Expand Your Processing Options with Hydrophilic Interaction Chromatography

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The term hydrophilic interaction chromatography (HILIC) was coined only recently: 1990, but its origins date back more than 30 years. The lag results from the time that it took to (1) recognize hydrophilic interaction as a discrete adsorption mechanism and (2) discover how to exploit it in a chromatography format. With the exception of one company, HILIC has been overlooked by chromatography media manufacturers. Applications are consequently uncommon. Nevertheless, the technique is already being employed for commercial purification of proteins, and it offers valuable purification process options.

The mechanism of HILIC relies on the behavior of salts and other solutes in proximity to hydrated surfaces. Some salts are strongly excluded from protein surfaces. These are referrred to as strong "structure-forming" salts. They are easily identified by their high rankings in the Hofmeister series of lyotropic and chaotropic ions. Examples include ammonium sulfate, sodium sulfate, and potassium phosphate -- all popular protein-precipitating agents. Some organic solutes are similarly excluded, notably including polyethylene glycol (PEG), which is also used for protein precipitation.

With these solutes excluded from the proteins' hydration shells, those shells are composed of pure water. This creates a discontinuity between the shells and the surrounding bulk solvent, which is highly concentrated in the excluded solute. Entropically, this is a highly unfavorable situation. It resolves itself by individual proteins associating to share their hydration shells. This is called cohydration. It reduces the surface area of pure protein-hydration water in contact with the bulk solution, and also liberates some protein hydration water back to the bulk solvent. The higher the concentration of excluded solute in the bulk solution, the stronger the entropic drive toward protein self-association.

In free solution, with an increasing concentration of excluded solute, this situation eventually progresses to the point where the associations among proteins are so stable and so large that the proteins precipitate, leaving the highly solute-concentrated bulk solvent energetically "at rest." This is the thermodynamic basis of both salt precipitation and PEG precipitation. If you add another component to the system, namely a *very* strongly hydrated solid phase in the form of a chromatography support, proteins will preferentially share their hydration shells with the solid phase rather than with one another. They adsorb to the chromatography support. This process, driven by high concentrations of exluded solutes, is HILIC. Figure 2. The proteins can then be selectively eluted at high resolution in a descending gradient of the excluded solute.

If this sounds suspiciously similar to what you've read about Hydrophobic interaction chromatography (HIC) you shouldn't be surprised. However, the revelation that has begun to emerge over the last few years is that HIC is actually a mixed-mode mechanism (expect it to be another 10 years before this shows up in textbooks -- if ever). New research has revealed that direct interactions between hydrophobic surfaces are a strong attractive force in solution, hundreds of times stronger than van der Waals forces. In other words, HIC is not a purely entropically driven process. This is heresy, but like many heresies, it's true. Even so, hydrophobic interactions are still not strong enough, or long-range enough, for most HIC ligands to pluck

a protein out of physiological solution. Nevertheless, if you apply an external associative force -- such as the HILIC effect -- hydrophobic ligands are able to enhance protein adsorption in proportion to ligand hydrophobicity. Likewise, since the enhancement is hydrophobic in nature, retention behavior of experimental proteins shows a correlation with their surface hydrophobic characteristics.

It is worth note that the more hydrophobic a chromatography ligand, the more weakly it is hydrated. This means that as you increase hydrophobicity through a series of ligands, the contribution of the HILIC effect is proprotionally diminished. Less excluded solute is required to promote binding, and selectivity becomes more dominantly hydrophobic. By the time you get to reverse-phase ligands, the HILIC effect is no longer necessary to promote binding at all. However, it still influences protein behavior and so, even on C-18 columns, it still influences selectivty.

As expected, the HILIC effect has also been shown to enhance adsorption with other chromatography mechanisms. For example, IgG binding to protein A can be enhanced by various excluded solutes added to the mobile phase -- either salts or PEG. Mechanisms that can't tolerate salt can be enhanced with PEG, for example ion exchange. In this case, adding PEG to the mobile phase allows proteins to bind that would otherwise fail to do so. For those proteins that normally do bind, it strengthens the association, as indicated by higher capacity and later elution in salt gradients. A similar pattern is seen with hydroxyapatite and a number of biological affinity mechanisms. Overall, you should expect the HILIC effect to enhance binding with any adsorption mechanism. This turns out to have real practical significance. Even if you don't wish to use HILIC as a pure mechanism, situations often arise where it would be beneficial to enhance binding with some other method.

Protein selectivity . The most highly hydrated proteins naturally bind at lower excluded solute concentrations than less hydrated proteins. All other things being equal, larger proteins are more highly hydrated than smaller ones, so in HILIC it is usual to see a larger protein retained more readily than smaller one. Within a size class, the proteins with the most developed hydration sheaths bind most readily. These are the proteins with the highest proportion of charged groups. It doesn't matter what the charge is. It is simply that charged residues bind more hydration water (and bind it more strongly) than uncharged residues.

