Green, Aqueous Two-Phase Systems Based on Cholinium Aminoate Ionic Liquids with Tunable Hydrophobicity and Charge Density

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ABSTRACT: Cholinium-based ionic liquids made of aminoate anions ([Ch][AA] ILs) have been proven to have low toxicity and to be readily biodegradable. This class of ILs has great potential as a novel phase-forming component for aqueous two-phase system (ATPS) used in protein separation. In this study, environmentally benign ATPSs were prepared by using a series of [Ch][AA] ILs (where [AA] = lysinate, β-alaninate, glycinate, serinate) with a thermo-separable polypropylene glycol 400 (PPG 400). These [AA] anion components differ in both hydrophobicity and acid/base behavior, allowing a tunable response to the pH of the ATPS. By using the effective excluded volume theory, the phase-separation abilities of the different [Ch][AA]-based ATPSs were determined, and the trends showed good agreement with the [AA] anion hydration capacities. The partition behavior of a model protein, i.e., bovine serum albumin (BSA), was investigated by measuring the IL-rich (bottom) phase extraction efficiency and partition coefficient. The results showed that the partition behavior of the protein was governed by the hydrophobicity of the [AA] anion, whereby the BSA showed a greater affinity toward the IL-rich phase comprising a less hydrophobic [AA] anion. Furthermore, the effect of pH on the partition behavior of model proteins (i.e., BSA and trypsin) was investigated. When the pH of the system is greater than the isoelectric point pI of the model proteins and the [AA] anion, the model proteins are found to be mainly partitioned to the bottom phase. However, at a pH below the pI of the model proteins and the pI of the [AA] anion, the partitioning of model proteins favored the polymer-rich top phase. Overall, our findings demonstrate the flexibility possible in directing the target protein to a desired phase in ATPS through a proper selection of the IL’s hydrophobicity and [AA] anion.

KEYWORDS: Aqueous two-phase system, cholinium aminoate ionic liquid, tunable hydrophobicity, controllable charge density, liquid–liquid equilibrium, partition behavior

INTRODUCTION

Aqueous two-phase systems (ATPSs) are a promising protein purification technique used in the biotechnology industry due to their efficiency and simplicity in operation. This variant of liquid–liquid extraction is usually biocompatible due to the high water content present in both phases. Therefore, it has been widely applied in the downstream processing of biological compounds such as proteins, cells, organelles, and even genetic materials. Although the conventional dual-polymer or polymer + salt ATPSs are commonly used in these applications, the limited range of the polarities of coexisting phases has become a bottleneck in the use of these ATPSs. Over the past decade, considerable effort has been devoted to the use of ionic liquids (ILs) as an alternative phase-forming component in ATPS. ILs are often described as “green” solvents because they present two outstanding properties, namely the negligible volatility and the nonflammability under ambient conditions. In the context of ATPS formation, one of the IL’s main properties of interest, as compared to the organic salts with a higher melting point, is its extended solubility range in a multicomponent ATPS. In addition, the polarities and affinities of the IL can be tailored by a specific manipulation of the cation or anion groups, thus providing a wide range of hydrophobicity. It is known that the hydrophobic interaction is one of the primary forces driving the partition of proteins in ATPS. Hence, an IL-based ATPS can be designed in a way to permit a strong interaction between the IL-rich phase and the target compound during the separation process. Moreover, IL-based
ATPSs have shown higher extraction efficiencies than the conventional ATPS in the separation of biocompounds such as testosterone, epitestosterone, opium alkaloids, and bovine serum albumin (BSA).9–12

Despite the fascinating potential of IL-based ATPSs in the application of protein recovery, ILs used in the formation of ATPS are mostly derived from the cation components based on imidazolium or pyridinium and from the anion components such as halides. These ionic components have been found to be toxic and/or poorly biodegradable.13 The hydrophobicity of these traditional ILs can be tuned by changing the alkyl chain length of the IL cation.14 However, increasing alkyl chain length in this manner causes an increase in the toxicity and a decrease in biodegradability.15 Furthermore, these ILs are difficult to separate completely from the aqueous solution. Thus, the discharge of wastewater containing these ILs into aquatic ecosystems could inevitably lead to serious environmental pollution. In view of this, the synthesis of more environmentally friendly ILs with components featuring an enhanced biocompatibility and a reasonable environmental footprint has been proposed in recent years.16,17

