Chapter X

Phase-separated Systems for Bioseparations

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Most of the time, populations of different bioobjects/materials coexist and further processes, such as analyses and use of these materials, usually require homogeneous populations. In order to have more reliable outcomes, there is a need for cost-effective, efficient, and easy-to-use separation methods. Currently, sub-groups of heterogeneous populations are separated based on their specific properties such as affinity to certain targets, size, shape, and density. There is a continuous interest on better separation methods; this chapter aims to provide A) a brief background on affinity-based bioseparation techniques with a special focus on aqueous two-phase systems, B) density-based separation of objects in phase-separated media and C) dynamic separations in phase-separated media.

1.1 AFFINITY-BASED BIOSEPARATIONS

1.1.1 Chromatography Techniques

Affinity between molecules can be described as the tendency of these molecules to bond or aggregate based on attractive interactions such as the electrostatic, hydrophobic/hydrophilic, and ligand-ligand. For

separation, affinity-based methods rely on the interaction between the target to be isolated and the system that is designed to capture that specific target. Chromatography is the most common affinity-based separation method such that the separation is based on differential partitioning or adsorption of the targets in mobile phase while they are passing through a stationary phase. The stationary phase can be organic or synthetic polymers, inorganic materials, and composites, which are packed into a column to establish a desired rate of flow, provide enough binding sites, and sustain an appropriate pore size [1]. Chromatographic methods are widely employed for separation of proteins, antibodies, and enzymes [2-3]. Usually, specific ligands or functional groups, which can form complexes with the target molecules, are immobilized on the stationary phase. Membranes are also used as stationary phase regarding the same principles with column chromatography techniques and speculated to be more effective than column chromatography due to the increased surface area arising from their porous structure [4].

In chromatography processes, for large scale purifications and separations the operating costs mostly arise from the packing of the columns and large amounts of buffers/organic solvents for downstream processing [5] . There may also exist other problems in the reusability of chromatography columns due to the potential decrease in binding sites in each cycle and possible cracking of the packed material in the column [6-8]. In addition, autoclaving for sterilization and even basic sanitization can damage the columns, and basic sanitization; nonetheless, chromatography provides high selectivity and simplicity for downstream processes in bioseparation [5].

1.1.2 Partitioning

Another widely used method for bioseparation is partitioning. Partitioning is based on the manipulation of the distribution of biomolecules in a medium composed of parts that are designed to localize only specific molecules. In the partition chromatography, there is a liquid stationary phase and a mobile phase that can be liquid (liquid-liquid chromatography) or gas (gas-liquid chromatography)[9]; the target molecules can be captured in the stationary or mobile phase. Like other chromatography techniques, antibiotics, proteins, and other

biomolecules are enriched through partition chromatography [10]. Phase-separated systems, such as water-organic solvent systems, offer two chemically distinct bins to collect materials depending on their affinity to each phase. These systems were utilized to collect polar (water phase) and non-polar (organic solvent phase) molecules such as separation of kerosene from aromatics to produce a better fuel [11]and aspartic acid from lysine [12]. Their use as bioconverters or biocatalysts was also exploited to extract ethanol, which inhibits the yeast activity, during fermentation [13]. The integrity and the biological activity of cells are, however, affected by the presence of the organic phase [14]. Due to this kind of incompatibilities, the use of water-organic solvent systems in bioseparation is limited. A biocompatible single solvent system, which can provide chemically distinct zones, would be beneficial for bioseparations based on partitioning.

1.1.3 Affinity-based Bioseparations in Aqueous Two-Phase Systems (ATPS)

ATPS are formed by mixing two immiscible solutions of water-soluble polymers (e.g., poly(ethylene glycol) (PEG)-dextran [15], PEG-poly (acrylic acid)[16]), or a polymer and a salt (e.g., PEG-phosphate [15], PEG-sodium sulfate [17]), or a polymer and a surfactant (e.g., PEG-Triton [18]) followed by phase separation. These phase-separated systems in a common solvent offer two chemically and physically different zones to collect materials based on their partitioning behavior. The partitioning process in ATPS is as follows: i) a solution containing the target species is mixed with two immiscible components of ATPS until a homogeneous solution is obtained—the target has to have more affinity for one of the phases, ii) upon phase separation, the target prefers to stay in a specific phase, and iii) the target is recovered from the system through fractionation and further cleaned/purified by other methods, if necessary (Figure 1.1).

ATPS are designed to have high water content (e.g., 80–90%) in each phase for high degree of solubility of biomolecules [19-20], and due to the low interfacial tension between the phases, the biomolecules do not be harmed even if they need to pass the interface [21]. In addition, the pH and osmolality of ATPS can be adjusted by introducing chemical

reagents such as water-soluble salts to guarantee the biological activity of biomolecules are preserved. All of these properties ensure a biocompatible environment when inert and non-toxic components are used to form ATPS [15, 20, 22]. ATPS have been extensively used for the separation of cells and cellular components [15, 19, 23-25], DNA [26-31], proteins [15, 19, 32-36], and bioparticulates [37-43].

