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Review

Excluded Cosolvent in Chromatography

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ABSTRACT

The concept of cosolvent exclusion was developed by a group of Timasheff's laboratory in 1970-1990 and is currently used widely to explain the effects of a variety of cosolvents on the stability and solubility of macromolecules. Not surprisingly, these concepts have had substantial influence in the fields of formulation, protein folding and unfolding, but they have perhaps more surprisingly found their way into the field of chromatography. A variety of excluded cosolvents have been used to enhance binding and resolution of proteins and other macromolecules in ion exchange, hydroxyapatite, affinity, and hydrophobic interaction chromatography. These cosolvents include salting-out salts, amino acids and polymers, and frequently polyethylene glycol (PEG). A new mode of chromatography, termed "steric exclusion chromatography," was recently introduced. It employs hydroxylated solid phase surfaces. Steric exclusion of the PEG stabilizes the association of macromolecules with the solid phase. Elution is achieved by reducing the PEG concentration. Magnetic particles are also used in this chromatography. This review summarizes the concepts of preferential cosolvent exclusion and its applications in column chromatography.

Introduction

The theoretical foundation and supporting data on protein solvent interactions were developed primarily by pioneering work conducted in Timasheff's laboratory. Early studies focused on the mechanisms of protein denaturants, such as organic solvents and guanidine hydrochloride or urea, as they were found to play a critical role in understanding the unfolding and refolding pathways of proteins.[1-6] These additives (cosolvents) exerted their effects at very high concentrations, where conventional stoichiometric binding measurements were neither practical nor meaningful. Timasheff's group employed equilibrium experiments, for example, dialysis, which measured differences in cosolvent concentration between the vicinity (inside the dialysis bag) of protein molecule and the bulk phase (dialyzing solvent).[7] At high cosolvent concentrations, many different interactions between the protein surface and cosolvent were found to occur.[8-11] Water molecules, which were at high concentration in aqueous solution, were also bound to the protein or other macromolecules.[12,13] All these interactions could cause difference in cosolvent concentration around the protein surface.

These experiments provided a wealth of information about the effects of cosolvents on protein denaturation and stability in aqueous solution. Protein denaturants, for example, organic solvents and urea, were observed to bind to the proteins in the denatured, unfolded state at high cosolvent concentration, leading to stabilization of the denatured structure.[1-6] Protein stabilizers, for example, sugars and polyols, were found to show lower cosolvent concentration inside the dialysis membrane than the dialyzing solvent.[14-22] This showed that they were excluded from protein surfaces. Carpenter and Crowe and others[23-27] demonstrated that these excluded cosolvents also stabilized proteins against freeze-thaw stresses, and they have since been shown to prevent proteolysis.[28]

Other important applications of preferentially excluded cosolvents are found in the field of chromatography. Chromatography plays an essential role in purification of biopharmaceuticals such as proteins, peptides, nucleic acids, and viruses. Affinity and hydrophobic interaction chromatography (HIC) permit the application of various excluded salts to modulate binding and elution. Anion and cation exchange chromatography are less tolerant of salts but invite application of nonionic and zwitterionic excluded cosolvents such as polyethylene glycol (PEG) and glycine. Many of these applications have been found particularly to enhance fractionation of native proteins from fragments and aggregates.

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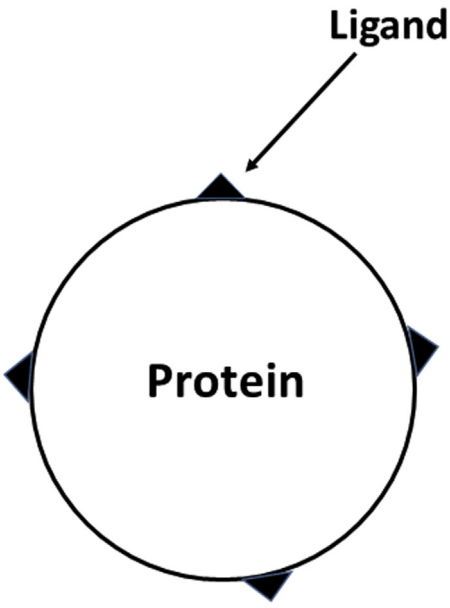

Figure 1. Specific ligand binding.

Excluded salts permit protein and virus species to be retained even on chemically unmodified solid phases such as size exclusion chromatography (SEC) media, which are generally considered not to be chemically interactive with biomolecules. These methods tend to be discussed in connection with HIC because of their use of precipitating salts, but they more properly belong in a classification that could be referred to as "preferential exclusion chromatography."

PEG has been found capable of promoting retention of large proteins and virus species even on solid phases that lack an active binding chemistry; no charge, no hydrophobicity, only hydroxylated surfaces. The effect is mediated by cosolvent exclusion of the polymer. This body of methods has been termed steric exclusion chromatography (SXC) in reference to the mechanism underlying cosolvent exclusion of polymers such as PEG from protein surfaces.[29-31] The cosolvent is excluded from the solid phase and the biomolecules. Instead of associating with each other, the biomolecules preferentially associate with the solid phase and remain there as long as the PEG concentration remains adequate.

This review describes the lifelong pioneering study of cosolvent exclusion by Professor Timasheff and the transition from academic research to practical applications in the field of biopharmaceutical chromatography.

