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Advances in aqueous biphasic systems for biotechnology applications

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Amongst the several processes investigated in the Biotechnology field, aqueous biphasic systems (ABSs) have been a target of relevant research, particularly in the downstream processing of high-value compounds of biological nature and, more recently, in emerging applications such as in the development of artificial cells and synthetic biology, micropatterning and 3D printing, and sample pretreatment and diagnosis. This review overviews and discusses the recent progress achieved in downstream processes using ABSs, namely by the development of integrated and continuous processes, and in emerging applications where ABSs seem to have strong potential, namely in cellular micropatterning and 3D printing, and in the pretreatment of biological samples to improve diagnosis. Advantages and bottlenecks of ABSs in all these applications are provided and discussed.

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Aqueous biphasic systems

Solvent-based systems to be applied in liquid–liquid extractions from water-rich biological media, particularly within the biotechnology field, are a relevant topic of research. To overcome sustainability concerns of some volatile organic solvents, new and more benign solvents and liquid–liquid systems have been proposed [1–4]. Among these, aqueous biphasic systems (ABSs) have received significant attention in the past decades because they can replace volatile organic solvents in liquid–liquid extractions. ABSs are at least ternary systems composed of water and two mostly nonvolatile compounds, such as polymers, salts, ionic liquids (ILs), among others. When pairs of these compounds are mixed at certain concentrations in water, two aqueous phases are formed, each one enriched in water and in one of the other two phase-forming components. Because ABSs are mainly composed of water, they may be tailored to display biocompatible features to be applied within the biotechnology field. Owing to these benefits, ABSs have been largely investigated in the downstream processing of high-value compounds, for example, biopharmaceuticals. The advantages [5] and disadvantages [6,7] of ABSs compared with conventional separation methods are presented in Fig. 1.

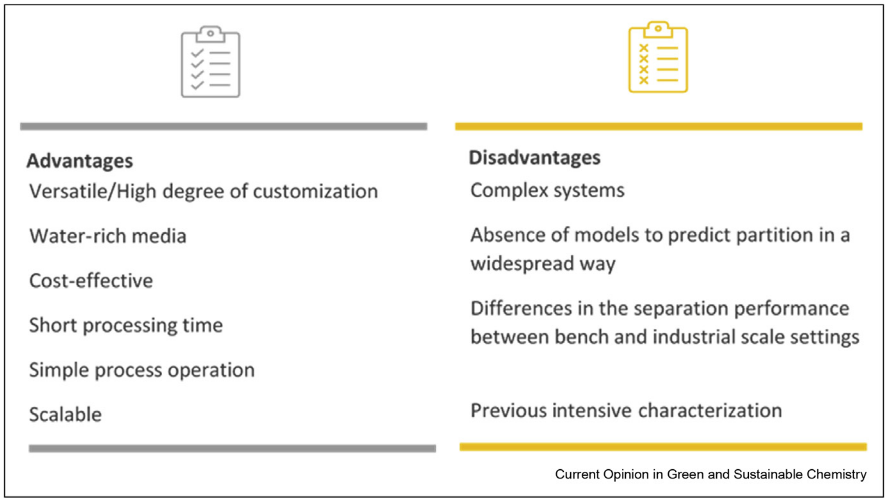
The most relevant recent examples on the application of ABSs in downstream processing are given in the following paragraphs, divided according to two topics required to achieve their industrial scale and widespread application: continuous processes and integrated processes. Despite the intensive investigation of ABSs in downstream processing, other recent applications of ABSs in Biotechnology with high potential to be a niche will be presented and discussed, particularly in what concerns their application in micropatterning, 3D printing, and biological sample pretreatment to improve diagnosis.

Biotechnology applications of ABSs

Downstream processing

Within the biotechnology field, ABSs have been investigated in the downstream processing of simple molecules such as amino acids, peptides and biobased dyes, to complex products such as proteins, enzymes, viruses, cells and nucleic acids [5,8]. Although encouraging results have been demonstrated in terms of purification performance, their application at industrial levels is far from being a reality. The main reason for this trend is that most biological products already have their production process well established; therefore, the introduction of an ABS to replace a given operation unit will lead to the need of adopting new infrastructures. Therefore, ABSs have higher potential to be integrated in not yet defined bioprocesses, such as in the purification of new developed molecules and bioproducts or in the purification of products that are still not purified by a cost-effective process. Whatever the case is, the purification and recovery of biomolecules in continuous mode and/or by applying integrated bioprocesses is essential to design competitive and more sustainable downstream processes than those used nowadays.

