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Review

# Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals

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ABSTRACT

The number of biotechnology-based pharmaceuticals in the late-stage pipeline has been increasing more than ever. As a result, there is an enhanced demand for more efficient and cost-effective processes. During the last years, the upstream technology for the production of biopharmaceuticals has been considerably improved. Continuous discoveries in molecular biology and genetics, combined with new advances in media and feed development, have significantly increased the production titres. In order to keep up this gain, it is now essential to design new, as well as to improve the existing downstream processes that remain an unresolved bottleneck. This review evaluates several alternatives to the currently established platforms for the downstream processing biopharmaceuticals, with main focus on aqueous two-phase extraction.



**Contents**



## 1. Introduction

The global pharmaceutical market is set to double in the coming years, being forecasted to reach US$1.3 trillion by 2020 [1]. Although small-molecule development declines, the global biopharmaceutical market is anticipated to continue to grow with expected revenues of US$100 billion by as early as 2010 [1]. Biopharmaceuticals have greatly improved the treatment of many diseases, and sometimes are the only approved therapies available for a particular disease. These biologic-based products, including recombinant therapeutic proteins, monoclonal antibody-based products for *in vivo* medical purposes and nucleic acid-based medicinal products, have shown to have application in several medicinal focus areas such as vaccination, immunisation, oncology, autoimmune, cardiovascular, inflammatory and neurological diseases (Table 1) [2,3].

Monoclonal antibodies (mAbs) represent the fastest growing biopharmaceutical market segment with a potential to reach total global sales of US$50 billion by the year 2013 [6]. More than 25 therapeutic mAbs have already been approved, several of which have reached blockbuster status, and many more are under development and in active clinical trials [7]. Besides their high rate of success, these biopharmaceuticals are also amongst the most expensive drugs available in the market. The annual cost per patient can reach up to US$40 thousand for antibodies that treat cancer conditions

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**Table 1**
Some biopharmaceutical products approved for human diseases [4,5].

| Biopharmaceutical | Company | Main indication | FDA approval date for first indication |
|---|---|---|---|
| Ilaris® (monoclonal antibody) | Novartis | Auto-inflammatory disease | 2009 |
| Ceprotin™ (human plasma-derived protein C) | Baxter Healthcare Corporation | Severe congenital protein C deficiency | 2007 |
| Lucentis™ (monoclonal antibody) | Genentech/Novartis | Age-related macular degeneration | 2006 |
| Fortical® (calcitonin) | Unigene | Postmenopausal osteoporosis | 2005 |
| Avastin® (monoclonal antibody) | Genentech | Cancer | 2004 |
| Xolair® (monoclonal antibody) | Genentech/Novartis | Allergic asthma | 2003 |
| Humira® (monoclonal antibody) | Abbott Laboratories | Rheumatoid arthritis | 2002 |
| Mylotarg® (monoclonal antibody) | Wyeth Pharmaceuticals | Acute myeloid leukaemia | 2000 |
| Remicade® (monoclonal antibody) | Centocor/J&J | Crohn's disease/rheumatoid arthritis | 1998 |
| Rituxan® (monoclonal antibody) | Genentech/Biogen Idec | Non-Hodgkin's lymphoma | 1997 |
| Betaseron® (β-interferon) | Berlex Inc./Chiron Corp. | Multiple sclerosis | 1993 |
| Proleukin® (interleukin 2) | Chiron Corp. | Cancer | 1992 |
| Actimmune® (γ-interferon) | Genentech/Intermune | Chronic granulomatous disease/osteopetrosis | 1990 |
| Epogen® (erythropoietin) | Amgen Inc. | Anaemia | 1989 |
| Orthoclone OKT® 3 (monoclonal antibody) | Ortho Biotech/Johnson&Johnson | Transplant rejection | 1986 |
| Intron A®/Roferon A® (α-interferon) | Schering Plough/Hoffman La Roche | Cancer/hepatitis | 1986 |
| Protropin® (human growth hormone) | Genentech | Growth deficiency | 1985 |
| Humulin® (human insulin) | Genentech/Eli Lilly | Diabetes | 1982 |

[8,9]. Given that, in contrast to other biopharmaceutical products, such as several vaccines, hormones and growth factors, mAbs may be administered to the patients in relatively large doses over a long period of time in order to achieve the desired level of efficacy, the treatment costs shoot even further [9].

In the past years, upstream processes have been receiving a considerable R&D investment and commercial attention. Remarkable advances in the cell culture technology, such as improved production media and feeding strategies, have boosted upstream productivity, with the yield of recombinant proteins increasing from a few milligrams per litre of cell culture to several grams per litre [10,11]. In addition, the maximum scale of mammalian cell bioreactors has been doubled in the last 10 years [12]. The downstream processes have, however, been overlooked causing a production bottleneck that is shifting the costs of production from up to downstream. Although the high titres can be handled by increasing the scale of the current purification platforms (e.g. using larger chromatography columns and filters), at some point, the physical limits of the existing facilities throughput and scalability will be reached [10,13]. The need to develop more efficient and cost-effective separation and purification processes is, thus, crucial in order to improve process efficiency and economics keeping the required high standards of quality for market approval. In this review, several alternatives to the currently established platform for the downstream processing of biopharmaceuticals, with main focus on aqueous two-phase extraction, are evaluated.