Preferential Interaction

Preferential interaction is in principle different from conventional ligand binding. Stoichiometric ligand binding is normally composed of small number of high-affinity binding, as schematically depicted in Figure 1. Such tight binding may make isolation of ligand/protein complex possible, influence the spectroscopic properties of the proteins (the magnitude of changes in spectroscopic properties increases with binding), or generate sufficient heat (the binding enthalpy increases with binding). On the contrary, preferential interaction ranges from weak, transient binding to strong binding of ligand (here called cosolvent) and water with the protein, all of which can occur at high concentration: it should be emphasized that in aqueous solution water is always at high concentration.

Figure 2 depicts preferential cosolvent interaction, where cosolvent molecules are represented by black circles. At high concentrations, cosolvent molecules show not only strong binding as shown in Figure 1, but also weak, transient binding as shown in Figure 2. Such a widely different binding can be measured from the difference in ligand (cosolvent) concentration between protein solution (m_L^P) and bulk phase (m_L^B), that is, $m_L^P - m_L^B$, which are in equilibrium achieved by dialysis or gel filtration. The value, thus, obtained is called "preferential cosolvent interaction" to indicate whether the cosolvents are bound or excluded. It is easier to understand this concept using an illustration in Figure 2a, where there is no preference of protein molecule for cosolvent or water (no preferential cosolvent binding). Figure 2b shows preferential cosolvent binding where there is excess of cosolvent molecules inside the dialysis membrane (dotted line), namely in the vicinity of protein molecules, relative to its concentration outside the membrane (in bulk phase). This situation is called preferential cosolvent interaction. Figure 2c depicts the opposite case, where the protein surface is depleted of cosolvent and surrounded by water molecules. A typical example of this case is salting-out salts, which are preferentially excluded from the protein surface. Why are they excluded from the surface? There appear to be 3 different mechanisms proposed. Obviously, strong hydration can lead to a negative cosolvent binding. Such hydration may not allow cosolvent molecules to penetrate the hydration layer, which should create excess water or deficient cosolvent concentration at the protein surface.

The second mechanism is exclusion of salts, sugars, amino acids, and polyols, which increase the surface tension of water. Traube[32] demonstrated the importance of surface tension in cosolvent effects on protein solubility. Gibbs[33] has shown that those solutes that raise the surface tension of water are depleted in the air-water interface. This can be explained from the hydration of salt ions. Hydrated solutes are more stable and more likely to remain in the

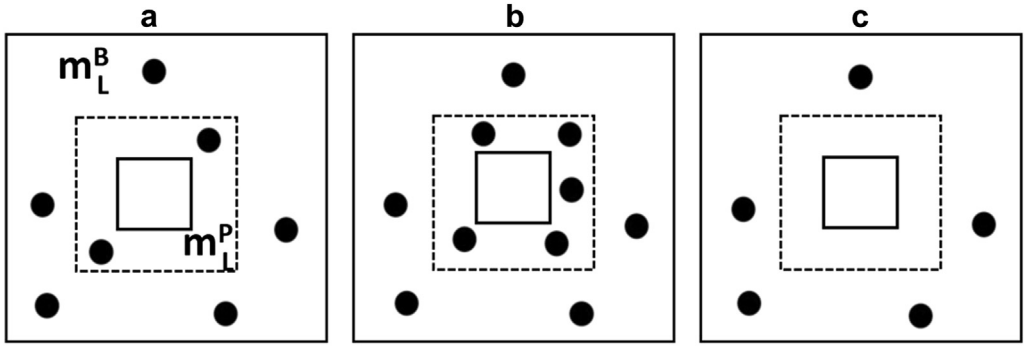

Figure 2. Preferential cosolvent interaction. (a) No preferential cosolvent binding; (b) preferential cosolvent binding; (c) preferential cosolvent exclusion. Square, protein molecule; dashed square, dialysis membrane; circles, cosolvent molecules.

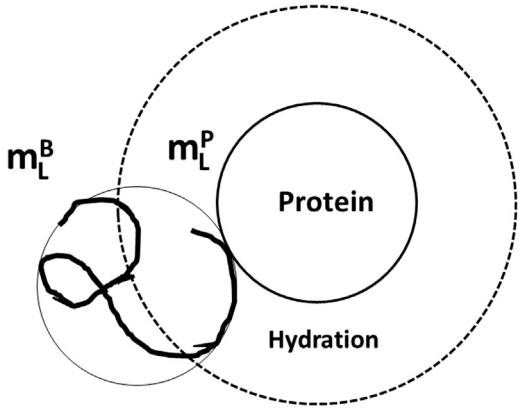

Figure 3. Steric exclusion of polymer.

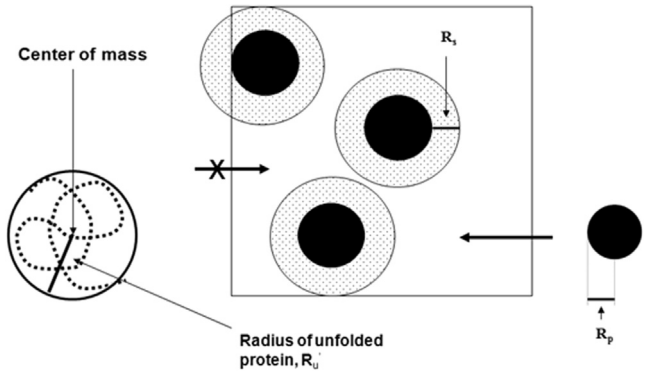

Figure 5. Stabilization mechanism of protein by the excluded polymer.

bulk phase than in the protein-bound hydration layer. This creates an uneven cosolvent concentration distribution, higher in the bulk phase and leading to $m_L^P - m_L^B < 0$. Thus, the salts, in particular more strongly hydrated salting-out salts, are preferentially excluded. This difference will increase when the cosolvent concentration is increased, as in the first case.

