GE Healthcare

WEBINAR

Protein characterization using size exclusion chromatography

Questions and answers from live sessions

These questions and answers are from the two live sessions of a webinar presented by GE's Åke Danielsson on October 26, 2016. After each presentations Åke participated in a live question and answer (Q&A) session. There was not enough time to answer the more than 90 questions that were asked. Åke has answered those questions, and we have compiled them in this document.

In order to help you find Q&As in areas of interest, we have organized them into the following categories:

Do you have additional questions?

Feel free to continue the discussion about analytical size exclusion chromatography (SEC) on our Discussion Forum http://proteins.gelifesciences.com/forum

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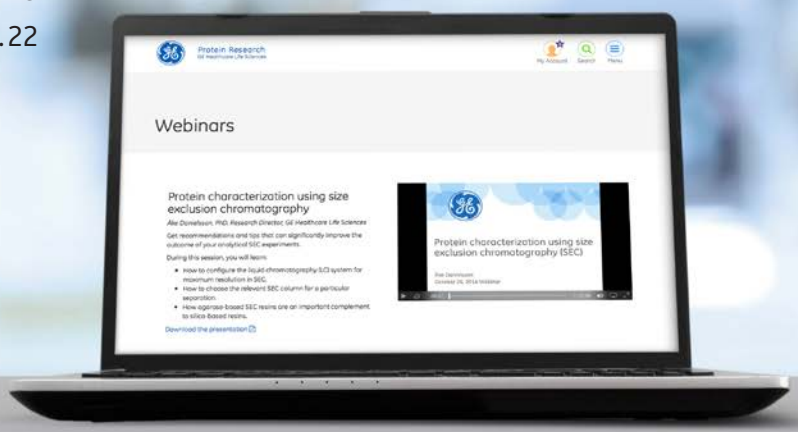

Aggregate analysis

Q. How can we analyze the monomer/dimer percentage in an aggregation-prone sample by SEC, if the protein is not visible under UV?

A. A purified sample is usually required, so that the dimer peak contains only dimer and the monomer peak contains only monomer. First, a suitable resin or prepacked column should be identified that can separate the dimer both from the monomer and from larger aggregates. Relative quantitation is done by integration of the peaks. If the protein does not adsorb at 280 nm, one can monitor absorption at 214 nm; all polypeptides absorb at this wavelength.

Q. Do you know how we can identify one aggregate from the other contaminants directly in the SEC?

A. Typically, you need purified protein samples to analyze for aggregation. One can analyze for aggregation in unpurified samples if the target protein is labeled (e.g., a green fluorescent protein [GFP] fusion protein or a protein that has been fluorescently labeled *in vitro*), by monitoring for the labeled protein.

Q. Does flow rate have an effect on protein aggregation?

A. Flow rate as such does not have an effect on aggregation. It has been reported that aggregates dissociate during a run, and this has been attributed to dilution that occurs after sample application and/or to the mobile phase having a different ionic strength than the sample. If such conditions promote dissociation of aggregates, then a slow flow rate will allow more time for dissociation. The phenomenon is described in Carpenter *et al.* (2009) J. of Pharmaceutical Sciences (DOI 10.102/jps).

Q. How is the % of aggregation calculated based on a SEC profile?

A. First, one needs to establish the elution volume(s) for the type(s) of aggregate(s) that need to be quantitated. Second, one needs to have a sample that contains the target protein and its aggregated forms but not any irrelevant components that potentially could contaminate the aggregate and monomer peaks. Third, the peaks (usually A_{280}) corresponding to aggregates and to monomer are quantitated by integrating the peak areas.

Q. I would like to know whether you can suggest guidelines to analyze protein complexes (for example to analyze the oligomeric state of proteins) by SEC using total cell lysates instead of purified proteins.

A. To study the oligomerization state of a particular protein in a complex sample using SEC, one would need to label that protein specifically (or have it recombinantly produced as a GFP fusion, for instance). One would then be able to monitor the elution position(s) for that particular protein with, for example, a fluorescence monitor even if there is a background of other proteins.

Q. How can I distinguish between aggregate formation and oligomer formation?

A. The best way to determine the molecular weight (MW) of the eluting components is to monitor the SEC separation with light scattering (in addition to UV absorption monitoring).

