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Phase behavior and protein partitioning in aqueous two-phase systems of cationic–anionic surfactant mixtures

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Abstract

Cationic–anionic surfactant mixtures can form aqueous two-phase systems. Such aqueous surfactant two-phase systems (ASTP systems) can be used for separation and purification of biomaterials. In this work we investigated the phase behavior and the partitioning of BSA and lysozyme in the ASTP system formed by mixtures of dodecyltriethylammonium bromide and sodium dodecylsulfate (SDS). The pseudo ternary phase diagram of these mixtures at low total surfactant concentrations contains two narrow two-phase regions, which represent two kinds of different ASTP systems formed when cationic and anionic surfactants are in excess, respectively (called ASTP-C and ASTP-A). The phase separation is associative, one phase is surfactant-rich, and the other phase is surfactant-depleted. Mechanisms behind the phase behavior are discussed. The phase behavior, especially phase separation time and phase volume ratio, is strongly influenced by total concentration and molar ratio of mixed surfactants. The effect of molar ratio is strong, which enables one to get desired phase systems also at very low total concentration by tuning the molar ratio of the surfactants. It was shown that the marked differences of surfactant concentration between the phases makes proteins distribute with different partitioning coefficients. The charges on the micellar surface, which can be adjusted by tuning the molar ratio of cationic surfactants to anionic surfactants, enhance the selectivity of protein partitioning by electrostatic effects. At pH 7.1, in the ASTP-C systems, negatively charged BSA is concentrated in the surfactant-rich phase and positively charged lysozyme in the surfactant-depleted phase, while in ASTP-A systems, a totally opposite partitioning was observed. It was shown that lysozyme could retain activity in ASTP systems.
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1. Introduction

When cationic and anionic surfactants are mixed at certain concentrations (much higher than CMC, but still very dilute), the solution separates spontaneously into two aqueous phases. An aqueous surfactant two-phase (ASTP) system is formed, where one phase is rich, and the other phase is poor in surfactants. A clear interfacial boundary exists between the two phases [1,2]. Such a phase separation, which has turned out to be common for cationic–anionic surfactant mixtures, used to be regarded as an undesired phenomenon during previous studies. The overwhelming aim for these studies of cationic–anionic surfactants has been to obtain stable homogeneous transparent solutions with high surface activity and stable organized molecular assemblies with special functions. It was not until 1994 when one of

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the present authors suggested in a short communication [1] that such a phase system might be used as a partitioning system for separation and purification of biomaterials. Since then some physicochemical properties of such systems have been studied [2]. Recently, different porphyrins and metalloporhyrins have been partitioned in ASTP systems consisting of dodecyltriethylammonium bromide (C_{12}NE) and sodium dodecylsulfate (SDS) [3]. The phase behavior of catanionic surfactants and catanionic mixtures in general has been reviewed [4,5].

On the contrary, the aqueous two-phase systems formed by polymers were recognized and exploited quite early. The polymer aqueous two-phase systems were first described by Beijerinck at the end of the last century [6], and were rediscovered by Albertsson as partitioning systems for separation and purification of biomaterials in the 1950s [7]. Now, extraction in aqueous polymer two-phase systems has been developed as an important fast, mild and easily scaled-up separation technique in biochemistry and biotechnology for separation of cells, particles and proteins [7–10]. Besides the most widely used poly(ethylene glycol) (PEG)–dextran and PEG–salt systems [7–10], many new phase-forming systems have been exploited, especially temperature-induced phase separation with random and block copolymers of ethylene oxide (EO) and propylene oxide (PO) [11–13]. Besides polymer systems, non-ionic surfactants such as Triton X-114 can form aqueous two-phases when they are heated above their cloud point temperatures. These mixtures have mainly been used for extraction of membrane-bound proteins [14–16], but have also been shown to be efficient for extraction of viruses [17]. Further improvement has been achieved by combining non-ionic surfactants with polymers, especially for partitioning of labile membrane proteins, due to two-phase separation between a micelle phase and a polymer phase [18,19].

Two-phase systems with cationic–anionic surfactant mixtures have a number of unique features that are of interest for separation of biomolecules:

(1) Micelles of surfactants can simultaneously offer hydrophobic and hydrophilic environments to solute species, which gives rise to a partitioning selectivity based on the hydrophobicity of biomaterials. It can be used for the partitioning of not only hydrophilic proteins, but also of water-insoluble proteins such as membrane proteins.

(2) The self-assembling natures of micelles of surfactant enables one to control and optimize the partitioning behavior by tuning micellar characteristics, including micellar shape and size.

(3) The charges on the micellar surface, which can been controlled by tuning molar ratio and total concentration of surfactants, can be used for the selective partitioning based on electrostatic interaction between micelles and proteins. This gives the possibility to separate different proteins based on their net charge [20].

(4) Surfactants can be removed from desired biomaterials after partitioning simply by diluting the surfactant-rich phase or by changing temperature. The precipitated surfactants can be recycled [21].

