Development of a Microfluidic Platform for R-Phycoerythrin Purification Using an Aqueous Micellar Two-Phase System

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ABSTRACT: Temperature-dependent aqueous micellar two-phase systems (AMTPSs) have recently been gaining attention in the isolation of high-added-value biomolecules from their natural sources. Despite their sustainability, aqueous two-phase systems, and particularly AMTPSs, have not been extensively applied in the industry, which might be changed by applying process integration and continuous manufacturing. Here, we report for the first time on an integrated microfluidic platform for fast and low-material-consuming development of continuous protein purification using an AMTPS. A system comprised of a microchannel incubated at high temperature, enabling instantaneous triggering of a two-phase system formation, and a microsettler, allowing complete phase separation at the outlets, is reported here. The separation of phycobiliproteins and particularly the purification of R-phycoerythrin from the contaminant proteins present in the aqueous crude extract obtained from fresh cells of Gracilaria gracilis were thereby achieved. The results from the developed microfluidic system revealed that the fractionation performance was maintained while reducing the processing time more than 20-fold when compared with the conventional lab-scale batch process. Furthermore, the integration of a miniaturized ultrafiltration module resulted in the complete removal of the surfactant from the bottom phase containing R-phycoerythrin, as well as in nearly twofold target protein concentration. The process setup successfully exploits the benefits of process intensification along with the integration of various downstream processes. Further transfer to a meso-scale integrated system would make such a system appropriate for the separation and purification of biomolecules with high commercial interest.

KEYWORDS: microfluidics, protein separation, ultrafiltration, process intensification, aqueous micellar two-phase systems

INTRODUCTION

Over the past two decades, microfluidics has significantly contributed to the development of many research areas and changed the paradigm of industrial chemical production design. It has been successfully applied in several fields including life sciences and medicine,1−3 as well as in process engineering.4,5 In recent years, its implementation in downstream processing has been gaining attention as it can enhance the separation rates and improve the purification efficiencies.6−8

Microfluidic units are devices that have at least one characteristic dimension under the size of one millimeter, typically in the range between 50 and 500 μm. One of the consequences of device miniaturization is an extremely high surface to volume ratio and, thus, a very efficient heat transport through the walls. Therefore, microfluidic devices can efficiently control the temperature, which can be crucial when dealing with highly endothermic or exothermic reactions, as well as for the process systems demanding fast temperature changes between the subsequent steps.9 Since microreactor volumes are typically in the microliter scale, less material and time is needed for
process optimization.\textsuperscript{10} Besides, diffusion takes place in a much shorter distance and mass transfer is thus significantly improved as compared to conventional reactors,\textsuperscript{11} especially in multiphase systems. Microflow processing allows also for efficient flow regime control and precise residence time determination; therefore, high process repeatability can be achieved.\textsuperscript{12} A continuous operation mode enables higher productivities and integration of several sequentially linked devices, so multiple steps can be performed without cleaning the devices during different stages of the process and transferring the material between the vessels.\textsuperscript{13−15} This further simplifies the downstream operation and speeds up the whole process.\textsuperscript{16−18}

Process intensification by device miniaturization can overcome crucial drawbacks of many conventional separation processes requiring large solvent consumption and long residence times due to poor mixing.\textsuperscript{17} Therefore, continuous processing and process intensification have been recognized as the key green engineering areas paving the way toward more sustainable technological platforms in pharma and fine chemicals production.\textsuperscript{19} However, despite its potential, the microfluidics-based preparative separation/purification of biomacromolecules is still seldom explored in the literature and even less on the industrial scale.\textsuperscript{20} For the latter, meso-flow devices where the throughput increase is achieved by internal "scale-out" or "numbering-up" present an ideal approach enabling fast transfer from lab- to industrial-scale production without losing the advantages of the microchannels.\textsuperscript{4,20}

Among the downstream process techniques suitable for separation and purification of biomolecules, downstream processes applying aqueous two-phase systems (ATPSs) could be an alternative to conventional chromatographic approaches.\textsuperscript{17,18,21−23} As coacervation systems, they mostly provide an aqueous environment and mild operational conditions, allowing the molecule to retain its native conformation, which makes them attractive alternatives also for enzymatic synthesis in flow.\textsuperscript{24} Recently, aqueous micellar two-phase systems (AMTPSs) as a special version of ATPSs have been introduced.\textsuperscript{17,18,21} They are composed of water and small amounts of a nonionic surfactant, creating a benign, more biocompatible, and cheap extraction system.\textsuperscript{25,26} Their application in the separation of proteins from cell extracts containing high amounts of water, such as macroalgae with water contents over 85%,\textsuperscript{27} has high potential. As recently reported, the use of AMTPSs may overcome some of the drawbacks associated with the processing of fresh algal cells, which is one of the recurrent bottlenecks associated with algae processing.\textsuperscript{17,18,26}