Q. Can you use a variety of ionic strengths and 6 M guanidinium HCl to reduce aggregation or run under denaturing conditions?

A. Yes, agarose-based SEC resins can be run in 6 M Gua-HCl or in 8 M urea, and they also can be run over a wide ionic strength range.

Q. If you have in a protein mixture an equilibrium between, for instance, a dimer and a tetramer of this protein, would it be possible to have only one elution peak corresponding to some kind of average between the two forms or ”sizes” (thus, to a trimeric form, depending of the ”speed” of the equilibrium between the two forms of the molecule)? Or would you always have two elution peaks corresponding in that case to a dimer and to a tetramer of the protein?

A. If the protein sample aggregates while it passes through the column or if aggregates dissociate during the passage, then the elution position will not give any indication of molecular size. One could perform experiments at different flow rates and with different sample concentrations to get an indication as to whether this is the case. The most reliable way to obtain molecular weight information is to use a light scattering monitor in addition to the UV monitor.

Detection

Q. If a protein cannot be detected by UV and we do not have a 220 nm detector, what can we do to get an analytical graph?

A. To monitor absorbance at 214 or 220 nm would be best as a general method to monitor proteins or peptides that do not contain aromatic amino acid residues. If it is an option to label the protein one could do that and then monitor the label (fluorescent monitor or absorption of visible light). If it is an option to collect fractions one could do that and then stain for protein with Bradford, Lowry, or similar chemistry.

Q. In your slide studying aggregation and degradation over time why are the absorbance values given as A_{220} rather than A_{280}?

A. Absorption at 280 nm is usually most convenient because buffer substances or additives typically do not absorb light at that wavelength. However, if the protein does not contain aromatic amino acids or if one needs a higher detection sensitivity, then 220 nm (or 214 nm) is an option. One needs to be careful in selecting buffer substances and additives that do not absorb light in the low UV range.

Q. Why do some of the chromatograms monitor protein at 220 nm instead of at 280 nm?

A. The peptide bond has an absorbance maximum near 220 nm. Aromatic amino acids have an absorbance maximum near 280 nm.

Experimental design

Q. Is resolution lower in a small column? If it is, can it be managed via increasing the flow time and column volumes (CV)?

A. Resolution will be improved by increasing resin bed height (for instance, by having two identical columns in tandem), if all other parameters are kept constant. If you don't want to increase bed height then lowering the flow rate can also improve resolution.

Q. What is the buffer specification for SEC – is it decided by the condition of the sample or by something else?

A. All commonly used buffers for protein work are compatible with agarose-based SEC resins, so the basic rule is to use the buffer that fits best for the sample or the target protein. Additives like arginine or low levels (typically 1–2%) of detergents could be included to minimize protein aggregation, for instance. The ionic strength of the buffer should be around 0.15 M to minimize unwanted protein adsorption.

Q. Is it necessary to equilibrate the column with the same flow rate as we are going to use after sample loading?

A. Equilibration can be performed at a higher flow rate, for example at the highest recommended flow rate for the column. A few minutes or so before sample application, one should switch back to the run flow rate.

Q. How is resolution affected in columns of different heights?

A. Resolution will be improved by increasing resin bed height (for instance, by having two identical columns in tandem), if all other parameters are kept constant.

Q. Could we take a high sensitivity narrow column and put many of them in tandem, so we will have high sensitivity but also high resolution?

A. Yes, that is a good way to improve resolution.

Q. (1) Is there a benefit or harm in using PEEK tubing or stainless steel for SEC separations? (2) How does temperature affect SEC resolutions?

A. (1) PEEK tubing is recommended, and it is commonly used in liquid chromatography (LC) systems that are optimized for protein separations. Stainless steel should be used with care, if at all, because salt-containing buffers and acidic buffers could cause corrosion.
(2) Temperature has no impact on retention according to SEC theory. Temperature could affect the conformation of proteins, which could change the size. Also, temperature will affect mobile phase viscosity (affecting the maximum flow rate to remain within pressure specifications for cold room runs) and analyte diffusivity.