## 2. Life beyond chromatography: What are the alternatives?

The efficient recovery and purification of biopharmaceuticals has been referred as a critical part of the production process [14]. The developed purification process must be robust, reliable, easily scaled-up and capable of removing both process-and product-related impurities as well as clearing virus in order to ensure product safety. The achieved purity, speed of process development, overall recovery yield and throughput are some of the main key parameters that must be taken into consideration during downstream process development [14]. The downstream section of a biopharmaceutical manufacturing plant usually encompasses three main sectors, namely, initial recovery, purification and polishing. The major process bottleneck has been found in the selective purification steps, currently dominated by packed-bed chromatography [15]. For example, the established platform for the purification of mAbs usually includes three chromatographic steps, in which the monoclonal is firstly adsorbed to an affinity resin, almost invariably a protein A affinity column, followed by two further chromatography steps, which will allow the removal of the host cell proteins, DNA, any leached protein A and aggregates as well as will provide an adequate level of overall viral clearance (Fig. 1) [16]. Although chromatography has been the workhorse of downstream processes due to its simplicity and high resolving power, at the same time, it has also been the major cost centre mainly due to media cost and relatively long cycle times [15]. In addition, higher-titre processes have been imposing practical limitations that will make the current technology platforms reach their limits of throughput and scalability [10]. Thömmes and Etzel presented a simple calculation for the capture of 100 kg of mAbs by protein A affinity chromatography to illustrate the chromatographic separations limitations at large scale [17]. If one assumes an optimal loading capacity of 50 g/L (close to the reported upper limits for the currently available resins [12]), a 3.2 m diameter and 25 cm bed height packed column would be required. Nevertheless, the largest industrial scale biochromatography columns used nowadays are just about 2 m in diameter and are operated at a 10–20 cm bed height. Very large columns can be as robust and reliable as small ones, but the high costs of the resins, buffers, and other consumables outstrip any upstream gains. In addition, large columns also suffer from scale-related packing problems, such as hysteresis, edge-effects and resin compression which may result in unpredictable fluid distribution and pressure drops [10]. The need for oversized columns can be overcome if several sequential cycles are used to process a single batch, common industrial practice for the protein A capturing of antibodies. This would reduce the initial capital investment but increase the operating costs as, besides a longer a process, more equilibration, wash, elution, regeneration and sanitisation steps would be required and the operational lifetime of the resin would be lower. Another option would be to use several smaller columns and operate them in parallel.

Several alternatives to the established platforms have generated long-standing interest either to replace column chromatography or to eliminate the number of chromatography steps by reducing the load of impurities in the feed stream [10]. Examples are flocculation, precipitation, crystallisation, high gradient magnetic fishing, membrane chromatography, filtration and liquid–liquid extraction [3,10,15–19]. Flocculation can be used in combination with the conventional used harvest and clarification unit operations in order to enhance the removal of both residual particulates and process-related impurities, such as host cell proteins and host cell DNA [10,17]. It is a similar process to coagulation, where suspended particles clump together due to attractive forces, which overcome

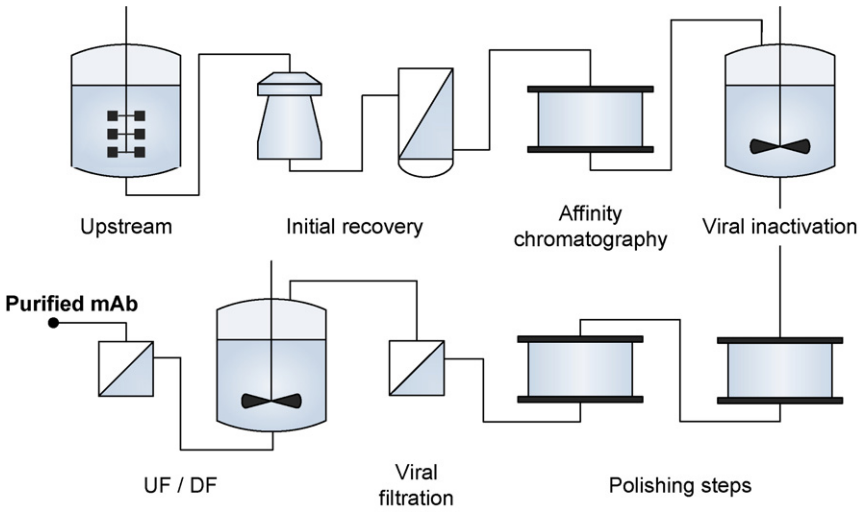


**Fig. 1.** Platform downstream process for mAbs.

any repulsive forces caused by like surface charges. These repulsive forces can be eliminated by the addition of inorganic electrolytes, which shield the surface charges, or polyelectrolytes that bind to and neutralize the surface charge. In addition, the agglomeration of particles can also be caused by a bridging effect exerted by the polymers that are adsorbed to more than one particle [18]. Polymers, such as polyethyleneimine, have been often added during cell harvesting in order to induce flocculation and to enhance the removal of cell debris by centrifugation. These polymers are, however, toxic and difficult to remove during the purification process [18]. The addition of non-toxic flocculating agents, such as calcium chloride and potassium phosphate, in combination with centrifugation has been proposed by Coffman et al. and Shpritzer et al. for the recovery of monoclonal antibodies [17]. A calcium phosphate precipitate was formed rapidly leading to the flocculation of the cell debris and allowing to obtain a clear supernatant after centrifugation. A subsequent filtration was still necessary for clarification, but requiring, however, less membrane area consequently reducing the process-related costs. In addition, the integration of flocculation in the process also led to a reduction of the burden on the subsequent downstream steps, allowing the number of chromatographic steps to be decreased [10,17].

Precipitation has been described as a mature technology and is among the simplest and less expensive fractionation methods. Under mild conditions, protein precipitation is reversible and subsequent redissolution can restore total activity. This technology can be used either to remove impurities or to isolate the target protein in a mixture [18]. In the first case, the differential solubility of proteins may be used for fractionation or to remove bulk proteins with different solubilities to that of the target product. The resulting precipitate should be treated as a particulate contaminant and removed during the recovery step. In the second case, the precipitation and subsequent redissolution of the target protein in a smaller volume of buffer not only reduces the processing volume, but also leads to a more concentrated target product pool free from the soluble contaminants. Precipitation has, however, been recognised as a low resolution separation technique due to its lack of specificity. In addition, in the large scale processes, the cost of the precipitating agents as well as the environmental impact of their disposal may also be an important bottleneck [20]. Affinity ligands can, nevertheless, be used as ligand carriers to improve the selectivity of this technology. Several polymers, including Eudragit [21], poly-*N*-isopropylacrylamide [22] and elastine-like protein [23], have been evaluated. The target protein adsorbs to the polymer in a homogeneous phase, after which, the entire affinity complex precipitates in a single step by manipulating the experimental conditions. After centrifugation, the affinity complex is separated from impurities and contaminants that remain in the supernatant. The target protein can, afterwards, be dissociated from the macroligand by manipulating the experimental conditions, in such a way that, the polymer will become insoluble and will precipitate. The target protein will be hence recovered from the supernatant, while the macroligand can be resolubilised for reuse [19].

