Journal of Pharmaceutical Sciences xxx (2016) 1-9

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

Novel Displacement Agents for Aqueous 2-Phase Extraction Can Be Estimated Based on Hybrid Shortcut Calculations

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ARTICLE INFO

Article history:
Received 17 March 2016
Revised 23 May 2016
Accepted 7 June 2016

Keywords:
albumin
IgG antibody
light scattering (static)
partition coefficient
precipitation
thermodynamics

ABSTRACT

The purification of therapeutic proteins is a challenging task with immediate need for optimization. Besides other techniques, aqueous 2-phase extraction (ATPE) of proteins has been shown to be a promising alternative to cost-intensive state-of-the-art chromatographic protein purification. Most likely, to enable a selective extraction, protein partitioning has to be influenced using a displacement agent to isolate the target protein from the impurities. In this work, a new displacement agent (lithium bromide [LiBr]) allowing for the selective separation of the target protein IgG from human serum albumin (represents the impurity) within a citrate–polyethylene glycol (PEG) ATPS is presented. In order to characterize the displacement suitability of LiBr on IgG, the mutual influence of LiBr and the phase formers on the aqueous 2-phase system (ATPS) and partitioning is investigated. Using osmotic virial coefficients (B_{22} and B_{23}) accessible by composition gradient multiangle light-scattering measurements, the precipitating effect of LiBr on both proteins and an estimation of both protein partition coefficients is estimated. The stabilizing effect of LiBr on both proteins was estimated based on B_{22} and experimentally validated within the citrate–PEG ATPS. Our approach contributes to an efficient implementation of ATPE within the downstream processing development of therapeutic proteins.

Introduction

Since the first biopharmaceutical drug (Humulin) was approved in 1982, the biopharmaceutical market has gained a significant growth with global revenues of 140 billion USD in 2013 and estimated sales of approximately 166 billion USD up to the year 2017.[1,2] Within the biopharmaceutical market, the production of mAbs contributes with global revenues of nearly 75 billion USD and thus accounts to almost 50% of all biopharmaceuticals produced.[3] With a current approval rate of 4 new mAbs a year, global sales on mAbs are estimated to reach nearly $125 billion by the year 2020.[3] The increased production of mAbs is caused by their enhanced demand as treatment for cancer, for immunologic disorders (e.g., multiple sclerosis, rheumatoid disorders, etc.) or against Alzheimer disease.[4,5]

Process innovations in the production of mAbs focused primary on the upstream processing, often disregarding the downstream processing. As a result, costs were shifted from upstream to downstream processing creating a bottleneck.[6] State-of-the-art downstream processing of mAbs bases almost exclusively on cost-intensive chromatographic steps and can account to 50%-80% of the total production cost.[7] Even though chromatography cannot be replaced as a key technology of the downstream processing to achieve purity >99.9%, disadvantages like enormous feedstock pretreatment and limitations in capacity require other purification steps for initial product capture.

A promising technology allowing for an efficient capture of the target protein in an early stage of the downstream processing is the continuous extraction using an aqueous 2-phase system (ATPS).[8-10] An ATPS is formed by either mixing 2 hydrophilic polymers (e.g., polyethylene glycol [PEG] and dextran) or a hydrophilic polymer and a kosmotropic salt (e.g., phosphate, sulfate, or citrate salt) exceeding a critical concentration.[11,12] APTSs in general exhibit a high biocompatibility, can easily be scaled up, and show high selectivity and recovery of the target protein.[8,11] The purification potential of ATPS for the extraction of biological compounds was first shown by Albertsson.[13] Several other research groups later showed that the protein-partitioning behavior within salt–PEG ATPS could be selectively influenced using the displacement agent

Abbreviations: ATPE, aqueous 2-phase extraction; ATPS, aqueous 2-phase system; CG-MALS, composition gradient multiangle light scattering; DA, displacement agent; ePC-SAFT, Electrolyte Perturbed-Chain Statistical Associating Fluid Theory; HSA, human serum albumin; PEG, polyethylene glycol; TLS, tie-line slope.

This article contains supplementary material available from the authors by request or via the Internet at http://dx.doi.org/10.1016/j.xphs.2016.06.012.

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http://dx.doi.org/10.1016/j.xphs.2016.06.006
0022-3549/

(DA) NaCl, allowing for a purification of IgG from the contaminants.[9,14-16] Further advances, substituting phosphate salts with the biodegradable citrate salt as a phase former enabled a purification of IgG from a hybridoma cell culture supernatant with a recovery yield of 99%.[15] Nevertheless, the selection of DAs enabling a selective and efficient IgG purification based on aqueous 2-phase extraction (ATPE) remained almost exclusively empirical and is still limited to chlorides, especially to NaCl.

The use of a DA has a direct impact on the phase composition of the ATPS. Unfortunately, the mutual influence between NaCl and the phase formers of the salt–PEG ATPS is often unattended with only few publications dealing with this topic.[17-21] These publications focus on the effect of NaCl on the ATPS binodal curves. It was shown that NaCl has a significant influence on the ATPS phase composition by steadily expanding the ATPS region with increasing NaCl concentration.[17-21]

To facilitate the selection of DAs and thus enable the application of the ATPE on an industrial level, suitable design tools have to be available allowing for the selection of suitable ATPS and DAs other than NaCl based on a minimal experimental screening effort.

