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INTEGRATED PLATFORMS FOR THE CLARIFICATION AND CAPTURE OF MONOCLONAL ANTIBODIES

PLATAFORMAS INTEGRALES PARA LA CLARIFICACIÓN Y CAPTURA DE ANTICUERPOS MONOCLONALES

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Abstract

Antibodies play an important role in both medicine and analytical biotechnology. Recently, the demand for monoclonal antibodies (mAbs) has been increasing exponentially, since their potential for therapeutic use was disclosed and improved techniques to produce them at higher titers have become available. Although upstream processing of mAbs has suffered a tremendous improvement, in the last decades, the downstream processing has been considered the bottleneck in providing antibodies at reliable costs, being, in fact, the major cost factor with 50 to 80% of total production costs. Therefore, alternative methods for mAbs's purification may be required. Aqueous two-phase systems (ATPS) have been described, for the last decade, as a viable alternative to the current establish platform, since clarification, concentration and purification can be achieved in just one step using a biocompatible environment. Therefore, the use of ATPS for integrative purification of mAbs it will be reviewed.

Keywords: antibodies, capture, purification, aqueous two-phase systems, integrated platform.

Resumen

Los anticuerpos tienen un papel importante tanto en la biotecnología médica como en la analítica. Desde que su potencial para uso terapéutico fue descubierto y las técnicas para producirlos en concentraciones más altas fueron mejoradas y se hicieron disponibles, la demanda de anticuerpos monoclonales (mAbs) ha estado incrementado exponencialmente. A pesar de que en las últimas décadas los procesos de fermentación de mAbs han mejorado notablemente, los procesos de recuperación y purificación han sido considerados el cuello de botella para la producción de anticuerpos a precios confiables. De hecho, es esta última etapa la que involucra un mayor costo abarcando del 50 al 80% de los costos totales de producción. Debido a esto, métodos alternativos de producción de anticuerpos son requeridos. Los sistemas de dos fases acuosas (SDFA) han sido descritos en la última década, como una alternativa viable a la plataforma actual establecida, dado que la clarificación, concentración y purificación pueden ser llevadas a cabo en un solo paso dentro de un ambiente biocompatible. Es por tanto que el uso de SDFA para una purificación integral será revisado.

Palabras clave: anticuerpos, captura, purificación, sistemas de dos fases acuosas, plataforma integral.

1 Introduction

Monoclonal antibodies (mAbs) are within the most important biopharmaceutical products of the pharmaceutical industry, with total sales expected to reach $58 billion by 2014 (Morrow, 2012). The therapeutic mAbs market is growing fast and their innovative and lucrative offerings has thus resulted in a positive revenue growth rate even during an economic recession (Frost and Sullivan, 2012).

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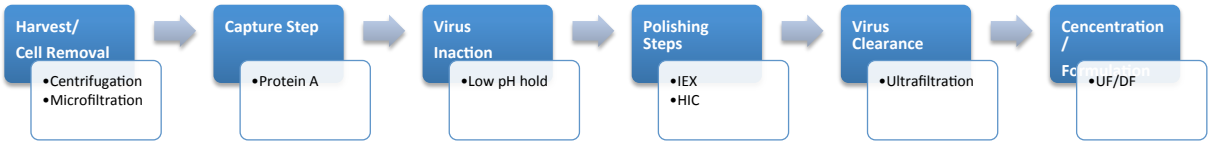

Fig. 1. A typical monoclonal antibody recovery process.

Up to 2013, 33 mAbs have received regulatory approval by the FDA for therapeutic and diagnostic purposes with the majority targeting diseases such as cancer and autoimmune disorders (PhRMA, 2013). According to the 2013 PhRMA's report, there are over 907 new biotechnology medicines in current development, targeting more than 100 diseases, including 338 monoclonal antibodies. Nevertheless, antibody therapeutics have some disadvantages, mainly their relatively expensive manufacture (Chan *et al*., 2009). Currently, research is being carried out on improving antibody efficacy, reducing production costs and improving affinity and specificity, with considerable success (Chan *et al*., 2009). Therefore, it is expected that the widespread use of antibodies will impel the high costs down.