Crystallisation is another inexpensive technology that has been recognised for many years as a powerful technology because of its ability to simultaneously concentrate, purify and stabilise the target product [10,17]. This process involves the formation of a regularly structured solid phase, which impedes the incorporation of contaminants or solvent molecules and, therefore, yields products of exceptional purity. The manufacturing process of the world's first successful biopharmaceutical, a recombinant human insulin, launched into the healthcare market in 1982, relied on a crystallisation step [17]. Some other commercial processes for therapeutic proteins manufacture have also been involving a crystallisation step either as a bulk storage step or as a replacement of a chromatography step [10,17]. Examples are the recombinant protease inhibitor aprotinin, a mutein of the bovine pancreatic trypsin inhibitor (BPTI) [24], and the protein Apo2L, a member of the tumor necrosis factor family of cytokines [25]. The next major milestone for this unit operation in the biopharmaceutical industry will be the development of a crystallisation process for a therapeutic monoclonal antibody as an alternative to a chromatography step. In fact, the implementation of crystallisation at process scale for separation and purification of therapeutic antibodies still remains a challenge mainly due to their large size, glycosylation and their high degree of segmental flexibility [16]. In addition, the inherent complexity of the process and process control difficulties are also relevant concerns [10].

The high gradient magnetic separation technology is also a promising approach that has been adapted from the chemical and mineral processing industries. Öskara et al. have prepared a novel magnetic poly(ethylene glycol dimethacrylate-*N*-methacryloly-L-histidine-methylester) support for the purification of antibodies in a magnetically stabilised fluidised bed [26]. Higher adsorption values of immunoglobulin G from human plasma of up to 320 mg/g and a purity of 87% were obtained. The main advantages of this pseudospecific affinity chromatography consist of its simplicity, stability and low cost [26].

Membrane chromatography is also emerging as an attractive alternative to traditional column chromatography [17]. However, it has some limitations that need to be overcome before routine successful process-scale production, such as distorted or poor inlet flow distribution, non-identical membrane pore size distribution, uneven membrane thickness and lower binding capacity [27,28]. The first three limitations can be minimised when using multiple-layer configurations [27]. Nevertheless, the low binding capacity, which can be attributed to lower surface to bed volume ratio as well as to flow distribution problems, is still a major concern in a bind/elute purification mode [27,28]. In a flow-through mode, this bottleneck is no longer considered as a major issue. Flow-through membrane chromatography has been successfully used to remove traces of process-related impurities, such as host cell proteins, DNA, RNA, endotoxins and viruses, at less than 1% concentration [28,29]. The demand for a flow-through high throughput polishing step in large scale antibody process has been a great opportunity for the application of this alternative technology. The disposable membrane chromatography systems can offer many advantages over packed-bed chromatography, including large scale columns packing-related problems, equipment and adsorbent cleaning, column lifetime, validation, flowrate, buffer usage and floor space [17,28]. Zhou and Tressel from Amgen have developed a disposable cost model for a 10-year process, which compares packed-bed chromatography to disposable membrane-based chromatography [28]. A 23% cost reduction, a significant 95% buffer consumption reduction and a faster processing time were attained when disposable membrane chromatography was used instead of the conventional packed-bed chromatography [28].

Conventional liquid–liquid extraction, using organic-aqueous phase systems, is a classical and versatile technology that has been established as a workhorse in the pharmaceutical industry. This unit operation, with all its advantages, has, however, not gained wide industrial recognition in the field of biotechnology due to the poor solubility and possible denaturation of the proteins in organic solvents [30]. Its use in biotechnology has been, thus, limited to the recovery of low molecular weight products, such as antibiotics and organic acids from fermentations broths. On the other hand, liquid–liquid extraction based on aqueous two-phase systems (ATPSs) has shown a great potential and versatility for the downstream processing of biopharmaceuticals, such as monoclonal antibodies [11,31–43], high density lipoproteins [44], hormones [45,46], cytokines [47,48], growth factors [49,50] and plasmid DNA [51,52]. 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 stabilising effect on the protein tertiary structure and biological activity [53,54]. Aqueous two-phase extraction (ATPE) has important advantages over the currently established packed-bed chromatography, as it can combine a high biocompatibility with an easy scale-up and continuous mode of operation [53]. In addition, it can overcome some of the technical drawbacks currently encountered with most chromatographic supports, such as high cost, low productivities, scale-related packing problems and diffusional limitations [11,14,53].

## 3. Aqueous two-phase systems

Aqueous two-phase systems result from the incompatibility between two aqueous solutions of structurally different components such as two polymers (e.g. polyethylene glycol and dextran), or a polymer and a salt (e.g. phosphate), above a certain critical concentration [53]. This phenomenon has been firstly reported in the literature by Beijerinck in the 19th century, who discovered that agar and gelatine formed two phases when mixed at certain concentration [55]. However, it was not until 1955 that Albertsson discovered that polyethylene glycol (PEG), potassium phosphate and water, and PEG, dextran and water formed two-phases and realised the potential use of these systems as an important separation technique in the downstream processing of biomolecules [55]. Besides advantageous economics and technological simplicity, ATPE can further be considered as an integrated process, in which the insoluble components can be removed while at the same time the target product is purified. The selective partitioning of a target product between the two phases is the basis of a two-phase system separation. This is controlled by a number of parameters relating to the system properties (e.g. polymer type, molecular weight and concentration, salts type and concentration, pH values, and ionic strength) and the target solute (e.g. charge, molecular weight, hydrophobicity, and conformational characteristics) and the interactions between the two. The complexity of the chemical and physical interactions involved in the partitioning process can make these systems very powerful in contrast to other established separation techniques, as a very high resolving power can be achieved just by manipulating the system properties. It is, hence, not surprising that successful applications of ATPSs have been reported in the literature for the downstream processing of several biopharmaceuticals. Table 2 presents the prototype ATPSs developed for the purification of some biopharmaceuticals.

