# Osmotic Virial Coefficients as Access to the Protein Partitioning in Aqueous Two-Phase Systems

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**ABSTRACT:** A promising alternative to state of the art chromatographic separations of therapeutic proteins is the extraction of the target protein using an aqueous two-phase system (ATPS). The use of an additional salt working as a displacement agent can influence the protein partitioning behavior in ATPS and thus enable a selective purification of the target protein. The selection of a suitable ATPS for protein extraction requires information concerning the protein–protein interactions (second osmotic virial coefficient $B_{22}$) as well as the interactions between protein and solute (displacement agent and phase-forming components) (cross virial coefficient $B_{23}$). In this work, the partitioning behavior and the precipitation affinity of immunoglobulin G (IgG) is considered within a polyethylene glycol (PEG)-phosphate ATPS. The influence on IgG partitioning upon addition of NaCl and $(NH_4)_2SO_4$ was investigated. In order to access the IgG precipitation affinity and the IgG partitioning behavior, the $B_{22}$ and $B_{23}$ values were determined for several combinations of solute [PEG, phosphate buffer, NaCl, and $(NH_4)_2SO_4$] and IgG based on static light scattering measurements. A qualitative estimation of the IgG precipitation affinity and the suitability of a solute as potential displacement agent within the PEG-phosphate ATPS on the basis of the measured $B_{22}$ and $B_{23}$ values is presented. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3703–3709, 2015
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## INTRODUCTION

The development of industrial scale bioprocesses has come into high scientific and industrial interest over the last decades. Although the biotechnological production of fine and bulk chemicals on an industrial scale is still in its infancy, the biotechnological production of pharmaceuticals has already entered the stage of industrial scale.[1] One class of biopharmaceuticals having a high contribution to the global sales are therapeutic proteins (e.g., monoclonal antibodies) used, for example, for Gaucher's disease, leukopenia, and cancer treatment.[2] Their production is already well understood and production titers of recombinant proteins in the upstream processing have steadily increased up to several grams per liter due to improvements in the mammalian cell culture technique.[3] Unfortunately process development has been focused almost exclusively on optimization of the upstream processing. The downstream processing in state of the art process development is often accomplished by packed bed chromatography.[4] A series of these cost-intensive chromatographic steps is necessary to account for the high purity of therapeutic proteins for final product formulation.[5] As a result, the production costs were shifted from up- to downstream processing.[6] In the field of red biotechnology, common cost for the downstream processing range from 50% to 80% of the total production cost of a monoclonal antibody.[7] One major reason for this is the limited capacity and missing scale-up potential of chromatographic separations.[4]

A promising alternative, circumventing the problem of chromatographic separations associated with capacity and scalability is protein extraction in aqueous two-phase systems (ATPS). ATPS are obtained by mixing either two hydrophilic polymers [e.g., polyethylene glycol (PEG) and dextran] or a polymer and a salt (e.g., PEG and phosphate-salt) above a critical concentration in water.[8,9] In recent studies, polymer-salt ATPS are favored over polymer–polymer ATPS due to the replacement of the second polymer (e.g., dextran) by the cheaper salt.[10] ATPS have major advantages over common organic aqueous extraction systems if used for protein purification. Because of the large amount of water in both phases (75%–90%, w/w), a high biocompatibility and low interfacial tension is provided yielding in a mild environment for proteins.[11,12] The scale-up can be easily accomplished, as tools and methods therefore can be directly adapted from the chemical industry.[13]

The partitioning of the target protein within the ATPS can be influenced directly by the choice of the phase-forming components (salt or polymer) and additionally by the use of displacement agents (e.g., neutral salts).[14] allowing for a selective partitioning of the target protein and the contaminants.[15] For a model system containing PEG, phosphate buffer and NaCl Azevedo et al.[16] showed that by selecting appropriate process conditions the target protein (antibody) can be recovered from a hybridoma cell-culture supernatant with a yield of 90% and a purification factor of 4.1. However, because of the complexity of these systems, a selection of appropriate phase-forming components and process conditions is often based on cost and time intensive screening procedures. This leads to the fact that extraction using ATPS is still not used in industrial applications. To decrease the amount of necessary screening experiments, and enable an efficient ATPS selection, a new method accessing the partitioning of proteins in ATPS has to be available.

In recent years, the thermodynamic modeling of ATPS has made considerable progress.[17,18] It was shown that a system containing water, two phase-forming components (organic salt–polymer, inorganic salt–polymer, and polymer–polymer), buffer,

**Abbreviations used:** ATPE, aqueous two-phase extraction; ATPS, aqueous two-phase system; DA, displacement agent; IC, ion chromatography; IgG, immunoglobulin G; PEG, polyethylene glycol; SLS, static light scattering.

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and displacement salt can be modeled accurately using the ePC-SAFT equation of state. Although the partitioning of small biomolecules (e.g., amino acids) is already accessible using this approach, calculations for proteins (e.g., monoclonal antibodies) are not satisfactory.

This is based on the fact that the partitioning behavior is dependent on the physico-chemical properties of the target protein, the contaminants, and their interactions with and within the ATPS.[15] Because of the non-spherical shape, the size, the surface properties, and water content, these interactions cannot be accounted for using models like PC-SAFT, where molecules are considered as chain of spherical segments.

