Chapter 12

Alternative Separation Methods: Crystallization and Aqueous Polymer Two-Phase Extraction

James M. Van Alstine*, Günter Jagschies†, Karol M. Łącki§

*JMVA Biotech AB, Stockholm, Sweden, †Royal Institute of Technology, Stockholm, Sweden, §GE Healthcare Life Sciences, Freiburg im Breisgau, Germany, §Karol Lacki Consulting AB, Höllviken, Sweden

12.1 INTRODUCTION

12.1.1 Two Classic Separation Methods Based on Phase Transfer

Twenty years ago it was uncommon for someone to speak at a recombinant biopharma processing conference in regard to large-scale purification of proteins using crystallization or aqueous polymer two-phase extraction (ATPE). The methods were known and respected, but not considered full of promise. This was somewhat surprising given that crystallization was well established in the pharmaceutical industry [1,2] and used in the processing of insulin and lipase proteins [3,4]. At the same time, two-phase extraction was also well established in the pharmaceutical industry and even had shown promise with one mAb (Rituxan), one interferon (Betaferon), some other recombinant proteins, as well as metric tons per year of amino acids and antibiotics [5–7] (Fig. 12.1). That situation has changed over the past few years with enhanced knowledge of general crystallization and protein crystallization [1,2,8–10], supporting progress in regard to the large-scale crystallization of mAbs and other proteins in micrometer-sized particles, for purification and formulation, by both batch and continuous methods [1,2,5,11]. At the same time ATPE—first developed by Per-Ake Albertsson et al. [12], and its potential for industrial scale purification—first championed by M. R. Kula et al. [13] followed a similar developmental path [14–16]. Appreciation is growing of its abilities to simultaneously effect clarification, target concentration, buffer exchange, and contaminant reduction in unit operations that can be performed continuously or in batch mode in simple containers. More recently, scientists have come to understand that protein-phase behavior in aqueous solutions, which is directly related to protein structure, is a common determinant of ATPE, crystallization, and other phase phenomena (such as gelation) [17–19] with many effects, such as those related to buffer salt types, buffer salt types common to both methods (see the following).

12.1.2 Related Notes From Previous Chapters in This Book

Chapter 10 provided an introduction to alternative separations methods, including crystallization and ATPE. Fig. 10.1 will help readers orient themselves in regard to unit operations in which crystallization or liquid-liquid extraction methods such as ATPE may be employed. Some review articles are noted in Chapter 10 (other reviews are noted in Section 12.1.1 herein). Fig. 10.3 provides rapid insight into crystallization and ATPE, and documents the industry perception that crystallization ranks with affinity chromatography in terms of target purification, and that it is relatively easy to scale, but that process development (in terms of identifying suitable, readily-scaled conditions) may be challenging. Given the myriad operational factors to be controlled (e.g., Table 12.2), ease of operation is also potentially challenging. The perception of ATPE is that its flexibility and selectivity are of interest, and that process development, scale-up, and operation are reasonably simple. Both methods are perceived to rank slightly lower than chromatography in regard to process economics [20,21].

Chapter 10, Table 10.3 will help the reader define performance attributes related to their particular challenges. If their main interest is in antibody processing, Table 10.4 provides a comparison of affinity chromatography, crystallization, several types of ATPE, and some other alternative separation methods. The present chapter contains specific subchapters in regard to both crystallization and ATPE. Each subchapter is prefaced with a “Strengths, Weaknesses, Opportunities,
and Threats” (SWOT) summary table. Examples of unit operations may be found in the individual subchapters. Use of ATPE in regard to clarification has been noted in Chapter 11 Section 11.3.4 (see also Table 11.3).

Both Chapters 10 and 11 contain notes and examples in regard to unit operation and process economics for other separation methods, including Protein A affinity chromatography. Ref. [4] provides valuable information and perspectives in regard to comparing the performance of different separation methods in both batch and continuous modes. The related data and methodology may be useful as a basis for evaluating crystallization and ATPE.

### 12.2 CRYSTALLIZATION

Table 12.1 presents an overview of the basic strengths, weaknesses, opportunities, and technical threats related to crystallization.

#### 12.2.1 Introduction

Crystallization is a classic method for protein purification with a history of more than 150 years [1,23] deeply tied to our understanding of protein hydration and self-association, proteins as unique molecular entities, as well as to protein structure and function [1,8,10,22,23]. McPherson and Gavira recently wrote an excellent introduction to protein crystal growth (PCG) with a focus on crystals for structural studies [8]. Hekmat [1] recently provided an excellent review with a focus on large-scale protein crystallization for use in purification and formulation. In 2003, two reviews by both Lee and Kim [24] and Klyuschnichenko [25] covered PCG from high-throughput screening (HTS) to kilogram scale production [25]. Refs. [1,24,25] discuss both theory and equipment.

Crystallization has the ability to purify, concentrate, hold in stable storage, and offer delivery modes for (protein) pharmaceuticals. Advantages include improved understanding of how to scale crystallization processes (see the preceding) and ease of employing in single-use, flexible-manufacturing formats (SWOT Table 12.1). Of the weaknesses noted in Table 12.1, the need for relatively pure starting material, relatively long process times (days in some cases), and developing unique conditions for each target are challenging. However, in Chapter 11 it was noted that flocculation and precipitation approaches can yield quite pure and concentrated target solutions amenable to depth and sterile filtration prior to initiation of crystallization. Such treatments may result in a sterile, target- and reagent-rich solution, which need only be slightly modified to promote crystallization. Appropriate conditions, including apparatus surface materials or coatings, can be established via high throughput screening (HTS) methods [3,24–27] even though, due to their significant surface-to-volume ratio, HTS chambers may cause some artifacts due to surface nucleation.

Protein crystallization can be a complex process involving a number of steps. Crystal growth is driven by supersaturation, in which target solution concentration exceeds solubility. However, crystal formation requires that proteins, which tend to be large and relatively slowly diffusing, spatially orient and contact in a manner that promotes crystal formation, and thereby excludes contaminants from the crystal. If such orientation is not possible, or if the driving force to come out of free solution in a precipitate or other phase is high, the protein may form an irregular precipitate or gel or other mesophase, which may include contaminants.

![FIG. 12.1](https://example.com/figure12_1.png)  
Fig. 12.2 shows a simplified protein phase diagram based on variation of a single type of protein and single type of crystal-growth-promoting component under otherwise fixed conditions. If protein and crystallization agent concentration are both increased so as to move along a 45 degrees line outward from the origin, conditions in the chamber will move from undersaturation through the solubility curve into a region of supersaturation where crystal nucleation may occur. If protein concentration is further increased, there is a chance to move into a region where phase separation may occur, creating a buffer-rich and a protein-rich (perhaps gel-like) phase. If instead of protein concentration, crystallization agent concentration is further increased, there is a chance to move into a region where precipitation may occur. One goal of larger-scale crystallization is to create uniform bioreactor conditions where neither precipitation or other forms of phase separation are favored over crystallization.

Every type of protein will exhibit its own phase diagram associated with different conditions. A wide range of process variables and influences are recognized (Table 12.2, see also [1,8,28]). In operation, many conditions will be fixed for practical reasons, such as a need to work at 4°C or within a pH range where the target protein will be stable over the time course required for significant crystal growth. Some key crystallization variables are protein concentration, crystallization agent type and concentration, pH, and temperature. The latter two are appreciated to warrant careful control in large-scale processes [1,3,8].

Fig. 12.2 illustrates several points relating to protein phase behavior [1,3,17]. First, that the crystallization promoting reagent(s) may have a defined concentration range of usefulness that exists between conditions where there is no crystal formation, and where other phase changes such as precipitation can occur. The second is that most proteins exert a physical influence on their suspending solutions in regard to buffering pH, viscosity, and free-water content. As a result, increasing protein concentration can lead to alteration in crystallization or even bulk phase separation. As in chromatography, effects related to protein aggregate formation may also be tied to protein concentration, as will abrupt changes in solution conditions, including temperature. To control crystal morphology, size, and quality, it may be advantageous to use temperature to perform nucleation at a higher supersaturation rate, but grow the crystals at a lower supersaturation rate [1].

McPherson noted various methods for creating supersaturation [8]. In addition to those noted herein they include direct mixing of protein and precipitant solution to create a supersaturated condition in batch mode, alteration of solution salts,

<table>
<thead>
<tr>
<th>TABLE 12.2 Factors Affecting Crystallization</th>
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<tbody>
<tr>
<td>Physical</td>
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<tr>
<td>1. Temperature and variation</td>
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<tr>
<td>2. Surfaces, nucleation condition</td>
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<tr>
<td>3. Methodology/approach to equilibrium</td>
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<tr>
<td>5. Chamber geometry</td>
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<tr>
<td>7. Pressure</td>
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<td>8. Time</td>
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<tr>
<td>10. Electrostatic/magnetic fields</td>
</tr>
<tr>
<td>11. Medium dielectric property</td>
</tr>
<tr>
<td>13. Rate of equilibration</td>
</tr>
<tr>
<td>14. Nontarget impurities</td>
</tr>
<tr>
<td>15. Chaotropes/Kosmotropes</td>
</tr>
<tr>
<td>17. History of the sample</td>
</tr>
</tbody>
</table>

addition of a ligand that changes target solubility, addition of organic solvents to change suspending solution dielectric properties, addition of protein cross-bridging agents, protein concentration by solution dehydration via filtration or dialysis of water out of the sample, and removal of solubilizing agents. The latter may occur, for example, via various means, including use of temperature or pH responsive solubilizing agents that self-associate above a certain temperature. However, approaches involving addition of complex chemicals may not be practical in regard to large-scale biopharmaceutical processing where the goal is target purification.