Chromatography media . As noted, HILIC requires a chromatography support with a highly hydrated surface. Ideally, it should be uncharged. Appropriate ligands include strongly hydroxylated polymers. These include sugars and polymeric carbohydrates like dextrans and cellulose, also polyethylene glycols. Several products are available on the market that can be used for HILIC, even though none of them were developed for this application. One class of products include size exclusion chromatography (SEC) supports with polyhydroxy "tentacles" of one sort or another inside the pores. These include the Superdex supports by Pharmacia, and the Tentacle SEC supports from E. Merck. Even traditional SEC supports can be used, but their net hydroxyl concentration tends to be lower than tentacle supports. Achieving the same selectivity therefore requires a higher concentration of excluded solute.

Another class of HILIC media includes HIC supports designed for use with very labile proteins. These products tend to use either PEG or PEG-like ligands. Examples include the ether-5pw and ether-Toyopearl lines from TosoHaas, and Pharmacia's Source-ether. Perseptive Biosystems recently suspended manufacture of their POROS-ether, but if you have some on hand, it works very well. Doing "HIC" on these supports has actually been HILIC all along. They don't have enough hydrophobicity to affect selectivity that much. This is why their selectivity

is so different from strongly hydrophobic supports like buty, octyl, and phenyl.

Choice of excluded solute. Like the corresponding precipitation techniques, salt-driven HILIC has a distinctly different selelectivity from PEG-driven HILIC. This is because salts block most of the charge interactions between proteins while PEG does not. If you want to use a salt to drive adsorption, ammonium sulfate, sodium sulfate, and potassium phosphate are all good candidates. They can be used with any of the supports listed. If you are considering using PEG, you need to be aware that PEG solutions are viscous. They increase operating pressure and decrease diffusivity. Decreasing diffusivity depresses both capacity and resolution. Smaller PEG polymers are less viscous on a molar basis, but you have to use such high concentrations that you lose more than you gain. PEG-6000 is a good weight to use. Polymers larger than that seem not to offer any benefits. You will be restricted to chromatography media designed to support high flow at high backpressures. Pharmacia Source-ether and TosoHaas ether-5pw are both suitable. If you try to use Superdex, you will likely have to reduce your flow rate.

Given its viscosity, why would you want to use PEG? PEG is nonionic. No matter what concentration of PEG your protein elutes in, you can take your product directly to any charge-based separation method, with only minimal sample preparation. For example, you can go directly to ion exchange after titrating your sample pH, or to hydroxyapatite. You can also go to affinity. Experimental results show in all cases, that the residual PEG enhances product binding during sample loading. After the column is loaded, the PEG washes through. There is no need to conduct a separate PEG removal step. Contrast these benefits with the problems of dealing with high residual salt concentrations.

One other limitation with PEG deserves note. You can't monitor it spectrophotometrically or fluormetrically. You can't monitor with pH.You can't monitor it with conductivity. You may be able to monitor it with refractive index, but most people working with protein separations don't have RI detectors. How then, can you generate a gradient trace? This is where PEG's viscosity actually has a positive feature. Most of the current generation chromatographs have pressure monitors. During a descending PEG elution gradient, you can generate a pressure trace as indicative of PEG concentration, as a conductivity trace is of salt concentration.

Can you mix salt and PEG to drive binding? Only for entertainment. There doesn't seem to be any beneift, and there is a potentially serious liability. Adding concentrated salt to PEG, or vice versa, causes a phase separation. It creates a dispersion of pure PEG mixed with the salt-concentrated aqueous phase. The salt concentrations that cause phase separation are high enough that you don't have to worry about eluting-salts causing a problem in conjuction with PEG enhancement of ion exchange, but you should always do an off-line qualifying experiment to establish compatibility before you mix concentrated salts and PEG on a column.

Sample preparation is a significant limitation with HILIC. In most cases, efficient adsorption requires a concentration of PEG or salt very near to, or slightly in excess of the concentration required to bring about precipitation in free solution. This means that you cannot equilibrate your sample off-line in advance. The only way to conduct sample loading is with a technique called on-line dilution. Briefly, on-line dilution involves feedng sample into a chromatograph through one line, feeding in a "binding-diluent" simultaneously through another line, and mixing them immediately upstream from the column. The pre-column contact time between the sample and diluent is too short to permit precipitation, even if the binding salt or PEG are at product-precipitating levels. The significance of this point is that it makes HILIC practical for preparative applications. Methodology for conducting on-line dilution is discussed at length in vol 2, no.1

of this Newsletter (1997), and in BioPharm 8(3) 21-27 (1995), both of which can be downloaded from our Electronic Library.

