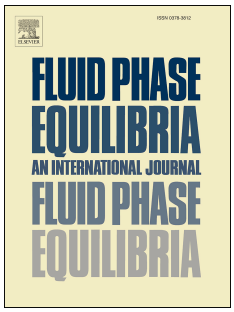

Aqueous two-phase systems: Towards novel and more disruptive applications

Jorge F.B. Pereira, Mara G. Freire, João A.P. Coutinho

PII: S0378-3812(19)30402-9

DOI: https://doi.org/10.1016/j.fluid.2019.112341

Reference: FLUID 112341

To appear in: *Fluid Phase Equilibria*

Received Date: 31 August 2019

Revised Date: 12 September 2019

Accepted Date: 1 October 2019

Please cite this article as: J.F.B. Pereira, M.G. Freire, Joã.A.P. Coutinho, Aqueous two-phase systems: Towards novel and more disruptive applications, *Fluid Phase Equilibria* (2019), doi: https://doi.org/10.1016/j.fluid.2019.112341.

Perspective

Aqueous Two-Phase Systems: Towards novel and more disruptive applications

Jorge F. B. Pereira[1*], Mara G. Freire[2] and João A. P. Coutinho[2]

[1]Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Rodovia Araraquara-Jaú/01, Campos Ville, 14800-903 – Araraquara, SP, Brazil.

[2]CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

[*]Corresponding author e-mail: jfb.pereira@unesp.br

Abstract

Aqueous two-phase systems (ATPS) have been mainly proposed as powerful platforms for the separation and purification of high-value biomolecules. However, after more than seven decades of research, ATPS are still a major academic curiosity, without their wide acceptance and implementation by industry, leading to the question whether ATPS should be mainly considered in downstream bioprocessing. Recently, due to their versatility and expansion of the Biotechnology and Material Science fields, these systems have been investigated in novel applications, such as in cellular micropatterning and bioprinting, microencapsulation, to mimic cells conditions, among others. This perspective aims to be a reflection on the current status of ATPS as separation platforms, while overviewing their applications, strengths and limitations. Novel applications, advantages and bottlenecks of ATPS are further discussed, indicating some directions on their use to create innovative industrial processes and commercial products.

Keywords:

Aqueous two-phase systems; aqueous biphasic systems; biotechnology; separation; purification; bioanalytics; biomaterials; bioprinting; micropatterning.

1. Contextualization

Aqueous two-phase systems (ATPS), also known as aqueous biphasic systems (ABS), are formed when at least two water-soluble components (*e.g.* polymers, ionic liquids, alcohols, salts, sugars, among others) are mixed in water above given concentrations, leading to the formation of a biphasic (liquid-liquid) system [1–4]. Although the formation of aqueous two-phase systems was originally recognized by Beijerinck in 1896 [5] during the cultivation of bacteria in media composed of gelatin, agar and water, it was only in the 1950s that the concept of ATPS as separation platforms was established by Albertsson [1]. This author extensively studied the use of ATPS composed of two polymers or polymers and salts for the separation of biological products (*e.g.* proteins, viruses, organelles, nucleic acids, etc.) [1]. In the more than 70 years of subsequent research, the number of ATPS-focused studies grew significantly, covering from the most fundamental aspects intended on the determination and characterization of the ATPS phase diagrams and their properties [6–12] to the evaluation of their use in a wide array of separation processes [1–4,13–20].

Due to formation of two immiscible aqueous phases, each enriched in one of the phase-forming components, their low interfacial tension and enhanced mass transport between the phases, ATPS have been widely applied in liquid-liquid extraction (LLE) processes. They have been found to be biocompatible, amenable and sustainable platforms for the extraction, separation and/or recovery of biological products [1,4]. In addition, most studies have shown ATPS as simple separation processes, highlighting their potential to be scaled-up and introduced in several downstream processing steps in industrial plants [14,15,21]. Despite all academic efforts emphasizing these advantages, their application is still predominantly confined to small lab and pilot scales processes, as confirmed in the large number of literature reviews focused on ATPS [2–4,13–15,18,21–27]. Therefore, it is about time to stop, rethink, and try to understand which drawbacks are limiting their wide application, and particularly which should be the next steps to make of ATPS a true industrial and/or commercial technology.

Over the last years, more than 100 studies were published per year reporting the use of ATPS for different purposes [3]. Some are focused on their application, while others on the understanding of the phase separation and partition mechanisms. In general, ATPS-application studies are divided in two categories: ATPS used in downstream processing operations (*e.g.* clarification, separation, extraction and purification, some of

which can be combined) [1,14,22,23,26], and ATPS used in pre-treatment strategies (mainly as a concentration step) to improve analytical characterizations [25,28]. In addition to these studies, recent works have been proposing ATPS for uses that cannot be categorized in any of these applications, being designed as ATPS emerging and non-conventional applications [24,28]. In **Fig. 1** a conceptual scheme representing the categorization of ATPS-applications, some of the most relevant examples and an overview of future perspectives is presented. As schematized in **Fig. 1**, the advances in the use of traditional ATPS depends also on innovative and disruptive technologies, which should provide advances on continuous or all-in-one processing, be cheaper but at same time more automatized and integrative, and allow the recyclability and polishing of solvents and target-compounds. On the other hand, recent advances in biotechnology and materials science have provided a new perspective for the use of ATPS, by using them in new and unexpected ways [24,29]. An illustrative example is the use of ATPS as a basis for micropatterning and bioprinting procedures. Another example of emerging applications is the introduction of ATPS in artificial cells production [24,30–35] and synthetic biology procedures [24,36,37].

Overall, we do believe that the future of ATPS will be highly dependent not only on the development of innovative technologies and science fields, but also in surpassing some of the limitations of current ATPS-based technologies. In the following sections, some examples and insights regarding the traditional applications and the development of innovative technologies involving ATPS are described and discussed.