Peter J. Leicester and Patrick D. Shaw Stewart of Douglas Instruments have undertaken some data mining of the Protein Data Bank (PDB) in order to understand various conditions used in the growth of crystals for the myriad of structures in the PDB [28]. For example, they provide lists of the Top 14 organic and inorganic precipitants—many polyethylene glycol polymer variants as well as kosmotropic salts, common temperatures used—often 4–8°C or 19–23°C, protein concentrations—from 1 to 300 mg/mL with many in the 5–30 mg/mL range [28]. Not surprisingly, many of the successful PCG examples noted as follows fit within these categories.

The preceding references provide insight to the effects of various crystallization reagents on protein crystal formation. Some reagents that are effective when growing crystals at small scale for protein structure analysis may be expensive or toxic and not suitable for use in bioprocessing. Insight into the molecular properties and interactions that govern protein crystal (as well as precipitate) formation are continually forthcoming (e.g., [17,18,29]) including basic understanding and prediction of salt effects [30] and electrostatic interactions [31] and nonelectrostatic forces [30–32]. Many of the effects and how they are modified by pH, ionic strength, temperature, and other variables such as addition of kosmotropic versus chaotropic salts, or addition of substances such as PEG that have a strong tendency to interact with water molecules. It may help to consider protein crystal growth in terms of self-association interaction with a crystal surface offering different (mixed mode) interactions.

Crystallization of small molecules has a long industrial history [2,9,33] and, as noted herein, is of significant interest for application of therapeutic proteins in bioprocessing (e.g., [34,35]). Thömmes and Eitzel have noted that in 1982 Eli Lilly produced the first recombinant biopharmaceutical (insulin) via a process including crystallization [36]. They also noted that Genentech used it in the scale-up manufacture of a tumor necrosis factor Apo2L [34]. In the related process, which eliminated a chromatography step, a temperature gradient from 25 to 4°C over 8 h was used. The relatively slow temperature reduction of a protein-rich solution containing PEG resulted in slow growth of large (150 μm) crystals readily recovered by filtration [34,36]. Bayer used a multifactorial approach to develop an aprotonin crystallization step, which was incorporated into a large-scale process as bulk storage step (Table 12.3, data from Ref. [35]). The proteins noted herein are of relatively small molecular weight. However, several large proteins (>150 kDa) have been crystallized [28]. Many protein crystallization studies have been done using lysozyme proteins from various sources. They tend to be relatively small, very highly charged proteins with very high pKas. As such, some caution might be exercised in relating results obtained with such proteins to other targets. Nevertheless, the pI range of proteins that have been crystallized is from 2 to 11 with most in the 4–9 range [28].

In practical use, a decision to explore crystallization as a method for protein purification may be driven by two different sets of circumstances. In one case, during a protein product’s initial development, it may be fortuitously noted that the

<table>
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<th>TABLE 12.3 Aprotinin Target Process</th>
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<tr>
<td>Step</td>
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<tr>
<td>Culture supernatant</td>
</tr>
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<td>Filtration</td>
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<td>CIEC</td>
</tr>
<tr>
<td>RPC</td>
</tr>
<tr>
<td>AIEC</td>
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<tr>
<td>UF/DF</td>
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<tr>
<td>Crystallization</td>
</tr>
</tbody>
</table>

AIEC = anion exchange chromatography, CIEC = cation exchange, RPC = reverse phase chromatography, UF/DF = ultrafiltration diafiltration.

protein can form crystals, readily precipitates, or otherwise shows a tendency to form mesophases. In that case, it may be wise to explore crystallization as it may replace one or more other unit operations [36]. In the second case, the purification of a protein may be challenging in regard to a contaminant protein similar in physical properties, and chromatographic behavior to the target protein. In such a case a crystallization step may help isolate targets from contaminants. Both precipitation and crystallization are liquid-solid (L-S) separations, which often involve similar reagents, and offer relatively similar advantages. Both can be problematic if they occur during chromatography or filtration. As such, scouting for conditions where they occur might be undertaken when characterizing the general properties of a target protein in regard to selecting an appropriate downstream purification method. Such scouting might include target-free media in an effort to identify conditions where target proteins can be readily separated from HCPs. An example of the latter is the use of 10 min, 70°C heat treatment to precipitate many (but not all) E. coli host cell proteins (HCPs), but which can leave even hydrophobic target proteins such as green fluorescent protein mutants in solution (e.g., [37]).

12.2.2 Preparative Small Protein Crystallization

Ref. [1] provides examples of preparative protein crystallization of many proteins including lysozyme, and lipase. As noted herein [34], an Apo2L/TRAIL protein was purified at a relatively large scale (800L) by stirred-tank batch crystallization. Purification of a lyase anticancer protein by batch crystallization has been performed at 100L scale from a partially purified solution. The target protein was expressed in E. coli at 2 g/L and accounted for 43% of the total protein. Crystals were obtained in a 100L vessel at 87% yield from a solution containing 9.0% polyethylene glycol 6000, 3.6% ammonium sulfate, and 0.18 M sodium chloride. The process involved several crystallization steps followed by anion exchange (to remove endotoxin) and gel filtration [38]. In the aprotonin crystallization example noted herein (Table 12.3 and Ref. [35]), the authors did not use a brute force screening kit (of various reagents), but a multifactorial approach to find new crystallization conditions that were compatible to the purification process, and stated that “Overall, the multifactorial approach turned out to be 10-fold more efficient. The new crystallization conditions were scaled up and implemented into the purification process as a bulk storage step.”

Hebel, Üdinger et al. [39] used lysozyme and lipase as test proteins in development of large-scale, high-yield crystallization processes. Other examples are to be found in the general reviews and books noted herein.

12.2.3 Monoclonal Antibody Fragment and Antibody Crystallization

Lewus et al. [18] and Wanga et al. [40] discussed interactions and phase behavior of a monoclonal antibody and effects of other proteins in mixtures. Early belief in the feasibility of using crystallization for large-scale purification of antibodies was supported by the long use of precipitation to purify serum antibodies and belief that such behavior was related to protein structure [41,42]. Over a decade ago Jion et al. studied the liquid-liquid (L-L) phase separation of a mixture of mAbs (1–20 g/L) and PEGs (1000–6000 at 5% to 11% w/w) into PEG-rich and protein-rich phases. Effects of temperature on the critical point of nucleation were also studied. Choice of critical point conditions (10.5 mg/mL IgG in 11% PEG 1000) resulted in formation of 100μm IgG crystals after 3 weeks at 30°C [43]. Such a long time at elevated temperature may create bioburden challenges. In addition, it is to be appreciated that in a protein mixture, contaminants, including proteins of opposite net charge to a target antibody, could affect such critical phenomena [40].

Hebel et al. [44] investigated mL-scale “stirred tank” crystallization of an antibody fragment (Fab). Ammonium sulfate 1.1 M was able to induce nucleation after 1 h and the yield was 82% after 4 h. Increasing ammonium sulfate to 1.6 M resulted in a 99% yield after 2 h. The crystals were washed with 4 M NaCl solution to remove the ammonium sulfate whose salting out tendencies might affect further handling of the crystals.

Huettmann et al. [45] described the preparative crystallization of a single-chain Ab using an aqueous two-phase system. Their approach took advantage of the formation of a liquid-liquid (L-L) two-phase system formed with PEG 2000 and sodium sulfate, and the fact that at relatively high PEG concentrations, 50μm protein crystals formed spontaneously at the phase interface and in the salt-rich denser phase. The large crystals later accumulated into the less dense, PEG-rich upper phase. “An increase of the PEG from 2% to 4% (w/v) increased the yield from ~63% to 87%, respectively.” Such phenomena, involving use of L-L two-phase systems to create conditions suitable for L-S based crystallization or precipitation may not be unique [46].

12.2.4 Monoclonal Antibody Crystallization Phase Diagrams

Researchers from Biberbach University and Boehringer Ingelheim [3] studied the vapor diffusion crystallization conditions of an intact monoclonal IgG4 antibody using PEG8000 as a primary precipitant. Studies under “microbatch” conditions
allowed construction of a phase diagram (Fig. 12.3). Fc-binding activity of the mAb was not affected by the crystallization. Model protein contaminants were excluded from the crystals with 95% efficiency. They concluded that “Removal of protein contaminations and virus was successful and comparable to Protein A chromatography. DNA removal, however, was marginal. This may be due to adsorption of DNA to the crystal surface by electrostatic interaction.” They noted that target protein mAb04c is positively charged at the pH of crystallization. However, this does not necessarily mean that the crystal has to be net cationic or that other DNA-crystal interactions would not be possible, as is the case for other polyacid interactions with proteins [32,42,47]. When Protein A purified protein (which would be low in contaminants) was used, 95% of the target protein could be crystallized. However, when a less-purified target protein solution was used, only 31% of target protein was crystallized [3].

The phase diagram in Fig. 12.3 resembles the generic phase diagram in Fig. 12.2. It suggests, as expected, that increasing the concentration of protein or PEG precipitant concentration can shift conditions from a region with little crystal formation to one of significant crystal formation. As expected, further increases in the PEG often resulted in precipitate formation. Phenomena such as the latter might be exploited in a dual separation like operation [46] where a concentration and purification of target protein is accomplished first by precipitation, and then after alteration of conditions, shifts experimental conditions into another region of the phase diagram where crystallization occurs.

