Novel downstream processes for the purification of monoclonal antibodies based on aqueous two phase partitioning and hydrophobic interaction chromatography

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ABSTRACT

Monoclonal antibodies have proven their potential as therapeutic molecules with applications as diverse as diagnosis, oncology and treatment of autoimmune diseases; the need for their supply is higher than it ever was, with the global market for monoclonal antibodies being expected to grow to nearly $58 billion in 2016. The main challenge for this growth remains the fact these products need to be manufactured cost-effectively to meet the current demand and although productive and efficient upstream processes have been developed, the downstream processing has become the new bottleneck for monoclonal antibody production. Thus, it is crucial to improve the existing purification platforms. To address this problem several alternatives were tested in, starting from the screening of new possible aqueous two phase systems (ATPS) for affinity partitioning of monoclonal antibodies with the LYTAG ligand, followed by the intensive testing of the most promising system (PEG-ammonium sulfate) with different parameters and finally a purification process combining ATPS with PEG6000-ammonium sulfate followed by hydrophobic interaction chromatography (HIC) with different commercially available resins. The introduction of an affinity ligand aimed to improve ATPS selectivity whilst taking advantage of its inherent qualities of cost effectiveness, scalability and high capacity however the tested systems were fruitless in achieving a desirable purification. The HIC was introduced to increase the selectivity, still representing an accessible substitute to the traditional protein A chromatography. The combination of a 10%PEG6000-12% Ammonium Sulfate and a chromatography with HiTrap Butyl FF achieved of the best results with extraction yields up to 99% and purities as high as 96.5%.

1. Introduction

Monoclonal antibodies are nowadays the fastest growing group of biotherapeutics in clinical trials, one of the key factors contributing to their market growth is the prevalence of cancer and the limitations of existing alternative treatments. In fact, approximately 286 monoclonal antibodies are in various stages of clinical development and oncology is the area of greatest activity, with approximately 150 new monoclonal antibodies being tested. In addition, some 70 monoclonal antibodies are in clinical development for treatment of inflammatory and autoimmune diseases, others are in clinical development for treatment of various metabolic disorders, central nervous system disorders, infectious diseases, cardiovascular diseases and transplant rejection (1). However, the existing production capacity of today’s industry for monoclonal antibodies comes short when faced with the ever growing demand. Although cell culture productivity was dramatically improved, meeting the desired production, the downstream processing was unable to accompany this evolution accounting for high costs and product losses and becoming the industry’s new bottleneck for monoclonal antibody production. The downstream processing meets the various requirements of purification by applying a series of platform technologies. The five main sections comprise harvest (usually performed by centrifugation and dead end filtration), primary recovery (with an ultrafiltration/diafiltration followed by affinity chromatography), viral clearance (by virus inactivation and nanofiltration),
purification and polishing (with a series of chromatographies namely hydrophobic interaction and ion exchange). Although the traditional purification platform has been improved to its theoretical optimum, the high amount of purification steps translates into higher product losses. The most limiting step is the affinity chromatography (usually with protein A resins) often accounting for about 50% of the total downstream costs due to the high price of the resins, which also leads to the use of smaller columns cycled several times, aggravating the second drawback of affinity chromatography as the bottleneck of downstream processing since large volumes of supernatant need to be loaded in relatively smaller columns. Therefore there is an urge to develop alternative purification techniques, especially to the affinity chromatography step (2).

The main objective of this work is to design an alternative purification process that could address these problems assuring the purification of monoclonal antibodies from their complex culture medium, guaranteeing the selective extraction of antibodies in a cost effective and scalable for high capacity matter, envisaging process integration and intensification. The technologies chosen to attain these goals were aqueous two phase systems (ATPS) and hydrophobic interaction chromatography (HIC). The choice of ATPS is based on the fact that it has the potential to overcome several of the mentioned drawbacks by being a fast, reliable, biocompatible, high capacity and low cost technique that can be used in continuous operation mode.