In particular, ILs composed of cholinium ([Ch], the cation component of the IL) are a type of environmentally friendly phase-forming candidate.18–20 A past study showed that the [Ch]-based ILs possess remarkable biodegradability as well as low toxicity toward filamentous fungi and freshwater crustacean Daphnia magna.21 However, it is important to realize that the toxicity of the IL is not solely contributed by the cation. Several past studies conducted on the cytotoxicity of the [Ch]-based ILs on macrophage17 and human cell lines22 showed that the anion component of the IL has a great influence on the overall toxicity. Amino acids (AA) are a suitable candidate as the anion component in the IL due to their abundance in nature and the reasonable cost of synthesizing ILs derived from AA.23 More importantly, it has been proven that ILs having an AA as the anion component exhibited properties such as a reduced toxicity24–26 and a good biodegradability.27,28 In addition, a broad range of hydrophobicity can be exhibited by [AA]-derived ILs, depending on the nature of the AA. Unlike imidazolium-based ILs, the hydrophobicity can be tuned without the need to alter the cation alkyl chain length. Therefore, ILs of the [Ch] cation paired with [AA] anions may emerge as a useful choice of IL in applications demanding a low toxicity and environmental friendliness.

In the present study, a series of [Ch][AA] ILs, namely cholinium lysinate ([Ch][Lys]), cholinium β-alanine ([Ch][β-Ala]), cholinium glycinate ([Ch][Gly]), and cholinium serinate ([Ch][Ser]), were used to form ATPSs with PPG 400. The PPG 400 is a thermo-responsive polymer having a lower critical solution temperature in water (LCST) of 46 °C.19 The PPG 400 solution thermo-separates into a biphasic solution at a temperature higher than its LCST; the thermo-separated polymer phase could be reused in the formation of a new ATPS, making this variant of ATPS even more sustainable and economically viable.19 The liquid–liquid equilibria of our PPG 400 + [Ch][AA] + water systems were determined at 25 °C experimentally. The binodal data were correlated using empirical equations that have been widely adopted for the conventional IL-based ATPSs. Based on the fitting accuracy of these empirical equations to the experimental binodal data, a model that best describes the binodal curve of this novel ATPS was determined. The experimental tie-line data were also validated using Othmer-Tobias and Bancroft equations, which have been widely used for the IL-based ATPSs.15,23,29 The phase-separation abilities of the [Ch][AA]s were evaluated using the effective excluded volume (EEV) theory. Then, the influence of the IL hydrophobicity on the partition behavior of a model protein (i.e., BSA) in these ATPSs was studied systematically. Moreover, the effect of pH on the partition behavior of proteins was investigated by using proteins (i.e., BSA and trypsin) with different isoelectric points (pI).

## EXPERIMENTAL SECTION

### Materials

Choline hydroxide [20% (w/w) in water], β-alanine (99.5% purity), serine (99% purity), and L-lysine (98% purity) were obtained from Sigma-Aldrich (Australia), while glycine (99% purity) was procured from Merck (Australia). PPG 400, BSA (98% purity), trypsin, and Nα-benzoyl-l-arginine ethyl ester (BAAE) were purchased from Sigma-Aldrich (St. Louis, USA). All of these chemicals were used without further purification. The details of the synthesis and characterization of [Ch][AA] ILs are given in the Supporting Information.

### Determination of Binodal Data

The binodal curves were determined using a turbidimetric titration method.30 A series of ATPSs were prepared by mixing the known mass fractions of [Ch][AA], PPG 400, and deionized water in 15-mL centrifuge tubes. The mixtures were initially turbid and cloudy. Then, deionized water was added dropwise followed by thorough mixing, until the mixtures became clear. For each system, the total weight of deionized water added was measured, and the mass fractions of IL and polymer at the phase-transition point were determined. The data were used to plot the binodal curves.