Applications. Labile proteins are likely to benefit most from HILIC. The presence of excluded solutes -- whether salts or organic polymers -- outside the hydration sheath of a protein, has a strong stabilizing influence on protein structure. In essence, the discontinuity between the pure water hydration sheath and the solute-concentrated bulk solvent creates an "exclusionary pressure" on the protein. This tends to compact its structure. Extensive studies show that proteins are actually smaller in such solutions than they are in physiological environments. This is the mechanism by which ammonium sulfate and PEG slurries stabilize enzymes. HILIC is therefore actively protein-stabilizing. This can be very useful with large labile proteins. Indeed, one of the applications where HILIC has proven most useful is with purification of "difficult" IgM monoclonals. It also works very well with Factor VIII. If you have a protein that is undergoing denaturation on other ligands, HILIC may give you the safe haven you need, without sacrificing separation performance.

Other than labile proteins, HILIC is well suited to purification of large poorly soluble proteins. These would be the ones that precipitate first in ammonium sulfate or PEG. When purifyng from crude sample, the smaller more soluble proteins pass through the column, leaving the bulk of the column's capacity for the product of interest. You can then fractionate it from other retained proteins with a descending linear gradient of the excluded solute. If you are going to use HILIC to fractionate smaller more soluble proteins, it is best to do so at a later process step, so that column capacity is not consumed by stronger adsorbing proteins.

If you are using the HILIC effect to enhance product adsorption with other methods, such as affinity or ion exchange, enhancement will be most pronounced with larger proteins more hydrated proteins. You should be aware that besides enhancing adsorption, adding excluded solutes also alters selectivity. For example if you have coeluting proteins of markedly different sizes on an ion exhanger, PEG may enhance binding of the larger one to a point that allows efficient separation.

In general, very small proteins, peptides, and polynulceotides are poorly suited to HILIC. Often, even saturated levels of the excluded solute are insufficient to achieve adsorption. Likewise, these products will be least influenced if you are attempting to use the "HILIC effect" to alter selectivity with other methods.

Another area to watch out for concerns strongly basic proteins that tend to form electrostatic complexes with DNA, endotoxin, and phospholipids in crude feedstreams at low pH. Unless you add 0.5M -1.0M sodium chloride to a PEG binding phase, you'll find that the complexes are stabilized by cohydration. The added salt is required to dissociate these complexes. Sodium chloride is not a strong enough "precipitating salt" in this concentration range to cause phase separation of the PEG. If you're using preciptiating salts -- instead of PEG -- to drive HILIC, the electrostatic complexation phenomenon is not an issue.

Protocols

I. Using salts as the excluded solute.

Column: Any of the supports mentoned above. 1-2 mL column for analytical screening. Desired volume for preparative work. Condition the column in advance by flowing binding buffer over it to compact the bed. Then lower the adapter accordingly. This is recommended to prevent creation of head space between the adapter and bed during column equilibration and sample loading.

Linear flow rate: 200-1500 cm/hr, depending on the media.

Buffer A: 1.0 M sodium sulfate or 2.0 M ammonium sulfate, or 2.0 M potassium phosphate, in the buffer of choice (Note

that ammonium sulfate should not be used above pH 7.5 due to evolution of denaturing caustic ammonia gas).
Buffer B : Buffer A minus the binding salt.

Equilibrate column:10-15 column volumes (cv) of buffer A.
Inject sample: up to 2.5% cv of unequilibrated sample for analytical-scale screening applications. Up to the capacity of the column, by on-line dilution, for preparative applications. With some media, capacity can exceed 25mg of adsorbed protein per ml of gel.
Wash: 5-10 cv buffer A
Elute: 10-20 cv linear gradient to buffer B
Strip: 5 cv buffer B

II. Using PEG-6000 as the excluded solute. As in protocol I, except restricting the column to media able to support high flow and high backpressure, and substituting 15% (up to 20%) PEG for the salt in buffer A. It is also advisable to include at least 0.05M sodium chloride in your eluting buffer to promote protein stability and discourage low ionic strength precipitation of large poorly soluble proteins.

Last but certainly not least, contact your chromatography media representatives and urge them to provide HILIC products. As process developers, we need all the tools we can lay our hands on. The technology to produce highly hydrated uncharged supports is certainly available. It's just a matter of suppliers perceiving that there's enough of a market to make their investment worthwhile. Development of HILIC-optimized supports should make it possible to use lower concentrations of exluded solutes, and possibly increase the diversity of such solutes to include common sugars and some amino acids.

Credits. The reference originally describing HILIC is by Andy Alpert, in the Journal of Chromatography, 1990, 499 177. To learn more about the influence of PEG on ion exchange, download a copy of the following poster from our electronic library: "A method for obtaining unique selectivities in ion exchange chromatography by addition of organic solvents to the mobile phase." This work is published in J. Chromatography, 1996, 743 51.

Several parts of this article are adapted from the book *Purification Tools for Monoclonal Antibodies* by Pete Gagnon (ISBN 0-9653515-9-9). Most of the key citations supporting the article are in Chapters 6 and 8, the latter of which discusses and illustrates examples of HILIC. Chaper 9 gives several detailed examples of HILIC enhancement of affinity chromatography.

This article was reprinted from the Autumn 1998 issue of Validated Biosystems Quarterly Resource Guide to Downstream Processing, http://www.validated.com.
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