Trilsky et al. [48] of Amgen undertook screening studies related to crystallization and L-L phase separation of monoclonal antibodies (mAbs) and Fc-fusion proteins. Twenty-two different protein targets were studied in the HTS format (microtiter plates) in order to evaluate the general applicability of various methodologies and identify conditions successful over a broad range of targets. Screening involved 100 conditions based on reagents that are “Generally Regarded As Safe (GRAS)” and thus could be applicable to processing of biopharmaceuticals. All of the proteins studied exhibited L-L phase separation behavior, indicating their possible amenity to being concentrated or purified by crystallization, ATPE, or precipitation. One Fc-fusion protein spontaneously concentrated in a mesophase to 150 g/L. Four of 16 IgG2s examined formed diffraction-quality crystals, with four others forming “crystal-like” particles. Three of the IgG2s that crystallized well did so within a few hours under the same set of inexpensive operating conditions (Table 12.4). A phase diagram related to one of these proteins (mAb A) is shown in Fig. 12.4.

Fig. 12.4 shows a phase diagram with logarithmic scales in order to accompany the range of concentrations studied under conditions where no evaporation took place. The figure summarizes the phase behavior of mAb A at pH 6 (shown for unseeded conditions in Table 12.4).

Trilsky et al. [4] concluded that, “Many of the conditions under which phase separation occurred could be encountered in typical downstream processes, so an understanding of solubility limits is needed for robustness of those processes.

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**FIG. 12.3** Phase diagram of mAb04c with PEG 8000 as precipitant. Buffer: 0.1 M imidazole, 0.2 M calcium acetate, pH 7.0, room temperature. From Y. Zang, B. Kammerer, M. Eisenkolb, K. Lohr, H. Kiefer, Towards protein crystallization as a process step in downstream processing of therapeutic antibodies: screening and optimization at microbatch scale, Plos ONE 6 (2011) e25282, https://doi.org/10.1371/journal.pone.002528, reproduced with permission.
In cases where the solubility limits are exceeded, phase-separation-based unit operations could be viable alternatives. It remains to be determined whether these unit operations compare favorably to the chromatography and filtration unit operations currently used for protein purification. Overall, phase separation is applicable to all monoclonal antibodies and Fc-fusion proteins and the conditions identified in this work provide an opportunity to evaluate the use of phase separation for purification, formulation, and bulk storage.

### 12.2.5 Crystallization From Mixtures of Proteins

Proteins or other macromolecular contaminants can affect target protein crystallization in several ways. They can interact with protein in solution to alter its solubility [40] as well as its mechanical ability to interact with a crystal’s surface. They may also adsorb to the protein crystal surface and affect crystal growth [3]. This may be a particular problem when, at the operant pH, the target and contaminant proteins or nucleic acids or endotoxins have opposite net charge (see Table 11.2 in Chapter 11).
However, crystals of target proteins such as ovalbumin, lysozyme, lipase, and green fluorescent protein have been grown from mixtures of pure proteins, or clarified and filtered fermentation feed, which have included up to 15% HCPs (for discussion see Ref. [1]).

12.2.6 Crystallization of mAb From Clarified Feed

Smejkal et al. [49] succeeded with crystallization of a therapeutic IgG1 mAb from clarified CHO culture feed in unstirred microbatch experiments and then successfully scaled to 1 L. Yield was 90% and purity was >98% based on a 17 fold reduction in HCP and significant reduction in host cell DNA to below a 2 ppm detection limit. The first crystallization step reduced HCP from ≈200,000 to ≈40,000 ppm with a second crystallization step reducing HCP to ≈17,000 ppm. Molecular modeling of the protein suggested an Fv domain rich in negative electrostatic and hydrophobic patch regions, which promoted a tendency to interact with positive and hydrophobic Fc patch regions and promote crystallization. As these regions are often conserved, their work suggests an approach to screen for such propensity in other mAbs and Fabs [48]. These same scientists reported varying mAb concentrations from 150 to 15 g/L altered mean crystal sizes from 6 to 44 μm [49]. Crystals of such size are significant because, while structural studies may be undertaken on relatively small well-formed crystals, bioprocessing should be undertaken with crystals large enough to be easily washed, centrifuged, or filtered.

12.2.7 Scale-Up of Protein Crystallization

Hekmat [1] recently reviewed the work of Hekmat, Smejkal, Hebel et al. into the scale-up of protein crystallization [38,49,50] who have noted “little systematic know-how exists for technical-scale protein crystallization in stirred vessels” [50]. Schmidt et al. previously noted an inverse relation between hydrodynamic shear forces and mean crystal size [51]. In their recent work, Hekmat et al. used impellers developed to allow for gentle stirring in mammalian cell cultures, and employed them in a “scoping” mode that prevented build-up of crystal sediments [50]. One-liter scale-up studies with lysozyme [39] indicated that as stirrer speed went from 50 to 100 to 200 rpm, protein crystals went from large agglomerates, to favorable sized crystals with a large-size distribution, to uniform tetragonal crystals of uniform size. A maximum local energy dissipation of “2.2 W kg⁻¹” led to the fastest onset of crystallization over two orders of magnitude of volume (6 mL, 100 mL, and 1 L) [1,50]. This chapter’s authors believe such work promotes optimism in regard to future single-use bioreactor-type batch crystallization chambers, although other continuous formats [2,11] may also be attractive if employing single-use containers and equipment.

12.2.8 Discussion and Conclusions

Crystallography is improving both in regard to practical experience, molecular level understanding, and chemical engineering, and as with precipitation, the method should be amenable to application in continuous mode and employment with single-use apparatus. Given the size and shape of many protein crystals, there is already significant filtration know-how, and products to effect good crystal recovery and washing. However, there appears to be significant room for innovation in regard to protein crystal recovery, especially in continuous processing, as regards the use of methods such as elutriation, which rely on crystal density, or methods such as ATPE interfacial adsorption, which are dependent on crystal size. It is perhaps not surprising that in comparing their 2008 and 2013 surveys of industry attitudes to separation methods, Tran et al. [20,21] noted a four-fold increase in interest in crystallization. What we can expect from the future is that many of the weaknesses noted in Table 12.1 will be reduced either via direct research, or as a result of general scientific progress in understanding of protein structure, protein hydration, and chemical engineering of large-scale and continuous protein crystal growth. Genetic engineering may have a significant role to play if recombinant amino acid residue sequences can be identified that enhance crystallization without affecting other desired properties. Flocculation and precipitation operations prior to crystal growth may have an interesting role to play if the reagents used can also be employed in the crystal growth processes. Zhang et al. [3] discussed the possible reduction in crystal growth caused by adsorption of contaminant nucleic acid. This might suggest crystallization pretreatments involving charged or mixed-mode membrane filtration or use of calcium chloride to induce DNA flocculation [52].

12.3 AQUEOUS POLYMER TWO-PHASE EXTRACTION

For readers who want to refresh their memories, or quickly come to a basic understanding of aqueous polymer two-phase extraction (ATPE), Chapter 10 summarizes reviews comparing it with other alternative separation methods. Table 10.3
presents important attributes for chromatography, crystallization, precipitation, and three major classes of ATPE systems. Table 10.4 offers an overview of the comparative mAb purification performance of various methods. Table 11.2 reviews net charge properties of bioprocess targets and contaminants, and Table 11.3 presents some aspects of bioprocess feed clarification by ATPE in comparison with flocculation/precipitation methods.

An overview of the basic strengths, weaknesses, opportunities, and technical threats related to ATPE is given in Table 12.5, some elements of which were taken from Ref. [16].

### 12.3.1 Introduction

There are a large number of pairs of compounds that are able to generate liquid-liquid (L-L) two-phase systems. In each case, buffered aqueous solutions may be the third “component.” They can be divided into six groups that differ regarding the chemical nature of the generated phases. These are (a) polymer-polymer systems, which can comprise polymers of both nonionic character and polyelectrolytes; (b) polymer-salt systems, typically formed using a kosmotropic anion containing salt such as phosphate, citrate, or sulfate; (c) EOPO and other thermosensitive, and other single, responsive, polymer-water systems; (d) alcohol-salt systems; (e) polymer-ionic liquid systems, and (f) surfactant-water systems. The latter are often designated as aqueous micellar two-phase systems. Much literature exists on each of these six types of L-L systems. Rituximab and some other pharmaceuticals are processed using L-L extraction systems that are not aqueous polymer systems (Fig. 12.1). However, aqueous polymer two-phase systems show much promise in regard to bioprocessing, and are the topic of this subchapter.