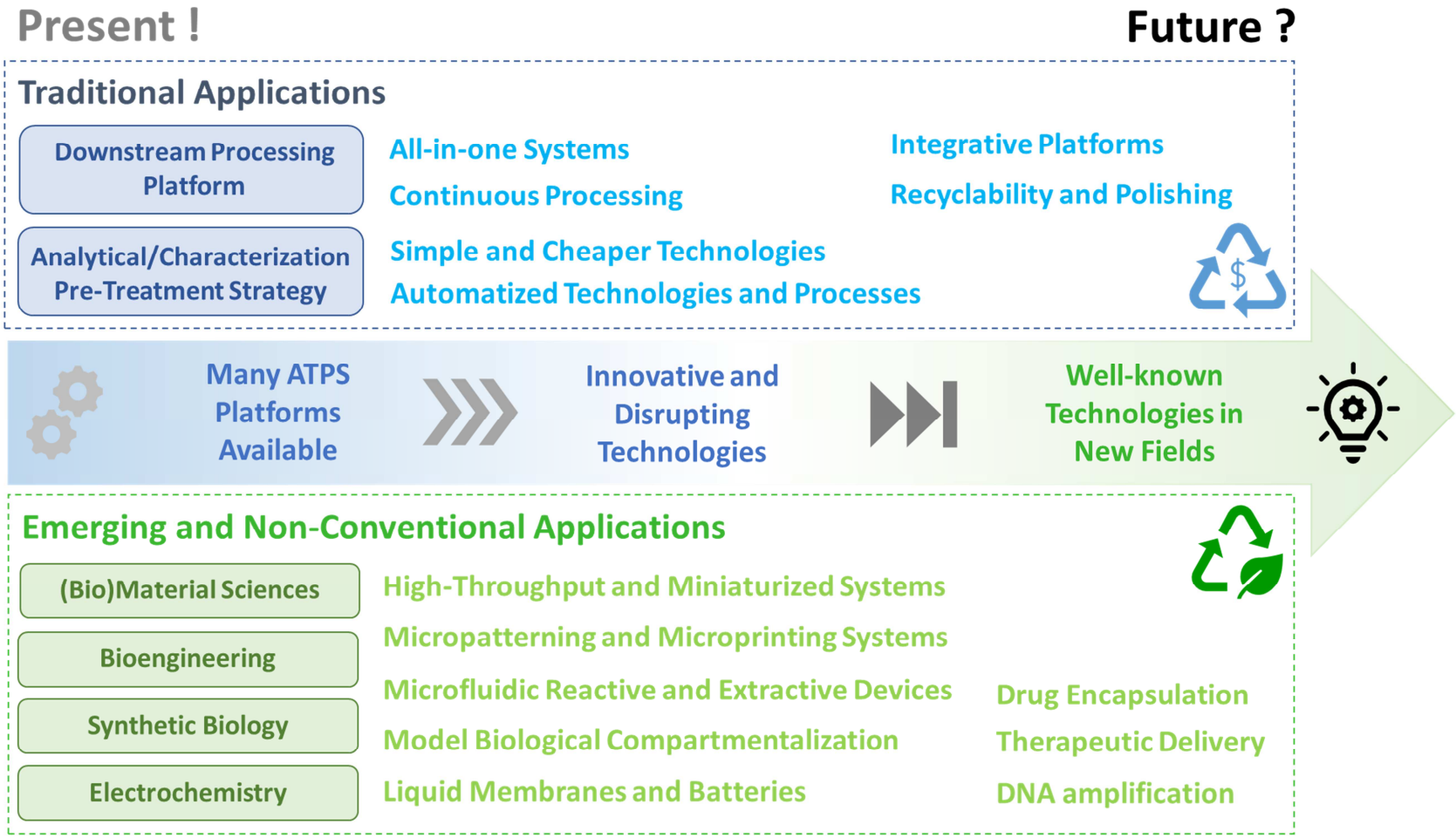

Fig. 1. Representative scheme of ATPS-based applications, examples and future perspectives.

2. Perspectives in the Traditional Applications of ATPS

2.1. Downstream Bioprocessing

ATPS were initially proposed as interesting platforms for feed clarification, recovery and partial purification of biological products. Simplicity, selectivity, efficiency and scalability were always pointed out as strengths, but ATPS have not been effectively implemented in industrial bioprocessing [21], thus raising the following question: *Is it time to stop academic studies focused on ATPS for the separation and purification of bioproducts?* We do believe that this type of studies should not be stopped and that there are still interesting opportunities in the field of bioseparations; however, a revolution on the way these studies are performed is required. It is senseless to continue to carry out lab-scale studies in which the partition of a specific biomolecule is optimized at different conditions (*e.g.* pH, temperature, phase-forming components, etc.) and to expect that the corresponding ATPS will be then easily employed at an industrial scale. Biotechnology industries have already well-stablished downstream processing plants, mostly combining several filtration and chromatography units. Moreover, and as happened with ATPS, also the number and efficiency of chromatography and ultrafiltration technologies for the recovery and purification of biological products, particularly high-value biopharmaceuticals, have significantly increased over the past years [38].

Soares et al. [21] performed a comprehensive analysis of strengths, weaknesses, opportunities and threats (SWOT) of the biomolecules' partitioning in ATPS. The authors highlighted as major strengths, the integrability, scalability, biocompatibility, selectivity and continuous operation possibility, while identifying as weaknesses the limited predictive design, handling capacity (processing of large volumes) and the lack of know-how regarding the installation, validation and operation of ATPS-based technologies [21]. Authors have further highlighted the use of ATPS in the primary clarification of monoclonal antibodies (mAb) cultures, purification of plasmid DNA, recombinant proteins and small biomolecules, as well as the process simplification and integration by coupling ATPS and chromatography. Recently, González-Valdez et al. [28] emphasized some novel aspects and possibilities to maximize the effective use and implementation of ATPS in downstream processing. The authors highlighted their

implementation in a continuous processing mode and process integration as the two major drawbacks, which, if overcome, can turn these systems into a reality.

As summarized in **Fig. 1**, it is fundamental to find the "optimal" ATPS-based platform to perform the complete extraction and purification of a given bioproduct, and, ideally, to be able to integrate several upstream and/or downstream stages and recycle the phase-forming components. Studies where ATPS are used for the reduction of downstream processing stages, preferentially as all-in-one or as continuous processing units, can reduce the "distance" that separates these systems from the industrial reality. Furthermore, ATPS may find a relevant place in the purification of bioproducts that are not easily purified by conventional methods. Finally, only by addressing and guaranteeing the phase-forming components recycling it will be possible to be economically competitive with other well-established techniques. Overall, ATPS-based studies should describe and demonstrate all their advantages over the already stablished techniques, and authors should still be aware of the reluctance of the commercial and industrial partners to adopt their use.

2.1.1. Continuous processing

Most published works in ATPS were developed in a batch mode, as single and non-integrated platforms. Thus, besides the excellent extraction and purification performance commonly reported, their implementation in continuous or semi-continuous industrial operations still represents logistical and processual issues to be solved [28]. Some studies presented however the use of ATPS in continuous mode, namely by using: (*i*) mixer-settler units and column contactors; and (*ii*) continuous countercurrent distribution, centrifugal partition chromatography (CPC) or countercurrent chromatography (CCC).

The first type integrates the mixing, coalescence and separation stages into a single step, basically combining mixing stages (tanks, columns, and pumps) with settling/separating devices (settlers, decanters, columns, and centrifuges). Mixer-settler units are common industrial units in conventional aqueous/organic liquid-liquid extraction. They were one of the first devices used for the continuous ATPS processing [28], particularly as early stages of purification (low resolution). Espitia-Saloma et al. [23] extensively revised this type of devices for ATPS continuous processing, providing a full analysis of operational and design parameters. Although some successful examples (in lab- and pilot-scales) by using ATPS-based mixed-settler units were

reported, such as for the recovery and purification of enzymes [39] or antibodies [40], further efforts are still required for the development of these technologies. Due to the high number of ATPS types and highly distinct properties (viscosity, density, interfacial tension, etc.), the hydrodynamics of mixing and separation are unpredictable and case-by-case dependent. Moreover, the mechanisms behind the partition of the target-solutes and contaminants/impurities must be addressed and clarified.

The second category, based on chromatographic principles, was originally stablished for aqueous/organic and organic/organic liquid-liquid systems [41]. Although the number of studies focused on ATPS-based processes using CCC-related modes has been growing in the recent years [42,43], particularly because of the high-resolution, selectivity and automatization of these technologies, these are still scarce. Similar to CCC, centrifugal partition chromatography (CPC) was recently used by Santos et al. [44,45] to convert ATPS into scalable large-scale processes. In the first report, the authors demonstrated the separation of three phenolic acids and of an aldehyde-rich fraction applying a polymer-based ATPS in CPC [45]. In addition to the efficient separation achieved, the authors also implemented an integrated platform to reuse the polymer-rich phases of the ATPS, decreasing the carbon footprint by 36% and by increasing the sustainability of the entire process [45]. More recently, the same group shown the effective use of ATPS in fast CPC for the purification of PEGylated cytochrome c conjugates [44]. A sustainable ATPS-FCPC process was designed in continuous regime not only to isolate the bioconjugates, but also to allow the recovery and reuse of both the unreacted cytochrome c and ATPS phases [44].

