High-throughput screening of aqueous biphasic systems with ionic liquids as additives for extraction and purification of enveloped virus-like particles

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Due to their unique properties, virus-like particles (VLPs) have been portrayed as a promising high-value biopharmaceutical in VLP-based vaccination and cancer therapy. Nevertheless, due to limited physical and economical capabilities of the current downstream processing of VLPs, their production is still difficult and seen as a major problem that needs to be tackled. In this work, high-throughput screening on a liquid handling station (LHS) has been implemented for efficient selection of adequate polymer-salt aqueous biphasic systems (ABS) for extraction and purification of enveloped Hepatitis C virus pseudoparticles (HCVpp). The effect of polyethylene glycol (PEG) molecular weight and salt type (citrate, sulfate, and phosphate) was first evaluated. Furthermore, to optimize extraction parameters, the effect of pH and tie-line lengths (TLL) was also addressed. For the most promising ABS and extraction conditions, the addition of ten ionic liquids (ILs) as adjuvants was investigated. Insights on the chemical features of ILs that impact HCV-VLPs partitioning are highlighted. Finally, the potential of studied ABS containing ILs as adjuvants in the extraction and purification of VLPs from cell culture supernatants was addressed. The 100% extraction efficiency of VLPs in the PEG-rich phase was achieved, and VLP purity was increased compared to the system without IL. In conclusion, these promising results show that ILs can be very effective in modulating the phase properties of polymer-salt ABS, achieving high HCV-VLP purification.

KEYWORDS
aqueous biphasic systems, high-throughput screening, ionic liquids, virus-like particles

1 | INTRODUCTION

Virus-like particles (VLPs) are multimeric nanostructures resembling structural proteins of the virus. However, contrary to the virus, they lack genetic material.¹ Therefore, these particles are not able to self-replicate, but still possess functional viral proteins, which enable them to penetrate and efficiently enter the cells.² Because of these properties, VLPs have been largely used for biotechnological applications, such as VLP-based vaccination or cancer therapy.³⁴ However,
there are still viruses for which there is no vaccine available. One of these is Hepatitis C virus (HCV). HCV infection worldwide represents a large problem for the public health system, with up to 1.75 million new infections per year. Overall, it is estimated that nearly 80 million people are infected worldwide. HCV infects liver cells and causes liver disease and cirrhosis, and may ultimately lead to hepatic failure and death. In this scenario, taking into account the limited production of VLP-based vaccines, the low purification yields, and the low stability of enveloped VLPs, alternative purification methods are hence constantly sought.

Nowadays, chromatography dominates in downstream processing (DSP) of VLPs. However, upstream process development is pushing it towards its physical and economic limits. In general, DSP needs to meet high recovery efficiencies and purity of VLPs obtained in intact form. In addition, to be economically viable, it should minimize its process time and reduce the reagent consumption. Currently used separation techniques for purification of VLPs rely on the relatively big size of the VLPs and are mainly based on gradient ultracentrifugation, ultrafiltration, precipitation, or size-exclusion chromatography. Nevertheless, all these methods do not satisfy the economic requirements because they are time consuming, yield low product recovery, and are very difficult to scale up. For instance, very common sucrose or CsCl2 density-gradient ultracentrifugation provides low yields; some impurities are still retained, and the implementation of the process is very difficult due to problems with scaling up. The precipitation methods (with polyethylene glycol (PEG), salts (such as sulfate or phosphate) possess low selectivity toward VLPs, and in ultra- or microfiltration membrane clogging occurs and large impurities are also often retained and co-concentrated.

Considering all these limitations, novel, cost effective, and efficient purification techniques are hence urgently required. Aqueous biphasic systems (ABS) are one of the possible, and well-recognized, alternatives. Aqueous biphasic systems are recognized as a versatile and biocompatible separation/purification platform. Aqueous biphasic systems comprise two immiscible aqueous-rich phases composed of polymer-polymer, polymer-salt or salt-salt combinations. Moreover, both phases are mainly composed of water, which opens the door to these systems as a biocompatible medium for a diverse range of biomolecules. In addition, ABS display many advantages, such as easy scalability, continuous operation mode, and possibility of combination of several steps into one-unit operation. On the other hand, the ABS usually display low selectivity and polarity differences between the two phases, which affects the purity of the desired product. To overcome these drawbacks, ionic liquids (ILs) have been introduced as phase forming compounds of ABS, enabling to finely tune the properties of the aqueous phases in equilibrium, hence greatly enhancing the purity and stability for a large range of biomolecules, such as proteins, enzymes, antibodies, and DNA. Beside the use of ILs as phase-forming components, they can also be used in low amounts as adjuvants. It was already reported that biomolecules (ie, enzymes and proteins) keep its structure and function in presence of ILs aqueous solutions, enabling more efficient and more selective extraction and purification of high-value biopharmaceuticals, such as VLPs.

Separation and purification of VLPs using ABS have been reported. Andrews et al purified yeast VLPs by a two-step strategy, which included PEG 400 or 600 and ammonium sulfate ABS for debris separation and a PEG 4000 or 8000 and (NH4)_2SO4 with added sodium chloride or phosphate for VLP purification from the proteins. Benavides et al used PEG–potassium phosphate system for the recovery and primary purification of double layered Rotavirus-like particles (dRLP) produced using baculovirus expression system. Jacinto et al reported on ABS purification of recombinant HIV-VLPs from a cell supernatant, and Lueachau et al studied the recovery of B19 virus-like particles using PEG 1000-magnesium sulfate ABS. However, until now the use of ILs in implementation of ABS to improve VLPs separation and purification has not been reported.

Because the partitioning of biomolecules is influenced by various factors, such as biomolecule hydrophobicity, charge, structure, size, as well as ABS features (PEG molecular weight, salt type, pH, additives), considering the lack of predictive models for ABS selection envisaging a specific purification problem, a lot of experiments for each ABS are necessary in order to gain sufficient insights. Given that, an automated approach in obtaining these insights is highly beneficial. Using high-throughput screening (HTS), the whole process is expedited and miniaturized, thus reducing resources and allowing to increase the number of replicates and conditions that can be tested. This approach has already been used for non-enveloped VLPs DSP development using ABS. In this work, high-throughput screening on liquid handling station (LHS) has been used as a tool for rapid selection of adequate ABS for extraction and purification of chimeric retroviral particles pseudotyped with HCV envelope proteins. Here, ABS composed of PEG and salts were chosen and the effect of PEG molecular weight (400, 600, 1000 g/mol) and salt type (citrate, sulfate, and phosphate) was studied. Furthermore, to optimize the extraction parameters, the effect of pH and tie-line lengths (TLL) was also evaluated. The effect of addition of 10 ILs as adjuvants to the most efficient ABS was investigated in terms of the extraction and recovery of HCVpp. Finally, the potential of the most promising ABS in purification of VLPs from cell culture supernatants was evaluated.
2 MATERIALS AND METHODS

2.1 Chemicals and biologicals
Polyethylene glycol with molecular weights of 400, 600, and 1000 g/mol (hereafter described as PEG 400, PEG 600, and PEG 1000, respectively); potassium phosphate monobasic anhydrous; potassium phosphate dibasic anhydrous; ammonium sulfate; citric acid monohydrate; and tripotassium citrate monohydrate were all purchased from Sigma–Aldrich (purity ≥99% (w/w)). Polyethylene glycol and salt solutions were prepared and used as follows: 70% (w/w) PEG 400, 70% (w/w) PEG 600, 70% (w/w) PEG 1000, 40% (w/w) KH₂PO₄, 40% (w/w) K₂HPO₄, 50% (w/w) citric acid, 50% (w/w) tripotassium citrate, and 40% (w/w) ammonium sulfate. Mono and dibasic phosphate and citrate stock solutions were mixed at different proportions to yield the desired pH values. pH values of ammonium sulfate stock solutions were adjusted with hydrochloric acid and sodium hydroxide.

