# Affinity ATPS Strategies for the Selective Fractionation of Biomolecules

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## Abbreviations

| | |
|---|---|
| AL | Affinity ligand |
| ATPAP | Aqueous two-phase affinity partitioning |
| ATPS | Aqueous two-phase systems |
| CMC | Critical micelle concentration |
| DEX | Dextran |
| DS | Degree of saturation |
| EBA | Expanded bed adsorption chromatography |
| EF | Extractive fermentation |
| $K_p$ | Partitioning coefficient |
| mAbs | Monoclonal antibodies |
| MACS | Magnetic-activated cell sorting |
| mPEG | Monomethylether PEG |
| PAP | Papain |
| PEG | Polyethylene glycol |
| TLL | Tie-line length |

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TM Target molecule
UCB Umbilical cord blood
$V_R$ Volume ratio

## 6.1 Introduction

The past 20 years have been decisive for biotechnology in terms of technology development and transfer, novel protocols, optimization tools based on software utilization, and new unit operations in order to establish alternative approaches for final product design and further commercialization. In this context, the biotech industry has experienced a series of challenges that have evolved in the past decades, particularly in the downstream processing area, commonly known as the bioseparation methods division. One of the most important opportunity areas within the actual established processes is the extremely high purity required for specific bioactive compounds (e.g., human therapeutics that require purities >99.9%) (Knäblein 2005). In addition, these products need to be separated from a complex matrix of contaminants typically including whole cells, cell fragments such as membranes and organelles, polysaccharides, proteins, and nucleic acids. These aspects contribute directly to an important increase in bioprocessing costs, since a high number of unit operations are usually required to ensure product quality (i.e., purity, final presentation).

Literature reports state that the typical bioprocess cost is divided into upstream and downstream operations, the latter accounting for up to 80% of the total capital invested (Cunha et al. 2003; Knäblein 2005). In this context, there is a constant need to establish scalable and bioselective methods for product recovery and purification which finally result in the implementation of novel bioengineering strategies in order to accomplish new technical objectives and designs in bioprocesses in the biotech industry. Two common strategies that seek to contribute to the potential of a bioprocessing design are intensification and integration. Bioprocess intensification intends to maximize process efficiency increasing the throughput given in a single or group of unit operations by a careful characterization and selection of process parameters (Benavides and Rito-Palomares 2004; Hernández-Mireles and Rito-Palomares 2006; Schügerl and Hubbuch 2005). Following this strategy consumables and raw materials quantities can be reduced, thus favoring the economic and technical dimensions of the operation. On the other hand, bioprocess integration attempts to merge two or more unit operations in one, thus reducing the total number of stages in the downstream section and finally improving overall recovery and purity yields (Benavides and Rito-Palomares 2008; Schügerl and Hubbuch 2005).

Considering the advances and importance of process integration and intensification, research efforts have developed important techniques that present important benefits for novel bioprocess engineering designs. One of the most employed technologies is extractive fermentation (EF), which consists in the in situ removal of selected components with the aid of a biocompatible extracting agent (e.g., typically

an ad hoc solvent) during a fermentation step (Costa and Badino 2015). As stated by Gutiérrez et al., EF allows microbiological processes carried out in batch mode to significantly increase production yields by constantly removing end products like acetic acid or lactate in commercial fermentation steps, hence improving the effective growth rate of the host cell employed (Gutiérrez et al. 2013). A similar approach has been reported for in situ purification of high-added value products, in which a two-phase extraction system formed with polyethylene (PEG) and dextran was used to grow *Synechocystis* sp. PCC 6803 and concentrate the biomass and β-carotene in the top phase and lutein in the bottom dextran phase. The results of this study demonstrated the potential application of a two-phase extraction procedure that allowed production and in situ recovery of these cyanobacterial products, reducing the necessary steps for their final purification (Chavez-Santoscoy et al. 2010).

Another whole-broth processing technique that is fairly recent and allows recovery and purification of biomolecules from crude extracts (e.g., fermentation broths, natural sources such as vegetable or food matrixes) is expanded bed adsorption (EBA) chromatography. According to Lihme et al., if the proper adaptations are given to this technology in the current biotech industry, successful high-throughput methodologies for mass production of highly sophisticated pharmaceuticals and even low-cost industrial enzymes could be implemented (Lihme et al. 2000). The use of EBA-related approaches is attractive since it allows, in many cases, the combination of traditional separation effects obtained by centrifugation, filtration, concentration, and purification in a single step, thus reducing the number of steps and production time, increasing yields, and reducing bioprocessing costs.

Liquid-liquid extraction techniques such as aqueous two-phase systems (ATPS) constitute also an important alternative to traditional fractionation and purification techniques, primarily chromatographic-based procedures, in order to develop a scalable and economic process with integration and intensification potential. These systems are obtained when two phase-forming components (e.g., two polymers, polymer-salt, ionic liquid-salt, alcohol-salt, detergents) are mixed over certain critical conditions, thus forming a two-phase system with immiscible phases, each with different physicochemical and biochemical properties (Park et al. 2007; Silvério et al. 2008). Noticeable advantages of this technique include improved biocompatible environment for biological products, scalability and ease of operation, possibility of continuous operation for high-throughput recovery of target products, and positive bioprocess economy (Benavides and Rito-Palomares 2008; Cordes et al. 1987; Rito-Palomares 2004). In this context, ATPS have been used for the primary recovery, concentration, and purification of different types of bioproducts such as proteins, nucleic acids, whole cells and cell fragments, and molecular weight metabolites. The molecular mechanisms underlining the partition behavior in ATPS are complex, and a delicate balance between process parameters (e.g., temperature and pH, volume ratio ($V_R$), tie-line length (TLL), system constituents selection/concentration) and solute characteristics (e.g., molecular weight, hydrophobicity, superficial charge, isoelectric point, solubility) determines the efficiency of a particular system within an specific extraction and/or purification procedure (Benavides and Rito-Palomares 2008; Schindler and Nothwang 2006).

One of the major disadvantages of ATPS partitioning is their lack of specificity. Even though numerous researches have established effective partitioning of high-added value molecules by carefully designed experiments, the manipulation of system parameters does not usually provide high enough specificity to maximize target product separation from a complex sample for advanced biotechnological applications. In this tenor, several strategies could be employed to improve this major drawback, and one that has exhibited potential and positive feedback within the scientific community will be discussed in the following sections.

The objective of this chapter is to present the concept of affinity-based two-phase partitioning in order to overcome selectivity issues in traditional ATPS. The main definitions of affinity-enhanced liquid-liquid partitioning, additional variables, and challenges to be considered within these systems, along with successful examples and challenges of actual applications of this technology, will be thoroughly discussed.

## 6.2 Affinity Partitioning

### 6.2.1 Definition and Conventional Applications

Biomolecule partitioning in two-phase systems can be carried out if the preference of these molecules toward the phases in the systems is unequal, i.e., when their partitioning coefficient ($K_p$), the concentration of target product in top phase divided by the concentration of target product in the bottom phase, is different than 1. In most cases $K_p$ values of target biomolecules partitioned in ATPS are not extremely high or low ($10 < K_p > 0.1$), and therefore, the application of such systems for processes oriented to high-purity products (such as molecular diagnostics and point-of-care assays) is hampered.