2 Production and purification of mAbs

There are two critical issues in the process development of a biopharmaceutical product, such as mAb. The first is to minimize the time to reach the clinical studies and the second is to develop a process which can deliver sufficient amounts of therapeutic product to meet market demands at an acceptable price per dose (Birch and Racher, 2006).

Improvements in the upstream processing of mAbs have been responsible for an increase of 100 fold in cell productivity, in the last 15 years (Birch and Racher, 2006). For example, scientists working at PERCIVIA (PER. C6® technology platform) reached a record level titer of 27 g/L at harvest for an antibody product, which is a very important milestone in the production of monoclonal antibodies (DSM Press Release, 2008). However, with increasing upstream concentrations, increases also the need to find better downstream recovery processes, which can become a significant proportion of total manufacturing cost and can as well limit the overall plant throughput (Birch and Racher, 2006). Therefore, the greatest constraints of the current manufacturing platforms of mAbs are currentely found at the downstream level. Although these high titers can be managed by increasing the scale of the present purification platforms (e.g. using larger chromatography columns and filters), at some point, the physical limits of the existing facilities and scalability will be reached (Gottschalk, 2008). There is therefore a need to find better solutions, which ultimately can bring their prices down. In order to find alternative downstream processing methodologies to the traditional downstream processes (dsp), it is critical evaluate the traditional unit operations currently used by the biopharmaceutical companies to understand their limitations.

The downstream process of a biopharmaceutical product aims to remove all the impurities from the feeding streams and obtain a purified product with quality enough to meet the regulatory agencies requirements. Therefore, impurities such as host cell protein, DNA, adventitious and endogenous viruses, endotoxin, aggregates and other species must be removed while an acceptable yield is maintained. In addition, impurities introduced during the purification process must be removed as well (Liu *et al*., 2010). The basic operations in a typical platform of downstream processing of mAbs, include clarification, concentration, selective purification and virus inactivation and removal, as illustrated in Fig. 1.

2.1 Cell harvesting

The first unit operation, in a mAb recovery process, is the removal of cells and cell debris from the culture broth and clarification of the cell culture supernatant that contains the antibody product. This is generally accomplished using a centrifugation, depth filtration or tangential flow microfiltration. In particular, the disc-stack continuous centrifugation coupled with depth filtration is the current preferred process (Liu *et al*., 2010).

2.2 MAb's capture and virus inactivation

The next operation is a capture step, which is achieved by affinity chromatography. This is the most selective type of chromatography used in biotechnology, since it separates proteins based on reversible interactions between a protein and a specific ligand covalently

coupled to chromatography matrix (Liu *et al*., 2010). Specifically, for the purification of human mAbs, the affinity ligand usually involves a recombinant Protein A (Gottschalk, 2008), which has high affinity for the Fc region of IgG-type antibodies, resulting in a high degree of purity and recovery in a single step (Liu *et al*., 2010). This chromatography involves the passage of the clarified cell culture supernatant over the column at pH 6-8. At these conditions, antibodies bind to the column and the unwanted components flow through, these may include host cell proteins, nucleic acids, cell culture media components and, eventually, viruses. An optional intermediate wash step may be carried out to remove non-specifically bound impurities from the column, followed by elution of the product at pH 2.5-4, which allows also inactivation of the remaining virus (Liu *et al*., 2010).

2.3 Polishing steps and virus removal

For polishing steps, one or two additional chromatography steps are commonly employed. Most mAb purification processes will include at least one ion exchange chromatography step, generally a cation exchange column (CEX) for intermediate purification and an anion exchange column (AEX) operating in flow-through mode to remove negatively charged impurities, such as DNA, host cell proteins, endotoxins, and endogenous and adventitious viruses. In addition, hydrophobic interaction chromatography (HIC), mixed mode chromatography or hydroxyapatite chromatography may be chosen as well (Liu *et al*., 2010). Membrane chromatography can also be used instead the traditional packed-bed chromatography, especially for the polishing flow-through steps, as will be described in the next section.