Polymers are also excluded from the protein surface as depicted in Figure 3. As shown in Figure 3, there is excess water (hydration) at the protein surface in polymer solution due to steric exclusion of the polymer (thick black line). This leads to a negative value of $m_L^P - m_L^B$. The steric exclusion principle was formulated as molecular crowding by Minton and Winzor.[34-36] The interaction of native protein with the inert crowding polymers has been described by the excluded volume effect. Protein stability, due to crowding, has also been explained based on the difference in excluded volume effect between the native and unfolded structures.

The same effect on self-association of colloidal particles by polymer exclusion has been explained by the depletion interaction mechanism.[37-40] Colloidal particles are surrounded by an exclusion layer, due to the inability of the polymer to approach the colloidal surface (vertical lines) within the distance of the polymer radius, Rp, as depicted in Figure 4. When 2 colloidal particles approach each other within a distance of 2 Rp, the polymer cannot enter the space between the 2 surfaces and thus are excluded from the space: the space becomes free of polymers. Therefore, a force, equivalent to the osmotic pressure of the solution (due to the exclusion of the polymer), acts on the colloidal particles, and its magnitude increases with polymer concentration. This is called "depletion effect" and forces the 2 colloidal particles to associate. Such depletion effect, thus osmotic pressure, always operates in the presence of excluded polymers between colloidal particles.

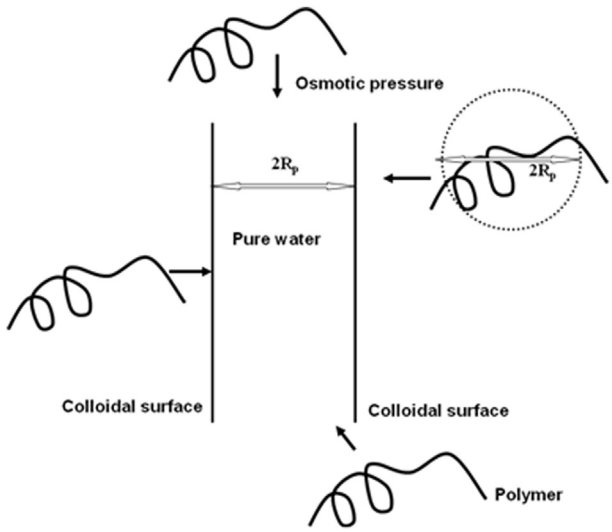

Figure 4. Depletion effect of polymer.

Molecular crowding effects should also occur at high concentrations of macromolecules. In other words, protein molecules should be more stable at higher protein concentration, suggesting that the shelf life of proteins can be enhanced at higher protein concentration. Figure 5 shows a protein molecule occupying a finite space, which is represented by black circles. If another solute, which has no volume, is dissolved in water, then the entire space unoccupied by the protein is available. Introduction of another protein molecule (also shown in black circle), with the radius Rp, is much more restricted than the small molecule because its center of mass cannot approach the surface (Rs) of the protein within the newly introduced protein, as depicted by the hatched area. More specifically, the interaction of protein molecules themselves is unfavorable because of their excluded volume. As shown in Figure 5, it would be much more difficult to place the unfolded protein that has a greater radius (Ru).

A variety of cosolvents also show preferential exclusion. Certain amino acids, such as glycine, alanine, and sodium glutamate, showed moderate exclusion.[17-19] Trimethylamine *N*-oxide, which is most commonly found in osmolytic cells and organisms, is also excluded.[19] This cosolvent is nontoxic and used to increase the cellular osmotic pressure against external high salt concentration.[41] Sugars and polyols enhance protein stability in aqueous solution.[15,16] The mechanism of their exclusion is more likely between the aforementioned cases. These cosolvents are highly water soluble and have a hydrodynamic radius much larger than the size of water.

Preferential exclusion of salts, polymers and other small cosolvents is a thermodynamically unfavorable condition. If the surface of the protein can be reduced, this unfavorable condition is alleviated. This may be achieved in 2 ways, both of which result in smaller surface areas. First case is folding-unfolding equilibrium. In this equilibrium, the unfolded state has a larger surface area than the folded state and excludes cosolvent more strongly. Preferentially excluded cosolvents shift the equilibrium toward the folded state, stabilizing the protein structure. The second case is molecular association. Excluded cosolvents enhance intermolecular association (that can lead to precipitation) or surface adsorption. The associated state in both cases has smaller surface area per molecule.

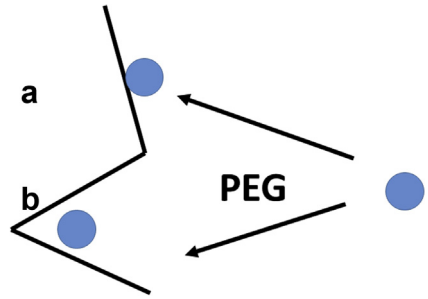

Figure 6. The effects of excluded PEG on protein binding (a and b) and retention in SEC.