The predictive design of ATPE processes and respective application at process scale has been, however, hindered by the complexity of the systems combined with the fact that the partition mechanisms are poorly understood and method development is relatively empirical [54,59]. A more or less elaborate screening of polymer and buffer concentration, polymer and buffer type, pH or temperature had to be performed for the most of the processes presented in Table 2. The time consuming, random screening approach as well as the overall low predictability concerning the impact of potential process changes on process performance can, thus, be considered as a major drawback for this unit operation to be implemented at large scale. The design of experiments (DoE) can be used as a powerful strategy to find the optimal purification process conditions, to analyse the influence of the different experimental parameters involved and to better evaluate the interactions between them [36]. Alternatively, a high throughput and automated screening approach allowing a rapid parameter evaluation can be used. Using this approach, it is possible to establish conditions for effective separation of target molecules and also to test process robustness and parameter dependencies in a short time [59].

## 4. Developing a manufacturing aqueous two-phase extraction process

Practical strategies for the ATPE processes design and implementation at large scale are essential to overcome the poor understanding of the molecular mechanism behind the solute partitioning in ATPS and, consequently, minimise the required time for the ATPE process design [60]. Typically, general process conditions can be selected based on the acquired experience in the partitioning of the target solute in ATPS. This represents, however, a major bottleneck for the generic and wide application of this technique as, in one hand a large number of experiments would be necessary, and in the other hand the researchers interested in the use of ATPE would need to become experts in the area before starting the process design. A practical strategy for the ATPE processes development has been suggested by Benavides and Rito-Palomares [60,61]. This strategy can be divided into four main stages: (i) initial physicochemical characterisation of the feedstock; (ii) selection of the type of ATPS; (iii) selection of system parameters and (iv) evaluation of

**Table 2**
Aqueous two-phase extraction of biopharmaceuticals.

| Biopharmaceutical | Production source | ATPS | Recovery yield (%) | Purity (%) | PF | Reference |
|---|---|---|---|---|---|---|
| Monoclonal antibody (human immunoglobulin G) | Chinese hamster ovary cells | PEG/phosphate | 88 | – | 4.3 | [38] |
| | | PEG/dextran | 96 | 95 | – | [42] |
| | | PEG-$(COOH)_2$/dextran | 82 | 96 | 3.6 | [43] |
| | | EOPO/dextran | 85 | 88 | – | [39] |
| | | PEG/citrate | 97 | 76 | 1.8 | [40] |
| Human interleukin-18 binding protein (IL-18BP) | Chinese hamster ovary cells | PEG/sulphate | 98 | 92 | 2.3 | [56] |
| Human monoclonal anti-human immunodeficiency virus (HIV) 2F5 | Transgenic tobacco extract | PEG/phosphate | 95 | – | 3–4 | [35] |
| Human growth hormone (hGH) | *Escherichia coli* | EOPO/starch | 70 | – | 5.0 | [46] |
| Apolipoprotein A (Apo A) | *E. coli* | Reppal/EOPO | 82 | – | 3.0 | [57] |
| Human growth hormone antagonist | *E. coli* | PEG/sulphate | 83 | – | – | [45] |
| Monoclonal antibody (murine immunoglobulin G1) | Hybridoma cells | PEG/phosphate | 90 | 80 | 5.9 | [32] |
| Human recombinant interferon α1 (rhIFN-α1) | *E. coli* | PEG/phosphate ester | 76 | – | 25 | [48] |
| Human insulin-like growth factor I (IGF-I) | *E. coli* | PEG/sulphate | 70 | 97 | – | [50] |
| Monoclonal antibody (immunoglobulin G) | Hybridoma cells | PEG/phosphate | 90 | – | 6.2 | [31] |
| | | PEG/citrate | 99 | 96 | 3.3 | [41] |
| Hepatitis B surface antigen (HBsAg) | Yeast cells | PEG/phosphate PEG/sulphate | 89 | – | 20.5 | [58] |
| α-1-Antitrypsin (AAT) | Yeast cells | PEG/phosphate | 100 | – | 3.6 | [58] |

PF, purification factor; EOPO, ethylene oxide/propylene oxide copolymer.

the influence of process parameters upon product recovery/purity [60,61]. The process scale-up is, however, not included in this practical strategy.

Fig. 2 presents an overview of a possible practical strategy, which can be used to facilitate the development and scale-up of ATPE processes. The partition behaviour of a defined solute in ATPSs may be strongly influenced by its physicochemical properties, such as molecular weight, isoelectric point and hydrophobicity. It is, thus, crucial to carry out a physicochemical characterisation of the target product and major contaminants present in the feedstock as a first step in the process design. Once the product of interest and major contaminants are characterised, the ATPS bottom and top phase-forming components must be selected. In general, there are two major types of ATPSs, polymer/salt and polymer/polymer. The initial selection of a polymer/salt ATPS (e.g. PEG/phosphate) has been preferred due to several process advantages including low cost, low viscosity, short phase separation time and possible recycling strategy of both polymer and salt [60–64]. When the polymer/salt ATPSs are not suitable and the cost of the product of interest is considerable, a polymer/polymer ATPS (e.g. PEG/dextran) may be considered. Alternative phase-forming chemicals, such as crude dextran, hydroxypropyl starch or waxy starch [46,61], have been used in an attempt to reduce process-related costs. No major research on the thermodynamic characterisation of these alternative ATPSs has, however, been conducted.