Aqueous two phase systems are made by mutual incompatibility of different solutes in aqueous solution, namely polymers and/or salts. The two phases are formed spontaneously when the concentrations of usually two of these components go beyond the critical composition resulting in two phases, each one enriched mainly with one of the components (3). The partitioning of molecules between the phases is influenced by several parameters that can be adjusted to favor the selective migration of the target protein in order to achieve its purification namely: the type of polymer, its molecular weight and size, the system’s polymer and salt concentrations, the addition of a third element as a salt like NaCl that can increase the hydrophobicity based separation, the pH, temperature or ionic strength. Nevertheless even with the optimization of these parameters these systems often show a lack of selectivity, making it usually impossible to use ATPS as a single step purification technology as desirable (4) (5). Aqueous two phase affinity partitioning counters this problem either by chemically modifying a phase forming component of the system, by attaching to it an affinity ligand with specificity for the molecule of interest, or by the addition of free ligands in solution, in both cases, the ligand will assure the binding between the protein of interest and one of the phases of the system (6). One of these ligands, LYTAG, has an affinity for PEG and Choline, it is an improved version of the C-LytA, the C-terminal module of LytA amidase, a hydrolase from Streptococcus Pneumoniae that catalyzes the cleavage of the N-acetylmuramoyl-L-alanine bond of the peptidoglycan backbone (7). Biomedal provides a variant of LYTAG, the LYTAG-1Z that is particularly suited for monoclonal antibody purification; it is a C-LytA improved mutant hybrid with the Z domain of protein A from Staphylococcus aureus making it able to bind to Immunoglobulin G. LYTAG-1Z could purify antibodies in ATPS by bonding to them through the Z domain and then selectively take them to the PEG rich phase or choline rich phase (8).

Another alternative is to combine ATPS with a secondary operation in order to improve the purity. Namely Hydrophobic interaction chromatography (HIC), it is a powerful bioseparation tool for the purification of proteins that has been intensively developed in the latest years, possessing a higher capacity and lower cost than the traditional protein A chromatography. HIC takes advantage of the hydrophobicity of proteins promoting their separation on the basis of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins. The adsorption requires a high salt concentration in the mobile phase in order to achieve the salting out of the proteins thus assuring their binding to the column, and the elution is achieved by decreasing the salt concentration of the eluent, thus, it can be successfully used after a polymer/salt ATPS to furtherly purify the non-polymer phase taking advantage of the elevated salt concentrations for the adsorption. HIC separation is mainly influenced by the type of ligand (its hydrophobicity) and the type (regarding its salting out ability) and concentration of salt in the mobile phase, these parameters can be adjusted to optimize the separation (9).

<table>
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<th>Nomenclature</th>
<th>Description</th>
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<td>ATPS</td>
<td>Aqueous two phase partitioning</td>
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<td>HIC</td>
<td>Hydrophobic interaction chromatography</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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2. Materials and methods

Chemicals and biologicals

In this work several types of PEG were used, the ones with molecular weight of 400, 600, 3350 Da were provided by Sigma-Aldrich (St. Louis, MO, USA), the ones with 800, 1000, 1500, 2000 and 6000 Da molecular weight were purchased from Fulka (Buchs, Switzerland). Choline chloride was first obtained from Alfa Aesar and then Acros Organics (New Jersey, USA), Choline dihydrogenocitrate was bought from Sigma-Aldrich. Phenol red was provided by Labbox (Barcelona Spain),Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium di-hydrogen phosphate
anhydrous, di-sodium hydrogen phosphate anhydrous and ammonium sulfate came from Panreac (Darmstadt Germany). DL-dithiothreitol 1 M solution in water, ammonium persulfate and N,N,N',N'-tetramethylethlenediamine were purchased from Sigma. Acrylamide/bisacrylamide, Coomassie blue (for electrophoresis) and the silver staining kit came from Bio-Rad (Hercules, CA, USA). Bovine serum albumin (BSA) standards (2 mg/mL) and Coomassie Plus (Bradford) Protein Assay were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Milli-Q water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals were from analytical grade and none of them has undergone any further purification. The GFP-LYTAG used in the partitioning experiments was produced by E. coli REG-1 strain transformed with the plasmid pALEX2Ca-GFP supplied by Biomedal (Spain).

LYTAG-12 was provided by Biomedal with a purity of 90% and a concentration of 3.6 mg/mL. The antibodies used for the spiking of serum and supernatant were Gammanorm antibodies from Octapharma. The fetal bovine serum used in the partitioning experiments was a low IgG variant provided by Gibco Life Technologies (USA). The fetal bovine serum and supernatant were Gammanorm antibodies from Octapharma. The fetal bovine serum used in the partitioning experiments was a low IgG variant provided by Gibco Life Technologies (USA). The fetal bovine serum was 10 g, and was composed of 3g and Several PEG-Phosphate-Choline combinations (24% of PEG 3350, 7% phosphate and 5.5% of choline chloride at pH 6, 7 and 8, 24% of PEG 3350, 2% phosphate and 15% phosphate and a repetition of the first system with PEG 2000 instead of PEG 3350). 3g PEG-Ammonium sulfate systems used for ATPS combined ammonium sulfate with PEG 600, 1000, 3350 and 6000 in concentrations ranging from 10 to 15% (w/w), experiments were also conducted with 15% of NaCl and at pH7. LYTAG was added to the PEG 3350 and 6000, in the amounts of 5% w/w, 2 µL, 30 µL, 60 µL and 90 µL. The systems applied to the HIC had 10 g, and were composed of 10% PEG 6000, and 10%, 12%, 15% or 20% of ammonium sulfate, the loads of 20% were either spiked serum or supernatant.