In order to enhance ATPS selectivity toward recovery and purification of high-added value products, aqueous two-phase affinity partitioning (ATPAP) has emerged. This technique, as presented in Fig. 6.1, consists in the chemical modification of at least one of the phase-forming components (e.g., typically polymers such as PEG or dextran) in order to attach an affinity ligand (AL) which will exhibit high specificity toward a particular molecule (Cordes et al. 1987). A second approach consists in the addition of ligands free in solution (Fig. 6.1B), thus avoiding a phase-component activation protocol, which will also have specific affinity to the target product (Azevedo et al. 2009). The final outcome of these strategies is an improved selectivity of the system between the selected ALs and the particle to be partitioned to the modified or AL-enriched phase, thus significantly increasing/decreasing $K_p$ values and providing a different scope for novel downstream processing applications.

The first theoretical and practical approach of ATPAP was developed by Flanagan and Barondes in 1975. They proposed a scheme called "affinity partitioning" which included modified versions of polyethylene oxide (PEG) with ethylenediamine and dinitrofluorobenzene in order to have an active dinitrophenyl-PEG (dnp-PEG).

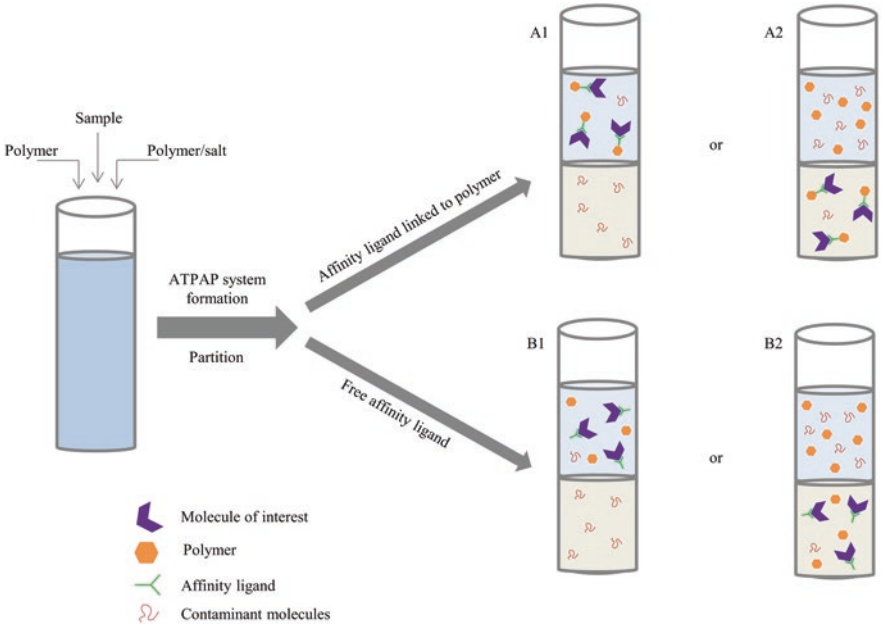


**Fig. 6.1** (*A1* and *A2*) ATPAP systems with affinity ligand (AL) chemically coupled to phase-forming chemicals in top and bottom phases, respectively. (*B1* and *B2*) ATPAP systems with ALs free in solution concentrating in top or bottom phase, respectively

This active derivative allowed a 150% increase in the partitioning coefficient of the target product, S-23 myeloma proteins, in a PEG 6000-dextran 500 system. In addition, one of the most important conclusions of the authors was that affinity interactions were easily reversible, since the addition of a simple competitor of dnp-PEG, e.g., *N*-Dnp-lysine, could reverse the effect of the partitioning behavior (Flanagan and Barondes 1975). From this point on, there has been a notable increase in ATPAP applications for the recovery of high-added value compounds in order to propose commercial applications for them or as sample-preparation protocols to be used in further analytical assays.

### 6.2.2 New Implications and Variables in ATPAP

Affinity partitioning exhibits a partitioning behavior similar to the one traditional ATPS have; nevertheless a more complex set of equilibria and variables are present in ATPAP systems, as presented in Fig. 6.2. Since the final objective of ATPAP is to modify the "natural" partition behavior of solutes, a simple thermodynamic approach may be considered in order to determine the final potential for particle partition toward a specific phase. The association/dissociation constants between all the solutes must be considered (Fig. 6.2), which include the following: (i) Polymer-AL, (ii) Polymer-AL-target molecule (TM), and (iii) AL-TM when ligands

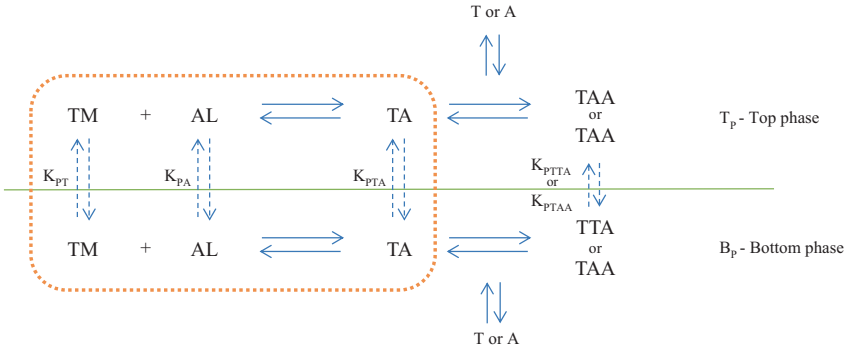


**Fig. 6.2** Scheme that highlights the possible interactions of a target product (TM or T) and an affinity ligand (AL or A) in a two-phase system with an interface in between. These reactions can apply to a single interaction between a ligand and target product (information inside the *dotted box*) or additional ligand or target product molecules can be bound consequently. The partition coefficients ($K_p$) of the complexes (TA, TAA, or TTA) require a net change in Gibbs free energy ($\Delta G$), which takes into account the concentration of both the AL, the TM, the employed phase-forming chemicals, and the conditions taken place in the ATPAP procedure, in order to finally determine the energy needed to transfer 1 mole of complex from one phase to the other

free in solution are used. If it is assumed that no products are adsorbed in the interface, the free energy required ($\Delta G_7$ in Fig. 6.2) to transfer 1 mole of complex (e.g., polymer-AL-TM or AL-TM) from one phase to the other can be calculated from the values of the $\Delta G$ changes for dissociating the complex in one phase, transferring the involved species across the interface, and finally reassembling the complex in the opposite phase (Asenjo and Andrews 2008; Johansson 2000). Following the same thermodynamic path presented by Flanagan and Barondes, the estimation of the partition coefficient of a particle in an ATPAP system can be obtained and is presented here in Eq. (6.1).

$$K_p = \frac{\left[P_f\right]^{TP} + \sum_{n=1}^{N}\left[P_n AL\right]^{TP}}{\left[P_f\right]^{BP} + \sum_{n=1}^{N}\left[P_n AL\right]^{BP}} \tag{6.1}$$

The first term, $P_f$, in Eq. (6.1) is defined as the concentration of target product free in solution in either the top or bottom phase, and the second terms are the complex aggregates between the products coupled to a selected AL, where $N$ is defined as the total number of ligands (recognition groups) in the affinity molecule. A simplified model would consider the basic interaction of 1 target molecule bound to 1 AL ($N = 1$), but this scenario does not always take place.