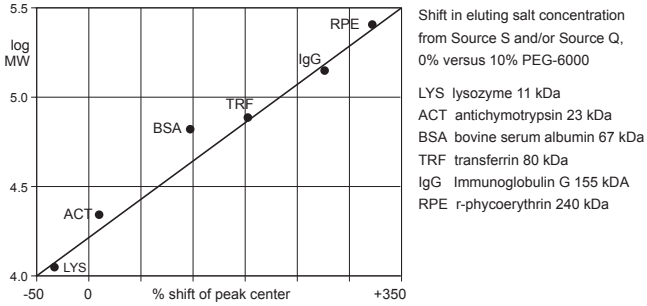

Figure 7. Linearity of enhanced retention in PEG as a function of protein size. Cited from the study by Gagnon et al.[51]

Preferential Exclusion and Chromatography

Chromatography brings another element into the equation, where agents preferentially excluded from protein surfaces are also excluded from the surfaces of chromatographic solid phases. The relatively large surface of the solid phase makes it the dominant component of the system so that under conditions where cosolvents are strongly excluded, proteins tend to associate with the chromatography surface rather than with each other. As with cosolvent exclusion in free solution, the phenomenon of preferential exclusion itself does not alter the fundamental interaction of the protein with the chemistry of the solid phase. It contributes to retention independently; its effects are additive.

Also as in free solution, the contribution of preferential exclusion to retention is related to solute size. Retention of larger biomolecules and supramolecular assemblages is enhanced to a greater degree than small biomolecules. Retention of aggregates is enhanced to a greater degree than native proteins. Retention of viruses is enhanced to a greater degree than proteins. In essence, preferential exclusion imposes size selectivity on top of the native selectivity of the solid phase. This has significant practical value for a variety of chromatography methods.

Size Exclusion Chromatography

One of the first reports of preferential exclusion having significant effects on chromatography involved SEC. When protein samples containing 5% PEG (average molecular weight of 6000-7500) were applied to Sephadex G-75 in the absence of the PEG, their elution volumes, especially larger proteins, greatly increased compared to those without PEG in the applied samples.[42] When PEG was included in the elution buffer as well, their elution volumes further increased to an extent that the elution volume showed no correlation with their molecular weight. A similar observation was made with Bio-Gel P-60. Thus, as depicted in Figure 6a, the presence of PEG in the applied sample or in both the applied sample and the elution buffer promoted association of the proteins with the surface of the SEC media surface.

Second, protein molecules partition between the inside and outside of the particles pores, as shown in Figure 6b. The thermodynamically unfavorable exclusion of PEG occurs to a larger extent for the protein outside the pores, thereby stabilizing the protein inside the pores. These mechanisms cause proteins to elute later than they should based on their actual size.[43] PEGs are also problematical even when present only in the applied samples because its size overlaps with many proteins, causing it to migrate through SEC media at about the same rate as small-to-midsized proteins. This prolongs and compounds its effects, creating a possibility of erroneous determination of the molecular weight when the applied samples contain residual PEG carried over from the preceding steps. PEG is further problematical because it increases viscosity. This reduces diffusivity in turn with the result that its presence contributes to protein peak broadening in addition to retarding protein transport through the column.

SEC is somewhat more tolerant of excluded salts in applied samples because their small size allows them to be quickly separated from the proteins and they contribute less to viscosity, but they can still cause proteins to elute later than in the absence of such salts. This is a key reason why including high concentrations of strongly excluded salts such as citrates, phosphates, and sulfates is discouraged in SEC mobile phases.[44,45] Indeed, inclusion of preferentially excluded salts has been documented to achieve stable retention of proteins on SEC media[44-46] and even on ion exchange media.[47-49] An interesting parallel was observed on monoliths with a hydrophilic hydroxylated surface. High concentrations of precipitating salts were able to achieve high-capacity binding and purification of viruses.[50]

Ion Exchange Chromatography

Ion exchange chromatography largely precludes practical use of preferentially excluded salts because they interfere directly with the mechanism of electrostatic binding. PEG, however, is nonionic and does not interfere. Basic experimental evaluation in the 1990s re-affirmed the basic phenomena observed in free solution. Retention of proteins was enhanced in proportion to their size.[51] Larger PEG polymers had a larger effect than smaller PEG polymers and higher concentrations of a given PEG size increased the magnitude of the response.[51] Figure 7 illustrates the response of model proteins ranging from 11 kDa (LYS, lysozyme) to 240 kDa (RPE, r-phycoerythrin) to the presence of 10% PEG-4600 during elution from anion exchange Source Q (for antichymotrypsin, bovine serum albumin, transferrin, and r-phycoerythrin) and cation exchange Source S (for lysozyme and IgG). Degree of enhancement is expressed as the percentage increase in the amount of NaCl required to achieve elution, measured at peak center.

Given that preferential exclusion is independent of ion exchange, enhancement should have been equivalent, regardless of whether the experiments were conducted on cation exchangers or anion exchangers. This was observed as expected (Fig. 7). Despite some of the proteins being run on a cation exchanger and some on an ion exchanger, the overall response across both exchanger types was linear. It is evident that enhancement of protein binding by 10% PEG-6000 became stronger for larger proteins, requiring higher salt concentration. On the negative side, loss of diffusivity due to viscosity of the PEG caused peak broadening in proportion to protein size. This was believed to result from the depression of diffusivity due to the viscosity of PEG in combination with slower diffusion constants of the larger proteins.

