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Protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer

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Abstract

The phase behavior of a thermoseparating cationic hydrophobically modified ethylene oxide polymer (HM-EO) containing tertiary amines has been investigated at different pH, salt and sodium dodecyl sulfate (SDS) concentrations, in order to find a water/HM-EO two-phase system suitable for protein partitioning. The used polymer forms micellar aggregates that can be charged. By changing pH and SDS concentrations the netcharge of the SDS/HM-EO aggregate can be shifted from positive to negative. Bovine serum albumin (BSA) and lysozyme were partitioned in the thermoseparated two-phase systems of the cationic polymer at different pH, salt and SDS concentrations. The dominant attractive interactions between the polymer aggregates and the studied proteins were shown to be of electrostatic (Coulomb) nature rather than hydrophobic interaction. At low ionic strength the positively charged polymeric aggregates attracted negatively charged BSA and repelled positively charged lysozyme. Upon addition of SDS the negatively charged aggregates attracted lysozyme and repelled BSA. Thus, it was possible to direct proteins with different charges to the polymeric phase and redirect them to a polymer-depleted phase by changing the netcharge of the polymeric aggregates. The effect of different salts on the partitioning of BSA in a system of slightly positively charged HM-EO was studied. NaCl and KBr have a significant effect on driving the BSA to the polymer-depleted phase, whereas KF and K_2SO_4 have a smaller effect on the partitioning. The cloud point temperature of the charged polymer decreased upon addition of SDS near the isoelectric molar ratio of SDS to polymer and also upon salt addition. In the latter case the decrease was smaller than expected from model calculations based on Flory–Huggins theory, which were performed for a charged thermoseparating polymer at different charges and salt concentrations.

Keywords: Mixed micelles; Temperature effects; Aqueous two-phase systems; Thermoseparation modelling; Protein partitioning; Proteins; Poly(ethylene oxide)

1. Introduction

Aqueous two-phase systems have been used for separation and purification of biomolecules since the 1950s [1,2]. New polymeric systems containing thermoseparating polymers, which have been developed during the last 10 years, can be used for purification of different types of biomolecules [3–6]. A thermoseparating polymer displays a lower critical solution temperature (LCST) in water solutions [7]. Above the LCST the polymer forms a two-phase system where one of the phases (usually the top phase) is polymer depleted and the other is polymer

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enriched. Ethylene oxide containing polymers as poly(ethylene glycol), and copolymers of ethylene oxide (EO) and propylene oxide (PO) are usually thermoseparating. In the latter example the LCST decreases as the content of PO increases [8]. Generally these polymers form highly polymer enriched phases upon thermoseparation. However, in order to efficiently partition proteins to the polymeric phase in a one-polymer two-phase system the polymeric phase should not be too concentrated. This type of system can by achieved by using hydrophobically modified EOPO polymer (HM-EOPO) [9].

In this study we investigate the possibility of using a thermoseparating cationic hydrophobically modified ethylene oxide polymer (HM-EO) as a two-phase forming component where the electrostatic interaction between proteins and polymer can be modulated, by changing pH, addition of sodium dodecyl sulfate (SDS) and salt. The cationic HM-EO polymer is a comb polymer composed of strands of ethylene oxide, interspersed by aliphatic tertiary amines, where the aliphatic chains ($C_{12}H_{25}$) are coupled to the amines. The EO-amine blocks are in turn connected by isophorone diisocyanate (IPDI) groups, Fig. 1. A detailed description of how the polymer was produced has been recently described by Thuresson et al. [10]. The cationic HM-EO displays properties similar to the uncharged HM-EOPO, in terms of micellization and phase compositions of the two-phase system. The thermosepa-