Considering the information previously presented for ATPAP systems, these protocols provide a biocompatible environment for biological molecules and samples which can be manipulated in order to develop more predictable purification strategies. As a result, ATPAP techniques combine the bioselectivity of an affinity technique with the robustness and scalability of well-known liquid-liquid fractionation

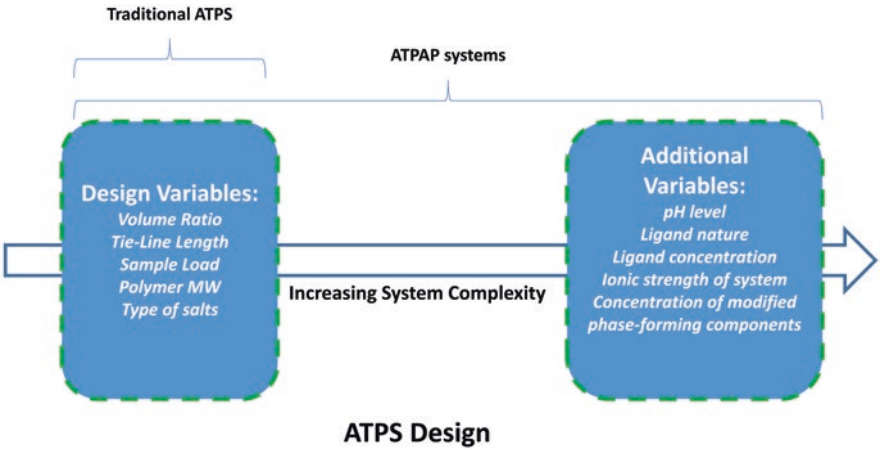


**Fig. 6.3** Variables involved in ATPS design. In the *left section*, traditional variables needed for system characterization and product partitioning. In the *right section*, important additional variables that our research group has identified in order to design a novel and effective ATPAP partitioning strategy are presented

systems, hence representing a valuable approach for the recovery and purification of high-added value biologicals. Nevertheless, a new set of process parameters must be taken into account in ATPAP protocols in order to consider the affinity interactions present. In a recent research conducted by our group, we defined key elements to be considered in liquid-liquid affinity partitioning, and now along with additional variables, these are presented in Fig. 6.3. Based on scientific literature, it can be stated that affinity-enhanced interactions in ATPAP systems are directly affected by the ionic state of both the selected ALs and the target products to be recovered (González-González and Rito-Palomares 2014). In this tenor, the pH level and ionic strength of selected systems must be carefully monitored and studied, in order to design an efficient extraction protocol. In the same subject, information regarding AL concentration and modified phase-component concentration needs to be considered. ATPAP strategies include an important term, defined as the degree of saturation (DS) (i.e., number of ligand molecules/number of polymer molecules) which constitutes a pivotal role in target product capture in a selected phase of the system. The major impact that DS poses within product capture resides in the establishment of the selective basis for product capture, since a high saturation content may inhibit system efficiency by means of a hook-type effect. Considering this, a high concentration of ALs or modified phase components could introduce shielding effects toward target molecules, generating crowding behaviors (i.e., steric hindrance) which could finally represent a problem between target products and active receptor sites. These scenarios need to be considered always if an efficient ATPAP strategy is to be designed and implemented.

## 6.3 Phase-Forming Components Activation Procedures

In order to obtain higher affinity on the target molecule, it is convenient to use specific ligands compatible with the nature of the biomolecule. The use of affinity ligands to increase the efficiency of purification can be used in two ways: (1) free affinity ligands or (2) covalently linked affinity ligands. Free affinity ligands in solution are used in order to avoid the chemical surface functionalization of the polymer (Fig. 6.1). In the case of free ligands, it is highly recommended to consider the partitioning behavior of the ligand in the ATPS before the interaction analysis between the ligand and the target molecule. On the other hand, one of the main strategies is the chemical modification of the polymer used. The surface functionalization of the polymer (e.g., polyethylene glycol (PEG) and/or dextran (DEX)) allows a covalent binding with the affinity ligand. Typically, the covalent binding between the polymer and the target molecules is achieved through hydroxyl groups of the polymer. In the case of PEG, the monomethylether of PEG (mPEG) is frequently used to cleave one target molecule per polymer chain and/or to link multiple PEG chains to avoid a cross-linking reaction (Zalipsky 1995). In this regard, the first step of the downstream processing of a biomolecule is to find a suitable functionalization of the polymer.

There are different methodologies that can be used in surface functionalization of the polymer (Fig. 6.4). In the particular case of PEG and DEX, surface functionalization is achieved by the activation of hydroxyl groups present in those polymers. There are two main approaches for the functionalization of the polymer's surface: (1) direct transformation of OH groups and (2) reaction of the molecule with a bifunctional molecule which means that one function will form the attachment to the polymer and the other remains available for further chemical reactions (Zalipsky 1995). The most accurate methodology should be selected according to the nature and properties of the target molecule. For molecules with tryptophan residues, the benzoate activation methodology seems more suitable because the benzoyl groups in the polymer act as an affinity ligand and recognize the tryptophan residues from the protein. This process is appropriate due to the integration of the affinity ligand to the polymer with the activation of it, which will lead to the reduction of chemical reactions.

One of the main drawbacks of surface functionalization techniques is the number of reactions and the time required to achieve the activation of functional groups in the polymer. The ethylenediamine and butanediol diglycidyl methodologies require from 65 to 110 h of consecutive reactions in order to activate the surface. The activation of hydroxyl groups thought these techniques facilitates the binding of proteins, peptides, and amino acids (Flanagan and Barondes 1975; Persson and Jergil 1995). On the contrary, methodologies such as periodate and tresyl-chloride provide a shorter protocol for surface functionalization (less than 24 h), but they facilitate the coupling of nucleophilic substitutions and additions to the polymer such as proteins, cofactors, or carbohydrate-binding modules (De Gouveia and Kilikian 2000; Ekblad et al. 1998; Persson and Jergil 1995). In the case of the benzoate activation methodology, it takes just up to 5 h to functionalize the surface.

