

Influence of acid solutes on the phase behaviour of aqueous two-phase systems, containing poly(ethylene glycol) and poly(ethylene imine)

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This paper is part of a study on the possibility of using aqueous two-phase systems (ATPSs) for separation of low-molar-mass organic acids in the biotechnology industry. We present for the first time experimental data on the influence of titrating organic acids on the phase behaviour of the ATPSs, containing poly(ethyleneglycol) (PEG) and poly(ethyleneimine). The underlying hypothesis of the mechanism advanced is discussed from the point of view of the acid structure, and is validated by the observed ATPSs phase behaviour. Finally, we demonstrate the opposing influence of the lactic acid and titrating acids in these systems.

Key words: ATPS, poly(ethyleneglycol), poly(ethyleneimine), organic acid solutes, lactic acid

1. INTRODUCTION

Aqueous two-phase systems (ATPSs), consisting of two structurally different polymers like poly(ethyleneimine) (PEI) and either poly(ethyleneglycol) (PEG) or hydroxyethylcellulose (HEC), are extensively employed for the bioseparation of low-molar-mass organic acids [1 – 4] since they provide the possibility to obtain high equilibrium concentration of the desired product in the PEI-rich phase through acid-base association.

In recent contributions [5, 6], devoted to a systematic study on ATPSs for the separation of lactic acid (LA), we have examined the phase behaviour of (PEG+PEI) ATPSs as a function of key variables such as temperature, pH and lactic acid concentration. Our work was motivated by the fact that PEI forms a two-phase system with a neutral (uncharged) polymer, such as PEG, only when strong bivalent or polyvalent counter-ions to the charges on the polycation are introduced [1,2,4]. To the best of our knowledge, our paper [6] was the first to illuminate in details the influence of anions such as phosphate and sulphate on the phase diagrams of (PEI + PEG) ATPSs and to propose a mechanism, explaining the experimentally observed phase behaviour.

The purpose of this work is to contribute further to the experimental evidence and expand the ideas, advocated in [6] by offering new insights on the influence of an acid solute on the phase behaviour

of (PEI+PEG) ATPSs.

To realize that, we will firstly focus on the experimental validation of the suggested mechanism by examining yet again how the nature of an inorganic acid solute, used in titrating the PEI, influences the phase formation of the (PEI+PEG) ATPSs. According to the assumptions of our hypothesis [6] which have been confirmed experimentally, a higher valency titrating acid expands the two-phase region, while mono-valent acids (such as HCl and HClO₄) do not form an ATPS with the neutral PEG at any concentrations (we have tried up to 30 mass percent of each polymer). To substantiate further these ideas by new experimental data we have chosen to examine the influence of boric acid because it is of uncertain valency in water solutions. Thus, by experimentally locating where the binodal of the boric acid titrated ATPS will be situated in the compositional phase space (relevant to the ATPSs of the other two acid solutes), we will test the viability of the phase-formation mechanism advocated.

Then we expand further the scope of the mechanism application by studying how an organic acid solute will influence the (PEG+PEI) ATPSs phase formation. The reasons behind that are three-fold: *i*) To obtain new experimental evidence. To the best of our knowledge, so far, organic acids have not been used as PEI titrating acids, and hence there are no data on how they might influence the phase behaviour of the (PEG+PEI) ATPSs; *ii*) From theoretical point of view it is important to investigate how solutes with different molecular structures, representatives of the very large variety

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of organic acids, will affect the ATPSs phase formation; *iii*) To expand the verification of the (PEG+PEI) ATPSs phase formation mechanism, applying the new experimental data obtained.

In the view of this, and by analogy with the inorganic acid solutes, we have chosen the following organic acids: citric, oxalic, succinic, malic, and tartaric ones (Figure 1). Thus, the influence of the nature of the organic solutes, manifested either by the acid chain length (i.e. molecular structure influence) or by the number of present OH groups (i.e. the ability to form additional H⁺ bonds), will be examined. In the first case, oxalic and succinic acids will be applied as titrating acids, while in the second – citric, succinic, malic, and tartaric acids will be studied.