A crucial feature of AMTPSs is their temperature dependency as they only form two phases by the proper temperature manipulation.\textsuperscript{25,28} When the temperature reaches a value above the cloud point, two macroscopic phases are formed, allowing the creation of two very distinct environments in terms of hydrophobicity,\textsuperscript{28} which can be further manipulated by the introduction of salts, cosurfactants, or ionic liquids.\textsuperscript{25,29} AMTPSs have proven to be helpful in the purification of antibodies,\textsuperscript{30} natural colorants,\textsuperscript{27} and proteins,\textsuperscript{16,28,31} and included in the latter, R-phycocerythrin (R-PE).\textsuperscript{28} R-PE is one of the most abundant phycobiliproteins with the highest commercial importance. In the last decades, natural photosynthetic pigments like R-PE have been recognized as biologically active molecules with many health benefits.\textsuperscript{32,33} R-PE is a fluorescent protein found in red seaweed.\textsuperscript{34} It acts as an auxiliary photosynthetic pigment in red macroalgae, showing also recognized anti-inflammatory and antioxidant activities, and can thus be incorporated into pharmaceutical and cosmetic formulations.\textsuperscript{35} It has been used as a fluorescence-based marker in cell biology and immunology and as a natural food colorant.\textsuperscript{35} R-PE is thus a valuable and expensive protein when in a highly pure form (from 155 €·mg⁻¹ as a colorant and up to 664 €·mg⁻¹ as antibody–R-PE complexes).\textsuperscript{36} It is currently produced by a lengthy and costly multistep approach, consisting of water leaching, ammonium sulfate staged precipitation, and ionic exchange chromatography.\textsuperscript{37,38} In our previous work, the extraction and purification of R-PE from fresh macroalgae were performed using an AMTPS on a macroscale batch system, achieving an extraction yield of 82.6 ± 0.4% and a selectivity of 5.9.\textsuperscript{26} Yet, the separation using AMTPSs has never been performed in a continuous operation mode, which could improve the potential application of AMTPSs at an industrial scale.

To harness the benefits of continuous operation and process intensification by miniaturization, this work aimed to develop an AMTPS for the purification of R-PE from algal extracts using a microfluidic system comprising several process units. R-PE was used in this work as a model biomolecule. The main reason for using it was the previously gathered knowledge regarding its purification using AMTPS in a batch process.\textsuperscript{26} Moreover, the use of the extract obtained from the red macroalgae allowed us to study the effects of a complex real sample instead of using a synthetic protein solution, contrary to the common practice that has been adopted.\textsuperscript{18} As the use of microfluidics enables precise temperature control and consequent fast temperature change needed for the phase separation when AMTPSs are applied, as well as the integration of subsequent phase separation, significant improvements in the R-PE purification process were expected. Furthermore, the microfluidic setup developed in this study can be used as a high-throughput screening platform for further optimization of the AMTPS for R-PE purification, as well as for other biomolecules of interest. Merging these green solvent systems with such novel process development techniques might significantly contribute to their more extensive use both in industry and in research.39

### EXPERIMENTAL SECTION

**Materials.** The red macroalgae 	extit{Gracilaria gracilis} were kindly provided by ALGAPlus, Lda, Ilhavo, Portugal. Tergitol 15-S-7 (laboratory grade) was acquired from Sigma-Aldrich (St. Louis), while the McIlvaine buffer components, namely, Na₃HPO₄·2H₂O (≥99.5%), citric acid monohydrate (≥99.5%), and R-PE standard aqueous solution (≥100 mg·mL⁻¹), were obtained from Merck (Darmstadt, Germany). Milli Q (MQ) water was used for the preparation of all solutions and extracts. For the microfluidic setup, glass microchips (Micronit Microfluidics B.V., Enschede, the Netherlands) were used with channel dimensions of 150 μm width, 150 μm height, and 332 or 676 mm length; two microsettlers (V = 380 and 1290 μL) and an ultrafiltration (UF) module (V = 87 μL) were both developed in-house. The settlers consisted of a stainless-steel plate into which a triangular hole with 2 mm depth was incised, and covered with a poly(methyl methacrylate) (PMMA) plate. For the UF module, the housing was from two PMMA plates, the gaskets were cut from an expanded poly(tetrafluoroethylene) (ePTFE) film (GORE-TEX, Gore & Associates, Inc.) of 0.5 mm thickness, while the membranes used were either made of regenerated cellulose (RC) or polyethersulfone (PES), both with a molecular weight cutoff (MWCO) of 100 kDa Ultragel PLH90095 (RC) and Biomax PBHK07610 (PES), both from Millipore Company, Billerica. Perfluoroalkoxy (PFA) tubes with an inner diameter of 500 μm (VICI AG International, Schenkon, Switzerland) were used, and the syringe pumps PHD 4400 were used.
supplied by Harvard Apparatus, Holliston. The tubes were connected with microsettlers and a UF module via high-pressure polyether-etherketone (PEEK) tube fittings (Vici AG International, Schenkon, Switzerland). UV–vis spectrophotometer Shimadzu UV-2600 (Shimadzu Scientific Instruments, Columbia, MA) was used for the absorption spectra measurements.

