Chromatography Today Help Desk

To buffer or not

One of the focus areas of this issue of Chromatography Today is on the analysis of large molecules. One approach that has gained significant interest to allow better classification of large molecules is the use of size exclusion chromatography. From a theoretical perspective it is one of the simplest separation mechanisms to understand as it relies on a physical segregation process instead of the traditional mechanisms using chemical interactions between analytes and stationary phases commonly seen in other modes of separation [1,2]. The differentiating mechanism is based strictly on the cross sectional area of the analytes and what pores of the stationary phase these molecules are able to access.

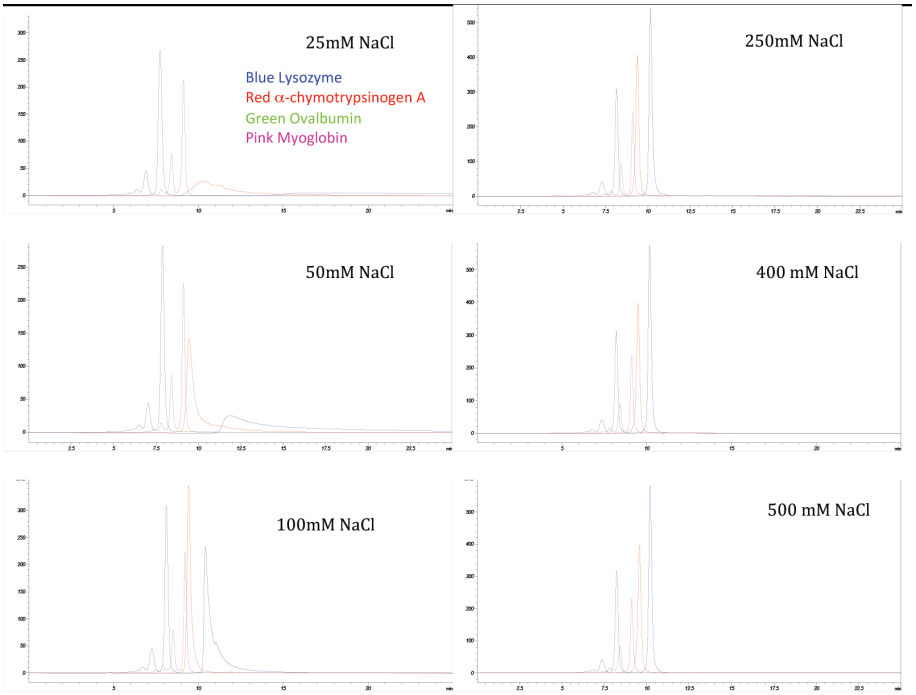

Figure 1. The effect of varying salt concentration for the separation and elution of blue – lysozyme, red - α -chymotrypsinogen A, green – ovalbumin, pink – myoglobin using SEC. Column is 300 x 4.6 mm flow rate 0.35 mL/min.

Aqueous size exclusion chromatography uses a non-interactive stationary phase coupled with an aqueous mobile phase. However, with the wide range of molecules being analysed by this powerful technique, there are occasions when secondary interactions between the stationary phase and the analyte occur. These undesirable interactions alter elution times and also result in peak shape deformation away from a true Gaussian profile. Antibody drug conjugates (ADC's) are particularly prone to these secondary interactions which is not particularly surprising since most secondary interactions are polar in nature and ADC's as a class of compounds are in fact known for their high degree of polarity [3,4]. The ADC's high degree of polarity [5] is one of their attributes that contributes to their retention and ideal chromatographic behaviour on diol stationary phases.

There are some obvious solutions to reducing the amount of secondary interactions between the analyte and the stationary phase in SEC. One approach is to use buffers while another approach is the addition of an organic additive to the mobile phase. In both situations, the desired result is for the compound to not chemically interact with the surface of the column and instead elutes based on the pore volume of the stationary phase that it is able to traverse through. So the obvious question what effect does using buffers and organic solvents have on the analysis of proteins using SEC?

To understand the unrealised benefit of using this approach let's examine the structural properties of a protein [6] as well as how proteins interact with other molecules. Proteins typically have three definable levels of structure: the primary structure relates to the arrangement of the amino acids or the amino acid sequence, the secondary structure is due to regularly repeating local structures stabilised by hydrogen bonds, and finally a tertiary structure which is obtained by nonlocal interactions, most commonly the formation of a hydrophobic core, but also through salt bridges, hydrogen bonds, disulphide bonds, and even post-translational modifications.

In the presence of different solvents, the secondary and tertiary structures can be disrupted resulting in a change in the shape of the protein, often referred to as denaturing. As the protein changes its shape, the cross-sectional area is altered which results in a different retention time when the protein is analysed via SEC. To ensure consistency when noting retention times of proteins in SEC, it is necessary to note the solvent system used as this can impact the spatial arrangement. Frequently, it is the native size of the protein which is important, to ensure that the chromatographic result is accurate, meaningful, and reproducible. The addition of a high level of buffer or an organic solvent can effectively alter the shape of the molecule, and so the data that is produced will not be correct, but it will also potentially affect the chromatography. This is shown in Figure 1 which provides an example of altered chromatographic performance of 4 molecules due to changing the buffer composition of the mobile phase. It can be clearly seen that the peak shape and retention time for lysozyme and α-chymotrypsinogen vary with different buffer concentrations, with retention time and peak width increasing as the buffer concentration is decreased. While these changes could be the result of secondary interactions with the stationary phase, the shift in retention time could also be caused by changes to the configuration

of the protein. This can happen in one of two ways [7,8], either through disruption of the molecular bonds known as the chaotropic effect, or through promotion of the molecular bonding known as the kosmotropic effect. Thus, chaotropic salts interfere with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic interactions, which, at high co-solvent concentrations, results in protein denaturation. Kosmotropic salts, cause water molecules to favourably interact which stabilises intermolecular interactions in proteins. The salt molecules readily interact with water from the protein's hydration shell and remove it from the protein surface, which produces thermodynamically unfavourable interactions that are reduced when proteins associate to form complexes. Increased salt concentration results in protein precipitation (salting out) which is the principle for hydrophobic interaction chromatography (HIC) [9].

The use of buffers in SEC is standard, but the chromatographer should be aware of the effect that the buffer has on the protein, and on the way the protein interacts with the stationary phase.

The use of organic solvents in SEC is generally not to be recommended, since many common organic solvents are chaotropic in nature, however there are occasions when the use of organic solvent can be beneficial [10,11]. In situations where it is not feasible to separate the large molecules that are very close to each other in terms of their native size, then the use of organic solvent can result in differential changes in the hydrodynamic radius, which can improve a separation, however the compounds that are being separated are not in the native form and so the relationship between the retention time and the molecular mass is invalidated.

Conclusion

One of the greatest challenges that faces analytical chemists is to ensure that the sample result being reported is representative of the sample being analysed. Although this may seem a trivial component of the whole analytical process, without correct sampling the integrity of the resulting data is highly questionable and in fact meaningless. This is particularly the case when looking at the analysis of larger molecules where protein transformations are commonplace and readily induced through a variety of external parameters, including pH, solvent composition and temperature changes. It is incumbent on the analytical scientist to be aware of the changes that can occur and ensure that interpretation of the resulting data incorporates the modification that have been made to the analytes during the analytical procedure. However, the very modifications that could potentially invalidate the analytical process can be employed by the analytical chemist to drive a separation that would previously not be feasible using the native forms of the protein molecule.

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