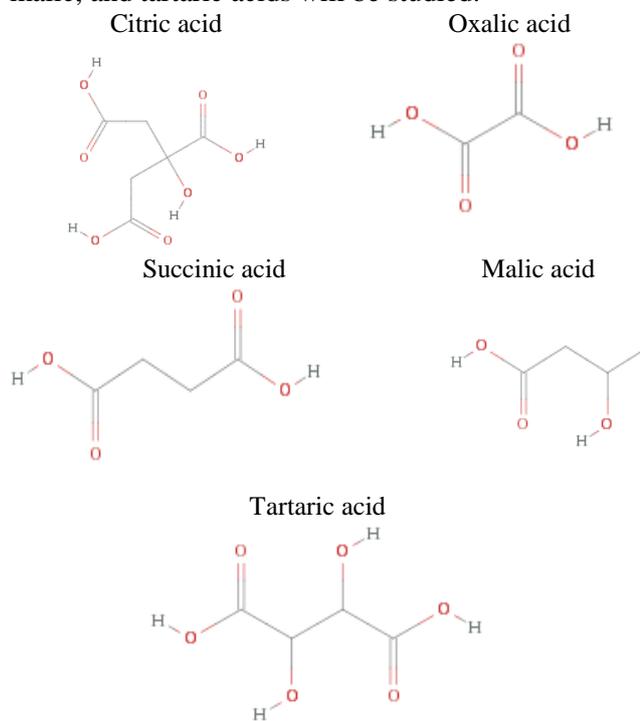


Figure 1. Structure of the organic acids, applied in this study.

Finally, the effect of addition of a second acid, lactic acid, on the phase behaviour will be examined and compared for the cases of H₂SO₄ and tartaric acid, used to titrate the PEI.

2. EXPERIMENTS

2.1. Materials and Methods

PEG 4000 was obtained from Fluka. It is a narrowly distributed technical product, with a polydispersity index of about 1.05 as determined in this study by gel permeation chromatography (GPC). PEI was obtained from Aldrich. According to the manufacturer, the number-average molar mass M_n of the PEI was approximately 10000

g·mol⁻¹ by GPC, and the mass-average molar mass M_w was approximately 25000 g·mol⁻¹ by light scattering; so the PEI sample was characterised by a polydispersity index of about 2.5.

Stock solutions of the polymers in distilled water were prepared gravimetrically on an analytical balance. For the PEG, the mass fraction (w) of the stock solution was 0.4, while for PEI a mass fraction of 0.2 was chosen. The PEI solutions were titrated with the inorganic acid solutes (H₂SO₄, H₃PO₄ and H₃BO₃), and with the organic acid solutes to pH = 7.5; in all cases with uncertainties of approximately ±0.05 pH units.

Since concentrated lactic acid solution contains high levels of dimers, it was first diluted tenfold and then boiled under reflux for 8 to 10 h to hydrolyse the dimers. The resulting solution, containing about 130 g·L⁻¹ lactic acid, was further diluted to obtain a stock solution of known concentration for use in the preparation of the quaternary ATPS.

All stock solutions were prepared gravimetrically on an analytical balance with ± 0.0001 g accuracy. The expanded uncertainty of all gravimetric measurements was ± 5.3·10⁻⁴ g with coverage factor of 2.

2.2. Experimental Procedure.

ATPS Preparation

All ATPSs were prepared gravimetrically by mixing appropriate quantities of the necessary stock solutions with distilled water in 50 ml beakers. For determination of the binodal curves, a glass vessel with a volume of 125 ml was used. It was provided with an external jacket through which water at a constant temperature of (36.0 ± 0.1) °C was circulated.

For the preparation of the quaternary ATPSs, stock solutions for PEG and PEI were made like the above but with the addition of either 4.3 or 8.6 g·L⁻¹ of lactic acid, when the PEI was titrated with H₂SO₄, or with addition of either 4.85 or 8.70 g·L⁻¹ of lactic acid when the PEI was titrated with tartaric acid. These particular concentrations were chosen to exemplify the concentration range in which the inhibitory effect of lactic acid was manifested.