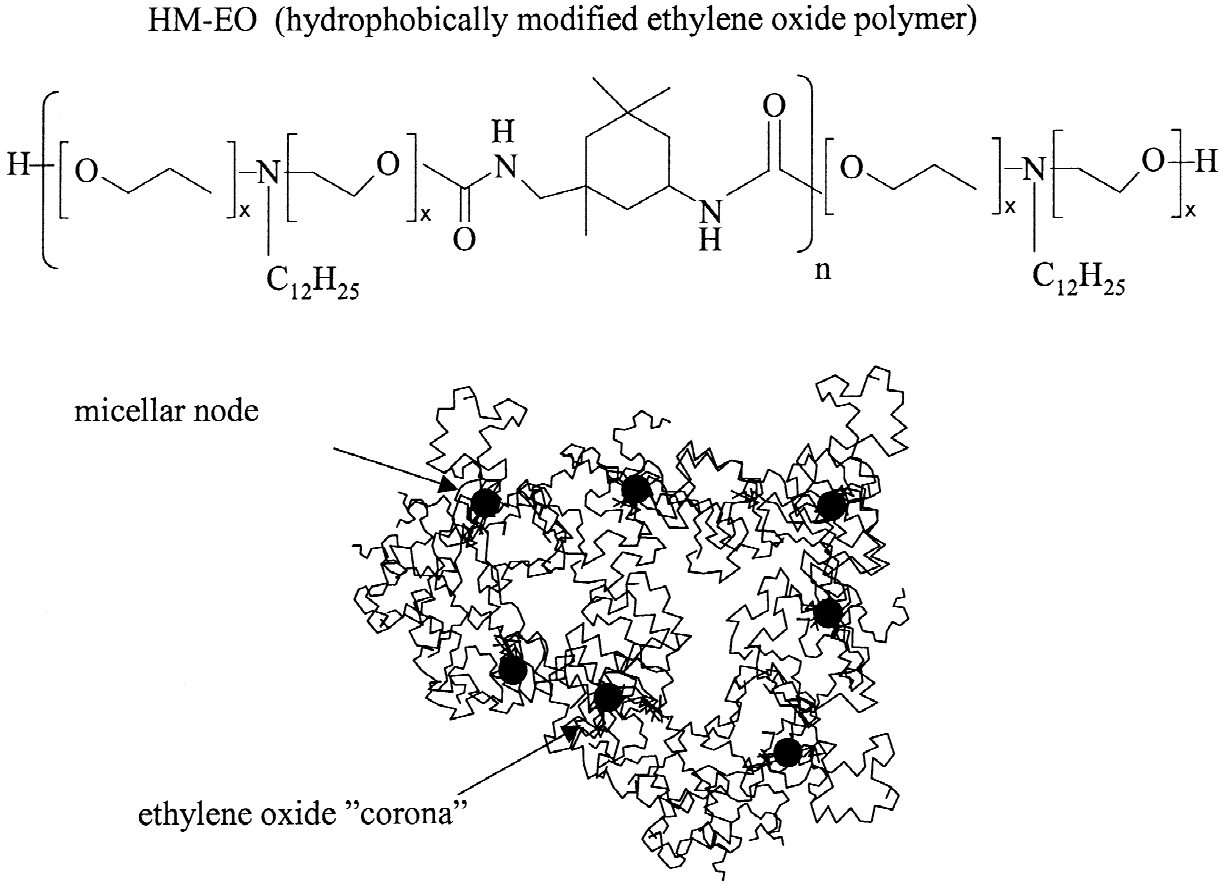

Fig. 1. The HM-EO polymer consists of an ethylene oxide backbone with interspersed isophorone diisocyanate (IPDI) and amine groups. Aliphatic chains of 12 carbons are grafted to the amine groups. In water solutions the polymer forms aggregates with micellar aliphatic nodes linked by hydrophilic ethylene oxide chains.

ration of the cationic polymer is, however, dependent on pH and ionic strength of the solution. The thermoseparating water/HM-EO system differs from previously studied aqueous two-phase systems by encompassing several properties that affect the partitioning of biomolecules and properties that can be modulated by environmental variables: thermoseparation (which affect the tie-line of the system), polymer-charge at different pH, micro-compartmentalization of hydrophobic micellar region and polar aqueous-ethylene oxide rich regions. The latter exerts also an entropic repulsion, which facilitates exclusion of the protein from the polymer rich phase. In contrast to water/non-ionic surfactant systems, where the surfactant is of the C_xEO_y type, this water/HM-EO system contains large ethylene-oxide rich regions, which may potentially attract many different proteins that have preference for slightly hydrophobic phases [i.e. poly(ethylene oxide) rich phases] [11]. Charged thermoseparating polymers have been used previously but only in two-polymer systems (phase separation based on polymer–polymer segregation) [12]. The charged thermoseparating polymer in the latter system was a linear copolymer containing many chargeable groups. Thus our polymeric system differs from the referred systems by being a one-polymer thermoseparated system and by the localization of charges to specific micellar like regions.

The aim of this work was partly to understand how the thermoseparation changes with polymer charge and salt concentration (by comparison of model calculations and experiments), and partly to find conditions were it is possible to direct proteins with different charges to the thermoseparated polymeric phase and redirect them to a polymer-depleted phase. As model proteins BSA and lysozyme have been chosen. BSA with isoelectric point (pI)~5.5 and lysozyme with pI~11 are negatively, respectively, positively charged in the studied pH interval (pH 7–11). In the systems containing SDS, the anionic aliphatic detergents associate with the cationic polymer micelles and decrease the netcharge of the polymer complex. Depending on the amount of SDS in the system and pH, it is possible to shift the polymer–detergent complex netcharge from positive to negative. Adding aliphatic SDS may also increase the hydrophobicity of the polymer/detergent complex. This creates a possibility to favor or disfavor the partitioning of charged biomolecules as well as molecules with different hydrophobicity to the polymeric phase [13].

In order to understand the phase behavior of the polymer and the partition behavior of proteins in the water/HM-EO system we have modelled this polymer in the framework of the Flory–Huggins theory of polymer solutions [14]. An extension of the Flory–Huggins model, developed by Karlström, has been used in this study to model thermoseparating ethylene oxide containing polymers [15].

2. Flory–Huggins based model of thermoseparating polymer

The Flory–Huggins model is useful for studying fundamental effects and obtaining insights in biomolecule partitioning and phase behavior of thermoseparating polymers and aqueous two-phase systems [16,17]. The simple Flory–Huggins theory predicts increased solubility of the polymer at increased temperature. Thermoseparating polymers, however, phase separate in a high temperature interval. This effect is reproduced in the modeling by adding a term describing the internal entropy of the polymer segments. The model of Karlström divides the polymer segments into two classes: nonpolar and polar segments, where the nonpolar segments have a higher number of possible internal states than the polar segments [15]. The condition for equilibrium between the different segment states is $\partial\Delta G_{\mathrm{mix}}/\partial P = 0$, where P is the fraction of polar segments in the chain. The enthalpy and entropy of mixing, for a three-component system, can then be described as:

$$\Delta H_{\mathrm{mix}} = n^{\mathrm{T}}\Big\{ \Phi_1\Phi_2(Pw_{1\mathrm{p}} + (1-P)w_{1\mathrm{u}}) + \Phi_2^2\Big[P(P-1)w_{\mathrm{pu}} + \frac{(1-P)^2}{2}w_{\mathrm{uu}}\Big] + \Phi_1\Phi_3 w_{13} + \Phi_2\Phi_3\big[w_{\mathrm{p3}}P + w_{\mathrm{u3}}(1-P)\big]\Big\} \quad (1)$$

and

$$\Delta S_{mix} = -n^{T}R\left[\frac{\Phi_1}{M_1}\ln\Phi_1 + \frac{\Phi_2}{M_2}\ln\Phi_2 + \Phi_2 \times\left(P\ln P + (1-P)\ln\frac{1-P}{f}\right) + \frac{\Phi_3}{M_3}\ln\Phi_3\right] \quad (2)$$

The total number of lattice sites are given by n^{T}, Φ_i describes the lattice concentration (i.e. volume fractions) of a component *i* and M_i is the degree of polymerisation in lattice units. The fraction of polar segments, *P*, is a function of the interaction parameters w_{ij}, Φ_i and the ratio between the number of possible conformations of the nonpolar segments and the polar polymer segments. This ratio is the parameter, *f*, in Eq. (2), which in our model has the value 8. Subscripts *u* and *p* refer to the nonpolar and the polar segments, respectively. The free energy of the solution can then be written as:

$$\Delta G_{mix} = \Delta H_{mix} - T\,\Delta S_{mix} \quad (3)$$

The composition of each phase in a separated system is determined by minimizing the free energy of mixing of the whole system. This is obtained by moving trial amounts of components between the phases until the lowest free energy is reached with the constraints of mass conservation of the system and electroneutrality of each phase.

