Novel Polymer–Polymer Conjugates for Recovery of Lactic Acid by Aqueous Two-Phase Extraction

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Abstract. A new family of polymer conjugates is proposed to overcome constraints in the applicability of aqueous two-phase systems for the recovery of lactic acid. Polyethylene glycol–polyethylenimine (PEI) conjugates and ethylene oxide propylene oxide–PEI (EOPO-PEI) conjugates were synthesized. Aqueous two-phase systems were generated when the conjugates were mixed with fractionated dextran or crude hydrolyzed starch. With 2% phosphate buffer in the systems, phase diagrams with critical points of 3.9% EOPO-PEI–3.8% dextran (DEX) and 3.5% EOPO-PEI–7.9% crude starch were obtained. The phase separation temperature of 10% EOPO-PEI solutions titrated with lactic acid to pH 6 was 35°C at 5% phosphate, and increased linearly to 63°C at 2% phosphate. Lactic acid partitioned to the top conjugate-rich phase of the new aqueous two-phase systems. In particular, the lactic acid partition coefficient was 2.1 in 10% EOPO-PEI–8% DEX systems containing 2% phosphate. In the same systems, the partitioning of the lactic acid bacterium, *Lactococcus lactis* subsp. *lactis*, was 0.45. The partitioning of propionic, succinic, and citric acids was also determined in the new aqueous two-phase systems. *Biotechnol Bioeng* **66**: 211–218, 1999.
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INTRODUCTION

Lactic acid remains a speciality chemical despite its potential as a large-scale chemical intermediate and raw material for the production of the biodegradable polymer polylactic acid (PLA). Two factors are known to influence the economy of lactic acid production by microorganisms: (i) lactic acid inhibition, which decreases fermentation productivity; and (ii) the efficiency in the recovery of the lactic acid. At present, the most widely used process for the recovery of lactic acid involves precipitation with calcium hydroxide and separation by filtration of the calcium salt of the acid (Atkinson and Mavituna, 1991). Treatment of the precipitate with sulfuric acid leads to preferential precipitation of $CaSO_4$, which is filtered off. Concentration by water evaporation and purification by several chromatographic steps are used to achieve the final product specifications. For each ton of lactic acid produced, this method irreversibly consumes about 400 kg of $Ca(OH)_2$ and 540 kg of H_2SO_4, which are converted to about 760 kg of $CaSO_4$ waste.

Alternative techniques, such as extraction and sorption, have been developed. Extraction of lactic acid with high-molecular-weight aliphatic imines has been studied (Dai and King, 1996; Kertes and King, 1985; Tamada et al., 1990; Tamada and King, 1990; Yang et al., 1991), technically improved (Han and Hong, 1996; Miller et al., 1996; San-Martín et al., 1996; Thom et al., 1996), and patented (Baniel et al., 1996; Dalcanale et al., 1992). A serious drawback is the toxic effect of the organic solvents and imines on microorganisms (Honda et al., 1995; Yabannavar and Wang, 1991). Therefore, immobilization of the amine-based compounds in solid sorbents has been used in lactic acid production (Córdoba et al., 1996; Kaufman et al., 1996). The low capacity of the resins, typically between 0.1 and 0.2 g lactic acid/g resin, and the difficulties associated with handling of solids during fermentation, make these processes difficult to operate.

Aqueous two-phase systems (ATPS) have been used for liquid–liquid extraction (Hustedt et al., 1986) and for bioconversion of different substances. The uneven partitioning of cells in an ATPS allows the removal of the product from the cell-free phase. ATPS have been used for the production of bulk chemicals (Drouin and Cooper, 1992; Jarzebski et al., 1992), and specifically for the production of lactic acid (Katzbauer et al., 1995; Kwon et al., 1996; Planas et al., 1996, 1997). However, an even distribution of lactic acid between the two phases, together with the cost of the poly-

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mers, makes this process concept economically nonviable. To improve the performance and economy of ATPS as extractive fermentation systems, three main problems must be addressed: (i) the use of phase systems in which lactic acid partitions preferentially to the cell-poor phase; (ii) the use of crude carbohydrates as one of the phase-forming polymers; and (iii) the recycling of the polymers.

In the present study, a new family of polymer–polymer conjugates is presented. It consists of a PEI tail covalently bound to a core of polyethylene glycol (PEG) or ethylene oxide propylene oxide (EOPO) copolymer. The phase formation capacity of the conjugates when mixed with carbohydrate polymers was investigated, and the partitioning of organic acids, in particular lactic acid, was determined. Moreover, the thermoseparating performance of the new conjugates was compared with that of EOPO. Thermoseparating EOPO copolymers have been used as recyclable polymers in protein purifications using ATPS (Alred et al., 1992; Harris et al., 1991).