Determination of the Binodal Curves

In our experiments, the binodal curve was approximated by the cloud-point curve, determined by dropwise addition of water to the well stirred ATPSs, prepared as described above, until turbidity

Table 1. The coefficients of equation (1) for the binodal curves of the (PEG+PEI) ATPSs in which PEI was titrated with different acids to pH = 7.5.

Titrating Acids	Coefficients		
	<i>a</i>	<i>b</i>	<i>c</i>
Phosphoric acid	16.5771	1.3533	$1.6446 \cdot 10^{-4}$
Sulphuric acid (no LA)	15.2028	0.6665	$7.5751 \cdot 10^{-4}$
$c_{\text{LA}} = 4.3 \text{ g}\cdot\text{L}^{-1}$	51.1574	0.9620	$7.0714 \cdot 10^{-4}$
$c_{\text{LA}} = 8.6 \text{ g}\cdot\text{L}^{-1}$	59.9603	0.8618	$9.7091 \cdot 10^{-4}$
Boric acid	50.3827	0.8100	$3.8722 \cdot 10^{-4}$
Tartaric acid (no LA)	15.2028	0.6665	$7.5751 \cdot 10^{-4}$
$c_{\text{LA}} = 4.85 \text{ g}\cdot\text{L}^{-1}$	40.2332	0.8226	$7.9867 \cdot 10^{-4}$
$c_{\text{LA}} = 8.7 \text{ g}\cdot\text{L}^{-1}$	36.0429	0.7231	$1.0540 \cdot 10^{-4}$
Oxalyc acid	17.0781	0.6581	$4.8062 \cdot 10^{-4}$
Succinic acid	22.6295	0.5867	$8.2416 \cdot 10^{-4}$
Malic acid	17.9718	0.6123	$1.2048 \cdot 10^{-4}$

disappeared and the solution became transparent, which indicated the transition from heterogeneous to homogeneous phase behaviour. All solutions were maintained at $(36.0 \pm 0.1)^\circ\text{C}$ in a temperature controlled glass vessel. The composition of the last point in the two-phase region and the composition of the first point in the homogeneous region were determined gravimetrically, and the mean value of the two was taken as a point on the binodal. This typically introduced an ambiguity not worse than $\pm 0.005 \cdot w_i$ in the binodal composition.

Then a polymer solution (either PEI or PEG) was added to the homogeneous phase in a drop wise manner until turbidity appeared. The system was allowed to rest until a complete separation was assured before water was added again to transfer it to the homogeneous region. The process was repeated until the binodal was determined. This mechanism of the binodal determination assures a clear-cut distinction of phase separation and excludes precipitation/aggregation.

The binodal curves of the quaternary ATPSs were determined in the same way. However, lactic acid stock solution was used throughout in place of pure water in order to maintain a constant lactic acid concentration, either $c_{\text{LA}} = 4.3$ or $8.6 \text{ g}\cdot\text{L}^{-1}$, when the PEI was titrated with H_2SO_4 , or $c_{\text{LA}} = 4.85, 8.70 \text{ g}\cdot\text{L}^{-1}$ when the PEI was titrated with tartaric acid.

All experiments and measurements were conducted at least in triplicates.

For the ATPSs, examined in this study, the binodal curves were correlated with the following equation [7]:

$$w_2 = a \exp(-bw_1^{0.5} - cw_1^3) \quad (1)$$

where w_1 and w_2 are the mass fractions of PEG and PEI, respectively, and a , b and c are coefficients.

The coefficients of Eq. 1 for the binodal curves of the ATPSs in which PEI was titrated with different acids at pH = 7.5, are given in Table 1.

3. RESULTS aND DISCUSSION

Influence of the PEI Titrating Acid

The assumptions of the mechanism, explaining the experimentally observed phase behaviour of (PEG+PEI) ATPSs [6], can be briefly summarized as follows: PEI is a strong polybase which behaves markedly different from the corresponding monomers and other polybases, even at high ionic strength, and its behaviour is accounted for by: (a) the branched structure of the polymer, containing three different types of amine group; (b) strong inter- and intra-molecular electrostatic and hydrogen-bonding interactions between the various amine groups; and (c) the pH-dependent structure of the polymer in aqueous solution [8]. At low pH values, mutual charge repulsion leads to expansion of the polyion, while in the higher pH range, the polymer contracts due to hydrogen bonding. Further protonation for $\text{pH} < 3$ is precluded by strong electrostatic repulsions between the charged groups, which, depending on the type of acid used, corresponds to approximately 70 % protonation [9]. These changes in the PEI molecule conformation

will influence the interaction between the charged polymer and the solvent (water) and the uncharged polymer (PEG), and consequently will expand or contract the two-phase region of the phase diagram.