Different ILs were used, namely, 1-ethyl-3-methylimidazolium chloride, [C₂mim]Cl; 1-butyl-3-methylimidazolium chloride, [C₄mim]Cl; 1-hexyl-3-methylimidazolium chloride, [C₆mim]Cl; 1-butyl-3-methylimidazolium bromide, [C₄mim]Br; 1-butyl-3-methylimidazolium acetate, [C₄mim][CH₃CO₂]; 1-butyl-3-methylimidazolium thiocyanate, [C₄mim][SCN]; tetramethylammonium bromide, [N₁₁₁₁]Br; tetraethylammonium bromide, [N₂₂₂₂]Br; tetrapropylammonium bromide, [N₃₃₃₃]Br; tetrabutylammonium bromide, [N₄₄₄₄]Br. Imidazolium-based ILs were acquired from Iolitec, and ammonium-based ILs were acquired from Sigma-Aldrich. The purity level of all ILs were > 98% (w/w). The water content in the ILs was checked before use by the Karl Fisher titration method (model Metrohm 831 Karl Fisher coulometer) and taken into consideration in the calculations. The chemical structures of the studied ILs are shown in Figure A1 in the Supporting Information.

HEK 293 (ATCC CRL-1573) cell line was used to produce HCVpp. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco) and kept in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

2.2 Production and purification of HCV pseudoparticles
For HCVpp production, HEK 293 cells with stable expression of all viral components (retroviral core proteins, E1-E2 glycoproteins, p7-NS2, and green fluorescent protein (GFP) marker gene) were seeded in 225-cm² tissue-culture flasks (Sarstedt, Nümbrecht, Germany) and cultured until 80% of cellular confluence was reached. Dulbecco's Modified Eagle's Medium was then changed with a fresh one and VLPs produced during the subsequent 24 h. Supernatants were harvested and filtered through 0.45-μm syringe filters for clarification and finally purified by a two-step ultracentrifugation: first, at 200 000 × g (42 000 × rpm in a 45Ti rotor) for 2 hours and, second, at 300 000 × g (60 000 × rpm in a 90Ti rotor) 20% (w/v) sucrose cushion for 2 hours.

2.3 Liquid handling station
Liquid handling station, Tecan Freedom EVO® 200 (Tecan, Crailsheim, Germany), was used in this work as liquid handling platform. LHS was equipped with an 8-channel liquid handling arm, a centric gripper, a shaker (Te-shake, Tecan, Crailsheim, Germany), a centrifuge (Rotanta 46RSC, Hettich, Tuttlingen, Germany), and an Infinite200 spectrophotometer (Tecan, Crailsheim, Germany). The LHS was controlled using Evoware 2.3 software (Tecan, Crailsheim, Germany).

2.3.1 Automated phase diagrams and tie-line determination procedure
All presented binodal phase diagrams were determined using the LHS. For that, ultrapure MilliQ water, salt, and PEG stock solutions were pipetted into a 96-well microplate to yield different combinations of PEG/salt concentrations. The final volume of prepared systems was 300 μL per well. Next, the plate was mixed at 1000 rpm for 5 minutes. Afterwards, the formation of two-phase system was determined by visual inspection. The biphasic system was indeed easily detectable as soon as the solution became turbid while being mixed; on the contrary, one-phase systems stayed transparent. In accordance with the data available in the literature, 12 different salt concentrations were chosen and combined with eight different PEG concentrations. A binodal point for each salt concentration was defined as the mean value between two neighboring systems of which one displayed the formation of the biphasic system. The obtained binodal curves were fitted to the Merchuk equation:

\[ Y = A \exp \left[ (BX^{0.5}) - (CX^3) \right], \]
where $Y$ and $X$ are the PEG and the salt mass fraction percentages, and $A$, $B$, and $C$ are constants obtained from fitting of the experimental binodal data.

Tie-lines were obtained with the lever arm rule using volume ratios ($V_r$) calculated from mass balances, as reported in the literature.\textsuperscript{34,35} Briefly, systems within biphasic region were prepared by pipetting water and salt into 1.2 mL deep-well plates, followed by the addition of 70 μL of methyl orange. Finally, PEG was added, and the plate was mixed. Afterwards, the plate was transferred to the centrifuge and centrifuged at $4000 \times g$ for 5 minutes, allowing for the separation of the phases. All ABS were prepared yielding final volumes of 650 μL. Fifty μL samples from the top phases were carefully collected using the pipetting tips liquid level detection function, diluted in 200 μL of water, and the absorbance at 470 nm was measured. The methyl orange exclusively partitions into the upper phase, hence, the absorbance measured at 470 nm in a sample of the upper phase correlates to the phase volume. For determination of tie-lines the system initial mixture composition, top and bottom phase volumes and the mathematical representation of the curve fitted to Equation (1) were combined. Note that in the studied ABS, the top (upper) phase corresponds to the PEG-rich phase, while the bottom phase to the salt-rich phase.

### 2.3.2 Extraction of purified HCVpp using PEG-salt-based ABS

ABSs with volume ratios near 1 (in order to have a sufficient amount of phases for further analysis) were chosen in VLPs partitioning experiments. In order to explain the main factors that rule the HCVpp partitioning in studied PEG-salt ABS, different parameters, such as PEG molecular weight, salt type, pH, TLL, were studied. Six hundred fifty μL ABS were prepared by pipetting stock solutions of phase forming compounds, water and 100 μL previously purified VLPs with known concentrations into 96 deep-well plates. Aqueous biphasic systems were mixed, incubated (30 minutes), and centrifuged (at $4000 \times g$ for 5 minutes) to allow complete phase separation. Samples of top and bottom phases (50 μL) were taken, diluted and analysed for VLP content using an Infinite200 spectrophotometer at the wavelength of 280 nm. Virus-like particles (VLPs) partitioning studies were always performed in duplicate. Partitioning of VLPs was calculated by multiplying the measured VLPs concentration in the top and bottom phases by the respective phase volumes. The interference of the salt and PEG was taken into account through the use of blank control samples.