In bioprocessing, the controlled partition of substances between aqueous polymer two-phase systems is typically termed aqueous polymer two-phase extraction, or ATPE. ATPE systems include those formed with a host of polymer mixtures [12], or polymers in solutions with relatively high concentrations of kosmotropic salts [16,55,56]. Typical polymer pairs

| TABLE 12.5 SWOT Overview of ATPE |
|-----------------|-----------------|
| Traits          | Comments         |
| **Strengths**   |                  |
| Theory of partitioning is well understood | |
| Ease of scalability and integration incl. use in continuous processing | |
| Systems are flexible, robust, and biocompatible | |
| Ideal for high-throughput screening | |
| Wide range of targets (proteins, nucleic acids, virus, cells) | |
| Inexpensive L-L nature is ideal for single-use processing and HTS | |
| Targets can be maintained in solution | |
| 1-step clarification, target capture, and contaminant reduction | |
| Can employ EOPO and other polymers already used in industry | |
| **Weaknesses**  |                  |
| Less-developed predictive design—could be improved | |
| High cost of some classic ATPE system polymers like dextran | |
| Protein solubility (capacity) of some ATPE systems <10 g/L | |
| No vendor specializing in the technology or specialized single-use devices (e.g., continuous flow phase separators) | |
| Industry knowledge on installation, validation, and operation | |
| **Opportunities** |                  |
| Predictive modeling based on primary structure | |
| Can take advantage of molecular target understanding (e.g., [18,19,53,54]) | |
| Primary clarification of dense feeds with continuous flow to column | |
| Operation in back partition or three-phase modes. | |
| New systems offering inexpensive polymers (e.g., EOPO or PAA)* | |
| New systems offering much higher capacities (e.g., 30 g mAb/L)* | |
| New PAA and EOPO systems offer recycling of polymers | |
| ATPE dedicated single-use equipment | |
| **Technological threats** | |
| Other novel L-L approaches to bioseparation (e.g., ionic liquids) | |
| Improved methods of clarification by centrifugation and filtration | |
| Improved clarification and primary capture by EBA | |
| Clarification by polymer induced flocculation (see Chapter 11) | |

involve polyethylene glycol (PEG) and dextran [12], each at 5%–10% (w/w) in solution. However, useful phases will form with PEG and hydroxypropyl starch, Ucon (an “EOPO” polymer) and dextran, PEG and polyvinylalcohol (PVA) [57], or PEG and polyacrylic acid (PAA) [58] or a range of other polymers [12–14]. Many of these same polymers will also form ATPE systems when mixed with high concentrations (e.g., often >0.5 M) of salts. In addition to polymer-polymer and polymer-salt aqueous two-phase systems, one can form two aqueous phases when the solubility thermo-sensitive EOPO polymers (such as Ucon, Tergitol, Breox and some Pluronic polymers) are raised above their solution-critical temperature (Tc) in low-salt concentrated solutions [57–60]. Various attributes of the three major types of aqueous polymer two-phase systems are noted in Table 10.3. In general, polymer-polymer systems are more expensive, can have viscous phases, and offer reasonable protein target solubility (i.e., capacity) of 5–10 g protein/L in the target-containing phase. Although polymer salt systems can be much less expensive, they typically offer significantly reduced target solubility (e.g., 1–3 g/L), single-polymer systems can offer much higher target solubility (>30 g/L). In general, the order of selectivity (such as differentiating between host cell proteins and target proteins) is polymer-polymer > polymer-salt > single-polymer Tc, for systems in which selectivity is not enhanced via the use of polymer-coupled affinity ligands [61–64]. Aqueous polymer-phase systems and their use for ATPE have been studied for almost seventy years [12], and a number of excellent books [61–64] and reviews (e.g., [14,65–69]) have been published, including a number outlining partitioning theory [12,59,70,71].

Key bioprocessing methods, such as centrifugation for clarification, followed by chromatography and filtration for purification, offer excellent performance in the laboratory; however, when they are scaled, significant challenges appear. They include ease of integration of different unit operations with the others, significant hydrodynamic pressure and pressure drops, loss of efficiency due to feed viscosity, fouling, time dependence of target diffusion limited operations, challenging mass transport modeling, and apparatus with which costs scale exponentially with target mass per batch. These challenges have been solved by innovative products and processes. However, they have become amplified in the past decade due to significant increases process scales, fermentation cell densities, and product titers. Aqueous polymer two-phase extraction (ATPE) is largely devoid of such challenges (as reviewed in [15,16,68,69]). It addition, it is suitable for targets from small molecules to proteins, nucleic acids, virus, cell organelles, bacteria, and eukaryotic cells [12]. Its potential to be used for larger-scale bioprocessing was recognized more than thirty years ago by Maria Regina Kula et al., Bo Mattiasson, Rajni Hatti-Kaul et al., Per-Åke Albertsson, Folke Tjerneld et al., Juan Asenjo, Barbara Andrews, et al. and others in academia [13,61–63,72] as well as Heinsohn et al. at Genencor [73] and Hart et al. at Genentech [7]. As noted further on in this chapter, research into the potential of ATPE continues today both in the biopharma industry and in academic groups such as Aires-Barros et al., Asenjo et al., Bülow et al., Hubbuch et al., and Rito-Palomares et al.  

### 12.3.2 Phase Systems, Phase Diagrams and Partition Coefficients

When pairs of polymers such as 500,000 MW dextran (T500) and 8000 MW poly(ethylene glycol) (PEG) PEG are dissolved in aqueous biological buffers at approximately >5% (w/w) each, two immiscible liquid phases often form. Each phase is enriched in one of the polymers. Such systems possess low liquid-liquid interfacial tensions (Table 12.6) and readily emulsify upon gentle agitation. Upon mixing, one phase is suspended as droplets in the complementary (continuous or suspending) phase. Which phase become continuous is determined by several factors, including relative phase volumes and viscosity. The systems are typically abbreviated in the fashion “(X, Y)” [12] where the first number represents the total system (not phase) concentration of the denser phase polymer (e.g., dextran, as % w/w). The second number refers to concentration of the other polymer. The systems are usually made up in standard buffer systems, which in many cases are isotonic for cells. In some cases the polymers may be added directly to culture media to form a two-phase system. Mixtures of divalent ions and PEG can sometimes precipitate, so filtration may be required prior to use. In dextran and PEG systems, the less dense PEG-rich phase is typically the suspending phase. If the systems are formed from polymer and salt, such as 15% PEG and 10% sodium citrate, the salt-rich phase is often the denser phase. PEG salt systems often have 10X higher interfacial tension and higher polymer-rich phase viscosities [76,77]. In both types of systems, phase droplets in the emulsion rapidly coalesce and grow to a size where the denser-phase droplets quickly sediment. As a result, the phases demix rapidly. The rate of demixing is typically dependent on phase ratios, phase viscosities, and the height (not the volume) of the chamber. Relatively large-volume phase systems (e.g., 1000 L in a chamber 30 cm high × 200 cm wide × 200 cm long) can often significantly demix in an hour or two.

Systems enriched in NaCl typically exhibit a chloride ion concentration phase ratio close to 1, with negligible interfacial potential. Phosphates, citrates, sulfates, and other kosmotropic ions partition asymmetrically between the phases, resulting in electrostatic potentials that can be measured with open-tipped capillaries, which are top-phase positive relative to the bottom phase (Table 12.6). Two-phase systems compounded with two polymers and buffer systems enriched in NaCl tend to show target partition coefficient selectivity (i.e., sensitivity) to noncharge properties of proteins, cells, and other targets.
### TABLE 12.6 Physicochemical Properties of Some Dextran and PEG Two-Phase Systems

<table>
<thead>
<tr>
<th>#</th>
<th>System</th>
<th>Buffer Solution Type</th>
<th>Phase Viscosity (mPa·s)</th>
<th>Interfacial Potential (mV)</th>
<th>Interfacial Tension (μN/m)</th>
<th>Phase Density (g/cc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dex</td>
<td>PEG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(7*, 5)</td>
<td>109,35,0</td>
<td>14.6</td>
<td>5.7</td>
<td>14.3</td>
<td>1.064</td>
</tr>
<tr>
<td>2</td>
<td>(5, 3.5)</td>
<td>109,35,0</td>
<td>24.3</td>
<td>3.9</td>
<td>1.93±0.21</td>
<td>1.66±0.17</td>
</tr>
<tr>
<td>3</td>
<td>(5, 4)</td>
<td>109,35,0</td>
<td>32.4</td>
<td>4.0</td>
<td>2.10±0.13</td>
<td>11.00±0.20</td>
</tr>
<tr>
<td>4</td>
<td>(5, 4)</td>
<td>91,29,25</td>
<td></td>
<td></td>
<td>1.48±0.07</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(5, 4)</td>
<td>73,23,50</td>
<td></td>
<td></td>
<td>1.35±0.08</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>(5, 4)</td>
<td>55,17,75</td>
<td></td>
<td></td>
<td>1.14±0.05</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>(5, 4)</td>
<td>34,11,100</td>
<td></td>
<td></td>
<td>0.78±0.03</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>(5, 4)</td>
<td>18,6,125</td>
<td></td>
<td></td>
<td>0.50±0.03</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>(5, 3.5)</td>
<td>7,3,150</td>
<td></td>
<td></td>
<td>0.70±0.28</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(5, 4)</td>
<td>7,3,150</td>
<td>0.08±0.14</td>
<td></td>
<td>4.99±0.06</td>
<td>10.26</td>
</tr>
<tr>
<td>11</td>
<td>(6, 4)</td>
<td>7,3,150</td>
<td></td>
<td></td>
<td>10.87±0.07</td>
<td>12.40</td>
</tr>
<tr>
<td>12</td>
<td>(6.5, 4)</td>
<td>7,3,150</td>
<td></td>
<td></td>
<td>14.36±0.06</td>
<td>13.26</td>
</tr>
<tr>
<td>13</td>
<td>(7, 4)</td>
<td>7,3,150</td>
<td>0.20±0.04</td>
<td></td>
<td>17.22±0.12</td>
<td>14.14</td>
</tr>
<tr>
<td>14</td>
<td>(8, 4)</td>
<td>109,35,0</td>
<td>69.1</td>
<td>4.4</td>
<td>34.7</td>
<td>1.070</td>
</tr>
<tr>
<td>15</td>
<td>(8, 4)</td>
<td>7,3,150</td>
<td></td>
<td></td>
<td>26.55±0.21</td>
<td>15.68</td>
</tr>
<tr>
<td>16</td>
<td>(5, 4)</td>
<td>*55,55,0</td>
<td></td>
<td></td>
<td>1.57±0.16</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>(5, 4)</td>
<td>*45,45,30</td>
<td></td>
<td></td>
<td>1.44±0.10</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>(5, 4)</td>
<td>*30,30,75</td>
<td></td>
<td></td>
<td>0.65±0.06</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>(5, 4)</td>
<td>*15,15,120</td>
<td></td>
<td></td>
<td>0.26±0.05</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>(5, 4)</td>
<td>*5,5,150</td>
<td></td>
<td></td>
<td>0.05±0.05</td>
<td></td>
</tr>
</tbody>
</table>