**Algae Preparation and Solid–Liquid Extraction.** Fresh *G. gracilis* biomass used in this work was cultivated by ALGAPlus—Produção e Comercialização de Algas e seus Derivados, a company specialized in the production of marine macroalgae, located in Ilhavo, Portugal. The algae grew in a land-based integrated multitrophic aquaculture system with high nitrogen content due to the use of aquaculture in the wintertime when the R-PE content is higher than that during the summer. Samples were washed three times with freshwater and once with demineralized water. The biomass was then stored at –20 °C until further use. The solid–liquid extraction using MQ water was carried out as previously described.25

**AMTPS Characterization.** The density of the phases before and after separation was measured using a benchtop density meter DMA 5000 (Anton Paar GmbH, Graz, Austria). The rheograms were obtained using a rotational rheometer Physica MCR 302 comprising a concentric-cylinder measuring system and a CP50/1° cone-plate (Anton Paar GmbH, Graz, Austria).

**AMTPS-Based Batch Purification.** A tergitol 15-S-7-based AMTPS was prepared by mixing 10 or 20 wt % algal crude extract, 10 wt % surfactant, and 0.18 M McIlvaine buffer. AMTPS was prepared by mixing 10 or 20 wt % algal crude extract, 10 wt % surfactant, and 0.18 M McIlvaine buffer.

**AMTPS-Based Purification in a Microfluidic System.** As shown in Figure S1 in the Supporting Information, a syringe containing a homogeneous micellar solution inside a syringe at 25 °C. After separation, the two phases were then carefully separated, and their weights and volumes were determined.

**Ultrafiltration.** To further concentrate the R-PE solution, miniaturized tangential ultrafiltration (UF) module was constructed.
as schematically represented in Figure S3 in the Supporting Information. A membrane of different materials was placed between the two spacers carved out of a PTFE film, which were further squeezed between two PMMA plates with two inlets and two outlets to form a 300 μm depth channel on each side of the membrane. The membrane had a filtration surface of 144 mm² and the UF module had a volume of 87 μL. For the UF, the bottom solution from the microsettler was pumped to the module, the retentate was pumped out using the syringe pump, and both aqueous phases were determined to estimate the apparent viscosity and density data of the homogeneous phase after purification had been stopped.

The efficiency of the UF was evaluated by the rejection coefficient of the UF membrane, which was determined according to eq 5.

\[ \text{rejection coefficient} = 1 - \frac{[\text{R-PE}_{\text{permeate}}]}{[\text{R-PE}_{\text{retentate}}]} \quad (5) \]

where \([\text{R-PE}_{\text{permeate}}]\) and \([\text{R-PE}_{\text{retentate}}]\) are the R-PE concentrations (mg·mL⁻¹) of the permeate and the retentate, respectively. The concentration factor was calculated as the ratio between the R-PE concentration in the retentate and its concentration in the bottom phase after purification had been stopped.

**Visual Characterization of the Formation of an AMTPS.** For visual characterization of the two-phase system formation, a 0.5 mm wide PFA tube incubated in a water bath at 40 °C and connected with the exit of the microchannel incubated at 60 °C was positioned under a light microscope with an integrated high-speed camera Motion-Scope HSMS-226-2 (Motion Scope, Lukovica, Slovenia).

**R-PE Concentration Analysis.** Estimation of the R-PE concentration (mg·mL⁻¹) in samples based on the measurements of absorbance spectra between 200 and 700 nm was assessed using a UV-vis spectrophotometer. Since R-PE has the characteristic maximum peak at 564 nm and minimum peaks at 455 and 592 nm, these absorbances were used to estimate its concentration in an aqueous solution as suggested by Beer and Eshel.41 Due to the interference of AMTPS compounds, the concentration of R-PE was calculated using the modified Beer and Eshel equation

\[ [\text{R-PE}] = \frac{1}{[\text{A}_{564} - A_{492}]} \times 0.20437(A_{492} - A_{455}) \epsilon \quad (6) \]

where the absorptivity coefficient (ε) values were determined to be 0.12355, 0.13270, and 0.12840 for the inlet solution, top, and bottom phases, respectively. The absorptivity coefficient for each phase was defined using an R-PE standard solution in the concentration range from 0.05 to 0.2 mg·mL⁻¹. A blank control was used for each phase, i.e., an identical system with water instead of the sample containing R-PE.