### Determination of Tie-Line Data

A series of ATPSs were first prepared by mixing the appropriate amount of [Ch][AA], PPG 400, and deionized water in 2-mL microcentrifuge tubes. The mixtures were then centrifuged at 6000 rpm for 10 min to ensure a complete phase separation. Samples from both phases were carefully withdrawn. The concentrations of IL in both phases were determined using an UV–vis spectrophotometer (Cary 100 UV–vis, Agilent Technologies) at 277 nm. To quantitate the water content of the top and bottom phases, the samples were subjected to vacuum-drying at −92 °C for 24 h using a freeze-dryer (CoolSafe series, ScanVac).31 The weight loss in the sample was equivalent to the mass of the water in the system. The polymer mass fraction was calculated by difference. The tie-line length (TLL) and the slope of the tie-line (STL) were calculated using eqs 1 and 2 as shown below:

\[
\text{TLL} = 
\left( w_1^b - w_1^w \right) + \left( w_2^b - w_2^w \right) \times 0.5
\]

\[
\text{STL} = \frac{w_1^b - w_1^w}{w_2^b - w_2^w}
\]

where \( w_1^b, w_2^b, w_1^w, \) and \( w_2^w \) represent the equilibrium mass fractions (w) of the polymer (1) and [Ch][AA] IL (2) in the top phase (t) and bottom phase (b).

### Partitioning of Model Proteins in ATPSs

The protein solution was prepared by dissolving 2 mg of the model protein in 1 mL of deionized water. Then, several ATPSs were prepared in 2-mL microcentrifuge tubes by adding 50% (w/w) PPG 400, 4% (w/w) IL, 20% (w/w) protein solution, and deionized water to make up a final weight of 2 g. The desired pH of the ATPS was adjusted using 1 M hydrochloric acid. All of the systems were mixed vigorously by using a vortex mixer before being allowed to settle at 25 °C for 3 h to attain equilibrium of the two phases. Then, the ATPSs were centrifuged for 5 min at 2000 rpm in order to ensure complete phase separation. The volumes of the top phase (\( V_T \)) and bottom phase (\( V_B \)) were measured. For the determination of BSA concentration, a Bradford assay was used. In brief, the dye reagent was first prepared by diluting with deionized water (one part dye reagent to four parts of deionized water). Then, 10 μL of the sample was mixed with 200 μL of the diluted dye reagent in a 96-well microplate. Subsequently, the mixture was incubated at room temperature for 5 min. The absorbance of the
change in time expressed in min,

0.5 mM of BAEE, 50 mM of Tris bu

mixture was measured at 595 nm using a microplate reader (Sunrise, Tecan). In the trypsin case, the concentration of trypsin was

Table 1. Values of Parameters of eqs 6–8 Used in the Fitting of Binodal Data of PPG 400 + [Ch][AA] + Water Systems at 25°C

<table>
<thead>
<tr>
<th>equation</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>R²</th>
<th>sd²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPG 400 + [Ch][Lys] + water</td>
<td>6</td>
<td>149.2</td>
<td>−0.633</td>
<td>−6.855 (× 10⁻³)</td>
<td>0.9996</td>
<td>1.343</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.674</td>
<td>0.785</td>
<td>−0.399</td>
<td>0.008</td>
<td>0.9998</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>104.3</td>
<td>−36.15</td>
<td>2.502</td>
<td>0.041</td>
<td>0.9992</td>
</tr>
<tr>
<td>PPG 400 + [Ch][Ser] + water</td>
<td>6</td>
<td>181.1</td>
<td>−0.711</td>
<td>−2.784 (× 10⁻³)</td>
<td>0.9991</td>
<td>0.632</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.756</td>
<td>−0.250</td>
<td>−0.130</td>
<td>0.003</td>
<td>0.9996</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>138.4</td>
<td>−65.69</td>
<td>9.495</td>
<td>−0.063</td>
<td>0.9966</td>
</tr>
<tr>
<td>PPG 400 + [Ch][Gly] + water</td>
<td>6</td>
<td>162.6</td>
<td>−0.710</td>
<td>2.831 (× 10⁻³)</td>
<td>0.9983</td>
<td>1.339</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.109</td>
<td>0.338</td>
<td>−0.294</td>
<td>0.006</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>119.1</td>
<td>−52.85</td>
<td>6.761</td>
<td>−0.026</td>
<td>0.9961</td>
</tr>
<tr>
<td>PPG 400 + [Ch][β-Ala] + water</td>
<td>6</td>
<td>143.5</td>
<td>−0.621</td>
<td>2.481 (× 10⁻³)</td>
<td>0.9992</td>
<td>1.302</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.972</td>
<td>0.428</td>
<td>−0.288</td>
<td>0.005</td>
<td>0.9997</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>103.9</td>
<td>−37.33</td>
<td>3.109</td>
<td>0.019</td>
<td>0.9985</td>
</tr>
</tbody>
</table>