The extraction efficiency of HCV-VLPs into to PEG-rich phase, $EE_{VLPs}$, is the percentage ratio between the amount of VLPs in the PEG-rich phase to the total VLPs amount in the two phases in equilibrium, according to the following equation:

$$EE_{VLPs} = \frac{w_{PEG}^{VLPs}}{w_{PEG}^{VLPs} + w_{salt}^{VLPs}} \times 100,$$

where $w_{PEG}^{VLPs}$ and $w_{salt}^{VLPs}$ are the total weight of VLPs in the PEG-rich phase and in the salt-rich phase, respectively. The recovery yield of VLPs into PEG-rich phase, $Y_{VLPs}$, is the percentage ratio between the amount of VLPs in the PEG-rich aqueous phase ($w_{PEG}^{VLPs}$) to that added in the initial mixture ($w_{initial}^{VLPs}$), according to the following equation:

$$Y_{VLPs} = \frac{w_{PEG}^{VLPs}}{w_{initial}^{VLPs}} \times 100.$$

### 2.3.3 Extraction of purified HCVpp using PEG-salt-based ABS with ILs as adjuvants

After the optimization procedures (Section 2.3.2.), PEG 400 + citrate buffer pH ≈ 7 (TLL of 40) ABS was selected as the most promising system in terms of extraction efficiencies and VLPs recoveries. To this system, 5% (w/w) of each IL was added, and the effect of ILs on partition behavior of HCV-VLPs was studied. Because the addition of ILs to the ABS usually affects binodal phase diagram, TLs and TLL, the volume of the phases in equilibrium was always determined and considered in all calculations. In order to evaluate the effect of the IL anion and the IL cation alkyl side chain length, 10 different ILs were used. The ILs selected for the study of the anion effect were $[C_4\text{mim}]Cl$, $[C_4\text{mim}]Br$, $[C_4\text{mim}][CH_3CO_2]$, and $[C_4\text{mim}][SCN]$, while to study the effect of the cation alkyl side chain length the ammonium-based ($[N_{1111}]Br$; $[N_{2222}]Br$; $[N_{3333}]Br$; $[N_{4444}]Br$) and imidazolium-based ($[C_2\text{mim}]Cl$, $[C_4\text{mim}]Cl$, and $[C_6\text{mim}]Cl$) ILs were used. Six hundred fifty μL ABS were built by adding stock solutions of PEG 400, citrate buffer, 100 μL previously purified VLPs, ILs, and water into 96 deep-well plates. The plate was then mixed, incubated (30 minutes), and centrifuged (at $4000 \times g$ for 5 minutes) to achieve the complete partitioning of VLPs between the two phases. Samples of top and bottom phases were taken (50 μL), diluted, and analysed for VLP content as previously described. $EE_{VLPs}$ and $Y_{VLPs}$ were determined using Equations (2) and (3).
2.4 Extraction and purification of HCVpp from cell supernatants using PEG-salt-based ABS with ILs as adjuvants

The potential of the most promising systems identified in Sections 2.3.2 and 2.3.3 was evaluated in terms of VLPs recovery and purification from cell culture supernatants, where besides the VLPs, impurities are also present. Aqueous biphasic systems formed by PEG 400 and citrate buffer pH ≈ 7 (TLL of 40) were selected, and several ILs, namely, [C4mim]Cl, [C4mim][CH3CO2], and [N4444]Br at 5% (w/w), were added. Five thousand μL ABS were built by mixing stock solutions of phase-forming components, water, and 750 μL of cell supernatants (which correspond to 20% loading). Aqueous biphasic systems were stirred, incubated (30 minutes), and centrifuged (at 4000 × g for 5 minutes) to reach phase equilibrium. Samples of top and bottom phases were taken, diluted, and analysed for VLP content. The HCV-VLPs and contaminant proteins were identified using reversed-phase ultra-high-performance-liquid chromatography (RP-UHPLC, UltiMate® 3000 RSLC × 2 Dual system). Sample analysis was done on a Waters Acquity BEH300 C4 1.7 m column (diameter × length = 2.1 mm × 50 mm) at a flow rate of 0.45 mL/min and a column temperature of 60°C. Mobile phase buffers were ultrapure water containing 0.1% [v/v] trifluoroacetic acid (TFA) (solvent A) and acetonitrile containing 0.1% [v/v] TFA (solvent B). Protein analysis was conducted as follows: the column was equilibrated with 5% B for 1.4 minutes, followed by an increase to 23.5% B in 0.1 minutes, followed by a gradient of 23.5% to 63.5% over 4.7 minutes and an increase to 95% in 0.1 minutes, held for 0.5 minutes. The total time of analysis was 6.8 minutes per sample. The purity of VLPs is the ratio between the HPLC peak area corresponding to HCV-VLPs and the total area of all peaks corresponding to all proteins present at the top phase, at a UV wavelength of 280 nm, according to the following equation:

\[
\text{Purity} = \frac{\text{Area}_{\text{HCV-VLPs}}}{\text{Area}_{\text{All proteins}}}
\]

2.5 Analytical methods

2.5.1 Dynamic light scattering measurements

Dynamic light scattering measurements were used to determine the size of VLPs in diameter and its zeta potential. For that, a Zetasizer Nano ZS from Malvern Instruments was used. Measurements were carried out at 25°C. Three consecutive measurements of each sample were performed, and average results were presented.

Structural integrity of VLPs

Structural integrity of the viral particles was assessed during apparent hydrodynamic diameter (dₜₐ₇) measurements. The equipment allows to define the particle size distribution after VLPs separation using aqueous biphasic systems, which when compared with the control VLPs, gives a direct evaluation of the structural integrity of the particle. The Zetasizer Nano ZS uses the Brownian motion of particles in solution where the light from a laser beam is directed through the moving particles in suspension, and the light scattered by the particles is detected and its intensity converted to the size distribution, based on the volume or the number of particles in each size class. In all measurements, the chromatography flow standard operating procedure of the Zetasizer software (Malvern Instruments) was used. The data were acquired in a back-scattering geometry at 173°. The measurements were carried out using a sample volume of 45 μl in a 3×3-mm quartz cuvette (Hellma Analytics, Müllheim, DE) at 25°C. Three consecutive measurements of each sample consisting of 12 to 14 10-second runs were done.

2.5.2 Examination of VLP activity

HuH-7 cells were used for infection assays. Briefly, HuH-7 cells were seeded in 12-well plates at a density of 0.15×10⁶ cells per well and incubated overnight at 37°C. After 24 hours, fresh DMEM with 2% (v/v) FBS and 5% (v/v) Human Serum and 8 μg/mL of polybrene and supplemented with HCVpp recovered from top phase (PEG-rich) of ABS by sucrose gradient ultracentrifugation were added to the cells. Control cells were prepared in a similar manner except that instead of DMEM with HCVpp, fresh DMEM was added. After infection, the cells were maintained for 72 hours at 37°C before analysis.

To evaluate infection efficiencies, infected HuH-7 cells were analysed using fluorescence microscopy and flow cytometry. Fluorescence microscopy allows for a qualitative and fast assessment of GFP-positive cells. For the quantification of GFP-positive cells, flow cytometry was used. For that, cells were harvested from culture using standard cell-culture methods and stained with ice cold FACS buffer. Cells were examined using CyFlow-space (Sysmex-Partec, Görlitz, Germany) equipment and results analysed using FlowJo software (Oregon, USA).
3 | RESULTS AND DISCUSSION

3.1 | Binodal curves and tie-lines

Determination of binodal curves and tie-lines is needed to characterize the two-phase region that can be used in ABS extraction and partitioning experiments. Phase diagrams and tie-lines presented throughout this work were obtained on a liquid handling station. Binodals and tie-lines of three different water-soluble PEGs (PEG 400, PEG 600, and PEG 1000) and three buffered salts (citrate, phosphate, sulfate) at pH $\approx 7$ were determined. In Figure 1, examples of the binodals are shown for PEG 400-, PEG 600-, and PEG 1000-citrate buffer systems at pH $\approx 7$. All other determined binodal phase diagrams and tie-lines can be found in the Supporting Information in Figures A2 to A15.

Moreover, all obtained correlation coefficients are summarized in Table A1 in the Supporting Information. Good correlation parameters above $R^2 = 0.99$ were obtained, which validates the methodology used in this work. When possible, the obtained phase diagrams were confirmed and shown to be in good agreement with the binodal curves already reported in the literature and done in batch scale.36-41

In all binodal curves, the region above the solubility curve represents the two-phase region. Therefore, the larger this region is, the higher is the ability of PEG to form two-phase system in aqueous media. As can be seen from Figure 1, the biphasic region increases with the PEG molecular weight. This is a well-known phenomenon42: the increase of molecular weight of PEGs results in the increase of its hydrophobicity, making them more easily excluded to a second liquid phase by the salting-out species.