Q. If time is an issue for sample degradation, then how can I consider using SEC for a sample run?

A. SEC has some benefits compared to other methods in that respect. SEC can be performed with buffers that contain protease inhibitor cocktails. Also, SEC runs could be achieved in as little as 10 min or so. Furthermore, the target protein starts being separated from proteases (if there is a sufficient size difference between the two) as soon as the sample reaches the resin bed.

Q. Which kind of standard proteins would you recommend for calibrating a Superose™ 6 increase 5/150 column? I am looking particularly for larger sizes close to 1000 kDa.

A. Unfortunately, there are not really any large proteins that fulfill the criteria (e.g., regarding cost, availability, and stability) for a good standard protein. Thyroglobulin (M_r 669 000) and IgM (M_r 750 000) are examples of molecular weight standards below M_r 1 000 000 that have been used.

Q. For unfolded protein, what do you recommend as molecular weight (MW) standards?

A. The recommendation would be to establish a standard curve under denaturing conditions (e.g., in 6 M Gua-HCl), and then one could use denatured, reduced, and alkylated globular (monomeric) proteins as standards.

Q. You mentioned that the smaller the sample, the better the resolution. However, I have found that my protein gets diluted out too far to be detectable on SDS-PAGE. I also cannot concentrate my protein to more than ~ 0.75 mg/ml. I had assumed that adding a 1 ml or 5 ml loop would solve this problem. Do you have any ideas for this?

A. When sensitivity or low protein concentrations is the issue, then it is best to use a narrow inner diameter (i.d.) column. The difference in peak height (i.e., concentration of the eluted protein) is dramatic between using a 3.2 mm i.d. column and a 10 mm i.d. column. The recommendation would be to use a 3.2/300 (3.2 mm i.d. × 300 mm bed height) column with the relevant resin for your separation. Sample volume should be no more than 50 µL, and fraction sizes should be between 50 and 100 µL over the relevant elution range.

Q. Does amount of protein loaded onto the column impact the resolution? How much protein is usually loaded for analysis?

A. Sample volume is critical for resolution, but (mass) amount is not critical as long as sample viscosity is not too high. For proteins, sample concentrations up to 20 mg/mL will typically provide good results. If one uses A_{280} monitoring and if one has access to sufficient sample quantities, then application of a 1–3 mg/mL solution of protein (in the relevant volume for the column that is used) is a good starting point.

Q. How can I separate my target protein (99 kDa) from my contaminant (66 kDa)? I have in my lab a Superdex™ 200 Increase 10/300 column.

A. Provided that the two proteins have a similar shape, I would expect that they will be reasonably well separated (but not baseline separated) if you apply 50 µL of sample and run at 0.5 mL/min.

Q. How can I make a double load in a run?

A. It is not recommended to make a second injection before a run is finished. Although it might be possible to save time in this way when a large number of highly similar, one-component samples are being analyzed, the drawback is that data interpretation becomes exceedingly hard if anything unexpected happens during the run series.

Q. Is there any difference by running at high flow rate and at lower flow rate?

A. Resolution is flow rate dependent. A lower flow rate results in higher resolution.

Q. Should I run SEC in cold conditions?

A. It depends on the stability of the sample; the separation technique as such performs equally well in the cold room as at ambient temperature. For analytical protein separations it is common to run at ambient temperature.

Q. How can I know the void volume (V_o) of prepacked and hand-packed SEC columns?

A. The void volume is best determined experimentally. A good void volume marker needs to be sufficiently large to be completely excluded from the pores, it should show no tendency to adsorb to the resin, and it should also be straightforward to monitor. The resin's exclusion volume is provided by the vendor, and a sufficiently large void volume marker should be selected accordingly. Dextran fractions with a defined molecular weight distribution are often used.

Q. How does sample concentration affect resolution?

A. Sample concentration is not critical as long as sample viscosity is not too high. For proteins, sample concentrations up to 20 mg/mL will typically provide good results.

Q. How do I prepare an antibody under reducing conditions for SEC?

A. One can use standard protocols for reducing and alkylating proteins if one wishes to run SEC under denaturing conditions. For analytical purposes, it is more common to use SDS-PAGE for reducing conditions and to use SEC for analysis under native conditions (i.e., to analyze for oligomerization and aggregation).