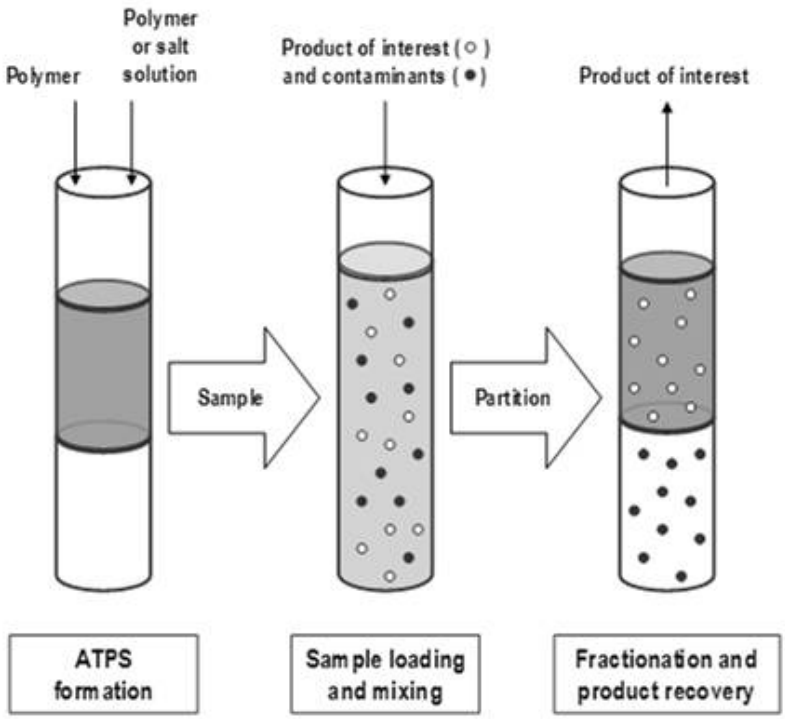

Figure 1.1. Schematic depiction of partitioning-based separation of materials in ATPS (adapted from reference [40]).

The partitioning coefficient of the bioobjects in ATPS is defined as the proportion of concentration of these objects in the top phase to the bottom phase [15]. This coefficient was found empirically to be affected by molecular weights of the polymers that are used to form these

systems and the availability and the nature of salts/surfactants [44]. The molecular weight of the polymer directly influences the phase diagram (i.e., the composition) of the system. In addition, the bioobjects have a tendency to be partitioned into the phase that is composed of polymers with lower molecular weight due to the excluded volume effect [36, 44]. Although, both polymers forming the ATPS are hydrophilic; yet, there is still a difference in their hydrophobic character—this difference is exploited for effective partitioning. The hydrophobic resolution of ATPS, which is the ability to separate molecules depending on their hydrophobic character, is, therefore, high [22]. The hydrophobic behavior of the polymers can be modified by the introduction of hydrophobic groups to the structure of the polymer. Tjerneld and Lu demonstrated that in a PEG-dextran two-phase system, tryptophan, a protein with a hydrophobic surface, partitions into the PEG-rich phase, however, when hydrophobically modified dextran is used (i.e., benzoyl and valeryl dextran), tryptophan prefers the modified dextran-rich phase [45]. Albertsson and coworkers showed an increase in the affinity of chloroplasts to the polymers when the polymers are modified with deoxycholate and palmitate—both hydrophobic groups—in a PEG-dextran system as well [46].

Surfactants can be employed to modify the hydrophobic/hydrophilic behavior of proteins; in general, the hydrophobic parts of the surfactants associate with the hydrophobic parts of the proteins exposing the hydrophilic parts of the protein to the aqueous environment [47]. In most of the cases, the surfactants (e.g., Triton, Tween, and Brij) contain PEG as the hydrophilic part rendering a "PEG'lated" protein which prefers the PEG-rich phase [47]. Covalent PEG'lation of proteins have also been shown by grafting PEG molecules to the protein structure to increase the partitioning coefficient of the protein to the PEG-rich phase [48].

Bioobjects can also be enriched in ATPS based on their electrostatic behavior (i.e., the Coulomb's Law based on the net charge on their surface). For polymer-salt ATPS, concentration of salts and introduction of other salts alter the partitioning behavior of bioobjects. In a polymer-salt system, the ionic-strength of the salt-rich phase is higher, this phase sequesters more water molecules and hydrophilic biomolecules are pushed to the interface or to the polymer-rich phase—this phenomenon is known as "salting-out" [26]. For instance, in two different ATPS, PEG-

ammonium sulfate and PEG-sodium citrate, more biomolecules, a set of enzymes, proteases that are produced by *C. perfringens*, are found in the PEG-rich phase of PEG-sodium citrate since ammonium sulfate has more ionic strength at the same percentage of salt and polymer (molecular weight of ammonium sulfate is lower than sodium citrate) [26]. (Similarly, at higher percentages of the same salt and/or at lower molecular weights of PEG, the partitioning coefficient of the enzymes increases [33].The strength of interaction between the polymer and the salt depends also on the type of ions that the salt introduces; for example, compared to sodium, lithium interacts more strongly with PEG and offers a higher positive charge density on the polymer chain (i.e., PEG becomes a pseudo-polycation); in turn, carmine dye, due to its negative surface charge, partitions more into the PEG-rich phase [49]. For purification of proteins, their isoelectronic points should also be taken into account, since the sign of their charge changes at a specific pH value (e.g.,separation of green fluorescent protein from *Escherichia coli [*35]). In some cases, salts can be used to suppress the effects the electrostatic behavior of molecules; for instance, normally in a PEG-sodium polyacrylate system, plasmid DNA is expected to partition into the PEG-rich phase since DNA and poly(acrylate) have similar net surface charge; however, at higher concentrations of salt, due to the salting-out effect arising from the hydrophilic surface of DNA, it can partition into the poly(acrylate)-rich phase [29]. Another effect observed due to the increase in the salt concentration is the change in the hydrophobic behavior of polymers; PEG becomes more hydrophobic at higher concentrations of salt due to the suppression of electrostatic forces and start interacting strongly with the non-polar regions of the target protein—hydrophobic forces become more pronounced [50].