**Aqueous two phase extraction**

The aqueous two phase systems were prepared in 10 ml graduated test tubes, the phase components were mixed with a vortex agitator (Ika, Staufen, Germany) and centrifuged for 3 min at 3800 rpm (fixed angle rotor centrifuge from Eppendorf, Hamburg, Germany) to separate the phases. The top phases were extracted with micropipettes and the bottom phases with 5 ml syringes.

**Hydrophobic interaction chromatography**

HiTrap Octyl FF, Butyl FF, Phenyl FF, Butyl HP and Phenyl HP (GE Healthcare Biosciences, Uppsala, Sweden) columns prepacked with Sepharose media were used. They have 1 ml of bed volume and bed dimensions of 0.7 cm x 2.5 cm. The Fast Flow (FF) and High Performance (HP) columns differ in the particle size (45-165 µm for the FF and 24-44 µm for the HP) and on the amount of ligand bound to the matrix (HiTrap Butyl FF: 40 µmol butyl/ml gel in 4, HiTrap Butyl HP: 50 µmol butyl/ml gel, HiTrap Octyl FF: 5 µmol octyl/ml gel, HiTrap Phenyl FF: 25 µmol phenyl/ ml gel with 6% cross-linked agarose, HiTrap Phenyl HP:25 µmol phenyl/ml gel). All the chromatographic separations were performed on an Åkta Purifier system from GE Healthcare at a flow rate of 1 ml/min. Each column was first washed then equilibrated with 5 column volumes of the adsorption buffer (1.5 M of ammonium sulfate, 10 mM of phosphate at pH7), before the injection of the ATPS bottom phase with a 1 ml sample loop. The unbound
compounds were washed with 2 column volumes of adsorption buffer, a 10 mM phosphate elution buffer at pH 7 was used for the elution with a 13 column volumes. The column's flowthrough the eluate were recovered in 1 ml and 0.5 ml fractions using a FRAC 950 collector (GE Healthcare).

**Analytical methods**

Purity assessment by protein gel electrophoresis
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine the qualitative purity of the samples. The spiked serum and supernatant samples were diluted 5 or 10 fold in milli-Q water, all samples were prepared by mixing 20 µL of diluted sample, 25 µL of Bio-Rad loading buffer and 5 µL of DTT followed by denaturation in a water bath at 100°C for 5 minutes. A 20 µL of each sample and 5 µL of molecular marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad) were applied to 12% acrylamide gels, prepared with a 40% acrylamide/bisacrylamide stock solution. The gels were run at 100 mV with a running buffer composed of 25 mM Tris-HCl, 192 mM of glycine and 0.1% (w/v) SDS with pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250 for one hour and destained successive washes with a solution of 30% ethanol, 10% acetic acid, 60% water (v/v).

Total protein quantification with Bradford essay
The total concentration of proteins was measured by the Bradford essay with a Coomassie Plus kit (Pierce, Rockford, IL, USA). Bovine Serum albumin was used as a standard and the sample’s absorptions were measured in 96-well polystyrene microplates with a Spectramax 384 Plus microplate (Molecular Devices, Sunnyvale, CA, USA) at 595 nm. Blanks the top and bottom phases and adsorption and elution buffers were made to subtract the interference from polymers and salts.

**Antibody quantification by HPLC**
The sample’s antibody concentrations were assessed by affinity chromatography with an analytical POROS Protein G Affinity column with 2.1 x 30 mm dimensions (Applied Biosystems, Foster City, CA, USA). Prior to quantification, the ATPS samples were diluted 10 times and the HIC samples 5 times with the adsorption buffer.

The chromatographic runs were performed with an ÄKTA™ 10 Purifier system (GE Healthcare Biosciences, Uppsala, Sweden). With a 50 mM sodium phosphate, 150 mM NaCl adsorption buffer at pH 7.4 and a 12 mM HCl, 150 mM NaCl elution buffer. UV absorbance at 215 nm was monitored during the operation.