Beside the ability of PEG to undergo liquid–liquid demixing, two-phase formation in a polymer-salt ABS also relies upon the salting-out ability of the salt. For the three PEGs under study, the binodal curve composed of PEG-phosphate was closer to the axes as compared to PEG-citrate and PEG-sulfate. The following order in salting-out ability of the salts can be deducted: phosphate $> $ sulfate $> $ citrate. In this context, the lowest concentrations of PEG and salt are needed for phosphate-based ABS formation.

The pH effect on the formation of ABS was also studied using the PEG 400-citrate buffer ABS (as it shall be seen and discussed later, this ABS displayed the highest potential in VLPs extraction and recovery) in a pH range from six to eight. The respective binodal phase diagrams are shown in the Supporting Information (Figure A4). In general, with an increase of the pH, a larger binodal region is observed. This fact is related with an increased salting-out ability of the salt as pH increases.43

3.2 | Extraction of purified HCVpp using PEG-salt-based ABS

3.2.1 | Effect of salt and polymer molecular weight

The first step before choosing the most promising PEG-salt biphasic system was the identification of the salt and molecular weight of PEG, which were best suited for HCV-VLPs extraction. Three different buffered salts, namely, citrate, phosphate, and sulfate (at a fixed pH, pH $\approx 7$), combined with three PEGs, namely, PEG 400, PEG 600, and PEG 1000, were tested in ABS partitioning experiments, and their performance was evaluated in terms of extraction efficiency and recovery yield of VLPs. The obtained results are presented in Figure 2A-C.
FIGURE 2  Extraction efficiency (EE_{VLP\%}, scatter plot) and recovery yield (Y_{VLP\%}, bars) of HCV-VLPs in the systems composed of (A) PEGs + citrate + H_2O at pH ≈ 7, (B) PEGs + phosphate + H_2O at pH ≈ 7, and (C) PEGs + sulfate + H_2O at pH ≈ 7. Error bars correspond to standard deviations obtained from replicates. PEG, polyethylene glycol.

All two-phase system compositions used in this part of the study correspond to a similar TLL of ca. 30. The respective initial mixture compositions of the extraction points can be found in Table A2 in the Supporting Information. In all ABS extraction experiments with VLPs, the small liquid-liquid interface between the top and bottom phase was observed. Therefore, it was concluded that the interfacial partitioning due to precipitation occurred and the amount of precipitated VLPs, within experimental uncertainty, was calculated from the mass balance for VLPs. Detailed data for the extraction efficiency and recovery yields of VLPs, as well as the amount of VLPs precipitated in the interface, are reported in Table A3 in the Supporting Information.

The gathered results reveal the preferential partitioning of VLPs to the PEG-rich phase for all the studied systems, with the exception of the system composed of PEG 1000 and phosphate. Nevertheless, complete partitioning of VLPs was never attained. Looking closely at Figure 2A-C, it can be concluded that citrate showed the highest recoveries of HCVpp in the PEG-rich phase, for all PEGs tested. The highest recovery of 49.8% in the PEG-rich phase was obtained for the PEG 400 + citrate ABS. Moreover, good VLPs recoveries of 36.4% have been achieved in the bottom phase of the latter ABS, indicating that citrate salt is a good candidate as a phase-forming component in ABS for extraction of VLPs. Furthermore, in all studied systems composed of PEG-ammonium sulfate at pH ≈ 7, a large decrease in VLPs recoveries in the bottom (salt-rich) phase (8.2% and 2.9% for the PEG 400- and PEG 600-(NH_4)_2SO_4 ABS, respectively) was observed. The reason was most likely precipitation of VLPs and interfacial partitioning because (NH_4)_2SO_4 is commonly used in precipitation purification methods. Better results were achieved for the PEG-phosphate ABS, where top (PEG-rich) phase recoveries of 46.2% and 45.2%, and bottom (salt-rich) phase recoveries of 35.4% and 27.9%, were obtained for the PEG 400- and PEG 600-phosphate systems, respectively. As mentioned earlier, a different partitioning behavior was observed for the PEG 1000-phosphate ABS. It seems that effect of size-exclusion of PEG resulted in VLPs preferring the bottom salt-rich phase, with 31.5% VLPs recovery in bottom (salt-rich) phase. In addition, around 60% of VLPs were lost during the extraction process, due to salt-induced precipitation of phosphate.

A decrease in top-phase partitioning of VLPs was observed with increase of the molecular weight of PEG (49.8%, 49.2%, and 29.7% for PEG 400-, 600-, and 1000-citrate ABS, respectively; 46.2%, 45.2%, and 8.5% for PEG 400-, 600-, and
Taking into account the relatively large size of VLPs, this fact is probably due to the increase in excluded volume of PEG with increase of its molecular weight. Furthermore, PEGs with higher molecular weight led to a decrease in VLP solubility in the top phase. Considering the overall results obtained for EEVLPs% and YVLPs% and the fact that the citrate anion is biodegradable and nontoxic,44 further optimizations were carried out in the PEG 400–citrate system.

3.2.2 Effect of pH

In the next step, the effect of pH, in the range of six to eight, on the phase equilibria for the ABS composed of PEG 400-citrate and on VLPs partitioning was evaluated. All experiments were done at the same TLL of ca. 30 (respective initial mixture compositions of the extraction points can be found in Table A2 in the Supporting Information), to enable a fair comparison and more accurate interpretations. In Figure 3, we can see that shifting to more acidic or basic pH, resulted in decrease in recovery yields of VLPs (detailed data can be found in Table A4 in the Supporting Information). This was especially observed for the system with pH ≈ 6, where a decrease in VLPs yield in the top phase from 49.8% at pH ≈ 7 to only 15.5% at pH ≈ 6 was observed. This indicates that at more acidic pH values, precipitation of VLPs occurs. This effect is considerably smaller for the more basic system at pH ≈ 8. At this pH, a decrease in VLPs recovery was also observed (39.1% VLPs recovery), however not as pronounced as at pH ≈ 6. Because the isoelectric point (IEP) of VLPs determined in this work is 3.3 (details can be found in the Supporting Information), VLPs have negative net charge in all studied ABS. Taking into account all of gathered results, further experiments were performed at neutral pH of 7.

3.2.3 Effect of the TLL

One of the main parameters influencing the partitioning of solutes in ABS is the TLL. By increasing the TLL, the composition of both phases becomes very distinct, increasing the hydrophobicity of the top phase and the interfacial potential between the two phases.42 To evaluate how the TLL affects partitioning of HCV-VLPs, the ABS composed of PEG 400-citrate buffer at pH ≈ 7 was chosen and TLL varied between 30 and 50. The results are shown in Figure 4, and further details are reported in Table A5 in the Supporting Information.

From Figure 4A-B, it can be observed that the partitioning of VLPs in PEG 400-citrate ABS is highly dependent on the TLL. With an increase of the TLL, an increase in both EEVLPs% and YVLPs% is observed until TLL of 40. This effect of TLL on HCV-VLPs partitioning is related with the higher ionic strength created by the increase in salt concentration, which improves the extraction of VLPs to the PEG rich phase due to electrostatic repulsion effects.45 On the other hand, for TLL longer than 40, a large decrease in VLPs recovery is observed (from 84.9% for TLL of 40 to only 39.2% for TLL of 45). This is most likely due to the fact that the VLPs are no longer soluble and stable in such high PEG and salt concentrations. Nevertheless, at TLL of 40, it is possible to extract VLPs with 88.8% efficiency and 84.9% recovery, envisaging the potential of these systems in concentration of VLPs from aqueous solutions.