To modify the partitioning behavior, it is also possible to add affinity ligands to the ATPS—this type of process is generally referred as affinity-enhanced partitioning. PEG is commonly derivatized to include a ligand which can behave like the target molecule. Gavanese and Gaikar showed the enhanced extraction of *penicillin acylase* in the presence of PEG-benzoate and PEG-phenylacetamide derivatives in a PEG-salt two-phase system [51].

There also exist other novel approaches to tune the partitioning behavior of target population. For the recovery of recombinant proteins, Viede, Nilsson, and coworkers introduced partitioning peptide sequences to

their protein of interest and observed a dramatic increase in the partitioning coefficient of this protein in a PEG-potassium phosphate two-phase system [52]. Flanagan and Barondes showed that proteins experience higher partitioning coefficients if they have specific receptor sites for the polymers comprising the two-phase systems [53].
In terms of cost, the price of materials can be significantly lowered, compared to chromatography, when bulk quantities are purchased. The partitioning in ATPS is also scalable and can be used to separate high volume solutions. There are also other studies to develop a more sustainable process such as the use recyclable salts (e.g., sodium citrate) [54] and replacing some polymers with cheaper alternatives (e.g., exchanging dextran with starch) [55]. Finally, compared to affinity chromatography in which the target molecules bind to the receptor on a solid surface, the partitioning in ATPS takes place in a three-dimensional medium (i.e., a volume) that can potentially offer more sites for binding/affinity.

1.1.4 The Use of ATPS in Microfluidics and Droplet Formation

The use of ATPS in the separation and selection of cells or biomaterials from heterogeneous mixtures in microfluidic channels holds a promise for a sensitive and continuous separation process [56]. In addition, since the direction of flow in a microfluidic system is perpendicular to gravity, possible effects of gravity in partitioning behavior of cells in ATPS [24]. PEG and dextran two-phase systems are the most commonly used ATPS in microfludics. Usually, following phase-separation, the two immiscible phases are separated from each other and fed into a microfluidic system along with the suspension/solution to be enriched; the species to be enriched can also be introduced into the system in one of the phases. The low interfacial tension between the phases prevents the droplet formation and ensures a stable flow.
Partitioning in microfluidics systems is the miniaturized version of macroscale purifications in ATPS and especially useful when there are only limited quantities of target material. Singh and coworkers introduced hydrophobic partitioning tags to their target protein that significantly favors the PEG-rich phase in a PEG-salt system and managed to purify this protein from sub-microliters of cell lysate [57].

Animal cells are also enriched in microfluidic devices through ATPS; Chang and coworkers demonstrated the continuous extraction of chinese hamster ovary cells to the PEG-rich phase in a PEG-dextran system by increasing the hydrophobicity of these cells in weak acidic conditions [58]. SooHoo and Walker showed the concentration of leukocyctes from the whole blood by exploiting the differences in surface properties of leukocyctes and erytrocyctes [59]. Seki and coworkers underlined the advantages of using microfludic systems by demonstrating the enrichment of large cell aggregates in a PEG-dextran two-phase system in the presence of lithium sulfate—the macroscale partitioning of these cells in ATPS was not successful due to their size such that the cells ended up at the interface due to their density [24].

Although, the stability of flow in ATPS is useful for partitioning, the creation and manipulation of droplets in microfluidic channels for sorting, mass transfer, and extraction of biomolecules are also desirable [60]. To create droplets, two separated-phases are injected into microfluidic channels individually, one to act as dispersed and another to form a continuous phase. Due to the low interfacial tension between the phases, however, mechanical perturbation (e.g., pieozoelectric actuation [61] or mechanical vibration [62]) is necessary to form the droplets.

Further manipulation of the droplets, such as polymerization to fabricate microshperes [61] and inducing interfacial precipitation or gelation to improve encapsulation efficiency [62], was also shown. In droplet microfluidics, monodispersity is a sought property for quantitative analysis of biomolecules; Shum and Song employed monodisperse PEG droplets as templates to obtain PEG-dextran core-shell particles with a polydispersity less than 4% [63]. Above a critical flow rate, it is also possible to obtain/manipulate corrugated patterns of flow by tuning the frequency of perturbations and the flow rates of two phases [64].

1.2 DENSITY-BASED SEPARATION OF OBJECTS IN PHASE SEPARATED MEDIA

Until now, ATPS have only been used for the separation of bioobjects/molecules based on their partitioning behavior, in this section, we will discuss an alternative use of these systems for separations.