It was previously shown by Herhut et al.[19] and George and Wilson[20] that the intermolecular interactions between protein molecules in solution, as well as the interactions of protein molecules with other molecules in solution can be used to predict process conditions for protein precipitation/crystallization. Within the work of George and Wilson[20] and Bonneté et al.,[21] the second osmotic virial coefficient $B_{22}$ is used to characterize the interactions between molecules of one species (e.g., protein–protein). Protein precipitation is favored if the protein–protein interactions increase due to decreasing distance between protein molecules indicated by negative values of $B_{22}$.[21] One big advantage using this approach is that process conditions like pH, temperature, solute, and solute concentration are captured in one measure which enables a quick characterization of the different influence factors.

Within this work, we extend this approach in order to enable an ATPS selection and estimation of the protein distribution behavior based on a minimum of experimental effort. Therefore, the second osmotic virial coefficient $B_{22}$ is used to account for the interactions between the protein molecules itself. Based on the $B_{22}$ values, a qualitative estimation of the precipitating effect of the phase-formers on the protein within the ATPS is accessible. Furthermore, an estimation of the precipitating affinity of the protein IgG due to an addition of the displacement agents NaCl and $(NH_4)_2SO_4$ is possible on the same basis. The cross virial coefficient $B_{23}$ is used in order to quantify the interactions between the different solutes of the ATPS (phase-formers and displacement agent) and the protein. By means of the $B_{23}$ values, the suitability of a solute as potential displacement agent can be evaluated.

The osmotic virial coefficients $B_{22}$ and $B_{23}$ in different buffer solutions were measured via static light scattering (SLS). This enables a nondestructive and quick characterization of the various interactions. In order to validate the $B_{22}$ and $B_{23}$ results, several PEG-phosphate ATPS containing the protein immunoglobulin G were evaluated with respect to the partition coefficient and the precipitated amount of the protein. The influence of NaCl and $(NH_4)_2SO_4$ acting as displacement agents on the partitioning of IgG was also investigated. These results will contribute to the selection of suitable ATPS and thus simplify the downstream processing development of therapeutic proteins.

## MATERIALS AND METHODS

### Materials

The monoclonal antibody IgG (CAS: 9007–83–4) was purchased from Octapharma (Langenfeld, Germany) in a 10 mL solution containing 165 mg/mL IgG with a purity of 95%. Potassium phosphate dibasic ($K_2HPO_4$, CAS: 7758–11–4), sodium phosphate monobasic ($NaH_2PO_4$, CAS: 7558–80–7), sodium chloride (NaCl, CAS: 7647–14–5) and ammonium sulfate $(NH_4)_2SO_4$, CAS: 7783–20–2) were obtained from VWR BDH Prolabo (Leuven, Belgium). PEG (CAS: 25322–68–3) with a molecular weight of 2000 Da were purchased from Merck (Darmstadt, Germany).

### Sample Preparation

#### *Preparation of ATPS*

The ATPS were prepared from stock solutions of PEG (50%, w/w), phosphate buffer (40%, w/w), and water. A mixture of $K_2HPO_4$ and $NaH_2PO_4$ was used to adjust the desired pH value of 7 of the phosphate buffer solution. NaCl or $(NH_4)_2SO_4$ was added as solid material. In order to obtain a final IgG concentration of 0.06% (w/w) in each ATPS an IgG stock solution of 1% (w/w) protein was applied. The addition of the IgG stock solution was performed after complete dissolution of the salts. All components were mixed in a 15 mL high performance centrifuge tube (VWR International, LLC, Radnor, Pennsylvania) to a total mass of 9 g. Afterward, the centrifuge tubes were vortexed for 2 min with the Tube rocker (Ika, Staufen, Germany). All IgG partition experiments were performed at 25°C and pH 7. ATPS-containing centrifuge tubes were placed in a ThermoMixer C (Eppendorf, Hamburg, Germany) for at least 14 h to ensure the phase-separation under constant temperature. In order to guarantee the entire phase separation, the tubes were centrifuged for 10 min (25°C, 2700 g) with an Eppendorf Centrifuge 5804 R (Hamburg, Germany).

#### *Measurement of the Protein Partition Coefficient*

At least 1 mL sample of the top and bottom phase was removed from the centrifuge tubes for a determination of the IgG concentration. The volume of the top phase was removed directly. The bottom phase was removed by punching a cannula through the tube wall preventing a contamination of the sample by the top phase. The IgG concentration was measured after syringe filtration with an Infinite 200 PRO plate reader from Tecan (Crailsheim, Germany) by UV absorption at a wavelength of 280 nm. The IgG extinction coefficient $\varepsilon_{280}$ at 280 nm was determined by serial dilution to 1.334 mL $cm^{-1}$ $g^{-1}$. Blank systems without IgG were assayed to account for the absorbance of PEG, phosphate buffer, NaCl and $(NH_4)_2SO_4$.

#### *Measurement of the ATPS Phase Compositions*

The weight fraction of each salt [e.g., NaCl, phosphate buffer, $(NH_4)_2SO_4$] was determined by ion chromatography (IC) by measuring the amount of every anion species. Therefore, a calibration for $Cl^-$, $PO_4^{3-}$, and $SO_4^{2-}$ was performed. The ion chromatography system ICS-2100 of Thermo Scientific Dionex (Germering, Germany) is composed of a RFIC IonPaC AS22 column and an ASRS 300 suppressor unit. The amount of water in each phase was measured by Karl Fischer titration using the 915 KF Ti-Touch device of Metrohm (Filderstadt, Germany). The weight fraction of PEG in each phase was subsequently determined by mass balance.

### Static Light Scattering

The determination of the second osmotic virial coefficient $B_{22}$ and the cross virial coefficient $B_{23}$ was realized via SLS. The SLS experiments were performed as described by Herhut