Clarification and capture of monoclonal antibodies from complex media using aqueous two-phase

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Monoclonal antibodies (mAbs) are within the most important biopharmaceutical products of the pharmaceutical industry. The demand to efficiently supply the biopharmaceutical market with mAbs, led to the need for production processes that rapidly produce large quantities of pharmaceutical monoclonal antibodies at moderate costs and in a consistent and reproducible manner. Major limitations in current manufacturing platforms of mAbs are no longer found upstream but in the downstream processing (DSP). Challenges in purification include developing robust purification processes with integration of the upstream and downstream, allowing efficient, sustainable and cost-effective processes. Aqueous-two phase systems (ATPS) shown to be a valuable alternative to the established platforms due to its relatively easy scalability, capacity of continuous operation and high capacity. Besides that, clarification, concentration and purification can also be achieved in just one step, using a biocompatible environment.

In this work, the design of an innovative downstream process was developed based on an aqueous two-phase systems step for the purification of mAbs from a complex medium, comprising cell separation and antibody selective extraction, envisaging process integration and intensification. Subsequently, it was also performed a polishing step of cation exchange chromatography, in order to increase the purity of the antibody. Partition studies of mAbs from CHO cell supernatants were investigated using different types of ATPSs, namely Polyethylene glycol (PEG)- Sodium polyacrylate (NaPA), PEG-dextran and PEG-Choline chloride. The effect of the ligand LYTAG-ProA was also evaluated in the partition of the antibodies. PEG-NaPA systems showed high yields of extraction of mAbs (without the use of ligand-LYTAG ProA) and shown to be an optimal system for the clarification of cells, with 100% of elimination of cells from the IgG rich phase.

Keywords: Aqueous two-phase systems; monoclonal antibodies; downstream processing; cell clarification

Introduction

Nowadays with major rapid advancements in genetic sequencing and the translation of basic medical sciences research into clinical practice, humanized mAbs are now the fastest growing group of biotechnology-derived molecules in clinical trials. Thanks to robust market demand, approval of new products and new indications as well as launch of mAbs generic drugs, the global mAbs market size will ascend by more than 12% in 2013-2017, hitting in 2017 $141 billion. By July 2014, a total of 36 mAbs were approved by the FDA and 7 were still under review for use in humans for treating several diseases and conditions including: cancer, chronic inflammatory diseases, transplantation, infectious diseases and cardio-vascular diseases.

However, besides their high rate of success, these biopharmaceuticals are also amongst the most expensive drugs available in the market. The increasing product demands in combination with a market introduction of biosimilars call for less and less expensive products in order to remain competitive. Hence, the current rather expensive production processes need to be improved significantly and the downstream processing area needs to adopt new efficient and cost effective separation and purification methodologies.

Significant progress has been achieved by optimization of upstream processing (USP) in the last two decades: process efficiency, achievable cell densities and product titers were increased enormously in cell culture processes by developing recombinant technologies as well as media and process control strategies. So, the greatest capacity constraints in current manufacturing platforms of mAbs, are no longer found in the upstream production processes, but in the downstream purification (DSP) area. DSP has been considered responsible for the major cost factor with 50-80% of total production costs.

Challenges in the purification of bioproducts include developing robust purification processes that allow integration of the upstream and downstream, in order to develop efficient, sustainable and cost-effective processes.

Currently, the established platform for the purification of mAbs usually includes three chromatographic steps, in which the mAb is firstly adsorbed to an affinity resin, almost invariably a protein A (ProA) affinity column, followed by two further chromatography steps, which will allow the removal of the remaining host cell proteins, DNA, leached proA and aggregates, as well as providing an adequate level of overall viral clearance. ProA chromatography takes advantage of the highly specific interaction between the Fc region of mAbs and immobilized ProA, which is a cell wall component of Staphylococcus aureus, rendering purities greater than 98% in a single step. However, ProA affinity chromatography does suffer from several limitations, being the high cost of the resin the worst enemy – which can be up to 10 times as expensive as conventional chromatographic supports.

In an attempt to overcome the limitations posed by ProA affinity chromatography, various non-chromatographic alternatives purification protocols have generated long-standing interest. Flocculation, precipitation, two-phase extraction, membrane processing and crystallization are non-chromatographic techniques that can also be used for the purpose.

Liquid–liquid extraction based on aqueous two-phase systems (ATPSs) has shown a great potential and promising choice for
the downstream processing of biopharmaceuticals, such as monoclonal antibodies, high density lipoproteins, hormones, cytokines, growth factors and plasmid DNA.

The first report in the literature of biphasic systems composed mainly by water was in 1986 by Beijerinck. He noticed a certain immiscibility in solutions of agar with soluble starch or gelatin, and upon mixing he observed that they separated into two immiscible phases. Lately, in 1958, Per-Åke Albertsson reported ATPSs as a good method for separation and purification of cell particles and biomolecules.

Aqueous two-phase systems are formed when two particular chemically different components, such as two polymers (e.g. PEG and dextran) or a polymer and a salt (e.g. PEG and phosphate) are mixed at a concentration higher than a critical value, so that they separate into two phases at equilibrium. In contrast to the conventional organic-aqueous phase systems, these systems form a gentle environment for biomolecules as, in one hand, the bulk of both phases consists mainly of water and, in the other hand, most of the polymers used have a stabilizing effect on the protein tertiary structure and biological activity. Furthermore, good resolution and yields can be obtained simply by varying certain experimental conditions such as pH, ionic strength, and polymer molecular weight. If properly optimized, this technique also provides integration of clarification (removal of cells debris), concentration, and partial purification, reducing the number of downstream processing steps and improving the overall yields and costs of the recovery process, which is especially important in the production of high valuable recombinant proteins.