MATERIALS AND METHODS

Chemicals

Polymer stock solutions were prepared in ultrapure water (Milli-RO4 water purification system, Millipore, Bedford, MA). $EO_{50}PO_{50}$ (50% ethylene oxide, 50% propylene oxide random copolymer [UCON 50HB5100], average molecular weight 4 kDa) was obtained from Union Carbide (New York, NY), and dextran T500 (average MW 500 kDa) (DEX) was purchased from Pharmacia Biotech (Uppsala, Sweden). Acid-hydrolyzed maize starch with an average molecular weight of 250 kDa (waxy maize [WM]) was obtained from Lyckeby Reppe (Växjö, Sweden). WM stock solutions were prepared by resuspending the dry powder in water to a ~30% (w/w) suspension, and boiling for 10 min to obtain a stable solution.

DL-Lactic acid (2-hydroxypropionic acid) 85% in water was obtained from Acros Organics (Pittsburgh, PA). Propionic acid for synthesis grade, and citric acid monohydrate (GR) were obtained from Merck (Darmstadt, Germany). Succinic acid was obtained from Sigma Co., (St. Louis, MO). NaOH pellets (GR) from Merck were used to adjust pH of the acid stock solutions. *m*-Phosphoric acid (pro analysis grade), sodium dihydrogen phosphate (extra pure grade), and disodium hydrogen phosphate (extra pure grade), also from Merck, were used to prepare phosphate stock solutions of different pH values.

Polymer Conjugates

Two PEG–PEI conjugates based on PEG (5 kDa) and PEI (10 kDa), and an EOPO–PEI conjugate based on $EO_{50}PO_{50}$ and PEI (600 Da) were synthesized.

A mixture of M-PEG (5 kDa) epoxide (Shearwater Polymers, Huntsville, AL) (10 g, 0.00200 mol), PEI (10 kDa, 5 g, 0.00050 mol), ethyl alcohol (50 g), and triethylamine (2 mL) was heated for 65 h at 40°C in a nitrogen atmosphere under agitation. Ethyl alcohol was then distilled off under reduced pressure (0.2 mm Hg, 80°C). The reaction yielded 13.8 g of PEG (5 kDa)–PEI (10 kDa) conjugate (molar ratio 4:1, weight ratio 2:1, Lot AK 127-2), which will be referred to as PEG-PEI(2:1). ^{1}H-NMR (DMSO-D6) analysis was performed. One hundred percent conversion was confirmed. No residual epoxy groups were detected.

A mixture of M-PEG (5 kDa) epoxide (Shearwater Polymers; 10 g, 0.00200 mol), PEI (10 kDa, 2.5 g, 0.00025 mol), ethyl alcohol (50 g), and triethylamine (2 mL) was heated for 65 h at 40°C in a nitrogen atmosphere under agitation. Ethyl alcohol was then distilled off under reduced pressure (0.2 mm Hg, 80°C). The reaction yielded 11.2 g of PEG (5 kDa)–PEI (10 kDa) conjugate (molar ratio 8:1, weight ratio 4:1, Lot AK 127-3), which will be referred to as PEG-PEI(4:1). ^{1}H-NMR (DMSO-D6) analysis was performed. One hundred percent conversion was confirmed. No residual epoxy groups were detected.

A mixture of $EO_{50}PO_{50}$ (120 g, 0.30 mol), epichlorohydrin (100 g, 1.08 mol), sodium hydroxide (25 g, 0.625 mol), and water (3.1 g, 0.12 g per gram NaOH) was heated for 20 h at 60°C in a nitrogen atmosphere under agitation. After cooling to room temperature, 500 mL of dichloromethane and 300 mL of distilled water were added to the reaction mixture. Solid NaH_2PO_4 was added to adjust the pH to 7.0. The organic phase was separated and dried with anhydrous magnesium sulfate. After drying, the solvents were evaporated under reduced pressure (0.2 mm Hg, 60°C) and the final product obtained was a viscous, colorless oil, which will be referred to as EOPO epoxide. The reaction yielded 103.4 g (87.4% of the theoretical yield) of the product. ^{1}H-NMR (DMSO-D6) analysis at 0.72 ppm (t, CH3CH2CH2CH2O—), 1.05 ppm (d,—OCH2CH(CH3)O—),1.43 ppm (m, CH3CH2CH2CH2O—), 2.55 ppm (m, CH2 epoxy), 2.72 ppm (m, CH2 epoxy), 3.35 ppm (m, OCH2CH(CH3)O—), and 3.51 ppm (s, —OCH2CH2O—, —OCH2CH(CH3)O—, H3CH2CH2CH2O) confirmed 100% substitution.