3.3 Extraction of purified HCVpp using PEG-salt-IL-based ABS with ILs as adjuvants

3.3.1 Effect of the alkyl side chain length in the IL cation

After identification of the most promising polymer-salt ABS and optimization procedures, PEG 400 + citrate, pH ≈ 7, and TLL of ca. 40, the effect of the alkyl side chain length on the IL cation on the partitioning of HCV-VLPs was studied. For that, three imidazolium-based ILs with a fixed anion (Cl⁻) combined with the cations \([C_2\text{mim}]^+\), \([C_4\text{mim}]^+\), \([C_6\text{mim}]^+\),
and four ammonium-based ILs with a fixed anion (Br\(^-\)) combined with the cations \(\text{[N}_{1111}\text{]}^+\), \(\text{[N}_{2222}\text{]}^+\), \(\text{[N}_{3333}\text{]}^+\), \(\text{[N}_{4444}\text{]}^+\), were selected and added in a total concentration of 5% (w/w) to the ABS composed of PEG 400 and citrate, at pH \(\approx 7\), at TLL of 40. The obtained results are displayed in Figure 5A-B and further details are reported in Table A6 in the Supporting Information.

As it can be observed, the recovery yields of VLPs for these systems were smaller than those obtained for the reference system without IL. It seems that VLPs become less soluble and stable with the presence of these ILs. Nevertheless, the extraction efficiency of the ABS where \(\text{[C}_{4\text{mim}}\text{]}\text{Cl}\) (91.1%) and \(\text{[N}_{4444}\text{]}\text{Br}\) (91.3%) were added is slightly higher (although within standard deviations) when comparing with the system without IL (87.9%). It is also worth mentioning that
ammonium-based ILs possess high ability to affect the extraction efficiency and recovery yields of VLPs within PEG-salt ABS. For ammonium-based ILs with an increase of the alkyl chain length, an increase in EEVLPs% and YVLPs% is observed, which is probably related to the increased hydrophobicity of the IL with the increase of the carbon atoms in the alkyl chain of the IL cation. Because the viral particles are highly hydrophobic and their hydrophobicity is higher than most of the proteins, more hydrophobic ILs can increase the hydrophobic interactions between VLPs and ILs present in the PEG-rich phase, leading to a higher extraction. On the other hand, alkyl side chain of imidazolium-based ILs affected the separation performance of the studied ABSs to a smaller extent. For these ILs, no major differences are observed neither in the extraction efficiencies nor in the recovery yields. Finally, no clear conclusions can be drawn regarding the influence of the increase of the alkyl chain length (from ethyl to hexyl) in imidazolium-based ILs on the partitioning of HCV-VLPs.

3.3.2 Effect of the IL anion

Figure 6 presents a comparison between the extraction efficiencies and VLP recovery yields for the systems containing ILs with common \([\text{C}_4\text{mim}]^+\) cation and different anions with the objective of understanding the influence of the IL anion structure on VLPs extraction for ABS composed of PEG 400 and citrate buffer (pH \(\approx 7\)) at initial TLL of 40. Results for the addition of \([\text{C}_4\text{mim}][\text{Br}], [\text{C}_4\text{mim}][\text{Cl}], [\text{C}_4\text{mim}][\text{CH}_3\text{CO}_2],\) and \([\text{C}_4\text{mim}][\text{SCN}]\) are presented, as well as results for the system without IL, for comparison purposes. Detailed data for extraction efficiency and recovery yields of VLPs are reported in Table A6 in the Supporting Information.

It can be seen that for the system containing \([\text{C}_4\text{mim}][\text{CH}_3\text{CO}_2]\), the increase of both average EEVLPs% and YVLPs% from 87.9% and 84.9% (for the reference ABS without IL) to 90.3% and 88.5%, respectively, was observed. Furthermore, the ABS containing \([\text{C}_4\text{mim}][\text{Cl}]\), albeit displaying a bit lower VLPs recovery, also led to an increase in the VLPs extraction efficiency up to 91.1%. The obtained values of YVLPs% follow the anion’s order: \([\text{CH}_3\text{CO}_2]^- \geq \text{Cl}^- > \text{Br}^- > [\text{SCN}]^-\). This trend is in line with the hydrogen-bond basicity (\(\beta\)) of the IL and therefore enhanced ability to accept protons \((\text{[C}_4\text{mim}][\text{CH}_3\text{CO}_2]) (\beta = 1.20) > [\text{C}_4\text{mim}][\text{Cl}] (\beta = 0.95) > [\text{C}_4\text{mim}][\text{Br}] (\beta = 0.87) > [\text{C}_4\text{mim}][\text{SCN}] (\beta = 0.71))\). In general, it seems that the ability of the anions to accept protons from VLPs is ruling the partitioning behavior in the studied ABS. Indeed, ILs with a highest hydrogen bonding basicity—\([\text{C}_4\text{mim}][\text{CH}_3\text{CO}_2]\) and \([\text{C}_4\text{mim}][\text{Cl}]\)—are capable to improve the partition of HCV-VLPs to the PEG-rich phase, in which these ILs are mainly present (extraction efficiencies of ILs higher than 80%) in the studied ABS. The obtained results further confirm the observation that in such a quaternary PEG-salt-ILs ABS, the IL anion hydrogen-bond basicity plays a major role in biomolecules partitioning, as it was previously reported for immunoglobulin G and myoglobin. It seems that the ability of the IL anion to accept protons from VLPs is ruling the partitioning behavior in these systems.

Nevertheless, the partitioning results obtained for the systems with 5% (w/w) ILs added show that no significant improvement in extraction efficiencies and recovery yields can be achieved with the use of studied ILs. However, these ILs are capable to significantly enhance the selectivity toward VLPs as further discussed in Section 3.4.

3.4 Extraction and purification of HCVpp from cell culture supernatants using PEG-salt-based ABS with ILs as adjuvants

Finally, the potential of the studied systems in extraction and purification of HCV-VLPs from cell culture supernatants was evaluated. The capacity to purify VLPs of the ABS containing the most promising ILs selected in the previous step was
evaluated and compared with the same ABS without IL. Aqueous biphasic system formed by PEG 400 and citrate buffer pH $\approx 7$ (TLL of 40) was chosen and 5% (w/w) of several ILs (or no IL) added. The examples of RP-UHPLC chromatograms are shown in Figure 7, and chromatograms for the other systems can be found in Figures A16 to A18 in the Supporting Information.

According to the results obtained for purified VLPs sample, the retention time of HCVpp was found to be around 3.0 minutes. As mentioned before, and observed here for the purified VLPs, the VLPs preferentially partition to the PEG-rich phase. Furthermore, for all the studied systems, the presence of VLPs in the salt-rich phase was not observed. Complete extraction of VLPs present in cell culture supernatants to the top phase was achieved. The contaminant proteins present in cell culture supernatants were found to elute at around 0.8, 1.6, and 2.75 minutes; therefore, it allowed us to define purity of the VLPs in the PEG-rich phase. The purity values calculated using Equation (4) are gathered in Table 1. The obtained purity of VLPs in the PEG-rich phase of PEG 400-citrate ABS with no IL added was found to be 53%. Nevertheless, the purity of VLPs in the systems with 5% (w/w) of each one of the selected ILs ranged between 40 and 70%, and the highest purity of 70% was observed for the system with [C$_4$ mim]Cl added.

