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Single stage aqueous two-phase extraction for monoclonal antibody purification from cell supernatant

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ABSTRACT

The current work investigates the extraction of a monoclonal antibody immunoglobulin G1 from a Chinese hamster ovary cell supernatant. Solubility of biologic substances was figured out as the most limiting factor during aqueous two-phase extraction. Therefore, it was majorly considered for system choice and for the determination of an operating window to prevent product loss due to precipitation. The solubility of immunoglobulin G1 was screened for different solutions of phase forming components. Best solubility was observed for a polyethylene glycol 2000 – phosphate aqueous two-phase system at pH six. The influence of additional sodium chloride and cell supernatant loading on the purification was investigated. Most promising extraction conditions were determined to either include no or a high amount of sodium chloride. A decrease of product phase (immunoglobulin G1-rich phase) volume further improves the purification resulting purification factors of up to 3.1 with an immunoglobulin G1 yield of higher than 90% within a single extraction step.

1. Introduction

Aqueous two-phase extraction (ATPE) is a promising method for the purification of mAbs and other biological compounds. Major advantage of ATPE in comparison to chromatography is a continuous application at high capacity which is comparably easy to scale up leading to low process time and cost [1]. Aqueous two-phase systems (ATPS) conventionally comprise two hydrophilic polymers or a hydrophilic polymer and a salt dissolved in water. Above a certain concentration two phases form, both having an aqueous character [2].

The first application of ATPS for mAb purification was reported by Andrews et al. in 1990 [3]. Activated polyethylene glycol (PEG) molecules were bound to Protein A or anti-bovine serum albumin (BSA) ligands to enhance the affinity towards mAb and thus influence partitioning. Because of the high cost of this procedure, Andrews et al. presented a different approach in 1996 [4]. They purified murine immunoglobulin G (IgG) from hybridoma cells with ATPS consisting of PEG 1450, phosphate salt (phosphate) and water. The extraction was carried out at 15 wt.% PEG 1450, 14 wt.% phosphate and 12 wt.% additional sodium chloride (NaCl) at pH 5.5. Though IgG was mainly recovered in the top phase, the authors already mentioned protein precipitation which had to be minimized by an adjustment of extraction conditions. Since that time, many investigations on mAb purification by ATPE have been published mainly applying PEG–phosphate ATPS for purification.

In 2007, Rosa et al. [5] analyzed the purification of IgG from synthetic protein mixtures containing BSA and myoglobin by a PEG – phosphate ATPS. Various PEG molecular weights in a range of 1000–20000 g/mol were investigated and optimal conditions were found for a system composition of 8 wt.% PEG 3350, 10 wt.% phosphate salt and 15 wt.% additional NaCl. The yield of native IgG in the top phase was reported to be 97% with a purity of 99%. Azevedo et al. [6] extended the work of Rosa et al. and applied PEG–phosphate ATPS for the purification of IgG from synthetic media, Chinese hamster ovary (CHO) cell supernatant and hybridoma supernatant. They reported optimal conditions for high IgG recovery at 12 wt.% PEG 6000, 10 wt.% phosphate and 15 wt.% additional NaCl at pH 6. At the given conditions, 88% of IgG from CHO cell supernatant were recovered with a purification factor of 4.3 (see definition in Eq. (3)). Furthermore, Azevedo et al. [7] investigated the applicability of PEG/citrate ATPS for the

Abbreviations: ATPE, aqueous two-phase extraction; ATPS, aqueous two-phase system; BP, bottom phase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CS, cell supernatant; IgG1, immunoglobulin G1; KOH, potassium hydroxide; mAB, monoclonal antibody; MP, mixing point; NaCl, sodium hydroxide; PEG, polyethylene glycol; PFC, phase forming component; Phosphate, phosphate salt; PP, product phase; SEC, size-exclusion chromatography; TP, top phase.

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Nomenclature

Greek letters
Φ Phase ratio (−)

Symbols
c concentration (mg/kg)
K partition coefficient (−)
M mass (g)
P total purity (%)
PF purification factor (−)
w weight fraction (g/g)
Y yield (%)

Subscripts
BP bottom phase
CS cell supernatant
i component i
IgG1 immunoglobulin G1
j sample j
NaCl sodium chloride
PEG polyethylene glycol
phos phosphate
PP product phase
total total
TP top phase

purification of IgG from hybridoma cell supernatant. The extraction step was carried out at 8 wt.% PEG 3350, 8 wt.% citrate, 15 wt.% additional NaCl and pH 6 leading to an IgG recovery of 99% and a purity of 44%. In 2006 Platis et al. [8] reported a specific application of PEG/phosphate ATPS. The authors presented the first ATPE for plant derived mAbs. They extracted human anti-human immunodeficiency virus (HIV) mAb 2F5 from tobacco extract using an ATPS that consisted of 12 wt.% PEG 1500 and 13 wt.% phosphate at pH 5. MAb was recovered with a purity of 95% in the bottom phase.