Although these chromatography-related continuous processes allow high yields and purification factors, sometimes even higher than traditional chromatographic methods, several processual issues remain to be solved, namely: (*i*) the retention of the stationary phase and back pressure issues – equipment commercially available was designed for organic-based separations, and thus designed to work with phases with distinct properties, *i.e.* high interfacial tension, low phases' viscosities and high difference in the density values of the coexisting phases; (*ii*) the equipment maintenance and associated cost – apparatus (pump, connection, tubes, etc.) are poorly prepared to handle with the high concentration of the phase-forming components (mainly salts, ionic liquids and polymers) used in aqueous media.

We believe that one of the challenges for the downstream bioprocessing using ATPS is the development of continuous and automatized technologies, able to provide

competitive advantages over the traditional downstream processing units, *e.g.* by reducing the processing time and cost, by allowing the solvents' recyclability, and by improving the separation performance and yield. Ideally, the integration with preceding upstream stages, such as continuous bioreactors or cell disruption high-pressure homogenizers, will bring significant advantages to ATPS applications.

In addition to batch experiments, we advise the scientific community to move on with the characterization, optimization, modelling and automatization but using ATPS in continuous operation mode. It should be however remarked that the down-scale of ATPS to a micro-scale by using microfluidic devices could also find interesting applications and induce relevant progress in the understanding of the mechanisms underlying the continuous processes, providing the development of adequate models to simulate and optimize the processing parameters (more insights regarding microfluidic devices is provided below). Moreover, instead of adapting ATPS-based processes to the equipment commercially available, which are designed for conventional organic/aqueous liquid-liquid systems, we do believe that the design of new apparatus to meet the ATPS properties and characteristics is fundamental aiming at turning these systems in an industrial reality.

2.1.2 Process integration

The integration of the downstream units with previous upstream or clarification stages or following high-resolution downstream or polishing stages are key issues for the reduction of costs. Accordingly, the flexibility of ATPS-based platforms in this respect has been regarded as a major advantage to support their industrial application [21,28]. Other bioprocessing stages have been integrated with the ATPS separation step, namely: (*i*) production (extractive fermentation or fermentative extraction; direct or *in-situ* extraction) [46,47]; (*ii*) enzymatic reactions [48,49]; (*iii*) cell disruption [50–52]; (*iv*) *in-situ* refolding [53,54]; (*v*) clarification or primary recovery (centrifugation, precipitation) [55,56]; (*vi*) high-resolution purification operations (chromatography, ultrafiltration) [26,57]; (*vii*) polishing stages (crystallization, lyophilization, drying) [58,59].

Several reports have pointed new perspectives regarding process integration with ATPS. Gonzalez-Valdez et al. [28] identified extractive fermentation, *in-situ* refolding, enzymatic reaction, and cell disruption as major steps that can be integrated in ATPS together with the separation step. Soares et al. [21] pointed out the feed clarification,

complex three "compartment" separations (cells or cellular debris tends to form a macroscopic phase at the interface of ATPS), and initial (post-fermentation) concentration units (accommodation of high loads of the target compound and large handling volumes) as ideal stages for integration in ATPS in downstream bioprocessing. Particularly, the authors noted that, under the correct conditions, ATPS can work as multi-task units for the recovery of bioproducts from complex broths, *i.e.* clarifying, concentrating and purifying the target compound [21]. Moreover, since the target compound(s) and contaminants or impurities can be distributed between different phases, differently from solid-liquid, precipitation and crystallization operation units, ATPS do not require additional separation procedures before further processing steps.

Regarding the feed clarification, an interesting approach comprising the integration of ATPS principles and solvent sublimation, known as aqueous-two flotation systems (ATPF), was proposed. ATPF is a relatively new technique, allowing to achieve high recovery and purity yields, with simple operation, environmental and economic advantages, possible recyclability and further integration with cell disruption processes. However, its integration with subsequent downstream platforms is still required. A complete overview on recent advances and future directions regarding ATPF-based processes was recently published by Sankaran et al. [60].

Soares et al [21] highlighted back-extraction procedures, by integrating two ATPS. Emphasis was given to the "*more elegant*" approaches using ATPS composed of thermo-responsive polymers, which undergo phase separation by temperature change. We fully agree that thermo-responsive (or thermo-separating) ATPS have unique properties. These systems can be integrated not only in back-extraction procedures but also as an advanced technology in extractive bioconversion or as a pre-chromatographic stage. Recently, Ferreira et al. [61] demonstrated the potential of thermoreversible ATPS composed of ammonium-based zwitterions (ZI) and polymers as integrated bioreaction-separation processes. The laccase biocatalytic reaction occurs in the homogeneous medium, after which a change in temperature induces the two-phase formation and the complete separation of the enzyme and the biocatalysis products [61]. It is important to note that these thermo-responsive ATPS allowed not only the integration of two units, but also the maintenance of the enzyme stability (thermoreversibility occurs at amenable temperatures) and the recyclability of both the ZI and enzyme. More information on these ATPS can be found in the work published by Leong et al [62].

An interesting demonstration of process integration/intensification was shown by Münchow et al. [63,64], which proposed the use of continuous microfluidics aqueous two-phase (PEG/Dextran aqueous solutions) electrophoresis for protein transport, concentration, and partition. Proteins can be partitioned according to their affinity to one of the ATPS coexisting phases, while the application of an electric field perpendicular to the phase boundary enhance, extend or reverse the separation process [63]. For example, the transport of charged biomolecules by electroextraction is faster than by diffusion, and thus, a fine adjustment of the yield and selectivity of the method according to the charge of the biomolecules can be achieved.

As final example in this field, a simultaneous preparative crystallization of single chain antibody using an ATPS was proposed by Huettmann et al. [58]. The authors demonstrated the effective integration of two steps, namely by designing an ATPS that favors the crystallization of proteins by using PEG and sodium sulfate. The authors observed that at sufficiently high concentrations of PEG, a second immiscible phase is formed with the simultaneous crystallization of the target protein. The crystal nucleation occurs in the salt-rich phase (at the phase boundary), whereas the protein crystal growth progress mainly in the PEG-rich (top) phase where the crystals are partitioned [58]. In our opinion, this is a relevant example of the high potential of ATPS as an integrative platform since protein crystallization processes are well-established in the pharmaceutical and food industries. Despite these approaches' relevance, additional efforts to demonstrate the phases' recycling and protein polishing studies are still required.

2.1.3 Challenges for the implementation in industrial bioprocessing

We fully agree that ATPS are highly versatile and flexible and can be designed to act as integrative platforms. This possibility is however not new, as described in the book *Separation Using Aqueous Phase Systems*, edited by Fisher and Sutherland in 1989 [65]. Therefore, a question remains: *What failed in the ATPS implementation at a large scale*? In our opinion, the problem is that only isolated ATPS-related studies are found, and even these are still scarce taking account the large number of phase-forming components that can be combined to create ATPS, operational conditions that can be changed, and target products to separate. Only by doing an exhaustive and systematic study it will be possible to achieve relevant insights that could allow the design of effective ATPS for a given application. Furthermore, if it is difficult to implement

single-operation ATPS at an industrial scale, it will be even more difficult to implement them as integrated platforms due to the studies and adjustments required in equipment and infrastructures. A big gap remains between the bench and pilot-scale apparatus and/or procedures and the reality of industrial bioprocessing. We do understand that some ATPS-based studies are of a more fundamental nature, while others are devoted to application of individual case studies. Therefore, independently of the purpose, before starting "yet another" work on the partition/separation/purification of some exciting biological product, authors should consider the knowledge that can be generated and how these processes could be implemented at an industrial scale, as well as if it is a competitive process compared to the well-established ones.