The phases of ATPS order according to their density; therefore, in addition to offering zones with different chemical properties, they provide two zones with different densities. The interface between these two zones can act as a filter to capture objects based-on their density—this interface is, in fact, a step-in density. Overall, an aqueous two-phase system provides three distinct steps-in-density: i) the interface between two phases, ii) the interface at the liquid/air boundary, and iii) the interface at the liquid/container boundary.

Density is frequently used to divide materials into homogeneous groups—the applications vary from classification of cells [65-67] to sorting different crystal structures [68]. A two-phase system can be used to separate three sub-populations from each other; however, greater number of sub-populations requires more interfaces. Current method to separate multiple number of sub-populations based on their density is to sort them in a layered media, in which each layer is intended to provide a zone with different density. This type of media, density gradient systems (DGS), is prepared by careful overlayering the solutions of a single chemical (e.g., Ficoll or sucrose) at different concentrations (i.e., different densities) in a common solvent. The boundary between these layers with different densities is intended to provide a step-in-density to classify objects. A variety of biomolecules such as liposomes of different densities [69], cellular components [70-71], and the contents of seminal fluid [72], have been sorted using DGS. However, since the zones in these gradient systems are not at thermodynamic equilibrium with each other, the density profile offered by this type of medium changes over time due to diffusion—they cannot sustain molecularly sharp steps-in-density.

Phase-separated systems with more than two-phases (multiphase systems, MuPSs) in a common solvent will offer additional interfaces to be utilized in the separation of multiple sub-populations allowing the collection of more than three populations with different densities. Conceptually, this kind of systems can be treated as stable stacks of liquid membranes for density-based separation. In the Whitesides group at Harvard University, we have observed that combinations of some water-soluble polymers and surfactants, settling under gravity or through centrifugation, form multiphase systems (MuPSs). Although, Albertsson suggested the idea—sets of immiscible phases can form higher order systems—several years ago, until our work, there was only

one three-phase system and one four-phase system [14]. In our work, we listed 112 three-phase systems, 73 four-phase systems, 31 five-phase systems and 3 six-phase systems [73].

We have demonstrated the density-based separations in these systems by separating density standard beads (Figure 1.2) and different formulations of Nylon. In MuPSs, the difference in densities between adjacent phases can be very small ($\Delta\rho$ ~ 0.001 g/cm^3) and the steps-in-density can be tuned by the addition of water-soluble salts and water-miscible solvents.

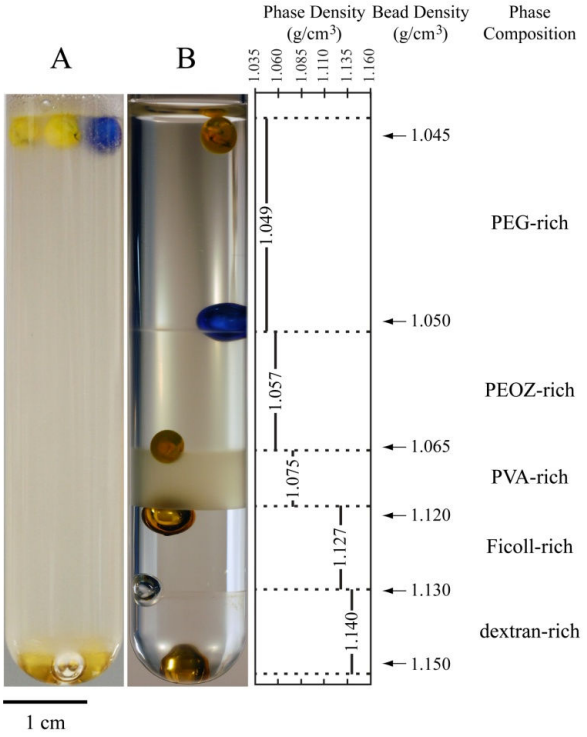

Figure 1.2. Preparation of MuPSs and the use of MuPSs in density-based separation. A) A five-phase system is prepared by mixing the aqueous solutions of PEG, poly (2-etyhyl 2-oxazoline) (PEOZ), poly(vinyl alcohol) (PVA), Ficoll, and dextran. The system can settle under gravity or centrifugation. B) Upon phase-separation under centrifugation, each density standard bead moves to the relevant interface (reprinted from reference [73]).

For density-based separation, compared to layered but miscible media, MuPSs offer five advantages: (i) the steps-in-density of MuPSs are thermodynamically stable; (ii) the collection of samples after the separation is convenient due to the sharp interfaces; (iii) the viscosities of each phase can be decoupled from density (i.e., at a given density, it is possible to access a range of viscosities by changing the components of MuPSs); (iv) the MuPSs can be prepared in advance of use and stored; and (v) the MuPSs reform readily by centrifugation or gravity, if perturbed or even mixed. These stable stacks of steps-in-density offer the ability to isolate and collect target species with high resolution and will potentially be useful for the separation of biological materials.

1.3 DYNAMIC SEPARATIONS OF OBJECTS IN PHASE-SEPARATED MEDIA

Rate-zonal centrifugation exploits the differences between hydrodynamic behaviors of objects for separation. This method is useful for classifying objects that are denser than the centrifugation medium and frequently used for the separation of nanoparticles. Currently, DGS, which are described in the previous section, are utilized as separation media to facilitate the recovery of the separated products since the multiple zones in DGS can potentially limit sub-groups of nanoparticles to narrow regions [68, 74-77].