Sometimes integration of ATPSs with other different techniques to accomplish more effective purification of enzymes is a common procedure. In mAbs downstream processing, after mAb capture, ion exchange chromatography can be used as a polishing step. Nevertheless, the application of ATPSs at process scale has been hampered by the complexity of the system combined with the fact partition mechanisms are poorly understood and that the method development is fairly empirical.

Affinity partitioning is based on the preferential/biospecific interaction between the molecule and affinity ligands. This interaction results in a biomolecule-affinity ligand complex which selectively partitions to one of the phases leaving the contaminating substances or proteins in the other phase.

In 2008, Maestro et al. described a novel procedure for affinity partitioning of recombinant proteins fused to the choline-binding module C-LytA (C-terminal module of LytA) in ATPS containing PEG. C-LytA constitutes the C-terminal part of the LytA amidase from Streptococcus pneumonia, an enzyme that catalyses the cleavage of the N-acetylmuramoyl-L-alanine bond of the peptidoglycan backbone. The C-LytA module is the major representative of the choline-binding domain family. Hence the affinity of C-LytA for choline and choline structural analogues allows its use as an efficient affinity tag for single-step purification of hybrid proteins.

Maestro et al. showed that C-LytA may bind to PEG molecules in the ChBS, which can be used to accumulate C-LytA or C-LytA-tagged proteins in the PEG phase, whereas addition of choline reverses this interaction and directs the protein to the PEG-poor phase.

LYTAG two-phase purification system is a protein purification system, patented by Biomedal, based on the use of two aqueous components. The method relies on the affinity of the protein tag LYTAG (an improved mutant of C-LytA) for one of the two-phase components, allowing recombinant protein separation and purification from cellular extracts or culture media. In the procedure, the LYTAG-fused protein is retained in one of the aqueous phases while most of the undesired proteins can be removed by simply discarding the opposite phase. After replenishing the system with fresh phase, the protein of interest can be easily recovered in it, with high purity, by reversing its localization with the addition of choline, the specific LYTAG ligand. This system is particularly well suited for biotech industries and laboratory specialized in protein separation and purification, as it is time saving (separation can be completed within few minutes, minimizing the effect of proteases), simple, cost efficient, and highly versatile for scaling up protein purification process of recombinant LYTAG fusion proteins, representing a convenient alternative to solid resins.

By fusing LYTAG ligand with Protein A, a potential process for selective purification of mAbs from a supernatant or a CHO cell culture is created. LYTAG-ProA-mAb complexes will accumulate in PEG rich-phase, with a high purity grade, while further proteins will partitioning to the other phase.

Materials and Methods

Chemicals and Biologicals

Polyethylene glycol (PEG) with molecular weight of 3350 and sodium polycrylate (NaPA) with molecular weight 8000 (45% in H₂O) were purchased from Sigma (St. Louis, MO, USA). Dextran 500 kDa was purchased from Fluka (Buchs, Switzerland). All polymers were used without further purification. Potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium phosphate dibasic anhydrous, sodium phosphate monobasic anhydrous, sodium chloride, DL-dithiothreitol (DTT), ammonium persulfate (APS) and N.N.N',N'tetramethylethylenediamine (TEMED) were obtained from Sigma. Choline chloride was purchased from Alfa Aesar (Karlsruhe, Germany). Hydrochloric acid (HCl) 37% were obtained from Fluka (Buchs, Switzerland). Sodium acetate was obtained from Merck (Darmstadt, Germany). Acetic acid 100% was purchased from Fisher Scientific (Hampton, New Hampshire, USA). All other chemicals were of analytical grade. Water used in all experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

Human immunoglobulin G (IgG) for therapeutic administration (product name: Gammanorm®) was obtained from Octapharma (Lachen, Switzerland), as a 165 mg/mL solution containing 95% of IgG. Bovine serum albumin (BSA) standards (2 mg/mL) and Coomassie Plus (Bradford) Protein Assay were purchased from Thermo Scientific Pfizer (Rockford, IL, USA).

CHO cell supernatant was produced and delivered by the research team of IcoSagen SA, partner of the European Project – INTENSO. This supernatant contains a humanized monoclonal antibody, from IgG1 class. This antibody is derived from mouse anti-hepatitis C virus subtype 1b NS5B (nonstructural protein 5B) monoclonal antibody 9A2 expressed in mouse hybridoma culture. cDNA of antibody variable regions were isolated and cloned into the human IgG1 constant region-containing antibody expression vector. The CHO cells were grown in a mix of two serum-free growth media, the CD CHO Medium (Gibco®, Carlsbad, CA) and the 293 SFM II Medium (Gibco®). Final concentration of IgG is approximately 0.300 g/L.

Hybridoma cell culture were supplied by research assistance Sara Rosa from TagusPark IST. The cells were grown in a mix of two different media. The mixture is composed of 25% (v/v) of Dulbecco’s modified Eagle’s medium (DMEM) (Gibco®) and 75% (v/v) of CD Hybridoma Medium (Gibco®). Both media were filtered with a 0.22 µm of diameter. Due to the low concentration of IgG in this supernatant, pure IgG was added to a final concentration of 0.300 g/L in order have the same concentration of IgG in supernatant. Human IgG for
therapeutic administration from Octapharma was used for this supplementation.