To compare the effects of a thermoseparating charged polymer to an uncharged polymer, calculations of polymers with different number of charges were performed. An uncharged polymer, a polymer with one charge and one with three charges were used in these calculations. In all three cases the same interaction parameters were used (see Table 1). The indices of the interaction parameters are: 1 is water, p and u the polar and nonpolar segments of the polymer respectively, 3 the negative counter-ion and 4 positive co-ions. The interaction parameters between the salt and the co-solutes, polymer and water, were set to keep the effective interaction to zero, i.e. the ions will not affect the cloud point temperature (CPT) significantly in any other way than to keep the phases neutral. Thus the added salt is a ''theoretical salt'' whose effect is to facilitate partitioning of the charged polymer. It has no enthalpic ''salting-out'' effect on the polymer. The purpose of adding the theoretical salt is to disentangle entropic from enthalpic cloud point-decreasing effects from the salt.

Table 1
Model parameters

w_{13}	0
w_{14}	0
w_{1p}	501
w_{1u}	10 381
w_{3p}	0
w_{3u}	4701
w_{4p}	0
w_{4u}	4701
w_{up}	5105
w_{uu}	9402
w_{34}	0

Below are the interaction parameters, w_{ij}, used in the model calculations given in J/mol. Subscript 1 refers to water, 3 to the anion, 4 to the cation and u and p to the non-polar and polar segments of the polymer, respectively. The ratio of possible non-polar conformations to polar conformations, *f*, is set to 8. The degree of polymerization in lattice units of the polymer M_{pol} is 200. All others components have M_i equal to 1.

To illustrate how the theoretical salt affects the thermoseparation of a charged polymer, model calculations were performed using different amounts of salt. First a system of charged polymer (+3) and its counter-ion, the latter having a concentration of 0.3% v/v was calculated. Subsequently the ion concentration was increased by further addition of 0.6, 1.2 and 2.4% v/v salt (where the salt is a 1-1 electrolyte composed of equal volumes of cations and anions).

3. Materials and methods

3.1. Materials

The polymer used in this study was a comb polymer with a polymer backbone composed of EO blocks separated by tertiary amines with an aliphatic C_{12} group, see Fig. 1. In a recent publication the molecular mass of this polymer was estimated by size exclusion chromatography to be ~25 000 [10]. The HM-EO polymer is a gift from Akzo Nobel (Sweden). The batch from which the experiments were performed contained 30% w/w polymer with a pH of 5.4. Most of the polymer was used without further purification for the phase diagram and the protein partitioning studies. However, some polymer was purified by repeating phase separation (10 times) at pH 11 and then freeze-dried. The latter polymer

was used for determination of the number of chargeable groups of the polymer by titration. Proteins used were BSA and chicken egg white lysozyme, both obtained from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade. Millipore Milli-Q water was used in all solutions and buffers.

3.2. Number of chargeable groups per HM-EO polymer

The maximum number of charges or number of tertiary amine groups was determined by titration. The pH was first set to 11 with NaOH in order to neutralize the polymer. The polymer was then titrated with HCl and pH was measured in order to obtain a titration curve, from which the average number of charges per polymer could be calculated. The purified HM-EO was used in this study.

3.3. Cloud point temperatures

The cloud point temperature (CPT) was measured to determine the border between the two-phase region and the one-phase region. This was performed by heating the samples of different polymer concentrations in a water bath at a rate of 0.2 °C/min. The cloud point was determined visually at the temperature at which the solution became turbid. NaOH was added to raise the pH from 5.4 of the polymer solution prepared from the batch polymer to the desired pH. The CPT was determined at different pH for a 2% w/w HM-EO solution. The CPT dependence on NaCl concentration at pH 7 (containing 10 m*M* potassium phosphate buffer) and pH 12 (pH adjusted with NaOH) was determined by adding different amounts of NaCl to a 2% w/w polymer solution. CPT investigations were also performed for 3.6% w/w polymer solutions at pH 7 by adding different amounts of SDS.

3.4. Separation of phases

Phase separation was performed by centrifuging at a defined temperature. The centrifuge was a water bath-heated 4233ECT Centrifuge (Cologno Monzese, Italy). In order to compare protein partitioning of different systems with different CPT, the phase separation was performed at a temperature $T = \text{CPT}$ (of the actual system) + 10 °C. This means that all systems will have the similar relative position in a phase diagram, assuming that the shape of the phase diagrams are similar and the difference is only in CPT. All bottom phases formed by thermoseparation were polymer enriched. The viscosity of the bottom phases was not measured but was low enough to be transferred with a Pasteur pipette to a new test tube.