A mixture of EOPO epoxide (20 g, 0.005 mol), PEI (600 Da, 2.5 g, 0.00417 mol), ethyl alcohol (60 g), and triethylamine (2 mL) was heated for 66 h at 40°C in a nitrogen atmosphere under agitation. Ethyl alcohol was then distilled off under reduced pressure (0.2 mm Hg, 80°C). The reaction yielded 22 g of EOPO-PEI 600 conjugate (molar ratio 1.2:1, weight ratio 8:1, Lot AK-097-1). ^{1}H-NMR (DMSO-D6) analysis was performed. One hundred percent conversion was confirmed. No residual epoxy groups were detected.

Phase Diagrams and Partitioning Studies

ATPS were made up by mixing appropriate amounts of polymer stock solutions, and carboxylic acid stock solutions, which had been pH-adjusted previously (PHM82 standard pH meter, Radiometer, Copenhagen, Denmark), phosphate stock solutions, and distilled water to 3.0 g. Par-

titioning in the conjugate-based systems was performed by mixing the appropriate amounts of DEX stock solution and conjugate/carboxylic acid stock solution. After adding the appropriate amount of phosphate stock solution and bacterial suspension, distilled water was added to a final weight of 3.0 g. The tubes were capped and shaken gently for 30 min at 30°C. Phase separation was allowed to progress for 5 h at 30°C. Conjugate/carboxylic acid stock solutions were obtained by titrating the polymer conjugate with the carboxylic acid to the desired pH. Each phase system was made in duplicate, samples from the top and bottom phases were removed, and the concentration of carboxylic acid, and, when possible, phase-forming polymers, was determined.

Lactococcus lactis subsp. *lactis* 19435 from the American Type Culture Collection (Rockville, MD) was grown overnight in M17 medium (Merck). Bacterial cells were harvested, washed, and resuspended in phosphate buffer (2% [w/w]) at pH 6 to a final concentration of about 14 g dry weight/L. One milliliter of the cell suspension was added to the phase systems, resulting in a final concentration of about 9×10^9 colony forming units (cfu)/mL. The partition coefficient was defined as the ratio between the cell concentration (cfu/mL) in the top and in the bottom phases, as determined by plate counting.

The pH values reported in the Results section correspond to those of the original stock solutions, which may not correspond exactly to those of the final ATPS, because the dilution of polyelectrolytes often involves pH changes.

Analysis

Separation of carboxylic acids and of phase components of EOPO-PEI–DEX and EOPO-PEI–WM systems, was performed by HPLC using a prepacked gel exclusion chromatography column (Ultrahydrogel 500, 300 × 7.8 mm i.d.; Waters, Millipore Corp., Milford, MA) at 22°C (Planas et al., 1997). The mobile phase was a 0.005 *M* H_2SO_4 water solution. The eluent flow was set at 0.6 mL/min using an LC pump (Model LC 6A, Shimazu Corp., Kyoto, Japan). Samples were injected into the flow line by an autosampler (Marathon, Spark Holland, Emmen, The Netherlands). The analytes were detected with a differential refractometer (Waters 410 differential refractometer, Millipore).

RESULTS

Three different polymer conjugates were synthesized: an EOPO-PEI conjugate and two PEG-PEI conjugates [PEG-PEI(2:1) and PEG-PEI(4:1)]. PEG-PEI conjugates and dextran could not be separated by HPLC, and therefore PEG-PEI–DEX systems were used to study the effect of different parameters only on K_{lac}. Because the concentration of EOPO-PEI conjugate and DEX could be determined simultaneously in the analytical system, more detailed studies could be performed in these systems.

Phase Diagrams

To be able to use ATPS for practical applications in downstream processing, or for analytical purposes, it is necessary to know the extent and basic characteristics of the two-phase region of aqueous polymer–polymer mixtures. Furthermore, the two-phase area can be affected by different factors such as temperature, pH, or salt content. Thus, when studying the partition performance of any solute or particle in ATPS in different conditions, it is necessary to distinguish between the net effect of these different conditions on the partitioning, and the effect obtained through changes in the composition of the phases.

Phase diagrams for systems containing EOPO-PEI conjugate titrated with lactic acid and DEX were obtained at different pH values (Fig. 1). The critical points decreased with decreasing pH. At pH 6, the critical point was about 5.7% EOPO-PEI–5.2% DEX, which changed to 5.6%–5.1% at pH 5, to 5.3%–5.1% at pH 4, to 4.9%–5.2% at pH 3, and to 4.3%–5.0% at pH 2. The increase in the two-phase area with the decrease in pH did not affect the relative concentrations of the polymers in the two phases, as shown by the constant slope of the tielines over the whole pH range studied (Table I). The maximum concentration of lactate in the phase systems at pH 6 was between 1.6% (w/w) and 0.98%, the lactate partition coefficients (K_{lac}) varied between 1.3 and 1.0 in systems far from the critical point and close to the critical point, respectively. In the systems at pH 5, the lactate concentrations were between 2.0 and 1.3 and K_{lac} between 1.3 and 1.2; at pH 4, the lactate concentrations were between 3.3% and 1.8% and K_{lac} between 1.3 and 1.1; at pH 3, lactic acid concentrations increased to 7.9% and 4.4%, and K_{lac} remained between 1.4 and 1.1. Finally, in systems at pH 2, the lactic acid concentrations were as high as 26.9% and 16.9%, and K_{lac} between 1.5 and 1.2, respectively.