Q. What is the minimum protein concentration for injection?

A. The minimum concentration is defined by the sensitivity of the monitoring system. A narrow column provides higher sensitivity. As an example, 60 µg of different standard proteins in 20 µL sample volume gave A_{280} nm peaks around 0.2 AU after separation over a narrow i.d. column (3.2 mm i.d. × 300 mm bed height).

LC system configuration

Q. What do you mean by a 14 µL column valve?

A. In the talk, the aim with that particular example was to illustrate how any component in the flow path contributes to peak broadening. This particular component has a 14 µL ”dead volume”. It happened to be a column valve (which is used to divert the flow either to the column or directly to waste), but any component in the flow path that has a ”dead volume” (e.g., the UV cell or a conductivity monitor) has a similar effect on peak broadening. One needs to minimize the ”dead volumes”, because they act as mixing chambers that contribute to peak broadening and hence loss of resolution.

Q. What is considered a large dead space (volume after column) in an LC system (Superdex 200 Increase 10/300)? Is 300 µL large enough to expect an effect on resolution?

A. A 300 µL tubing volume between the column outlet and the UV cell is on the high side. That volume corresponds to 1.5 m length of 0.5 mm i.d. tubing, which seems to be much longer tubing than should be necessary to connect the column with the UV cell. One should expect some impact on resolution for the mentioned column (which contains 24 mL of resin and has a peak volume around 1 mL).

Q. Why does the tubing diameter and length affect the resolution?

A. The resolution is obtained over the column, but if the sample is diluted before it reaches the column, then effectively the sample volume will be larger than intended because the sample will have been diluted as it passed through the capillary. The wider and longer the tubing, the more the sample will have been diluted. A smaller sample volume provides better resolution than does a larger volume. Similarly, the resolution that has been achieved over the column will be partly destroyed if there is a mixing chamber (i.e., an unnecessarily wide or long capillary) between the column outlet and the monitor.

Mobile phase

Q. What's the detergent tolerance between different resin matrices?

A. Agarose-based resins tolerate detergents, so both ionic and nonionic detergents can be used (e.g., 1% SDS).

Q. Do the buffers require salt (e.g., 200 mM) to minimize protein binding to the resin?

A. Yes, it is recommended to use salt at around 150 mM for that reason.

Q. What about using detergents with the SEC column? Do you know something about using sarkosyl on it?

A. Ionic and nonionic detergents can be used with agarose-based SEC resins, so sarcosyl (N-Lauroylsarcosine) should be fine to use.

Q. Can you run isopropanol through the columns?

A. Agarose-based SEC columns can be run long term with 5% isopropanol. Short term (e.g., for cleaning) 30% isopropanol can be used.

Q. Can I inject samples in organic solvents, such as DMSO? Is it detrimental to the resin?

A. It is not recommended to use DMSO with prepacked agarose resins.

Resin and column selection

Q. What is the best column for separation of a 5.8 kDa protein such as insulin?

A. If you want to separate it primarily from sample components with a lower molecular weight, Superdex Peptide columns would be the best agarose-based option. If you want to separate it primarily from sample components with a higher molecular weight (or from both high and low molecular weight components), then Superdex 75 Increase would be the best choice.

Q. What if the protein is small and a contaminant is below 100 daltons? How wil the contaminants be separated?

A. I would suggest using Superdex Peptide. For instance, aprotinin (M_r 6512) can be clearly separated from vitamin B12 (M_r 1355) and cytochrome C (M_r 12 384). In addition, glycine (M_r 75) can be separated from tri-glycine (M_r 189; see GE Data file 18116379).

Q. What is the difference between Superdex 75 and 200?

A. They have different pore sizes and hence different selectivities. Superdex 75 Increase is preferred for separations in the (protein) molecular weight range ~ 3000 to ~ 70 000, while Superdex 200 Increase has its optimal separation range between M_r ~ 10 000 and ~ 600 000.

Q. Which Superdex Increase column is better for QC analytical labs that perform many runs in one day, for example 20–50 samples per day?

A. That throughput is achievable with a 15 cm bed height SEC column (e.g., Superdex 75 Increase 5/150 or Superdex 200 Increase 5/150).

Q. Since there is an overlap between the optimal separation range for Superdex 200 Increase and Superdex 75 Increase, are they equal in performance in their overlapping ranges?