Figure 1



Advantages and disadvantages of ABSs when applied in downstream processing. ABSs, aqueous biphasic systems.

Integrated bioprocesses

The integration of several downstream processing steps can be achieved using ABSs, for example, combining clarification with prepurification, clarification with capture, capture with polishing, among others. Furthermore, upstream and downstream steps can be integrated as well [6,9,10]. Overall, from the production to polishing steps, ABSs can be designed to reduce the process steps while increasing the purity of the target bioproduct. Examples of integrated bioprocesses investigated in the past 2 years include extractive fermentation [11,12], extractive bioconversion [13–16], cell disruption and extraction [12,17,18], clarification or primary recovery and purification [19], and extraction with high-resolution recovery (i.e. chromatography) [20–22]. Fig. 2 shows a summary of the main advances reached in the integration of bioprocesses using ABSs.

In extractive fermentation/bioconversion, cells or enzymes are "immobilized" in one of the ABS phases, while the target compound being produced is continuously partitioned to the opposite phase, combining thus production and primary purification steps. In this field, ABSs composed of polyethylene glycol (PEG) and dextran 500 were used to produce pullulan by *Aureobasidium pullulans* and simultaneously separate this product from the fermentation broth [11]. The pullulan migrated to the dextran-rich phase with a 95.2% recovery, while the biomass was concentrated at the PEG-rich phase [11]. Within extractive bioconversion, Meyer et al. [13] applied a thermoreversible ABS composed of imidazolium-based ILs and sodium phosphate to carry out a biocatalytic reaction by lipase. The enzyme was enriched and reused 6 times in the salt-rich phase, while the reaction product (1-phenylethanol, an important chiral building block) was recovered at the IL-rich phase [14].

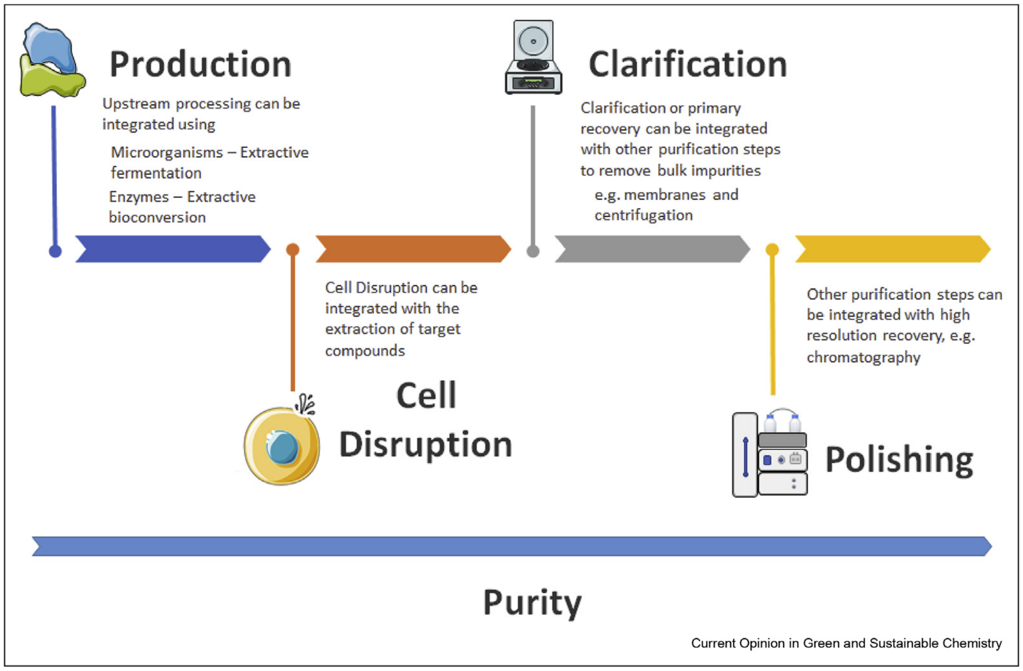
ABSs also demonstrated to allow the integration of cells disruption and recovery of the target intracellular bioproduct. Leong et al. [12] applied ABSs composed of thermoseparating polymers (copolymers of ethylene oxide–propylene oxide) for the purification of poly(R)-3-hydroxybutyrate from *Cupriavidus necator*. After ultrasonic cells disruption, the temperature was increased to create a two-phase system, in which one of the phases contains poly(R)-3-hydroxybutyrate with a yield of 97.6% and a purification factor of 1.36 [12].