misture was measured at 595 nm using a microplate reader (Sunrise, Tecan). In the trypsin case, the concentration of trypsin was determined using the assay developed by Schert and Takenaka. Briefly, the sample was added to a 3-mL aqueous solution containing 0.5 mM of BAEE, 50 mM of Tris buffer, and 10 mM of calcium chloride (CaCl₂) at pH 7.6 and 25 °C, followed by an immediate mixing. The absorption of solution was measured using a UV–vis spectrophotometer at 253 nm for 5 min at an interval of 0.5 min. The BAEE units, U/mL of trypsin were calculated using eq 3 as shown below:

\[
\text{BAEE units, U/mL of trypsin} = \left[ \frac{(\Delta A_{253}/\Delta t)_{\text{test}} - (\Delta A_{253}/\Delta t)_{\text{blank}}}{(K_v)(V_v)} \right] 
\]

where \(\Delta A_{253}\) is the difference in absorbance at 253 nm, \(\Delta t\) is the change in time expressed in min, \(d_i\) is the dilution factor, \(K_v\) is the constant for the change in \(\Delta A_{253}/\Delta t\) per trypsin unit (the \(K_v\) value was given to be 0.001 at pH 7.6 and 25 °C in a 3.2-mL reaction mixture), and \(V_v\) is the volume (mL) of the enzyme used. To assess the degree of protein separation achieved in the ATPS, the partition coefficient (K) of model protein was calculated using eq 4. K is the ratio of equilibrium concentration of the protein in the bottom phase (\(C_B\)) to the equilibrium concentration of the protein in the top phase (\(C_T\)):

\[
K = \frac{C_B}{C_T} 
\]

The bottom-phase extraction efficiency (\(E_B\)) of the model proteins was calculated using eq 5 as shown below:

\[
E_B(\%) = \frac{C_B V_B}{C_T V_T + C_B V_B} \times 100 
\]

where \(V_T\) and \(V_B\) are the volumes of top and bottom phases, respectively.
RESULTS AND DISCUSSION

**LLE Data and Correlations.** The binodal data for PPG 400 + [Ch][AA] + water systems at 25 °C are tabulated in Table S1 (in the Supporting Information). All the binodal data were correlated using the Merchuk equation \(^35\) [eq 6] and nonlinear empirical expressions \(^29,36\) with four fitted parameters [eqs 7 and 8] as shown below:

\[
\begin{align*}
\ln w_1 &= a \exp(b w_2^{0.5} - c w_2^3) \\
\ln w_1 &= a + b w_2^{0.5} + c w_2 + d w_2^2 \\
\ln w_1 &= a + b w_2^{0.5} + c w_2 + d w_2^2 \\
\end{align*}
\]

where \( w_1 \) and \( w_2 \) are the mass fractions of the [Ch][AA] and PPG 400, respectively, while \( a, b, c, d \) and \( e \) are the fitting parameters. These equations have been widely applied to the correlation of binodal data of IL-based ATPSs. \(^37,38\)

A least-squares regression of the experimental binodal data was used to determine the fitting parameters expressed in the nonlinear empirical equations. The values of the parameters in eqs 6-8, along with the corresponding coefficients of determination \( (R^2) \) and standard deviation \( (sd) \), are tabulated in Table 1. On the basis of the obtained \( R^2 \) and sd, eq 7, which is a four-parameter correlation equation, was found to be the best fit to the binodal data of all the investigated systems.