A. The two resins have different pore size distribution, so their performance in the overlapping range is not identical. However, both resins give satisfactory separations over the overlapping range.

Q. Which resin is recommended to fractionate a mix of proteins with different molecular weights ranging from 5 kDa to 250 kDa, with the majority being between 20 and 75 kDa? The purpose would be get different fractions with high resolution to further apply to cell culture.

A. I would recommend Superdex 200 Increase. I would expect that Superdex 75 Increase will also give an adequate separation over that range.

Q. What is the best column for rabies virus purification?

A. While SEC could be contained for separation of hazardous samples, it is probably not the method of choice for that particular separation.

Q. What are the main differences between the Increase and previous generation of columns?

A. The Increase family provides higher resolution than the previous resin generation, when run times are equal. Also, the Increase columns can be operated approximately 2× faster while still providing the resolution that the previous resin generation did.

Q. Can any of the columns you talked about today be used with an ÄKTA™ FPLC system?

A. Yes, the 10/300 columns (10 mm i.d. × 300 mm bed height) could be used with the ÄKTAFPLC system. Superdex 75 Increase, Superdex 200 Increase, and Superose 6 Increase are all available as prepacked 10/300 columns.

Q. To separate amino acids and peptides with low size (< 3 kDa), which is the best column? My separation system is HPLC.

A. Superdex Peptide is an agarose-based resin that was optimized for such separations.

Q. Would a Superose 6 loaded column be suitable for separating viral capsids around 30 nm diameter?

A. Yes, it would be useful to test Superose 6 Increase for that separation.

Q. Which resin would you recommend to separate Mabs (150 kDa) from Mab-based complexes (~ 450 kDa)?

A. I would recommend Superdex 200 Increase.

Sample injection

Q. I didn't understand the difference between sample volume and valve volume.

A. The sample loop (injection loop) will hold a certain volume, for instance 100 µL. However, if one has a syringe with exactly 100 µL of sample and injects that into the sample loop, then the sample loop will not contain 100 µL of sample. This can be explained by fluid dynamics. The sample does not move like a rigid cylinder through capillaries. The sample will be distributed over a longer capillary stretch because the head and tail will be diluted.The important point is that even if 100 µL sample is injected into a 100 µL loop, less than 100 µL sample will subsequently be applied to the column.

Q. If the sample loop is 2 mL and I load 1.5 mL, is there any possibility to inject a bubble to the column as I load 1.5 mL?

A. Yes, that is one way to make sure that all of the sample is subsequently applied to the column. One thing to consider: the bubbles need to be sufficiently small so that the gas is dissolved when the bubbles reach the resin bed. Any gas bubbles in the resin bed will disturb chromatography performance.

Q. How can I configure the valve volume? If I fill the injection loop with a bigger volume, part of the sample is going to waste. Is this the best way to guarantee analytical separation and resolution?

A. Yes, it is the preferred way for reproducible results.

Q. You were talking about filling the sample loop. I have a question regarding it. If I want to load the whole sample from the sample loop, do I have to inject 200 µL although the volume of the sample loop is only 100 µL?

Yes, that is the preferred way for reproducible results.

Q. What's the optimum value for "empty loop" parameter relative to sample loop volume?

A. It is recommended to use at least three sample loop volumes of eluent in order to completely empty the sample loop.

SEC principles

Q. What is meant by an exclusion limit of 20 000 bp for DNA in the case of GE's Sephacryl™ S-1000 resin?

A. "Exclusion limit" means the size of the smallest molecule that is completely excluded from the pores in the resin beads. So for Sephacryl S-1000, ~ 20 000 bp DNA (or larger) does not enter into the pores. In practical terms, this means that with this resin you would be able to separate 20 000 bp DNA from smaller DNA species, but you would not be able to get any separation at all between 20 000 bp DNA and larger DNA.

Q. Why do SEC resins have different bead sizes? Can we calculate how much beads of different sizes are available in a specific volume of resin?

A. SEC resins are produced with different bead sizes to target different types of applications. Smaller beads provide higher resolution, if all other parameters are equal. But larger beads cost less and are straightforward to use at larger (preparative) scale. Most commercial resins have a bead size distribution. If one wishes to find out the bead size distribution, it is best to check the technical information that is supplied with the product or to contact the manufacturer.