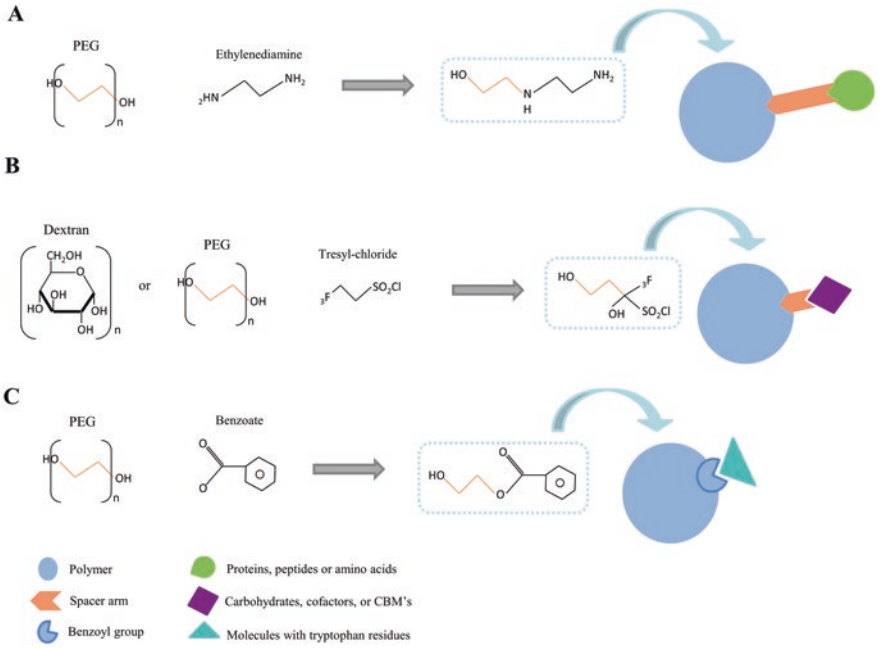


**Fig. 6.4 Polymer surface functionalization**. Three of the most common and used techniques to activate OH groups phase-forming polymers are presented. (**a**) Ethylenediamine technique provides a longer spacer arm between the polymer and the affinity ligand, which enhances the bioseparation process due to the higher interaction of the affinity ligand and the target molecules such as proteins, peptides, and amino acids. (**b**) Tresyl-chloride technique generates shorter spacer arms between the polymer and the affinity ligand. Nevertheless, the main advantage of this kind of technique is the short time required to activate the polymer. This technique allows the binding of carbohydrates, carbohydrate-binding modules (CBMs), and cofactors. (**c**) Benzoate techniques involve an integration affinity process because it enables the polymer to act as an affinity ligand itself. Additionally, this specific technique has a second advantage of short reaction time (5 h) in comparison of the above techniques that can take from 20 to 110 h reaction activation processes. Polymers activated with this technique will be able to link proteins with tryptophan residues. Hence, the benzoate technique is a feasible methodology for the activation of polymers for ATPAP systems

Another relevant aspect to be considered is the selection of the spacer arm between the polymer and the affinity ligand, as its length dictates at least in some degree the level of interaction between both constituents. Despite that ethylenediamine and butanediol diglycidyl methodologies have longer reaction times; they provide a longer arm between the polymer and the affinity ligand which will allow to the target molecule to have a better interaction with the polymer. In contrast to this, periodate and tresyl-chloride methodologies provide a shorter space arm which decreases the flexibility of the polymer-affinity ligand complex and reduces the efficiency of the separation process.

It must be emphasized that modifications of other functional groups can be achieved in order to enhance the efficiency of purification processes through ATPAP

protocols. The biochemical configuration of the target molecule will govern the addition or modification of certain functional group. The most common group modifications can be done to hydroxyl groups, carboxyl groups, guanido groups, carboxamido groups, and specific amino residue modification in proteins (Bonara and Drioli 2009). In this tenor, it should be considered the operational conditions of each methodology such as pH, temperature, and salt concentration in order to maintain the stability of the protein. Additional information of the advantages of these groups modifications are described in the literature (Bonara and Drioli 2009; Yang et al. 1995; Zalipsky 1995). Rocha et al. studied the feasibility to use alginate as a macroligand to recover papain (PAP) from *Carica papaya.* The aim of this macroligand is to direct the target molecule to the polymer-rich phase. Specifically, alginate was used as a free affinity ligand; hence no polymer surface functionalization was done. The results obtained from this study revealed that ATPS formed with PEG-salt sodium citrate could recover only 20% of PAP in the PEG-rich phase. The addition of alginate (0.1% w/w) enhanced the recovery of the enzyme to 72% (Rocha et al. 2016). Other interesting study focuses on the separation of histidine, histidine derivatives, and amino acids using metal affinity (Oshima et al. 2015). The separation of histidine derivatives was due to the specific compatibility between functional groups of the biomolecules and divalent metal ions such as Co (II), Ni (II), Cu (II), or Zn (II). Oshima and colleagues used an intermediary (chelating ligand) to create a stronger hydrophobic complex which will distribute in the PEG-rich phase. Among these divalent metal ions, the best extraction was reached with Cu (II) due to the strong hydrophobic interaction with the intermediary. The chelating ligand alizarin complexone (3-[N,N-bis(carboxymethyl)amino methyl]-1,2-dihydroxy anthraquinone, AC) and the metal affinity ligand were used in a free form. The extraction of histidine in the presence of Cu (II)-AC was 73% in comparison with the 13% of extraction using Cu (II)-free. With this novel protocol, histidine and histidine derivatives can be separated with a high selectivity over other amino acids due to the specific interactions with the imidazole group of histidine.

## 6.4 Affinity Liquid-Liquid Partitioning: Polymer-Polymer Systems

Considering the typical phase-forming components of ATPS, traditional polymer-polymer systems are among the most employed and versatile for product isolation (selected examples in Table 6.1). Systems formed by PEG and DEX have been exploited to recover high-value molecules that justify the cost of the chemical-forming phases, particularly dextran. In addition, affinity-enhanced polymer-polymer systems provide a wide scope for the recovery of biological products. In these affinity-based strategies, the polymer-ligand complex added to the system mainly partitions toward the phase rich on that specific polymer (e.g., DEX-ligand molecules partition to the DEX-rich phase). Purified or isolated molecules in these systems include monoclonal antibodies (mAbs) (Azevedo et al. 2009; Rosa et al. 2007), nucleic acids (Barbosa et al. 2008, 2010), industrial enzymes (Ekblad et al.

**Table 6.1** Selected examples of affinity partitioning in polymer-polymer aqueous two-phase systems

| Product | System | Modification/AL | Performance | Reference |
|---|---|---|---|---|
| Plasma membranes | DEX 500/ PEG 3350 | DEX-wheat agglutinin | >91% recovery BP | Ekblad et al. (2000) |
| β-Galactosidase | DEX 500/ Valeryl DEX 500 | DEX-benzoyl | 4X increase in $K_p$ | Lu and Tjerneld (1997) |
| Therapeutic plasmid | DEX 40/PEG 600 | GST+ zinc finger | 5900X increase in $K_p$ | Barbosa et al. (2008) |
| Red blood cells | DEX 500/ PEG 6000 | MPEG-IDA (II) | >95% recovery BP | Laboureau and Vijayalakshmi (1998) |
| $CD133^+$ stem cells | DEX 70/ Ficoll 400 | Anti-CD133 AB | >60% recovery TP | González-González and Rito-Palomares (2015) |
| Human IgG | DEX 500/ PEG 1500 | PEG-HRP | 90% recovery in TP | Hye-Mee et al. (2007) |
| Trypsin | DEX 500/ PEG 8000 | PEG-trypsin inhibitor | 32X increase in $K_p$ | Andrews et al. (1990) |
| Proteases from *Penicillium restrictum* | NaPA/PEG 2000 | PEG-benzoyl | 18X increase in $K_p$ | Gomes Barros et al. (2014) |

1998; Ling et al. 2010; Teotia et al. 2004), cellular membranes (Barinaga-Rementeria Ramírez et al. 2002), human cells (Laboureau and Vijayalakshmi 1998), and recently human stem cells (González-González and Rito-Palomares 2014; González-González et al. 2016; Sousa et al. 2011). In this section selected examples of bioproducts effectively recovered with ATPAP technologies are described and discussed.