Since mammalian cells used in the manufacture of mAbs produce endogenous retroviruses and are occasionally infected with adventitious viruses during the upstream processing, a virus clearance step is also required, prior to the final product formulation. This virus removal is normally accomplished by a filtration operation using a membrane with a pore size adequate to the type of the virus. Finally, an ultrafiltration can be used for protein concentration and buffer exchange (Liu *et al*., 2010).

2.4 Alternative capture steps

Currently, the downstream processing of mAb's is focused on chromatography, which is a critical and widely used separation and purification technology due to its high resolution (Gottschalk, 2008). However, chromatographic processes have some limitations specially when are used at large scale. Thus, other alternatives have to be considered in order to increase the manufacturing capacity and to decrease cost of goods, especially those that allow process integration and intensification.

Other alternative unit operations may include flocculation, precipitation, two-phase extraction, membrane chromatography and crystallization (Gottschalk, 2008). Flocculation and precipitation can be used in combination with conventional cell separation techniques, such as centrifugation and microfiltration, to remove residual particulates and soluble impurities, which might otherwise increase the burden on polishing steps, allowing the reducing on the number of chromatographic steps. Crystallization, despite its limited application to mAb downstream process due to its low yields, is another inexpensive technology that in some cases can simultaneously purify, concentrate, and stabilize the protein. Membranes are already being used in many bioprocesses, mostly for filtration rather than chromatography. However there is a huge benefit of membrane chromatography over conventional bead chromatography, mainly because pore diffusion is minimal. Membrane chromatography behaves similarly to packed chromatography columns, but in the format of conventional filtration modules, which usually has multiple layers containing functional ligands attached to the internal pore surface throughout the membrane structure (Gottschalk, 2008; Liu *et al*., 2010).

Hence, there is a need to study innovative solutions, which can include the review of simpler and less expensive separation technologies, such as aqueous two-phase systems (ATPS), the use of disposable modules and the development of process integration platforms.

3 Aqueous two-phase extraction (ATPE)

In the last years, liquid-liquid extraction using aqueous two-phase systems (ATPS), has been evaluated and developed in order to obtain an alternative primary stage unit operation in downstream processing. However, despite the potentials of this technique, nowadays aqueous two-phase extraction (ATPE) is only considered as a complementary technique and not a replacement of a unit operation in the dsp platform

(Rosa *et al*., 2007). Nevertheless, ATPE has been described as an effective method for the purification of different biological products, such as cells, virus, RNA, plasmids and proteins (Azevedo *et al*., 2007).

ATPS are formed spontaneously when hydrophilic solutes, such as two different polymers or a polymer and a certain salt, are mixed together in an aqueous solution above a certain critical concentration, as a result of mutual incompatibility (Azevedo *et al*., 2007; Azevedo *et al*., 2009a; Rosa *et al*., 2007; Azevedo *et al*., 2009b; Ferreira *et al*. 2008; Rito-Palomares, 2004). Beijerinck, in 1896, upon mixing agar and gelatin, was the first to report this phenomenon. However, only sixty years later (in 1956) was shown that proteins are suitable to be partitioned into ATPS (Albertsson, 1956).

Well-studied ATPSs include polyethylene glycol (PEG)/dextran and PEG/phosphate, where each phase generally contains 80-90% (w/w) water (Azevedo *et al*., 2009a). Therefore, since ATPS are mainly constituted by water, and most used polymers have a stabilizing effect on the protein tertiary structure, these systems provide a biocompatible environment for biomolecules, such as maps (Azevedo *et al*., 2009a; Azevedo *et al*., 2009b). Besides providing biocompatibility for recombinant proteins, this technique also allows integration of clarification, concentration and partial purification in just one step (Schügerl and Hubbuch, 2005). In addition, this process is cost-effective and its scale-up is very easy and reliable (Azevedo *et al*., 2009b).

3.1 How to purify mAbs in ATPSs?

The partition of a target compound, mAbs for instance, in ATPS depends on both intrinsic and extrinsic properties. Intrinsic properties include size, electrochemical properties, surface hydrophobicity and hydrophilicity, and conformational characteristics. While extrinsic properties include type, molecular weight and concentration of phase forming components, ionic strength, pH and temperature, among others (Azevedo *et al*., 2009b). These extrinsic properties can be manipulated in such way that the target mAb can be recovered in the phase that contains fewer impurities. Also, it is possible to increase the predictability and selectivity of an ATPS extraction by attaching affinity-ligands covalently to one of the phase-forming components, thus the target biomolecule (mAb) can be partitioned to the phase containing the ligand (e.g. protein A) (Rosa *et al*., 2007).