3.5. Protein partitioning

Partitioning of proteins in two-phase systems is described by a partition coefficient, *K*, which is the ratio of protein concentration in the top phase (C_t) to that of the bottom phase (C_b), $K = C_t/C_b$. The polymer concentration of the used systems was 3.6% w/w. Partitioning was performed in systems with or without the addition of NaCl (100 m*M*). The pH was adjusted by adding NaOH to the polymer stock solutions. All proteins were dissolved in buffers (NaOH–glycine or potassium phosphate, both 10 m*M*) of the actual pH before adding them to the polymer solutions. In systems containing SDS, the SDS solution was added to the polymer and mixed before the addition of protein. The total concentration of lysozyme in the two-phase systems was 1 mg/g sample and BSA concentrations were 5 mg/g sample. Phase separation was performed as described above and the protein concentrations of bottom and top phase were determined by measuring absorption at 280 nm. Corresponding systems without protein were used as blanks. Duplicates of each sample were made. Activity of lysozyme was determined by using an activity-solution which was prepared by dissolving 24 mg of *Micrococcus lysodeikticus* in 80 ml of 0.1 *M* potassium phosphate buffer, pH 7 [18]. To 1 ml of activity solution approximately 50 μl of sample was added. The change in absorption was measured at 450 nm, for 1 min. For best results this change should fall in the range of 0.0015–0.0040 absorption units per minute. The spectrophotometer was a UV-2101PC from Schimadzu (Kyoto, Japan).

3.6. HM-EO, SDS, NaCl and water content in the phase systems

Phase composition was determined with respect to

HM-EO, SDS, Cl^- ions and water concentrations. In order to determine SDS concentrations, the bottom phases were diluted 50–60 times for systems containing 0.15% w/w SDS and 60–70 times for systems containing 0.2% w/w SDS. The top phases were used without dilution. A volume of 1.0 ml of the diluted sample, 50 μl 100 m*M* of Methylene Blue and 2.0 ml of ethyl acetate (100%) was added to a 10 ml test tube. The mixture was thoroughly mixed by vortex and then centrifuged for 2–3 min in a table centrifuge at 4500 rpm. After centrifugation the test tube contained two liquid phases. The top phase, containing ethyl acetate and extracted SDS–Methylene Blue complex was removed and collected in a test tube. Another 2.0 ml of pure ethylene acetate was then added to the remaining (bottom phase) sample and centrifuged as above. This procedure was repeated 5–7 times until no Methylene Blue was further extracted to the ethylene acetate phase. The amount of Methylene Blue was then determined in the collected ethylene acetate phase by measuring absorbance at 655 nm. Dilutions were made by adding 95% aqueous ethanol. Methylene Blue and SDS was extracted to the ethylene acetate phase by the molar ratio of 1:1, which confirmed the usefulness of this method. No interference of the cationic HM-EO polymer was observed.

Cl^- concentrations in top and bottom phases were determined by standard Mohr titrations. To the samples 5 drops of 5% w/w K_2CrO_4 and 20–40 mg of $NaHCO_3$ was added. The samples were then titrated with 10 m*M* $AgNO_3$ until an orange precipitate was observed. No interference from either SDS or HM-EO was observed.

Finally the top and bottom phases were freeze dried in order to determine the water content and the total mass of the non-volatiles of the samples. Thus it was possible to derive the concentration of HM-EO in the top and bottom phases of the systems, by subtracting the known amounts of NaCl and SDS determined from previous measurements.

4. Results

4.1. Number of charges of HM-EO

Purified polymer at pH 11 was titrated, requiring 60.0 μmol and 28.0 μmol HCl to neutralize 0.298 g and 0.148 g of HM-EO, respectively. These values imply that there are 5 respectively 4.7 charges per polymer chain, assuming a molecular mass of 25 000. According to the manufacturer and a study published recently the polymer molecule contains 4–5 amines [10].

4.2. Cloud point temperature of HM-EO

The CPT at different polymer concentrations at pH 11 is shown in Fig. 2. The lowest CPT was obtained at 29 °C for polymer concentrations between 0.5 and 3% w/w. In the study of Thuresson et al. the lowest CPT of the uncharged polymer was 20 °C [10]. The CPT at different pH (in Fig. 3) was determined for polymer concentrations of 2% w/w. The CPT remains constant at pH above 10 where the polymer becomes uncharged. At pH below 9, the CPT increases rapidly. In the system of pH 7 the CPT was studied at different salt concentrations in order to screen the electrostatics (see Fig. 4). The charged polymer has a relatively high cloud point even at high salt concentration. At 1 *M* NaCl the CPT is 35 °C. Included in the diagram (Fig. 4) are also two systems at pH 12. One with 1 *M* of NaCl where CPT is 13 °C, and the other without salt, where CPT is

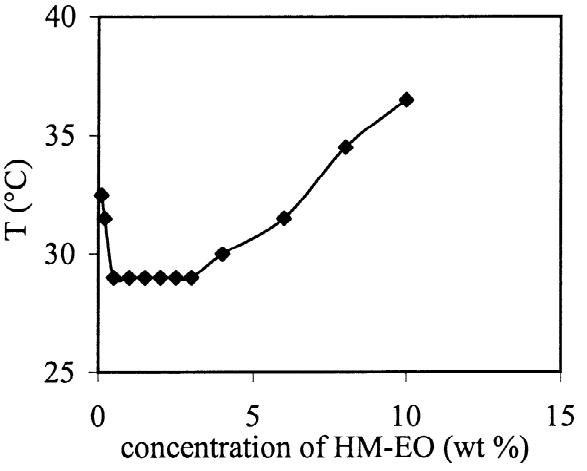

Fig. 2. Cloud point temperatures at different concentrations of uncharged HM-EO at pH 11. The area above the curve represents the two-phase area, where a polymer-rich bottom phase and a water-rich top phase coexist. Below the curve the system is a homogenous mixture of polymer and water.