We also advanced the hypothesis that the change in PEI molecular conformation alone is not sufficient to lead to two-phase formation and that there is another factor, most probably related to the nature of the titrating acid. Thus, we suggested that the addition of polyvalent anions, as part of the titrating acid, influences the ability of PEI molecules to form aggregates either by H-bonding or by cross-linking. Aggregation of PEI molecules would clearly enhance the incompatibility of the two polymers and would lead, in its turn, to an expansion of the two-phase region. Obviously, different titrating acids will stabilize (promote) to a different extent the aggregation process [6].

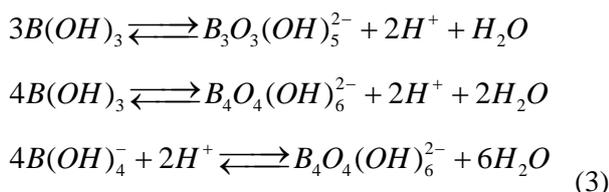
3.1. Inorganic Acid Solutes

We have shown experimentally that when phosphoric acid is used to titrate the PEI, the resulting ATPS has a larger two-phase region compared to the case when sulphuric acid is applied, because the aggregation of PEI molecules (via cross-linking) in the case of H₂SO₄ is less pronounced [6]. Thus, the question is how titrating the PEI with boric acid will influence the phase behaviour of the PEG+PEI ATPS.

It is well known that boric acid, H₃BO₃, (or better boron trihydroxide B(OH)₃) is a weak acid. It behaves more like a Lewis, than a Brønsted acid. In an aqueous solution boric acid does not dissociate but interacts with water molecules to form tetrahydroxyborate ion:



Monomeric species can exist under the limited conditions where the total concentration of boron is less than 0.025 M, while polymeric ions prevail at higher concentration and at high pH. Different polyborate ions can be formed, for example [10, 11]:



According to the above, in the conditions of our experiment (pH = 7.5 and high boric acid concentration), boric acid anions with valency of 1 and 2 are present in the solution. Hence, within the

light of the phase formation mechanism advocated, it is expected that the phase behaviour of the boric acid PEI titrated ATPS will be an intermediate one, namely between that of an HCl/HClO₄ titrated PEI (no two phase region) and H₂SO₄ titrated PEI. This is because the PEI cross linking will be even less pronounced than when H₂SO₄ is used, and therefore the two-phase region will be smaller. Our experiments confirmed that, the boric acid PEI titrated ATPS' binodal is situated in the concentration phase space to the right of the H₂SO₄ phase.

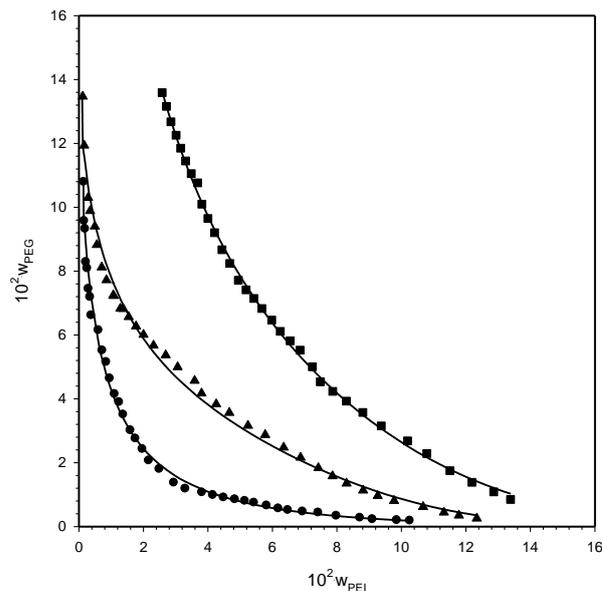


Fig. 2. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36$ °C, experimental: (●) H₃PO₄; (▲) H₂SO₄; (■) H₃BO₃; (—) calculated binodals from Eq 1.