Systems expressed as (X, Y) Z where X is %, w/w Dextran (Dex) 500000 (GE Healthcare) or + Dextran 40000; Y is %, w/w PEG 8000 Sentry Grade (Union Carbide) and Z buffer solution (NaP mM, Na2P mM, NaCl, pH 7.2 or *pH 6.8) approx. isotonic for cells. TLL (% w/w) tie line length. Measured at 22°C and with ten determinations. Blank entries not determined. Results are expected to vary somewhat with polymer lot. Modified from [75] and for related methods see references [74] and [73], as well as [62,63] and [76].
This is illustrated in Fig. 12.5 where cell partition is expressed as a partition coefficient $K$ related to the ratio of cells in the upper phase to cells not in the upper phase (i.e., at the phase interface or in the lower phase) [75]. As evidenced in Fig. 12.5B there are often direct relationships between log $K$ and various key system parameters in regard to both cell and macromolecular partition. The effects of added salts on target partition often vary in regard to the Hoffmeister series [61–64,67]. Hans-Olof Johansson et al., have studied such effects [78], which relate to ions affecting polymer-polymer, polymer-target, and target-target interactions [30].

Systems formulated with two polymers, or one polymer and an excess of kosmotropic salt(s), tend to be sensitive to protein or cell surface charge properties, including protein $pI$ (Fig. 12.5B). Much has been written on such interactions [12,55–71]. In addition to charge and noncharge based partitions, practitioners recognize that coupling of various affinity ligands or target binding substances such as proteins or lectins to phase polymers can alter target partition between the phases on the basis of hydrophobic affinity, metal ion affinity, immuno-affinity, substrate enzyme affinity, and lectin-target affinity ([79,80] and [61,62,64]). Such polymer-affinity ligands can sometimes be very effective at significantly shifting partition coefficients even at micromolar concentrations, which may relate to binding one polymer unit per target [16]. It is beyond the scope of this review to delve further into these effects, other than to note that that some PEG-alkyl ligand surfactants (e.g., Brij 58 palmitate) can serve as effective hydrophobic affinity ligands [79]. In addition, PEG-Protein A has been used to shift the partition of antibodies and antibody-target complexes to the PEG-rich phase [81].

Fig. 12.6 shows a typical isothermal phase diagram for a polymer-polymer or polymer-salt (compounds $C_1$ and $C_2$) aqueous polymer two-phase system, in which traditionally the water/buffer axis is not shown [12]. The solid line represents...
the critical concentration curve beyond which two phases exist at equilibrium. By convention, the compound yielding the
less-dense phase is shown on the y-axis. The lowest total concentration of compounds yielding two phases is the critical
point C, which varies with compound type, temperature, pH, conductivity, etc. The two-phase system denoted by point A
is characterized by the “tie-line” XY (Table 12.6) such that the volume ratio of upper-phase to the more-dense lower phase
is given by the length ratio of XA to AY.

Many phase system properties, such as phase interfacial tension, vary with system XY tie-line length (TLL). Examples
of this are shown in Fig. 12.7A and B in regard to PEG1000-NaCitrate polymer-salt two-phase systems [76]. Not surpris-
ingly, there is a direct inverse relationship between a cell partition expressed as Log K and interfacial tension that acts to
hold cells to the phase droplet interface where they are transported to the bulk macroscopic interface that forms between
the phases as they separate (Fig. 12.8).

\[
K = \exp[-\Delta E / kT]
\]

(12.1)

Given Eq. (12.1), it is expected that log K should vary directly with the system and solute properties determining the
partition (e.g., Figs. 12.5B and 12.8). This has been shown in many examples over the past 70 years [67–72]. Under ideal
circumstances (e.g., absence of time-dependent target aggregation, degradation, or denaturation), one expects macromol-
ecule K values to be independent of time and other factors, such as partition vessel geometry.

Fig. 12.8 is a replot of data from a partition optimization study by Mao et al. at Biogen IDEC Corporation [14]. The
results involve the partition of a monoclonal antibody (mAb) in various phase systems containing 14% (w/w) PEG 3350

![Graph A](image1.png)

![Graph B](image2.png)

**FIG. 12.7** Relationship between TLL and (A) phase density or (B) phase viscosity, for polymer-salt two phase systems formed using PEG1000 and sodium
citrate. From H. Yuan, Y. Liu W. Wei, Y. Zhao, Aqueous two-phase systems with phase separation behavior and system properties of polyethylene glycol and

![Graph C](image3.png)

**FIG. 12.8** Partition of mAb in 14% PEG 3350, pH 7.2 systems containing mutually increasing concentrations of NaCitrate (7.5%–10.1%, w/w) and
NaCl (5.1%–11.5%, w/w) [14]. Data from L. N. Mao, J. K. Rogers, M. Westoby, L. Conley, J. Pieracci, Downstream antibody purification using aqueous
and mutually increasing concentrations of NaCitrate (range 7.5%–10.1%, w/w) and NaCl (range 5.1%–11.5%, w/w) at pH 7.2. As expected [55, 56], over these salt concentration ranges, K changed significantly (i.e., from 1 to 66). Mao et al. saw less effect on K due to NaCitrate concentration change, than for the larger concentration change in NaCl [14]. The large change in K may relate to significant changes in TLL (e.g., [76], see also Fig. 12.7) more than alteration in (citrate-induced) interfacial potential in these NaCl-rich systems. Repplotting data from Ref. [14] as Fig. 12.8 would appear to confirm this.

Much has been written in regard to protein partition in ATPE with Walter and Johansson providing classic summaries of the related effects and the influences of system pH and salt composition, as well as protein interaction with polymer molecules; including polymers modified with affinity ligands [62, 67].

The relationship in Eq. (12.1) is not expected to hold for cell and other bio-particles, as interfacial tension acts to hold such particles at the phase interfaces [12, 70]. Eq. (12.1) can be rewritten in regard to free energy of interfacial adsorption ($\Delta G^o$) [12] with

$$ K = \exp \left( \frac{\Delta G^o}{kT} \right) \tag{12.2} $$

It should be possible to calculate $\Delta G^o$ by measuring the equilibrium contact angle $\theta$ formed between the line tangent to the surface and the two-phase boundary. For a spherical particle of radius $r_c$ in a system with liquid-liquid interfacial tension of $\gamma_{TB}$, Young’s equation holds [70], and

$$ \Delta G^o = -\gamma_{TB} r_c^2 \left( 1 - \cos \theta \right) \tag{12.3} $$

Equations (12.2), (12.3) suggest that with all other system factors being equal, log K should vary linearly with interfacial tension, or various other factors that affect the free energy of phase-cell interaction (as reflected by $\theta$). Table 12.6 systems #9–13 and #15 span over two orders of magnitude in interfacial tension, while their dextran concentrations only vary between 5% and 8%, yet demonstrate the expected “direct” first approximation relationship (Fig. 12.9).

Micron-sized particles may be large and dense enough to sediment, even in the relatively viscous aqueous polymer phases, and their K values can be dependent, to some extent, on the time between phase mixing and sampling. Thankfully in most systems, phase demixing (to a reasonably defined planar interface) generally occurs at a more rapid rate than cell sedimentation [75]. In addition to sedimentation, cell partition is influenced by a number of other “nonthermodynamic factors.” Cells are generally large enough to exhibit negligible diffusion and possess significant surface area. As a result, they show a tendency to partition to, and remain at, the phase interface that has significant, albeit relatively small, interfacial tension (Table 12.6). Phase droplet interface adsorbed cells can affect droplet fusion and sedimentation. As such, cell concentration in phase systems can affect phase demixing rates when the cell mass increases higher than 1% (w/w). However, ATPE can be effective, and provide reproducible results, even when handling feeds of 30% (w/w) cell mass [13, 60, 82].

![FIG. 12.9](image-url) Influence of interfacial tension on log K partition of rat erythrocytes (filled triangles) and Acholeplasma laidlawii cells (filled circles) in (X, 4) V systems with low interfacial potential (i.e., #9 to 13, Table 12.6). From J. M. Van Alstine, Eukaryotic cell partition, in: R. Hatti-Kaul, (Ed.), Chapter 11 in Aqueous Two-Phase Systems: Methods and Protocols, Book 11, Methods of Biotechnology Series, Humana Press, New Jersey, 2000, pp. 119–142, used with permission.
Because $\Delta G^\circ$ (Eq. 12.2) refers to free energy of transfer of solute from one phase to another, it can be expanded to include separate terms related to charge-related, noncharge-related general target solute-phase interactions, as well as specific affinity-related target solute-phase polymer interactions. In many cases alterations in $\Delta G^\circ$, as reflected in log $K$, can be directly related to system properties or molecular target surface structure (e.g., [53,54,70,71,78]).