**RESULTS AND DISCUSSION**

**AMTPS Characterization.** As mentioned above, AMTPSs are temperature-dependent systems and thus the phase separation is triggered by the temperature increase. The binodal curve of the Tergitol-based AMTPS has been previously reported and has shown that temperatures above 38 °C result in a two-phase system creation at the surfactant concentration and pH selected in this study.25 In this sense, a temperature of 40 °C was selected to perform the batch purification of R-PE. Additionally, our previous results have shown that R-PE maintains its structural integrity at this temperature.26 Besides, the apparent viscosity and density data of the homogeneous phase and both aqueous phases were determined to estimate the fluid flow regime in the channels.

As evidenced from Table 1, the density of all three phases was close to the values for water, while the viscosity of the top phase, which is rich in surfactant, was more than 20 times higher than that measured for the bottom/surfactant-poor phase.

**AMTPS-Based Batch Purification.** AMTPS-based batch purification was performed using two different concentrations of crude algal extracts in the system, namely, 10 and 20 wt %. At least half an hour of incubation time at 40 °C was required for the phase separation to be observed macroscopically, which was in accordance with our previous work with a Tergitol 15-S-7-based AMTPS.26 Table 2 shows the results of recovery of R-PE in the bottom/surfactant-poor phases after the complete separation of phases for 4 h (or 240 min) of incubation at 40 °C. These data are compared for both crude algal extract concentrations. The main results suggest an increase in the amount of phycobiliproteins in the system, which led to an increase in both the recovery and selectivity parameters. However, the relatively slow phase separation could be accelerated by expediting the temperature increase to reach the temperature of a two-phase formation, but at the same time prevent protein denaturation by fast cooling, which, in a batch system, is hampered by the relatively inefficient heat transfer.

**AMTPS Formation and Separation in a Microfluidic Device.** Microscale systems have several orders of magnitude higher surface to volume ratios than conventional batch vessels, which enables very efficient heat transfer and, consequently, fast temperature changes. Thus, our approach was developed by exposing the homogeneous crude extract solution to a temperature shock at 60 °C for just a few seconds. This was expected to be enough to trigger and accelerate the phase separation without compromising the viability of the phycobiliproteins, and particularly, R-PE.

The change from batch to a continuous process was first performed using the microflow system presented in Figure S1 in the Supporting Information comprising a 676 mm long microchannel incubated at 60 °C, a 97 cm long PFA tube, and a microsettler of 380 μL volume, all incubated at 40 °C. Regarding the flow rates used, the residence times in the microchannel incubated at 60 °C were between 30 and 91 s. Further phase separation occurred in a tube connected with a vertically positioned microsettler (Figure S1 in the Supporting Information), yielding an overall residence time between 18.9 and 58 min. The top/surfactant-rich phase was pumped out from the microsettler using a syringe pump due to its much higher viscosity in comparison with the bottom/surfactant-poor phase.
phase (Table 1), which freely flows from the system. However, at flow rates above 30 μL·min⁻¹, the phase separation was not efficient, as the bottom phase contaminated the outlet of the top phase. This was related to the very small difference in densities between both phases (Table 1) and the small size and right-triangle geometry of the microsettler.

To overcome this problem, a larger microsettler with a final volume of 1290 μL was designed as an isosceles triangle, where the outlets were located at the vertices of both long sides (see the close-up in Figures 1 and S2 in the Supporting Information). By increasing the microsettler volume by 3.4-fold and, despite the size reduction of the connecting tube (44 cm), the phase separation was efficient up to a flow rate of 120 μL·min⁻¹. At this flow rate, it was possible to achieve a total residence time close to 12 min, of which 4 s were inside the microchip at 60 °C, 43 s inside the PFA tube at 40 °C, and 10.8 min inside the microsettler.

As shown in Figure 2 and in the Video Supporting Information presenting the annular flow regime with small droplets in a PFA tube exiting the microchip, only 4 s incubation in a microflow system at 60 °C enabled the formation of two phases. By following the fluid flow at various distances from the entrance in the PFA tube and thereby at various residence times, it is evident that the more viscous top phase, which is also more hydrophobic due to the higher surfactant concentration than the bottom phase, positions toward the hydrophobic PFA wall with residence times from the entrance of the microchannel to the point of visual inspection in a tube incubated at 40 °C were (a) 19 s, (b) 29 s, and (c) 49 s.

Figure 2. Photo of a two-phase flow in a tube connected with the exit from the microchannel, incubated for 4 s at 60 °C, confirms the formation of an AMTPS. Applying the flow velocity of 120 μL·min⁻¹, the residence times from the entrance of the microchannel to the point of visual inspection in a tube incubated at 40 °C were (a) 19 s, (b) 29 s, and (c) 49 s.