LYTAG-Protein A is a recombinant protein produced, purified and delivered by Biomedal (partner of the European Project – INTENSO). The protein was produced and purified using the “LYTAG Two-Phase Purification System” developed by Biomedal®. The LYTAG-Protein A delivered by Biomedal has only one Z domain – the LYTAG-1Z.

**Aqueous Two-Phase Systems**

Aqueous two-phase systems of a final weight of 5 g and 10 g were prepared by weighing out appropriate amounts of components from stock solutions. Stock solutions of 50% (w/w) PEG 3350, 25% (w/w) dextran 500 kDa and 50% (w/w) choline chloride were prepared by dissolving the appropriate amounts of the polymers in milli-Q water.

**PEG-NaPA Systems**—The concentration of PEG and NaPA in the system was 8% and 6%, respectively. 11% of a 5 M NaCl stock solution was added to the systems, making a final concentration of 550 mM of NaCl in the ATPS. The loading of the protein feedstock solution (pure IgG, Icosagen supernatant or hybridoma cell culture) represents 35% of the mass of the system. Finally, water is added in order to achieve the desired final system composition. To study the effect and effectiveness of the ligand LYTAG-ProA, systems containing 15% (w/w) of the ligand were also prepared.

**PEG-Dextran Systems**—The concentration of PEG 3350 and dextran 500k in the system was 6% and 7%, respectively. The loading of the protein feedstock solution (pure IgG, Icosagen supernatant or hybridoma cell culture) represents 35% of the mass of the system. Finally, water is added in order to achieve the desired final system composition. To study the effect and effectiveness of the ligand LYTAG-ProA, systems containing 15% (w/w) of the ligand were also prepared.

**PEG-Choline chloride Systems**—The concentration of PEG 3350, choline chloride and phosphate buffer in the system was 24%, 7% and 5.5%, respectively. The loading of the protein feedstock solution (pure IgG, Icosagen supernatant or hybridoma cell culture) represents 35% of the mass of the system. Finally, water is added in order to achieve the desired final system composition. To study the effect and effectiveness of the ligand LYTAG-ProA, systems containing 5% (w/w) of the ligand were also prepared.

All ATPS were prepared in 15 ml graduated falcon tubes in order to measure properly the volume of the phases after the separation. For each system, a blank was also prepared, which has the same phase forming components compositions, but without the protein loading (with water instead). The phase components were thoroughly mixed on a vortex agitator (Ika, Staufen, Germany). Then, the tubes were left to equilibrate for a few minutes at room temperature and, afterwards, were centrifuged for 5 min in a fixed angle rotor centrifuge (Eppendorf, Hamburg, Germany) at 4000 rpm, to ensure total phase separation. For systems containing hybridoma cells, the centrifugation step was skipped, since the goal is to remove the cells without using a centrifugation step. Finally, phase volumes were measured and top and bottom phases were carefully separated and taken for further analysis.

**Cation Exchange Chromatography**

For this polishing step two different chromatographic media were used: a strong cation exchange fibrous column and a HiTrap SP FF pre-packed column. Strong cation exchange fibrous column were delivered by Jacobs University Bremen, in Germany, where they were fabricated. They are Biotoolomics 1 mL column with 3.3 cm of bed height and 0.62 cm of column inner diameter. The adsorbent material of these columns is mostly cellulose-based, but some are non-woven composites of natural and synthetic fibers. The OH- groups are quite prevalent in these adsorbents, terminal epoxy groups are used to bring in IEX functionalities. HiTrap SP FF is prepacked with SP Sepharose Fast Flow and is a strong cation exchanger for small-scale protein purifications purchased from GE Healthcare (Uppsala, Sweden). It has 1 mL of bed volume and bed dimensions of 0.7 cm × 2.5 cm. The matrix is composed by 6% highly cross-linked agarose and has a particle size distribution of 45 –165 µm. The ligand is Sulphopropyl.

The chromatography runs using both columns were performed on an Äkta™ Purifier 10 system from GE Healthcare. The data collection and processing was accomplished using Unicorn 5.1 software. Conductivity, pH and UV absorbance at 280 nm of the samples were continuously measured.

The columns were equilibrated with 5 column volumes (CV) of 20 mM sodium acetate buffer pH 5, 6 or 7 (depending on the runs), prior to injection at 1 mL/min. ATPS phases containing IgG were injected at 1 mL/min using a 1 mL sample loop. After washing unbound compounds with 5 CV of the equilibration buffer, elution of bound components was triggered by increasing salt concentration from 0 to 1 M NaCl at 1 mL/min. Column flowthrough and eluate were continuously collected as 1 and 0.5 mL fractions, respectively, in a FRAC 950 fraction collector from GE Healthcare. The recovery yield of IgG was determined by the ratio between the mass of IgG in the eluted fractions and the mass of IgG initially loaded in the column.