References to more in-depth theory [70], and mathematical analysis and modeling abound. There is still discussion on many issues of significance in regard to chemical engineering with references from Hatton et al. [71], Cabezas, Jr. et al. [83,84], Prausnitz et al. [85], and Asenjo et al. (e.g., [69]) and of note as regards macromolecule partitioning, or Rito-Paloma et al. [86] and Cabal [87] in regard to cell partition.

12.3.3 Recent Trends in ATPE

Ratanapongleka has reviewed ten relatively recent studies related to partition of different enzymes of commercial interest, and eight in which ATP systems were used for extractive bioconversion [65], both applications having been frequently studied previously [12,61–64]. The studies reviewed primarily involved PEG-salt systems with typical recoveries of 80%–100% and purification factors from 2 to 10 [65]. Rosa et al. have reviewed the potential for ATPE to be used for the large-scale purification of several proteins [15]. Such reviews indicate the power of this simple, yet elegant, technology.

The tendency for partitioning studies to be revisited occurs every decade or two, driven by advances in theory, analytical capabilities, target characterization, and modeling. This has a tendency to eliminate perceived weaknesses of the technology, while developing a large body of empirical results. As a result, ATPE is moving from a technology seen as more of an art than a science, to a promising separation tool for use in bioprocessing [14–16,20,21].

Recent trends in ATPE research that further support increased interest in the technology are:

- Newer, less expensive, and better characterized polymers, including functionalized polymers and surfactants for use in affinity partitioning [57–59].
- Thermosensitive and other polymers offering features such as polymer recycling [57–60]. See also Refs. [71–75] in Chapter 10.
- Enhanced understanding of salt effects [30,53,54,78].
- Improved understanding of protein L-L phase separation and related protein physical properties [17,18,45,48,53,54,59,88]
- High-throughput screening (HTS) and high-throughput process development (HTPD) methods; including use of HTS for phase diagram construction [78,88–92].
- Better understanding of process variables that exert significant influence over yield and purity [14,16,68,93,94].
- Combination of partitioning with precipitation, crystallization, or chromatography [45,46,48,60,95–97].
- Work on targets emerging as particularly challenging in bioprocessing, including nucleic acids [95,98] and stem cells [86,87,99].
- Continuous processing using ATPE [96,100–103].
- Continued industrial interest and industrially focused academic research [7,14,15,73,96–98,102,103].

12.3.4 Key References on the Partitioning of Monoclonal Antibodies

As one might expect, much recent research on protein partitioning has been in regard to mAb processing. This includes reference [14] related to Fig. 12.8, as well as a number of other Refs. [15,16,55,56,60,90,94,96,97,101–103]. In reviewing this literature, it is interesting to note that the optimal PEG-salt partition system identified twenty years ago by Andrews, Nielsen, and Asenjo [55] is similar to those identified in more recent screening efforts (e.g., [14,56,97,101,103,104]). Andrews et al. also noted the use of back extraction approaches to increase process efficiency, and the limitations of PEG-salt system-based processes imposed by the 1–2 g/L protein solubility in the phases. As regards PEG-salt systems, target solubility issues are of concern; however, other results are impressive. Rosa et al. [104] reported a PEG, NaPhosphate, NaCl system which, in experimental studies, related to IgG purification with serum albumin and myoglobin as representative contaminant proteins that resulted in a 97% single-operation yield with a target at 99% purity.

12.3.5 General Considerations Related to Scouting and Choice of Phase System

Much information regarding choices of phase systems for specific applications can be found in the main book Refs. [12,13,62,64] as well as specific references in Section 12.3.5. All ATPE systems (polymer-polymer, polymer-salt, and single-polymer Tc) are capable of effecting good primary clarification and target capture, although due to their high-target capacity and ability to be generated within fermentation samples, the latter systems may offer particular advantages (Chapter 11,
Section 11.3.4). Classic two-polymer ATPE systems, which typically offer higher-target capacity than PEG-salt systems, often feature PEG and dextran polymers. However, the cost of dextran has led to research involving PEG-salt systems, which are less expensive, but somewhat compromised by the high salt concentrations required [12,14,17,55,56,65,76,77]. A desire to replace dextran has also led to several attempts to explore less-expensive two-polymer systems. These typically involve PEG plus a dextran substitute such as hydroxypropyl starch or polyacrylic acid (PAA) [12,16,57–60,102,105]. PAA systems are discussed in more detail below. As noted in Section 12.3.3, the main replacement considered for PEG (often termed polyethyleneoxide or polyEO) polymers in polymer-salt or two-polymer phase systems has been thermotropic analogues, which are so-called polyethylene oxide-polypropylene oxide (EOPO) random, or block copolymers [16,57,59,60,98]. These polymers become more hydrophobic and self-associate above a critical temperature (Tc). Such polymers include several nonionic detergents already used in bioprocessing and food processing (e.g., Ucon, Tergitol, Breox and some Pluronic polymers). They will behave as PEG in regard to ATPE systems; however, the EOPO phase can be isolated, and its temperature raised above its Tc to generate another two-phase system and effect a secondary extraction [16,57,59,60,98].

As noted in Chapters 10 and 11, precipitation and ATPE lend themselves to relatively easy modeling and scale-up [14], even if used in continuous processing [96,101,103,106]. Benavides and Rito-Palomares addressed the extensive experimental work required to establish “optimal” ATPE operation parameters by proposing general rules to facilitate the establishment of processes based on lessons learned from practical experiments involving a wide range of targets [68,93]. This work (Fig. 12.10) was adapted by Rosa, Aires Barros, et al. ([15], see also [96]).

### 12.3.6 Clarification in Single-Polymer EOPO Phase System

EOPO polymers can be used on their own to generate two-phase systems, without pairing with added salt or another polymer, as long as in the presence of some kosmotropic salt such as 100 mM NaCitrate or NaPhosphate. They can be added to almost any aqueous system (high salt concentration is not required) and used to generate ATPE systems. This is one reason they can offer high capacity (protein solubility) (>25 g/L). As noted in Chapter 11 (Section 11.3.4, Fig. 11.7), this allows them to be used to partially clarify even dense feeds in a manner whereby 95% of protein target will end up in a

---

water-enriched phase containing only 1%–2% of a neutral hydrophilic polymer. The rest of the polymer will be in a dense polymer-rich phase [45–47]. The basic attributes of such systems are [60]:

1. Inexpensive (1 USD/L), GRAS compatible.
2. 10X present capacity, i.e., 25–50 g/L.
3. High enough interfacial tension to significantly clarify ferments.
4. Low polymer concentration (1%–2%) in the target-rich phase.
5. Some selectivity (i.e., reduction of DNA, HCP) based on added salts.
6. Some target concentration, so no net change in process volumes.
7. Low conductivity in target-rich phase allows direct application in chromatography.
8. Compatible with varied follow-on chromatographic steps.

Studies conducted by GE Healthcare have shown that Breox 50 A 1000 (approx. MW 3900, Tc approx. 40°C) added to CHO cell culture (mAb at 1.3 g/L) in a bioreactor at 10% (w/w) together with 0.1–0.2 M NaPhosphate added as stock solution or as solids (to reduce feed dilution) resulted in a two-phase system with a phase volume ratio (U/L) of 1.7. One-liter test systems in 30-cm high containers (commensurate with large- scale WAVE bioreactor processing) phase clarified in 1–2 h. Upper-phase mAb recovery was typically >95%, without significantly reducing HCP (by enzyme-linked immunosorbent assay) or aggregates (by Protein A and SEC). It was readily filtered prior to being applied to a MabSelect SuRe (Protein A-based) affinity column. Six separate test runs indicated that EOPO partition did not appear to affect mAb sample aggregation, or MabSelect performance, compared with control (direct column application of centrifuged and filtered feed). MabSelect offered 99% HCP reduction and a full mAb recovery, plus 30% reduction of aggregates. The 1% residual polymer expected in upper-phase [59,60] should not adversely affect column capacity; however, its effect on column lifetime is presently unknown. Experiments with polyclonal Ig samples showed the method worked for feed solutions containing 25 g/L mAb. Other EOPO polymers gave similar results at T > Tc (not shown). Similar results were also seen with Ab fragments and other targets and a variety of other feeds [60].

12.3.7 Partition of Proteins in Two-Polymer Phase Systems Containing Polyacrylate

Polyacrylic acid (PAA) polymer-polymer two-phase systems have been pioneered by Han-Olof Johansson et al. [58,107,108]. In these systems, which typically contain NaPAA of 8000 or 15,000 MW and 0.1 M kosmotropic salt plus another polymer such as PEG or an EOPO polymer, the polyacrylate behaves similar to dextran and can offer impressive partition results (Fig. 12.11). As a result, much is already known about such systems and how they can be employed. In the systems in Fig. 12.11, nucleic acids tended to favor the lower phase [58]. In addition to offering cost reductions compared with dextran, the major advantages of using PAA are that the lower phase is very clear, which is ideal for HTS screening using optical analytical methods; the phases are lower in viscosity, which speeds up phase separation time; the PAA is of relatively lower MW and can be readily filtered; protein solubility (system capacity) is improved [58]; and by lowering the pH to 3 or 4, the PAA will protonate, and, in becoming electrically neutral, will self-associate and come out of solution. This latter property creates exciting possibilities in regard to polymer removal and recycling.