![Figure 7](image)

**FIGURE 7** Chromatograms from RP-UHPLC of (A) PEG-rich phase and (B) salt-rich phase of the system composed of polyethylene glycol (PEG) 400 + citrate pH 7 + H$_2$O + 5% (w/w) of [C$_4$ mim][CH$_3$CO$_2$]. RP-UHPLC, reversed-phase ultra-high-performance-liquid chromatography; VLP, virus-like particle.

| IL          | Purity of VLPs, % |
|-------------|-------------------|
| No IL       | 53                |
| [C$_4$mim]Cl| 70                |
| [C$_4$mim][CH$_3$CO$_2$] | 40               |
| [N$_{4444}$]Br | 55               |

Abbreviations: ABS, aqueous biphasic systems; IL, ionic liquid; PEG, polyethylene glycol; RP-UHPLC, reversed-phase ultra-high-performance-liquid chromatography; VLP, virus-like particle.

**TABLE 1** The RP-UHPLC purity of VLPs extracted from cell culture supernatants in the PEG-rich phase of ABS composed of PEG 400 + citrate +5% (w/w) of ILs at pH 7 at TLL of 40% (w/w)
Although the ABS without IL added showed to be highly efficient in extracting the VLPs, it was not able to separate VLPs from majority of contaminants present in cell culture supernatants. The most abundant contaminant present in cell culture supernatants is bovine serum albumin (BSA), which is derived from the FBS commonly used in growth medium supplementation. In previous study, it was shown that in the systems composed of PEG and citrate, the BSA preferentially partitions to the PEG-rich phase, when relatively low molecular weight PEG (PEG 1000) is used, and reversed partitioning behavior was observed for the systems with high molecular weight PEG, from PEG 2000 onwards. Because ABS used in this work are composed of low molecular weight PEG 400, the BSA indeed mainly partitioned to the PEG-rich phase, as confirmed by RP-UHPLC. It was further demonstrated that it is possible to back-extract BSA from PEG-rich phase by changing the pH of the citrate to 4.5. Nevertheless, based on the results gathered in this work, acidic pH has negative effect on VLPs recovery, thus making this approach unviable for the viral particles used in this study. However, when ILs are present, even at low concentrations, this scenario changes for each IL in a specific manner. For instance, when [C₄mim][CH₃CO₂] was added, lower VLP purity level in top phase than with the system without IL was observed. On the other hand, the addition of both [C₄mim]Cl and [N₄₄₄₄]Br enhanced the VLPs purity. The decrease in VLP purity when the most hydrophilic IL, [C₄mim][CH₃CO₂], is used, is probably related to the fact that it decreases the hydrophobicity of the PEG-rich phase and, therefore, improves partitioning of more hydrophilic contaminant proteins to this phase. On the other hand, very promising results were obtained with the system containing 5% (w/w) of [C₄mim]Cl, where 32% enhancement in purity of VLPs compared to the systems when no IL was used, was attained. The less hydrophilic ILs are able to increase VLPs purity in the top phase. However, it seems that more important than hydrophobicity/hydrophilicity balance are the specific interactions, hydrogen bonds established between the VLPs and ILs, which play a crucial role when [C₄mim]Cl is used.

Even though the addition of ILs does not significantly improve the extraction efficiency and recovery yields of VLPs (as discussed in Section 3.3), it is possible to significantly enhance the purity of VLPs through implementation of adequate IL as adjuvant. Therefore, the proposed systems can be used in the rapid concentration and primary purification of VLPs from cell culture supernatants.

### 3.5 Structural and functional stability of HCV-VLPs

Dynamic light scattering measurements of apparent hydrodynamic diameter ($d_h$) were used to determine the size of particles most commonly present in each sample. For that, samples from top and bottom phases of studied ABSs were collected and analysed. For comparison purposes, the average size of purified VLPs diluted in water was also measured. The obtained results are gathered in Table 2.

The result obtained for samples collected from the PEG-rich phase of ABS showed to be in good accordance with the particle size estimated for HCV-VLPs dispersed in water, for most of the systems. Only in the system formed with PEG 1000-citrate and in the system where [C₄mim][SCN] was added, larger particles were present, suggesting that VLPs formed aggregates. This is in good agreement with the recovery yields obtained for these ABS. The results also imply,

### Table 2

| Sample source | Average particle size, nm |
|---------------|---------------------------|
| VLPs in H₂O   | 81.5 ± 6.1                |
| PEG 400 + citrate pH ≈ 7 | 81.9 ± 7.9  |
| PEG 600 + citrate pH ≈ 7 | 82.5 ± 15.0  |
| PEG 1000 + citrate pH ≈ 7 | 226.2 ± 24.3  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim]Cl | 81.8 ± 10.6  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim]Cl | 108.0 ± 10.3  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim]Cl | 98.5 ± 12.5  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim]Br | 89.8 ± 19.6  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim][CH₃CO₂] | 85.7 ± 7.9  |
| PEG 400 + citrate pH ≈ 7 + [C₄mim][SCN] | 230.5 ± 15.2  |
| PEG 400 + citrate pH ≈ 7 + [N₄₄₄₄]Br | 82.7 ± 16.1  |
| PEG 400 + citrate pH ≈ 7 + [N₄₄₄₄]Br | 94.0 ± 9.9  |
| PEG 400 + citrate pH ≈ 7 + [N₄₄₄₄]Br | 103.1 ± 7.9  |
| PEG 400 + citrate pH ≈ 7 + [N₄₄₄₄]Br | 86.9 ± 5.5  |

Abbreviations: ABS, aqueous biphasic systems; IL, ionic liquid; PEG, polyethylene glycol; VLP, virus-like particle.
FIGURE 8  Functional validation of extracted HCVpp. Microscopy green-fluorescence images and percentages of GFP (+) HuH-7 cells infected with VLPs recovered from PEG-rich phases of the ABS composed of PEG 400 + citrate pH \(\approx 7 + H_2O + 5\% \text{ (w/w)}\) ionic liquids (ILs) (when present). GFP, green fluorescent protein; PEG, polyethylene glycol; VLP, virus-like particle

given the similar size of particles in water and PEG-rich phase of most of the ABS, that the VLPs maintain their structural integrity and self-assembly properties after extraction. On the other hand, the particle size in the bottom phase is much higher than in the top phase, indicating that larger VLP aggregates form in the salt-rich phase.

Because in this work biological functional HCVpp with an eGFP reporter gene were used,\(^{51}\) it was possible to evaluate if they kept their functionality after separation using ABS. Incorporation of GFP marker gene in the viral particles allowed simple evaluation of the specific infectivity of HCVpp after a one-round infection process using microscopy and flow cytometry. As it can be seen in Figure 8, control HCVpp and those recovered from top phases of ABS composed of PEG 400-citrate pH \(\approx 7\) at TLL of 40\% (w/w) with or without addition of IL are capable to infect HuH-7 cells. These results validate the suitability of the proposed systems in extraction and purification of HCV-VLPs. When comparing the percentages of GFP (+) cells infected with growth medium supplemented with untreated, control VLPs, and those recovered after extraction process, it can be observed that the addition of 5\% (w/w) of IL results in a small decrease in infectivity of HCVpp. However, interesting results were obtained with the viral particles recovered from PEG-rich phase of the system without IL added. These particles were more infectious than the control VLPs. It was previously reported that the presence of relatively low concentrations of PEG increases the thermal stability of virus and does not compromise immunogenicity in vivo.\(^{52}\)

Overall, the results obtained in this work prove that after extraction the VLPs maintained their structural and functional properties, and thus, the investigated ABS can be a viable alternative purification platform for high-value biopharmaceuticals, such as VLPs.

