts by attaching to and damaging the DNA of replicating cancer cells, possibly through a number of proteins that bind to the damaged DNA, and ultimately inducing the apoptosis (programmed death) of the cancer cells. By developing a method for easily purifying known, and isolating hitherto unknown, cisplatin-damaged DNA-binding proteins, the researchers hope to further understand the action of the drug that might ultimately open the way to better cancer therapeutics.

Affinity chromatography using bound substrates as a retention tool can also be used to study the behavior of enzymes under a variety of physiological conditions, including drug treatments. In one recent example, S. Altikat and colleagues at Ataturk University (Erzurum, Turkey) studied the in vitro effects of anesthetic drugs on the enzyme glucose 6-phosphate dehydrogenase (G6PD) isolated from human red blood cells (*5*). G6PD deficiency is a hereditary disorder affecting 400 million people worldwide, with its highest incidence in the Jewish Kurd population of Turkey (62% of males are affected). The researchers determined that it would be appropriate to study the effect of inhalation anesthetics on this enzyme, for fear that any inhibitory effects

A selection of companies offering affinity resins

Company	Website
Affiland	www.affiland.com
Amersham Biosciences	www.amershambiosciences.com
BD Biosciences Clontech	www.clontech.com
BIA Separations	www.biaseparations.com
Bio-Rad	www.bio-rad.com
Calbiochem	www.calbiochem.com
IBA	www.iba-go.com
Invitrogen	www.invitrogen.com
Millipore	www.millipore.com
MoBiTec	www.mobitec.de
Molecular Probes	www.probes.com
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Prolinx	www.prolinx.com
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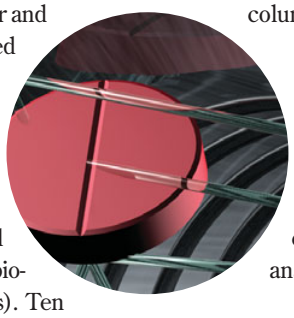
could be significantly damaging to populations with a decreased amount of active enzyme already in the blood.

They did their studies by purifying the enzyme using a 2′5′-ADP-Sepharose 4B affinity gel, which acted as a substrate binder. The gel provided enzyme of sufficient purity to determine kinetic behavior and inhibitory effects. Their results showed that four of the seven anesthetics tested exhibited significant inhibition of the enzyme in vitro.

Historically, some of the earliest uses of selective chromatographic binding for the purification of enzymes using a synthetic ligand instead of the natural substrate have included triazine dyes. Such dyes have been used to purify a wide variety of dehydrogenases, glycolytic enzymes, and kinases. Recently, Sergio Streitenberger and colleagues at the University of Murcia (Spain) showed that lactate oxidase could be purified 41-fold from crude extracts of *Aerococcus viridans* using reactive blue H-ERD bound to Sepharose; this dye bound 92% of the enzyme and released 35% of it upon elution (*6*). This research demonstrates the potential difficulties of finding an appropriate ligand (especially an artificial one—as is becoming more common in the search for biomimetic compounds similar to already-known drugs). Ten dyes were tested, and their affinities for their targets varied widely; some of the best binders were so highly selective that the bound enzyme could not subsequently be eluted.



MIPs, monoliths, and more

But affinity chromatography today does not just rely on traditional techniques for its effectiveness. Researchers are constantly honing and improving the methods, and coming up with new and varied applications. One significant addition to the battery of methods is the use of molecular imprinted polymer (MIP) methodology. Recently, Jianchun Xie and colleagues at Peking University (Beijing, China) coupled the technique to MS to purify and identify known and putative antitumor compounds from the plant species *Peganum nigellastrum* (*7*).

Molecular imprinting relies on the use of the final desired compound or its analogue during synthesis of the column polymers to create recognition sites of defined specificity. "The technique involves the formation of definable interactions between a given template molecule and polymerizable functional monomers during polymerization with excess cross-linking agents," wrote the researchers. "Subsequent removal of the template from the resulting polymer results in complementary binding sites (imprints), which consist of functional groups with a particular arrangement fit for the corresponding templates." Using harman, the structural analogue of the known antitumor compounds harmaline and harmine, the researchers created MIP columns that were capable of specifically isolating these agents and a structurally related compound.

The MIP method has tremendous potential in its ability to use stable and removable structural analogues to known natural products to produce binding columns in the absence of known targets. Even more hopeful for the future would be its ability to use structures predicted from molecular modeling as templates for creating columns.

Karin Pflegerl and colleagues at the University of Agricultural Sciences (Vienna, Austria) and BIA Separations (Ljubljana, Slovenia) recently reported another new twist on the use and production of affinity chromatography supports (*8*). They directly synthesized the desired peptide to act as a subsequent affinity ligand on a monolithic chromatography column using combinatorial chemistry. The CIM (Convective Interaction Media) column used was a macroporous monolith containing epoxy functional groups that are activated to attach the desired ligand. The researchers used this functional group as the attachment site for solid-phase combinatorial synthesis of a defined peptide targeted to bind human blood coagulation factor VIII, elegantly taking advantage of the flow-through nature of the column to add amino acids sequentially.

On other fronts, progress is continually being made on new column supports, new spacer arm chemistries, new ligands, and especially new ways to miniaturize and adapt the techniques for use with a variety of automation systems. And this is not to mention the intense work, as shown in some of the examples above, on adapting methods for direct use with various MS analysis techniques.

The final word

From traditional antibody techniques to the use of MIP methods, affinity chromatography remains one of the most important bioanalytical techniques at the heart of the drug discovery and development process. The more selective the affinity web, the greater the efficiency of the entire enterprise—a critical requirement when the cost of analyzing the "excess" productivity of combinatorial methods is skyrocketing.

Movements toward molecular modeling, improvements in protein engineering, and the information derived from comparative genomics seem likely to produce more and more potential drug targets. And in an era of limited resources, the use of these targets in affinity systems to isolate new drug candidates may become valuable insurance that research dollars will not be needlessly wasted. Likewise, the use of known drug compounds and analogues to isolate potential target proteins and genes may provide hope for a new understanding of the underlying mechanisms of drug behavior, and give us economically effective high-throughput tools for screening combinatorial and natural products libraries.

Given this web of high potential, affinity chromatography looms large as a good technology to stick with.

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