European Journal of Pharmaceutics and Biopharmaceutics 112 (2017) 38–44

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Solubilization of proteins in aqueous two-phase extraction through combinations of phase-formers and displacement agents

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ARTICLE INFO

Article history:
Received 1 August 2016
Revised 21 October 2016
Accepted in revised form 14 November 2016
Available online 17 November 2016

Keywords:
Downstream processing
Aqueous two-phase extraction
Protein partitioning
Protein interactions
Protein solubilization

ABSTRACT

The aqueous two-phase extraction (ATPE) of therapeutic proteins is a promising separation alternative to cost-intensive chromatography, still being the workhorse of nowadays downstream processing. As shown in many publications, using NaCl as displacement agent in salt-polymer ATPE allows for a selective purification of the target protein immunoglobulin G (*IgG*) from human serum albumin (*HSA*, represents the impurity). However a high yield of the target protein is only achievable as long as the protein is stabilized in solution and not precipitated. In this work the combined influence of NaCl and polyethylene glycol (Mw = 2000 g/mol) on the *IgG-IgG* interactions was determined using composition gradient multi-angle light scattering (CG-MALS) demonstrating that NaCl induces a solubilization of *IgG* in polyethylene glycol 2000 solution. Moreover it is shown that the displacement agent NaCl has a significant and beneficial influence on the *IgG* solubility in polyethylene glycol 2000-citrate aqueous two-phase system (ATPS) which can also be accessed by these advanced B_{22} measurements. By simultaneous consideration of *IgG* solubility data with results of the ATPS phase behavior (especially volume fraction of the respective phases) allows for the selection of process tailored ATPS including identification of the maximum protein feed concentration. Through this approach an ATPS optimization is accessible providing high yields and selectivity of the target protein (*IgG*).

1. Introduction

With global sales of approximately 75 billion USD in 2013 and predicted sales of nearly 125 billion USD in 2020 monoclonal antibodies (mAbs) play a major role within today's biopharmaceutical market [1]. The mAbs growth potential is driven by the increased demand in therapeutics in order to fight cancer, autoimmune- and infectious diseases [2–4].

In contrast to the downstream processing which has often been neglected in process optimization, considerable improvements in upstream productivity have been achieved within the last 30 years [5] creating purification bottlenecks. This leads to the fact that for mAbs up to 80% of the total production costs are incurred by the downstream processing [6,7]. Chromatography still being the state of the art key purification technology turns out to be the main cost driver within the downstream processing of mAbs. This drawback can be circumvented by both optimization and innovations in column techniques or by novel purification approaches [8,9].

One efficient integration of capture, concentration and selective purification of pharmaceutical proteins like mAbs in one continuous step, and thus a promising alternative to chromatography, is the aqueous two-phase extraction (ATPE) [6,10,11]. In contrast to chromatography ATPE is cost-efficient and can be scaled up easily [11,12]. However, the implementation of ATPE for the biopharmaceutical purification at process scale has only been shown in a few cases [6,13,14] as methods and design approaches determining the complex protein partitioning are mainly empirical [15,16].

Beneficially, aqueous two-phase system (ATPS) phase composition (polymer-polymer, polymer-salt) in the absence of a protein can be calculated based on thermodynamic models like the ePC-SAFT equation of state [17–19]. However, due to the complexity of therapeutic proteins their partitioning in ATPS is not accessible based on ePC-SAFT. In order to overcome this drawback and

Abbreviations: ATPE, aqueous two-phase extraction; ATPS, aqueous two-phase system; CG-MALS, composition gradient multi-angle light scattering; DA, displacement agent; ePC-SAFT, electrolyte Perturbed-Chain Statistical Associating Fluid Theory; HSA, human serum albumin; IgG, immunoglobulin G; mAb, monoclonal antibody; PEG, polyethylene glycol; TLL, tie line length; TLS, tie line slope; UV, ultra violet.

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http://dx.doi.org/10.1016/j.ejpb.2016.11.016
0939-6411/

Nomenclature

Latin symbols

B_{22}	second osmotic virial coefficient of protein (mol m^3 g^{-2})
B_{23}	second osmotic cross virial coefficient (mol m^3 g^{-2})
c_i	mass concentration of component i (g m^{-3})
K_i	partition coefficient of component i based on c_i (-)
M_i	molar mass of component i (g mol^{-1})
m_i	mass of component i (g)
S_{IgG}	selectivity of *IgG* (-)
w_i	weight fraction of component i (-)
Y_i	yield of component i (-)

Greek symbols

$\varepsilon_{IgG,280}$	*IgG* extinction coefficient at 280 nm (m^2 g^{-1})
$\varepsilon_{HSA,280}$	*HSA* extinction coefficient at 280 nm (m^2 g^{-1})

Superscripts

bot	bottom phase
sat	saturated
top	top phase

Subscripts

2	protein
3	solute (phase-former or displacement agent)
HSA	human serum albumin
IgG	immunoglobulin G
prot	protein

estimate the protein partitioning, hybrid shortcut calculations have been applied recently [20,21].