However, similar kind of problems due to the instability of these systems occurs in rate-zonal centrifugations—these systems are vulnerable against the mechanical disruption during centrifugation and collecting the enriched products, due to the absence of a clear interface, is challenging. As listed above, compared to DGS, MuPSs provide stability, storability and ease-of-preparation/collection. One additional advantage is the superior tenability of MuPSs—in DGS, the viscosity of layers correlates with density; thus, viscosity and density of a layer cannot be decoupled from each other. MuPSs offer access to a range of viscosities at the same density since different polymers can be combined to obtain a specific density as well as additives can be used to alter the physical properties of MuPSs.

In Whitesides group, we have also utilized MuPSs for the purification of the products of a gold nanorod synthesis to exemplify the potential use of phase-separated systems in rate-zonal centrifugation [78]. In a three-phase system composed of PEOZ, Ficoll and Brij 35 (a non-ionic PEG-based surfactant, we have enriched the target population, gold nanorods, from 48% to 99% with a benchtop centrifuge in 10 minutes (Figure 1.3). One thing to consider while separating inorganic or metallic particles in MuPSs is choosing the components of MuPSs from the set of polymers/surfactants that won't interact with the surface coatings of these particles. In our work, for instance, to enrich cetyl trimethylammonium bromide-stabilized gold nanorods, we eliminated the ionic and charged polymers and surfactants from consideration since they caused gelation or precipitation of these nanorods.

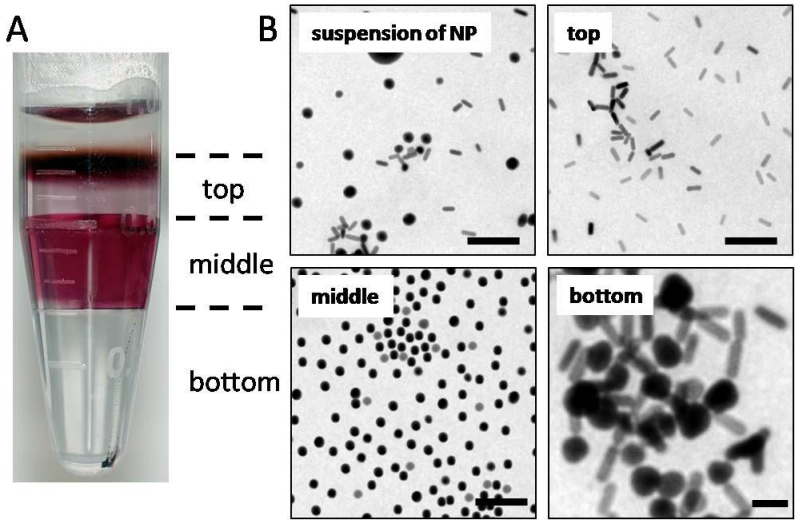

Figure 1.3. The separation of gold nanorod in a three-phase system through centrifugation. A) An image of an aqueous three-phase system composed of PEOZ, Ficoll and Brij 35after separating the reaction products of a gold nanoparticle synthesis for 8 min at 16 000g. B) Transmisson electron microscopy images of suspension of nanoparticles (suspension of NP) and samples collected from the layers as shown in (A). The scale bar in each image corresponds to 200 nm (adapted from reference [78]).

We expect this new centrifugation medium, MuPSs, to be used in dynamic separations (e.g., rate-zonal centrifugation) more conveniently than traditional DGS.

References

1. Jungbauer, A. (2005). Chromatographic media for bioseparation, *J. Chromatogr. A*, **5**, pp. 1233–1239.
2. Poole, C. F. (2003) The Essence of Chromatography, 1st Ed. (Elsevier Science, The Netherlands).
3. Janson, J. C. (2011) Protein Purification, Principles, High Resolution Methods, and Applications, 3rd Ed. (John Wiley &Sons, Inc. , USA).
4. Zeng, X.,Ruckenstein, E. (1999). Membrane chromatography: preparation and applications to protein separation, Biotechnol. Prog., 15, pp. 1003–1019.
5. Gottschalk, U. (2008). Bioseparation in antibody manufacturing: the good, the bad and the ugly, Biotechnol. Prog., 24, pp. 496–503.
6. Azevedo, A. M.,Rosa, P. A. J.,Ferreira, I. F.,Aires-Barros, M. R. (2009). Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing, Trends Biotechnol., 27, pp. 240–247.
7. Thömmes, J.,Etzel, M. (2007). Alternatives to chromatographic separations, Biotechnol. Prog., 23, pp. 42–45.
8. Ghosh, R. (2002). Protein separation using membrane chromatography: opportunities and challenges, J. Chromatogr. A, 952, pp. 13–27.
9. Ahuja, S. (2003) Chromatography and Separation Science, 1st. Ed. (Academic Press, USA).
10. Hatti-Kaul, R. (2000) Aqueous Two-Phase Systems: Methods and Protocols, (Humana Press, New Jersey).
11. Rydberg, J.,Cox, M.,Musikas, C.,Choppin, G. R. (2004) Solvent Extraction Principles and Practive, 2nd Ed. (Taylor & Francis Group, USA).
12. Schügerl, K. (1994) Solvent Extraction in Biotechnology: recovery of primary and secondary metabolites, (Springer-Verlag, Berlin).
13. Wang, H. Y.,Robinson, F. M.,Lee, S. S. (1981). Enhanced alcohol production through on-line extraction, Bitech. Bioeng. Symp., 11, pp. 555–565.
14. Albertsson, P. A. (1986) Partition of Cell Particles and Macromolecules, 3rd Ed. (John Wiley & Sons, New York).
15. Albertsson, P. A. (1958). Particle fractionation in liquid two phase systems, the composition of some phase systems and the behavior of some model

