APPLICATION NOTE

AN3007: Estimating protein partitioning in multicomponent aqueous two-phase extraction based on CG-MALS measurements

Christian Kress, Ph.D. and Christoph Brandenbusch, Ph.D., Technical University of Dortmund

Summary

This work presents a new application of osmotic coefficients from CG-MALS measurements. The coefficients are used to predict partitioning behavior and estimate the precipitation affinity of immunoglobulin G (IgG) within a PEG-phosphate aqueous two-phase system (ATPS). Furthermore, the influence of NaCl and $(NH_4)_2SO_4$ acting as displacement agents on IgG partitioning was investigated.

B_{22} and B_{23} values were determined for several combinations of solute (PEG, phosphate buffer, NaCl and $(NH_4)_2SO_4$) and IgG using a Calypso® system. The results show that CG-MALS measurements (B_{22} and B_{23}) serve as a qualitative estimate of IgG precipitation affinity and predict the suitability of a solute as potential displacement agent within the PEG-phosphate ATPS[1].

Introduction

The aqueous two-phase system (ATPS) is a promising method of protein purification. It is obtained by mixing two hydrophilic polymers—e.g. polyethylene glycol (PEG) and dextran) or a polymer/salt combination (e.g. PEG and phosphate-salt)—above a critical concentration in water.[1,2] Some major advantages of an ATPS for protein purification over common organic aqueous extraction systems are the large fraction of water in both phases (75-90% w/w), high biocompatibility and low interfacial tension.[3,4] Scale-up is readily accomplished, as applicable tools and methods can be directly adapted from the chemical industry.[5]

Selective partitioning of the target protein and the contaminants within the ATPS is influenced by the choice of phase-forming components (salt or polymer), displacement agents (e.g. neutral salts) and process conditions. However, due to the complex parameter space of these systems, the selection is often based on cost- and time-intensive screening procedures. To decrease the amount of necessary screening experiments, and enable an efficient ATPS selection, a new method for predicting the partitioning of proteins in ATPS is sought.

We have developed a strategy to estimate the protein distribution behavior with a minimum of experimental effort. The new approach is based on measurements of the osmotic self- and cross-virial coefficients, B_{22} and B_{23} over a series of displacement agents such as NaCl and $(NH_4)_2SO_4$ (Figure 1). B_{22} accounts for self-interactions of the protein molecules, providing a qualitative estimate of the precipitation affinity of the protein IgG. The cross-virial coefficient B_{23} quantifies interactions between the protein and various solutes in the ATPS, i.e. phase formers and displacement agents. The suitability of a solute as displacement agent can be evaluated from the B_{23} values.

[1] A portion of the content of this application note was published in Kress and Brandenbusch, "Osmotic virial coefficients as access to the protein partitioning in aqueous two-phase systems", *J. Pharm. Sci.* (2015), DOI: 10.1002/jps.24602

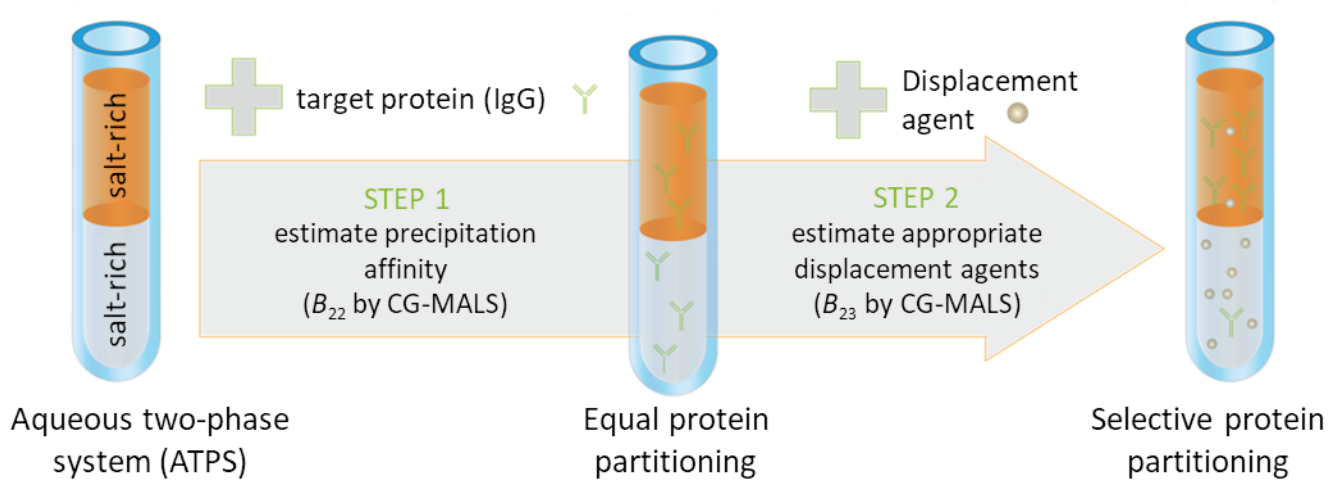

Figure 1: Concept for estimating protein partitioning in ATPS using osmotic virial coefficients from CG-MALS measurements.