![FIG. 12.11 Partitioning of mAbs and test contaminant proteins (myoglobin and bovine serum albumin) at 0.2 g/L in 6% (w/w) PEG4000, 6% NaPAA15000 systems, 20 mM NaPhosphate systems containing 0.25 or 0.30 M NaCl, at 20°C. Reproduced with permission from R. Hjorth, K. Lacki, E. Macedo, M. Malmquist, J. Shanagar, J.M. Van Alstine, Separation method using polymermulti-phase systems, GE Healthcare W02008156409 A1.](image-url)
12.3.8 Partition of Recombinant Proteins in EOPO Two-Polymer Phase Systems

Persson et al. [105] investigated the use of EOPO polymer and hydroxypropyl starch (HPS) phase systems for the primary recovery of E. coli-derived recombinant proteins from centrifugally clarified feed using recombinant human growth hormone (rhGH) as a test target. The test process was to proceed to anion exchange chromatography (AIEC) following partition. Some results are summarized in Table 12.7. Two-ATPE systems were studied; one offering a higher concentration of EOPO, and thus higher cost and phase viscosity. They reported effective DNA removal and good secondary clarification from either system with some interesting trade-offs in yield, purification factor, and follow-on AIEC volume performance between the two systems (Table 12.7).

Several different EOPO polymer-containing, two-polymer phase systems have been investigated for a variety of different separation modes and targets [98,109,110]. This includes PEG-EOPO systems, EOPO-PAA systems, and EOPO-HM EOPO systems employing hydrophobically modified EOPO polymers (see the preceding refs.)

12.3.9 Single-Polymer ATPE Clarification Followed by Polyacid Precipitation

The EOPO thermoresponsive, single-polymer, two-phase system described for clarification and initial target recovery (Section 12.3.8) can be combined with the polyacrylic acid precipitation method described in Chapter 11 (Sections 11.4.3 and 11.6.1). After addition of the EOPO polymer and 50 mM NaPhosphate to a CHO-cell mAb fermentation, an ATPE system was rapidly formed with a protein-free, polymer-rich (e.g., 70% w/w) phase at the bottom and a less-dense polymer-poor (e.g., 1% w/w) water-rich phase on top. Target mAb protein was almost totally recovered in the water-rich phase. Electrophoresis and analytical assays showed minor reduction of host cell proteins (HCP) and DNA—both of which, under the conditions studied, also partitioned to the upper phase. Polyacrylic acid was therefore added to the filtered upper (water-rich) phase, which led to preferential precipitation of the mAb target due to its high pI and a significant reduction of negatively-charged HCP and nucleic acid, which remained in solution. This two-stage process yielded a 90% recovery of the mAb together with a 95% reduction of both HCP and DNA. If the precipitation was done in the presence of >50 mM NaPhosphate or NaCitrate or similar chaotropic salt, the precipitated target protein was easily resuspended in a 1:1 volume mixture of aqueous buffer, and could be added directly to a chromatographic column after simple filtration [60,111].

The ability of the EOPO polymer to form a two-phase system in, and preliminarily clarify, almost any fermentation (e.g., even at 30% cell mass and 20 g/L target protein), coupled with the ability of the resulting target in the upper phase to be effectively precipitated, in one or more sequential steps by polyacrylic acid (NaPAA) precipitation is of interest. The precipitation can isolate target protein from the bulk of nucleic acid and HCP contaminants, and leaves it in a readily re-suspended and chromatographed precipitate. This suggests a possible robust generic upstream alternative separation-based bioprocessing approach.

12.3.10 ATPE Versus Classical Protein A-Based Bioprocessing of mAbs

It is always difficult to undertake cost and other (e.g., environmental impact) comparisons involving two very different unit operations such as ATPE versus Protein A affinity chromatography as results can be very affected by variables such as feed, scale, ability to use existing facilities, etc. Such studies should be done, but the results can only serve as a rough guideline to what might be experienced in practice under one set of circumstances compared with another.

### Table 12.7 ATPE of E. Coli-Derived rhGH Target in Two EOPO-HPS Two-Phase System

<table>
<thead>
<tr>
<th>Sample</th>
<th>hGH Yield (%)</th>
<th>Purification Factor</th>
<th>DNA (μg/mL)</th>
<th>Viscosity (CPS)</th>
<th>Turbidity (NTS)</th>
<th>AIEC Vol. (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>100</td>
<td>1.0</td>
<td>700</td>
<td>3.0</td>
<td>461</td>
<td>4200</td>
</tr>
<tr>
<td>TP1⁶</td>
<td>95</td>
<td>1.9</td>
<td>30</td>
<td>3.6</td>
<td>3</td>
<td>2500</td>
</tr>
<tr>
<td>TP2⁶</td>
<td>70</td>
<td>5.0</td>
<td>1.3</td>
<td>14.0</td>
<td>54</td>
<td>900</td>
</tr>
</tbody>
</table>

⁴No recycling.
⁶TP 1 is top phase (8.2% w/w EOPO), TP 2 is top phase (20% EOPO).

The past decade has seen order of magnitude increases in fermentation cell densities (e.g., 1% v/v to >10% v/v) and target concentration in feed (e.g., 1 to >10 g/L), but in many cases, less dramatic improvements in downstream processing methods, capacities, or productivity. This suggests a role for alternative separation methods to augment or replace certain conventional unit operations. Due to their selectivity and resolution, there should continue to be a major role for affinity and ion exchange chromatographic separation methods; however, other methods, particularly those such as ATPE, which are complementary to chromatography, are of growing interest. This is especially true in regard to new targets that lack effective high-capacity, high-resolution affinity chromatography methods of purification. In this regard, it is of interest to develop and test ATPE-based processes in comparison with conventional processes, including those involving mAb purification and Protein A-based affinity purifications. The literature is starting to see articles related to comparative economic analyses of alternative separation and more standard protein purification methods. Three recent efforts, with a focus on mAb processing by alternative methods versus standard Protein A chromatography-based approaches include papers by Hammerschmidt et al., with colleagues from Novartis on “Economics of recombinant antibody production processes at various scales: Industry-standard compared to continuous precipitation” [106]; Rosa et al. with colleagues from Bayer Technologies on “Aqueous two-phase extraction as a platform in the biomanufacturing industry: Economical and environmental sustainability” ([94], see also [96,112]); and Eggersglueß et al. with colleagues from Boehringer Ingelheim on “Integration of aqueous two-phase extraction into downstream processing” [97,103]. The Hammerschmidt et al. work was presented in Chapter 11. Some insights into the Rosa et al., and Eggersglueß et al. studies are given herein.

Paula Rosa et al. [94] compared results for a single-product production plant producing 2.1 tons of mAb per year (17.5 kg/batch) based on three bioreactors of 7000 L volume. The complex feed contained mAb target at 2.5 g/L, corresponding to ~20% of the total UV 215 nm absorbance. A single reactor harvest was processed at a time with three batch runs processed per week. In this study, it was assumed that the initial target recovery/clarification steps (typically disk-stack centrifuge followed by depth filtration) would be performed prior to both ATPE and Protein A affinity chromatography. As such, the clarification ability of ATPE was not exploited. The model assumed a mAb yield of 95% at 95% purity. The ATPE process, which was based on a PEG 3350-Phosphate, polymer-high salt, two-phase system, had previously been validated at pilot scale and consisted of extraction, back extraction, and washing steps. Three batches were processed in one ATPE-based operation. The process design took into account the tanks and pumps and mixer-settlers required for the ATPE related operations. The Protein A-related operations assumed multiple runs in a column with a 1.2 m diameter and a 20 cm-high bed. Resin price was assumed to be 16 KUSD/L and dynamic binding capacity was assumed to be 30 g/L, with practical resin lifetime set at 100 cycles (Fig. 12.12; Table 12.8).

The total capital investment costs of 12 ± 1 MUSD were similar for the two processes. Protein A was seen as having higher overall mAb processing costs. However, today the Protein A dynamic binding capacity for 6 min residence time, and the column practical lifetime, might be doubled to 60 g/L and >200 cycles [103]. This would significantly impact overall

operating costs (reduction by 2.5 fold) and also capital investment. However, as noted elsewhere in this chapter, the use of different phase systems than PEG-Phosphate could significantly (e.g., 10X) increase the system capacity in terms of mAb solubility. This would also reduce operating and capital investment costs. Rosa et al. found that the ATPE process could be more ecologically responsible in regard to total costs, including the volumes of chromatography resin that needed to be used, but that conclusion could be affected by improvements in the performance of both modern chromatography resins, and ATPE systems. Higher waste treatment and disposal costs for the ATPE-based process were due to the high concentrations of PEG. These might be reduced by using an EOPO polymer that could be more readily recovered by thermally-induced precipitation. It should also be noted that the analysis did not account for the cost of subsequent purification steps, as the two approaches did not produce that same material. Hopefully Rosa et al. will revisit such modeling in the future.

Jan Eggersgluess et al. [103] investigated a complete ATPE-based process sequence with a variety of processing options. They then conducted an economic evaluation between ATPE and Protein A chromatography based on more recent performance specs. Part of the attraction of the ATPE-based processing was that it was potentially applicable to a wide range of targets, including those beyond monoclonal antibodies, and the minor alterations in phase system composition necessary to accommodate different targets would not be expected to significantly alter process performance or economics. The clarification potential of ATPE was not exploited, but was also of interest. The initial ATPE system was 12% PEG 400, 18% Phosphate, pH 6. Target feed was Chinese hamster ovary (CHO) cell culture.