particles in them application to the isolation of cell walls from microorganisms, Biochim. Biophys. Acta 27, pp. 378–395.
16. Saravanan, S.,Rao, J. R.,Nair, B. U.,Ramasami, T. (2008). Aqueous two-phase poly(ethylene glycol)- poly(acrylic acid) system for protein partitioning: influence of molecular weight, pH and temperature, Process Biochem., 43, pp. 905–911.
17. Ananthapadmanabhan, K. P.,Goddard, E. D. (1987). Aqueous biphase formation in polyethylene oxide-inorganic salt systems, Langmuir, 3, pp. 25–31.
18. Sojo, M. M.,Nunez-Delicado, E.,Garcia-Carmona, F.,Sanchez-Ferrer, A. (1998). Partial purification of a banana polyphenol oxidase using Triton x-114 and PEG 8000 for removal of polyphenols, J. Agric. Food Chem., 46, pp. 4924–4930.
19. Albertsson, P. A. (1973). Application of the phase partition method to a hydrophobic membrane protein, phospholipase A1 from Escherichia coli, Biochemistry-US, 12, pp. 2525–2530.
20. Abbott, N. L.,Blankschtein, D.,Hatton, T. A. (1991). Protein partitioning in two-phase aqueous polymer systems. 1. Novel physical pictures and a scaling-thermodynamic formulation, Macromolecules, 24, pp. 4334–4348.
21. Albertsson, P. A. (1978). Partition between polymer phases, *J. Chromatogr.*, **159**, pp. 111–122.
22. Asenjo, J. A.,Andrews, B. A. (2011). Aqueous two-phase systems for protein separation: a perspective, *J. Chromatogr. A.*, **1218**, pp. 8826–8835.
23. Albertsson, P. A. (1956). Chromatography and partition of cells and cell fragments, *Nature*, **177**, pp. 771–774.
24. Yamada, M.,Kasim, V.,Nakashima, M.,Edahiro, J.,Seki, M. (2004). Continuous cell partitioning using an aqueous two-phase flow system in microfluidic devices, *Biotechnol. Bioeng.*, **88**, pp. 489–494.
25. Edahiro, J.,Yamada, M.,Seike, S.,Kakigi, Y.,Miyanaga, K.,Nakamura, M.,Kanamori, T.,Seki, M. (2005). Separation of cultured strawberry cells producing anthocyanins in aqueous two-phase system, *J. Biosci. Bioeng.*, **100**, pp. 449–454.
26. Gomes, G. A.,Azevedo, A. M.,Aires-Barros, M. R.,Prazeres, D. M. F. (2009). Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate, *Sep. Purif. Technol.*, **65**, pp. 22–30.
27. Hatti-Kaul, R. (2001). Aqueous two phase systems, *Mol. Biotechnol.*, **19**, pp. 269–277.
28. Luechau, F.,Ling, T. C.,Lyddiatt, A. (2009). Partition of plasmid DNA in polymer-salt aqueous two-phase systems, *Sep. Purif. Technol.*, **66**, pp. 397–404.