The osmotic virial coefficients were measured via CG-MALS in a series of buffer solutions. CG-MALS enables non-destructive and rapid characterization of the various interactions. The results contribute to the selection of an optimal ATPS and thus simplify the downstream process development of therapeutic proteins.

Materials and Methods

Materials

Monoclonal antibody *IgG* was obtained in a 165 mg/mL solution with a purity of 95%. Polyethylene glycol (PEG) had a molecular weight of 2000 Da.

CG-MALS

B_{22} and B_{23} were determined via static light scattering (SLS) as described in Reference 6. The system comprised a Calypso automated composition-gradient system, an Optilab® differential refractometer and a DAWN multi-angle light scattering (MALS) instrument. Instrument control, data acquisition and analysis were accomplished with CALYPSO™ software.

Measurement of protein B_{22}

B_{22} of the protein in a specific solution can be determined according to Equation 1: [7]

$$\frac{\Delta R(c_2)}{C_{\mathrm{SLS}}} = \left(\frac{dn}{dc_2}\right)^2 \cdot \frac{M_2 \cdot c_2}{1 + 2 \cdot B_{22} \cdot M_2 \cdot c_2} \qquad (1)$$

In Equation 1, dn/dc_2 is the refractive index increment of the protein in the solution, c_2 represents the protein concentration, and M_2 the molar mass of the protein. $\Delta R(c_2)$ is the excess Raleigh ratio, principally the intensity of scattered light. C_{SLS} is a system constant related to the wavelength of the incident light and the refractive index of the solvent.

In order to determine dn/dc_2 the refractive index was measured at several different protein concentrations by the Optilab and a linear regression applied to the data. In order to determine B_{22} the scattered light was measured at several different protein concentrations by the DAWN and Equation (1) was fit to the light scattering data.

Determining the solute B_{33}

Analogous to the refractive index increment of the protein, dn/dc_3 of the solute can be determined with the Optilab. B_{33} of a relatively large polymeric solute (PEG) can be measured similarly to B_{22} of the protein.

In case of a small solute such as phosphate buffer salts, NaCl, or $(NH_4)_2SO_4$, the scattered light intensity is too low to make meaningful B_{22} measurements. Therefore, we calculated B_{33} from literature values of the concentration-dependent osmotic coefficient φ using Equation 2:

$$\varphi = B_{33} \cdot M_3 \cdot c_3 \qquad (2)$$

Measurement of the cross-virial coefficient B_{23}

The cross-virial coefficient B_{23} between the protein (index 2) and another solute (index 3) in a given solution can be determined according to Equation 3:[8]

$$\frac{\Delta R(c_2, c_3)}{C_{SLS}} = \left(\frac{dn}{dc_2}\right)^2 \cdot \frac{M_2 \cdot c_2}{1 + 2 \cdot B_{22} \cdot M_2 \cdot c_2} + \left(\frac{dn}{dc_3}\right)^2 \cdot \frac{M_3 \cdot c_3}{1 + 2 \cdot B_{33} \cdot M_3 \cdot c_3} - 4 \cdot \left(\frac{dn}{dc_2}\right) \cdot \left(\frac{dn}{dc_3}\right) \cdot B_{23} \cdot M_2 \cdot M_3 \cdot c_2 \cdot c_3 \quad (3)$$

In Equation 3 M_3 is the molar mass, c_3 the solute concentration, dn/dc_3 the refractive index increment and B_{33} the second osmotic virial coefficient of solute 3 (e.g. PEG, salt).

The experimental determination of the cross-virial coefficient B_{23} can be divided into three parts, illustrated in Figure 2. In the first part B_{22} and the refractive index increment dn/dc_2 of the protein are determined. The third part represents the determination of B_{33} and dn/dc_3. In case of small solutes B_{33} is determined from literature values as described previously. B_{23} is determined by varying the ratio of c_2/c_3 and measuring $\Delta R(c_2,c_3)$ in the overlapping central part of Figure 2, and fitting Equation 3 to the data.