Since proteins (including Abs) are usually hydrophilic molecules they will have higher affinity for the more hydrophilic phase, which in polymer-polymer systems, such as PEG/dextran systems, is normally the dextran-rich phase, and in a polymer-salt systems, like PEG/phosphate, is the also the bottom phase (phosphate).

In polymer-polymer system, a typical strategy to enhance mAbs affinity towards the more hydrophobic phase (PEG-rich phase) is by introducing an affinity ligand in the systems, i.e., in the PEG rich phase. This can be achieved by adding the ligand to the system without coupling (free ligand) or by modifying the two terminal hydroxyl groups of PEG molecules with the affinity ligand (Azevedo *et al*., 2009a).

The polymer-salt systems have been widely used and are an attractive cost-effective alternative to polymer-polymer systems, due to the use of a relatively inexpensive phase component (salt) (Azevedo *et al*., 2009a). In this type of systems the selective extraction of antibodies can be achieved by using low polymer molecular weights, high Ionic strengths through the addition of a neutral salt (e.g. NaCl), lowering the pH and even by introducing affinity ligands. However, the high concentration of salt usually masks the affinity or electrostatic interaction between the ligand and biomolecule, which limits the applicability of this last methodology (Azevedo *et al*., 2009a).

3.2 ATPE vs chromatographic separation

At smaller scale (low mAbs titers), protein A (ProA) chromatography is very advantageous comparing with other processes, since it is possible to obtain more than 95% of purity with a simpler process (Shukla *et al*., 2007). On the other hand, ProA chromatography also accounts for the major costs in the whole production process of mAbs (Rosa *et al*., 2010). The main disadvantages of using ProA chromatography are thus essentially related to the high cost of ProA resins, which can be up to 10 times as expensive as conventional chromatographic supports (Shukla *et al*., 2007), and most importantly, to the possible leakage of ligand from the matrix and consequent contamination of the final product with a Staphylococcus protein (Azevedo *et al*., 2008). In addition, higher-titer processes have been imposing practical limitations, namely the use of larger columns, which will make the current technology platforms reach their limit in terms of throughput and scalability (Gottschalk, 2008). Another key limitation of this type of

chromatography is the need to carry out product elution at low pHs (Shukla *et al*., 2007), in order to break the interaction between protein A and Fc region of Ab. Additionally, some antibodies demonstrate a higher binding affinity than others, requiring a lower elution pH (Liu *et al*., 2010). An exposure to low pH conditions can result in the formation of soluble high molecular weight aggregates and/or insoluble precipitate formation during product elution. Insoluble aggregate formation can reduce the column lifetime if precipitation occurs during elution, which might be threatening the product activity (Shukla *et al*., 2007).

ATPS on the other hand, constitutes an interesting alternative to the traditional chromatography since clarification, concentration and purification can be combined in only one operation using a non-toxic phase environment (Azevedo *et al*., 2008). In addition, it can overcome some of the technical drawbacks currently encountered in chromatographic process (Azevedo *et al*., 2009a; Shukla *et al*., 2007). One of the major advantages of large-scale ATPE is its easy scale-up together with the possibility of continuous operation using traditional liquid-liquid extractors. Besides, the equilibrium in ATPS is rapidly reached and the recovery of proteins is facilitated, since the system operates with fewer theoretical plates than a chromatographic system (Azevedo *et al*., 2009a). Nevertheless, the handling, storage and disposal of the large amounts of raw materials required at a process scale may also constitute a disadvantage. Although PEG is biodegradable and non-toxic, salt disposal (e.g. phosphate) can be an issue, which can be minimized by recycling the polymers and salts used in the process. The recycled raw materials have, however, to fulfill the purity requirements to guarantee the operating consistency and reproducibility of batch-to-batch ATPS (Rosa *et al*., 2010).