The compositions of the experimental tie-line at 25 °C along with the corresponding calculated TLL and STL are tabulated in Table 2. In relation to the STLs, the variation is not prominent for each of the systems, suggesting that the obtained tie-lines are near parallel. The STLs for the PPG 400 + [Ch][Lys] + water system are the steepest among the investigated systems. The top and bottom phases of all the systems were mostly saturated with the PPG 400 (polymer-rich) and the [Ch][AA] (IL-rich), respectively. An increase in TLL of the system led to an increase in the concentrations of PPG 400 and [Ch][AA] in the respective top and bottom phases.

The LLE data of the PPG 400 + [Ch][AA] + water systems are presented in Figure 1. The empirical correlation equations given by Othmer-Tobias [eq 9] and Bancroft [eq 10] were used to fit the tie-line data:

\[
\begin{align*}
1 - w_1^t &= k_1 \left[ 1 - \frac{w_2^b}{w_2^t} \right]^n \\
\frac{w_2^b}{w_2^t} &= k_2 \left[ \frac{w_1^t}{w_1^b} \right]^r \\
\end{align*}
\]

where \( w_1^t \) is the mass fraction of the polymer (1) in the top phase, \( w_2^b \) is the mass fraction of ILs (2) in the bottom phase, and \( w_2^t \) and \( w_2^b \) are the mass fractions of water (3) in the bottom and top phases, respectively. \( k_1, n, k_2, \) and \( r \) are the fitting parameters. The linear dependence plot of \( \log((1 - w_1^t)/w_1^t) \) against \( \log((1 - w_2^b)/w_2^b) \) from eq 9 [in Figure S5a-d, Supporting Information] and the linear dependence plot of \( \log(w_2^b/w_2^t) \) against \( \log(w_1^t/w_1^b) \) from eq 10 [in Figure S6a-d, Supporting Information] indicated an acceptable consistency of the experimental results. The values of the fitting parameters together with the \( R^2 \) values are presented in Table S2 (in the Supporting Information).
Phase Separation Ability of [Ch][AA]-based ATPSs.

The experimental binodal curves for all four types of PPG 400 + [Ch][AA] + water systems at 25 °C are plotted in Figure 2.

![Figure 2](image_url)

Figure 2. Binodal curves for PPG 400 + [Ch][AA] + water systems plotted on a molality basis at 25 °C.  ● [Ch][Lys];  ○ [Ch][Ser];  ▲ [Ch][Gly];  ■ [Ch][β-Ala].

where the concentrations of PPG 400 and [Ch][AA] are expressed in molality (mol/kg). Considering the fact that PPG 400 and the [Ch] cation are present in all the investigated ATPSs, the relative phase-forming ability due to the [AA] anion could be postulated from the position of the binodal curve in the phase diagram. The closer the binodal curve is to the origin of the phase diagram, the lower the concentrations of phase-forming components required for the formation of the ATPS. Based on Figure 2, the ability of the [AA] anion to induce phase separation follows the order of [Lys] > [Gly] > [Ser] > [β-Ala]. However, this observation was unable to distinguish the phase-forming ability between [Gly], [Ser], and [β-Ala] ions as their binodal curves are mostly at the same position. Since the phase-forming abilities of the IL anions could not be determined distinctively from the plotted binodal curves, the EEV theory was used to correlate the binodal data in order to further distinguish the phase-separation ability of the [AA] anions.

EEV theory is based on the concept that all the compositions along a binodal are geometrically saturated solutions in which any molecules within them are randomly distributed at the macroscopic level. This theory could be used to identify the phase-separation ability of the component present in the system. Recently, studies have shown that EEV theory is also applicable to ATPSs composed of ILs and salt.

The EEV equation used to correlate the binodal data is shown below:

\[
\ln \left( \frac{V_{f13}}{M_2} \right) + f_{313} = 0
\]  (11)

where \( V_{f13} \) is the scaled EEV of the IL and \( f_{313} \) is the volume fraction of the unfilled effective available volume after tight packing of IL molecules into the network of the polymer aqueous solution. \( w_1, w_2, M_1, \) and \( M_2 \) are the mass fractions and molecular weights for the polymer and ILs, respectively. The coefficients of eq 11 along with the corresponding sd for PPG 400 + [Ch][AA] + water systems are presented in Table 3.