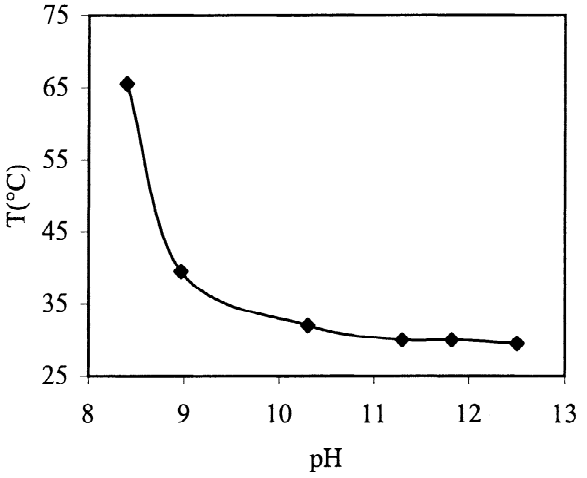

Fig. 3. Cloud point temperatures of HM-EO at different pH. The concentration of HM-EO is 2% w/w. pH was set by adding NaOH to the polymer solutions.

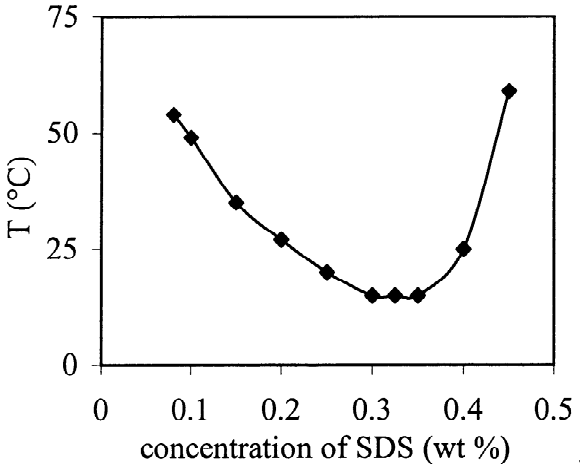

Fig. 5. Cloud point temperatures of HM-EO at different SDS concentrations. The concentration of HM-EO was 3.6% w/w. pH was set to 7.0 by adding NaOH to the polymer solution.

29 °C (black circles). In Fig. 5 the effect of SDS on the CPT is shown for systems where pH is 7. There is a minimum in CPT at 0.3–0.35% w/w SDS, which may well correspond to the isoelectric molar ratio of SDS to the cationic polymer. Above or below this concentration the CPT rises significantly. The rise in CPT is stronger at higher rather than lower SDS concentrations. According to the determination of number of charges on HM-EO, however, only 0.21% w/w of SDS is needed to neutralize 3.6% w/w of fully charged HM-EO. Thuresson et al. [15] report that the batch polymer may contain free aliphatic amines, which is an impurity from the production process. Thus, by using the batch polymer more SDS is needed to create an isoelectric SDS/HM-EO complex.

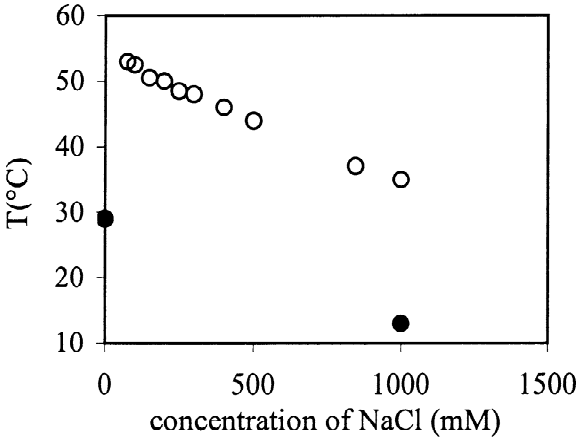

Fig. 4. Cloud point temperatures of HM-EO at different NaCl concentrations. The concentration of HM-EO is 2% w/w. White circles (○) correspond to systems at pH 7 (containing 10 m*M* phosphate buffer) and the black circles (●) correspond to a system of pH 12 (pH set by NaOH).

4.3. BSA and lysozyme partitioning

In systems without SDS lysozyme slightly prefers the top phase (the polymer depleted phase), while in systems with SDS lysozyme is partitioned to the bottom phase (Fig. 6). By decreasing the pH in systems with SDS larger K values are obtained, although lysozyme still partitions to the bottom phase. Systems with salt results in a less extreme partitioning of lysozyme compared to the corresponding salt-free system. An even partitioning of lysozyme is obtained in the system of pH 7, 0.2% w/w SDS and 100 m*M* NaCl.

In a salt-free system at pH 11, where both HM-EO and lysozyme are uncharged, lysozyme prefers the top phase, with $K = 1.9$ (not shown in the diagram). The activity measurements showed that 80–100% of the lysozyme activity remained after the partitioning, indicating that no significant denaturation of the enzyme occurred, consistent with previous work [19].

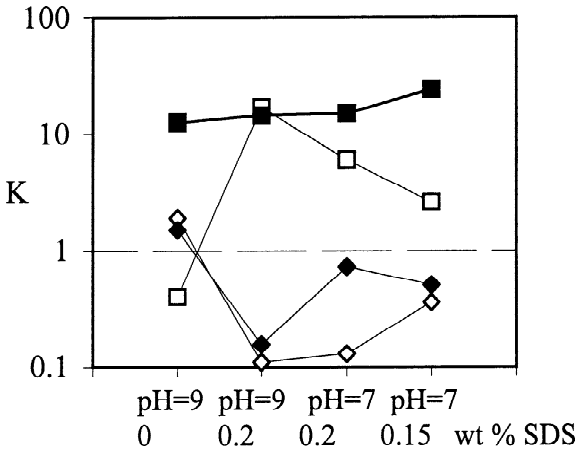

Fig. 6. The effect of SDS and pH on the partition coefficient of lysozyme and BSA in thermoseparated water–HM-EO two-phases systems. The concentration of HM-EO was 3.6% w/w. Buffer composition was 10 m*M* NaOH–glycine at pH 9 or 10 m*M* potassium phosphate at pH 7. The NaCl concentration was 0 or 100 m*M*. Systems: (1) pH 9, no SDS; (2) pH 9, 0.2% w/w SDS; (3) pH 7, 0.2% w/w SDS; (4) pH 7, 0.15% w/w SDS. Symbols: squares: BSA; diamonds: lysozyme; white: no NaCl; black: 100 m*M* NaCl. The separation temperatures are listed in Table 2.