29. Johansson, H.,Matos, T.,Luz, J. S.,Feitosa, E.,Oliveira, C. C.,Pessoa Jr., A.,Bülow, L.,Tjerneld, F. (2012). Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems, *J. Chromatogr. A*, **1233**, pp.
30. Ribeiro, S. C.,Monteiro, G. A.,Martinho, G.,Cabral, J. M. S.,Prazeres, D. M. F. (2000). Quantitation of plasmid DNA in aqueous two-phase systems by fluorescence analysis, *Biotechnol. Lett.*, **22**, pp. 1101–1104.
31. Rahimpour, F.,Feyzi, F.,Maghsoudi, S.,Hatti-Kaul, R. (2006). Purification of plasmid DNA with polymer-salt aqueous two-phase system:optimization using response surface methodology, *Biotechnol. Bioeng.*, **95**, pp. 627–637.
32. da Silva, M. A. O.,Arruda, M. A. Z. (2009). An aqueous two-phase system as a strategy for serum albumin depletion, *Talanta*, **77**, pp. 985–990.
33. Porto, T. S.,Medeiros e Silva, G. M.,Porto, C. S.,Cavalcanti, M. T. H.,Neto, B. B.,Lima-Filho, J. L.,Converti, A.,Porto, A. L. F.,Pessoa Jr., A. (2008). Liquid-liquid extraction of proteases from fermented broth by PEG/citrate aqueous two-phase system, *Chem. Eng. Process.*, **47**, pp. 716–721.
34. Linder, M. B.,Qiao, M.,Laumen, F.,Selber, K.,Hyytia, T.,Nakari-Setala, T.,Penttila, M. E. (2004). Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems, *Biochemistry*, **43**, pp. 11873–11882.
35. Johansson, H.,Ishii, M.,Minaguti, M.,Feitosa, E.,Vessoni Penna, T. C.,Pessoa Jr., A. (2008). Separation and partitioning of green fluorescent protein from Escherichia coli homogenate in poly(ethylene glycol)/ sodium-poly(acrylate) aqueous two-phase systems, Sep. Purif. Technol., 62, pp. 166–174.
36. Albertsson, P. A.,Cajarville, A.,Brooks, D. E.,Tjerneld, F. (1987). Partition of proteins in aqueous two-phase systems and the effect of molecular weight of the polymer, Biochim. Biophys. Acta, 926, pp. 87–93.
37. Albertsson, P. A.,Frick, G. (1960). Partition of virus particles in a liquid two-phase system, Biochim. Biophys. Acta 37, pp. 230–237.
38. Walker, S. G.,Lyddiatt, A. (1999). Processing of nanoparticle bioproducts: application and optimisation of aqueous two-phase systems, J. Chem. Technol. Biotechnol., 74, pp. 250–255.
39. Benavides, J.,Mena, J. A.,Cisneros-Ruiz, M.,Ramirez, O. T.,Palomares, L. A.,Rito-Palomares, M. (2006). Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems, J. Chromatogr. B., 842, pp. 48–57.
40. Benavides, J.,Aguilar, O.,Lapizco-Encinas, B. H.,Rito-Palomares, M. (2008). Extraction and purification of bioproducts and nanoparticles using aqueous two-phase systems strategies, Chem. Eng. Technol., 31, pp. 838–845.
41. Helfrich, M. R.,El-Kouudi, M.,Etherton, M. R.,Keating, C. D. (2005). Partitioning and assembly of metal particles and their bioconjugates in aqueous two-phase systems, Langmuir, 21, pp. 8478–8486.

42. Negrete, A.,Ling, T. C.,Lyddiatt, A. (2007). Aqueous two-phase recovery of bio-nanoparticles: a miniaturization study for the recovery of bacteriophage T4, J. Chromatogr. B, 854, pp. 13–19.
43. Long, M. S.,Keating, C. D. (2006). Nanoparticle conjugation increases protein partitioning in aqueous two-phase systems, Anal. Chem., 78, pp. 379–386.
44. Baskir, J. N.,Hatton, T. A.,Ulrich, W. S. (1989). Protein partitioning in two-phase aqueous polymer systems, Biotechnol. Bioeng., 34, pp. 541–558.
45. Lu, M.,Tjerneld, F. (1997). Interaction between tryptophan residues and hydrophobically modified dextran effect on partitioning of peptides and proteins in aqueous two-phase systems, J. Chromatogr. A, 766, pp. 99–108.
46. Westrin, H.,Albertsson, P. A.,Johansson, G. (1976). Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems, Biochim. Biophys. Acta., 436, pp. 696–706.
47. Albertsson, P. A.,Andersson, B. (1981). Separation of membrane components by partition in detergent-containing polymer phase systems: isolation of the light harvesting chlorophyll al b protein, J. Chromatogr. A, 215, pp. 131–141.
48. Karr, L. J.,Shafer, S. G.,Harris, J. M. (1986). Immuno-affinity partition of cells in aqueous polymer two-phase systems, J. Chromatogr. A, 354, pp. 269–282.
49. Mageste, A. B.,de Lemos, L. R.,Ferreira, G. M. D.,da Silva, M. C. H.,da Silva, L. H. M.,Bonomo, R. C. F.,Minim, L. A. (2009). Aqueous two-phase systems: an efficient, environmentally safe and economically viable method for purification of natural dye carmine, J. Chromatogr. A, 1216, pp. 7623–7629.
50. Gunduz, U.,Korkmaz, K. (2000). Bovine serum albumin partitioning in an aqueous two-phase system: effect of pH and sodium chloride concentration, J. Chromatogr. B, 743, pp. 255–258.
51. Gavasane, M. R.,Gaikar, V. G. (2003). Aqueous two-phase affinity partitioning of penicillin acylase from E. coli in presence of PEG-derivatives, Enzyme Microb. Tech., 32, pp. 665–675.
52. Köhler, K.,Ljungquist, C.,Kondo, A.,Veide, A.,Nilsson, B. (1991). Engineering proteins to enhance their partition coefficients in aqueous two-phase systems, Nat. Biotechnol., 9, pp. 642–646.
53. Flanagan, S. D.,Barondes, S. H. (1975). A method for purification of proteins using specific polymer-ligands in aqueous polymer two-phase systems, J. Biol. Chem., 250, pp. 1484–1489.
54. Kula, M. R. (1990). Trends and future prospoects of aqueous two-phase extraction, Bioseparation, 1, pp. 181–189.
55. Venancio, A.,Teixeira, J. A. (1993). Evaluation of crude hydroxypropyl starch as a bioseparation aqueous-phase-forming polymer, Biotechnol. Prog., 9, pp. 635–639.
56. Hardt, S.,Hahn, T. (2012). Microfluidics with aqueous two-phase systems, Lab Chip, 12, pp. 434–442.