**Diafiltration**

Due to the high conductivity of the bottom phases of PEG-choline chloride ATPS, the phases had to be subjected to a diafiltration step utilizing an Amicon Ultra 0.5 mL (Millipore, Bedford, MA, USA) centrifugal filters containing a 10 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane. Approximately 0.5 mL of bottom phase solution was placed in the filter unit and centrifuged (Eppendorf, Hamburg, Germany) at 14000 g for 30 minutes. After this first centrifugation the permeate solution was collected and approximately 0.5 mL of the adsorption buffer, used in the chromatographic purification, was added to the filter unit and centrifuged using the same centrifuge at the same conditions. This step is repeated two times more. In total, 4 centrifugation steps are performed. The solution was maintained at 4°C during the centrifugation process. In the end of the last centrifugation, the 0.5 mL of adsorption buffer are added to the filter unit, resuspended with pipette several times and then collected to an eppendorf for further use.

**IgG Quantification**

The concentration of IgG was determined by affinity chromatography in ÄKTA™ 10 Purifier system from GE Healthcare using an analytical POROS Protein G affinity column (2.1 x 30 mm) from Applied Biosystems (Foster City, CA, USA). Samples containing IgG were diluted 10 times in the adsorption buffer – 50 mM sodium phosphate buffer at pH 7.4 containing 150 mM NaCl – and 0.5 mL were injected in the column using an autosampler A-900 from GE Healthcare. Elution was triggered by decreasing the pH to 2–3, using 12 mM HCl with 150 mM NaCl during. Absorbance was monitored at 280 nm.

**Total Protein Quantification**

Total protein concentration was determined by the Bradford method using a Coomassie Plus kit from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA) was used a standard for protein calibration. The assays were set up in 96-well polystyrene microplates and the absorbance was read at 595 nm in a Spectramax 384 Plus microplate reader from Molecular Devices (Sunnyvale, CA, USA). To avoid interference from PEG and NaPA, all samples from top and bottom phases were analyzed against blanks systems containing the same phase composition. The IgG purity was determined by the ratio between the concentration of IgG
determine from Protein A HPLC and the total protein concentration.

**Protein Gel Electrophoresis**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to evaluate qualitatively the purity of the ATPS and CEX fractions. Samples were diluted in a sample buffer containing 62.5 mM Tris-HCl pH 6.2, 2% SDS, 0.01% bromophenol blue and 10% glycerol and denatured in reducing conditions (0.1 M DTT), at 100°C for 5 minutes. Samples were applied in n a 12% acrylamide gel, prepared from a 40% acrylamide/bisacrylamide stock solution (29:1), and run at 90 mV using a running buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS at pH 8.3. Gels were stained with Coomassie Brilliant Blue R-250 for 1 hour and destained by successively washing the gels with 30% (v/v) ethanol and 10% (w/v) acetic acid solution.

**Cell Counting**

To determine the total number of cells present in the top and bottom phase, the cells present in both phases (live or dead) were counted using a hemocytometer under an optical microscope Leica DMLB (Wetzlar, Germany) by means of trypan blue dye (Gibco®) exclusion method. After counting, phases were also visualized in an Olympus CKX41 (Tokyo, Japan) microscope by directly putting the phases in a 96 well plate in order to make a live streaming visualizations of the phases. Digital images acquisition was done with CellSens Entry software (Tokyo, Japan) and treated with ImageJ software. 

**Extraction Performance Parameters ATPS**

To evaluate the extraction performance of the ATPS, different parameters are used. These include the partition coefficient, Kp, which is the ratio of the concentration of the protein in the top phase to that in the bottom phase; the recovery yield of IgG in the top phase, Y_{top}, defined as the ratio between the mass of IgG on the top phase and the total mass of IgG added to the ATPS; the recovery yield of IgG in the bottom phase, Y_{bottom}, defined as the ratio between the mass of IgG on the bottom phase and the total mass of IgG added to the ATPS; the purity of IgG in the top and bottom phase, P_{top} and P_{bottom}, given by the ratio between the concentration of IgG (top or bottom phase) and the concentration of total protein in that same phase, expressed in percentage and the volume ratio, VR, defined as the volume ratio between the top and bottom phase.

**Results**

The partition of pure IgG, IgG from Icosagen supernatant and IgG from Hybridoma cell culture was analyzed in PEG-NaPA, PEG-Dextran and PEG-Choline chloride ATPS in the presence, and absence of LYTAG-ProA ligand. IgG partition coefficients, recovery yield and purities were assessed.

**Evaluation of PEG-NaPA ATPS**

Systems with 5 g total and composed of 8% PEG 3350, 6% of NaPA 8000, 550 mM of NaCl and 10 mM of phosphate buffer at pH 7 were prepared to assess the partitioning of mAbs between the two phases. These conditions have been previously established as the best conditions for this ATPS\(^2\). Experiments with and without LYTAG-ProA were conducted and for each feedstock triplicates were made.

It is important to acknowledge that in PEG-NaPA ATPSs top phase is the PEG-rich phase and the bottom phase is the NaPA-rich phase.

According Figure 1, it is evident that regardless of the type of feedstock used in the ATPS, mAbs partitions toward the top phase, i.e., the PEG-rich phase. This is evidenced by values of Log Kp higher than 1. 

![Figure 1](image1)

**Figure 1** — Effect of LYTAG-ProA on IgG partitioning (Log Kp) in PEG-NaPA systems. System composition: 8% PEG 3350, 6% NaPA 8000, 10 mM of phosphate buffer pH 7, 500 mM of NaCl, 35% of feedstock and 15% of LYTAG-ProA (only for systems with ligand, marked as + LYTAG). Results were obtained after quantification of IgG in each phase, by affinity chromatography.