Figure 2 shows the influence of H₃PO₄, H₂SO₄ and boric acid as PEI titrating acids on the (PEG+PEI) ATPS phase formation.

3.2. Organic Acid Solutes

To examine how organic acid solutes influence the ATPSs formation we firstly chose tartaric and citric acids as analogues (same number of H, same valency of the anions) of H₂SO₄ and H₃PO₄. It should be noticed that we were not able to determine the ATPS binodal curve when citric acid was used to titrate the PEI. The reason behind this is the high concentration of the citric acid in the PEI solution (needed to obtain the required pH) when PEG was added. The resulting system was at least three phase (at this concentration citric acid forms ATPS with PEG). Applying the technique outlined above the binodal curve could not be determined.

When tartaric acid was used to titrate the PEI, the binodal curve of the resulting (PEG+PEI) ATPS was determined without any difficulty. Moreover, when compared with the binodal of the (PEG + H₂SO₄ PEI titrated) ATPS, the two-phase regions of the two ATPSs completely coincided (**Figure 3**). Although this fact might be unexpected at a first glance, still it does not contradict the assumptions of our mechanism contributing the phase formation in this particular case to the PEI cross-linking.

However, we believed that this line of behaviour

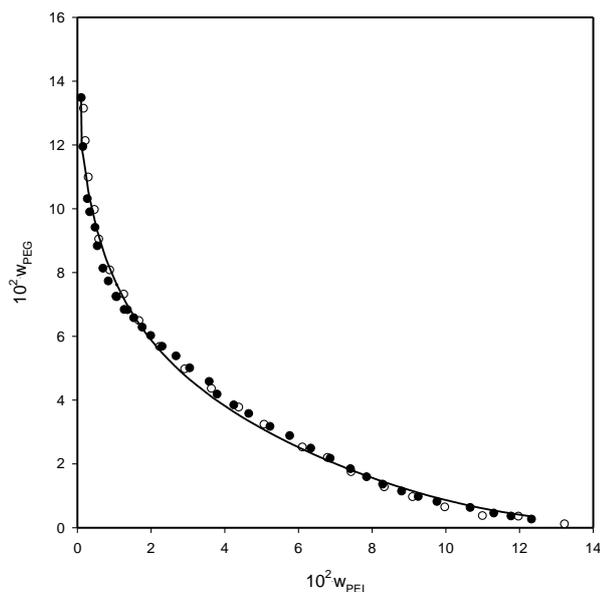


Fig. 3. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36$ °C, experimental: (●) H₂SO₄; (○) tartaric acid; (—) calculated binodals from E. 1.

should be further examined in more details. In view of this, new series of experiments with other organic acids, used to titrate the PEI solution, were performed. The organic acid solutes were chosen in such a way that will represent a diversified selection of acids with either different chain length or different number of OH groups. We believe that those two factors play a dominant role in the ATPSs phase formation mechanism: the chain length represents the difference in the molecular configuration of the acid solutes, and has an important influence on the strength of the PEI aggregates (the latter are a result of a bridge formation between two polymer chains), while the number of the OH groups influence on the aggregation via H-bonding.

3.2.1. Influence of the Chain Length

We chose two acids, succinic and oxalic, for our study of the influence of chain length on the ATPS phase formation. Each of them has two carboxylic groups but different chain length (Figure 1). The

resulting binodals are presented on Figure 4. The oxalic acid PEI titrated ATPS has a larger two-phase region than the one with the succinic acid titrated PEI but smaller than the tartaric acid titrated PEI (Figure 4). The explanation within the light of the mechanism suggested is the following: the shorter the acid solute's chain, the more rigid is the polymer aggregate formed. Hence the two polymers become more incompatible which, in its turn, leads to an expansion of the two-phase region.