Regarding ATPS (polymer–polymer, polymer–salt), including a DA in the absence of proteins, thermodynamic modeling is already feasible using the Electrolyte Perturbed-Chain Statistical Associating Fluid Theory (ePC-SAFT) equation of state.[22-24] Moreover, the influence of different process parameters like temperature, type of phase former, and phase former concentration on the ATPS phase composition is accessible.

However, protein partitioning (e.g., IgG) within the ATPS cannot be calculated using ePC-SAFT due to their nonspherical shape, surface characteristics, size, and solvent content. As molecules are regarded as chains of spherical segments within ePC-SAFT, the complex physicochemical properties of the proteins including their interaction with the surrounding molecules cannot be accessed sufficiently. In our approach, these protein–protein and protein–solute interactions are captured by the second osmotic virial coefficients B_{22} and the cross virial coefficient B_{23} accessible via composition gradient multiangle light-scattering (CG-MALS) measurements.

In our previous publication,[25] a hybrid shortcut calculation based on the cross virial coefficient B_{23} allowing for the estimation of the protein partition coefficients within salt (citrate, phosphate)–PEG2000 ATPS as a function of the NaCl and phase former concentration was successfully presented. In this work, this hybrid shortcut estimation is applied to citrate–PEG ATPS including the new DA lithium bromide (LiBr) to access the partition coefficients of IgG and human serum albumin (HSA).

LiBr allowed for a selective purification of the target protein IgG from the contaminant HSA (already identified as a representative impurity by the studies[6,15,26]) within a 14.5% (w/w) citrate and 8% (w/w) PEG2000 ATPS. The displacement suitability of LiBr with regard to IgG was investigated with respect to the mutual influence of LiBr and the phase formers (citrate, PEG) in combination with the cross virial coefficient B_{23}. The influence of different amounts of LiBr on the tie-line slope (TLS) and on the 2-phase regime of the ATPS was determined and illustrated in phase diagrams.

Furthermore, as the precipitation of the target protein has to be avoided to circumvent a loss of product,[27] the ability to estimate the precipitating effect of a DA on the target proteins within the ATPS is crucial. Protein precipitation/crystallization in solution is related to the protein–protein interactions which can be expressed by the second osmotic virial coefficient B_{22}.[28,29] Protein precipitation and crystallization is generally favored with increasing attractive protein–protein interactions indicated by negative B_{22} values.[30]

In this work, the precipitating effect of LiBr on both proteins is estimated based on the second osmotic virial coefficient B_{22} also determined via CG-MALS measurements.

The results of this work elucidate that our hybrid shortcut calculation could be efficiently applied to ATPS containing the DA LiBr in addition to NaCl.

The osmotic virial coefficients B_{22} and B_{23} represent the protein–protein and protein–solute interactions, respectively and are key parameters for the protein-phase behavior within ATPS. In combination with our hybrid shortcut calculation and knowledge of the mutual influence between DAs and phase formers, a selection of suitable ATPS is provided in an early stage of downstream processing development.

Materials and Methods

Materials

The proteins of this work are IgG (CAS: 9007-83-4) and HSA (CAS: 70024-90-7), both purchased from Sigma-Aldrich (Steinheim, Germany). 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 trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$, CAS: 6132-04-3) were delivered from VWR BDH Prolabo (Leuven, Belgium). PEG (CAS: 25322-68-3) with a molecular weight of 2000 Da was obtained from Merck (Darmstadt, Germany), and LiBr (CAS: 7550-35-8) was purchased from Sigma-Aldrich.

Sample Preparation

Preparation of ATPSs

The preparation of all ATPSs was performed by pipetting the required amounts of stock solutions of PEG (50% w/w), NaCl (25% w/w), citrate buffer (40% w/w), and water. To adjust the pH of the buffered citrate solution (40% w/w) to a value of 7, solid citric acid was added to the trisodium citrate stock solution. In case of LiBr and high NaCl concentrations, solid salt was added to the ATPS. To provide the final concentration of 0.06% (w/w) of each protein within the ATPS, stock solutions containing 1% (w/w) IgG and HSA were used. The final protein partition experiment including mixing of all stock solutions and centrifugation was performed as described in Kress and Brandenbusch.[31]

Measurement of Protein Partition Coefficients

For measurements of the protein partition coefficients, the removal of top and bottom phase for sampling and the determination of the protein concentration in clear solution by UV absorption at 280 nm were performed as described in Kress and Brandenbusch.[31] The protein extinction coefficients at 280 nm $\varepsilon_{prot,280}$ were determined to 0.538 mL cm^{-1} mg^{-1} in case of HSA and to 1.334 mL cm^{-1} mg^{-1} in case of IgG.[25] Blank systems without protein were used to account for the absorbance of PEG, citrate buffer, NaCl, and LiBr.

Measurement of the ATPS Phase Compositions

To determine the ATPS phase composition, the weight fraction of each salt (e.g., NaCl, citrate buffer, LiBr) was measured by ion chromatography. The amount of water of each phase was determined by Karl Fischer titration as described in Kress and Brandenbusch.[31] The weight fraction of PEG was calculated by mass balance.

Static Light Scattering

The second osmotic virial coefficient B_{22} and the cross virial coefficient B_{23} were determined by CG-MALS with a system