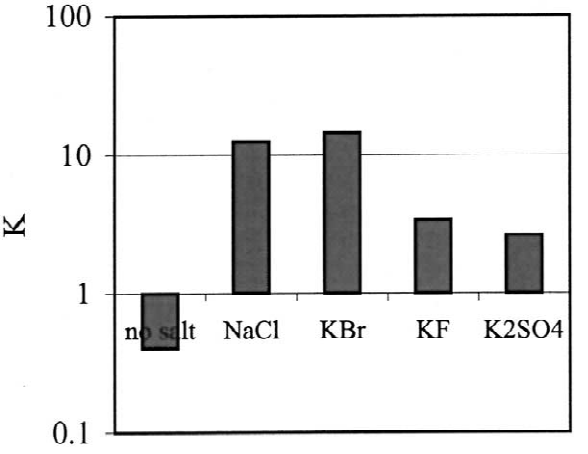

Fig. 7. The effect of different salts on the partition coefficient of BSA in thermoseparated water/HM-EO two-phases systems. The concentration of HM-EO was 3.6% w/w. Buffer composition was 10 m*M* NaOH–glycine at pH 9. The salt concentration was 100 m*M* in all systems with salt. The separation temperatures for the different systems were 50 °C without salt, 47 °C with NaCl, 49 °C with KBr, 46 °C with KF and 36 °C with K_2SO_4.

The trend of the partitioning behavior of the negatively charged BSA was almost the opposite that of the positively charged lysozyme. In the SDS-free and salt-free system BSA partitions to the bottom phase (polymer enriched phase), whereas in systems containing SDS, BSA prefers the top (Fig. 6). At pH 9 in a SDS-free system the addition of salt will change the preference of BSA from the bottom phase to the top phase. In all of the studied systems containing salt, BSA partitioned to the top phase. As pH is decreased from 9 to 7 the *K* values decrease in systems without salt. The *K* value is also decreased when decreasing the SDS concentration from 0.2% w/w to 0.15% w/w. The *K* values of BSA in systems with salt, however, are not strongly affected by the change in pH and SDS.

4.4. Effects of different salts on partitioning of BSA

In Fig. 7 the partitioning of BSA is shown for systems with different salts, at pH 9. All the salts drive BSA to the top phase, while BSA partitions to the bottom phase in the salt-free system. NaCl and KBr drive the partitioning of BSA to the top phase more than KF and K_2SO_4. In a salt-free system at pH 11 (not shown in the diagram), where BSA is highly charged while the polymer is uncharged, BSA partitions to the water phase (top phase), with $K = 2.0$, which is similar to the partitioning of lysozyme in the corresponding system.

4.5. Partitioning of HM-EO, water, SDS and NaCl in water/HM-EO systems used for protein partitioning

The *K* values for the components HM-EO, SDS, Cl^- and water for different systems are given in Table 2. Cl^- ions partition to the top phase, slightly more than water. The composition of Na^+ ion in the phase was not determined. Presumably the Na^+ ion distribution follows the Cl^- ion distribution due to the electro-neutrality of the phases. The partitioning of HM-EO and SDS is enhanced towards the bottom phase when adding salt to the system. Interestingly SDS has a smaller *K* value than HM-EO, i.e. the molar SDS/HM-EO ratio is larger in the bottom phase than in the top phase.

Table 2
Partition coefficients (K) of the phase forming components in thermoseparated two-phase systems used for partitioning of BSA and lysozyme

pH	SDS (% w/w)	NaCl (mM)	$K_{HM\text{-}EO}$	K_{SDS}	K_{H_2O}	K_{Cl}	Volume ratio V_{top}/V_{bottom}	Separation temperature (°C)
9	0.0	0	0.08	–	1.1	–	3.4	50
9	0.2	0	0.13	0.12	1.1	–	1.7	40
7	0.2	0	0.11	0.05	1.1	–	3.3	36
7	0.15	0	0.14	0.04	1.1	–	3.9	45
9	0.0	100	0.08	–	1.1	1.2	3.4	47
9	0.2	100	0.11	0.03	1.1	1.2	2.2	28
7	0.2	100	0.10	0.02	1.1	1.3	3.8	36
7	0.15	100	0.10	0.01	1.1	1.3	3.6	45

K values of the four main components in the phase systems: HM-EO, SDS, water and Cl^- are presented in the columns. The separation temperature is 10 °C above the cloud point temperature of the system. All systems contained 3.6% w/w HM-EO. The pH and concentrations of SDS and NaCl are given in the first column. The buffers in the systems are: NaOH–glycine, 10 mM, pH 9 or potassium phosphate, 10 mM, pH 7.

4.6. Model calculations of charged thermoseparating polymers

In Fig. 8 the calculated phase diagrams of a model thermoseparating polymer with different charges are shown. The interaction parameters of all components are the same in all calculations presented in this work. The polymer, however, have a variable fraction (which is temperature dependent) of non-polar segments. The uncharged form of the polymer exhibits a LCST at 28 °C. The same polymer with one charge has a LCST of 35 °C and with three charges the LCST is 52 °C. The two-phase area decreases as the charges of the polymer is increased. The polymer concentration in both the top phase and the bottom phase increases as the charge of the polymer increases. Phase systems were also calculated for a polymer with three charges and different amount of salt, the results are shown in Fig. 9. The LCST is decreased as the amount of salt is increased and approaches the LCST of the uncharged polymer for systems with large amounts of salt. In the system where the only ionic components are the polymer (+3) and its counter-ions the LCST is 52 °C, same as in Fig. 8. In the system with the further addition of 0.6% v/v salt the LCST is 39 °C and increasing the salt concentration to 1.2 and 2.4% v/v decreases the LCST further to 35 °C and 32 °C, respectively.