The effect of 2% (w/w) phosphate buffer on the EOPO-PEI–DEX phase diagram was studied at pH 6 (Fig. 2). The critical point decreased to 3.9% EOPO-PEI–3.8% DEX

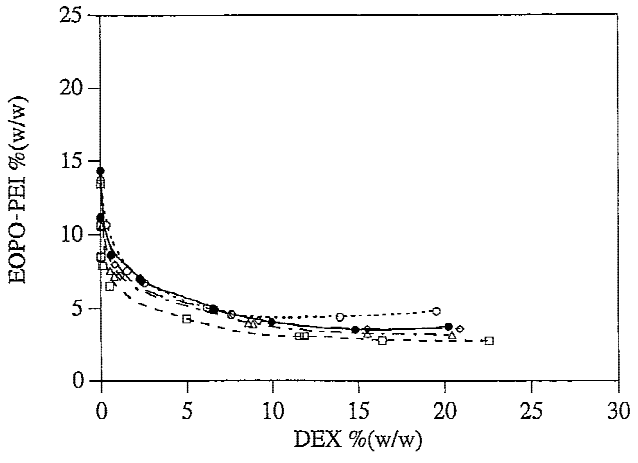

Figure 1. Binodials of the EOPO-PEI–DEX systems titrated with lactic acid to pH 6 (—○—) pH 5; (····○····) pH 4; (--◇--) pH 3; (–·–△–·–), and pH 2 (---□---). The phase systems were formed at 30°C.

Table Ia. Characteristics of EOPO-PEI-based ATPS where EOPO-PEI was titrated with lactic acid to different pH values: slopes of tielines.

Polymer concentrations	pH 6	pH 5	pH 4	pH 3	pH 2
10% $EO_{50}PO_{50}$-PEI–8% DEX	−0.52 ± 0.01	−0.45 ± 0.02	−0.51 ± 0.01	−0.53 ± 0.01	−0.47 ± 0.01
8% $EO_{50}PO_{50}$-PEI–6% DEX	−0.51 ± 0.01	−0.45 ± 0.01	−0.48 ± 0.01	−0.47 ± 0.01	−0.48 ± 0.01
6% $EO_{50}PO_{50}$-PEI–5% DEX	−0.48 ± 0.01[a]	−0.48 ± 0.01	−0.45 ± 0.01	−0.44 ± 0.02	−0.42 ± 0.01
10% $EO_{50}PO_{50}$-PEI–10% DEX + 2% phosphate	−0.66 ± 0.03				
10% $EO_{50}PO_{50}$-PEI–10% WM + 2% phosphate	−0.51 ± 0.01				

The phase diagrams of these phase systems are shown on Figures 1, 2, and 3.
[a]Phase composition 6.5% EOPO-PEI–5% DEX.

compared with the phase diagram obtained in the absence of phosphate, where it was about 5.7% EOPO-PEI–5.2% DEX. The decrease in the critical point concentrations was accompanied by a shift of the binodial toward the abscissa. Thus, in the diagram obtained from systems not containing phosphate (Fig. 1), the segment of the binodial close to the abscissa remained at about 3.5% EOPO-PEI, whereas, in the diagram obtained from systems containing phosphate, it remained at about 2% EOPO-PEI. The slopes of the tielines of the phase diagram obtained from systems containing phosphate were constant over the whole phase diagram (Fig. 2), but they were steeper (~−0.66) than the slopes of the tielines of the phase diagram obtained from systems not containing phosphate (~−0.50) (Table I).

The capacity of the new EOPO-PEI conjugate to form ATPS with crude hydrolyzed starch from maize (WM) was investigated in systems containing 2% phosphate at pH 6 (Fig. 3). Phase separation occurred and the critical point was at about 3.5% EOPO-PEI–7.9% WM. The slopes of the tielines in the EOPO-PEI–WM diagram decreased as the phase systems were brought to the critical point, from −0.51 ± 0.01 at 10.0% EOPO-PEI–9.5% WM to −0.30 ± 0.01 at 4.85% EOPO-PEI–4.56% WM.