Fundamental studies are a must since useful insights on the molecular-level mechanisms ruling solute partition and/or phases' separation mechanisms can be provided. Only by addressing this knowledge, effective ATPS for a target application can be designed. Furthermore, the effect of impurities on both the solute partition and phases' equilibrium and compositions should be appraised. When envisioning a large-scale process, the purity of the phase-forming components may not be as high as at the laboratory scale, and related economic issues need to be considered. Recently, Patel et al [66] carried out a dynamic modelling of ATPS to quantify the impact of bioprocessing design, operation and variability. The authors highlighted the lack of experimental studies that consider the impurities as individual components as one of the main modelling limitations of ATPS, suggesting the use of high throughput analysis to overcome this issue. Furthermore, when dealing with salt-salt ATPS ion-exchange may occurs, leading to more complex systems involving more species in equilibrium, and requiring the quantification of all ions present in each phase (that according to the Gibbs Phase Rule and respective degrees of freedom can be more than two since more than 3 species are present). In addition, pH effects and phase-forming components speciation should always be considered as these change the ATPS formation capacity and solutes partitioning. Although negligible ion-exchange effects were previously demonstrated in ATPS formed by ionic liquids and strong salting-out salts [67,68], such as aluminum, and potassium-based salts, the same may not apply to systems involving pairs of salts with similar cohesion energy [69] or more complex ATPS involving or protic-ILs [70] or polyvalent ions [71].

As schematized in **Fig. 2**, applied ATPS-based studies focused on the extraction/purification of a target compound should also consider their use in

continuous mode and integration of several operation units, ideally designing and simulating an industrial plant. Moreover, their effective implementation is dependent on the recyclability, biocompatibility and sustainability (economic and environmental issues). In our opinion, the effective implementation of ATPS in industrial bioprocessing is highly dependent of its overall sustainability, mainly associated with the recyclability of the ATPS components (which can be responsible for more than 50% of the total process cost [72]). It is important to note that the recycling/reusing procedures should guarantee the reproducibility, purity requirements of the original extraction/purification stage, as well as the economic and environmental sustainability of the overall bioprocess. These features should be always considered in the design, scale-up, control and automatization of the respective ATPS.

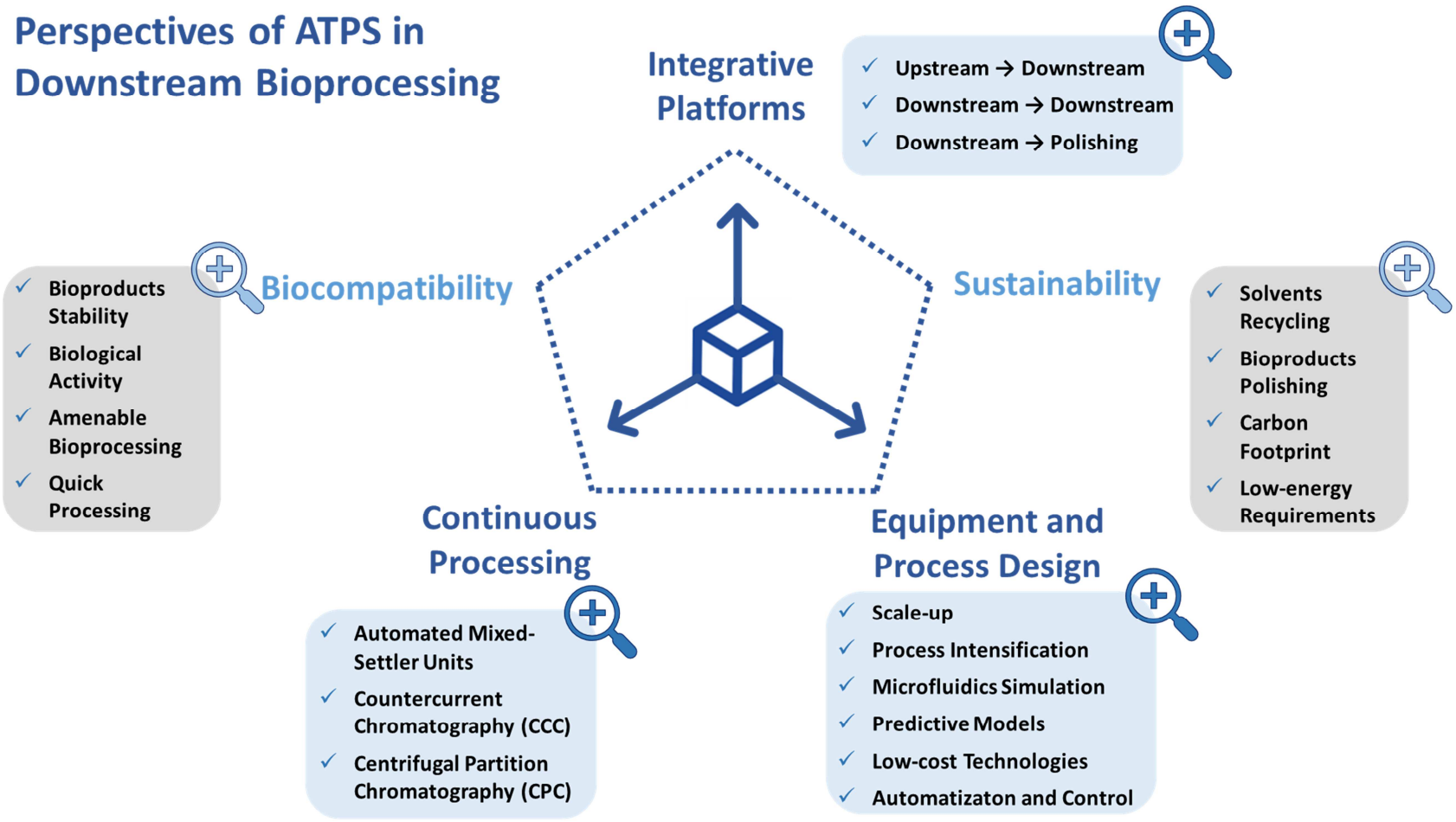

Fig. 2. Representative scheme of the perspectives for the traditional application of ATPS in downstream bioprocessing.

2.2. Pre-treatment Strategies for Analytical Purposes

Following the use of ATPS in downstream bioprocessing, their use for analytical purposes appears as the most popular. ATPS have been use as pre-treatment techniques to concentrate (to overcome the detection limits of the analytical technique) of residual drugs [73] and pollutants in water [73–76] and food samples [75,77,78]. In the same line, ATPS have been used to treat complex samples aiming the improvement of (bio)analytical and (bio)characterization purposes [25,79].

A growing number of contaminants and pollutants (some with serious health and environmental impact) are continuously released to the aqueous environment by human activities. However, these pollutants are usually present in trace levels in complex environmental samples, turning their accurate quantification and identification a great challenge. Thus, also taking advantage of the concentration capacity of ATPS by changing the volumes of the two phases, these can be applied as pre-treatment/concentration techniques, both to eliminate interferences and enrich the target pollutants. Representative examples of the potential of ATPS for environmental monitoring were shown by Freire and co-workers [73,76,80], namely using IL-based ATPS to improve the detection and quantification of the human endocrine disruptor bisphenol A [76], synthetic hormones [80], and other active pharmaceutical ingredients [73]. It is important to highlight that the key feature of ATPS as pre-treatment techniques for environmental monitoring is their ability to significantly enhance the pollutant concentration by simply adjusting the compositions of the ATPS components along a given tie-line while guaranteeing the non-saturation of the respective phase.