The partitioning of some biomolecules, although uneven, is not as one-sided as might be expected and wished. In these cases, the addition of a neutral salt or an affinity ligand can improve the partitioning of the target biomolecule, increasing significantly both the process yield and selectivity [53,65]. The effect of adding salts to an ATPS is dependent on the type of system and salt that is used. For example, in polymer/polymer systems, the uneven partition of an added salt between the top and bottom phase may alter the partitioning of the target biomolecule depending on the system pH [53]. In general, water structure making ions ($Li^+$, $Na^+$, $NH_4^+$, $Ca^{2+}$, $Mg^{2+}$, $F^-$, $SO_4^{2-}$, $CO_3^{2-}$, $PO_4^{3-}$, $CH_3COO^-$) favour the more hydrophilic phase, whereas water structure breaking ions ($K^+$, $Rb^+$, $Cs^+$, $Cl^-$, $Br^-$, $I^-$, $SCN^-$, $NO_3^-$, $ClO_4^-$) favour the more hydrophobic phase [53]. At higher salt concentration, e.g. polymer/salt systems, the addition of a neutral salt, such as NaCl, may increase the hydrophobicity difference between the phases due to the decrease of the amount of bound water [66]. This may result in the exposure of hydrophobic patches on the protein surface, which will promote hydrophobic interactions with the polymer phase [32,36,38,66], therefore, enhancing the partitioning coefficient.

Selective ligands, with specific affinity for the biomolecule of interest, can be attached covalently to one of the phase-forming components in order to make the extraction in ATPS more predictable and selective. This strategy is mainly used for polymer/polymer ATPSs, as, in polymer/salt systems, the high salt concentration usually masks an affinity or electrostatic interaction between the ligand and the target biomolecule [11,37]. Both PEG and dextran have been modified with different type of ligands to enhance the partition of target molecules. Dextran is a relatively hydrophilic polymer whose properties can be changed by introducing hydrophobic groups, as benzoyl and valeryl [67], or charged groups, such as sulphate and diethylaminoethyl. However, the PEG molecule has been the preferred target of modification as, due to its terminal hydroxyl groups, it is easily amenable to derivatisation [68]. A wide variety of hydrophobic, charged and biospecific ligand molecules have been coupled to both PEG and ethylene oxide/propylene oxide copolymer (EOPO) molecules in order to enhance the affinity of biopharmaceuticals towards the top phase [33,34,37,39,42,43,47].

Once the ATPS has been defined, a phase diagram must be obtained in order to preliminary evaluate the influence of the different system parameters, such as tieline length, phase volume ratio and pH, on the partitioning of the target product. If acceptable recovery yields and purities are attained, an experimental design methodology can be used to optimise the purification of the target molecule in the selected ATPS. The high and low experimental design set points must be defined in accordance to the preliminary partitioning results and phase diagrams. When the optimal conditions are obtained, a prototype ATPE process can be defined being, however, still necessary to further characterise the process parameters including the number of stages, phase separation, multi-stage equipment type and recycling of phase-forming components. In many ATPE processes, more than one theoretical stage may be necessary to reach the desired yield and level of purity at the lowest cost and raw materials and time consumption. The liquid–liquid

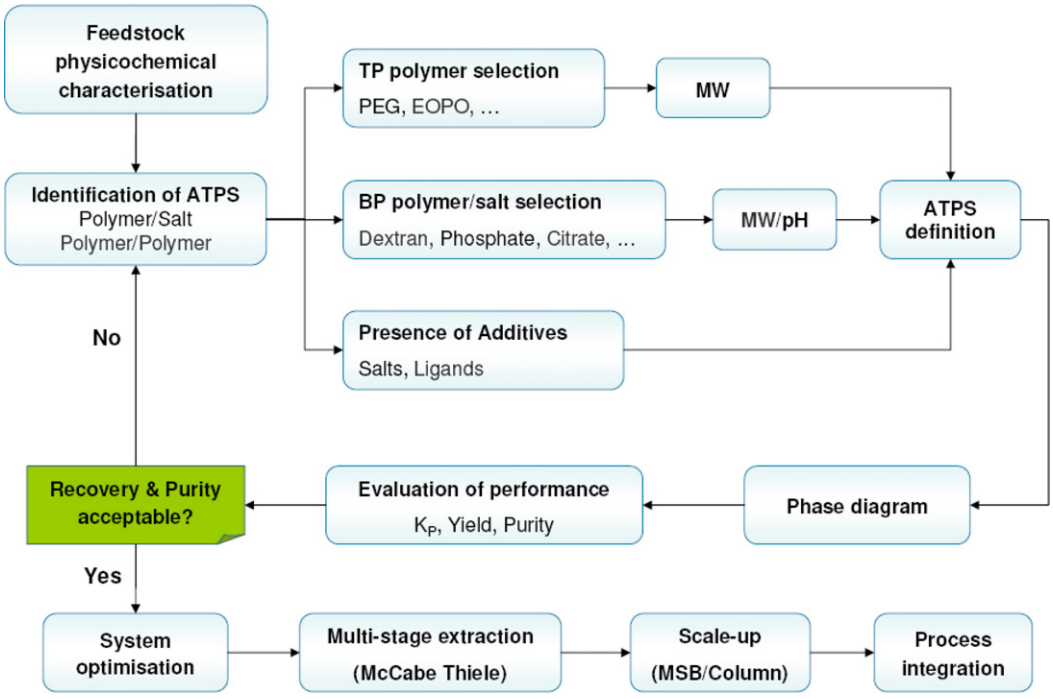


**Fig. 2.** Practical strategy to ATPE process development and scale-up for the downstream processing of biopharmaceuticals (TP, top phase; BP, bottom phase; MW, molecular weight; $K_P$, partition coefficient; MSB, mixer-settler battery) (adapted from [60,61]).

equilibrium (LLE) data should, thus, be obtained in order to determine the number of required theoretical stages and, consequently, better characterise the multi-stage liquid–liquid ATPE process. The McCabe Thiele method is typically used as a graphical solution for the determination of the number of theoretical stages [69–71], In order to use this method, a single equilibrium curve must be obtained based on the LLE data and an operating line determined according to the solute mass balance. The energy and mass balances should be also automatically satisfied [69–71].