Continuous AMTPS-Based Purification of R-PE in a Microfluidic Device. The R-PE purification from the phycobiliprotein crude extract was investigated in the microfluidic system presented in Figure 1. The performance was evaluated by the R-PE recovery and selectivity in the bottom/surfactant-poor phase and compared with the batch system (Table 2). In the microflow device, the purification was achieved in only 12 min. This allowed us to conclude that comparable selectivity and recovery data were obtained for the miniaturized system when compared with the batch system (240 min) but requiring much lower times (Figure 3). On comparing the two tested crude algal extract concentrations, again the systems showed similar performances for both modes of operation. In both cases, it was possible to process higher amounts of crude extract without compromising system performance. Thus, 20 wt % of the crude extract with R-PE was selected for further studies. New studies of the microflow-based purification system were performed at various flow rates, for which the results of our previous work with Tergitol 15-S-7-based AMTPS were used for comparison.²⁶ The performance of the continuous system was evaluated by the R-PE recovery in the surfactant-poor and surfactant-rich phases (Table 3). As expected, at longer process times, the R-PE recovery in the surfactant-poor phase was higher, reaching a maximum of 79 ± 6%. Yet, by increasing the flow rate to 120 μL·min⁻¹, it was possible to reduce the processing time to almost 6 min while still maintaining an R-PE recovery in the top phase above 75%. However, when the processing time was further reduced by increasing the flow rate to 200 μL·min⁻¹, the outlet phases could not be efficiently separated.

Higher flow rates resulted also in ca. 30% of R-PE losses since R-PE partitions toward the top phase (Table 3). As shown in Figure 4, the phase separation in the microsettler at various inlet solution flow rates, it was impossible to establish a clear interface at flow rates above 120 μL·min⁻¹, which was confirmed by the evidence of incomplete phase separation at the outlets. Again, a very small difference in the density could be used to explain less effective phase separation at lower residence times in the microsettler. Therefore, the flow rate of 120 μL·min⁻¹ was selected for further experiments.

Table 3. Flow Rate Influence on the Extraction Time and R-PE Recovery in the Top and Bottom Phases Tested for 20 wt % of the Extract

| flow rate (μL·min⁻¹) | extraction time (min) | bottom (surfactant-poor) phase | top (surfactant-rich) phase |
|----------------------|-----------------------|-------------------------------|-----------------------------|
| 80                   | 17.3                  | 79 ± 6                        | 21 ± 6                      |
| 120                  | 11.6                  | 76 ± 7                        | 24 ± 7                      |
| 160                  | 8.7                   | 73 ± 7                        | 27 ± 7                      |
| 200                  | 7.4                   | 72 ± 6                        | 28 ± 6                      |

Figure 3. R-PE recovery in the bottom/surfactant-poor phase for 10 and 20 wt % of aqueous phycobiliprotein crude extracts considering continuous versus batch processing. The line represents the selectivity of R-PE.
Setup of a Microflow Ultrafiltration Module and Its Characterization. Envisioning higher purity levels required by some applications, after the purification of R-PE, polishing of the target protein was achieved by the integration of an ultrafiltration module at the surfactant-poor phase outlet. With this, the system allowed us to separate the phycobiliproteins from the solvent, further concentrating and purifying them. During the experimental setup, different layouts of the ultrafiltration module were tested regarding the position of the feeding solution, the flow rates applied, and the membranes used. The best results were obtained when the module was standing upright with the feeding solution coming from the upper side and the retentate being pumped out from the underside with half the flow of the feeding solution. Further details on this study can be obtained in the Supporting Information (Figure S3).

The formation of a protein layer near the membrane is a complex process influenced by electrostatic and hydrophobic interactions as well as by the hydrodynamic properties of the module.41 When the flow rate at the retentate side was decreased in comparison to inflow, the protein layer became thicker and the mass transfer to the permeate side was aggravated. The instability of surface deposition was assumed since the white membrane displayed an intense pink color at the bottom of the module after the flow had been stopped for some time (Figure S4 in the Supporting Information). When the flow of the filtered solution came from the upper side, some of the unstable protein deposits fell off the membrane, leaving the filtration module at the bottom side, where they were withdrawn directly to the retentate container. This way, the protein layer maintains approximately the same thickness over a longer period. Once the ultrafiltration module was set up, two different types of membranes were studied, namely, one composed of polyethersulfone (PES) and another made of regenerated cellulose (RC), both with a molecular weight cutoff (MWCO) of 100 kDa. The results presented in Table 4 showed that the PES membrane displayed better performance than the membrane based on RC, which allowed the transport of considerable amounts of R-PE to the permeate side, as shown in Figure 5. In this case, the permeate was pinkish, which was not the case for the PES membrane.

In both cases, the theoretical concentration factor was 2, while the obtained concentration factors were lower (Table 4). This was due to the losses associated with the permeation through the

Table 4. Comparison of All Tested Membranes Using 20 wt % of Phycobiliprotein Crude Extract

| material      | membrane cutoff (kDa) | rejection coefficient$^a$ | concentration factor$^b$ | selectivity |
|---------------|-----------------------|---------------------------|--------------------------|-------------|
| regenerated cellulose | 100                   | 0.7 ± 0.1                 | 1.4 ± 0.1                | 3.4 ± 0.1   |
| polyethersulfone    | 100                   | 0.93 ± 0.03               | 1.78 ± 0.02              | 4.1 ± 0.2   |

$^a$Percentage of R-PE retained by the membrane in the retentate side. $^b$Ratio between R-PE concentrations in the retentate and feed.
membrane or retention on and within the membrane itself. The best result was obtained for the PES membrane with losses of R-PE lower than 7%. A smaller concentration factor in the case of the RC membrane can be attributed to the leakage on the permeate side (around 33%).