One of the most important areas in which recent breakthroughs in bioseparations engineering have been proposed regarding liquid-liquid extraction techniques is that of stem cells separation. Research by Joaquim Cabral and Raquel Aires-Barros placed the platform for stem cell partitioning in ATPAP systems (Sousa et al. 2011) back in 2011. Since the development of an effective and robust separation system for large-scale cell separation with high throughput, purity, and yield was not yet established, an imminent challenge in this area was identified. In this research, an ATPAP system composed of PEG 8000 and DEX 500,000 was used for the selective isolation of $CD34^+$ stem cells from umbilical cord blood (UCB). In order to favor cell partition toward one of the phases, a macroligand free in solution (monoclonal antibody against the CD34 antigen marker) was employed. Since the selected ligand exhibited a partition toward the PEG-rich phase, a recovery yield of 95% and a purification factor of 245 were obtained for the studied stem cells. The authors determined that the presence of the affinity ligand demonstrated separation efficiencies comparable to the most common techniques for hematopoietic stem cell

purification upon a mononuclear cell fraction such as magnetic-activated cell sorting (MACS). One of the major concerns within this research is that of cell viability and pluripotency, since the selective separation of stem cells through the use of a mAbs targeted for outer protein markers could enhance cell differentiation in the separation process. Up to date, this particular effect has not been characterized effectively in ATPAP partitioning studies and thus represents an important study area in order to establish effective methodologies for stem cells isolation.

In the past 5 years, extensive research has established a defined path toward the recovery of CD 133$^+$ stem cells with means of establishing a novel, robust, scalable, and economic recovery platform for these target products. CD 133$^+$ stem cells have been successfully transplanted in humans for treatment of cirrhosis, leukemia, and ischemia (González-González and Rito-Palomares 2015), results that emphasize the importance of this particular line of cells. A recent study presented an ATPAP process for the selective recovery of CD 133$^+$ stem cells (González-González and Rito-Palomares 2015) from UCB. The use of traditional (PEG-DEX and Ficoll-DEX) and novel (UCON-DEX) two-phase systems was studied in the presence and absence of a macroligand free in solution, a CD 133/2 antibody, in order to favor partition of cells to a specific phase in the system. The novel system employed showed an increased partition of the ligand to the bottom phase (DEX), result that was validated with SDS-PAGE electrophoresis, and thus provided evidence to support the idea of allowing increased partition of CD 133$^+$ stem cells to the dextran phase. Traditional systems did not present a marked preference of the antibody toward neither phase. Even though the UCON systems selected exhibited ligand partitioning, the results did now presented an enhanced decrease or increase of the partition coefficient of stem cells. The authors concluded that additional strategies are needed to be considered in order to concentrate the antibodies more effectively in the top phase, which could finally result in the partitioning of stem cells to the target phase. In this tenor, one important characteristic that has to be considered is the DS, since the number of molecules/target product might play a vital role in selective partitioning. Considering the approach herein discussed, the use of cheap and recombinant mAbs might be a solution in order to reduce expense costs of these ATPAP strategies if they are to be implemented in the future.

The intensified partition of cell membranes and liposomes with a biotin spacer arm was studied in liquid-liquid affinity systems with DEX 40/DEX 2000 and PEG 3350, respectively (Barinaga-Rementeria Ramírez et al. 2002). Purification of membranes (outer/inner) represents an important research area for drug delivery strategies (Barinaga-Rementeria Ramírez et al. 2002). Target products were recovered in ATPAP systems which had deglycosylated avidin (D-avidin) as affinity ligand coupled to DEX molecules that partitioned to the bottom phase. Liposomes partition was first studied in order to determine optimal parameters for further purification of biotinylated membrane fractions. In systems with the absence of D-avidin, more than 80% of the liposomes partitioned to the PEG-rich phase. Addition of chemically active DEX phase induced a notable shift of biotinylated liposomes to the dextran-rich phase, from 85% in PEG-rich phase (ligand absence) to 90% in the dextran bottom phase. The authors concluded that the interaction between the biotin

spacer arm and D-avidin in liposomes and membranes determines the partitioning behavior of these molecules. This biological interaction represents a valuable approach for the purification of biotinylated products and also an important positive result for ATPAP for the recovery of high-added value cell fragments intended for therapeutic processes.

Polymer-polymer ATPAP systems offer enhanced versatility for biotechnology-based applications since both phases could be chemically active in order to further attach affinity ligands. In this tenor, an important characteristic within polymer activation techniques and ligand coupling is the nature of the spacer arm between the active phase component and the selected ligand. These spacers tend to have different lengths according to the protocols employed in chemical activation, and careful selection of the derivatization methodology would give an increased interaction of the ligand and the desired target molecule by means of positive mobility of the molecular bridging at hand. Also important to consider is the ionic force and pH of the systems in order to favor product partition. Even though marked shifts in salt concentration or pH may favor affinity interactions between target molecules and affinity ligands, these phenomena have not been deeply studied in ATPAP applications, and thus an important research niche can be identified in this area since positive effects considering these variables have been reported for protein partitioning in the past.

Even though polymer-polymer systems offer increased versatility for ligand coupling strategies, the most important protocols for ATPAP systems include free ligands in solution (González-González et al. 2016; Sousa et al. 2011). Nevertheless, a proper study of ligand partitioning must be undertaken in order to characterize its partitioning behavior and thus determine its true potential in affinity extraction procedures. Biochemical modification of affinity ligands can be carried out in order to favor their partitioning to a specific phase in the system. One of the most recent and common modification techniques in ATPAP systems is PEGylation, since it has been positively used in the past for efficient molecule modification. PEGylation reactions have been well studied and characterized and allow the coupling of a PEG moiety to a particular compound, in this case an affinity ligand (Barbosa et al. 2008). Because of the chemical nature of the modified ligand, it has been demonstrated that partitioning behaviors toward PEG-rich phases are greatly favored (Barbosa et al. 2008). This methodology can exploit modification of protein molecules (used as ALs) and thus aid in the design of efficient operations for the recovery of high-added value compounds. It is important to emphasize that no in-depth studies about PEGylation in ATPAP have been reported; however, new applications of this technology with ATPAP can be expected. Due to the stability of the systems and low ionic force, polymer-polymer systems offer important versatility for ATPAP strategies in recovery of whole particles and macromolecules (Table 6.1). One of the principal aspects to be considered when using these systems is the final cost, since PEG and particularly DEX may increase the final bioprocessing cost at pilot or industrial scales. Potential chemical-forming phases recycling strategies could minimize the negative perception of these systems for the generic application at large scale.