Thermal Separation Characteristics of EOPO-PEI Conjugate

At a certain temperature, aqueous solutions of EOPO copolymers and EOPO-PEI conjugates phase-separate into two phases: a polymer-rich bottom phase and a polymer-poor top phase. This property of the thermoseparating polymers has been used for practical applications; therefore, the separation temperature of the top phases of the 10% EOPO–8% DEX ATPS containing lactic, propionic, and succinic acids was determined. These values were compared with the phase-separation temperatures obtained for the top phases of 10% EOPO-PEI–8% DEX ATPS containing lactic, propionic, succinic, and citric acids, respectively (Table II). The separation temperatures of the EOPO-PEI-based top phases were all higher than those of the EOPO-based top phases. In the case of lactic acid-containing systems, clouding was not observed, even at 110°C. For propionic and succinic acid the separation temperature increased by 33°C (from 40°C to 73°C) and 41°C (from 32°C to 73°C), respectively.

Because the top phase of the system containing lactic acid did not show phase separation at any temperature up to 110°C, the effect of phosphate on the thermal separation characteristics of 10% EOPO-PEI solutions titrated with lactic acid to pH 6 was also determined (Fig. 4). At 5% phosphate, the separation temperature was 35°C and increased linearly to 63°C at 2% phosphate. At this point, the slope of the curve changed and the separation point increased to 103°C for solutions containing 1% phosphate.

Table Ib. Characteristics of EOPO-PEI-based ATPS where EOPO-PEI was titrated with lactic acid to different pH values: maximum concentration of lactic acid (C_{lac}) and K_{lac}.

	pH 2	pH 3	pH 4	pH 5	pH 6
C_{lac} (%)[a]	16.9–26.9	4.4–7.9	1.8–3.3	1.3–2.0	0.98–1.6
K_{lac}[a]	1.2–1.5	1.1–1.4	1.1–1.3	1.2–1.3	1.0–1.3

[a]Values refer to points in the phase diagram close to and far away from the critical point, respectively.

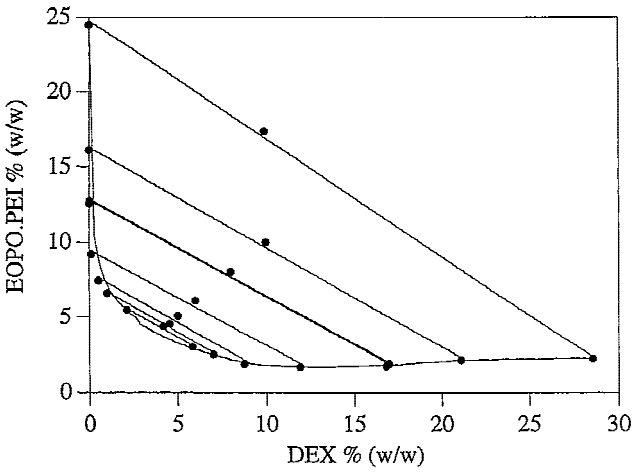

Figure 2. Phase diagram of the EOPO-PEI–DEX systems containing 2% phosphate buffer titrated with lactic acid to pH 6. The phase systems were formed at 30°C.

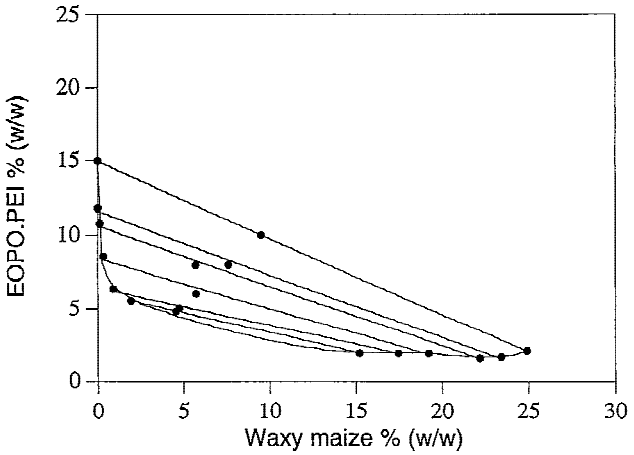

Figure 3. Phase diagram of the EOPO-PEI–waxy maize systems containing 2% phosphate buffer titrated with lactic acid to pH 6. The phase systems were formed at 30°C.

Lactic Acid Partitioning in PEG-PEI-Based ATPS

The partitioning of lactic acid was investigated in PEG-PEI(2:1)–DEX and PEG-PEI(4:1)–DEX ATPS. The influence of 2% (w/w) (~120 m*M*) phosphate in the ATPS was investigated at different pH values for systems containing 10% PEG-PEI(2:1)–8% DEX (Table III). The presence of phosphate resulted in a 1.4- to 1.7-fold increase in K_{lac} together with a more than twofold increase in the volume ratio. Increasing K_{lac} values were also observed with decreasing pH values, an effect that was more pronounced in systems containing phosphate than in those without phosphate. The highest K_{lac} value (1.66) was obtained in a system containing phosphate at pH 4.3, and the lowest K_{lac} value (0.9) was obtained at pH 6.4 in a system not containing phosphate.