One of the major advantages of the ATPE processes is related to the fact that the scale-up is possible through the use of conventional extraction equipment typically used in the chemical industry. The selection of an appropriate extractor is based on a large number of factors, such as systems properties, number of stages, throughput and floor space [72]. Fig. 3 shows a possible selection scheme for commercial extractors, where the most important parameters have been taken into consideration. Nevertheless, since the various liquid properties and the presence of certain impurities may have a strong effect on the extractors performance, one should notice that the final selection and design can be based only on experiments [72].

## 5. Large scale aqueous two-phase extraction: A tool for the purification of biopharmaceuticals

The success of ATPSs in the efficient generation of bench-scale prototype processes with commercial application has been reported for the recovery of a large number of biopharmaceutical products as presented in Table 2. A key feature of these systems is that they can be easily scaled-up from laboratory scale to industrial scale with performance data retained. Technical feasibility has been reported up to 100 000 L scale for the purification of proteins [30]. The biopharmaceutical industries have, however, still been reluctant to embrace this unit operation as a part of their own downstream processes, which can also be partially attributed to scale-up uncertainties and lack of know-how in terms of installation, process validation and operation [12,52,60]. 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. This drawback may be minimised by recycling the polymers and salts used in the process. The recycled raw materials have, however, to fulfil the purity requirements to guarantee the operating consistency and reproducibility of batch-to-batch ATPSs. The large amount of pure water required for the application of these systems in the downstream processing of biopharmaceuticals can also be an important concern. Nevertheless, this constraint can be easily overcome as the estimated costs of pure water for large biotech companies can be as low as US$ 0.2/L [28]. In the following subsections, the application of ATPSs on the downstream processing of biopharmaceuticals is illustrated by a selection of case studies.

### 5.1. Interleukin-18-binding protein

Interleukin-18 (IL-18), a member of the interleukin-1 (IL-1) cytokine superfamily, has been recognised as an important regulator of innate and acquired immune responses. IL-18 is expressed at sites of chronic inflammation, in autoimmune diseases, in a variety of cancers, and in the context of numerous infectious diseases [73]. Interleukin-18 binding protein (IL-18BP) is a naturally secreted protein that inhibits the IL-18 activity. In fact, IL-18BP is constitutively present in many cells and circulates in healthy humans, representing a unique phenomenon in cytokine biology. Due to the high affinity of IL-18BP to IL-18 as well as the high concentration of IL-18BP found in the circulation, it has been speculated that most, if not all of the IL-18 molecules in the circulation are bound to IL-18BP [74]. IL-18BP has, thus, been suggested as a therapeutic protein in a number of diseases and disorders, such as psoriasis, Crohn's disease, rheumatoid arthritis, psoriatic arthritis, liver injury, sepsis, atherosclerosis, allergies and others [56]. IL-18BP was initially purified from urine on an IL-18 affinity column. Extraction in ATPS offers, however, an alternative for the purification of IL-18BP. Kornmann and Baer performed an extensive screening of several PEG/salt ATPSs by using several cycles of factorial design