Similarly, ATPS can be used as a pretreatment/presorting stage in proteomic studies, separating and concentrating large fractions of proteins from crude extracts, which can be subsequently identified and characterized by 2D electrophoresis [81], mass spectrometry techniques [82,83], among others. From our perspective, the implementation of ATPS as a pre-treatment stage in proteins detection/characterization is one of their most appellative applications. However, as highlighted below, the intensification by automatization and miniaturization of ATPS can bring further advances in this field.

The most successful “bioanalytical-related approach” using ATPS is the solvent interaction analysis (SIA) method, proposed by Zaslavsky et al. [25], which is based on the solutes partitioning in ATPS. The authors [25] have recently reviewed the SIA

analytical progresses, highlighting the strong potential of ATPS to improve the characterization and analysis of individual proteins and protein-partner interactions in biological fluids, as well as the new opportunities to discover and monitor protein biomarkers. The SIA technique is a simple, robust and inexpensive method to detect changes in the protein structure and protein-protein in biological samples, *i.e.* serum or plasma proteome clinical samples, as a function of their partition coefficients. These findings represent the basis for the company Cleveland Diagnostics, Inc., which is about to commercialize the SIA technology, as IsoPSA^{TM} assay, for the early detection of prostate cancer. Considering that clinical proteomics and disease biomarkers are in general proteins, we believe that ATPS can be used as a powerful tool to improve diagnosis of a wide variety of diseases.

A representative scheme summarizing the uses and perspectives of ATPS as pre-treatment strategies for analytical purposes is shown in **Fig. 3**. Although it is evident that (bio)analytical analysis and pre-preparation (concentration) sampling procedures are regarded as relevant opportunities for the application of ATPS, for example on environmental monitoring and clinical purposes, commercial ATPS-based kits or technologies are yet scarce. The design of automatized and robotic devices, as well as miniaturized sample preparation and pre-concentration kits, will make a turnover in the field of analytical ATPS-based technologies. In our opinion, studies regarding the use of ATPS in the analytical field should focus on the development of disruptive and non-conventional technologies, for example in microarrays and biosensors for fast biological and genetic analysis. Additional information on the use of ATPS in emerging analytical applications is provided below.

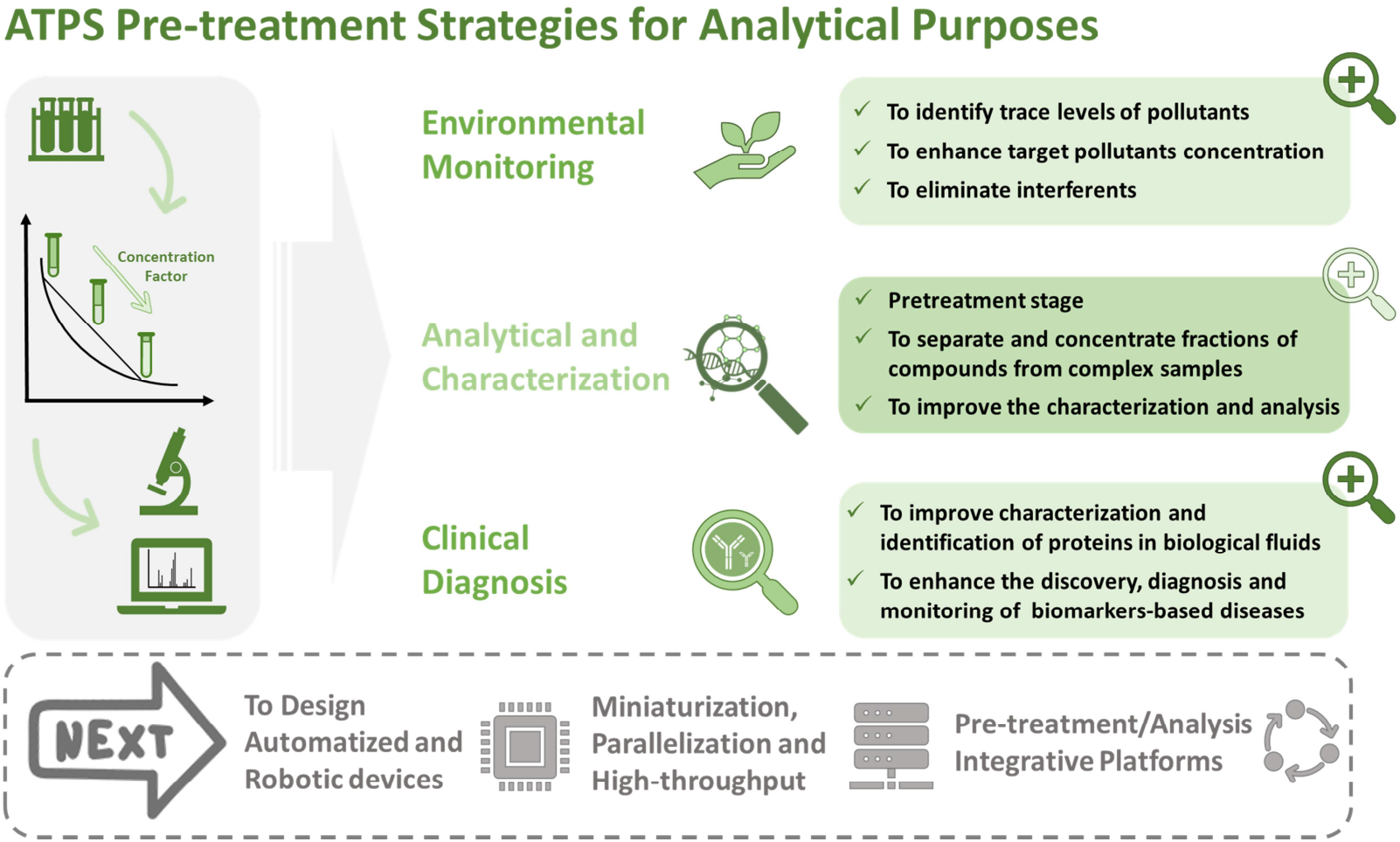

Fig. 3. Representative scheme of the uses and perspectives of ATPS as pre-treatment strategies for analytical purposes.

2.3. Other Applications

In the previous sections the two most explored applications of ATPS were overviewed. However, there are other small niches for the application of ATPS, such as in hydrometallurgy, urban mining, biorefinery and environmental bioremediation. ATPS have been demonstrated to be powerful tools for the selective extraction of metal ions [84,85] and, more recently, as integrative platforms for leaching and hydrometallurgical treatment of critical metals [86,87]. Pioneering works used polymer/salt ATPS; in particular, the use of triblock-copolymers have attracted the interest from metal-based industries [27,85]. However, and as recently stated by Karmakar and Sen [84], due to the environmental problems concerning the disposal of polymer and salt-concentrated waste streams, polymer-based ATPS were not effectively employed at a large scale. Therefore, the development of suitable regeneration (components recycling and metals leaching) methods and leaching/extraction/refining integrative processes could enhance the potential of this field. Significant advances were achieved with the development of ionic-liquid-based (IL-based) ATPS [2,86–88]. The work of Schaeffer et al. [86] is the perfect example of the potential of IL-ATPS as an efficient, flexible and integrated extraction-separation-purification platform for the hydrometallurgical treatment of critical metals, from leaching to electrodeposition. As shown by the authors, the “one pot” hydrometallurgical selective recovery of cobalt from nickel metal hydride batteries can be achieved using an acidic ATPS, allowing the sequential leaching, solvent extraction and electrodeposition of metals. Considering current environmental concerns and the relevance of urban mining, we do believe that ATPS will play a relevant role on this field. However, the process integration and intensification, solvents recycling and scaled-up viability, as well as the understanding of the fundamental mechanisms behind metal extraction, are crucial goals to be still achieved.