Evaluative parameters
Several parameters were used to evaluate the effectiveness of the separations. The partition coefficient, Kp, is used to measure the degree of separation of the antibody between the two phases of the ATPS, it is given by the quotient of the antibody’s equilibrium top and bottom phase’s concentrations. ATPS’s yields of extraction for the top and bottom phases are given by the mass of antibody in the respective phase divided by the sum of the masses of antibody in both phases. The flowthrough yield is given by the mass of antibody present in the flowthrough divided by the sum of the masses of antibody in both flow through and eluate, the yield of the elution is obtained analogously. The purity is given by the concentration of antibody in the system, divided by the total protein concentration in the system.

3. Results

**Assessment of possible systems for affinity ATPS**
PEG-Choline chloride systems were studied to assess their applicability for affinity two phase partitioning. However, it was observed that systems using PEG 400, 600 and 1000 would only form at very high concentrations (55% to 50% PEG, 30% choline chloride) rendering them unviable at industrial level. Since systems with polymers with higher molecular weight typically require lower concentrations for phase formation, PEG 1500, 3350, 6000 and 8000 were then tested permitting indeed the formation of systems with 30% PEG and 30% choline but at two phase forming concentrations jellification was observed.

![Figure 1 - PEG 1000 (A), 600 (B) and 400 (C) choline systems form only at high concentrations. PEG 1500-choline chloride system: short after the dissolution of the components and the phase formation, the jellification of the bottom phase is observed (D), 24 hours later both phases are jellified (E) and a small addition of water (3 drops) permits the dissolution but without the formation of two distinct phases(F).](image)

Therefore, PEG-Choline chloride systems were abandoned as they could not constitute scalable ATPE alternatives.

Both Phosphate-Choline chloride, PEG-Phosphate-Choline chloride at various pHs (6, 7 and 6), with PEGs 3350 and 600 and PEG-Phosphate-Choline citrate systems were tested with GFP and GFP-LYTAG to assess their viability for affinity ATPS but in all cases both GFP, GFP-LYTAG and most of the impurities were found in the same phase (the choline rich one) indicating no advantage for the use of LYTAG in these systems. These results suggest that most of the impurities present might be attracted to choline itself, as it is an essential nutrient and a methyl donor involved in many physiological processes, including normal metabolism and transport of lipids, methylation reactions, it has to be able to interact with a vast specter of biomolecules (10).

**PEG ammonium sulfate**
Systems with PEG 600, 1000 and 3350 with ammonium sulfate were tested and analyzed; the results are presented in the following figure.
As shown in the figure, this system has given an advantage to the use of LYTAG most similar to the ideal scenario in the case of PEG 1000 where the value of log(Kp) for GFP is negative meaning that it partitions preferably to the bottom phase, and positive for GFP-LYTAG meaning that it partitions preferably to the top, polymer rich, phase. PEG 600 is a low molecular weight polymer therefore its size exclusion effects on the proteins are negligible, the dominant factor in the separation being then the salting out effect caused by the sulfate which causes, in both cases, the proteins to migrate to the PEG-rich phase. PEG 1000 possesses a higher molecular weight increasing the steric effects causing GFP to migrate preferably to the bottom, salt rich phase; the fact that GFP-LYTAG displays a higher presence in the top phase must result from LYTAG’s selective binding to the polymer. However the steric exclusion effect is manifested in the fact that the yield is lower for the top phase with GFP-LYTAG than for the bottom phase with GFP. Finally, with PEG 3350, due to its greater molecular weight, the steric effects dominate all others and both proteins partition mainly to the bottom phase. In this case, the higher yields and Kp of GFP-LYTAG relatively to GFP are probably due to its superior molecular weight. Although the PEG 1000 system presented the biggest LYTAG inducing discrepancy, the PEG 3350 system showed both higher yields and Kp. In terms of purities the impurities migrate mostly to the same phase as GFP and GFP-LYTAG but in the case of the PEG 1000 system stronger bands were observable for GFP-LYTAG in the upper phase than for the remaining impurities which might indicate some extent of purification, encouraging the further testing of these systems with monoclonal antibodies.