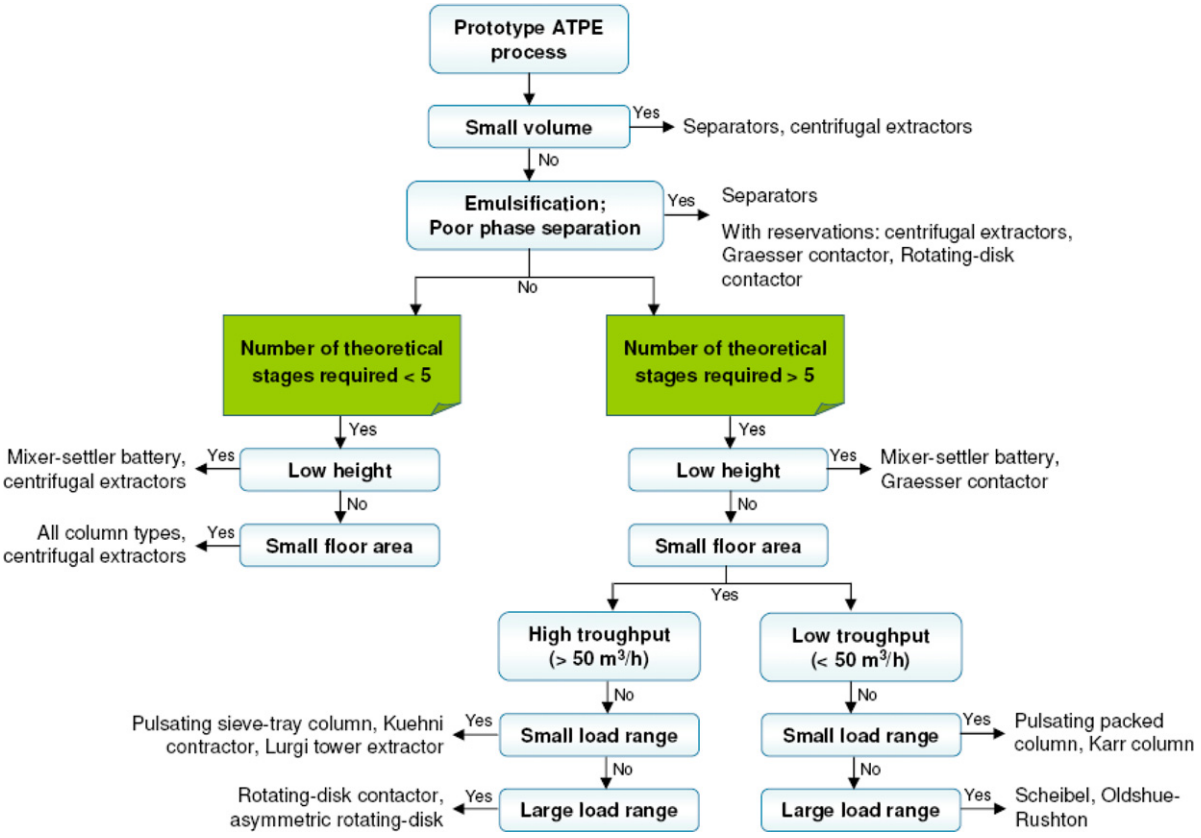


**Fig. 3.** Preliminary selection scheme for commercial extractors (adapted from [72]).

experiments. The initial experiments were performed at 2 mL scale, and it was possible to observe that the PEG/sulphate ATPSs were the best performing systems for the enhanced partitioning of IL-18BP to the polymer-rich phase. A second cycle of factorial design experiments was performed at 10 mL scale in order to select the appropriate conditions (pH, concentrations) that allow the highest recovery and purification of IL-18BP from serum-free CHO cells supernatant. The best performing ATPS was composed of 11.25% PEG 10 000/11.25% $Na_2SO_4$ at pH 5. This ATPS allowed a recovery of 98% of IL-18BP in the PEG-rich phase with a final purity of 92% [56]. The direct purification of IL-18BP from crude unclarified harvest was also assessed and it was observed that the presence of cells did not influence significantly the process performance [56]. The optimised ATPS has been 100-fold scaled-up, and no large differences were observed in the process performance. All the IL-18BP could be recovered with a final purity of 86% [56]. The developed ATPE process was still compared with two chromatographic steps typically used for the capture of IL-18BP, Q-sepharose fast flow and fractogel trimethylaminoethyl (TMAE) ion exchange chromatography. ATPE has shown to be a simpler and faster process, which can provide a higher purity and recovery yield [56].

### 5.2. Human growth hormone and respective antagonists

Human growth hormone (hGH) and antagonists for hGH, growth hormone antagonists (GHA), are examples of proteins that are useful for a variety of therapeutic applications. hGH has been used for the treatment of hypopituitary dwarfism and all other conditions resulting from low levels of hGH production. This hormone has also shown to improve the recovery of bum victims and other hospitalized patients. On the other hand, GHA has been used to treat acromegaly, a form of gigantism caused by overproduction of hGH. In addition, GHA has been used for other medical indications, such as the prevention of retinopathy in diabetes patients and the treatment of cancer patients with tumors overexpressing receptors that bind growth hormone [45]. In 2002, Hayenga and Valex have developed a multi-phase extraction process for the isolation of human growth hormone, growth hormone antagonist or a homologue of either from a biological source [45]. A two-stage extraction process using a PEG 4600/ammonium sulphate ATPS has been proposed for the recovery and purification of recombinant GHA from *Escherichia coli* cells homogenate. A first extraction with 8% PEG 4600 and 10% ammonium sulphate was performed at a 30 g scale, followed by a reextraction of the remaining GHA by adding a 40% PEG solution to the bottom phase. It was possible to recover 89% of GHA in the PEG-rich phase with high purity. The developed process has been scaled-up about 1000-fold and operated in an Alfa Laval LAPX 202 continuous disk stack centrifuge. The centrifuge has been modified as a purifier with one feed, two liquid effluents and axial solids ejection. An optimal feed rate of around 450 mL/min has been used with a discharge interval of 15–20 min. All the GHA could be recovered in the PEG-rich phase after the two-stage extraction process. The first extraction step was further scaled-up and operated in an Alfa Laval BPTX 205 disk stack centrifuge at a feed rate of about 3–5 L/min, which allowed the continuous processing of 1500 L of *E. coli* cells homogenate [45].

### 5.3. Insulin-like growth factor I

Insulin-like growth factor I (IGF-I) is a single-chain peptide produced in many tissues that is similar in molecular structure to insulin. This growth factor has a major role on the growth, survival and metabolism regulation and it has shown to have high therapeutic efficacy on the efficient control of many acute inflammatory conditions and improvement of metabolic conditions, including type 1 diabetes [75]. The ATPE process developed

by Genentech in the nineties for the recovery of periplasmic IGF-I has been reported in the literature as one of the few industrial cases known, in which the application of ATPSs for the recovery of a biological product has been successfully achieved [60]. When expressed in *E. coli*, IGF-I accumulates in both folded and aggregated forms in the fermentation medium and cellular periplasmic space. Due to its heterogeneity in production form and location, low recovery yields were obtained when using the typical clarification operations, cell disruption followed centrifugation. Hart and collaborators have proposed a new procedure, involving the addition of a chaotrope and reductant, to solubilise and extract IGF-I from cells while in fermentation broth [50]. This method, called *in situ* solubilisation, improved the recovery yield of IGF-I from the fermentation broth, but decreased the efficiency of the clarification for cell remnants removal. This was mainly related to the enhanced suspension viscosity, which increased the resistance to particle sedimentation, and the increased density of the bulk medium, which decreased the density difference driving force for solids sedimentation. To improve clarification performance and IGF-I recovery yield, an ATPE procedure was developed, which enabled the partitioning of soluble non-native IGF-I and biomass solids into separate liquid phases. The best results were obtained with systems composed of sodium sulphate and PEG 8000, which allowed the recovery of about 90% of solubilised IGF-I in the PEG-rich phase. The reliability of the developed process scale-up was also evaluated. The performance of the developed process, solubilisation followed by aqueous two-phase extraction, was reproducible at scales ranging from 10 to 1000 L. IGF-I could be recovered in PEG-rich phase with an overall recovery yield of 70% and a final purity of 97% [50].