Though there have been several publications on the field of mAb purification by ATPE, the design of an ATPE process is a challenging procedure. The ATPS applied for ATPE of mAbs all varied in PEG molecular weights, pH values and composition of phase systems. Furthermore, there might be differences in properties of the cell supernatants from which the mAb is extracted. Because of the complexity of ATPS and poorly understood partition mechanisms, up to now the design of an ATPE is completely empirical. General process conditions need to be selected upon experiences and require a certain knowledge of protein partitioning and behavior due to the system complexity [9,10]. Therefore, ATPE of mAbs is worth to be extended with regard to other ATPS and other cell supernatants.

In the scope of this work an immunoglobulin G1 (IgG1), against cholera toxin is purified from a CHO cell supernatant by single stage ATPE. PEG–phosphate systems have repeatedly been proven as a successful example for mAb extraction in ATPS [4,7,8,11]. They circumvent the comparably high raw material cost [9,12] low density difference between both phases and high viscosity of a polymer–polymer ATPS, which are all disadvantageous for continuous application [13]. The transferability of ATPE to another cell supernatant derived from a CHO cell line is analyzed. Results achieved are comparable to the mentioned publications, although extraction conditions have to be changed to reduce occurring precipitation of proteins. Since the protein solubility in ATPS is known to be limited [11,12], the solubility of IgG1 in pure solutions of the phase forming components is investigated. A combination of solubility screening and pure component partitioning experiments is applied to determine ATPS conditions like PEG molecular weight and pH value.

Due to the low solubility of CHO cell supernatant proteins in the ATPS, high PEG molecular weights applied in literature [5] are not applicable for the current purification task. The PEG molecular weight was therefore varied over a range of 2000, 3000 and 4000 g/mol offering the opportunity of lower viscosity which is favorable for continuous application. The pH value was adjusted to six, seven and eight. Since additional NaCl and sample loading of cell supernatant were determined to significantly influence the purification of mAbs in ATPS [4–6], their influence on IgG1 purification and on the phase equilibrium is investigated. Finally, the influence of phase ratio for the best determined conditions is investigated to define promising extraction conditions for the purification of IgG1 from cell supernatant.

2. Materials and methods

2.1. Materials

For the preparation of single stage ATPE experiments, stock solutions of both types of phase forming components (PFC) were prepared. For that purpose, PEG 2000 for synthesis with an average molecular weight ranging from 1900–2200 g/mol, PEG 3000 for synthesis with an average molecular weight ranging from 2700–3300 g/mol and PEG 4000 for synthesis with an average molecular weight ranging from 3500–4500 g/mol were purchased from Merck (Darmstadt, Germany). A 50 wt.% PEG-stock solution of each PEG molecular weight was prepared using the corresponding PEG and Millipore water in equal amounts.

The phosphate-stock solutions were prepared using two different types of phosphate-salts. Sodium dihydrogen phosphate dihydrate with a purity >99.8% was purchased from VWR Prolabo (Darmstadt, Germany). Furthermore, dipotassium phosphate trihydrate with a purity >99% was purchased from AppliChem (Darmstadt, Germany). Two different types of salt were used due to an improved solubility in water and the possibility of buffering the stock solution at various pH values depending on the ratio of salts. Three 25 wt.% phosphate-stock solutions were prepared at different pH values of six, seven and eight. For ATPE experiments investigating the influence of an additional entrainer salt, NaCl with a purity >99.8% was purchased from Sigma–Aldrich (Steinheim, Germany).

A CHO cell supernatant containing IgG1 against cholera toxin with a molecular weight of around 150 kDa and an isoelectric point of 8.33 was provided by Bayer Healthcare (Leverkusen, Germany). IgG1 concentration in the cell supernatant amounted to 440 mg/kg. Furthermore pure IgG1 against cholera toxin with a concentration of 10 g/kg buffered in phosphate buffered saline was applied for ATPE experiments and calibration of Protein A chromatography. Pure IgG1 was provided by Bayer Healthcare.

2.2. Analytics

Each top phase (TP) and bottom phase (BP) of an ATPE experiment was analyzed with regard to PFC- and IgG1-content. Furthermore, the purity of a sample was determined. Analytical methods mainly comprise the analysis by high performance liquid chromatography for the detection of PEG of any molecular weight, phosphate, chloride, IgG1 and purity. Phase masses were measured using a scale AB304-S by Mettler-Toledo (Greifensee, Switzerland) with an accuracy of ±0.001 g. Density was measured with a Densito 30PX Portable Density Meter by Mettler-Toledo (Greifensee, Switzerland) with an accuracy of ±0.001 g/cm^3. The pH value