## 6.5 Affinity Liquid-Liquid Partitioning: Polymer-Salt Systems

The use of polymer-salt systems has been reported for several decades for the purification of macromolecules, specifically proteins. Regarding ATPAP strategies, phase-forming components including salt phases are not very common for the recovery of a wide array of molecules, the most notable ones being industrial-grade enzymes (Table 6.2). The major drawbacks of these ATPAP systems lie within their highly ionic nature. The use of certain salts has proven to increase partition of molecules in ATPS, but above certain critical concentrations, a negative pattern of solubility for proteins and other biological products can be observed. In this context, important shifts in the ionic force of the system might hamper essential ionic interactions between ALs and target molecules, thus significantly reducing the affinity potential of ATPAP protocols. Nevertheless successful research has been reported in this area, and some important examples are here presented and discussed.

One of the most important areas in biotechnology engineering is that of food development including designs with new or novel ingredients. Asenjo and Andrews performed the selective purification of thaumatin, a flavor enhancer and sweetener protein from the *Thaumatococcus daniellii* fruit. Since thaumatin and some derivatives can exhibit a 2000-fold increase in sweetness when compared to dextrose, efficient operations for protein characterization and isolation have been proposed. PEG 8000-potassium phosphate systems were employed in a protocol which included functionalized PEG molecules through an epoxy-oxirane method in order to couple glutathione as AL. The addition of 8% of active PEG in the ATPAP system proved enough to increase the protein concentration in the PEG phase from 0.82 to 2.46 g/L (threefold increase). This result demonstrated that the purification and concentration of active proteins can be dramatically improved when using biospecific ligands attached to a phase-forming component. The authors also concluded that the DS plays a vital role in product partitioning, since in this research it was found that high concentrations of coupled glutathione further reduced the amount of recovered thaumatin (Asenjo and Andrews 2008).

Production of pharmaceutical compounds is a major research area within bioprocess engineering. During the past three decades, antibiotic synthesis procedures have been optimized through the efficient use of enzymes. One important example is that of penicillin acylase (PA) presented by Gavasane and Gaikar. This enzyme is involved in the production of 6-amino-penicillianic acid, a key intermediate in the production of semisynthetic penicillins (Hamilton-Miller 1966). Four active PEGs were developed: PEG-phenylacetamide (PEG-paa), PEG-trimethylamine (PEG-tma), PEG-benzoate (PEG-bz), and PEG-palmitate (PEG-pmt). Interaction between the target enzyme and PEG-bz or PEG-paa favored product recovery. The authors determined that high affinity was favored since the intermolecular energy between the active PEGs and PA was lower to the interaction energy between PA and its natural substrate. The enzyme exhibited a 15-fold increase in partition coefficient and 60% product recovery when PEG-paa was used. These functionalized PEGs proved increased interaction between the amide or phenyl groups with specific

**Table 6.2** Affinity partitioning of bioproducts in polymer-salt two-phase systems

| Product | ATPS | Affinity approach | Performance | Reference |
|---|---|---|---|---|
| Penicillin acylase | PEG 4000/$KPO_4$ | PEG-palmitate | >81% recovery in TP after optimization runs, $K_p$ 15X increase | Gavasane and Gaikar (2003) |
| Thaumatin | Glucoamylase | PEG-glutathione | 3.5X increase in $K_p$ after modification | Andrews et al. (1990) |
| Histidine derivatives | PEG 8000/$Li_2SO_4$ | Alizarin as AL | >75% recovery in TP when the chelating ligand was present in the ATPS | Oshima et al. (2015) |
| Phospholipase | PEG 6000/$KPO_4$ | Alginate as AL | ~80-fold increase in purification factor and 85% global recovery in TP | Teotia and Gupta (2004) |
| Phenolic acids | PEG 200–600/ $Na_2SO_4$ | CMIM derivatives | 5% w/w of CMIM allowed up to 99% recovery of gallic and vanillic acid | Almeida et al. (2014) |
| Glucoamylase | PEG 300–400/ $KPO_4$ | Starch as AL | 9X $K_p$ decrease when partitioned to the salt phase | Gouveia and Kilikian (2000) |
| Papain | PEG 8000/ Sodium Citrate | Alginate as AL | >70% recovery and 2.41 purification factor achieved | Rocha et al. (2016) |

residues in PA (Gavasane and Gaikar 2003). One key element discussed and emphasized in this research was the molecular simulation of ligand interactions, in order to provide enhanced bioselectivity for product retrieval in future applications. This approach can be exploited for future experiments using other types of ligands and target products in order to design robust, economical, and efficient downstream operations based on ATPAP technology.

Scientific advances in medicine are often possible due to availability of novel substrates or molecules that will help design further researches. The purification of phospholipase D (PLD) from *Arachis hypogaea* and *Daucus carota* with ATPAP systems was presented by Teotia and Gupta. This enzyme is implicated in several vital cellular processes including membrane transport and cytoskeletal recognition in mammalian cells. PEG 6000-potassium phosphate systems were constructed adding alginate as a ligand free in solution that partitioned almost exclusively to the PEG-rich phase (>95%). It was determined that, when alginate (0.2% w/v) was added to the system, an increase in the purification factor of PLD of 78-fold with more than 80% recovery was observed. These values represent a significant improvement when compared with those achieved in traditional ATPS (30 purity fold and 50% recovery). The main benefits of this research were that a high-value product could be recovered without preprocessing or clarification steps, with the

implementation of an economical PEG-salt system (Teotia and Gupta 2004). The most important contribution of this research is the affinity interaction between alginate, a naturally occurring anionic polysaccharide, and the target product. The interaction of molecules with phase-forming components has not been extensively studied in two-phase partitioning protocols. Hence, this could provide an interesting approach toward the design of novel separation techniques in APTAP through the visualization of potential affinity interactions of molecules and natural polymers.

Partitioning in polymer-salt systems presents important advantages for affinity-based strategies. Regarding the physicochemical characteristics of the systems, reduced viscosity and density for the phases can be seen, which increases the industrial potential and feasibility for ATPAP strategies to be scaled-up in commercial processes. In addition, the bulk cost per gram of phase-forming component/final phase is significantly lower when compared to polymer-polymer systems, specifically those which include dextran. Nevertheless, a set of future challenges can also be described for these systems. At a first glance, a deep and adequate characterization of the biospecific, electrostatic, and hydrophobic interactions between PEG molecules, ions, ALs employed, and target products to be recovered or purified is needed. Very few studies or comments have been reported in literature in this subject (Hamilton-Miller 1966; Teotia and Gupta 2004), situation that clearly highlights an important research niche in this area. Also to be considered is the complexity of the molecules or components that might aid or hinder the partition of a selected particle, since hydrophobic, ionic, or pH shifts can be achieved using a vast array of reagents, small molecules, or macroligands.

An important approach that must be considered when designing ATPAP strategies is the implementation of ordered and efficient statistical tools that provide positive information regarding unit operation performance. Thus, appropriate experimental designs coupled to optimization methodologies (i.e., response surface analysis) might prove useful in order to identify key elements or variables in ATPAP operations (e.g., Fig. 6.3) and thus establish feasible strategies to be applied at industrial level. The primary benefits from these studies would reside in the elaboration and elucidation of predictive mathematical models which could greatly reduce capital expenses, human hours, and time spent on research experiments involving the design and characterization of the operation at hand. In addition, if enough attention is given to the information presented in Table 6.2 and Fig. 6.4, polymer-salt affinity systems may provide interesting solutions for the recovery and purification of industrial enzymes that commonly withstand harsh processing conditions, such as shifting pH and temperature settings. Hence, cheaper and selective liquid-liquid systems for these bioproducts could be designed and massively implemented at industrial scale if the abovementioned challenges or suggestions are to be considered and addressed by biotechnology companies worldwide.