There are still some questions that still need to be answered until ATPE can be adopted by the biopharmaceutical companies (Rosa *et al*., 2010). One concern is the maximum capacity of these systems, specifically, whether they can handle high titer supernatants or whether the throughput will be limited by solubility problems (Azevedo *et al*., 2009a). The other constrain is related to the limited predictive design of this process, due to the poor understanding of the responsible mechanisms for the behavior of biomolecules in ATPS. The economical and environmental sustainability of an ATPS-based capture process has been recently evaluated and compared to the currently established platform and it was shown that an ATPS alternative platform can process and purify continuously the same amount of mAbs reducing significantly the costs, especially if higher titers of mAbs are used. The ATPS platform can also be environmentally validated if the process comprises the recycling of the phase forming phases (PEG and salt) and operates at high titer cell culture supernatants (more than 2.5 g/L) (Rosa *et al*., 2011).

4 Integration and intensification of process using ATPS

One of the major factors that have been interesting researchers about ATPS is that it allows process integration as the simultaneous separation and concentration of the target protein can be achieved, with posterior removal and recycle of the polymer (Cunha and Aires-Barros, 2002). Furthermore, ATPS can also provide solutions for purification of mAbs at large scale in a continuous and multi-stage operation.

The productivity, yield and economy of bioprocesses can be considerably improved by process integration (Schügerl and Hubbuch, 2005). Therefore, the manufacturers of bioproducts, such as mAbs, have a considerable interest to achieve process integration of the upstream operations of fermentation and especially the downstream recovery processes, to facilitate the development of scalable and efficient bioprocesses (Rito-Palomares, 2004). Thus, there is nowadays a strong demand for intensification and integration of process steps to increase yield, reduce the process time and cut down in running costs and capital expenditure. Besides these advantages, integrated processes still wait for broad industrial application, since it has a complex development, and thus there is a need for detailed process knowledge of the applicant (Schügerl and Hubbuch, 2005).

Process integration attempts to achieve specific objectives not efficiently met by discrete processes by combining two operations into one. There are considered three major areas of research: the extractive bioconversion, the extractive fermentation and the integration of cell disruption and primary purification step (Rito-Palomares, 2004). The latter is a key strategy to intensify the manufacturing of biologics, since it could enhance product yield and quality (Figure 2). The use of ATPS represents an atractive technlogy, since it is possible, by extracting the proteins from crude feedstock, to concentrate and purify the product in just one step (Schügerl and Hubbuch, 2005).

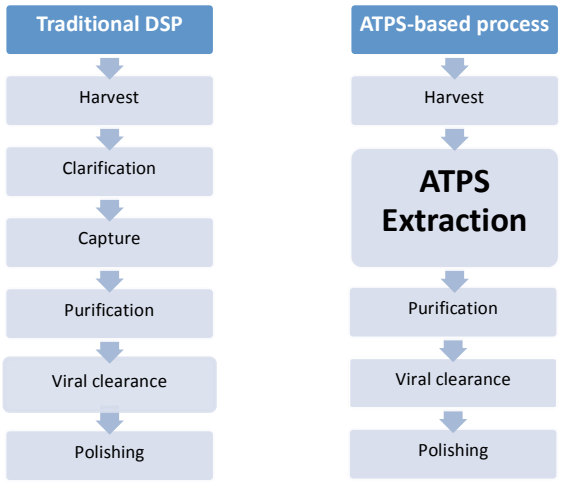

Fig. 2. Integration of clarification and capture in an ATPS-based process.

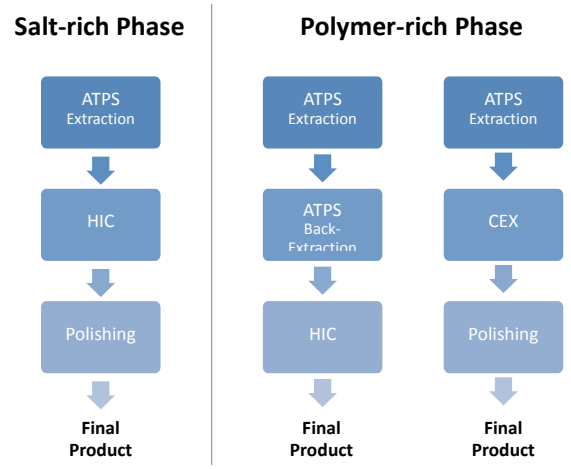

Fig. 3. Integration strategies of ATPS with other unit operations in order to avoid pre-conditioning steps depending whether the product is recovered in a salt-rich phase or in a polymer-rich phase.