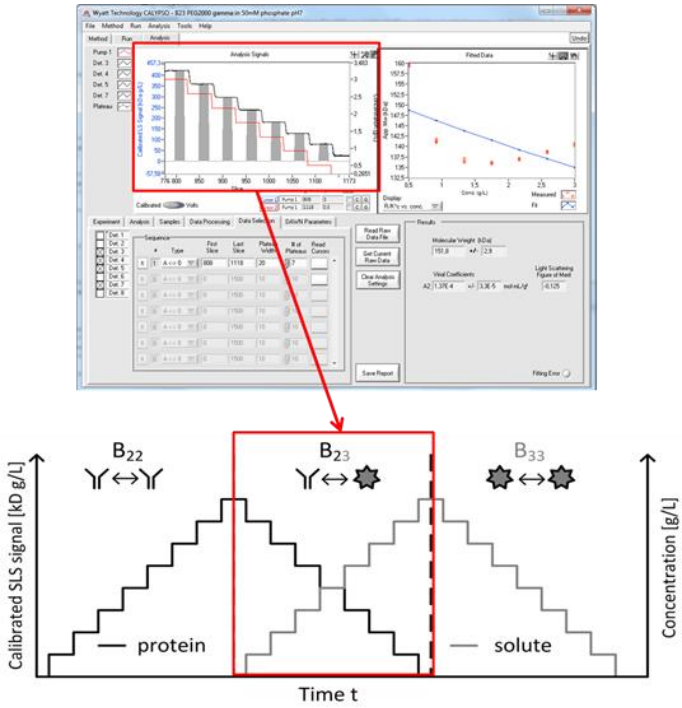

Figure 2: Bottom: Schematic method design for the characterization on the cross-virial coefficient B_{23} according to the method published in Reference 8. Top: Screenshot of CG-MALS measurement.

Results and Discussion

Protein precipitation affinity can be estimated by B_{22}

Protein partitioning within an ATPS is often accompanied by protein precipitation. In order to investigate the effect of the displacement agents on protein-protein interactions, the second osmotic virial coefficients B_{22} of IgG were measured by CG-MALS at pH 7 and 25°C in 0.05 M phosphate buffer, in the presence of varying concentrations of NaCl and $(NH_4)_2SO_4$. At the dilute buffer condition of only 0.05 M, a significant influence of the phosphate ions on the IgG-IgG interaction could be neglected. The results of B_{22} of IgG for the different solutes considered are shown in Figure 3.

For both salts, all B_{22} values are negative and decrease with increasing salt concentration. This indicates increasingly attractive protein-protein interactions with increasing salt concentration, favoring protein precipitation.[9] As shown in Figure 3, NaCl has a lower influence on B_{22} values than $(NH_4)_2SO_4$ and is thus expected to induce less protein precipitation.

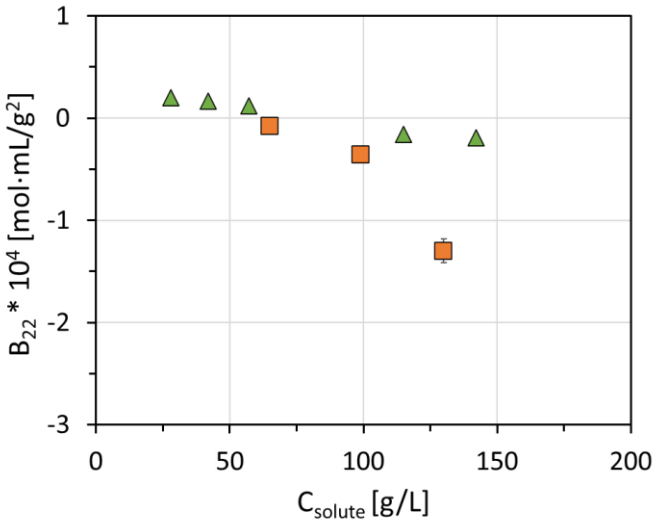

Figure 3: Dependency of the second osmotic virial coefficient B_{22} of IgG on the solute concentration. Measured in an aqueous buffer, 0.05 M K_2HPO_4-NaH_2PO_4, pH 7, at 298 K. Δ: solute is NaCl; □: solute is $(NH_4)_2SO_4$.

In order to validate the prediction of IgG precipitation affinity based on B_{22}, the precipitated amount of IgG was measured in an ATPS composed of 7% w/w PEG2000 and 14% w/w phosphate buffer pH 7 at 298 K, with and without the addition of displacement salts NaCl or $(NH_4)_2SO_4$.

The results of the IgG-precipitation within the ATPS are shown in Figure 4.