4  | CONCLUSIONS

In this work, high-throughput screening of polymer-salt ABS with ILs as adjuvants in the extraction and purification of HCV-VLPs was studied on a LHS. Although these systems had been advantageously used for many other biomolecules, their true potential for HCV-VLPs purification had never been tested. The implementation of a LHS allowed the reduction in time and resources needed to find and optimize an adequate ABS for VLPs separation. The results obtained in this work show a high affinity of enveloped VLPs to the PEG-rich phase in most of the polymer-salt ABS studied. Furthermore, ABS composed of low molecular PEG 400 and citrate buffer pH \(\approx 7\) at TLL of 40 was shown to be the best system in terms of VLP extraction efficiencies and recovery yields (87.9\% and 84.9\% respectively). It was further demonstrated that with the addition of 5\% (w/w) of adequate IL (\([C_4\text{mim}][\text{Cl}]\)), extraction efficiencies of VLPs can be enhanced up to 91.1\%. Moreover, IL with a highest hydrogen bonding basicity and ability to accept protons—\([C_4\text{mim}][\text{CH}_3\text{CO}_2]\)—is capable to not only increase extraction efficiency (EE\(_\text{VLPs}\% = 90.3\%\)), but also to improve VLPs recovery yield to 88.5\%. The structural and
The functional integrity of HCV-VLPs in the top phase of these systems has been maintained after the extraction process, with the exception of the system formed with PEG 1000 and in the presence of [C4mim][SCN].

The proposed ABS are capable to achieve 100% extraction of VLPs present in cell culture supernatants in a single-step and the adequate IL can also increase selectivity for VLPs. Although, further studies are still required, such as different ILs or evaluation of the effect of the IL concentration in VLPs partitioning, the use of small amounts of ILs in the polymer-salt ABS is a promising approach for VLP purification for VLP-based vaccines.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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REFERENCES

1. Ong HK, Tan WS, Ho KL. Virus like particles as a platform for cancer vaccine development. PeerJ. 2017;5:e4053-e4053. https://doi.org/10.7717/peerj.4053

2. Chroboczek J, Szurgot I, Szolajska E. Virus-like particles as vaccine. Acta Biochim Pol. 2014;61(3):531-539.

3. Donaldson B, Lateef Z, Walker GF, Young SL, Ward VK. Virus-like particle vaccines: immunology and formulation for clinical translation. Expert Rev Vaccines. 2018;17(9):833-849. https://doi.org/10.1080/14760584.2018.1516552

4. Rohovie MJ, Nagasawa M, Swartz JR. Virus-like particles: next-generation nanoparticles for targeted therapeutic delivery. Bioeng Transl Med. 2017;2(1):43-57. https://doi.org/10.1002/btm2.10049

5. WHO. Global Hepatitis Report, 2017. 2017. https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/

6. Blach S, Zeuzem S, Manns M, et al. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. Lancet Gastroenterol Hepatol. 2017;2(3):161-176. https://doi.org/10.1016/s2468-1253(16)30181-9

7. Kamal SM. Acute hepatitis C: a systematic review. Am J Gastroenterol. 2008;103:1283. https://doi.org/10.1111/j.1572-0241.2008.01825.x

8. Wolf MW, Reichl U. Downstream processing of cell culture-derived virus particles. Expert Rev Vaccines. 2011;10(10):1451-1475. https://doi.org/10.1586/erv.11.111

9. Vicente T, Roldão A, Peixoto C, Carrondo MJT, Alves PM. Large-scale production and purification of VLP-based vaccines. J Invertebr Pathol. 2011;107:S42-S48. https://doi.org/10.1016/j.jip.2011.05.004

10. Huhti L, Blazevic V, Nurminen K, Koho T, Hytönen VP, Vesikari T. A comparison of methods for purification and concentration of norovirus GII-4 capsid virus-like particles. Arch Virol. 2010;155(11):1855-1858. https://doi.org/10.1007/s00705-010-0768-z

11. Liu C-C, Guo M-S, Lin FH-Y, et al. Purification and characterization of Enterovirus 71 viral particles produced from Vero cells grown in a serum-free microcarrier bioreactor system. Arch Virol. 2011;65(5):e20005. https://doi.org/10.10371/journal.pone.0020005

12. Branston S, Stanley E, Keshavarz-Moore E, Ward J. Precipitation of filamentous bacteriophages for their selective recovery in primary purification. Biotechnology Progress. 2012;28(1):129-136. https://doi.org/10.1002/btp.705

13. Hu J, Ni Y, Dryman BA, Meng XJ, Zhang C. Purification of porcine reproductive and respiratory syndrome virus from cell culture using ultrafiltration and heparin affinity chromatography. J Chromatogr A. 2010;1217(21):3489-3493. https://doi.org/10.1016/j.chroma.2010.03.023

14. Raghavarao KSMS, Ranganathan TV, Srinivas ND, Barhate RS. Aqueous two phase extraction—an environmentally benign technique. Clean Techn Environ Policy. 2003;5(2):136-141. https://doi.org/10.1007/s10098-003-0193-z

15. Rito-Palomares M, Middelberg APJ. Aqueous two-phase systems for the recovery of a recombinant viral coat protein from Escherichia coli. J Chem Technol Biotechnol. 2002;77(9):1025-1029. https://doi.org/10.1002/jctb.673

16. Silva MFF, Fernandes-Platzgummer A, Aires-Barros MR, Azevedo AM. Integrated purification of monoclonal antibodies directly from cell culture medium with aqueous two-phase systems. Sep Purif Technol. 2014;132:330-335. https://doi.org/10.1016/j.seppur.2014.05.041

17. Gutowski KE, Broker GA, Willauer HD, et al. Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. J Am Chem Soc. 2003;125(22):6632-6633. https://doi.org/10.1021/ja0351802

18. Freire MG, Cláudio AFM, Araújo JMM, et al. Aqueous biphasic systems: a boost brought about by using ionic liquids. Chem Soc Rev. 2012;41(14):4966-4995. https://doi.org/10.1039/c2cs35151j
19. Du Z, Yu Y-L, Wang J-H. Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system. *Chem A Eur J*. 2007;13(7):2130-2137. https://doi.org/10.1002/chem.200601234

20. Deive FJ, Rodriguez A, Pereiro AB, et al. Ionic liquid-based aqueous biphasic system for lipase extraction. *Green Chem*. 2011;13(2):390-396. https://doi.org/10.1039/c0gc0075b

21. Taha M, Almeida MR, Silva FAE, et al. Novel biocompatible and self-buffering ionic liquids for biopharmaceutical applications. *Chemistry*. 2015;21(12):4781-4788. https://doi.org/10.1002/chem.201405693

22. Xu P, Wang Y, Chen J, et al. A novel aqueous biphasic system formed by deep eutectic solvent and ionic liquid for DNA partitioning. *Talanta*. 2018;189:467-479. https://doi.org/10.1016/j.talanta.2018.07.035

23. Pereira JFB, Lima AS, Freire MG, Coutinho JAP. Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems. *Green Chemistry*. 2010;12(9):1661-1669. https://doi.org/10.1039/c003578e

24. Desai RK, Streefland M, Wijffels RH, Eppink MHM. Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems. *Green Chemistry*. 2014;16(5):2670-2679. https://doi.org/10.1039/c3gc42631a

25. Pei Y, Wang J, Wu K, Xuan X, Lu X. Ionic liquid-based aqueous two-phase extraction of selected proteins. *Sep Purif Technol*. 2009;64(3):288-295. https://doi.org/10.1016/j.seppur.2008.10.010

26. Andrews BA, Huang RB, Asenjo JA. Purification of virus-like particles from yeast cells using aqueous-two phase systems. *Bioseparation*. 1995;5:105-112.