Similar to metal recovery, the search for more sustainable approaches for the environmental remediation of industrial aqueous effluents opened a new window for the use of ATPS-based technologies. ATPS have been used in the removal of toxic and carcinogenic dye-related compounds from textile industrial wastewaters [89–91], of aromatic compounds from industrial and environmental settlings [92], and of non-metabolized drugs [93] from domestic effluent treatment plants. We will not provide a detailed discussion on this application. We have doubts that ATPS are effective

platforms for sustainable environmental remediation purposes. Even considering 100% of removal of the target pollutants, the cross-contamination of the aqueous phase aimed to be treated by the ATPS phase-forming components will have a stronger impact, *i.e.* the ATPS phase-forming components become contaminants. This attempt should always consider the use of additional ultrafiltration of adsorption processes able to remove the ATPS phase-forming components, so that ATPS could be more effective and sustainable than the commonly applied remediation techniques.

3. Emerging and Non-Conventional Applications of ATPS

Most of the studies involving ATPS aim the development of technologies that can be scaled up and used industrially. However, and as briefly highlighted above, the most promising and emerging applications of ATPS do not require scaling-up, and sometimes need instead to be scaled-down [24]. Regarding these alternative uses of ATPS, Teixeira et al. [24] published a comprehensive review on their modern biotechnological applications, namely in:

i) cell micropatterning and microtissue engineering;

ii) solution microarrays for biochemical analysis;

iii) microfluidic devices;

iv) synthetic biology approaches.

The emergence of rapid, accessible and simple prototyping techniques (particularly 3D printers) for the fabrication of a range of versatile microfluidic devices led to the exploration of new fields of application for ATPS [94]. Microfluidics are portable systems that allow the precise handling of solvents, reagents and cells, intensifying processes (*i.e.* reduce costs and processing times) and improving the amount/quality of the data processed [24,94,95]. A relevant number of reviews [24,94–97] compiling most of the fundamentals, characteristics, limitations, applications, and advances of ATPS-related microfluidics platforms have been published. Representative examples of emerging applications for microfluidic ATPS platforms are detailed below. However, the large number of possibilities raises the question: *Is microfluidics the basis of the next generation of ATPS-based technologies?* It is evident that microfluidics provides solutions for some of the current ATPS' limitations, for example, by being more cost effective, and by allowing the high-throughput screening, parallelized and reproducible processes. However, novel ATPS-based technologies will be also dependent of proper

automatization and integration with current miniaturized sample preparation and analytical devices.

Teixeira et al. [24] anticipated five major areas of future growth in applied ATPS research, while providing some considerations on the technological and design needs, namely: (*i*) polymer design for target ATPS applications; (*ii*) stimuli-responsive systems; (*iii*) scaling industrial separation reactions; (*iv*) therapeutic microencapsulation and drug delivery; and (*v*) artificial cells and synthetic biology. We completely agree with the authors and the relevance of these new applications. However, these are yet quite immature and huge efforts are necessary to fill their technological gaps. **Fig. 4** summarizes some examples of ATPS-emerging applications, emphasizing those with promising future, as well as the barriers that need to be overcome before its commercial and industrial application. As summarized in **Fig. 4**, the use of ATPS in emerging and non-conventional applications is a result of multidisciplinary teams and knowledge. Researchers explore these systems at micro to nanoliter scales, employing robotized and sophisticated technologies, in fields that range from biotechnology, to health sciences, to material sciences (including biomaterials), to electrochemistry. The next sub-sections compile some of the most exciting applications described for ATPS, as well as some perspectives to overcome their current limitations.

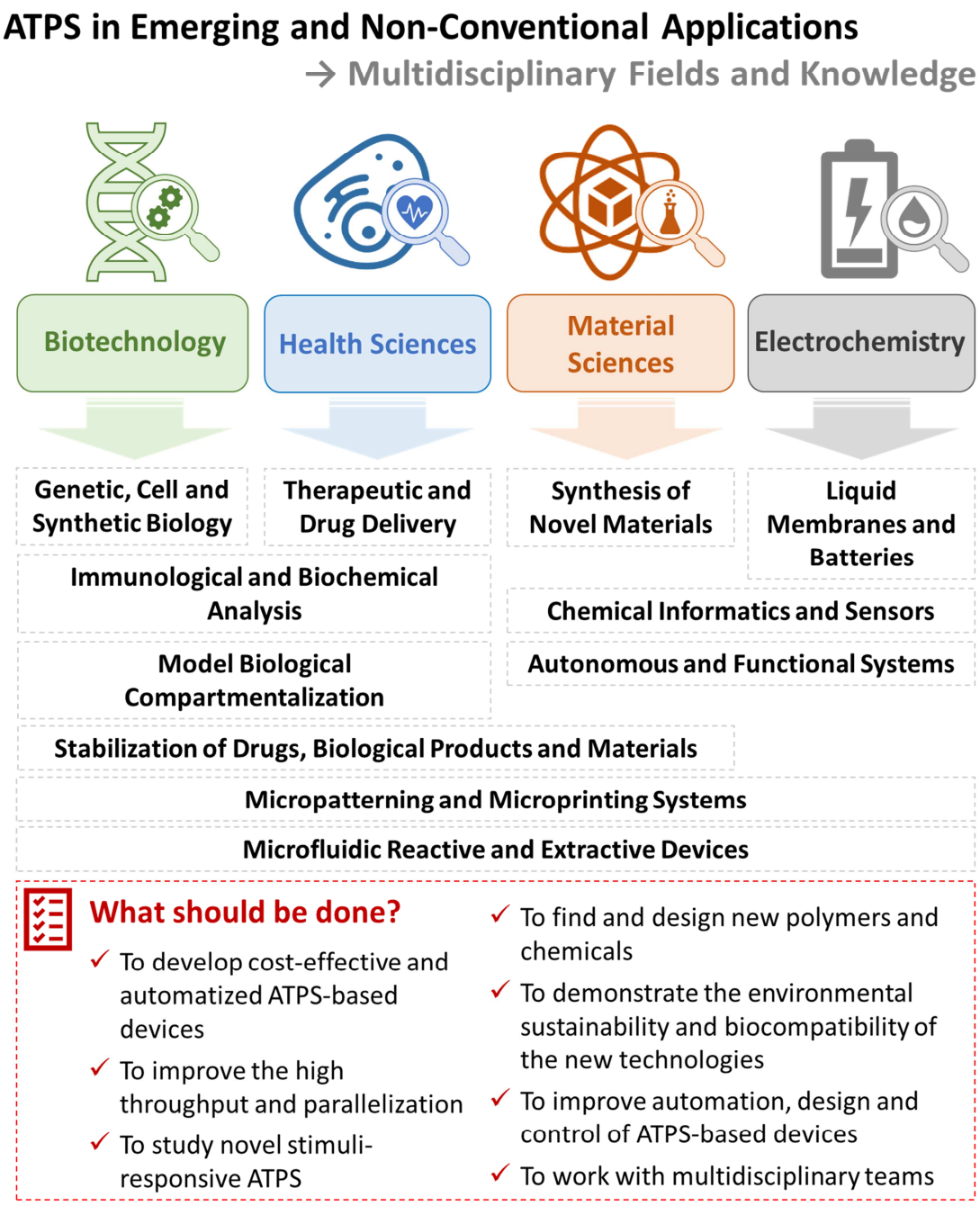

Fig. 4. Representative scheme of the emerging and non-conventional applications of ATPS and perspectives to overcome their current limitations.