Q. So SEC columns separate by size, not molecular weight (MW). Yet, in calibration runs provided in the column manual, proteins are always characterized by their MW. Is there a way to predict the hydrodynamic radius and based on that the elution volume of my protein? Especially when working with membrane proteins, I think I cannot assume that, for instance, all 200 kDa proteins will elute at the same volume. And how do I find out what kind of aggregates or oligomers are present in a given peak?

A. Determination of the hydrodynamic radius is not really straightforward. It depends on the molecular weight and the shape of the protein. As long as the separated proteins have a similar shape (e.g., if they are all globular proteins) it is reasonable to assume that they elute in the order of decreasing molecular weight. Confirmation of molecular weights can be done by monitoring SEC with light scattering.

Q. What's the best equation to relate K_{av} and MW when trying to estimate the MW of a protein, based on experimental K_{av} values of the test protein and of standard proteins?

A. K_{av} is plotted versus log molecular weight, and then a curve is fitted to the experimental data. That equation will be relevant for that particular column (dependent primarily on resin type and resin bed height).

Q. Can you compare the molecular weight of membrane proteins with soluble protein standards?

A. No. One can only say that the membrane protein elutes at a position corresponding to the elution position of an XX kDa globular protein. To determine molecular mass one should use light scattering or mass spectroscopy.

Q. Why does protein elution occur before the full bed volume passes through the column?

A. Large proteins that are sterically excluded from the pores will elute first. These proteins will only be able to occupy the liquid volume between the beads (because they will not be able to enter into the pores). For an agarose-based resin, that volume is about one-third of the total resin bed volume.

Q. My gel filtration standard showed good separation on my Superdex 200 10/300 column. But when two proteins of different MW elute at nearly the same point what can be inferred about the size of the two proteins?

A. If protein X has a lower molecular weight than protein Y, and both elute at roughly the same elution position in SEC (and given that the elution position is not very close to either the void volume or the total volume of the column) then the most common explanation is that protein X has an elongated shape (which gives it a larger hydrodynamic radius compared to a globular protein). Glycosylation could also be a reason for a deviant (larger) apparent molecular weight.

Q. What is the difference between resolution and sensitivity?

A. Resolution is the (horizontal) distance between two peaks, relative to the peak widths. It describes how well two components have been separated from each other. Sensitivity relates to the level of detection, or peak height.

Specific applications – glycoproteins

Q. What is your experience on the effect of glycosylation of purified recombinant proteins on hydrodynamic size? Does it usually make a large difference or is it negligible?

A. Glycosylation can make a considerable difference. Interpretation of SEC data for proteins with large carbohydrate moieties should be done with caution, especially if attempting molecular weight comparisons with nonglycosylated standard proteins. There are examples where a monomeric glycoprotein has been mistaken for a dimer due to its large apparent molecular weight in SEC.

Specific applications – group separations

Q. How can we thoroughly clean small dyes (Cy™3/Cy5) from the column quickly after using a SEC column to separate Cy3/Cy5 labeled proteins?

A. That type of separation is best done by using disposable desalting columns (e.g., NAP-5 or PD-10).

Q. What about group separations?

A. In the webinar I only focused on analytical SEC, but SEC is also widely used for preparative purposes including group separations. The most popular resin for group separations with SEC (e.g.,. desalting or buffer exchange of protein samples) is probably Sephadex™ G-25, a dextran-based resin.

Specific applications – membrane proteins

Q. How do we choose an appropriate SEC column when purifying membrane proteins with detergent micelles? Normally, it is hard to know the size of micelles.

A. The detergent shield will add to size, but not to the extent that the size of the membrane protein-detergent complex will be, for instance, doubled compared to the size of the protein alone. SEC columns optimized for protein separations typically have broad separation ranges. For example, Superdex 200 Increase separates (soluble, globular) proteins between M_r ~ 10 000 and ~ 600 000, and Superose 6 Increase separates between M_r ~ 5000 and ~ 5 000 000. For membrane proteins up to M_r ~ 200 000 the first choice is Superdex 200 Increase; for larger membrane proteins the recommendation is to try Superose 6 Increase.