One of the first reports of using ATPS in an integrative process of mAb production and purification dates to 1996. Zijlstra *et al.* were able to design an ATPS which support the long-term growth of animal cells, namely the mAb's producing hybridoma cells, and concluded that with an ATPS constituted by PEG 35,000 and dextran 40,000 hybridoma cells partitioned almost completely to the lower phase. However, with this system the mAb also partitions into the lower phase. Nevertheless, preliminary work using an affinity ligand coupled to PEG indicated that the partition coefficient of the mAb could be considerably improved (Zijlstra *et al.*, 1996), making this study very important, since it opens the door for the integration of both production and purification of bioproducts by animal cell culture.

Process integration can also address a holistic approach to process design in such a way that the outlet streams from a certain unit operation can be directly used as inlets for the next unit operation, eliminating thus the need for feed preconditioning (Figure 3). In 2008, Azevedo *et al.* evaluated an integrated process using ATPE, HIC and SEC for the purification of human IgG from a CHO cell supernatant. These unit operations were designed to allow the removal of target impurities and also the integration of different process units without the need for any conditioning step between them. An ATPS composed of 10% (w/w) PEG 3350 and 12% (w/w) citrate, at pH 6, allowed the recovery of IgG with a 97% yield, 41% HPLC purity and 72% protein purity. This bottom phase was then directly loaded on a phenyl-Sepharose HIC column to further improve the purification of IgG from the remaining proteins and taking into account the high concentration of the citrate salt present in the bottom phase. In general, proteins bind to HIC columns at high salt concentration followed by elution with a descending salt concentration. Thus, it was used a citrate mobile phase and the antibody recovered in the elution fraction with 99% of recovery yield, 86% HPLC purity and 91% protein purity. Finally, SEC allowed the final polishing by removing IgG aggregates and, at the same time, to desalt the antibody solution. The fractions collected in the HIC step were thus directly loaded in a Superose 6 size-exclusion column, without any kind of treatment and run in isocratic mode, leading a 100% pure IgG solution with 90% yield (Azevedo *et al.*, 2008).

Another important aspect to intensify a process is related to its scale-up. Until very recently, most of the process that involve ATPS were performed in agitated vessels followed by centrifugation (Rosa *et al.*, 2009a), however this equipment assembly (agitated vessel + centrifuge) provides only one theoretical stage and is expensive at large scale (Srinivas *et al.*, 2002). From the total product recovery and economic view point, it is clear that the definition of one-stage ATPS primary recovery processes are ideally preferred (Rito-Palomares, 2004). Nevertheless, more than one theoretical stage are typically necessary to achieve the desired yield and purity at large scale (Rosa *et al.*, 2009a), since in order to achieve high recovery yields, higher volume ratios and larger amounts of phase forming components and ligand will be necessary,

leading to a highly diluted product with a low purity. Additionally, an extra concentration step may be required, leading, as was discussed before, to a less cost-effective process. Hence, it is necessary to find a suitable compromise between recovery yield and purity. This problem can be solved using a multi-stage process. Actually, in a multi-stage approach, it is not necessary to select a system composition that allows a very high recovery yield, since it can be achieved by increasing the number of stages, although it is important to select a system that allows a high purification factor instead, in order to extract the lowest amount of contaminants along the different stages. Thus, using a multi-stage process, it will be possible to achieve a more effective process in terms of selectivity, target product enrichment, raw materials consumption and throughput (Rosa *et al*., 2009b).