3.1. Bioengineering and Biotechnological Applications

Biotechnology has exponentially grown in the current century, where the manipulation of living organisms by using different biomolecular and bioengineering tools is a common procedure. With the growth of biomolecular and bioengineering tools and devices, new challenges and opportunities have also emerged, and in our opinion ATPS can provide simple and low-cost solutions in this arena.

3.1.1. Immunological and Biochemical Analysis

As emphasized before, the development of disruptive ATPS-based technologies, for example microfluidic platforms and microarray strategies, created a window of opportunities in bioanalytical/biomolecular clinical diagnosis. For instance, the detection of infectious agents is carried out via the polymerase chain reaction (PCR) or by using portable nucleic acid detection systems, *e.g.* isothermal DNA amplification techniques. While the first one is a gold standard test but limited to laboratory tests (point-of-care device), the second is a portable device that requires extensive sample preparation. Therefore, to overcome these limitations, the authors proposed the use of micellar ATPS as a single, integrative, sensitive and ease-of-use tool to achieve *E. coli* cell lysis, lysate processing (*i.e.* DNA sample preparation) and enhanced isothermal DNA amplification (by using the thermophilic helicase-dependent amplification method) [37]. A similar approach was carried by the same research group, by applying ATPS to spot immunoassays and detect foodborne illnesses [98]. In this work, an UCON/phosphate salt ATPS was used for the preconcentration of *E. coli* 0157:H7 cells, improving the detection limit of the spot test within 30 min and allowing the detection without upstream processing or dilution procedures.

Frampton et al. [99] shown a successful example on the use of ATPS-based strategies to eliminate antibody cross-reactions in conventional ELISA assays. The ATPS-ELISA method proposed by the authors takes advantage of a PEG/dextran system properties to confine the detection of antibodies at specific locations in fully aqueous environments. Although the procedure follows the standard ELISA procedures, it requires lower quantities of antibodies and volume of biological samples, thus appearing as an interesting alternative for the detection of biomarkers in biological samples. However, this approach still exhibits the same limitations of the ELISA workflow, namely the lack of automatization and high throughput, being a laborious and time-consuming method. Recent advances using micropatterning arrays can overcome some of these drawbacks, making the ATPS-based ELISA procedure simpler and quicker [24]. Other traditional immunoassays can take advantage of the ATPS concentration aptitude, for example by intensifying the sensitivity of lateral-flow immunoassays for the detection of proteins [100], viruses [101], among others [24].

The above described examples demonstrate the high potential of ATPS to overcome the traditional limitations of (bio)diagnosis. From our perspective there are still

numerous opportunities for the use of ATPS in the biochemical and analytical fields. In this field, ATPS can be used as a combined purification and concentration platform, and ideally should be miniaturized to reduce the volume of biological samples and the analysis cost.

3.1.2. Artificial Cells, Cell Biology and Synthetic Biology

A remarkable example of out-of-the-box applications for ATPS was proposed by Arns and Winter [36], who demonstrated the potential of ATPS to recover the stability of a DNA hairpin (hp) submitted to high pressure (> 1000 bar). Authors have shown the important role of polymeric ATPS to recover the biological function of DNA hp. This example demonstrated not only the applicability of ATPS in cell biology studies, but also the potential of stimulus-responsive ATPS (pressure-modulated) for the control of biocatalytic systems.

ATPS have also shown to be promising in artificial cells and cell biology fields. ATPS can be used to simulate organelles and nucleoli liquid-environments, by mimicking the compartmentalization of biological molecules in membrane-enclosed and membrane-free structures of living cells. The Keating's research group is one of the most active on this field [102–105], with a series of interesting works using ATPS as model systems for biological compartmentalization in early and modern cells, *i.e.* cell-like assemblies. An overview on the use of ATPS as cell models was published in 2011 [102], highlighting their potential to mimic some properties of biological cells, such as micro-compartmentalization, protein relocalization in response to stimulus, loss of symmetry, and asymmetric vesicle division. A successful example is the study of Strulson et al. [104], where the intracellular compartmentalization and macromolecular crowding for partitioning RNA was mimicked by using a PEG/dextran ATPS. Interestingly, the RNA was 3,000-fold concentrated in the dextran-rich phase, leading to approximately a 7-fold increase in the rate of ribozyme cleavage. It is important to highlight that the catalytic rate was enhanced by adjusting the relative volume ratio of the ATPS. In our opinion, this is a remarkable example on the use of ATPS in modern biology and biotechnological approaches.

3.2. (Bio)materials Applications

In the last years, new strategies (*e.g.* micropatterning, bioprinting) and materials (*e.g.* biopolymers, hydrogels) have been created, offering solutions for cell

micropatterning and microtissue engineering, new polymer-based technologies (*e.g.* stimuli-responsive, self-assembly structures), and development of novel chemical/electronic devices (discussed above). Teixeira et al. [24] compared and discussed the novel ATPS-based patterning and bioprinting techniques, emphasizing their potential for: (*i*) analysis of cellular growth and cell differentiation; (*ii*) cocultivation of mammalian engineered systems; (*iii*) cocultivation of microbial engineered systems; and (*iv*) self-assembly of cell and tissue construction. All the mentioned ATPS-based strategies take advantage of the interfacial tensions between the coexisting phases (polymer-based systems are the most studied) to confine cells into pre-specified configurations without damaging, by using simple and small-scale approaches. Although all details about these procedures can be found in the review of Teixeira et al. [24], we would like to highlight one or two examples that in our opinion demonstrate the high potential of ATPS over conventional micropatterning/bioprinting processes.

APTS micropatterning technology allows the patterning of multiples types of cells (*e.g.* mammalian and microbial), creating cell exclusion zones and cell islands, further allowing to obtain insights on cell-cell interactions [30]. Frampton et al. [106] mimicked the liver cells function through the cocultivation of hepatocytes and fibroblasts, observing higher production rates of albumin in cocultures than in monocultures of hepatocytes. Similarly, microenvironments and interactions occurring between eukaryotic and prokaryotic cells can also be mimicked by a proper deposition of the microbial cells within ATPS. This approach can overcome incompatible growth conditions of coexisting cells and prevent overgrowth since the cells are confined within the droplet phase. Besides the physical confinement of cells, small molecules and metabolites are freely diffused through the interface, replicating the complex microbial communities environments found in nature [24].

ATPS cell coculture is a promising *in-vitro* platform to reveal interactions between distinct cells (mammalian, microbial, and plant-based ones), for instance by providing insights on the effects of pathogenic bacteria over mammalian cells and by simulating heterocellular realistic environments for mammalian cell proliferation and migration. However, the most exciting perspectives for ATPS are 3D cell cultivation and 3D microtissue. An excellent example is the high-throughput polymeric ATPS-based technique developed by Tavana and collaborators [33,107,108] for printing tumor spheroids at a microscale. The ATPS-printing microtechnology allows the confinement

of cells within droplets of the bottom phase, which are formed by the titration of the bottom phase in a large volume of the top-phase solution. This approach avoids the risk of evaporation of the liquid medium (commonly observed in 2D culture) and allows to control the aggregation of cells into spheroids. The laborious processes of handling, drug treatment and spheroids' analysis were overcome by implementing a robotic system to microprint the spheroids in 384-microwell plates [33,107,108]. The impressive handling and high-throughput capacity of this microtechnology was demonstrated by screening 25 chemotherapeutics and molecular inhibitors against over 7000 tumor spheroids of three cancer cell lines [107]. Other examples of microtissue strategies using ATPS include the use of ATPS to print cell-containing contractile collagen microdroplets (*i.e.* hydrogel-based microtissues) [109], formation and manipulation of cell spheroids using a density adjusted PEG/Dextran ATPS [110], among others [24].