**Antibody and serum partitioning in aqueous two phase systems**

**Effect of pH**

The tested IgG have isoelectric points between 6.6 and 9 most of them having a pH closer to 7 (14) therefore at pH 7 most of them would be neutral which would incentive the migration to the neutral polymer phase. Although the serum is quite diverse in composition, its major component, the bovine serum albumin, has a pI of 4.8 and will thus be negatively charged at pH 7 which would facilitate its migration to the salt rich phase to interact with the positively charged ammonium ions. Therefore systems composed of PEG 1000, 3350 and 6000 with ammonium sulfate were studied at their native pH and at pH 7. Each system was evaluated for the partitioning of pure antibody and of the serum proteins. The PEG 1000 system exhibited precipitation of the antibody and serum at both pHs, PEG 3350 system just at normal pH for the antibody and PEG 6000 system at pH 7 from the antibody. In terms of the purification achieved, for the PEG 1000 system at native pH both the antibody and the serum are concentrated in the precipitated fraction and at pH 7 it is also slightly present in in the top phase, so in this specific case, pH variation was inconsequential and the precipitation observed is probably due to the high salt concentration used (15% of ammonium sulfate). For PEG 3350, without pH control, the antibody partitioned mostly to the top and precipitate fractions whereas serum partitioned to the bottom and slightly to the top, representing a more advantageous situation that the one found at pH 7 in which both IgG and serum proteins partitioned to the bottom phase. As for PEG 6000, both antibody and serum are found in the bottom phase except in the case of the antibody at pH 7 where it is found in the precipitate (figure 3). The solubility of the proteins is affected by the pH, being minimum at the pl, which explains the precipitation in some cases. In this type of systems, precipitation is a disadvantage as it can lead to product losses and the fact that the pH variations had a low effect on the partitioning indicates that the influence of other parameters must be dominant. Those parameters were furthermore analyzed.

**Effect of NaCl addition**

Contrarily to the pH, the addition of 15% NaCl had a clear influence switching the partitioning of both antibody to the top, polymer rich phase (figure 3). Whilst electrostatic interactions between the proteins and the phase forming components are probably responsible for the selective partitioning of the proteins to the bottom phase in the NaCl free systems, the addition of this salt increases the salting out effect and decreases the electrostatic potential difference between both phases, as NaCl distributed evenly between the phases, causing the proteins to migrate preferably to the more hydrophobic polymer phase (10). At low protein concentrations, that is, in this case, when the serum and antibody are used as separate feeds, no precipitation was observed after the NaCl addition but when the spiked serum was used as feed, resulting in a higher protein concentration, the addition of NaCl...
resulted in the precipitation of the proteins. This observation and the fact that the salt caused the partitioning of both antibody and serum to the same phase discouraged the use of NaCl.

**Effect of polymer addition**

As all the systems contemplated in this analysis were made with PEG, the polymer influence is related with its molecular weight which influences the partitioning through steric exclusion effects as bigger polymers tend to form more aggregated tangled structures making it harder for large proteins to partition to their phase. On the other hand, the longer the PEG chain length, the more ethylene oxide groups will be present per PEG molecule for the same concentration of polymer, making the top phase more hydrophobic. The combination of these two effects will be responsible for the influence of the polymer molecular weight on the partitioning (10). The most evident effect of the polymer variation was the precipitation of the proteins. Systems formed with PEG 1000 presented precipitation both at native and neutral pH for the antibody and the serum proteins, this is probably due to the fact that since the molecular weight of the polymer is low, a higher salt concentration is required for phase formation, which can induce the proteins to precipitate. PEG 3350 exhibited precipitation only for the antibody and spiked serum and PEG 6000 for none of them. Secondly, for the systems at native conditions, most of the proteins in the PEG 1000 systems were found in the precipitated fraction and top, polymer rich phase, for PEG 6000 and PEG 3350 the weight of the polymer enhances the steric exclusion effects making it harder for the proteins to move to the top phase and thus switching the preferential partitioning to the bottom phase (figure 3). In all analyzed systems both antibody and serum partitioned mainly to the same phase so just by changing the polymer the selective partitioning of the proteins was not achieved.