The influence of the presence of 2% (w/w) phosphate, the influence of temperature, and the influence of polymer concentration on K_{lac} were investigated in PEG-PEI(4:1)–DEX ATPS (Table IV). In 10% PEG-PEI(4:1)–8% DEX systems, the presence of phosphate resulted in a 1.1-fold increase in K_{lac} and a less than twofold increase in phase volume ratio. The difference in K_{lac} due to varying temperature was not significant. Increasing the polymer concentrations from 10% PEG-PEI(4:1)–8% DEX to 12% PEG-PEI(4:1)–10% DEX led to an increase of about 1.1-fold in K_{lac}, yielding a maximum value (1.75) at 30°C.

Partitioning of Carboxylic Acids and *Lactococcus lactis* in EOPO-PEI–DEX

The new family of polymer conjugates was designed for the recovery of lactic acid from solutions containing it. However, because the new polymers are positively charged in a broad range of pH, they can be used as liquid anion exchangers in ATPS. Thus, other organic acids could be recovered from fermentation broths in which they have been produced.

The new EOPO-PEI conjugate was evaluated in regard to its capacity to extract organic acids (Table II). The partitioning of lactic, propionic, and succinic acids (K_{AH}) was determined in neutral ATPS containing 10% EOPO–8% DEX and 2% phosphate. These results were compared with K_{AH} values obtained when lactic, propionic, succinic, and citric acids were partitioned in 10% EOPO-PEI–8% DEX containing 2% phosphate buffer (Table II). In EOPO-based systems, organic acids partitioned mainly to the bottom phase, whereas they partitioned to the top phase of EOPO-PEI-based systems. K_{AH} values for lactic, propionic, succinic, and citric acids in the systems studied were 2.1, 1.12, 1.15, and 1.30, respectively. These values represented 2.4-, 1.3-, and 4.6-fold increases of K_{AH} for lactic, propionic, and succinic acids, respectively, when EOPO was replaced by EOPO-PEI.

The partition coefficient of *L. lactis* was studied in systems containing 10% EOPO-PEI titrated with lactic acid, 8% DEX, and 2% phosphate, because the highest K_{lac} was obtained in these systems. The partition coefficient of *L. lactis* was 0.45.

DISCUSSION

Chemical modification of thermoseparating polymers has been described for many polymers and applications (Alred et al., 1992; Galaev and Mattiasson, 1993), but preservation of the thermoseparating capacities of a polymer–polymer conjugate has so far not been reported. Thus, the polymer–polymer conjugates presented here present new possibilities in the field of thermoseparating polymers. The partitioning of carboxylic acids to the conjugate-rich top phase was demonstrated, and can be further optimized.

The use of PEI-based conjugates as phase-forming polymers, together with polysaccharides as second polymers, successfully shifted the partitioning of lactic acid to the conjugate-rich top phase. Lactic acid distributes evenly (Katzbauer et al., 1995) or to the bottom phase (Planas et al., 1997) of PEG–DEX systems. The highest value of K_{lac} in EOPO–DEX systems, (i.e., K_{lac} 1.3) was observed at pH 2 and decreased to 0.4 at pH 5. When PEI (MW ≈ 2 kDa) was included in EOPO–DEX systems, K_{lac} decreased to 0.09 at pH 6 (Planas et al., 1998). PEI has been used previously as a phase-forming polymer, giving rise to ATPS when mixed with PEG, dextran, or (hydroxyethyl) cellulose (HEC) (Dissing and Mattiasson, 1993). ATPS formation was, however, subordinate to the titration of PEI with di- or trivalent mineral acids. In these systems, PEI was the bottom phase-forming polymer so lactic acid partitioned to this phase. In HEC–PEI ATPS, K_{lac} ranged from 0.75 to 0.48 (i.e., the concentration difference factor ranged from 1.3 to 2.1) (Kwon et al., 1996). With the new conjugates K_{lac} values between 1 and 2.1 were obtained.

The pK_a for lactic acid is 3.8. The equilibrium concentrations for the different molecular species (acid and lactate) are given by the Henderson–Hasselbach equation, pH = pK_a + log(base/acid), which directly indicates that, at pH

Table II. Partitioning of carboxylic acids (K_{AH+A^-}) in EOPO- and EOPO-PEI-based ATPS containing 2% phosphate.