57. Meagher, R. J.,Light, Y. K.,Singh, A. K. (2008). Rapid, continuous purification of proteins in a microfluidic device using genetically-engineered partition tags, Lab Chip, 8, pp. 501–632.
58. Nam, K.,Chang, W.,Hong, H.,Lim, S.,Kim, D.,Koo, Y. (2005). Continuous-flow fractionation of animal cells in microfluidic device using aqueous two-phase extraction, Biomed. Microdevices, 7, pp. 189–195.
59. SooHoo, J. R.,Walker, G. M. (2009). Microfluidic aqueous two phase system for leukocyte concentration from whole blood, Biomed Microdevices, pp. 323-329.
60. Lopez, M.,Graham, M. D. (2008). Enhancement of mixing and adsorption in microfluidic devices by shear-induced diffusion and topography-induced secondary flow, Phys. Fluids., 20, pp.
61. Ziemecka, I.,van Steijn, V.,Koper, G. J. M.,Rosso, M.,Brizard, A. M.,van Esch, J. H.,Kreutzer, M. T. (2011). Monodisperse hydrogel microspheres by forced droplet formation in aqueous two-phase systems, Lab Chip, 11, pp. 620–624.
62. Shum, H. C.,Varnell, J.,Weitz, D. A. (2012). Microfluidic fabrication of water-in-water (w/w) jets and emulsions, Biomicrofluidics, 6, pp. 012808.
63. Song, Y.,Shum, H. C. (2012). Monodisperse w/w/w double emulsion induced by phase separation, Langmuir, 28, pp. 12054–12059.
64. Sauret, A.,Spandagos, C.,Shum, H. C. (2012). Fluctuation-induced dynamics of multiphase liquid jets with ultra-low interfacial tension, Lab Chip, 12, pp. 3380–3386.
65. English, D.,Andersen, B. R. (1974). Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque, J. Clin. Immunol., 5, pp. 249–252.
66. Zucker, R. M.,Cassen, B. (1969). The separation of normal human leukocytes by density and classification by size, Blood, 34, pp. 591–600.
67. Pösel, C.,Möller, K.,Fröhlich, W.,Scholz, I.,Boltze, J.,Wagner, D. C. (2012). Density gradient centrifugation compromises bone marrow mononuclear cell yield, Plos One, 7 pp. 1–10.
68. Xiong, B.,Cheng, J.,Qiao, Y.,Zhou, R.,He, Y.,Yeung, E. (2011). Separation of nanorods by density gradient centrifugation, J. Chromatogr. A, 1218, pp. 3823–3829.
69. Dipali, S. R.,Kulkarni, S. B.,Betageri, G. V. (1996). Comparative study of separation of non-encapsulated drug from unilamellar liposomes by various methods, J .Pharm. Pharmacol., 48, pp. 1112–1115.
70. Gilbert, J. C.,Wyllie, M. G. (1976). Effects of anticonvulsant and convulsant drugs on the atpase activities of synaptosomes and their components, Br. J. Pharmac., 56, pp. 49–57.

71. Schröder, M.,Schafer, R.,Friedl, P. (1997). Spectrophotometric determination of iodixanol in subcellular fractions of mammalian cells, Anal. Biochem., 244, pp. 174–176.
72. Brahem, S.,Mehdi, M.,Elghezal, H.,Saad, A. (2009). Semen processing by density gradient centrifugation is useful in selecting sperm with higher double-strand DNA integrity, Andrologia, 43, pp. 196–202.
73. Mace, C. R.,Akbulut, O.,Kumar, A. A.,Shapiro, N. D.,Derda, R.,Patton, M. R.,Whitesides, G. M. (2012). Aqueous multiphase systems of polymers and surfactants provide self-assembling step-gradients in density, J. Am. Chem. Soc., 134, pp.
74. Bai, L.,Ma, X.,Liu, J.,Sun, X.,Zhao, D.,Evans, D. G. (2010). Rapid separation and purification of nanoparticles in organic density gradients, J. Am. Chem. Soc., 132, pp. 2333–2337.
75. Chen, G.,Wang, Y.,Tan, L. H.,Yang, M.,Tan, L. S.,Chen, Y.,Chen, H. (2009). High-purity separation of gold nanoparticle dimers and trimers, J. Am. Chem. Soc., 131, pp. 4218–4219.
76. Sun, X.,Tabakman, S. M.,Seo, W.,Zhang, L.,Zhang, G.,Sherlock, S.,Bai, L.,Dai, H. (2009). Separation of nanoparticles in a density gradient: FeCo@C and gold nanocrystals, Angew. Chem. Int. Ed., 48, pp. 939–942.
77. Qiu, P.,Mao, C. (2011). Viscosity gradient as a novel mechanism for the centrifugation-based separation of nanoparticles, *Adv. Mater.*, **2011**, pp. 4880–4885.
78. Akbulut, O.,Mace, C. R.,Martinez, R. V.,Kumar, A. A.,Nie, Z.,Patton, M. R.,Whitesides, G. M. (2012). Separation of nanoparticles in aqueous multiphase systems through centrifugation, *Nano Lett.*, **12**, pp. 4060–4064.