Downstream processing is a time-consuming multistage process, particularly when considering the production of biopharmaceuticals such as monoclonal antibodies (mAbs). To overcome this drawback, Kruse et al. [19,22] integrated the clarification and purification steps with ABSs composed of PEG 400 and phosphate buffer, achieving a recovery of 78% of mAbs [19]. The same group of researchers then proposed ABSs to integrate clarification, capture and purification of mAbs, preceeded by high-resolution steps, namely diafiltration and ion-exchange chromatography. In ABS-based processes, the yield of mAbs was 97%, however decreasing to 74% when considering the entire downstream process [22].

Continuous processes

For a broader implementation of ABSs in a large scale, it is mandatory to operate in a continuous mode. This mode has inherent economic advantages and will allow the technology scale-up [23]. The investigation made on ABSs applied as continuous processes was recently reviewed [10], and includes the use of microfluidics, counter-

Figure 2



Integrated bioprocesses with ABSs. ABSs, aqueous biphasic systems.

current chromatography (CCC) and centrifugal partition chromatography (CPC) [10]. Fig. 3 depicts illustrative examples of microfluidics and CCC/CPC settings and summarizes the advantages of each type of approach.

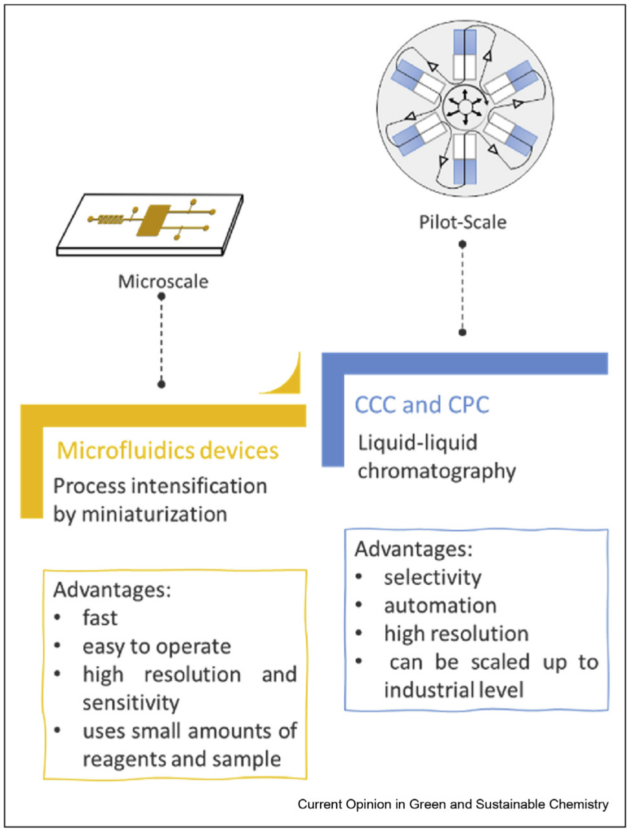
Microfluidics devices are based on a continuous liquid flow through narrow channels, operating at a micro scale [9,10]. These systems allow the process intensification [24]; however, they are particularly relevant to perform the characterization of a given solvent system [10]. This technology uses small amounts of reagents and samples, is fast, easy to operate and provides high resolution and sensitivity [9,10]. The use of ABSs in microfluidics in the continuous separation of biomacromolecules was recently reviewed by Vicente et al. [24]. Amongst recent achievements, Miličević et al. [25] applied ABSs constituted by potassium hydrogen phosphate and different deep eutectic solvents, formed by cholinium chloride and carbohydrates or polyols, in microfluidics to extract polyphenolic compounds from aqueous extracts of lemon balm and mint. With the best ABS, the authors reached an extraction of 94% of polyphenolic compounds in 3.65 s. With a different perspective, Vobecká et al. [16] studied the continuous enzymatic production of cephalexin, the product extraction and enzyme recycling in continuous-flow microfluidics, using ABSs composed of PEG and phosphate buffer.