Based on the results obtained with both membranes, it is assumed that most of the contaminant proteins present in the bottom phase are bigger than 100 kDa. Electrostatic and hydrophobic interactions play an important role in ultrafiltration since both membranes had the same MWCO and yet showed different performances. In sum, the nature of the membrane seems to be a crucial parameter, and for this case, the PES membrane was the best choice. When applying the RC membrane, the selectivity of the overall process was 7% worse when compared to the process without UF (selectivity = 3.6 ± 0.5). In contrast, using the PES membrane, the selectivity of the complete process has increased by 13%. Moreover, the dual function of the ultrafiltration unit was proven with the increase in the R-PE concentration and the complete removal of the surfactant from the fluorescent protein, as corroborated by FTIR analysis (see Figure S6).

**Integrated Process in a Microflow System.** To achieve a continuous phase separation and further concentration, an integrated process schematically presented in Figure 6 and visualized in Figure S5 (Supporting Information) was designed and tested. The process comprised of three main steps, namely, (i) (conventional) solid–liquid extraction to recover the phycobiliproteins from fresh *G. gracilis* cells, followed by the (ii) AMTPS microfluidic unit to separate the phycobiliproteins from the contaminant proteins, ending with (iii) an ultrafiltration unit to isolate the phycobiliproteins (and R-PE in particular) from the solvents and surfactants used. A detailed description and a photo are available in the Supporting Information (Figure S5). In brief, aqueous crude extract was mixed with the components to form the AMTPS inside the microfluidic device. The mixture was then introduced into the chip at a flow rate of 120 μL·min⁻¹, exposed to a temperature shock of 60 °C for 4 s, allowing phase separation (Figure 2) but preventing protein degradation and/or precipitation. Then, during 12 min of processing time, the system temperature was equilibrated at 40 °C to reach complete phase separation, while allowing the recovery of 79% of R-PE into the surfactant-poor phase and 44% of total proteins in the surfactant-rich phase, surpassing by 8% the batch process. The integration of an ultrafiltration module into the process further allowed to double the concentration of R-PE in the final solution and improved the selectivity of the process by 13% when using a PES membrane. Briefly, it means that in each gram of macroalgae samples having around 85–90% of water, 0.1–0.15 g of macroalgae cells is present. Considering the worst scenario of only 0.1 g of cells per gram of fresh macroalgae and, taking into account the proteomic analysis done in our previous work,²⁶ we may infer about the efficiency of the miniaturized process proposed. The initial extract used in this work had 59.4% of R-PE, which represents 0.0594 g of R-PE/0.1 g dry biomass. With losses of 30%, this process allowed us to obtain 416 mg of R-PE/g dry biomass.

Overall, the implementation of the microfluidic system allowed the development of a continuous process that was 20 times faster than the batch scale. Furthermore, the microfluidic technology offers the opportunity for an easier scale-up by numbering up the process.⁴,⁵,¹⁰,¹⁷,¹₈

**CONCLUSIONS**

This pioneering work reports the efficient implementation of a microfluidic system for the continuous AMTPS-based purification of R-PE from an algal extract by applying the process intensification and integration strategy. The possibility to achieve very fast temperature changes in a microchannel enabled us to trigger the instantaneous formation of an AMTPS, something impossible at a macroscale due to much slower heat transfer, which could also lead to protein denaturation. By developing an effective microsettler allowing high flow rates of inlet solution, the complete phase separation at the outlets of the system was successfully achieved. By making use of the advantages of the microfluid flow, we achieved the efficient purification of R-PE from a real crude extract in a continuous process. The data provided evidence that in the continuous system using microfluidics, it was not only possible to maintain the fractionation performance achieved in the batch process but also to reduce the processing time more than 20-fold. By further integrating a miniaturized ultrafiltration unit, the microflow system allowed the separation of the phycobiliproteins from the solvent, further concentrating them 1.8-fold and improving their selectivity by 13% in 20 times less time than in the batch process. The miniaturized purification system developed in this work is perfectly suited for further optimization and adaptable to other AMTPSs, with low material and time consumption. Furthermore, a transfer to a meso-scale integrated system such as that reported for the two-phase biocatalytic process²⁷ would enable a very efficient throughput increase, enabling larger-scale purification of this valuable protein or other industrially relevant biomacromolecules like antibodies or biobetters.
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c05042.