ATPS composition	Acid in the system (% w/w)	Separation temperature (°C)	K_{AH+A^-}	pK_a
10% $EO_{50}PO_{50}$–8% DEX	3.0 lactic	~39	0.88 ± 0.03	3.8
10% $EO_{50}PO_{50}$–8% DEX	3.0 propionic	~40	0.87 ± 0.02	4.9
10% $EO_{50}PO_{50}$–8% DEX	3.0 succinic	~32	0.25 ± 0.02	4.2, 5.6
10% $EO_{50}PO_{50}$-PEI–8% DEX	1.7 lactic	—[a]	2.1 ± 0.1	3.8
10% $EO_{50}PO_{50}$-PEI–8% DEX	1.0 propionic	~73	1.12 ± 0.03	4.9
10% $EO_{50}PO_{50}$-PEI–8% DEX	0.9 succinic	~73	1.15 ± 0.04	4.2, 5.6
10% $EO_{50}PO_{50}$-PEI–8% DEX	0.8 citric	~65	1.30 ± 0.02	3.1, 4.8, 6.4

Partitioning was performed at 30°C, pH 6.0. EOPO-PEI-based systems were titrated with the corresponding acid.

Values for the separation temperature were obtained by heating the resulting top phases of the first extraction system (defined by the composition given in lane 1).

[a]No temperature-induced phase separation of the primary top phase was observed, up to 110°C.

2.8, the acid reaches 90% in the uncharged form, and at pH 4.8 and higher more than 90% of the acid will be in the charged anionic form as lactate. For pH values higher than 5.8 more than 99% of the species will be present as lactate. For the partitioning equilibrium in two-phase systems the partition coefficient is defined as:

$$K_{lac} = (C_{lac})\text{top}/(C_{lac})\text{bottom}$$

where C_{lac} = [lactic acid] + [lactate]. At pH values higher than 5.8, the partitioning equilibrium can thus be written as K_{lac} = [lactate]top/[lactate]bottom; that is, partitioning of the negatively charged species is observed. This explains the relatively strong effect of the positively charged EOPO-PEI conjugate on the partitioning at pH 6.0 (Table II). The *K*-value was increased from 0.88 to 2.1 by including the conjugate in the phase system. The conjugate acts as a liquid ion-exchanger, which electrostatically attracts the dominating negatively charged lactate to the top phase.

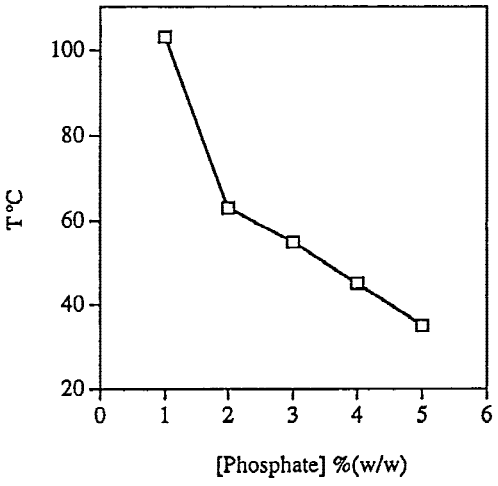

Figure 4. The influence of phosphate concentration on the separation temperature of the EOPO-PEI conjugate. Solutions containing 10% EOPO-PEI titrated to pH 6 with lactic acid.

The corresponding analyses for propionic, succinic, and citric acid (pK_a values in Table II) of the acid–base equilibria indicates that the dominating molecular form at pH 6.0 is the negatively charged base, for succinic acid in a 50:50 equilibrium between the -1 and -2 forms and for citric acid in a similar equilibrium between the -2 and -3 forms. Thus, the increased partitioning of all these acids to the top phase can be understood by the electrostatic interaction with the EOPO-PEI conjugate.

The variation in K_{lac} observed under different conditions reflects the fact that lactic acid partitioning is basically influenced by the polymer composition of the phases. Phosphate affected K_{lac} in all systems. Furthermore, phosphate decreased the solubility of the PEI-based conjugates in the bottom phase, as indicated by changes in the volume ratio in PEG-PEI(2:1)–DEX and PEG-PEI(4:1)–DEX systems, and changes in the slopes of the tielines in EOPO-PEI–DEX phase diagrams. The partitioning of the conjugate to the top phase increased K_{lac}. The effect of phosphate on K_{lac} has been described previously and related to the partitioning of PEI in EOPO–DEX ATPS (Planas et al., 1998). The extent to which the effects of phosphate on K_{lac} are related to the ionic balance between the phases needs further investigation. The effects of polymer concentration on K_{lac} in PEG-

Table III. Partitioning of lactic acid in ATPS containing 10.0% PEG-PEI (2:1) and 8.0% DEX at 30°C.

pH	2% Phosphate	Lactic acid (%)	Vol. ratio	K_{lac}
6.4	−	4.3	0.6	0.9 ± 0.1
	+	4.3	1.7	1.22 ± 0.01
4.9	−	5.2	0.7	0.99 ± 0.09
	+	5.2	2.0	1.54 ± 0.01
4.3	−	5.9	0.6	1.00 ± 0.02
	+	5.9	2.0	1.66 ± 0.08

pH was adjusted by titrating the PEG-PEI conjugate with lactic acid. The partition coefficient of lactic acid, and the volume ratio of the phases were studied at different pH values in the presence and absence of 2% (w/w) phosphate buffer.