CCC is a form of liquid—liquid chromatography that applies a strong centrifugal force to hold the liquid stationary phase counter to the flow of the liquid mobile phase. CPC, also known as hydrostatic CCC, on the other hand, uses the rotation of the column to retain the stationary phase [10]. CCC and CPC are relevant technologies because of their selectivity, automation, high resolution and the possibility to be scaled up to an industrial level [6]. In the past few years, bioproduct extraction and purification were achieved with ABSs used in CCC and CPC. For instance, efficient separation and recovery of three phenolic acids (65—87%) was accomplished with ABSs formed by PEG and sodium polyacrylate applied in CPC [26]. In addition, the reuse of the ABS polymer-rich phase allowed to reduce the carbon footprint of the process by 36% [26]. Polymer/salt-based ABSs were applied in CPC, where the addition of ILs as modifying agents led to a shift in the partition coefficient of proteins while achieving high stationary phase retention at reasonable flow rates [27]. Fast CPC using ABSs constituted by PEG and potassium phosphate allowed the purification of PEGylated cytochrome C conjugates, with recoveries between 88 and 100% and purities of ca. 100% [28]. Unreacted cytochrome C and solvents were successfully reused, reducing the E-factor and carbon footprint at ≈ 100 and 67%, respectively [28].

Challenges and opportunities in downstream processing

ABSs have potential to be applied in downstream processing. They are highly versatile and flexible,

Figure 3



Schematic illustration of microfluidics and CPC/CCC devices and related advantages. CPC, centrifugal partition chromatography; CCC, counter-current chromatography.

allowing their design to improve separation performance by changing the phase-forming components, compositions, temperature and pH values. However, these numerous options also raise limitations, requiring the ABS complete characterization, understanding of the mechanisms underlying their formation and product partitioning, and the development of models to predict their behaviour to allow their previous and adequate selection. Some of these challenges can be performed in microfluidic devices because they allow high-throughput screening. Furthermore, to be competitive, ABSs need to be cost-effective and more sustainable than the currently applied technologies. Therefore, environmental and economic analyses are mandatory aspects in the evaluation of the ABS potential in downstream processing. In this field, the recyclability and reuse of the ABS phase-forming components also is a required task. Significant advances have been demonstrated in the past few years with the application of ABSs in CPC, where high purities of high-value compounds have been achieved in continuous mode and significant reductions in the carbon footprint have been accomplished.

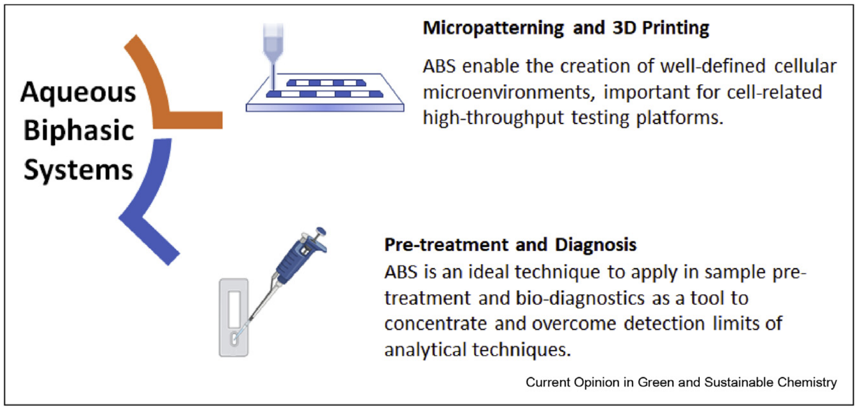
Emerging applications

Continuous advances in science have led to the proposal of other relevant applications of ABSs in addition to the downstream processing, some of which are summarized and discussed in recently published reviews [6,29,30]. The new applications of ABSs explored in biotechnology can be divided into 3 main areas: artificial cells and synthetic biology, micropatterning and 3D printing, and sample pretreatment and diagnosis. Artificial cells and synthetic biology are a highly interesting and innovative approach, where ABSs are used to construct artificial cells by compartmentation, allowing to mimic the intracellular environment and by creating cell-free systems that can be used for synthetic biology. Despite its relevance, in the past two years, this subject has been scarcely studied, and only with complex coacervates [31]; therefore, it is not a focus of this review. On the other hand, recent significant advances encountered in micropatterning and 3D printing, and in sample pretreatment and diagnosis using ABSs, are discussed in the following paragraphs. Fig. 4 summarizes promising applications of ABSs investigated in recent years.