**Notes**
The authors declare no competing financial interest.

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**ABBREVIATIONS**
AMTPS, aqueous micellar two-phase system; ePTFE, expanded polytetrafluoroethylene; MWCO, molecular weight cutoff; MQ, milli Q; PEEK, polyetheretherketone; PES, polyethersulfone; PMMA, poly(methyl methacrylate); RC, regenerated cellulose; R-PE, R-phycocerythrin; UF, ultrafiltration; V, volume

**REFERENCES**
(1) Suea-Ngam, A.; Howes, P. D.; Srissart, M.; de Mello, A. J. Droplet Microfluidics: From Proof-of-Concept to Real-World Utility? Chem. Commun. 2019, 55, 9895–9903.
(2) Mannino, R. G.; Pandian, N. K. R.; Jain, A.; Lam, W. A. Engineering ‘Endothelialized’ Microfluidics for Investigating Vascular and Hematologic Processes Using Non-Traditional Fabrication Techniques. Curr. Opin. Biomed. Eng. 2018, 5, 13–20.
(3) Whitesides, G. M. The Origins and the Future of Microfluidics. Nature 2006, 442, 368–373.
(4) Jensen, K. F. Flow Chemistry—Microreaction Technology Comes of Age. AIChE J. 2017, 63, 858–869.
(5) Žnidarič-Plazl, P. Biotransformations in Microflow Systems: Bridging the Gap between Academy and Industry. J. Flow Chem. 2017, 7, 111–117.
(6) Wang, K.; Luo, G. Microfluid Microextraction: A Review of Recent Development. Chem. Eng. Sci. 2017, 169, 18–33.
(7) Ferreira-Faria, D.; Aires-Barros, M. R.; Azevedo, M. Continuous Aqueous Two-Phase Extraction: From Microfluidics to Integrated Biomanoufacturing. Fluid Phase Equilib. 2020, 508, No. 112438.
(8) Rodríguez-Ruiz, I.; Babenko, V.; Martínez-Rodríguez, S.; Gavira, J. A. Protein Separation under a Microfluidic Regime. Analyst 2018, 143, 606–619.
(9) Pohar, A.; Plazl, I. Process Intensification through Microreactor Application. Chem. Biochem. Eng. Q. 2009, 23, 537–544.
(10) Žnidarič-Plazl, P. The Promises and the Challenges of Biotransformations in Microflow. Biotechnol. J. 2019, 14, No. 1800580.
(11) Günther, A.; Jensen, K. F. Multiphase Microfluidics: From Flow Characteristics to Chemical and Materials Synthesis. Lab Chip 2006, 6, 1487–1503.
(12) Atencia, J.; Beebe, D. J. Controlled Microfluidic Interfaces. Nature 2005, 437, 648–655.
(13) Wohlgemuth, R.; Plazl, I.; Žnidarič-Plazl, P.; Gernaey, K. V.; Woodley, J. M. Microscale Technology and Biocatalytic Processes: Opportunities and Challenges for Synthesis. Trends Biotechnol. 2015, 33, 302–314.
(14) Shallan, A. I.; Priest, C. Microfluidic Process Intensification for Synthesis and Formulation in the Pharmaceutical Industry. Chem. Eng. Prog. 2019, 142, No. 107559.
(15) Hood, R. R.; Vreeland, W. N.; De Voe, D. L. Microfluidic Remote Loading for Rapid Single-Step Liposomal Drug Preparation. Lab Chip 2014, 14, 3359–3367.
(16) Marques, M. P. C.; Fernandes, P. Microfluidic Devices: Useful Tools for Bioprocess Intensification. Molecules 2011, 16, 8368–8401.
(17) Angel, P.; Ortega, E. G.; Tsaoalidis, D.; Eare, M. Intensified Liquid-Liquid Extraction Technologies in Small Channels: A Review. Johnson Matthey Technol. Rev. 2019, 63, 299–310.
(18) Vicente, F. A.; Plazl, I.; Ventura, S. P. M.; Žnidarič-Plazl, P. Separation and Purification of Biomacromolecules Based on Microfluidics. Green Chem. 2020, 22, 4391–4410.
(19) Jiménez-González, C.; Poechhauer, P.; Brotkerman, Q. B.; Yang, B.-S.; am Ende, D.; Baird, J.; Bertsch, C.; Hannah, R. E.; Dell’Orco, P.; Noorman, H.; et al. Key Green Engineering Research Areas for Sustainable Manufacturing: A Perspective from Pharmaceutical and Industrial Perspectives. Lab Chip 2014, 14, 3359–3367.
Fine Chemicals Manufacturers. *Org. Process Res. Dev.* **2011**, *15*, 900–911.

(20) Lavric, E. D.; Woehr, P. Advanced-FlowTM Glass Reactors for Seamless Scale-Up. *Chem. Today* **2009**, *27*, 45–48.

(21) Glembin, P.; Racheva, R.; Kerner, M.; Smirnova, I. Micelle mediated extraction of fatty acids from microalgae cultures: Implementation for outdoor cultivation. *Sep. Purif. Technol.* **2014**, *135*, 127–134.