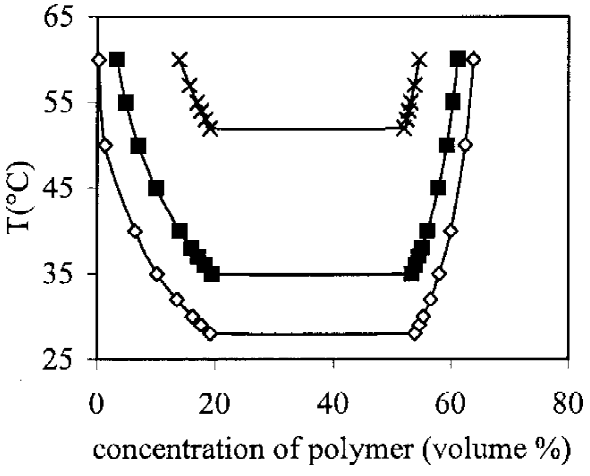

Fig. 8. Calculated cloud point temperature curves for charged theoretical thermoseparating polymer. The effect of increased polymer charge is illustrated. Symbols: (○) uncharged, (■) +1 charged, (×) +3 charged form of the polymer, respectively. The only ionic species in the system is the polymer and its counter-ions. The model parameters used are listed in Table 1.

5. Discussion

5.1. Physico-chemical properties of HM-EO

The amphiphilic nature of hydrophobically modified polymers tends to result in micellization or aggregation of the hydrophobic parts, while the more hydrophilic parts are localized on the surface of the aggregate or surround the micelles as linkers between the aggregates [19–21]. Critical aggregation

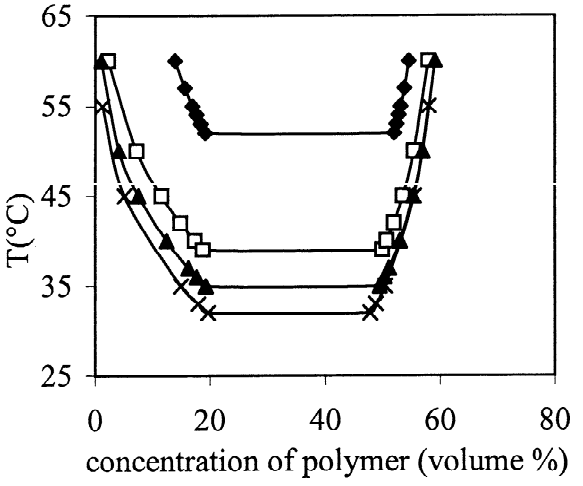

Fig. 9. Calculated cloud point temperature curves for a +3 charged theoretical thermoseparating polymer. Effect of increased salt (1-1 electrolyte, composed of equal volumes of anion and cation) concentration. Polymer concentration: 20% v/v. There are 0.3% w/w counter-ions to the polymer apart from the ions from the salt. Symbols: black diamonds (♦) no salt added (ions only from polymer and its counter ions), (□) 0.6% v/v salt, (▲) 1.2% v/v salt, and (×) 2.4% v/v salt. Model parameters used are listed in Table 1.

concentrations (CACs) or critical micelle concentrations (CMCs) have been reported to be between 0.1 and 1% w/w for a hydrophobically modified ethylene oxide polymer and 0.01% w/w for a HM-EOPO polymer [19,20]. Guillemet and Picullell have shown that in systems with a hydrophobically modified polycation and anionic SDS the CMC or rather the critical aggregation concentration occurs at even lower concentrations [22]. Thus we assume that our systems containing 3.6% w/w HM-EO are well above the CMC of the polymer and that the polymers in the systems exist as micelles, as shown in Fig. 1.

An additional feature of the cationic HM-EO is the solubility dependence on pH. HM-EO has a high CPT below pH 9 and a lower CPT at high pH. This is due to the increased polymer charge below pH 9 (Fig. 3). This increase in solubility with increasing charge is a well-known phenomenon that can be understood in terms of loss in entropy upon compartmentalization of a charged polymer and its counter-ions to one of the phases upon phase separation. The phenomenon is reproduced in the model calculations (Fig. 8), showing enhanced solubility (in terms of increased preference for one-phase formation instead of two-phase) as the charge of the polymer is increased.

5.2. Effect of NaCl and SDS on the CPT of HM-EO

Co-solutes like alcohols, surfactants and salts can strongly affect the CPT of hydrophobically modified polymers [23]. In this study we have used NaCl and SDS to show how the CPT of HM-EO can be changed. The decrease of CPT with increasing salt concentration in Fig. 4 can be explained by the decreased importance of the counter-ion entropy. The ion concentration difference between the phases decreases as salt is added to the system. Thus there cannot be a large loss of entropy upon compartmentalization of polymer and its counter-ions to one of the phases. This has also been predicted by calculations using the Flory–Huggins based model (Fig. 9). In the experiments, however, the cloud point of the charged HM-EO never reaches the cloud point of the uncharged polymer, while the model calculations predict that the LCST should rapidly decrease upon salt addition and reach the LCST of the uncharged polymer. Experimentally, the uncharged polymer had a cloud point of 29 °C in a solution without salt. In a solution of 1 *M* NaCl the charged polymer had a cloud point of 35 °C. Thus in the latter case, even though the salt concentration is very high and the entropic penalty of compartmentalization of polymer with its counter-ions is negligible, the CPT of the uncharged polymer is not reached. Apart from reducing the entropic penalty the salt has a salting-out effect, due to an effective repulsion between the salt and the polymer [24]. Hydrophilic salts will salt out polymers, which have significantly lower dielectric constant than water [25]. This well known fact can explain the decrease in cloud point of the uncharged polymer in Fig. 4 (compare the black circles in the diagram). Thus, considering these two effects it is surprising to find that the cloud point of the fully charged polymer (at pH 7) is as high as 35 °C at a NaCl concentration of 1 *M*. The relatively high CPT for a charged HM-EO polymer at high salt concentration can be understood by the effect of the charged micellar surface on the ethylene oxide coils compared to the neutral micellar

surface of the uncharged polymer. The EO chains cannot form a dense ''corona'' around the charged micellar surface without creating poor solvation conditions for the micellar charges and counter-ions. In order to overcome the increase in solvation energy it is therefore necessary to induce phase separation at a higher temperature.