## 6.6 Affinity Liquid-Liquid Partitioning: Alternative Systems

One area that has not been intensely studied, in the context of ATPAP, is that related to the characterization and use of natural (i.e., starch or gums) or alternative/intelligent polymers (thermosensitive polymers such as UCON). These systems have been poorly studied in their traditional two-phase configuration; hence, evidence for their applicability in affinity partitioning is limited. Nevertheless, considering that these alternative phase-forming components can offer important advantages over traditional two-phase-forming chemicals, strategies for the recovery and purification of several biological particles have been reported such as proteins or whole cells. This brief section will present a couple of successful cases in which ATPAP efficiently recovered biological products, along with a discussion of the primary challenges within this research area.

Affinity chromatography for monoclonal antibodies recovery constitutes the primary purification method of the biotechnological industries nowadays involved in mAbs production and commercialization. Traditional ATPS for mAbs recovery have been presented in the past with efficient yields (>90% recovery). An ATPAP strategy for human immunoglobulin (IgG) from CHO cells was reported by Ferreira et al., using non-conventional phase-forming components for the systems studied. The approach developed involved a system comprised by 6% w/w DEX 500,000, 8% w/w ethylene oxide/propylene oxide (UCON), and an AL free in solution in the form of triethylene glycol-diglutaric acid. With addition of TEG-COOH, an improvement on both extraction yield and purity of human IgG in the top phase was clearly observed. The significant increase in log $K_p$ (from −0.2 to 3.5) of IgG was attributed to electrostatic interactions between the positively charged IgG and the negative carboxylic groups of TEG-COOH (Ferreira et al. 2008). According to the optimization strategies employed by the authors (response surface methodologies), the optimal extraction system for the purification of the target product was determined to contain 5.5% w/w dextran, 10% w/w UCON, and 20% w/w ligand (TEG-COOH). In this particular case, the information provided by the authors supports one of the strategies previously mentioned in this chapter, which involves the elucidation of mathematical models for predictive behaviors of product partitioning and optimization.

Antov et al. employed a novel system formed by guar gum (a renewable polysaccharide galactomannan) and starch, in which carbohydrate-binding modules (CBMs) were used as affinity ligands since these tags are present in some proteins and enzymes and thus facilitate substrate location and coupling. The target product in this research was β-mannanase from *Cellulomonas fimi*, an enzyme with high affinity toward specific mannans because of its native CBM. The strategy consisted in understanding the affinity of the protein-binding module to the galactomannan polymer in the aqueous extraction system in order to determine the optimal partitioning conditions for mannanase. The natural polymers hydroxypropyl starch PES 200 and guar gum galactomannan (9% w/w and 3.6% w/w, respectively) were used as forming components. The selected system allowed a top phase recovery yield of 90% with a $K_p$ of 7.06 for β-mannanase. With the obtained results, the authors noted

that diverse CBMs could be employed for protein purification (Antov et al. 2006). Hence, a general extraction procedure for proteins or ad hoc molecules could be developed if each target molecule is fused with a specific binding module. This would represent an important advantage in the development of novel downstream processing strategies, in which an additional affinity behavior could represent an alternative for product purification.

Additional research results are presented in Table 6.3 in which successful applications of affinity partitioning have employed alternative phase-forming components. Among the results presented in this table, it must be considered that an interesting area that could prove important advantages in product recovery and

**Table 6.3** Affinity partitioning of bioproducts in alternative phase-component two-phase systems

| Product | ATPS | Affinity mechanism | Performance | Reference |
|---|---|---|---|---|
| β-Mannanase | PES 200-Guar Gum | Galactomannan as AL | 5.1X–fold increase in $K_p$ | Antov et al. (2006) |
| His-tag: GFP | Triton X114/water | Nickel-chelated TX | 20X–fold increase in $K_p$ when modified TX was used | Wang et al. (2013) |
| GFP | Triton X114/Ni-Triton X114 | Nickel-chelated TX | With use of EDTA to back-extract, 83% recovery yield and 70% purity achieved | Wang et al. (2014) |
| IgG | PEG 4000/ hydroxypropyl starch | NaCl in solution | Purification factor of 5.28 and extraction yield of 99.2% | Wu et al. (2013) |
| Cutinase | BREOX 100/PES 200 | TX 100 as AL | >11X–fold increase in $K_p$ when recombinant cutinase was purified | Mannesse et al. (1995) |
| Phenylalanine in racemic mixture | Chiral tropine ionic liquids (ILs) | Chirally active ILs | 65% of D-enantiomer partitioned to the TP rich in $Cu^{2+}$ backbone | Wu et al. (2015) |

purification is that of micellar preparations. These systems are formed when surfactants (ionic or no ionic detergents) are mixed with an organic/aqueous phase over a critical concentration known as the "critical micelle concentration" (CMC). When the resulting system is formed, the micelles can exhibit a hydrophobic or hydrophilic behavior (depending on the phase-forming chemicals). These particles can be chemically activated, as presented by Lam et al., in order to couple ALs and thus present potential for an ATPAP strategy. Nevertheless, the stability of the systems and the activation procedures for micelles have not been deeply studied, situation which highlights an important opportunity area for these protocols. The major challenge these systems suppose for product extraction resides in biocompatibility of the molecules to be recovered and the modified ATPAP system. In addition, since micellar systems can also significantly increase system costs (concentration and nature of surfactants), modification procedures to be employed in these strategies must be very selective and cheap and require little reaction time, as presented in Fig. 6.4, in order to maximize interaction between selected ligands and potential binding sites in the active micelles in the system. Considering the amphipathic nature of these systems, a careful and deep analysis of the interactions between the active sites, the involved ligands, and the target products should be first envisioned step in order to characterize positive strategies using this technology (Lam et al. 2005).

Considering the additional variables to be studied in affinity fractionation procedures as presented in Sects. 6.1 and 6.2, the idea of using a phase component (e.g., natural polymers) that may serve as an affinity molecule toward a target product, and at the same time constitute one of the phases in the system, could represent a significant approach for integration and intensification of unit operations. Nevertheless, certain challenges must be addressed within natural polymers and ATPAP partitioning. Considering the information discussed by Sturesson et al., some versions of industrial starches that are commercially available exhibit lower economical costs when compared to dextran or other synthetic polymers. However, the functional time of these natural polymers may be limited due to the biodegradable potential (Sturesson et al. 1990). Another important aspect that must be considered with natural polymers is their high viscosity. Even though these polymers (i.e., starch, guar gums) can be considered environmentally friendly in low concentrations, their high viscosities must be reduced in order to use them in ATPAP and traditional ATPS strategies. The most used approach in order to pretreat viscous polymers is that of enzymatic protocols (Antov et al. 2006), and heating in a minor scale, but the cost-effective approach of using a natural non-expensive polymer could be completely hampered. Finally, the industrial production schemes of such components must be standardized in order to produce quality batches with proved chemical stability, since current natural polymers solutions cannot be prepared and stored for prolonged periods of time.