Micropatterning and 3D printing

ABSs enable the creation of well-defined cellular microenvironments, which are important for cell-related high-throughput testing platforms. With this idea in mind, ABSs recently stood out in 2 main applications: micropatterning and 3D printing. For ABS-based micropatterning, the polymer–solvent system is dissolved into the cell culture medium to form ABSs. This strategy allows the formation of a cell island or colony that can be used to study migration, proliferation and differentiation of cells [32]. On the other hand, ABS-based 3D printing permits noncontact printing of cellular patterns and helps to overcome limitations of conventional methodologies, such as the production of uniformly sized spheroids [30]. To this end, ABSs were created using two immiscible polymers, where one was mixed in the matrix phase and the other was mixed in the ink [29,30,32]. Living cells can be introduced at any of these stages depending on the purpose, where the deposition of one phase to the other creates a 3D bioscaffold. As a new strategy of bioprinting, namely freeform reconfigurable embedded all-liquid, Luo et al. [33] investigated different polymer–polymer ABSs. Luo et al. [33] created an ABS-based 3D scaffold that was stabilized for weeks by a noncovalent membrane at the interface, enabling the compartmentalization of different cells. Ying et al. [34] developed a bioink containing ABSs composed of cells (human hepatocellular carcinoma cells, NIH/3T3 mouse embryonic fibroblasts and human umbilical vein endothelial cells), gelatin methacryloyl and poly(ethylene oxide). This ink was used to fabricate hydrogels, showing enhanced cell viability, spreading and proliferation when compared with standard hydrogels.

Figure 4



ABSs promising applications investigated in recent years. ABSs, aqueous biphasic system.

ABS-based biomedical approaches offer new opportunities to understand cells interaction and the formation of tissues with impact on health and therapeutics. In this line, Plaster et al. [35] used PEG/dextran ABSs to create 2D and 3D coculture of fibroblasts and breast cancer cells, and investigated the cellular interaction on the proliferation of breast cancer cells.

Sample pretreatment and diagnosis

Owing to the characteristics of ABSs that allow the extraction, purification and/or concentration of a given product in a single step, this is an ideal technique to apply in sample pretreatment and biodiagnostics. Within ABSs, ABS immunocytochemistry and ABS enzyme-linked immunosorbent assay (ABS-ELISA) are the two main techniques applied in biomarker analysis and early disease detection [30]. Kvas et al. [36] developed a cost-effective ABS-ELISA, where antibodies are confined in the bottom surfaces of assay plates by a PEG/dextran ABS. Compared with conventional ELISA, this technique reduced optical cross-talk effects, antibody quantities and analysis cost. Tongdee et al. [37] developed an one-incubation, 1-h multiplex PEG/dextran ABS-ELISA to detect cytokines, reducing by 4-fold the time required when compared with conventional ELISA.

The most successful diagnostic technique using ABSs is the solvent interaction analysis [6] because this method is simple and robust to monitor protein biomarkers. Accordingly, it has been commercially approved and applied in the early detection of prostate cancer using the prostate-specific antigen (PSA) as a biomarker [38]. PSA is usually quantified in serum; however, Pereira et al. [39] recently proposed an alternative approach based on the use of IL-based ABSs to carry out the pretreatment of urine samples and perform the diagnosis of prostate cancer by a less invasive method. ABSs composed of ILs and potassium citrate allowed the concentration of PSA by 250-fold in one step, thus allowing its quantification by more expedite equipment, as well as the identification of other PSA isoforms.

Owing to their characteristics, ABSs allow reducing reagent and sample volumes, analysis time and cost of analysis. Advances in the use of ABS-based techniques may contribute to the development of precision medicine. This type of application requires however equipment development, particularly to automatize the process [6], validation and regulatory approval [30].

Final remarks and future perspectives

ABSs were originally proposed for the extraction/purification of bioproducts in the 1950s and since then have been largely investigated in downstream processing. However, to find their place at the industrial level, it is still required to involve a multidisciplinary community to (1) create/improve the design of equipment/infrastructures to operate at a large scale; (2) develop models to predict the behaviour of ABSs and the partition of target bioproducts; (3) design continuous bioprocesses, including the reuse of the phase-forming components; (4) design ABSs to incorporate several operation units in downstream processing; and (5) perform economic and environmental analyses of all processes/methods developed. The recent examples given for integrated and continuous ABSs are relevant to show their promise as integrative platforms for biomolecules production and their potential to be scaled up. However, the works focused on these themes are still few and limited, and it would be naive to think that well-established or certified platforms in the production of bioproducts will change to accommodate ABS operation units. We believe that ABSs will have a place in the design of bioprocesses for new developed products, in which infrastructures and regulatory approval are not established yet, and in the purification of high-value compounds that still do not have an implemented production–purification process.