27. Benavides J, Mena JA, Cisneros-Ruiz M, Ramirez OT, Palomares LA, Rito-Palomares M. Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems. *J Chromatogr B*. 2006;842(1):48-57. https://doi.org/10.1016/j.jchromb.2006.05.006

28. Jacinto MJ, Soares RRG, Azevedo AM, et al. Optimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant. *Sep Purif Technol*. 2015;154:27-35. https://doi.org/10.1016/j.seppur.2015.09.006

29. Luechau F, Ling TC, Lyddiatt A. Recovery of B19 virus-like particles by aqueous two-phase systems. *Food Bioprod Process*. 2011;89(4):322-327. https://doi.org/10.1016/j.fbp.2010.10.008

30. Oelmeier SA, Ladd Effio C, Hubbuch J. High throughput screening based selection of phases for aqueous two-phase system-centrifugal partitioning chromatography of monoclonal antibodies. *J Chromatogr A*. 2012;1252:104-114. https://doi.org/10.1016/j.chroma.2012.06.075

31. Ladd Effio C, Wenger L, Ötes O, Oelmeier SA, Kneusel R, Hubbuch J. Downstream processing of virus-like particles: single-stage and multi-stage aqueous two-phase extraction. *J Chromatogr A*. 2015;1383:35-46. https://doi.org/10.1016/j.chroma.2015.01.007

32. Soares HR, Ferreira-Fernandes M, Marchel M, Alves PM, Coroadinha AS. Enhancing hepatitis C virus pseudoparticles infectivity through p7NS2 cellular expression. *J Virol Methods*. 2019.

33. Merchuk JC, Andrews BA, Asenjo JA. Aqueous two-phase systems for protein separation: studies on phase inversion. *J Chromatogr B Biomed Sci Appl*. 1998;711(1):285-293. https://doi.org/10.1016/S0378-4347(97)00594-X

34. Bensch M, Selbach B, Hubbuch J. High throughput screening technologies in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. *Chem Eng Sci*. 2007;62(7):2011-2021. https://doi.org/10.1016/j.ces.2006.12.053

35. Oelmeier SA, Dismer F, Hubbuch J. Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. *Biotechnol Bioeng*. 2011;108(1):69-81. https://doi.org/10.1002/bit.22900

36. Ferreira AM, Faustino VFM, Mondal D, Coutinho JAP, Freire MG. Improving the extraction and purification of immunoglobulin G by the use of ionic liquids as adjuvants in aqueous biphasic systems. *J Biotechnol*. 2016;236:166-175. https://doi.org/10.1016/j.jbiotec.2016.08.015

37. Gao Y-L, Peng, Q-H, Li Z-C, Li Y-G. Thermodynamics of ammonium sulfate—polyethylene glycol aqueous two-phase systems. Part 1. *Fluid Ph Equilibria*. 1991;63(1):157-171. https://doi.org/10.1016/0378-3812(91)80028-T

38. Murari GF, Penido JA, Machado PAL, et al. Phase diagrams of aqueous two-phase systems formed by polyethylene glycol+ammonium sulfate+water: equilibrium data and thermodynamic modeling. *Fluid Ph Equilibria*. 2015;406:61-69. https://doi.org/10.1016/j.fluid.2015.07.024

39. Suarez Ruiz CA, van den Berg C, Wijffels RH, Eppink MHM. Rubisco separation using biocompatible aqueous two-phase systems. *Sep Purif Technol*. 2018;196:254-261. https://doi.org/10.1016/j.seppur.2017.05.001

40. Voros N, Proust P, Fredenslund A. Liquid-liquid phase equilibria of aqueous two-phase systems containing salts and polyethylene glycol. *Fluid Ph Equilibria*. 1991;90(2):333-353. https://doi.org/10.1016/0378-3812(91)80027-9

41. Zaaslovsky BY, Gulaeva ND, Djararov S, Masimov EA, Miheeva LM. Phase separation in aqueous poly (ethylene glycol)-(NH4)2SO4 systems and some physicochemical properties of the phases. *J Colloid Interface Sci*. 1990;137(1):147-156. https://doi.org/10.1016/0021-9797(90)90051-O

42. Albertsson P-A. *Partition of Cell Particles and Macromolecules*. 3rd ed. New York, NY: Wiley; 1986.

43. Shahriari S, Neves CMSS, Freire MG, Coutinho JAP. Role of the Hofmeister series in the formation of ionic-liquid-based aqueous biphasic systems. *J Phys Chem B*. 2012;116(24):7252-7258. https://doi.org/10.1021/jp300874u

44. Vernau J, Kula MR. Extraction of proteins from biological raw material using aqueous poly (ethylene) glycol–citrate phase systems. *Biotechnol Appl Biochem*. 1990;12:397-404.

45. Saravanan S, Rao JR, Murugesan T, Nair BU, Ramasami T. Partition of tannery wastewater proteins in aqueous two-phase poly (ethylene glycol)-magnesium sulfate systems: effects of molecular weights and pH. *Chem Eng Sci*. 2007;62(4):969-978. https://doi.org/10.1016/j.ces.2006.10.025

46. Heldt CL, Zahid A, Vijayaragavan KS, Mi X. Experimental and computational surface hydrophobicity analysis of a non-enveloped virus and proteins. *Colloids Surf B: Biointerfaces*. 2017;153:77-84. https://doi.org/10.1016/j.colsurfb.2017.02.011
47. Johnson SA, Walsh A, Brown MR, et al. The step-wise framework to design a chromatography-based hydrophobicity assay for viral particles. *J Chromatogr B*. 2017;1061-1062:430-437. https://doi.org/10.1016/j.jchromb.2017.08.002

48. Cláudio AFM, Swift L, Hallett JP, Welton T, Coutinho JAP, Freire MG. Extended scale for the hydrogen-bond basicity of ionic liquids. *Phys Chem Chem Phys*. 2014;16(14):6593-6601. https://doi.org/10.1039/c3cp55285c

49. Marchel M, João KG, Marrucho IM. On the use of ionic liquids as adjuvants in PEG-(NH4)2SO4 aqueous biphasic systems: phase diagrams behavior and the effect of IL concentration on myoglobin partition. *Sep Purif Technol*. 2019;210:710-718. https://doi.org/10.1016/j.seppur.2018.08.058

50. Lu Y-M, Yang Y-Z, Zhao X-D, Xia C-B. Bovine serum albumin partitioning in polyethylene glycol (PEG)/potassium citrate aqueous two-phase systems. *Food Bioprod Process*. 2010;88(1):40-46. https://doi.org/10.1016/j.fbp.2009.12.002

51. Bartosch B, Dubuisson J, Cosset F-L. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. *J Exp Med*. 2003;197(5):633. https://doi.org/10.1084/jem.20021756

52. Pelliccia M, Andreozzi P, Paulo J, et al. Additives for vaccine storage to improve thermal stability of adenoviruses from hours to months. *Nat Commun*. 2016;7:13520. https://doi.org/10.1038/ncomms13520

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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