Q. I am trying to separate a 450 kDa membrane protein complex from aggregates and fragments of the same complex. If, even after following all your recommendations, a Superose 6 Increase column does not provide sufficient resolution, which column can I use as an alternative?

A. First, the selectivity range for Superose 6 Increase fits well with the outlined separation problem, so that is a relevant first choice. If there is incomplete separation in the lower molecular weight range (i.e., separation of the 450 kDa target from smaller proteins) then Superdex 200 Increase could be tried. It may also be worthwhile to check what the incompletely separated peaks actually contain, using a technique such as mass spectroscopy or light scattering. For instance, could it be that the 450 kDa protein is present as a dimer? Could there be degraded forms of aggregates so that intermediate size forms are present?

Q. Can detergents be used to solubilize membrane proteins to be analyzed? If so, how are they removed afterwards?

A. Ionic and nonionic detergents can be used in SEC with agarose-based resins at the relevant concentrations for keeping membrane proteins solubilized (typically 3–10 times the critical micelle concentration). For washing out detergent from the column, one should use larger volumes of detergent-free buffer than what one normally would use to change from one detergent-free buffer to another detergent-free buffer.

Specific applications – other

Q. I have a question on alpha-synuclein. When you ran SEC, did you dectect its conformation as a momomer (revealed by 14 kDa) or multimer? Did it come from recombinant or from animal brain?

A. The experiments were done with recombinant synuclein. The monomeric protein (M_r ~14 000) eluted earlier (i.e., had a larger apparent molecular weight) than a globular protein standard with the same molecular weight. The most likely reason is that synuclein was largely unfolded.

Q. Is it recommended to deplete the sample (serum) of the most abundant proteins prior to loading the column? What method do you recommend that will avoid losing low molecular weight proteins or decrease their loss?

A. The purpose of the separation determines whether high-abundant proteins like albumin and IgG should be removed first. Serum is an extremely complex sample, and the resulting SEC chromatogram, whether high abundant proteins have been removed or not beforehand, will also be very complex.

Q. Can salts and organic components like m-cresol be separated by SEC? which is the best column or condition to do so?

A. The agarose-based SEC resins were optimized for protein separations. Small molecules can also be separated by SEC, most commonly using silica-based resins and organic solvents.

Q. What do you think about an unfolded protein which has a theorical MW of 50 kDa and an apparent MW of 400 kDa on Superdex 200?

A. Depending on how the SEC column had been calibrated, I would consider the following: (1) If the column was calibrated with other unfolded proteins I would assume that oligomerization has occurred, or possibly that the target protein could be glycosylated and therefore has assumed an irregular shape. (2) If the standard proteins used for calibration of the column were folded, then I would argue that the difference is due to an unfolded protein being less compact and hence larger (and with a larger apparent molecular weight in SEC).

Q. Can SEC also be used with very high viscosity samples like starch solutions?

A. Highly viscous samples will affect chromatography performance negatively to yield poor chromatograms (distorted peaks). Also, the backpressure will increase, and it may be necessary to lower the flow rate in order to avoid exceeding the pressure limit for the column.

Q. What kind of SEC buffer can I use to analyze the protein oligomeric state that depends on the presence of ions of the main group VII of the periodic table? Is there a buffer I can use without these kinds of ions?

A. Provided that the ions are soluble in the selected buffer system, then it would be possible to use SEC to study protein interactions in the presence of these metal ions.

Specific applications – preparative SEC

Q. What is the feasibility for working with SEC for a large-scale operation?

A. Preparative SEC is well proven at large scale (using resin volumes well above 10 L). The main drawback compared to adsorptive chromatography techniques (e.g., affinity, ion exchange chromatography) is that there is a limit for the maximum sample volume.

Q. For best resolution in a sample run you told us the sample volume should not be more than 0.5% of total bed volume. Can I consider SEC for a large scale run with 100 L of sample? What is the feasibility to use SEC?

A. SEC is not practical for that sample volume. For preparative runs, the sample volume should typically not exceed 3% of the total resin bed volume. So for 100 L of sample one would need to use extreme volumes of resin, if at all doable.