**Figure 2** shows also that the partition of mAbs for the top phase is higher when LYTAG-ProA ligand is present in the system. This result was expected since the purpose of LYTAG-ProA is to pull mAbs to the PEG-rich phase (the polymer for which LYTAG has affinity). When comparing the three different feedstocks, it is perceived that Log Kp values are lower for Icosagen Supernatant, but in general the differences are minimal.

![Figure 2](image2)

**Figure 2** — Effect of LYTAG-ProA on the IgG recovery yield for each phase of PEG-NaPA systems. System composition: 8% PEG 3350, 6% NaPA 8000, 10 mM of phosphate buffer pH 7, 500 mM of NaCl, 35% of feedstock and 15% of LYTAG-ProA (only for systems with ligand). Results were obtained after quantification of IgG in each phase, by affinity chromatography. The IgG recovery yield in each phase was achieved by the ratio of the IgG mass in the phase by the IgG mass in the feed extract.

In respect of mAbs recovery yields (see Figure 2), PEG-NaPA ATPS without LYTAG-ProA have top phases yields of around 100% and bottom phase yields of about 5%. However, for systems with LYTAG-ProA, top phase yields are consistently lower. Slight decreases in top phase yields are verified while the low yields of the bottom phases remain unchanged. Global yields higher than 100% were obtained and might be due to interference of the polymers, namely NaPA, in the analytical determination of mAbs concentration. After multiple uses of the column, and due to the high viscosity of the NaPA, polymer debris can accumulate inside the analytical column and influence the affinity of the ligand to IgG. Therefore, one must be aware that the values may be slightly overestimated.

These results might suggest that, although LYTAG-ProA ligand is able to actually increase the partition of mAbs for the
PEG-rich phase, it may also be responsible for inducing protein precipitation, and therefore responsible for decreases in the total yields.

SDS-PAGE analyses (data not shown) also confirmed the presence of mAbs in the top phases, together with LYTAG-ProA and with other impurities (for systems with Icosagen and hybridoma cell culture). Moreover, no proteins were seen present in the NaPA rich-phases, which corroborates the 100% yields obtained in the top phases. For ATPSs with hybridoma cell culture feedstock, mAbs were also present in the top phase together with other impurity proteins but a few impurities seem to appear in the bottom phases.

According Bradford analyses, for Icosagen and Hybridoma cell culture systems without the ligand, top phase purities range between 47% and 49%. In the presence of the LYTAG-ProA, top-phase purities increase from 47% to 51% for Icosagen systems and from 49% to 51% in Hybridoma cell culture systems.

In this PEG-NaPA system there is no evidence that LYTAG-ProA has any beneficial effect on the partition, since the system itself already presents very good partition of IgG to the top phase. Moreover, the top phase yields obtained without LYTAG-ProA decrease in the presence of the ligand.

**Evaluation of PEG-Dextran ATPS**

ATPS of 5 g total mass and composed of 6% PEG 3350 and 7% of Dextran 500 kDa were prepared to assess how mAbs partition between the two phases. The conditions selected had been previously established as the best conditions for this ATPS\(^5\). Systems with and without LYTAG-ProA were also prepared and for each feedstock triplicates were made.

For the PEG-Dextran ATPS, it is observed that in the absence of the ligand the antibody partitions more or less evenly between the top and bottom phases. This is indicated by the Log Kp values very close to zero – varying between -0.2 and 0.3 (see Figure 3 A). Still, for pure IgG and Icosagen feedstock, IgG partitions preferentially to bottom dextran-rich phase, as expected. For hybridoma cell culture, IgG seems to partitions preferentially to the top PEG-rich phase. This difference of partitions may be due to the differences on the pH and salt concentration in the different feedstocks, two important parameters that have been shown to be significant for the partition between the two phases\(^5\).

When repeating the same systems in the presence of LYTAG-ProA ligand, a considerable improvement in the partition of the IgG to the top phase is noted. For all the three feedstocks Log Kp values increased substantially, being more evident for the case of hybridoma cell culture (Log Kp = 2.5). These results show that LYTAG-ProA can change the partition of antibodies in PEG-Dextran ATPS and can effectively push antibodies into the PEG-rich phase.

Concerning the recovery yields (see Figure 3 B), systems without LYTAG-ProA present similar yields in the top and bottom phases, for the three feedstocks (yields range from 40 to 60%). For systems with pure IgG and Icosagen supernatant low precipitation was noticed, but for systems with hybridoma cell culture total yields does not exceed 70%, indicating that around 30% of the antibody precipitates. When LYTAG-ProA is added, there is an increase of the top phase yield with a concomitant decrease of the bottom phase yield. However, and as previously observed in PEG-NaPA ATPS, the total yield is lower than the total yield observed systems without LYTAG-ProA, thus confirming that the ligand may induce antibody precipitation. Nevertheless, the use of LYTAG-ProA in this PEG-Dextran system is advantageous as it concentrates the majority of the antibody in the PEG phase. In PEG-NaPA systems, without the ligand, IgG already partitioned preferentially to the top phase, while in PEG-dextran systems, IgG is distributed in both phases. So, the presence of LYTAG-ProA enhances IgG partition to the top phase, enhancing the recovery yield of the antibody in the PEG-rich phase.

![Figure 3](image)

**Evaluation of PEG-Choline chloride ATPS**

Preliminary studies showed that the best PEG-choline composition was 24% of PEG 3350, 7% of choline chloride and 5.5% of phosphate buffer pH 8. It was seen that in the absence of phosphate buffer, the two phases only formed at higher concentrations of PEG. The experiments with and without LYTAG-ProA were conducted and for each feedstock triplicates were made.