The EEV theory was initially developed for (polymer + polymer)-based ATPSs. In the EEV formula, the \( f_{313} \) value was minute to the extent that it could be neglected due to the size difference between the two polymer components. However, with the presence of the \( f_{313} \) parameter, the EEV equation showed a better correlation with our binodal data. This suggests that the \( f_{313} \) value was indeed not so small as to be neglected for the PPG 400 + [Ch][AA] ATPSs. The increase in EEV indicates a greater phase-separation ability of the [Ch][AA] corresponding to a decrease in concentration of IL required for the formation of an ATPS. Based on the results shown in Table 3, we can conclude that the phase-separation of the [Ch][AA] ILs can be ranked in the order of [Ch][Lys] > [Ch][Ser] > [Ch][Gly] > [Ch][β-Ala]. Moreover, the hydrophobicity of the studied [AA] anions increased in the order of [Lys] > [Ser] > [Gly] > [β-Ala]. It is worth noting that this hydrophobicity scale of [AA] anions showed a similar trend to the phase-separation ability of the [AA] anions calculated using the EEV equation. This similarity implies that the [AA] anions possessing less hydrophobic groups have a greater affinity for water, thereby indirectly reducing the water available for hydrating PPG 400. Therefore, a more hydrophilic [AA] anion is a better salting-out agent and is more effective in promoting the formation of an ATPS.

Effect of Hydrophobicity of [Ch][AA] on Protein Partitioning. The partitioning of proteins in ATPSs is known to be governed by the interaction between the phase components and the physicochemical properties of the proteins, such as charge and surface hydrophobicity. A past study showed that the anionic nature of [Ch]-based ILs will affect the partitioning of protein in ATPS. However, the effect of [Ch]-based ILs’ hydrophobicity on the partitioning of a protein in ATPS has yet to be fully understood. In this study, the model protein, BSA, was partitioned in 50% (w/w) PPG 400 + 4% (w/w) [Ch][AA] + water systems (pH 11). The volume ratio (\( V_B/V_H \)) of all the prepared systems was set at 1. The values of \( E_B \) and \( K \) of BSA in the investigated ATPSs as well as the surface hydrophobicity of [AA] anions are summarized in Table 4. It was observed that the \( K \) value of BSA in all the ATPSs is greater than 5, indicating that the majority of the BSA was present in the IL-rich (bottom) phase. The \( K \) decreased as the hydrophobicity of the [AA] anions in [Ch][AA] increased (see Figure 3). The decrease in hydrophobicity of [AA] anions promoted the hydrophilic interaction between BSA and the IL-rich phase, which eventually enhanced the partition of BSA to the IL-rich phase.

Effect of pH on Protein Partitioning. Another possible mechanism which regulates the protein partitioning in the investigated ATPS is the electrostatic interaction between the surface charge of the protein and the phase components. Since the surface charge of the protein is determined by the pH of the ATPS, the partitioning behavior of protein as a function of pH is worthy of further investigation. In this study, the selected pH values covered both acidic and basic ranges (pH values ranging from 1.7 to 11). The pH was adjusted by the addition of HCl to
the moderately basic \([\text{Ch}][\text{AA}]\) mixture. The added acid has a primary role of protonating the \([\text{AA}]\) anion, returning it to the neutral (zwitterionic) form. When 1 mol of HCl per mole \([\text{Ch}][\text{AA}]\) has been added to the mixture, it is best thought of as a solution of \([\text{Ch}]\)Cl with an equimolar amount of the \(\text{H}[\text{AA}]\) anion in its zwitterionic form. During this process, the ionic strength of the mixture does not change. At still lower pH values, the \(\text{AA}\) further protonates, forming \([\text{HAAH}]^+\), and along with the additional \(\text{Cl}^-\), this increases the ionic strength of the solution.