**Effect of the ligand LYTAG-1Z**

As the manipulation of the previous parameters did not result in the selective partitioning of the antibody in the aqueous two phase tested, LYTAG-1Z addition appears as a decisive influential parameter for the viability of the one step purification with ATPS. Firstly, PEG 3350 and 6000 were tested with spiked serum and LYTAG-1Z as they were the ones that favored the partitioning of the proteins to the bottom phase, and the objective was to have most of the impurities in the bottom phase and the antibody driven to the top phase by LYTAG-1Z. Unfortunately, the addition of 5% LYTAG caused the precipitation of the antibody and part of the proteins of the serum and no antibody was found on the top phase. Precipitates are being undesirable, different amounts of the ligand were tested: 2, 30, 60 and 90 µL of LYTAG-1Z (corresponding to 0.06, 2 and 3 times the amount of antibody present in the system), but again, precipitation was observed in all cases, with the antibody being found in the bottom phase or precipitate (figure 4). As the evaluated PEGs had a high molecular weight, it was possible that they could be causing a steric exclusion of the antibody preventing it from going into the top phase even with the LYTAG-1Z affinity for PEG.
Figure 4 - Qualitative analysis by SDS-PAGE electrophoresis of the purity of both phases from 10% PEG 3350-10% Ammonium Sulfate (1 and 3) and 10 PEG 6000-10% systems (2, 5, 6 and 7). All systems were tested with Gammanorm antibody spiked fetal bovine serum (20% feed). 5% of LYTAG-1Z was added to systems 1 and 2, systems 3 and 4 received 2 µL of LYTAG each, system 5, 6 and 7 were supplied with 30, 60 and 90 µL of LYTAG each respectively. M stands for the molecular marker, T for the top phase, P for the precipitate and B for the bottom phase.

So a final trial with several different compositions of PEG 600 or 1000 and ammonium sulfate (PEG 600-ammonium sulfate: 18%-15%, 10%-20%, 27%-11%, PEG 1000-ammonium sulfate 15%-15%, 7%-18%, 26%-10%) was investigated in order to assess if by lowering the molecular weight of the PEG and changing PEG salt ratio one could adjust the steric and salting out effects to keep most of the impurities in the bottom phase whilst allowing the LYTAG to take the antibodies to the top phase. Nevertheless the phase containing most of the antibody also contained most of the albumin. For the 18% PEG 600 and the 7% PEG 1000 systems, the strongest bands were found on the top phase since the combination of lower concentrations of PEG and low polymer molecular weight, allowed the partitioning of the proteins to the top, while in the systems with higher PEG and salt concentration the combination of LYTAG induced precipitation and salting out effect were probably dominant causing the proteins to be found mostly in the precipitate (figure 5). Therefore, LYTAG-1Z did not permit the one step partitioning of monoclonal antibodies by affinity aqueous two phase partitioning. Nevertheless purification could be achieved by ATPS followed by a secondary operation.

As similar systems have been used for clarification (4) (15), PEG-ammonium sulfate systems could be maintained as a purification step for clarification, preliminary purification and concentration of the feed and the elevated salt concentration could be advantageously used in a polishing step by hydrophobic interaction chromatography (16).

Antibody recovery yield and purity with PEG 6000-ammonium sulfate ATPS
To verify the viability of the PEG-ammonium sulfate system as a preliminary purification step the extraction yield, kp and purity were determined. The extraction yield was 99.6%, the Kp 0.003734 and the purity of 14% which indicates that although some protein separation was achieved, there is the need for a second step purification process.

Dual purification with PEG 6000-ammonium sulfate ATPS and hydrophobic interaction chromatography
The salt concentration of the ATPS was optimized to favor the binding of the antibody to the column, the subsequent tests being effected with the bottom phases of 10% PEG 6000-12% ammonium sulfate systems fed with either spiked fetal bovine serum or spiked CHO supernatant. Each of the five columns being tested with both feeds, the obtained chromatograms, results and purities are shown in figure 6 and table 1. The columns being used in bind-elute mode the antibody was recovered mainly in the elution fraction.

Figure 5 - Qualitative analysis by SDS-PAGE electrophoresis of the purity of both phases from systems with various compositions made of PEG 600-Ammonium Sulfate (Polymer-salt compositions: 18%-15% (1), 10%-20% (5) and 27%-11% (6)) and PEG 1000-Ammonium Sulfate (Polymer-salt compositions: 15%-15% (2), 7%-18% (3) and 26%-10% (4)). Each system was fed with 20% Gammanorm antibody spiked fetal bovine serum and with 5% of LYTAG-1Z. M stands for the molecular marker, T for the top phase, P for the precipitate and B for the bottom phase.