(22) Racheva, R.; Tietgens, N.; Kerner, M.; Smirnova, I. In situ continuous countercurrent cloud point extraction of microalgae cultures. *Sep. Purif. Technol.* **2018**, *190*, 268–277.

(23) Roque, A. C. A.; Pina, A. S.; Azevedo, A. M.; Aires-Barros, R.; Jungbauer, A.; Di Profio, G.; Heng, J. Y. J.; Haigh, J.; Ottens, M. Anything but Conventional Chromatography Approaches in Bioseparation. *Biotechnol. J.* **2020**, *15*, No. 1900274.

(24) Vobecaká, L.; Ticha, L.; Atanasova, A.; Slouka, Z.; Hasal, P.; Pirbl, M. Enzyme synthesis of cephalxin in continuous-flow microfluidic device in ATPS environment. *Chem. Eng. J.* **2020**, *396*, No. 125236.

(25) Vicente, F. A.; Cardoso, I. S.; Sintra, T. E.; Lemus, J.; Marques, E. F.; Ventura, S. P. M.; Coutinho, J. A. P. Impact of Surface Active Ionic Liquids on the Cloud Points of Nonionic Surfactants and the Formation of Aqueous Micellar Two-Phase Systems. *J. Phys. Chem. B* **2017**, *121*, 8742–8755.

(26) Vicente, F. A.; Cardoso, I. S.; Martins, M.; Gonçalves, C. V. M.; Dias, A. C. R. V.; Domingues, P.; Coutinho, J. A. P.; Ventura, S. P. M. R-Phycocerythrin Extraction and Purification from Fresh Gracilaria Sp. Using Thermo-Responsive Systems. *Green Chem.* **2019**, *21*, 3816–3826.

(27) Roesijadi, G.; Jones, S. B.; Snowden-Swan, L. J.; Zhu, Y. *Macroalgae as a Biomass Feedstock: A Preliminary Analysis*; Pacific Northwest National Lab. (PNNL): Richland, WA (United States), 2010.

(28) Liu, C. L.; Nikas, Y. J.; Blankschtein, D. Novel Bioseparations Using Two-Phase Aqueous Micellar Systems. *Biotechnol. Bioeng.* **1996**, *52*, 185–192.

(29) Torres, F. A. E.; de Almeida Francisco, A. C.; Pereira, J. F. B.; de Carvalho Santos-Ebinuma, V. Imidazolium-Based Ionic Liquids as Co-Surfactants in Aqueous Micellar Two-Phase Systems Composed of Nonionic Surfactants and Their Aptitude for Recovery of Natural Colorants from Fermented Broth. *Sep. Purif. Technol.* **2018**, *196*, 262–269.

(30) Malpiedi, L. P.; Nerli, B. B.; Abdala, D. S. P.; de Alcântara Pessôa-Filho, P.; Pessoa, A. Aqueous Micellar Systems Containing Triton X-114 and *Pichia pastoris* Fermentation Supernatant: A Novel Alternative for Single-Chain-Antibody Fragment Purification. *Sep. Purif. Technol.* **2014**, *132*, 295–301.

(31) Tani, H.; Kamidate, T.; Watanabe, H. Aqueous Micellar Two-Phase Systems for Protein Separation. *Anal. Sci.* **1998**, *14*, 875–888.

(32) Pangestad, R.; Kim, S.-K. Biological Activities and Health Benefit Effects of Natural Pigments Derived from Marine Algae. *J. Funct. Foods* **2011**, *3*, 255–266.

(33) de Almeida, C. L. F.; Falcão, D. S.; Lima, D. M.; Montenegro, D. A.; Lira, N. S.; Athaye-Filho, D.; Petrónio, F.; Rodrigues, L. C.; de Souza, M. D. F. V.; Barbosa-Filho, J. M. Bioactivities from Marine Algae of the Genus Gracilaria. *Int. J. Mol. Sci.* **2011**, *12*, 4550–4573.

(34) Baghel, R. S.; Reddy, C. R. K.; Jha, B. Characterization of Agarophytes Seaweeds from the Biorefinery Context. *Bioresour. Technol.* **2014**, *159*, 280–285.

(35) Sonani, R. R.; Rastogi, R. P.; Patel, R.; Madamwar, D. Recent Advances in Production, Purification and Applications of Phycobiliproteins. *World J. Biol. Chem.* **2016**, *7*, 100–109.

(36) Aldrich-Merc, S. R-Phycocerythin. https://www.sigmaaldrich.com/catalog/product/sigma/52412?lang=en&pt=PT (accessed Sep 19, 2019).

(37) Separating and Purifying Technology for Micro Algae Phycoerythrin. CN Patent CN1587275A2004.

(38) Method for Fast Separating and Purifying R-Phycocerythrin, R-Phycocyanin. CN Patent CN10124009A2008.