![Figure 4](image)
notorious that in the presence of LYTAG-ProA the partition coefficients decrease, indicating that some IgG is pushed towards the top phase. These results were not the expected, since LYTAG-ProA has a natural affinity towards choline, which is in the bottom phase. However, since PEG mimics the natural ligand and is present in higher concentrations, part of the ligand LYTAG-ProA may partition towards the top phase, thus decreasing the partition coefficient. Another possibility is related to the fact that the top phase also contains a small concentration of choline salt and which will drive some antibody molecules to the top phase.

Figure 5 — Effect of LYTAG-ProA on the recovery yield (B) for each phase (T: Top phase; B: Bottom phase) in PEG-Choline chloride systems. System composition: 24% PEG 3350, 7% choline chloride, 5.5% of phosphate buffer at pH 8, 20% of feedstock and 5% of LYTAG-ProA (only for systems with ligand, marked as + LYTAG). Results obtained after quantification of IgG in each phase, by affinity chromatography. IgG concentration was determined from a calibration curve obtained using Gammanorn IgG as a standard. IgG recovery yields (Figure 5) in the bottom phase of around 90% are obtained with this systems and little or no precipitation seems to occurs. With LYTAG-ProA bottom phase yields decrease while top phase yields increase, being verified once again the possibility of LYTAG-ProA to be responsible for inducing protein precipitation. The use of LYTAG-ProA in this systems is not justified since IgG recovery yields decrease.

Purity of both phases with and without LYTAG-ProA was only evaluated through SDS-PAGE electrophoresis (data not shown), since the protein quantification by Bradford assays were subjected of interference by the phase forming components — probably derived to the present of phosphate and choline — giving origin to inconsistent results, with no observable pattern.

In the SDS-PAGE gels (data not shown) it was possible to confirm, for systems with pure IgG, that all the IgG is present in the bottom phase either in the presence and absence of the ligand. For Icosagen supernant systems, without LYTAG-ProA, very faint bands can be seen in the top phase but it is in the bottom phase that IgG and other impurities are concentrated. With LYTAG-ProA very faint bands are also seen but the stronger bands are in the bottom phase.

Clarification of Hybridoma Cell Culture

After studying the partition of the antibodies, the next step consists in evaluating the feasibility of the three systems for the clarification of the cell culture medium, envisaging process integration.

The development of an integrated purification of mAbs directly from the hybridoma cell culture medium will eliminate the clarification step typically performed by centrifugation or filtration, allowing the cell culture medium to be processed directly from the bioreactor to the ATPS system. In this way, not only expensive and time-consuming steps are eliminated but also many of the shortcomings of centrifugation and filtration that arise due to the high viscosity and heterogeneous distribution of particle size are circumvented. The main goal was thus the separation of cells from the mAb product.

Top and bottom phases and interfaces pictures of the three ATPS were captured (see Figure 6) and cells were counted (see Table 1).

Microscopy images show that, for PEG-NaPA ATPS, all the cells stay in the interface. No cells are seen neither in the top nor in the bottom phase. For PEG-Dextran, the majority of the cells stay in the interface, but it is also possible to observe a few cells in the top and bottom phases. Regarding the PEG-Choline chloride system, cells seem to be present in all the three phases, but mostly in the interface and in the bottom phase. For a quantitative analysis the cells were counted and the results are present in Table 1.

The cell counting corroborates what was observed in Figure 6. but it is important to note that the counting method may not be 100% accurate since it involves very small volumes of
heterogeneous solutions such as cell suspensions. Furthermore, when separating the phases it is almost impossible to assure that there is no contamination between phases.

Table 1 – Percentage of cells in top, bottom and interface and the concentration of IgG in top and bottom phase in PEG-NaPA, PEG-Dextran and PEG-Choline chloride ATPS. Results are displayed as mean ± STDV.

<table>
<thead>
<tr>
<th>PHASE</th>
<th>%Cells TOP PHASE</th>
<th>%Cells INTERFACE</th>
<th>%Cells BOTTOM PHASE</th>
<th>[IgG] mg/L TOP PHASE</th>
<th>[IgG] mg/L BOTTOM PHASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-NaPA</td>
<td>0.0 ± 100 ±</td>
<td>0.0 ± 0.0 ±</td>
<td>0.0 ± 5.1 ±</td>
<td>297.5 ±</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>PEG-Dextran</td>
<td>5.0 ± 81.0 ±</td>
<td>14.0 ± 0.5 ±</td>
<td>184.7 ± 4.4 ±</td>
<td>27.2 ±</td>
<td>4.3 ±</td>
</tr>
<tr>
<td>PEG-Choline</td>
<td>0.1 ± 69.0 ±</td>
<td>25.0 ± 0.2 ±</td>
<td>14.1 ± 0.6 ±</td>
<td>313.3 ±</td>
<td>5.6 ±</td>
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In PEG-NaPA systems 100% of the cells go to the interface and almost 100% of the IgG is in the top phase, which indicates PEG-NaPA ATPS presents itself as an excellent system for integrated clarification, with partitioning of cells and mAbs to opposite phases.