Eggersgluess et al. investigated twelve different scenarios related to integration of ATPE into downstream processing. Several options were studied including filtration, ion exchange, or further ATPE processing by back extraction. The latter considered a novel PEG-phosphate system, instead of the common PEG-phosphate and NaCl system approach. Two different types of ATPE processes were considered. The first was a fractional extraction where extraction and wash-extraction are interconnected.

The second (Fig. 12.13) was a process based on concentration by extraction and purification by wash extraction. The latter was judged to offer several advantages, including a potential five-fold reduction in load volumes without yield reduction, and a reduction in separation stages. When the ATPE was followed by ultrafiltration and then by cation exchange, mAb purity reached 98% with 90% overall yield, approaching Protein A performance (Chapter 11, Table 11.4). As in other studies, such as those by Mao et al. [14] or by Rosa et al. [94], one of most significant limitations related to use of PEG and high salt systems is related to the target solubility in the phases, which limits capacity. Eggersgluess et al.’s use of a phase system containing lower MW PEG can reduce this to some extent with their estimate that the process could be run without problems up to 7.5 g/L, and with 10 g/L requiring only some phase volume ratio adjustment. They considered various scenarios involving varied mAb concentration (1–10 g/L) and batch size (2–12 kL) and processing times. As in the case of Rosa et al. [94] Eggergluess’s process economic evaluation results indicated some scenarios that appeared to economically favor the use of ATPE. In some cases this was true even when Protein A resins were modeled as offering 300 cycles at a DBC of 50 g/L [103].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ATPE</th>
<th>Protein A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>Preclarified</td>
<td>Preclarified</td>
</tr>
<tr>
<td>Mode of operation</td>
<td>Continuous</td>
<td>Batch</td>
</tr>
<tr>
<td>MI process (kg/kg mAb)</td>
<td>4811</td>
<td>1068</td>
</tr>
<tr>
<td>Overall yield (%)</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Final purity (P_{HPC} %)</td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Final (mAb) g/L</td>
<td>2.0</td>
<td>11.4</td>
</tr>
<tr>
<td>Final mAb solution</td>
<td>Phosphate buffer, pH 6</td>
<td>Citrate buffer, pH 3–3.6</td>
</tr>
<tr>
<td>Postprocessing</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Overall mAb cost ($US/kg)</td>
<td>8.7 million</td>
<td>14.4 million</td>
</tr>
<tr>
<td>Total capital invest. ($US)</td>
<td>11 million</td>
<td>12.7 million</td>
</tr>
</tbody>
</table>

12.3.11 Elimination of Perceived Weaknesses of ATPE

In their review of adoption of alternative separation technologies, Tran et al. [22] noted that apparent risk associated with alternative technologies “must be addressed as they are a major impediment to the adoption of alternatives.” Such work is ongoing in regard to almost all alternative separation methods. By way of example, the following table notes how, over the past few years, solutions have emerged to the ten most common perceived weaknesses of ATPE, including those related to reagent cost, capacity (solubility), and process integration. The promise of early testing of ATPE in bioprocessing (e.g., [7]) was offset by the related biopharmaceuticals not making it to market; however, today, with even stronger arguments for its testing, several companies (e.g., Biogen IDEC, Boehringer Ingelheim, Bayer Technologies, GE Healthcare) have recently published papers showing continued industrial interest in the technology. One reason for this is that previously perceived weaknesses of ATPE have now largely been overcome (Table 12.9).

12.3.12 ATPE Discussion and Conclusions

In spite of the significant effort that has gone into comparing ATPE and Protein A-based chromatography as competitive methods, they are quite complementary (see Chapter 10). The same is true in regard to processing of non-mAb or Fc-fusion proteins by other forms of chromatography. ATPE is able to effect primary clarification, and reduce HCP, DNA, and other contaminants while isolating and, in some cases, concentrating mAbs, Fabs or other targets in aqueous neutral polymer-containing phases. ATPE may provide benefit by readily moving targets from a variety of feeds into mobile phases, which can then be further processed in generic (toolbox) processes. ATPE target-containing phases can often be readily filtered and applied directly to chromatography columns. Residual neutral hydrophilic polymers (e.g., PEG) in such phases may actually improve rather than reduce chromatographic performance [113]. Protein A chromatography often results in a 98+% pure product at 95% yield with follow-on processing addressing challenges related to removing residual contaminants. Use of ATPE upstream of a process’s first column step may improve its performance, and useful lifetime, and possibly even eliminate one of the subsequent downstream unit operations. More work needs to be done in regard to investigating such possibilities. In the meantime, we expect innovation in ATPE to continue as scientists explore its use with novel feeds [60], novel polymers and affinity ligands [114], novel liquid phases, such as those formed using ionic liquids [115], and novel chromatographic phase immobilization systems based on the approach of Müller [95]. In regard to the latter, the stainless-steel bead packed-column continuous ATPE method studied by Bayer Technology Services and the Technical University of Lisbon is noteworthy [112].
As noted in Chapter 10, studies in alternative separation methods related to downstream processing of mAbs provide the most complete data set for exploring the relative merits of different techniques. Tables 10.3 and 10.4 provide a comparison of various classic and alternative separation methods, including different forms of ATPE, in regard to general strengths and weaknesses and mAb purification performance. Table 10.4 suggests many methods are capable of offering good purity and yield, with other performance factors (e.g., concentration of antibody in feed, specific contaminants) being important discriminators. Similar performance considerations will be true for other targets, feeds, and purification goals. Chapter 10 also noted significant interest of the bioprocessing community in both crystallization and ATPE. The authors hope that this chapter convinces the reader that such interest is warranted. Alternative separation methods are complementary to methods such as chromatography and filtration. ATPE may find use upstream for primary clarification, as well as target concentration, buffer exchange, plus reduction of protein, endotoxin and other contaminant loads. In addition to its possible use as a downstream polishing step, crystallization may offer some advantages in regard to biopharmaceutical storage and even delivery [1,35]. It is noteworthy that we know of few studies where ATPE, which often reduces nucleic acid and HCP contaminants and results in target protein concentrated in a PEG-rich mobile phase, was studied in combination with precipitation or crystallization [45,46]. Research continues into both of these methods, and in the future, better theoretical understanding, process modeling, high-throughput process development, as well as new polymers and other reagents, will make them easier to utilize alone or in combination. Calculating the economic costs or benefits associated with use of ATPE or crystallization is still difficult. Some benefits, such as ATPE improvement in downstream column lifetime and performance, or crystallization providing stable intermediate storage, are hard to evaluate in silico. Future use of modern process characterization methods may help with this challenge [116].

### TABLE 12.9 Solutions to the ten most common perceived weaknesses of ATPE (Marco Rito-Palomares also addressed challenges and solutions in Ref. [68])

<table>
<thead>
<tr>
<th>Perceived Weaknesses</th>
<th>Modern Solutions Discussed in This Paper</th>
</tr>
</thead>
</table>
| 1  Expensive polymers, e.g., dextran, and need for two polymers | • Systems formed with less-expensive polymers such as polyacrylic acid or maltodextrin or starch replacing dextran  
  • Polymer recycling  
  • Single responsive polymer low-salt systems                                                                  |
| 2  Polymer variation in MW, branching, purity             | • In general these have all been improved upon while reducing polymer costs per kg                         |
| 3  Insufficient physical and theoretical understanding     | • Much improved over the past two decades                                                               |
| 4  Inability to model multiphase molecular phenomena       | • Much improved over the past two decades                                                               |
| 5  Many system variables difficult to screen              | • Modern high-throughput screening methods readily adaptable to ATPS                                      |
| 6  Capacity (target solubility) >2g/L                      | • Single responsive polymer systems can offer 10X improved capacity                                       |
| 7  Compatibility with follow-on chromatography or filtration| • Lower polymer concentration in modern systems reduces target-containing phase viscosity  
  • Modern (protein A) affinity and high salt-mixed mode chromatography resins more amenable to direct loading of target containing phases |
| 8  Difficult to recycle polymers                          | • Responsive polymers such as EOPO replace PEG, and PAA polymers to replace dextran make polymer recycling much more feasible |
| 9  Lack of suitable process equipment                     | • Modern single-use equipment and products are ideal matches for L-L separation methods  
  • Stainless-steel beds for L-L separation shown, Rosa et al. ([96], see also [95]).                        |
| 10 Inability to run continuously                         | • Continuous mixer-settlers demonstrated to work with ATP systems [96,100,101]                             |
ACKNOWLEDGEMENTS, NOTICES, AND DISCLAIMERS

The authors work for, or have previously worked for, General Electric Healthcare. Bioprocessing operations in general, and alternative separation technologies in particular, enjoy active patenting and the reader is advised to undertake “due diligence” before using any particular technology for commercial purposes. JMV A wishes to thank Raquel Aires-Barros, Rubín Soares, and Anna Azevedo for discussions regarding ATPE SWOT analysis during the writing of Ref. [16]. The authors regret that significant research over the past half-century by many scientific leaders and their colleagues could not be more fully covered here. Betaferon is a trademark of Bayer, Breox is a trademark of BASF, Brij is a trademark of Croda International, MabSelect is a trademark of GE Healthcare, Pluronic is a trademark of BASF, Rituxan is a trademark of Roche Genentech, Tergitol and Ucon are trademarks of Dow Chemical.

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