As illustrated in Figure 4, the BSA in the IL-rich phase gradually shifted to the polymer-rich phase as the system became more acidic. This phenomenon was indicated by the decreasing trends in both \(E_B\) and K values of BSA (pI = 4.9) with decreasing pH. Moreover, a drastic drop in \(E_B\) and K values of BSA (slope AB > slope BC, as shown in Figure 4) was noted when the pH of the system was below pH 4.4. In order to understand the impact of pH change on the protein surface charge, which in turn affects the protein’s partitioning behavior, another model protein (i.e., trypsin) having a pI of 10.5 was also tested in the PPG 400 + \([\text{Ch}][\text{Gly}]\) + water system under similar conditions. In general, it is found that \(E_B\) and K values of trypsin (as shown in Figure 5) were in a similar trend as BSA. A more pronounced decrease in both \(E_B\) and K values of trypsin (slope AB > slope BC, as shown in Figure 5) was observed when the pH of the system dropped below 6.5. According to Figures 4 and 5, the K values for both model proteins at pH 7 are postulated to be 8.4 and 8.5. A similar study has been conducted on the protein partition behavior using PPG 400 + \([\text{Ch}]\) carboxylates-based ILs + water systems in which the ranges of K for BSA and trypsin (at pH 7) were 3.38 – 9.31 and 4.17 – 14, respectively. Based on the comparison, the efficiencies of protein extraction in our ATPS are comparable to that of the reported PPG 400 + \([\text{Ch}]\) carboxylates-based ILs + water systems.

When the pH is below the pI of the protein, a net positive charge will be exhibited by the protein as its amine groups become protonated. Conversely, the protein will carry a net negative charge when the pH is above its pI as protons are lost from the carboxyl groups of the protein. At pH > pI, both BSA and trypsin were mostly distributed to the \([\text{Ch}][\text{Gly}]\)-rich bottom phase (\(E_B\) value >50%). This is due to the hydrophilic interactions between the model proteins and the [Ch] and [Gly] ions. However, when the pH of the system was gradually decreased, the proteins and the [Gly] ion (pI = 5.97) became less negatively charged and eventually positively charged when pH < pI. Thus, the electrostatic repulsion between the model protein and the [Ch] cation/[H][Gly]^+ ion shifted the model proteins from the IL-rich phase to the (PPG 400)-rich top.
phase (as indicated by the significant decrease in both $E_b$ and $K$ values). This can also be thought of as a pH-tunable "salting-out" effect, the overall ionic strength increasing below the pI of the [AA] anion. The increasing ionic strength in the IL-rich bottom phase can result in the dehydration of the protein, which causes an increase in the surface hydrophobicity of the protein.46 Therefore, the partition of the less-solvated protein to the polymer-rich (hydrophobic) phase will be promoted as a result of the hydrophobic interaction.

**CONCLUSION**

ILs based on [Ch] cation and [AA] anions were successfully employed, along with PPG 400, in the formation of a novel type of environmentally friendly ATPS for protein separation. The LLE data, including experimental binodal curves and tie-lines, were well correlated with a four-parameter empirical equation, and the Othmer-Tobias and Banko equations, respectively. For the first time, EEV theory was used to determine the phase-forming ability of the IL in polymer + IL ATPSs. The results obtained from EEV theory showed that the [Lys] anion possesses the greatest phase-forming ability among the studied [AA] anions. This finding was in good agreement with the hydration capacity of the respective anions. The partitioning behavior of BSA in the [Ch][AA]-based ATPSs was also found to be greatly influenced by the hydrophobic interactions between the protein and the system components. Thus, the partitioning behavior of a target protein could be easily achieved by tailoring the [Ch][AA]. Additionally, the ability to tune the charge of the [AA] anion species as a function of system pH allows a tuning of the interactions between the phase component and the model proteins (i.e., BSA and trypsin). Depending on the pI of both the protein and the [AA] anion, the electrostatic interaction can be altered via an adjustment of pH, thus promoting the selective distribution of a target protein in the ATPS. The pH-tunable "salting-out" effect induced by the IL-rich bottom phase at pH < pI[AA] can also enhance the hydrophobic interaction between protein and PPG polymer, thus improving the partitioning of protein to the PPG-rich top phase. Overall, these green [Ch][AA]-based ATPS systems can serve as a promising protein separation tool that meets the high separation efficiency and environmental friendliness requirements of the bioprocess industries.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.5b00881.

Information related to the synthesis procedure and characterization data ($^1$H and $^{13}$NMR) of [Ch][AA], the binodal data for PPG 400 + [Ch][AA] + water systems, the linear dependence plots of Othmer-Tobias and Banko equations, and the methodology used to determine the hydrophobicity index of AA (PDF)

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**Notes**

The authors declare no competing financial interest.

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