In PEG-Dextran 81% of the cells stay in the interface and 5% and 14% are in the top and bottom phase, respectively. Since only 5% of the cells are in the top phase – a number that can be considerable negligible – and almost of the IgG is in the top phase, this PEG-Dextran ATPS can be considered a very good system for integrated clarification.

For PEG-Choline chloride, the counting shows that the highest percentage of cells is in interface (69%) and only 6% of cells are in the top phase. In the bottom phase, where the IgG is concentrated, 25% of the cells are present. These 25% of cells in the IgG-rich phase are considered a quite high number and therefore, despite the very high recovery yields obtained in the ATPS extraction, this is not a well suited system for clarification of cells. In order to proceed with the polishing step with CEX chromatography bottom PEG-rich phase was subjected not only to a filtration step, to eliminate the cells of the bottom phase, but also to a diafiltration step in order to decrease the high conductivity (30,000 mS/cm) due to phosphate and choline chloride salts.

Polishing through CEX Chromatography

Cation exchange chromatography was selected for the second purification step. IgG-rich phases of the three types of ATPS were loaded on two different CEXs columns: i) Hitrap SP FF column and ii) Fibers column. All the fractions corresponding to the flowthrough peak were pooled into one (the FT fraction), as well as all the fractions corresponding to the elution peak (the E fraction).

PEG-NaPA—Top phases of PEG-NaPA ATPS were directly loaded on the two different CEXs columns. Obtained chromatograms are shown in Figure 7.

Chromatograms present in Figure 7 A and B both that all the majority of the sample components are eluted in the flowthrough step, which means that the proteins did not interact with the column. Off-line analysis also shown that the antibody was present on the flowthrough fraction and consequently the separation of IgG was not successful. This result can be explained considering the high conductivity values of the sample (around 40 mS/cm). On CEX columns charged molecules are only able to bind to ion exchange resins at low ionic strength, e.g. less than 5mS/cm. In the chromatograms is possible to see a considerable increase in the conductivity as the sample flows through the column and passes the conductivity detector. These high values of conductivity are probably to the presence of the NaCl added to the system (0.55 mM).

Hence, free salt PEG-NaPA ATPSs with the same composition were prepared but a two phase system was not formed. According the literature, at relatively low concentrations of polymers (below 10%) PEG-NaPA two-phase systems are only formed in the presence of a certain concentration of salt, due to entropy penalty upon compartmentalization of counter ions.

More concentrated systems systems were then prepared, including 10%PEG-10%NaPA, 10%PEG-12%NaPA and 12% PEG-12%NaPA ATPSs. Nevertheless, the yields obtained were much lower, a lot of protein precipitation was observed and consequently, it is not feasible to conduct a polishing step by CEX chromatography.

PEG-Dextran—Since PEG-Dextran systems are characterized by low ionic strengths, the feasibility of loading the ATPS top phases directly on the Hitrap SP FF column and Fibers columns was explored.

The chromatogram corresponding to the purification of IgG with the cationic fibers column, present in Figure 8, display a large peak found in the flowthrough region of the chromatographic profile, corresponding to the negatively charged proteins that were repelled by the negative charges of the resin. As the elution buffer is added and the conductivity increases, the positive charged proteins, which interacted with the resin, start to be eluted, and thus, the elution peak is obtained.

As it was seen in the beginning of the study, the major impurity of the hybridoma cell culture is the albumin, which has a pl of 4.8. At pH 7, this protein is negatively charged so, it is supposed to not interact with the resin and to be eluted in the flowthrough region.
The elutions peaks are very similar for both Lytag treatments. The presence of Lytag resulted in a slightly different band pattern compared to the control (no ligand treatment). Bands corresponding to the IgG are more intense and distinct in the presence of Lytag, indicating a higher purity of the purified IgG. This shows that Lytag aids in the separation and purification process, allowing for a clearer visualization of the target protein bands.

According to the chromatography results, the gel confirms the presence of albumin and other proteins in the lanes 3 and 6, i.e., FT fractions with and without the ligand. However, very faint bands corresponding to some impurity proteins near 75 kDa are also seen together with the IgG bands, in the eluate fractions (lanes 4 and 7). This means that IgG purity achieved in the elution pool is not as high as expected.

The presence of Lytag-ProA seems not to affect the purification. The elutions peaks are very similar for both ATPS treatments. The purification of IgG directly from the top phase of the PEG-Dextran ATPS was also performed with a cationic exchange column Hitrap SP FF, for comparison. The obtained chromatogram (data not shown) was very similar to the one obtained with the Fibers column and the SDS-PAGE analysis presented the same level of purity.

Bradford analyses (data not shown) gave IgG purities of 78% and 81% of the elution fractions with Fibers and Hitrap SP FF columns for systems without Lytag-ProA and 86% and 78% again for the elution fractions with Fibers and Hitrap SP FF columns of the ATPS with Lytag-ProA. Despite antibody not being totally pure, the results obtained with the two columns were quite similar, which might suggest that fiber resins may be as good as beads resins when talking about IgG purification.

**PEG-Choline Chloride**—Cation exchange chromatography was performed to improve the purity of the antibody extracts. Diafiltrated IgG bottom phases were injected in the two previously referred columns. Adsorption and elutions buffers at three different pHs (5, 6, and 7) were evaluated, and the different chromatographic profiles are depicted in Figure 10. All the fractions corresponding to the flowthrough peak were pooled into one (the FT fraction), as well as all the fractions corresponding to the elution peak (the E fraction). SDS-PAGE analyses of both phases of the ATPS and of the FT and E fractions from the chromatography run are presented in Figure 11.
flowthrough and eluate fractions at pH 5, respectively, show that albumin is present in both the flowthrough and eluate fractions. In the eluate fraction (Lane 5) the two bands of IgG are also present.