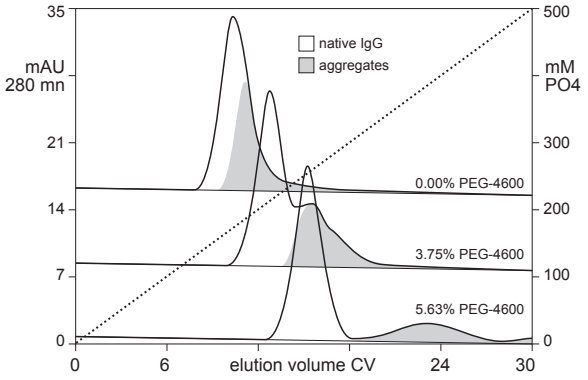

Figure 8. Enhancement of IgG aggregate removal from hydroxyapatite eluted with a phosphate gradient. Cited from the study by Gagnon.[63] CV, column volume.

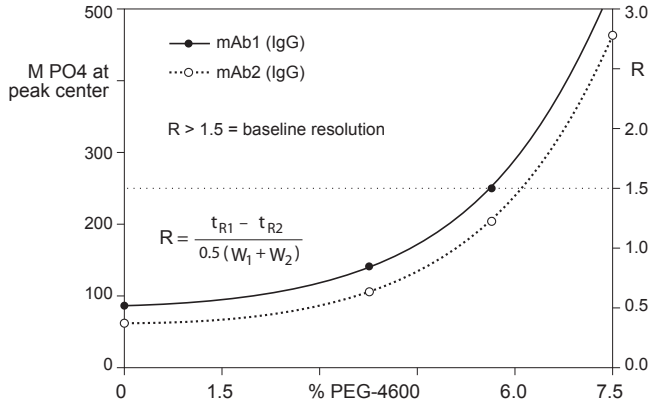

Figure 9. Parallel response of different antibodies to PEG in a phosphate gradient on hydroxyapatite. Cited from the study by Gagnon.[63]

Milby et al.[52] predicted a variety of practical applications of PEG in conjunction with ion exchange, including separation of fragments, monomers, dimers, and higher order aggregates. Indeed, Yoshimoto et al.[53] documented the ability of PEG to achieve baseline separation between BSA monomer and dimer populations on anion exchangers. Kluters et al.[54-56] demonstrated PEG's ability to enhance resolution of aggregates from native IgG on cation exchangers and have extended their findings to include PEG enhancement of resolution in pH gradients.

Examples from the field of virus purification show that PEG increases virus-binding capacity as well as separation of virus from smaller contaminants such as proteins. Zhou et al.[57] observed these effects with adeno-associated virus (AAV) serotype 9 on both anion and cation exchangers. In fact, virus purification could prove to be an even more fertile area for use of cosolvent exclusion than proteins. If PEG can be used to achieve baseline separations between protein monomers and dimers, its enhancement of fractionation between viruses (20-200 nm) and proteins (2-20 nm) should be of greater magnitude, and viruses offer an additional benefit: their larger size makes them more responsive to excluded cosolvents, which means that lower PEG concentrations and smaller PEG polymers can be used.[58] Lower PEG concentrations confer lower viscosity. Lower viscosity enables higher flow rates, which in turn give higher productivity.

Another practical feature of PEG and other nonionic or zwitterionic cosolvents is their ability to stabilize biological structures during chromatography. Feng et al.[59] used PEG to stabilize human tumor necrosis factor during fractionation by anion exchange chromatography. Lu et al.[60,61] used PEG in conjunction with anion exchange chromatography in flow-through mode to increase recovery and purity of hemoglobin. Mueller et al.[28] observed that high concentrations of either sorbitol or glycine blocked proteolysis of immunoglobulin M (IgM) even when proteases from various families were spiked into the test samples. Stabilization during ion exchange also works with virus particles. Zhou et al.[62] employed PEG to prevent dissociation of hepatitis B surface antigen virus-like particles during anion exchange chromatography.

Hydroxyapatite Chromatography

Retention of biomolecules by hydroxyapatite principally involves 2 adsorption mechanisms, cation exchange mediated through hydroxyapatite chromatography (HA) phosphate residues and metal affinity mediated through HA-calcium residues. The technique is especially known for its ability to separate IgG fragments and aggregates from native IgG, with the fragments eluting before native IgG and the aggregates eluting later. Cosolvent exclusion theory would tend to predict that the presence of PEG should increase that separation because aggregates are larger than native proteins. Experimental evidence confirmed the effect and showed that it was directly proportional to the size and concentration of the PEG.[63]

Inclusion of 5.6% PEG-4600 permitted baseline separation of aggregates that coeluted with native IgG in a phosphate gradient lacking PEG (Fig. 8).[63,64] Namely, there is no separation between the native IgG and aggregates in the absence of PEG, some separation in 3.75% PEG-4600 and near complete separation in 5.63% PEG-4600. The same technique was applied to 2 different IgG mAbs (mAb1 and mAb2), each with distinct elution characteristics: as shown in Figure 9, the mAb1 binds more strongly to the HA column and hence requires higher phosphate concentration for elution. Regardless of their differences, their responses in binding strength and aggregate resolution to the presence of PEG during the elution gradient were equivalent. Binding strength of both mAb1 and mAb2 similarly increased with increasing PEG-4600 concentration. Resolution (R) between native IgG and the smallest (first and major) aggregate peak was calculated as described in Figure 9 from the retention time and peak width of the native IgG peak (t_{R2} and W_2) and the aggregate peak (t_{R1} and W_1). The R value increased in parallel as a function of PEG concentration for both. Baseline resolution can be achieved for mAb1 above ~5.5% PEG-4600 and for weaker binder mAb2 above ~6% PEG-4600. This re-emphasized the independence of enhancement by preferential exclusion from the basic selectivity of the solid phase.