Although ABSs are not well established at an industrial level in the downstream processing and there is still a long path to cross, the work carried out up to date also led to the identification of more out-of-box applications of ABSs. From our perspective, emerging applications in other fields are an excellent opportunity and a niche for ABSs. The fact that the fields where these systems can be applied are new, namely in artificial cells and synthetic biology, micropatterning and 3D printing, and sample pretreatment and diagnosis, creates an opportunity for ABSs. In these fields, we still have time to make of the ABS technology the rule and not the exception. But the challenges also remain here, and to be applied widely, it is necessary first to overcome the faced drawbacks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

1. Chemat F, Vian MA, Ravi HK, Khadhraoui B, Hilali S, Perino S, Tixier ASF: **Review of alternative solvents for green extraction of food and natural products: panorama, principles, applications and prospects**. *Molecules* 2019, **24**:1–27. https://doi.org/10.3390/molecules24163007.

2. Pacheco-Fernández I, Pino V: **Green solvents in analytical chemistry**. *Curr Opin Green Sustain Chem.* 2019, **18**:42–50. https://doi.org/10.1016/j.cogsc.2018.12.010.

3. Clarke CJ, Tu WC, Levers O, Bröhl A, Hallett JP: **Green and sustainable solvents in chemical processes**. *Chem Rev* 2018, **118**:747–800. https://doi.org/10.1021/acs.chemrev.7b00571.

4. Häckl K, Kunz W: **Some aspects of green solvents**. *Compt Rendus Chem* 2018, **21**:572–580. https://doi.org/10.1016/j.crci.2018.03.010.

5. Phong WN, Show PL, Chow YH, Ling TC: **Recovery of biotechnological products using aqueous two phase systems**. *J Biosci Bioeng* 2018, **126**:273–281. https://doi.org/10.1016/j.jbiosc.2018.03.005.

6. * Pereira JFB, Freire MG, Coutinho JAP: **Aqueous two-phase systems: towards novel and more disruptive applications**. *Fluid Phase Equil J* 2020, **505**. https://doi.org/10.1016/j.fluid.2019.112341.

Status and future prospects of aqueous biphasic systems in biotechnology were reviewed

7. Soares RRG, Azevedo AM, Van Alstine JM, Aires-barros MR: **Partitioning in aqueous two-phase systems: analysis of strengths , weaknesses , opportunities and threats**. *Biotechnol J* 2015, **10**:1158–1169. https://doi.org/10.1002/biot.201400532.

8. Khoo KS, Leong HY, Chew KW, Lim J, Ling TC, Show PL, Yen H: **Liquid biphasic system: a recent bioseparation technology**. *Processes* 2020, **8**:1–22. https://doi.org/10.3390/pr8020149.

9. Kee PE, Ng T, Lan JC, Ng H: **Recent development of unconventional aqueous biphasic system: characteristics , mechanisms and applications**. *Crit Rev Biotechnol* 2020, **40**: 555–569. https://doi.org/10.1080/07388551.2020.1747388.

10. Ferreira-faria D, Aires-barros MR, Azevedo AM: **Continuous aqueous two-phase extraction: from micro fluidics to integrated biomanufacturing**. *Fluid Phase Equil* 2020, **508**:112438. https://doi.org/10.1016/j.fluid.2019.112438.

11. Badhwar P, Kumar P, Dubey KK: **Extractive fermentation for process integration and amplified pullulan production by A . Pullulans in aqueous two phase systems**. *Sci Rep* 2019, **9**:32. https://doi.org/10.1038/s41598-018-37314-y.

12. Leong YK, Show P-L, Lan JC-W, Krishnamoorthy R, Chu D-T, Nagarajan D, Yen H-W, Chang J-S: **Application of thermo-separating aqueous two-phase system in extractive bioconversion of polyhydroxyalkanoates by Cupriavidus necator H16, Bioresour**. *Technol* 2019, **287**:121474. https://doi.org/10.1016/j.biortech.2019.121474.