As the pH increases, albumin becomes more negative and does not interact with the column, being recovered in the flowthrough. Lanes 7 and 8 of the Figure 11 shows that no albumin is present in the elutions fractions. Hence, only the two bands corresponding to the two chains of the antibody appear to be present in the eluate fractions.

PEG-NaPA, PEG-Dextran and PEG-choline chloride ATPSs were evaluated for purification of mAbs and cell clarification, all in one step. Since high purity values were not obtained by ATPS, a polishing step through cation exchange chromatography was performed. The performance of two different columns was evaluated for this step: a cationic fibers column, a new material still being studied for purification of biological products, and a conventional cationic beads columns HiTrap SP FF.

Moreover, affinity ATPS driven by LYTAG-Pro A were conducted. LYTAG-Pro A is a recombinant protein with affinity for mAbs and PEG at the same time.

Evaluating the effect of LYTAG-Pro A ligand, the results were quite consistent. For all the three types of ATPSs LYTAG-Pro A proved effectively to increase the partition of antibodies to the PEG-rich phase. This is confirmed by higher Log Kp values of the ATPSs with the ligand when comparing with the ones obtained without the ligand. However, results also show that the total recovery yields of mAbs decrease when the ligand is present in the ATPSs. This might suggest that LYTAG-Pro A is responsible for inducing protein precipitation. Therefore, the use of this ligand was proven to be advantageous only when IgG is, evenly distributed in both phases (as is the case of PEG-dextran ATPS), because LYTAG-Pro A will shift the partitioning of IgG to the PEG-rich phase, and thus increase the PEG-rich phase yield. To sum up, LYTAG-Pro A showed only useful for PEG-dextran systems, while for PEG-NaPA and PEG-Choline chloride the results were better in the absence of ligand.

According cell clarification, PEG-NaPA systems proved to be the best system since no cells were present in the IgG-rich phase. For PEG-choline chloride systems, a partial clarification was achieved 95% of cell clearance in the IgG-rich phase. For this last system, a filtration step was necessary to perform, in order to remove the cells from the bottom phase.

Unfortunately the purities obtained after ATPS were very low for all the three systems, so a cation exchange chromatography polishing step was performed. For PEG-NaPA systems this step was not possible to be performed successfully due to the high conductivity of the top-phase. It would be interesting, in the future, to perform a diafiltration before the loading into the column to see how the purity would increase after the chromatographic step. Or try to apply other type of chromatography since the ideal is to apply the ATPS phases directly in the columns.

For PEG-dextran the chromatographic runs were performed and purities of around 80% were achieved. In fact the purity increased but SDS-PAGE revealed that some impurities were still present together with IgG. Since the runs were only performed at pH 7, it would be important to try other adsorption buffer with other pH values, in order to optimize the process.

For PEG-Choline, although it was not possible to load direct the phase into the column and not managing to quantitatively analyze the purities, the gels reveal only the two bands of the IgG in the eluate fraction, which suggests a high purity.

Conclusions

Nowadays with major rapid advancements in genetic sequencing and the translation of basic medical sciences research into clinical practice, mAbs are the fastest growing group of biotechnology-derived molecules in clinical trials. To keep up this growth rate new methodologies for separation and purification of mAbs, that are efficient and at the same time cost effective, need to be adopted.

The work herein presented shows that aqueous two-phase systems have proven to be a suitable technique for the recovery of biological products, namely mAbs, and as well for media clarification, proposing a valid solutions for the problem of downstream processing of biologicals.

The similarity of the chromatographic profiles and gels obtained with the two columns as well as recovery yields, might suggest that fiber resins may be as good as beads resins when talking about IgG purification.
Although with this system very good yields have been obtained, it would be interesting to test other choline salts as ATPS components, such as choline bicarbonate or choline acetate.

An important problem to solve is the interference that choline causes on the SDS-PAGE runs and most importantly in the Bradford assays. It would be important to qualitatively confirm the high purities obtained.

When comparing the performance of the two columns the fibers material appears to be a valuable alternative to bead resins. Similar purities and recovery yields were obtained either for PEG-Dextran and PEG-choline chloride. Nevertheless, further studies with fibers columns should be performed, namely with free polymer supernatant to access the real dynamic binding capacity.

This study herein presented revealed that there is no perfect ATPS. It was proved that some ATPSs are very good for recuperation of mAbs, others are ideal for cell clarification, but unfortunately, even when using an affinity ligand, very high purities, similar to the ones obtained by protein A affinity chromatography, are hard to accomplish with only an extraction ATPS step. Not only optimization is still needed as it is necessary to innovate and experiment new materials and compounds.

Aqueous two-phase systems per se will never be able to compete with the affinity chromatography, a second polishing step will always be necessary. As future work, potential solutions could include the investigation of new chromatographic media for polishing steps. For instance, the development of enhanced capacity adsorption systems with high throughput and low pressure devices like fibers. For example, it would be interesting to try to modify the properties of polymer-based fibrous materials according to tailor made specifications.

Implementation of non-chromatographic affinity technologies such as membrane affinity filtration, affinity cross-flow ultrafiltration or even and affinity precipitation, can be interesting approaches to explore in the future.

References