Elution of IgG from HA with a chloride gradient achieved substantial aggregate removal even in the absence of PEG but inclusion of PEG enhanced it further. This is shown in Figure 10, where near baseline separation was observed in the absence of PEG-4600, but additional separation was obtained in the presence of 3.75 and 7.50% PEG-4600. This leads to a conclusion that the greater the inherent aggregate separation achieved by a given method, the greater the degree of enhancement achieved by inclusion of PEG. This has a practical value. It recommends that instead of relying on brute-force enhancement, the native ability of the solid phase should be optimized first, and then PEG should be added. This also minimizes the amount of PEG that will be required to achieve the necessary resolution, which will minimize its depression of diffusivity.

The presence of PEG should logically have enhanced removal of virus from the native IgG as well. This was also confirmed experimentally. Removal of murine leukemia virus was increased about 5-fold over the level achieved in the absence of PEG.[65] Removal of minute virus of mice was increased about 10-fold. PEG has also been shown to modulate HA performance of recombinant AAV.[57]

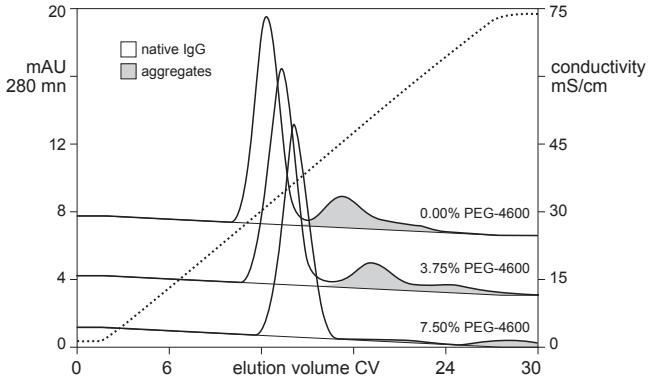

Figure 10. Enhancement of aggregate removal from hydroxyapatite eluted with a chloride gradient. Cited from the study by Lu et al.[61]

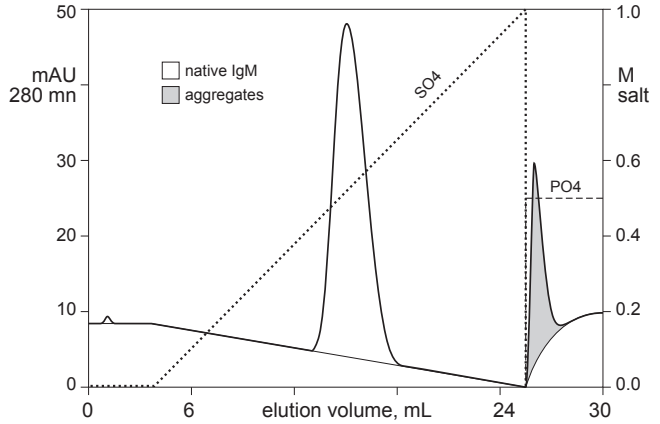

Figure 11. Enhancement of aggregate removal from hydroxyapatite with preferentially excluded salts. Cited from the study by Gagnon.[64]

Binding of AAV serotype 9 was greatly enhanced in the presence of 5% PEG-4000, leading to its delayed elution and improved separation from protein contaminants. PEG also increased virus-binding capacity on HA, as it did with anion exchangers.[57]

Unlike ion exchange, HA provides an opportunity to also evaluate the effects of preferentially excluded salts. Metal affinity interactions are known to be many times stronger than electrostatic interactions. In HA, for example, DNA remains bound even at 5 M NaCl, as long as dissolved phosphate is absent or at a low concentration. In fact, DNA retention increases in solutions of weakly excluded salts because they reduce the degree of electrostatic repulsion between DNA phosphates and HA phosphates.[66] This allows the DNA to more closely approach the HA surface and interact more strongly with the HA calcium residues.

Similar to PEG, sodium sulfate was found to selectively enhance retention of aggregates. An IgM mAb was used instead of IgG because HA-calcium binding of IgG was insufficiently strong. As shown in Figure 11, native IgM eluted about half way through a gradient to 1 M sodium sulfate. No aggregates eluted within the gradient. They remained bound and were subsequently removed with 500 mM sodium phosphate.[64]

Hydrophobic Interaction Chromatography

Preferentially excluded salts have been a critical contributor to retention in HIC throughout the evolution of the technique. The common HIC ligands such as phenyl and butyl are not sufficiently hydrophobic by themselves to achieve retention of proteins, in particular for native proteins, and more weakly hydrophobic ligands are even less able to do so. The force of preferential exclusion provides the additional increment of binding energy for HIC to achieve sufficient capacity to have practical utility. Naturally, the weaker the hydrophobicity of the ligand, the greater the contribution required by preferential exclusion. This is why weakly excluded salts such as potassium or sodium chloride can sometimes be used to achieve retention on phenyl or butyl ligands while strongly excluded salts such as ammonium sulfate, potassium phosphate, and sodium or potassium citrate are required on columns using immobilized polyethylene or PEG that is less hydrophobic. Indeed, strongly excluded salts can be used to achieve protein retention on SEC media,[44-46] monoliths with hydrophilic hydroxyl coatings,[50] and in some cases even on ion exchangers.[47-49]