The typically used multi-stage liquid-liquid extraction (LLE) equipments in chemical industry are known to provide more than one theoretical stage, in which high recovery yields and purities may be obtained. Conventional multi-stage extraction equipment such as spray columns, mixer-settler battery or column extractors, and non-conventional extraction equipment such as pulsed plates column, agitated plates columns or pulsed flower columns can be conveniently applied as an alternative to traditionally extraction equipment (agitated vessel + centrifuge) may be employed (Rosa *et al*., 2009a). In fact, continuous extraction has been used in the purification of proteins as this process increases the partition of target proteins compared with the batch ATPS process (Rito-Palomares, 2004). One example of an application of ATPS for a continuous operation in the recovery of a bioproduct is the work reported by Biazus *et al*. (2007), in which α- and β-amylase enzymes from Zea mays malt were recovered by continuous extraction using a PEG/$CaCl_2$.

Recently, in 2009, Rosa *et al*. were able to evaluate the feasibility of performing a multi-stage equilibrium ATPE of IgG and respective purification from the CHO cells supernatant impurities by simulating a cross-flow multi-stage extraction in test tubes. This study was later validated in a packed column and in a mixer-settler battery. In 2012, Rosa *et al*., evaluated the performance of a pilot scale packed differential contactor for the continuous counter-current ATPE of human IgG from a CHO cell supernatant. An IgG recovery yield of 85% was obtained with about 50% of total contaminants and more than 85% of contaminant proteins removal (Rosa *et al*., 2012). In 2013, the same authors showed the feasibility of using a mixer-settler battery to successfully purify human antibodies from a CHO and PER.C6 cells supernatants. A full process incorporating 6 extraction stages, 1 back-extraction stage and 3 washing stages was proposed allowing the removal of 97% of the impurities found in the CHO cell supernatant and 99% in the PER.C6 supernatant.

Final remarks

As a final conclusion, it is possible to state that ATPS represent an interesting alternative methodology in the downstream processing of biopharmaceutical products, mainly in mAbs, since they provide a biocompatible environment for biomolecules and it is possible to achieve at large scale clarification, recovery and purification of the target product in just one step. However, it is required some additional studies regarding the use of other polymers and conditions and also to design better strategies that allows process integration and intensification.

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References

Albertsson P.A. (1956). Chromatography and partition of cells and cell fragments. *Nature 4513*, 771-4.

Azevedo A.M., Rosa P.A.J., Ferreira I.F., Pisco A.M.M.O., de Vries J., Korporaal R., Visser T.J., y Aires-Barros M.R. (2009b). Affinity-enhanced Purification of Human Antibodies by Aqueous Two-phase Extraction. *Separation and Purification Technology 65*, 31-39.

Azevedo A.M., Rosa P.A.J., Ferreira I.F., y Aires-Barros M.R. (2007). Optimisation of Aqueous Two-phase Extraction of Human Antibodies. *Journal of Biotechnology 132*, 209-17.

Azevedo A.M., Rosa P.A.J., Ferreira I.F., y Aires-Barros M.R. (2009a). Chromatography-free Recovery of Biopharmaceuticals through Aqueous Two-phase Processing. *Trends in Biotechnology 27*, 240-47.

Azevedo A.M., Rosa P.A.J., Ferreira I.F., y Aires-Barros M.R. (2008). Integrated Process for the Purification of Antibodies Combining Aqueous Two-phase Extraction, Hydrophobic Interaction and Size-exclusion Chromatography. Journal of Chromatography A 1213, 154-61.

Biazus J.P.M., Santana J.C.C., Souza R.R., Jordão E., y Tambourgi E.B. (2007). Continuous extraction of α- and β-amylases from Zea mays malt in a PEG4000/$CaCl_2$ ATPS. *Journal of Chromatography B 858*, 227-33.

Birch J., y Racher A. (2006). Antibody Production. *Advanced Drug Delivery Reviews 58*, 671-85.

Brekke O.H., y Sandlie I. (2003). Therapeutic Antibodies for Human Diseases at the Dawn of the Twenty-first Century. *Nature Reviews Drug Discovery 2*, 52-62.

Chan C.E.Z., Chan A.H.Y., Hanson B.J., y Ooi E.E. (2009). The Use of Antibodies in the Treatment of Infectious Diseases. *Singapore Medical Journal 50*, 663-73.