Within previous works [20–22] the displacement ability of NaCl allowing for a selective immunoglobulin G (*IgG*) purification from human serum albumin (*HSA*) within polyethylene glycol (PEG)-salt (citrate, phosphate) ATPS could be efficiently estimated based on the cross virial coefficient B_{23}.

Furthermore the precipitating effect of one solute (phase-former and displacement agent) on *IgG* and *HSA* within salt (citrate, phosphate)-PEG2000 ATPS could be sufficiently estimated using the second osmotic virial coefficient B_{22}. However, B_{22} values of *IgG* and *HSA* were only determined as function of one solute.

Within these previous works, it was observed that for a constant protein feed concentration precipitation of the target protein *IgG* in a salt (phosphate/citrate)-PEG2000 ATPS decreased with increasing NaCl concentration [20,21]. This was unexpected as usually higher salt concentrations lead to an increase in precipitation.

In order to reveal the mutual influences leading to the decrease in protein precipitation and to further make this effect available for process development the combined influence of phase-forming PEG2000 and displacement agent NaCl on the *IgG-IgG* interactions (used to estimate precipitation affinity) in aqueous solution was investigated. Therefore B_{22} values of *IgG* were determined via CG-MALS (composition gradient multi-angle light scattering) as function of the PEG2000 concentration in the presence of NaCl.

Using this approach a solubilizing effect of NaCl on *IgG* in PEG2000 solution was identified.

Moreover *IgG* solubility (that is maximum achievable *IgG* concentration within the ATPS/ATPE) in top and bottom phases of ATPS composed of 13.5% (w/w) citrate and 8% (w/w) PEG2000 (best performing ATPS determined in previous works [22]) in dependency of the NaCl concentration (5, 7.5, 10, 12.5 and 13.5%) was determined for the first time.

It was shown that the addition of NaCl has significant and contrasting influence on the *IgG* solubility in top and bottom phases. *IgG* solubility in the top phase is increased with increasing NaCl concentration improving the displacement of *IgG* into the top phase. Furthermore by combining results on the *IgG* solubility within the ATPS and the shift in ATPS due to the addition of NaCl the maximum *IgG* ATPS feed concentration can be estimated. This information is crucial for the purpose of an efficient application of ATPE.

The results of this work outline that the solubilizing effect of NaCl on *IgG* in aqueous PEG solution can be accessed efficiently by B_{22} measurements in the presence of both solutes. Our approach allows for a quick and non-destructive selection of tailored ATPS characterized by high yields and selectivity of *IgG* whereby *IgG* is stabilized within the selected ATPS.

2. Materials and methods

2.1. Materials

Both proteins of this work, immunoglobulin G (IgG1) from human blood, prepared from Cohn Fraction II, III (*IgG*, CAS: 9007-83-4) and human serum albumin (*HSA*, CAS: 70024-90-7) were obtained 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 purchased from VWR BDH Prolabo (Leuven Belgium). Polyethylene glycol (PEG, CAS: 25322-68-3) with a molecular weight of 2000 Da was delivered from Merck (Darmstadt, Germany). Citric acid ($C_6H_8O_7$, CAS: 77-92-9) was purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Sample preparation

2.2.1. Preparation of aqueous two-phase systems

All ATPS were prepared by pipetting the required quantity of stock solutions of PEG (50% w/w), citrate buffer (40% w/w), NaCl (25% w/w) and water. The pH value of 7 of the buffered citrate solution (40% w/w) was adjusted by adding solid citric acid to the trisodium citrate stock solution. In order to achieve high NaCl concentrations solid NaCl was added to the appropriate ATPS. The final concentration of both proteins in every ATPS was equal between 0.06 and 0.13% (w/w) depending on *IgG* solubility and was provided using protein stock solutions containing 1% (w/w) *IgG* and *HSA*. The procedure of the subsequent protein partitioning including centrifugation was accomplished as described in [20].

2.2.2. Measurement of protein partition coefficients

The determination of the protein partition coefficients was performed by measuring the protein concentration in the clear solution after sample removal of top and bottom phase based on UV absorption at 280 nm as described in [20]. The protein extinction coefficient at 280 nm accounted to 1.334 mL cm^{-1} mg^{-1} in case of *IgG* ($\varepsilon_{IgG,280}$) and to 0.538 mL cm^{-1} mg^{-1} in case of *HSA* [21]. In order to account for the absorbance of PEG, citrate buffer and NaCl blank systems were used. Samples were checked on the appearance of precipitate by dynamic light scattering using a Dynapro Nanostar from Wyatt Technologies (Santa Barbara, USA) prior to