One of the limitations of using salts to promote retention in HIC is that it burdens follow-on chromatography steps in multistep purification procedures. The residual salt requires that HIC be followed with a salt-tolerant step or that the eluate be buffer exchanged to permit processing by ion exchange. PEG is not helpful in promoting retention on HIC columns because the viscosity requires reducing the flow rate and proteins precipitate in the column void. On the contrary, PEG may reduce hydrophobic force due to its own weak hydrophobicity.[31] There are certain organic solvents (e.g., dimethyl sulfoxide and 2-methyl-pentanediol), which are excluded from the native protein surface and yet have hydrophobic component, can destabilize the native structure and will weaken hydrophobic interactions. Another class of excluded agents avoids these problems. The amino acids glycine and alanine are excluded from protein surfaces.[17] Between the pH values of about 4.5 and 8.5, they are zwitterionic and do not contribute to conductivity. In one study, 2.0 M glycine was used to promote IgG binding on a phenyl column. The IgG was eluted in a reducing glycine gradient, and the IgG fraction was applied without buffer exchange directly to an anion exchanger.[67]

Other preferentially excluded amino acids have also been used successful to promote binding on HIC columns but in the form of salts. They include sodium glutamate and lysine hydrochloride.[68] As ionic compounds, they contribute to conductivity in the same way as traditional HIC salts.

Bioaffinity Chromatography

Enhancement of retention on SEC, ion exchangers, and hydroxyapatite suggests that the mechanism of preferential exclusion should also enhance retention by bioaffinity ligands. This has been confirmed but not extensively characterized. Murine IgG1 antibodies bind weakly to protein A; too weakly in most cases to support adequate binding capacity.[69] Application of murine IgG1 to protein A columns in increasing levels of preferentially excluded cosolvents showed higher binding efficiency. For a mAb that only achieved 30% binding in the absence of preferentially excluded agents, 98% binding was achieved by inclusion of either 8% PEG-6000 or 1.0 M sodium sulfate, as shown in Figure 12.[69] It is evident that the binding of the murine IgG1 is dependent on cosolvent concentration.

Steric Exclusion Chromatography

Exploitation of cosolvent exclusion with adsorptive chromatography methods is fairly straight forward. As mentioned previously for ion exchange and hydroxyapatite, the sample to be fractionated can be applied to the column, and then PEG or other excluded agents were introduced to impose size selectivity during the elution. This creates an implication that it might be possible to

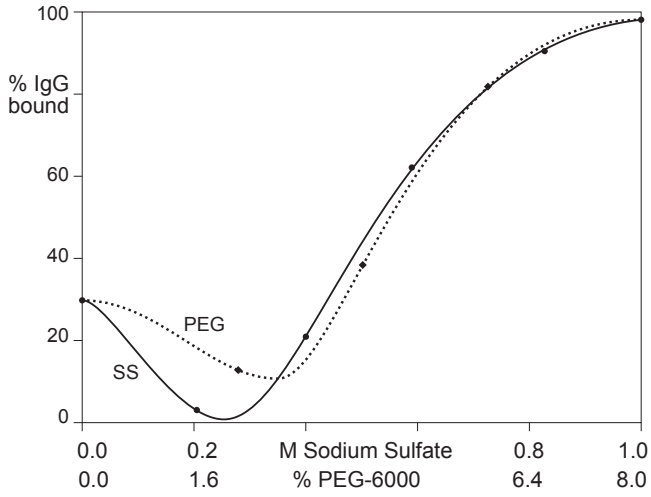

Figure 12. Enhanced binding efficiency of protein A affinity chromatography by preferentially excluded solvents and salts. Cited from the study by Gagnon.[67]

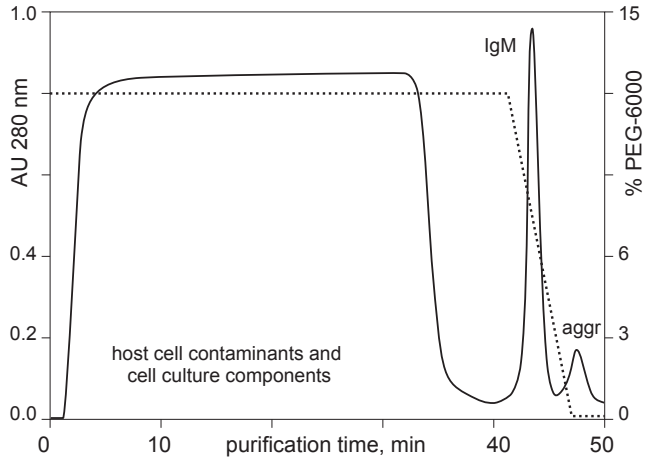

Figure 13. SXC purification of monoclonal IgM. Cited from the study by Lee et al.[69]

use PEG in combination with a nonadsorptive chromatography surface to achieve size-based separations of biomolecules, with the smallest species eluting first and larger species eluting later in proportion to their relative size. This would be a welcome addition to the field because there is no rapid high-capacity chromatography method capable of high-resolution size-based fractionation. However, there are 2 major barriers.

The first barrier is that the sample must be mixed with preferentially excluded agent before it is introduced into the column. The problem is that a concentration of excluded agent sufficient to achieve sample binding causes precipitation in the sample and clogs the column on contact. This particular problem has been overcome in the field of HIC by the technique of sample loading by in-line dilution. High-salt buffer is loaded continuously through one line, while sample is loaded continuously through another. They meet and mix immediately before entering the column. Depending on the chromatography system and valve setup, pre-column residence time of the protein in the high-salt buffer can be as brief as seconds, so there is insufficient time for the precipitates to form before column contact.