Specific applications – protein complexes

Q. What dissociation rates are acceptable for characterizing protein complexes using SEC and at what flow rates?

A. It is challenging to study unstable complexes with any method. SEC has the advantage that it is relatively quick and that a series of 10–20 different runs can be performed over a reasonable time period. It is important to perform the necessary controls, for example to check whether the elution profile is constant at different flow rates, to check whether the elution profile is identical for samples that have been stored for different times, to check whether the elution profile is identical for a low and high concentration sample, and perhaps to test different buffers and additives.

Specific applications – viruses

Q. Do virus particles show a peak in chromatography?

A. Virus particles contain protein, and proteins typically contain aromatic amino acid residues. Therefore, chromatography of viruses can be monitored by UV absorption at 280 nm.

Q. How can I consider the virus sample load to a chromatography column like Capto™ Core 700? What is the maximum height and diameter in this case?

A. Capto Core 700 is a resin that combines size separation with binding chromatography. It has an outer size-separating shell and an inner core with ligands that bind proteins. It is a hybrid technology, so SEC theory alone does not apply. Separations can be scaled, and bed heights around 20 cm are common. Please see GE application note 29000334AA for a description of virus purification with Capto Core 700.

Troubleshooting and maintenance

Q. Can NaCl block the pores of Sepharose CL-4B resin or any other resin?

A. Any solid particle (like salt crystals) could possibly block the pores, but a SEC experiment should never be designed so that such particles could be present in the resin bed. Sodium ions or chloride ions will not block the pores.

Q. If there is compression of the column beads, can the column be fixed and used?

A. If the resin bed has been compressed, one can reverse the flow and run the column slowly from the bottom to the top to see if the liquid-filled gap between the inlet filter and the resin bed disappears. Alternatively, if the column has a movable adapter at the top, one can move the adaptor downward until the liquid gap disappears. If this does not help, it is best to replace the column.

Q. Does having phenol red in the sample present a problem when using agarose-based resins? In other words, does it stick to the column?

A. To prolong the lifetime of the column, it is best to remove phenol red before the SEC separation. Larger volumes of phenol red can stain chromatography resins.

Q. When I do gel filtration, a wash volume between the sample injection and the elution phase always appears. Moreover, this volume varies and most likely is interfering with the elution of my protein. What could be happening?

A. Looking at the chromatogram, the volume between sample injection and the void volume (the elution position for a compound that is sterically excluded from the pores) is approximately one-third of the total resin bed volume for agarose-based resins. The presence of peaks in that volume (i.e., before the void volume) is an indication that there are technical issues. Such issues include, but are not limited to, a column that has not been properly equilibrated, carry-over from a previous run, or monitor problems.

Q. Why it is necessary to sanitize SEC resin as there is no binding in this resin?

A. There is very little adsorption to agarose-based SEC resins, but one can not expect that it is always zero. Furthermore, nonprotein components like lipids, if present in the sample, could be an issue. Also, there could be precipitation or large aggregate formation over the column if conditions are unfavorable. Regular cleaning with a pulse of NaOH is recommended for agarose-based resins.

Q. What would you recommend if samples are being partially absorbed by the Superdex inlet and outlet filters?

A. The inlet filter can be clogged if the sample contains particles (including large protein aggregates), but we are not aware of selective protein adsorption to the filters. The bed support is made of polyethylene, which is known to exhibit very low protein adsorption. If protein adsorption to hardware components is suspected, then one can try to alter the mobile phase (e.g., add 1–2% of detergent).

Q. Do we need to recalibrate the column each time after NaOH wash?

A. No, but it is good practice to check the efficiency (“number of theoretical plates”) of the column at regular intervals. This doesn’t have to be after every NaOH wash.

Q. What do you recommend if, unfortunately, the bead volume of a 24 ml column was compressed by ~ 1 ml? This seems to have happened because of a sudden increase in pressure and flow rate. Is it possible to reverse the effect?

A. If the resin bed has been compressed, one can reverse the flow and run the column slowly from the bottom to the top to see if the liquid-filled gap between the inlet filter and the resin bed disappears. Alternatively, if the column has a movable adapter at the top, one can move the adaptor downward until the liquid gap disappears. If this does not help, it is best to replace the column.

Q. What is the typical NaOH concentration for cleaning Superdex columns?

A. A column volume of 0.5 M NaOH is recommended for cleaning.

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