An additional interesting example of ATPS application on the biomaterials field is the use of the PEG/dextran system as an alternative strategy for the preparation of a cell-laden microgel [111]. This microgel is a biomaterial used for various biomedical purposes that is frequently produced using microfluidic oil-water systems. To overcome the poor distribution of crosslinking reagents in the oil phase and low microgel extraction rates, Liu et al. [111] used a co-flow microfluidic ATPS device to form uniform droplets of the microgel precursor reagent, which was then converted to a cell-laden microgel by horseradish peroxidase-catalyzed crosslinking.

The integration of high-throughput and robotic devices for 3D cell cultivation using ATPS demonstrates its potential as low-cost and simple technology for 3D cell cultivation and drug screening using *in vitro* cells models. This approach can offer more representative models of tissues and reduce the number of expensive, time-consuming and controversial animal tests for drug discovery. We think that these examples are good starting points to explore a plethora of opportunities for ATPS in the biomaterials-related fields.

ATPS can additionally be promising solutions for biomaterial synthesis using microfluidic systems. Since droplet microfluidic devices allow the formation and control of controlled-size droplets within a stationary phase entrapped in the microchannel, these have been applied for hydrogel preparation, bioassays, (bio)encapsulation and high-throughput dotted analysis. Previously, a high-throughput ATPS microfluidic approach for drug delivery in encapsulated tumor cells was

highlighted. Boreyko et al. [112] demonstrated the use of ATPS microdroplets with reversible phase transitions to mimic the dynamic and natural microcompartmentalization that occurs within the cytoplasm of cells. Reversible phase transitions from monophasic, biphasic, and core-shell microbead states were obtained by evaporation-induced dehydration and water rehydration.

3.3. Other Non-Conventional Applications

Most of the processes reviewed so-far are bio-based applications. However, few recent pioneering studies have proposed the use of ATPS for alternative and unexpected non-biological purposes. In one of these pioneering works it was proposed the use of IL-based ATPS as membrane-free batteries [113]. The technology proposed by Navalpotro et al. [113] is a remarkable advance on the electrochemical field, since the separation of electrolytes in batteries is not driven by expensive membranes but simply by the intrinsic immiscibility of the coexisting liquid phases. Further advantages of these ATPS-like batteries were demonstrated, such as the control of the cross-migration of the species in the membrane-free batteries, high battery voltage, and coulombic and energy efficiencies.

As the final example, we would like to highlight an ATPS-related technology, patented in 1997 by Rogers et al. [114], which was recently converted into an innovative commercial application for the separation and isolation of technetium radioisotopes (Tc^{99m}) from aqueous solutions containing radioactive or non-radioactive molybdate salts, the RadioGenix™ system, commercialized by NorthStar Medical Radioisotopes, LLC. As proposed by Rogers et al. [115] this application of ATPS resulted from the adaptation of a PEG-based ATPS to a solid Aqueous Biphasic Extraction Chromatography (ABEC®) mode, by grafting high molecular weights PEG polymers to a solid support and using high ionic strength salt solutions as the mobile phase. After almost 20 years, a polymer/salt ATPS is effectively used in a commercial application. As can be found in the website of the NorthStar Medical Radioisotopes, LLC company, *"NorthStar believes that FDA Approval of the RadioGenix® System (technetium Tc99m generator) and non-uranium based Mo-99 production technology marks a new era in nuclear medicine technology by providing a reliable, domestically produced and environmentally friendly Mo-99/Tc-99m supply for the United States."* From our perspective, this is the perfect example on how an original academic concept was transformed into a commercially viable application.

Final Remarks and Outlooks

An overview on the disruptive technologies and emerging applications of ATPS that, from our perspective, exhibit highest potential for future implementation or commercialization was here provided. As discussed in the various sections, ATPS share a series of outstanding properties that, even after 70 years of studies, still maintain their potential for the development of novel technologies. However, only a few examples of effective industrial application of ATPS are known. As with other bioseparation technologies, the implementation of new industrial/commercial processes faces the common risks and restrictions of installation, development, validation, and new operation costs. However, other ATPS barriers are also prevalent, particularly the poor understanding of the partition/separation mechanisms, limited number of predictive models and large-scale studies, commercial equipment for such a purpose, as well as the cost and large volume of some phase-forming components and their inability for recycling.

Future ATPS academic studies that aim to make the difference must address new approaches and applications, by incorporating the innovative and disruptive nature of the biotechnology, health and materials science fields to provide advanced and marketable technologies. From our perspective, the competitive advantages of ATPS, like process integration, continuous processing, biocompatibility, sustainability, and recyclability, need to be converted into simple, accessible and automatized industrial technologies/processes, which should bring effective (not residual) improvements in comparison to the current commercial technologies. For that purpose, we advise the scientific community to gather significant insights on molecular-level mechanisms ruling phase formation and solutes partition, to address the use of ATPS in continuous processes, to design new ATPS-specific and integrative apparatus, and to demonstrate the cost-effective and sustainable nature of ATPS over the well-known and established techniques. Continuous and integrated processes, high-throughput robotic, and life cycle assessment should be always considered to foster marketable applications.

We believe that new technological-based platforms using ATPS will effectively become commercial, particularly in the purification of some bio-based products (such as virus like particles, membrane proteins, cells, nucleic acids fragments) for which chromatographic solutions are currently noncompetitive, in integrative hydrometallurgy and urban mining procedures, as pre-treatment strategies for environmental monitoring,

and (bio)analytical and diagnostic approaches. It is however in new and emerging fields that relies the future of ATPS research. The number of ATPS non-conventional applications will certainly grow in the next years, attracting the interest of researchers from non-separation fields. Although we believe that the creativity and curiosity of these researchers, less-familiar with the concept of ATPS, can significantly contributes toward the development of "out-of-the-box" ATPS applications, we advise the following: *i*) to understand the fundamental and conceptual science behind the ATPS creation; *ii*) to select the most adequate systems according the application of interest from the extensive number of ATPS available in the literature; *iii*) to define the operational conditions and processual parameters considering the target-bioproduct or application – different conditions and parameters, such as temperature, pH, processing and sampling mode, volumes and quantities, affect directly the ATPS equilibrium and/or phases' separation time; *iv*) to be innovative but always considering the thermodynamic equilibrium and physicochemical properties of these systems; *v*) to take into account that the compound in higher content in ATPS should be water; *vi*) to address the biocompatibility and cost of the remaining phase-forming components; and *vii*) to consider and compare if the designed ATPS process is competitive with current technologies used for the same purpose.

We hope that this perspective will bring to the ATPS research community the enthusiasm to move forward to a new level and untrodden paths, as well as to stimulate researchers from other fields to bring creativity to this field.

Acknowledgements

This work was developed within the scope of the Young Researcher Project (2014/16424-7) funded by FAPESP (São Paulo Research Foundation Brazil). J.F.B. Pereira acknowledges financial support from CNPq (National Council for Scientific and Technological Development, Brazil) and CAPES (Coordination of Superior Level Staff Improvement, Brazil) (financial code 001). This work was also developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2019), financed by national funds through the FCT/MCTES, and Project POCI-01-0145-FEDER-030840 funded by FEDER, through COMPETE2020-Programa Operacional Competitividade e Internacionalização (POCI), and through FCT/MCTES.

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