13. Meyer LE, Gummesson A, Kragl U, Von Langermann J: **Development of ionic liquid - water - based thermomorphic solvent (TMS) - systems for biocatalytic reactions**. *Biotechnol J* 2019, **14**:1–7. https://doi.org/10.1002/biot.201900215.

14. Yu S, Zhang D, Jiang J, Cui Z, Xia W, Binks BP, Yang H: **Biphasic biocatalysis using a CO2-switchable Pickering emulsion**. *Green Chem* 2019. https://doi.org/10.1039/c8gc03879a.

15. Han X, Li W, Ma X, Fan D: **Enzymatic hydrolysis and extraction of ginsenoside recovered from deep eutectic solvent-salt aqueous two-phase system**. *J Biosci Bioeng* 2020, **130**: 390–396. https://doi.org/10.1016/j.jbiosc.2020.05.008.

16. * Vobecká L, Tichá L, Atanasova A, Hasal P, Michal P: **Enzyme synthesis of cephalexin in continuous- flow micro fluidic device in ATPS environment**. *Chem Eng J* 2020, **396**. https://doi.org/10.1016/j.cej.2020.125236.

Continuous integrated enzymatic production was developed

17. Lee KW, How CW, Chen L, Chen PT, Lan JCW, Ng HS: **Integrated extractive disruption of Gordonia terrae cells with direct recovery of carotenoids using alcohol/salt aqueous biphasic system**. *Separ Purif Technol* 2019, **223**:107–112. https://doi.org/10.1016/j.seppur.2019.04.031.

18. Suarez Ruiz CA, Martins M, Coutinho JAP, Wijffels RH, Eppink MHM, van den Berg C, Ventura SPM: **Neochloris oleoabundans biorefinery: integration of cell disruption and purification steps using aqueous biphasic systems-based in surface-active ionic liquids**. *Chem Eng J* 2020, **399**:125683. https://doi.org/10.1016/j.cej.2020.125683.

19. Kruse T, Schmidt A, Kampmann M, Strube J: **Integrated clarification and purification of monoclonal antibodies by membrane based separation of aqueous two-phase systems**. *Antibodies* 2019, **8**:1–17. https://doi.org/10.3390/antib8030040.

20. Balaraman HB, Rathnasamy SK: **International Journal of Biological Macromolecules High selective purification of IgY from quail egg: process design and quantification of deep eutectic solvent based ultrasound assisted liquid phase microextraction coupled with preparative chromatograph**. *Int J Biol Macromol* 2020, **146**:253–262. https://doi.org/10.1016/j.ijbiomac.2019.12.242.

21. Smith F, Mara C, Soares F, Nogueira M, De Cássia R, Miranda M, Bezerra E, Lucena R: **Simultaneous concentration and chromatographic detection of water pesticides traces using aqueous two-phase system composed of tetrahydrofuran**

and fructose. *Microchem J* 2019, **147**:303–310. https://doi.org/10.1016/j.microc.2019.03.033.

22. Kruse T, Kampmann M, Rüddel I, Greller G: **An alternative downstream process based on aqueous two-phase extraction for the purification of monoclonal antibodies**. *Biochem Eng J* 2020, **161**:107703. https://doi.org/10.1016/j.bej.2020.107703.

23. Fisher AC, Kamga M, Agarabi C, Brorson K, Lee SL: **The current scientific and regulatory landscape in advancing integrated continuous biopharmaceutical manufacturing**. *Trends Biotechnol* 2019, **37**:253–267. https://doi.org/10.1016/j.tibtech.2018.08.008.

24. Vicente FA, Plazl I, Znidarsic-Palazl P, Ventura SPM: **Separation and purification of biomacromolecules in microfluidics**. *Green Chem* 2020, **22**:4391–4410. https://doi.org/10.1039/C9GC04362D.

25. Miličević N, Panić M, Valinger D, Bubalo MC, Benković M, Jurina T, Kljusurić JG, Redovniković IR, Tušek AJ: **Development of continuously operated aqueous two-phase microextraction process using natural deep eutectic solvents**. *Separ Purif Technol* 2020, **244**:116746. https://doi.org/10.1016/j.seppur.2020.116746.