Table IV. Partitioning of lactic acid in ATPS containing PEG-PEI (4:1) and DEX titrated to pH 6.0 with lactic acid.

ATPS composition	T (°C)	2% Phosphate	Lactic acid (%)	Vol. ratio	K_{lac}
10.0% PEG-PEI–8.0% DEX	30	−	2.7	1.3	1.40 ± 0.03
10.0% PEG-PEI–8.0% DEX	30	+	2.7	2.4	1.59 ± 0.03
10.0% PEG-PEI–8.0% DEX	40	+	2.7	2.4	1.63 ± 0.01
12.0% PEG-PEI–10.0% DEX	30	+	3.0	2.4	1.75 ± 0.09
12.0% PEG-PEI–10.0% DEX	40	+	3.0	2.4	1.73 ± 0.02

Partition coefficient of lactic acid and volume ratio of phases were studied at different polymer concentrations and temperatures in the presence and absence of 2% (w/w) phosphate buffer.

PEI(4:1)–DEX systems can also be explained by changes in the phase system. Increasing polymer concentrations yield longer tielines, leading to more uneven partitioning (Albertsson, 1986).

The absolute amount of lactic acid contained in a phase system at a given polymer composition and pH depends on the mass ratio between the two polymers in the conjugate; that is, by increasing the PEI content in the conjugate, the basicity increases and more lactic acid is needed for titration. Thus, 10% PEG-PEI(2:1)–8% DEX systems contained 5.2% lactic acid at pH 4.9; 10% PEG-PEI(4:1)–8% DEX systems contained 2.7% lactic acid at pH 6; and 10% EOPO-PEI–DEX systems, in which the mass ratio between EOPO and PEI was 8:1, contained 1.7% lactic acid at pH 6. Because K_{lac} was shown to be independent of lactic acid concentration in the systems (Table Ib), the extractive capacity in terms of the total amount of lactic acid contained in the top phase increased with the decrease in mass ratio between the two polymers in the conjugate.

The economic competitiveness of ATPS as an extractive technique is highly dependent on the cost of the polymers, and the possibility of recycling them (Kroner et al., 1984). Both aspects were addressed in the design of the new PEI-based conjugates. The EOPO-PEI–WM phase diagram of systems with phosphate was similar to that of EOPO-PEI–DEX systems with phosphate. Replacement of dextran by crude starch resulted in an increase in the carbohydrate concentration in the bottom phase, and a decrease in the difference in concentration of EOPO-PEI between the phases. The use of fractionated polysaccharides, such as dextran, for large-scale purification processes in ATPS is far too costly. Hydroxypropylated starch has been proposed as an inexpensive polymer to overcome the viscosity problems associated with the use of crude dextran (Tjerneld et al., 1986; Venancio et al., 1993). Waxy maize starch is an amylopectin-rich starch, very stable in solution, which has been used as a phase-forming polymer after partial hydrolysis (Larsson and Mattiasson, 1988).

The relation between hydration of poly(ethylene oxide) in water, and temperature and salt concentration has long been known (Bailey and Koleske, 1976), but phase separation with changes in temperature and salt concentration have only recently been used for the recovery of proteins in aqueous two-phase systems (Alred et al., 1992, 1994; Harris et al., 1991; Johansson et al., 1996). The novel EOPO-PEI conjugate displayed temperature-induced phase separation only at 2% (w/w) phosphate, which is approximately fourfold higher than the phosphate concentrations used in growth media for lactic acid bacteria (Planas et al., 1996). The influence of enhanced phosphate concentrations on growth and lactic acid production needs to be established. It might also be useful to investigate if components of lactic acid bacteria growth media at appropriate concentrations could lower the separation for EOPO-PEI conjugates. If temperature-induced phase separation is not possible due to constraints of the bacterial growth medium, the most promising method is probably electrodialysis (ED) (Boniardi et al. 1997; Hariban et al., 1993). This method converts lactate to lactic acid and base after which lactic acid can be further concentrated by, for instance, evaporation. ED might be hampered by the high polymer concentration in the lactate containing the EOPO/PEI-rich top phase as well as by cells and proteins that will ultimately foul the ED membrane. This can be overcome by introducing an ultra- or nanofiltration membrane filter before the ED membrane.

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