Figure 6 – Representation of the characteristic FF (1) and HP (2) HIC (obtained with a Hitrap Phenyl FF and HiTrap Phenyl HP) for the separation of IgG from a 1ml sample of the ATPS bottom phases of a 10% PEG6000-12% Ammonium Sulfate system with spiked CHO supernatant (A) and fetal bovine serum (B) feeds. With a 15 M ammonium, 10 mM phosphate adsorption buffer and a 10mM phosphate elution buffer

When observing the chromatograms one can verify the presence of the characteristic flow-through and elution peaks
corresponding to the unbound proteins and the proteins that were bound to the column and then recovered in the elution. However there is a major difference between the FF and HP resins as the latter present a subdivision of the elution peak in two (that were analyzed separately). This phenomenon occurs due to the fact that proteins with lower hydrophobicity interact more loosely with the resin being eluted as soon as the salt concentration starts decreasing, more hydrophobic proteins, on the other hand bind strongly to the resin and only elute when the salt reaches a critical lower concentration. The HP columns possess more ligands which would permit the binding of more proteins, including the less hydrophobic ones which could be the reason behind this phenomenon. HP columns also have lower particle size allowing the resolution of the elution peak in two peaks.

Table 1 - Summary of the extraction yields (blue cells) and purities (yellow cells) obtained for spiked fetal bovine serum and CHO spiked supernatant feeds with the 10% PEG 6000-12% Ammonium sulphate ATPS and HIC with HiTrap Phenyl FF, Phenyl HP, Octyl FF, Butyl FF and Butyl HP.

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<th>Phenyl FF</th>
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Although there are result variations possibly originated either from the imprecisions of measurement associated to the Bradford method or from the differences in the feed or supernatant samples, clear behaviors are noticeable. First all the elution extraction yields for single peak elutions were high (98.3% to 100%) indicating that most of the antibody was effectively recovered with no significant discrepancy between columns. For the two peak elutions, the antibody was mainly recovered in the second fraction indication that it is amongst the most hydrophobic proteins, but this separation resulted in antibody losses since part of the antibody is still present in the first peak (the yields for the second fraction being 87.9 for Phenyl HP and 83.7 for Butyl HP). However the separation of the peaks resulted in a further purification for the second fraction, the purities going from 49.8 to 51.3 for the phenyl columns and from 60.1 to 62.4 for the butyl columns. But for the HP runs that did not form two peaks (for the supernatant) the switch to HP resulted in a decrease in purity probably due to its higher capture capacity resulting in the binding of more impurities.

Another relevant observation is that the purities are considerably higher for the supernatant fed systems (58.1% to 96.5%) than from the serum fed ones (49.8% to 62.4%). The reason for this is probably that the supernatant being a serum free medium of known composition is supplied only with the elements necessary for cell growth and protein production, thus containing less impurities than the supernatant. This is in fact one of the great advantages of using serum free media (17). Finally the purity of the fractions clearly increases with the decrease of the hydrophobicity of the ligands as it is known that phenyl is the strongest ligand presenting the lowest elution purities (49.8% and 58% for phenyl FF, 51.3% and 73.1% for phenyl HP with serum and supernatant feeds respectively), octyl with is the second strongest evidenced an increase in purity (55.9% and 76.5% for serum and supernatant) and the least hydrophobic ligand, butyl, presented the highest purities (60.1% and 96.5% for butyl FF and 62.4 and 80.4 for butyl HP for the supernatant and serum). The reason behind this is that higher strengths of interaction result in higher recovery of the target protein but can also mean a decrease in adsorption selectivity, the less hydrophobic ligands having a lower tendency bind to less hydrophobic impurities resulting in higher purities.