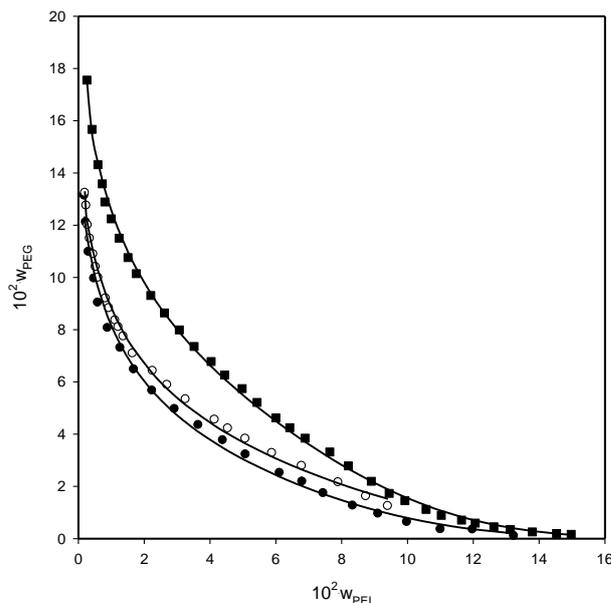


Fig. 4. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36$ °C, experimental: (●) tartaric acid; (○) oxalic acid; (■) succinic acid; (—) calculated binodals from Eq 1.

However, the comparison of the two binodals against the tartaric acid PEI titrated ATPS binodal, points to the fact that the chain length is not the single factor influencing the phase formation. Both, succinic and tartaric acids, have four C atoms. Obviously, both the presence of OH groups and their number, influence the ability of PEI molecules to aggregate via H-bonding.

3.2.2. Influence of the Number of OH Groups Present

In view of the above, we examined tartaric, malic and succinic acids. These acids have the same number of C atoms but different number of OH groups. The largest two-phase region is formed when PEI is titrated with tartaric acid, and the smallest – with the succinic (the tartaric has two OH groups and the succinic - none). The results obtained are shown on **Figure 5** and they comply completely with the mechanism, advocated by us.

Namely, the possibility of PEI molecules to form aggregates via H-bonding is greater when the number of the OH groups, present in the acid solute, is bigger. The latter either increases the rigidity of the PEI aggregates or their molar mass, which in its turn leads to increasing the incompatibility between the neutral PEG and the charged PEI, and expands the two-phase region.

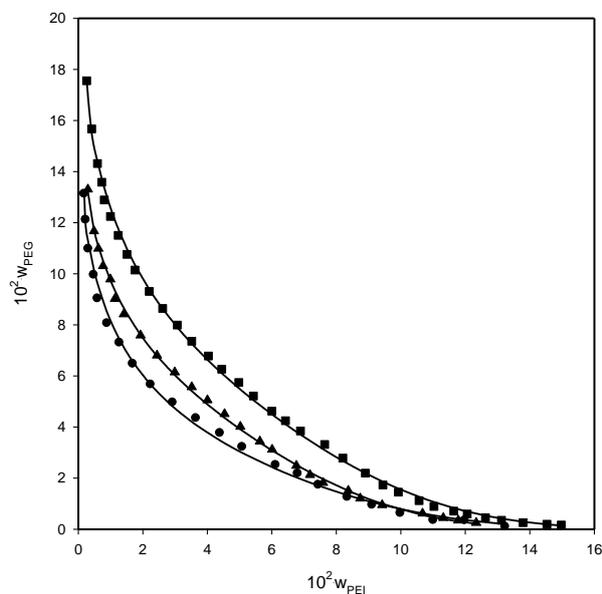


Fig. 5. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36\text{ }^{\circ}\text{C}$, experimental: (●) tartaric acid; (▲) - malic acid; (■) succinic acid; (—) calculated binodals from Eq. 1.