Cunha M.T., y Aires-Barros M.R. (2002). Large-Scale Extraction of Proteins. *Molecular Biotechnology 20*, 029-40.

DSM Press Release. (2008). DSM and Crucell Announce PER.C6® Technology Milestone.

Ferreira I.F., Azevedo A.M., Rosa P.A.J, y Aires-Barros M.R. (2008). Purification of human immunoglobulin G by thermoseparating aqueous two-phase systems. *Journal of Chromatography A 1195*, 94-100.

Frost and Sullivan. (2012). Frost & Sullivan Looks at the Future of Disease Therapeutics. Disponible en: http://www.prnewswire.com/news-releases/frost–sullivan-looks-at-the-future-of-disease-therapeutics-150142285.html. Accesado: 4 octubre 2012.

Gottschalk U. (2008). Bioseparation in Antibody Manufacturing: The Good, The Bad and The Ugly. *Biotechnology Progress 24*, 496-503.

Liu H.F., Ma J., Winter C., y Bayer R. (2010). Recovery and Purification Process Development for Monoclonal Antibody Production. *MAbs 2*, 480-99.

Morrow Jr. K.J. (2012). *The New Generation of Antibody Therapeutics: Current Status and Future Prospects - Overview*. Insight Pharma Reports, Cambridge Healthtech Institute, Needham, MA.

PhRMA (2013). Medicines in Development: Biologics. 2013 Report, Pharmaceutical Research and Manufacturers of America, Washington, DC.

Rito-Palomares M. (2004). Practical Application of Aqueous Two-phase Partition to Process Development for the Recovery of Biological Products. *Journal of Chromatography B 807*, 3-11.

Rosa P.A.J., Azevedo A.M., Ferreira I.F., de Vries J., Korporaal R., Verhoef H.J., Visser T.J., y Aires-Barros M.R. (2007). Affinity Partitioning of Human Antibodies in Aqueous Two-phase Systems. *Journal of Chromatography A 1162*, 103-13.

Rosa P.A.J., Azevedo A.M., Ferreira I.F., Sommerfeld S., Bäcker W., y Aires-Barros M.R. (2009b). Downstream processing of antibodies: Single-stage versus multi-stage aqueous two-phase extraction. *Journal of Chromatography A 1216*, 8741-49.

Rosa P.A.J., Azevedo A.M., Sommerfeld S., Bäcker W., y Aires-Barros M.R. (2011). Aqueous Two-phase Extraction as a Platform in the Biomanufacturing Industry: Economical and Environmental Sustainability. *Biotechnology Advances 29*, 559-67.

Rosa P.A.J., Azevedo A.M., Sommerfeld S., Bäcker W., y Aires-Barros M.R. (2012). Continuous aqueous two-phase extraction of human antibodies using a packed column. *Journal of Chromatography B 880*, 148-156.

Rosa P.A.J., Azevedo A.M., Sommerfeld S., Mutter M., Aires-Barros M.R., y Bäcker W. (2009a). Application of aqueous two-phase systems to antibody purification: A multi-stage approach. *Journal of Biotechnology 139*, 306-13.

Rosa P.A.J., Ferreira I.F., Azevedo A.M., y Aires-Barros M.R. (2010). Aqueous Two-phase Systems: A Viable Platform in the Manufacturing of Biopharmaceuticals. *Journal of Chromatography A 1217*, 2296-305.

Schügerl K., y Hubbuch J. (2005). Integrated Bioprocesses. *Current Opinion in Microbiology 8*, 294-300.

Shukla A.A., Hubbard B., Tressel T., Guhan S., y Low D. (2007). Downstream Processing of Monoclonal Antibodies-Application of Platform Approaches. *Journal of Chromatography B 848*, 28-39.

Srinivas N.D., Narayan A.V., y Raghavarao K.S.M.S. (2002). Mass Transfer in a Spray Column during Two-phase Extraction of Horseradish Peroxidase. *Process Biochemistry 38*, 387-91.

Zijlstra G.M., de Gooijer C.D., van der Pol L.A., y Tramper J. (1996). Design of aqueous two-phase systems supporting animal cell growth: A first step toward extractive bioconversions. *Enzyme and Microbial Technology 19*, 2-6.