4. Conclusions

The main objective of this work was to develop an alternative method for the purification of monoclonal antibodies that could provide a solution to the main problems of the current purification platform, namely regarding its capacity, effectiveness, and economy, to keep up with the advances in the production and exponential demand for monoclonal antibodies. The work developed in this thesis demonstrated that none of the analyzed ATPS systems could perform the desired one step purification with the LYTAG ligand. In fact PEG-choline systems would not form at industrially desirable concentrations, choline-phosphate and PEG-choline-phosphate systems conferred no significant advantage to the use of LYTAG as both GFP and GFP-LYTAG would partition to the same phase as well as most impurities. The best results for the preliminary experiences with GFP/GFP-LYTAG were obtained with PEG-
ammonium sulfate, more precisely with PEG 1000-ammonium sulfate which was the only system for which the LYTAG fused GFP partitioned to a different phase of the normal GFP, as well as most impurities. However, when proceeding to the tests with the spiked serum for the PEG-ammonium sulfate systems, the addition of LYTAG not only did not improve the partitioning but caused the precipitation of the samples for all the tested PEGs (600, 1000, 3350 and 6000 Da). This might be due to the formation lattices between the antibody and LYTAG-12 causing their precipitation by salting-out. Besides the fact that precipitation is undesirable because it leads to product losses, as molecules aggregate it is harder for them to migrate to the polymer phase as wanted. Therefore, the first suggestion for future work would be to use polymer-polymer systems to avoid precipitation by salting-out as the ionic strength of this type of systems is much lower than in polymer-salt systems. This is the most recommended idea since a one-step purification with such easily scalable and fast operation as ATPE, followed by the necessary polishing would be with no doubt the ideal scenario. As the intensive testing of the PEG-ammonium sulfate system by manipulation of the different partitioning affecting parameters; namely different molecular weight PEGs, salt concentrations, with and without NaCl and at different pHs, did not culminate in the selective partitioning of the antibody, the necessity of the addition of a functional affinity ligand to improve the partition in this system has been evidenced. Still in the topic of solely ATPS based separation other PEG based systems could be further explored, namely PEG-citrate and PEG-phosphate (that because of their higher salting out effect could be used to form systems with lower molecular weight PEGs thus favoring the migration of the LYTAG to the polymer phase which could also avoid the precipitation, citrate is also more environmentally friendly than the other two options), PEG-sodium sulfate or others to furtherly analyze the influence of the type of salt and also to explore the ATPS and “elution” by choline technique mentioned by Maestro et al (18). The mentioned technique consists in using a PEG-salt system to capture a LYTAG bound molecule (in mentioned experience it was GFP-LYTAG) into the PEG rich phase, as most impurities remain in the salt phase but some migrate preferentially to the polymer phase which is then removed and “eluted” with choline to which the LYTAG binds preferentially abandoning the PEG rich phase and resulting in higher purities. If the proposed alternatives are effective further testing would have to be performed towards scale up and process integration. Relatively to the PEG 6000-ammonium sulfate/ HIC combined system, the results were more encouraging. As it was previously mentioned, the ATPS system can be used for a preliminary purification and perhaps clarification and the chromatography permits the achievement of higher purities.

As evidenced by the elevated extraction yields, the recovery of the antibody was quite high for all columns indicating that the optimized salt concentration permitted the efficient binding of the antibody to the column. For the cases where two elution peaks were obtained, the fact that the antibody is present in higher amounts in the second peak (which also possesses higher purities) corroborates the fact that it is more hydrophobic that most the impurities, confirming that a hydrophobicity based separation is appropriate. The best overall results were obtained with the HiTrap Butyl FF column by combination of the purity and yields, with a 96.5% purity and 99.6% extraction yield for the supernatant, even though the purity obtained for the serum fed system was higher with the Butyl HP (62.4% vs 60.2%) the increase in purity is too low to justify the loss in product (the yield is only 83.7% if only the fraction with the highest purity is kept). The best results in terms of solely product recovery were obtained with the Phenyl resins but they also present the lowest purities and as the recoveries do not differ substantially, they are not as attractive purification wise. The low purities obtained for the serum separation (62.4% maximum) indicate that it is not the most appropriate technique to separate monoclonal antibodies from serum based medium, this is due to the higher amount of impurities present, for this type of systems, if this technique was to be adopted it would require further purification such as, for instance, an ion exchange chromatography prior to the polishing steps. However the results obtained for the supernatant are far more encouraging as the purity is substantially higher (especially the 96.5% for the Butyl FF resin) and the final purification could perhaps be integrated in the polishing steps. These results are particularly relevant since, as previously mentioned, the current tendency in the industry is to switch the production towards serum free culture media. The cost of this type of resin is lower than the one of affinity resins and the scale-up of column dimensions, while maintaining the resin performance, is feasible, therefore it does represent an attractive alternative to the currently used platform. It is recommended for the dual purification system to be furtherly tested for its robustness with other types of serum free media to verify the dimensions of its applicability. This system could also be tested with other salts not only for the HIC step but also for the ATPS, namely citrate or other sulfates (for its environmental benefits when compared to ammonium sulfate) or even phosphate as its higher salting out effect could allow for lower salt concentrations and to use perhaps lower molecular weight PEGs without risking precipitation thus encouraging the proteins to migrate to the PEG rich phase and decreasing the impurity burden of the bottom phase which might result in higher post chromatographic purities. Finally it would be important to furtherly asses complementary polishing/purification techniques envisaging process integration.

The present thesis revealed that the ATPS/HIC combined system with the 10%PEG 6000-ammonium sulfate system and the HiTrap Butyl FF column is the most promising of the studied
techniques for the purification of monoclonal antibodies, especially from serum free culture media. However, it still requires further studying, optimization and integration. Nevertheless, a one-step purification system with affinity ATPS would be more desirable in terms of economy, rapidity of separation and continuous operation and, therefore, the pursue for this kind of separation should also be continued.

REFERENCES