## 6.7 Current and Future Challenges for Liquid-Liquid Affinity Partitioning

Considering the major advances in bioprocess engineering regarding unit operations, purification protocols, and production schemes, one of the constant challenges and trends in this area is that of process integration and intensification. Considering the information presented throughout this chapter, the selective recovery of proteins (Effio et al. 2015; Flanagan and Barondes 1975), enzymes (Gomes Barros et al. 2014; Ling et al. 2010; Ramakrishnan et al. 2016; Teotia et al. 2004), recombinant proteins, cellular membranes (Laboureau and Vijayalakshmi 1998), or even human stem cells (González-González and Rito-Palomares 2015; González-González et al. 2016; Silva et al. 2014; Sousa et al. 2011) has been achieved with high recovery yields. Some of the major advantages of these success cases are the strong biocompatible environment, ease of operation, and economic feasibility of the designed ATPAP operations. In addition, the authors conclude that the reduction of chromatographic steps is a key element in these emerging bioprocessing areas. Nevertheless, considering the vast amount of information regarding chromatographic-based operations for biological recovery and purification, the well-established techniques and protocols at industrial level of these operations could possibly hinder ATPAP applications at commercial scales in the near future if some challenges (e.g., new phase-forming components, mathematical modeling, novel polymer modification techniques, versatile ligands) are not adequately studied and validated.

An assessment between ATPAP and chromatographic techniques was presented by our research group in the past (Ruiz-Ruiz et al. 2012). The main comparison among these operations must include the overall efficiency for recovery/purity yields and the economic burden of the protocol in the bioprocess at hand. One of the most interesting cases in which ATPAP posed an increased potential when compared to chromatographic-based separations is that of whole cells (CD $34^+$ stem cells) separation. Recovery yields tend to be above 90% after the employed protocols, but current separation techniques based on cell sorting methodologies (i.e., magnetic-activated cell sorting, MACS) will be insufficient in the future in order to meet the needs of stem cell therapies and treatments due to their low scalability potential and sample loading capacities (Huettmann et al. 2014). Since recovery yields are very comparable with those obtained with MACS devices, several reports have emphasized the simplicity and robustness ATPAP protocols pose within bioprocess design, including scale-up feasibility (González-González and Rito-Palomares 2015). The most important challenge in whole-cell recovery in ATPAP systems is the establishment of purity standards for the correct isolation of the target particles (e.g., red blood cells, stem cells), since these objectives have not been correctly addressed. Despite this, ATPAP methodologies seem promising since 96% recovery of CD $34^+$ stem cells as reported by Sousa et al. was obtained compared to 85% total extraction with a commercially available MACS procedure (Sousa et al. 2011).

Even though liquid affinity partitioning has not been extensively studied and characterized, important advantages such as feasible scale-up potential and intensification capabilities could position this recovery technique as a valuable component

of industrial bioprocess designs. In order to favor ATPAP coupling in current bioprocesses, an important challenge that must be addressed relies on the strict control and understanding of the electrostatic interaction between charged groups in target molecules and affinity ligands employed in the system at hand. This situation can be characterized by studying the optimal pH levels for protein/product stability and activity in a selected ATPAP system, since each molecule exhibits a particular partitioning behavior based on this variable. Another important aspect that must be considered is product partitioning maximized through hydrophobic interaction between ligands, system components, and particle/molecular structure (Benavides and Rito-Palomares 2008). Considering the aspects and practical experiences found in literature, the use of hydrophobic domains in AL could favor recovery of molecules with similar domains or active structures. In addition, this polarity-based effect could be positively studied and enhanced if the extraction procedure is made near the isoelectric point of a protein, since solubility will be low and thus facilitate hydrophobic interactions to take place.

One of the most important trends in ATPAP technology nowadays involves the study and characterization of affinity ligands. The advances and evolution of ALs in affinity separations have experienced dramatic changes in the past four to five decades, including those used in chromatographic and non-chromatographic techniques. Regarding ATPAP protocols, the first generation of ALs consisted in the careful synthesis of molecules in order to exert specific affinity toward a target product. During the past 15 years, research in this area has experienced a marked transition, which constitutes one of the most important trends in ATPAP technology, from chemical molecules to biological structures such as biological substrates, binding modules (i.e., CBMs), enzymes, antigens, or even antibody fragments. It can be expected that third-generation affinity ligands will mark an important increase in ATPAP designs in the near future, in which possibly molecularly engineered products (e.g., proteins, chimeric products, low molecular weight compounds) will be employed in dual-affinity strategies in order to favor product recovery in cheap and robust systems. Regarding also the importance within ligands in ATPAP, it can be expected that future applications of this extraction technique will favor the use of ALs free in solution since no polymer activation procedures would be needed in order to add the molecule to the system. The challenge of this approach resides in the proper and deep characterization of the partitioning of the selected ALs, aiming to not expect a change in the system characteristics such as $V_R$, TLL, and composition.

One last aspect that must be mentioned in this chapter is that one regarding all the biochemical or biological elution strategies of target products when partitioned with an ATPAP approach. Even though there is an important number of affinity partitioning research papers published in the last four decades (>40), desorption methodologies are rarely studied and discussed. This final step, even though simple and quick, could prove the most important challenge for ATPAP efficiency methods, since no clear methods have been characterized up to date. Some of the traditional methods employed for chromatographic-based separations have been proposed, such as ionic force shifting, pH adjustment, and addition of displacer

molecules (i.e., competitive substrates) to promote desorption of the target molecule from the affinity ligand used in an ATPAP strategy. Nevertheless, these protocols have not been properly studied, and thus an important opportunity area in this technology can be identified within displacing techniques for the final recovery and polishing steps in a selected bioprocess.

## 6.8 Concluding Remarks

The past two decades have witnessed a significant increase in the number of studies related to liquid-liquid affinity partitioning. It is evident that nucleic acid therapies and stem cell research could be positively enhanced by development of ATPAP technologies since specific interactions and high recovery yields could be expected. In addition, the possibility of designing extraction operations using natural resources, such as starch or gums, may give these systems an environmental-friendly dimension that could prove an advantage and trend within bioprocess engineering designs worldwide. Nevertheless, these systems continue to experience important challenges nowadays, as discussed in the past section, since the manipulation and high viscosity of the components has not yet allowed them to be commonly accepted and employed in fractionation research at lab and pilot scales. In this tenor, new research within material science could trigger the development of novel ATPAP designs in the near future, including at the same time the coupling of new affinity ligands, since the proper conjunction of efficient phase-forming chemicals and affinity-enhanced molecules is the perfect combination for the design of novel bioseparation breakthroughs. A last and important aspect to be considered within this topic involves the bioprocess economics in ATPAP strategies, specifically within ligand selection/coupling/production schemes. Capital investment in two-phase systems has been focused in the direct cost of the phase-forming chemicals. In this case, the relative costs of ligand inclusion should be permanently considered in order to establish competitive bioseparation ATPAP alternatives when compared to traditional chromatographic techniques and thus offer attractive technologies for future development of downstream processing strategies in the biotech industry.

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