### 5.4. Monoclonal antibodies

Monoclonal antibodies have emerged as one of the most exciting therapeutic agents in the biopharmaceutical industry. These biologicals constitute one of the most important lines of defence of the body immune system due to their high specificity and capacity of recognition and elimination of pathogenic and disease antigens. Extraction in ATPSs is an interesting alternative to the traditional downstream processing of antibodies. Several polymer/salt and polymer/polymer ATPSs have been screened and optimised for the purification of human antibodies from different complex cell culture media [11,31–43,76]. The first report describing the use of ATPSs for the purification of antibodies dates back to 1990, to the work of Andrews et al., who described an ATPE process based on a PEG molecule modified with protein A. This system was, however, not a viable approach due to the high cost of this ligand [76]. Later in the 1990s, Sulk et al. proposed a process using an aqueous two-phase extraction step and a thiophilic adsorption chromatography [31]. The process overall yield in immunoglobulin G1 (IgG1) was 71%, with 90% recovery in the ATPS step and a purification factor of 6.2. In 1996, Andrews et al. proposed a polymer/salt ATPS for the successful recovery of a murine IgG1 from a hybridoma cell supernatant in two steps [32]. The extraction of IgG to the PEG-rich phase was performed in an ATPS composed of PEG 1500, phosphate and NaCl, with a recovery yield of 90% and a purification factor of 2.7. IgG was then reextracted to a fresh phosphate solution, and the most hydrophobic compounds, which partitioned along side with IgG, were further removed by hydrophobic interaction chromatography, using ammonium sulphate as mobile phase [32]. At about the same time, Ziljstra and co-workers coupled triazine dye mimetic green to PEG in order to selectively recover IgG from hybridoma cells grown in the dextran-rich bottom phase [33,34]. More recently, the Aires-Barros group has reported the successful use of ATPSs for the extraction of antibodies from different cell culture supernatants using polymer/salt [36,38,40,41] and polymer/polymer [37,39,42,43] systems. The addition of NaCl to a PEG/phosphate enabled the separation of IgG from an artificial mixture of albumin and myoglobin [36] and the purification of IgG from both hybridoma and Chinese hamster ovary (CHO) cell supernatants [38]. PEG/citrate systems in the presence and absence of NaCl have also been successfully used for the recovery of human antibodies from both a hybridoma and a CHO cell supernatant [40,41]. The effect of coupling several hydrophobic, charged and biospecific ligands to different liquid supports, namely PEG and EOPO molecules, has been as well evaluated for the ability to bind IgG [37,39,42]. The best performing ligand was found to be glutaric acid and the best purification of IgG from a CHO cells supernatant was achieved using PEG/dextran ATPSs containing at least 1% (w/w) TEG-COOH. An IgG recovery yield of 96% and a total purity of 41% were obtained [37,42].

The success of ATPS in the efficient generation of bench-scale prototype processes for the downstream processing of antibodies has been clearly shown. High recovery yields and purities have been attained in just one stage, typically when high salt or affinity ligands concentrations or high volume ratios were used. In one hand, the use of high salt concentrations may be a concern during the scale-up of these systems to an industrial process as it will probably shorten the life time of the equipment due to corrosion problems. Also, lower loadings of supernatant may be purified in order to dissolve all the salt and it may cause more precipitation of the target product. On the other hand, the use of high ligand concentrations can result in more expensive processes, while the use of high volume ratios can lead to a highly diluted product with a low purity. A multi-stage counter-current ATPE strategy has recently been suggested by Aires-Barros group to avoid these limitations. The technical feasibility of multi-stage equilibrium ATPE of human IgG from a CHO cells supernatant has been evaluated and the performance compared to single-stage strategies [70,71]. A four stages cross-current operation was simulated in test tubes, and, according to the IgG equilibrium curves and respective McCabe Thiele diagrams, a predicted optimised scheme of a counter-current multi-stage ATPE was described [70,71]. Two different ATPSs have been evaluated, a PEG/phosphate and a PEG/dextran system. Significant improvements in both recovery yield and purity were observed when compared to a single-stage extraction step performed at the same experimental conditions [70,71]. An IgG recovery yield of 89% and a purity of 75% could be predicted when using a PEG/phosphate ATPS containing 10% NaCl, five stages and a volume ratio of 0.4. A single-stage extraction was carried out at the same experimental conditions (phase-forming components and NaCl concentrations, pH and volume ratio), and an IgG recovery yield of only 61% was attained with a purity of 55%, stressing the advantages of using multi-stage ATPE [70]. Based on the cells supernatant components equilibrium curves, it has as well been observed that the developed multi-stage ATPE process allowed the complete purification of IgG from the higher molecular weight contaminants and the partial purification from the lower molecular weight ones [70].

Similar results have been achieved when using PEG/dextran ATPSs [71]. High recovery yields and purities were achieved by finding a compromise between the ligand concentration, the number of stages and the volume ratio [71]. The best single-stage extraction conditions allowed the recovery of 96% of IgG in the PEG-rich phase with a final IgG concentration of 0.21 mg/mL, a protein purity of 87% and a total purity of 43%. Nevertheless, according to the optimised scheme of the counter-current multi-stage ATPE process, it was possible to predict that 95% of IgG could be purified in the PEG-rich phase with a final concentration of 1.04 mg/mL, a protein purity of 93% and a total purity of about 85%. The multi-stage process has shown to be more efficient than the

single-stage ATPE in terms of both IgG enrichment and removal of contaminants as well as raw materials consumption and throughput [71].

## 6. Conclusions and future challenges

During the last years, remarkable progresses in the upstream production have boosted productivity in the biomanufacturing industry. This has, however, led to bottlenecks in the downstream processing as the currently used platforms are becoming limited in terms of throughput and scalability [10]. ATPE has been reported as a valuable alternative to the established platforms due to its relatively easy scalability, capacity of continuous operation and high capacity [11,16]. The biopharmaceutical industries have, however, been reluctant to the application of this unit operation at large scale, mainly, due to the limited knowledge of the mechanism of solute partitioning in ATPS and lack of know-how in terms of installation, validation and operation [12,60,77]. In fact, three important questions still need to be addressed so that, ATPE is adopted by the biomanufacturing industries as an alternative platform. One is related to the maximum capacity of these systems, particularly whether they can process very high titre cells supernatants or whether the throughput will be limited by solubility problems. The second one concerns the limited predictive design of this process due to the poor understanding of the responsible mechanisms for the behaviour of biomolecules in ATPS. Experimental design methodology has been used as a strategy to screen and optimise the purification process conditions, allowing a fast evaluation of the different experimental parameters effect as well as their possible interactions [11,36]. Nevertheless, detailed models to predict the partition behaviour of both target product and contaminants based on solute–solvent interactions are currently lacking. The last important remaining question for this technology to be fully recognized as an alternative for the capture of biopharmaceuticals is how it compares to the currently established platforms in terms of economical and environmental sustainability.

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