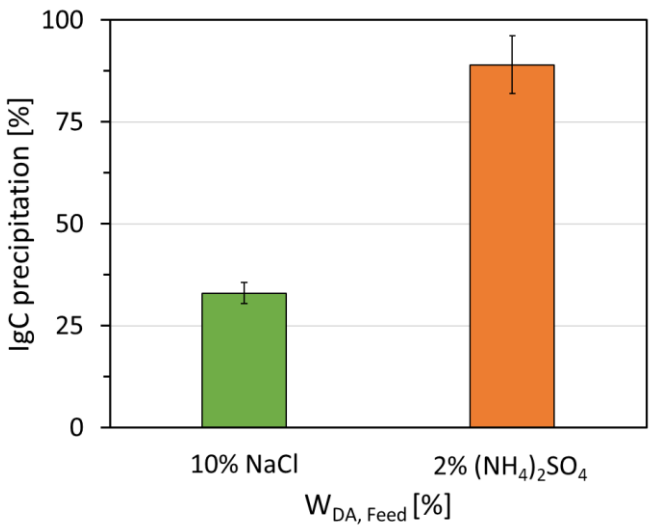

Figure 4: Dependency of precipitated immunoglobulin G on the concentration of NaCl and $(NH_4)_2SO_4$ added to the feed. The ATPS was composed of 7% w/w PEG2000 and 14% w/w phosphate buffer pH of 7 at 298 K.

Cross-virial coefficients measured between IgG and NaCl or $(NH_4)_2SO_4$ are significantly higher than between IgG and PEG2000 or phosphate. As a consequence, the IgG-solute interactions are expected to be stronger in the cases of NaCl and $(NH_4)_2SO_4$, and weaker in case of PEG2000 and phosphate. As NaCl and $(NH_4)_2SO_4$ enrich in the phosphate-rich bottom phase it is plausible that IgG will be displaced from the bottom to the top phase by both salts considered.

In order to validate our estimations of the IgG phase behavior based on osmotic coefficients, the IgG partition coefficient K_{IgG} was measured in an ATPS composed of 7% w/w PEG2000 and 14% w/w phosphate buffer pH 7, at 298 K, with and without displacement salts NaCl and $(NH_4)_2SO_4$.

In the absence of NaCl, the partition coefficient of IgG was measured as 0.09. Upon addition of 5% w/w NaCl, IgG partitions almost equally between both phases, yielding a value for K_{IgG} of about 1.18. Further addition of NaCl (10% w/w) dramatically increased the value of K_{IgG} to 38.50. Summarizing these results, in the absence of NaCl, IgG partitions into the bottom phase. Upon addition of 10 % w/w NaCl, IgG is displaced from the phosphate-rich bottom to the PEG-rich top phase. The IgG partition coefficient was also measured for $(NH_4)_2SO_4$ upon addition of 1% w/w and 2% w/w to the initial ATPS (7% w/w PEG2000 and 14% w/w phosphate buffer at pH 7).

As for the experiments using NaCl, without the presence of $(NH_4)_2SO_4$, the partition coefficient of IgG was measured to a value of 0.09. An addition of 2% w/w $(NH_4)_2SO_4$ to the initial ATPS resulted in an increase of K_{IgG} to a value of 1.08. $(NH_4)_2SO_4$ itself distributes to the phosphate-rich bottom phase as indicated by K_{AMS} of 0.26 and 0.23 in case of 1% w/w and 2% w/w, respectively.

Summarizing the results on the suitability of a displacement salt we showed that based on fast an easy B_{22} and B_{23} measurements we can estimate the partition behavior of a protein within an ATPS as well as the precipitating effect of the displacement salt used.

Conclusions

A new strategy for predicting the phase behavior of proteins within an ATPS was developed, based on comparisons of protein-protein interactions B_{22} and the interactions between the protein and the phase-forming components and displacement agent quantified as B_{23}. Osmotic self- and cross-virial coefficients are shown to be quite helpful in developing an ATPS protein extraction process. Selecting the displacement salts based on CG-MALS B_{22} and B_{23} measurements helps avoid protein precipitation.

Due to the strong repulsive interactions between IgG-NaCl and IgG-$(NH_4)_2SO_4$, indicated by high values of B_{23}, both salts were expected to be suitable displacement agents. This was confirmed experimentally in PEG-phosphate ATPS. In combination with B_{22} results, the precipitation affinity of IgG caused by the NaCl and $(NH_4)_2SO_4$ was estimated qualitatively.

Using a combination of B_{22} and B_{23} measurements, we cast the protein–protein and protein–solute interactions under dilute buffer conditions to the protein partitioning behavior in aqueous two-phase systems and developed a reliable estimation strategy.

The results open new possibilities for the development of an ATPS-based extraction step in the downstream processing of therapeutic proteins by reducing the experimental screening effort.

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