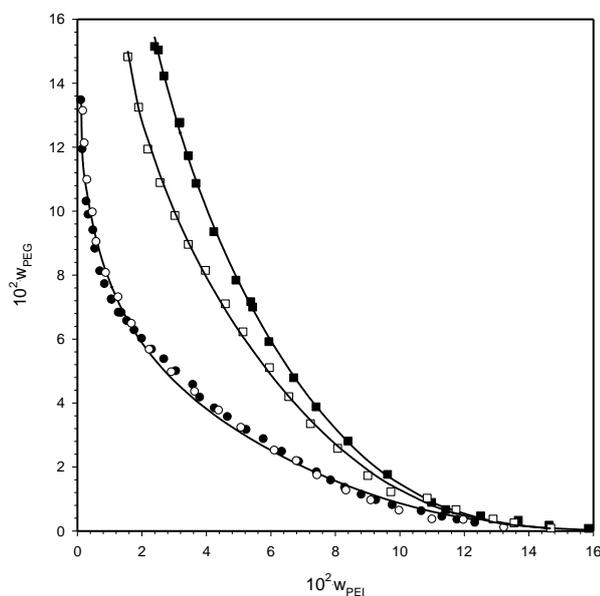


Fig. 6. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36\text{ }^{\circ}\text{C}$, experimental: (●) for the PEI titrated with H_2SO_4 , no lactic acid present; (○) for the PEI titrated with tartaric acid, no lactic acid present; (■) for the PEI titrated with H_2SO_4 and $c_{\text{LA}} = 8.6\text{ g}\cdot\text{L}^{-1}$; (□) for the PEI titrated with tartaric acid and $c_{\text{LA}} = 8.7\text{ g}\cdot\text{L}^{-1}$; (—) calculated binodals from Eq. 1.

3.3. Influence of the Lactic Acid

In our previous work [6] we demonstrated that the addition of Lactic Acid (LA) has a negative effect on the ATPS phase formation (the two-phase region

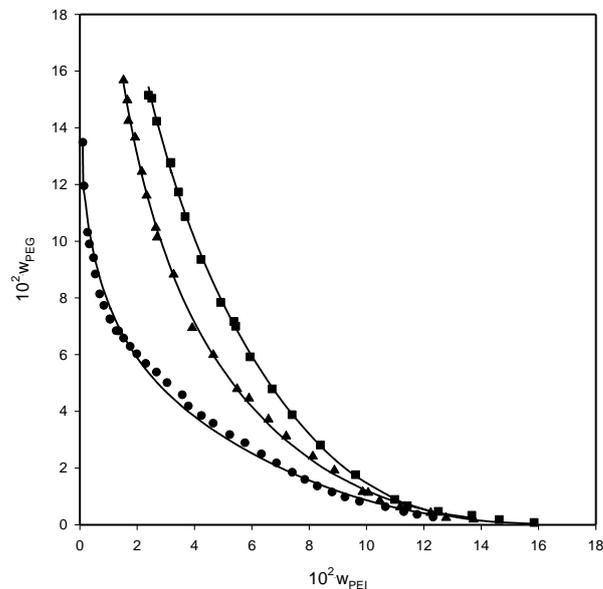


Fig. 7a. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36\text{ }^{\circ}\text{C}$, experimental: (●) for the PEI titrated with H_2SO_4 , no lactic acid present; (▲) $c_{\text{LA}} = 4.3\text{ g}\cdot\text{L}^{-1}$; (■) $c_{\text{LA}} = 8.6\text{ g}\cdot\text{L}^{-1}$; (—) calculated binodals from Eq. 1.