The addition of SDS to the cationic HM-EO polymer causes the formation of mixed micelles or aggregates of SDS and HM-EO, as discussed above. As we add SDS the netcharge of the SDS/HM-EO complex is decreased, which leads to a lower entropic penalty from the counterions, which in turn leads to a lower CPT, as shown in Fig. 5. The minimum in CPT at 0.3–0.35% w/w of SDS can be understood as the formation of an isoelectric SDS/HM-EO complex. As we add even more SDS the complex becomes negatively charged and the CPT increases. Another effect of adding SDS is the increase in hydrophobicity of the aggregate through the dodecyl chain. This explains why the neutral SDS/HM-EO complex has a CPT of only 15 °C compared to the 29 °C of the neutral polymer. In the previous discussion we claimed that solvation of micellar charges and counter-ions increase the CPT but in this case not only the absolute micellar charges are increased but also the hydrophobicity of the complex, and the latter accounts strongly for the decreases the CPT. This result is similar to the observations of Thuresson et al. who concluded that the CPT of a HM-EO polymer with a higher degree of aliphatic side chains have a lower CPT than a polymer with less aliphatic side chains [10].

5.3. Partitioning of proteins in salt-free systems

Since both proteins prefer the water phase ($K \sim 2$) at pH 11 where the polymer is electro-neutral, we can conclude that there are no major non-electrostatic attractive interactions between the polymer and the studied proteins. In the salt-free and SDS-free systems at pH 9 BSA and lysozyme are attracted and repelled, respectively, to the polymer-enriched phase, where the micellar aggregates are positively charged (Fig. 6). The addition of SDS to the system at pH 9 causes the formation of negatively charged mixed micelles of SDS and HM-EO. This explains why the phase preferences of the proteins are shifted. At pH 7 the mixed micelles are less negative and become even less negative by reducing the SDS concentration to 0.15% w/w. It was determined that 0.3% w/w of SDS is needed to neutralize 3.6% w/w of HM-EO at pH 7 (Fig. 5). BSA is, however, still partitioned to the top phase and lysozyme to the bottom phase when the SDS concentration is below 0.3% w/w. This is a somewhat surprising result that can be explained by the lower K value of SDS compared to that of HM-EO (Table 2), implying that the SDS/HM-EO ratio is higher in the bottom phase than the top phase. Thus, the mixed micelles are less positive in the bottom phase indicating that the driving force for positive proteins are towards the bottom phase and negative proteins should partition to the top phase, which agrees with the results.

5.4. Partitioning of proteins in systems with salt

In all systems with 100 mM salt BSA have high K values, while lysozyme partitions as in salt free systems but with K values closer to 1 (Fig. 6). The trend is that the salt results in a more extreme partitioning of BSA and a less extreme partitioning for lysozyme. From electrostatic reasons one would conclude that the partitioning would always be less extreme in systems with salt since the counter-ion entropy is less pronounced. However, since the different salt ions have different preferences for the phases they can influence the partitioning of charged macromolecules. Johansson et al. have shown that a positively charged polypeptide, composed of tryptophan and lysine, can be quantitatively partitioned to either a polymer phase or the water phase by changing the salt from $NaClO_4$ to Na_2SO_4 [26]. The salt effect on protein partitioning can be understood from an enthalpic effect [2]. The larger difference between the ions of a salt in terms of effective ion–solvent and ion–polymer interaction the larger is the effect of the salt. Both NaCl and KBr are salts that enhance the partitioning of the negatively charged BSA to the top phase, whereas the effect of adding KF or K_2SO_4 is smaller. The salts also cause an increased difference in polymer concentration of the phases. The partitioning of SDS and HM-EO is more pronounced towards the bottom phase in the salt containing systems than in the salt free systems, i.e. salt containing systems form denser polymeric

phases than salt-free systems (see Table 2). Thus there will be an entropic repulsion driving the partition of macromolecules towards the top phase, resulting in larger K values for both BSA and lysozyme. The relatively strong change in partitioning of the proteins when salt is added to the system may thus be due to the entropic repulsion caused by the ethylene oxide corona and the overall increased polymer concentration in salt-rich systems.

6. Conclusions

The cationic hydrophobically modified ethylene oxide polymer, HM-EO, forms thermoseparating aqueous two-phase systems that can be used for partitioning of charged molecules. It is possible to modulate the electrostatic interaction between proteins and HM-EO by changing pH, micellar netcharge and salt in these water/HM-EO systems. Negatively charged BSA, is efficiently partitioned to a positive polymer phase, whereas positively charged lysozyme is repelled from the positively charged polymeric phase. By changing the netcharge of the polymeric micellar aggregates with addition of SDS it is possible to reverse the partitioning of the positively and negatively charged proteins. The lysozyme activity is not destroyed indicating that SDS in these systems is localized in the micelles. Lysozyme and BSA interact only weakly with the uncharged HM-EO. The cationic HM-EO can therefore separate hydrophilic proteins with respect to the sign of their charge. Addition of salt counteracts the electrostatic effect of the cationic HM-EO polymer at pH 9. Mean field calculations based on the Flory–Huggins theory can be used to show how the solubility of a charged thermoseparated polymer increases as the charge increases. The calculations also illustrate how the thermoseparated charged polymer becomes less soluble as salt concentration is increased.

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