The second barrier is more restrictive. Most of the binding surfaces (i.e., functional ligand) in columns packed with porous particles resides within the particle pores. Proteins enter those pores by diffusion. Diffusion is slow. Because the viscosity of PEG reduces diffusivity in direct proportion, flow rate must be reduced to compensate. This creates in-column residence times of several minutes and the protein-PEG mixture precipitates in the void space between particles.[58] These 2 restrictions effectively prevent loading proteins at high concentrations of PEG on nonadsorptive columns packed with porous particles.

Chromatography media, such as monoliths, have fortunately evolved to include solid phases in which mass transport is convective instead of diffusive. Convective mass transport provides 3 key enabling benefits. Elimination of dependency on diffusion means that mass transport efficiency is unimpaired for large solutes with slow diffusion constants. Because diffusivity does not contribute to performance, the elevated viscosity of PEG is not a concern. Flow rates can be much faster than through porous particle columns. Rather than having pores with sizes of roughly 100 nm, monoliths have channels with sizes of 2-5 μm. They are commonly run at flow rates of 10 bed volumes per minute.

These benefits collectively enable a method now known as SXC, first performed with hydroxyl-coated monoliths.[56] Sample is introduced by in-line dilution, so that the solutes are already mixed with PEG as they enter the monolith. Large solutes are retained initially on the monolith channel surfaces and then deposited on already deposited solute. Channel diameter becomes gradually reduced as solute build-up on the surface until loading must be terminated to keep operating pressure within limits. The monolith was washed with clean PEG buffer and then eluted with a reducing linear gradient of PEG. Solutes elute in order of increasing size.

The original work on SXC was conducted with IgM, with a molecular mass of about 960 kDa, and bacteriophage M13, with a mass of about 16.7 MDa. The result for IgM is shown in Figure 13. IgM was loaded in 12% PEG-6000. During loading, a majority of UV absorbance flowed through the column. Descending gradient of PEG-6000 resulted in 90%-95% purity and 70% recovery despite the IgM being present at only 20 μg/mL in the filtered cell culture harvest applied to the monolith. Dynamic binding capacity for various IgMs ranged from 62 to 77 mg/mL of monolith. Aggregates were separated from native IgM with baseline resolution. Their later elution reinforces the point that the mechanism of greater PEG-enhanced binding of larger solutes operates the same way in SXC as in other chromatography methods.

Bacteriophage M13 was purified from *Escherichia coli* culture by the same technique. Dynamic binding capacity at 6% PEG-6000 was 9.9×10^{12} pfu/mL of monolith. As shown in Figure 14, following wash with 6% PEG-6000, descending gradient of the PEG resulted in virus recovery of 90%. Host cell proteins were reduced by 99.8%. High virus recovery was of special interest because M13 is

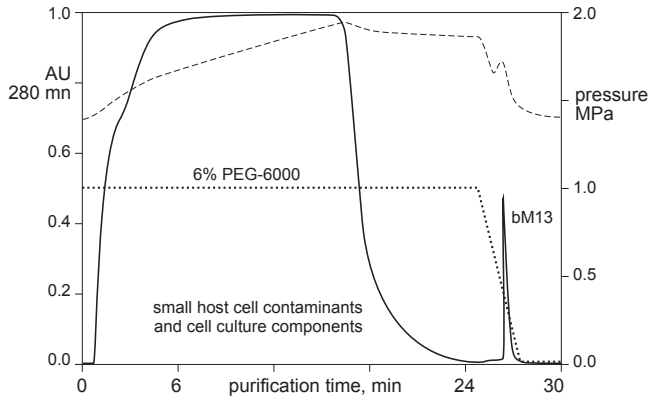

Figure 14. SXC purification of bacteriophage M13. Cited from the study by Lee et al.[69]

particularly vulnerable to shear forces. Shear is a known problem in porous particle columns where it is generated by eddy dispersion in the void space. It is not an issue in monoliths because flow through the convective channels is laminar. There are no eddies.

A variation of the technique was performed employing magnetic nanoparticles in solution rather than monoliths.[70] Magnetic nanoparticles were mixed with IgG followed by the addition of PEG. The particles were collected magnetically and washed with clean PEG buffer. IgG was eluted by suspending the particles in non-PEG buffer, and the particles were removed. Because there was no concern about accretion of IgG causing the narrowing of the channels observed with IgM on monolithic solid phases, the only restriction on capacity per particle was insulation of magnetic flux by the IgG layer on the particle surfaces. This permitted binding capacities of 58 g IgG per mL of nanoparticles—roughly 1000 times higher than the average capacity of commercial protein A affinity chromatography media and 250 times more than the highest capacity cation exchangers.

A follow-on polishing step with a conventional multimodal chromatography column reduced host cell proteins to 2 ppm, DNA to less than 1 ppm, and aggregate level to less than 0.05% and achieved an overall process recovery of 69%. Other research groups have used SXC to bind serum proteins to the surfaces of cryogel monoliths and visualized the retained solutes by scanning electron microscopy.[71] SXC has also been used to purify influenza virus on regenerated cellulose membrane adsorbers.[71]

Acknowledgments

This review is dedicated to Professor Serge N. Timasheff, whose life-long work on solvent interaction pave a way on application of cosolvents in pharmaceutical formulation and chromatography.

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