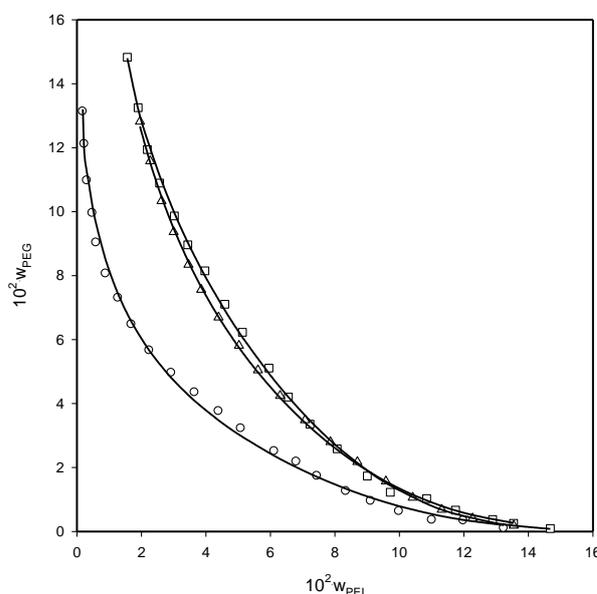


Fig. 7b. Binodal curves for the (PEG + PEI) ATPSs at pH = 7.5 and $T = 36\text{ }^{\circ}\text{C}$, experimental: (○) for the PEI titrated with tartaric acid, no lactic acid present; (Δ) $c_{\text{LA}} = 4.85\text{ g}\cdot\text{L}^{-1}$; (□) $c_{\text{LA}} = 8.7\text{ g}\cdot\text{L}^{-1}$; (—) calculated binodals from Eq. 1.

contracts). This was attributed to the destabilization of the PEI polymer aggregates, probably as a consequence of displacement of the polyvalent acid anions. As a result, the aggregating and cross-linking is decreased. To further explore how the addition of a second acid solute influences the ATPS phase formation we have compared the addition of LA to ATPSs titrated either with H₂SO₄ or with tartaric acid. As shown above, the two-phase regions of the two ATPSs coincide when there is no LA present (Figures 6 and 7).

The influence of LA on the phase formation of the ATPSs where PEI is titrated with tartaric acid is less pronounced (Figure 6). The two-phase region contracts to a lesser extent than that of the H₂SO₄ PEI titrated ATPS. The explanation of this fact within the light of the mechanism suggested is that though LA destabilizes the polymer aggregates by destructing the bridges between two polymer chains, still the additional stabilizing effects, caused by the H-bonding (presence of OH groups), prevail as demonstrated in the case of tartaric acid titrated PEI ATPS.

To further confirm the H-bonding effect we have studied the influence of different lactic acid concentrations on the phase formation of H₂SO₄ PEI titrated ATPSs and tartaric acid PEI titrated ATPSs (Figures 7a and 7b). Unlike the H₂SO₄ PEI titrated ATPSs, when different concentrations of LA are added, the change in the two-phase regions of the tartaric acid titrated ATPSs is negligible. The latter indicates that the stabilizing effect of the OH groups is more important and prevails over the destabilizing of the PEI polymer aggregates, caused by the addition of lactic acid.

4. CONCLUSIONS

This study offers new experimental data on the influence of organic acid solutes on the phase behaviour of aqueous solutions, containing PEG and PEI. Also, a detailed and thorough analysis of the application of the phase formation mechanism, advocated previously, is performed, and the influence of the chain length and number of OH groups of the organic acids is demonstrated. Finally, the effect of the addition of a second acid, the lactic acid, on the ATPS phase formation is also briefly discussed.

Our future research will be focused on obtaining further experimental evidence for the viability of the hypothesis advocated. One possible option is to explore how the molar mass of PEI solutions changes when titrated with a range of acid solutes (both organic and inorganic) to different pH. The

change in the molar mass will be calculated out from the changes in the viscosity of PEI solutions of different concentrations.

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ВЛИЯНИЕ НА КИСЕЛИНИ ВЪРХУ ФАЗОВОТО ПОВЕДЕНИЕ НА ВОДНИ ДВУФАЗНИ СИСТЕМИ, СЪДЪРЖАЩИ ПОЛИЕТИЛЕН ГЛИКОЛ И ПОЛИЕТИЛЕН ИМИН.

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(Резюме)

Тази статия е част от изследвания, посветени на изучаване на възможността за използване на водни двуфазни системи (ВДС) за разделяне на органични киселини с ниска молекулна маса. За първи път са представени експериментални данни за влиянието на титруващата киселина върху фазовото поведение на ВДС, съдържащи полиетилен гликол и полиетилен имин. Основната хипотеза на предложения механизъм е дискутирана от гледна точка на структурата на киселините и е потвърдена от наблюдаваното фазово поведение на ВДС. Показано е противоположното въздействие на млечната киселина и титруващата киселина в тези системи.