26. Santos JHPM, Almeida MR, Martins CIR, Dias ACRV, Freire MG, Coutinho JAP, Ventura SPM: **Separation of phenolic compounds by centrifugal partition chromatography**. *Green Chem* 2018, **20**:1900–1916. https://doi.org/10.1039/c8gc00179k.

27. Bezold F, Roehrer S, Minceva M: **Ionic liquids as modifying agents for protein separation in centrifugal partition chromatography**. *Chem Eng Technol* 2018, **42**:1–17. https://doi.org/10.1002/ceat.201800369.

28. * Santos JHPM, Ferreira AM, Almeida MR, Quinteiro PSGN, Dias ACRV, Coutinho JAP, Freire MG, Rangel-Yagui CO, Ventura SPM: **Continuous separation of cytochrome-c PEGylated conjugates by fast centrifugal partition chromatography**. *Green Chem* 2019, **21**:5501–5506. https://doi.org/10.1039/C9GC01063G.

Efficient extraction in Fast CPC of PEGylated cytochrome C conjugates with environmental analysis.

29. * Chao Y, Shum HC: **Emerging aqueous two-phase systems : from fundamentals of interfaces to biomedical**. *Chem Soc Rev* 2020, **49**:114–142. https://doi.org/10.1039/c9cs00466a.

Background, status, and applications of microfluidics in biomedical applications were reviewed

30. González-gonzález M, Rito-palomares M: **Cell-based aqueous two-phase systems for therapeutics**. *J Chem Technol Biotechnol* 2020, **95**:8–10. https://doi.org/10.1002/jctb.6173.

31. Poudyal RR, Keating CD, Bevilacqua PC: **Polyanion-Assisted ribozyme catalysis inside complex coacervates**. *ACS Chem Biol* 2019, **14**:1243–1248. https://doi.org/10.1021/acschembio.9b00205.

32. Ham SL, Tavana H: **Aqueous two-phase systems for micropatterning of cells and biomolecules**. In *Open-sp. microfluid*; 2018:249–272. https://doi.org/10.1002/9783527696789.ch12.

33. Luo G, Yu Y, Yuan Y, Chen X, Liu Z, Kong T: **Freeform , reconfigurable embedded printing of all-aqueous 3D architectures**. *Adv Mater* 2019, **1904631**:1–7. https://doi.org/10.1002/adma.201904631.

34. * Ying G, Jiang N, Maharjan S, Yin Y, Chai R, Cao X: **Aqueous two-phase emulsion bioink-enabled 3D bioprinting of porous hydrogels**. *Adv Healthc Mater* 2018, **30**:1–9. https://doi.org/10.1002/adma.201805460.

Bioink-based ABS were developed with relevant biological advantages

35. Plaster M, Singh S, Tavana H: **Fibroblasts promote proliferation and matrix invasion of breast cancer cells in Co-culture models**. *Adv Ther* 2019, **1900121**:1–8. https://doi.org/10.1002/adtp.201900121.

36. * Kvas ATM, Chiang B, Frampton J: **Aqueous two-phase system antibody confinement enables cost-effective, analysis of protein analytes by sandwich enzyme-linked immunosorbent assay with minimal optical crosstalk**. *Analyst* 2020, **145**:5458–5465. https://doi.org/10.1039/D0AN00699H.

Improved ABS-ELISA was developed with high economical advantages.

37. Tongdee M, Yamanishi C, Maeda M, Kojima T, Dishinger J, Chantiwas R, Takayama S: **One-incubation one-hour multiplex ELISA enabled by aqueous two-phase systems**. *Analyst* 2020, **145**:3517–3527. https://doi.org/10.1039/d0an00383b.

38. Mark S, K EA, Arnon C, Kannan M, S AJ, Mathew W, Martin D, Yair L, Alan P, Jack B, Aimee K, Prasad G, Null BZ: **Clinical validation of IsoPSA^{TM}, a single parameter, structure based assay for improved detection of high grade prostate cancer**. *J Urol* 2019, **201**:1115–1120. https://doi.org/10.1097/JU.0000000000000185.

39. Pereira MM, Calixto JD, Sousa ACA, Pereira BJ, Lima ÁS, Coutinho JAP, Freire MG: **Towards the differential diagnosis of prostate cancer by the pre-treatment of human urine using ionic liquids**. *Sci Rep* 2020:1–8. https://doi.org/10.1038/s41598-020-71925-8 (accepted).