(5) Multi-step partitioning procedures are possible, which can also be achieved by diluting the surfactants-rich phase with water or buffers, without the need for addition of new phase-forming surfactants [21].

(6) ASTP partitioning can readily be operated because of (i) low concentration of surfactants (it may be less than 1wt%); (ii) low phase viscosity of the surfactant-rich phase, while the viscosity of the surfactant-depleted phase is close to water; and (iii) fast phase separation time (some solutions can separate into two phases within 10 s without being centrifuged).

Compared to aqueous two-phase systems formed by non-ionic surfactants (temperature-induced aqueous two-phase systems), ASTP systems can be obtained also at low temperature. The phase separation of non-ionic surfactants takes place only above a critical temperature. So ASTP partitioning can be operated at desired temperatures, which is especially suitable for partitioning of thermo-sensitive proteins.

Based on the above features, ASTP systems may provide a useful alternative to aqueous two-phase systems formed by polymers and non-ionic surfactants for separation and purification of biomaterials. ASTP systems are similar to aqueous polymer two-phase systems in many aspects [2,22], so it might be possible to obtain information about the mechanism of partitioning in ASTP systems using the knowledge

that has been gained on partitioning in aqueous polymer two-phase systems. Johansson et al. [23] have reviewed driving forces for phase formation and a current understanding on mechanisms that drives partitioning of biomolecules in aqueous two-phase systems. Accordingly, it appears very important to identify as well as characterize the main driving forces and the underlying physical principles responsible for the observed partitioning behavior of biomaterials in aqueous two-phase surfactant systems.

In this work we have studied ASTP systems of mixtures of dodecyltriethylammonium bromide ($C_{12}NE$) and sodium dodecylsulfate (SDS), including phase behavior of the systems and partitioning of bovine serum albumin (BSA) and lysozyme in ASTP. BSA and lysozyme were selected as model proteins because both of them have been well characterized and they have quite different net charges, which enables one to investigate the effect of micellar charges and pH values on partitioning. Besides, the activity of lysozyme in ASTP systems has been measured and the interaction between proteins and mixed cationic–anionic surfactants are discussed.

2. Material and methods

2.1. Chemicals

Sodium dodecylsulfate ($C_{12}H_{25}SO_4Na$, SDS) was purchased from Fluka (Buchs, Switzerland), and was recrystallized from water–ethanol. Dodecyltriethylammonium bromide ($C_{12}H_{25}N(C_2H_5)_3Br$ ($C_{12}NE$) was prepared by refluxing the mixtures of dodecane bromide and triethylamine in methanol. Adding ether after most of solvent had been removed by distilling precipitated the crude product out. The crude product was recrystallized in mixed solvents of acetone–ether. No surface tension minimas were found for surfactants, which implies that no surface-active impurities exist in them.

Water was of Millipore quality. For measurement of surface tension, Millipore water was redistilled from alkaline permanganate, which insures that the surface tension of water is 71.5 mN M^{-1} at 25°C. All other chemicals were of analytical grade.

2.2. Proteins

Bovine serum albumin (BSA) (essentially fatty acid free) was obtained from Sigma (St. Louis, MO, USA). Lysozyme (EC 3.2.1.17) from hen egg white was obtained from Boehringer (Mannheim, Germany).

2.3. Determination of phase diagram

Solutions of surfactants were prepared by weight molar concentration (molar numbers of solute in 1000 g solution). Samples were prepared by mixing stock-solutions of cationic and anionic surfactant and were left to phase separation for at least 24 h at 20°C in a water bath. The phase separation was judged visually.

It should been pointed out that it is hard to give an exact definition of ASTP systems. In this paper, we defined ASTP as a phase system in which (i) both of the two phases are transparent or very weekly opalescent, and (ii) there is a clear interfacial boundary between the two phases. In some cases, especially when the composition of systems is very close to equimolar, the first prepared mixtures are turbid, which separate into two turbid phases or one turbid and one clear phase after standing. Such phase systems were excluded from ASTP in this work.

2.4. Determination of phase separation time

The phase separation time was determined by the appearance of a clear interfacial boundary that is vertical to the wall of the vessel.

2.5. Determination of concentration and composition of surfactants

Two methods were used to determine the concentration and composition of mixed surfactants in

the two phases. One was to measure the amount of nitrogen and sulfur in two phases, by which the concentrations of $C_{12}NE$ and SDS could be calculated. Mikro Kemi Laboratory (Uppsala, Sweden) carried out this measurement.

The second method was carried out by measurement of surface tension. The surface tensions of the two phases were measured at different dilutions. Surface tension values were plotted versus dilution factors. The cross point in the curve is the CMC (critical micelle concentration). It has been shown that CMCs of $C_{12}NE/SDS$, when expressed in terms of total concentrations of cationic–anionic surfactants, are nearly the same at a large molar ratio range, for example, the CMC of $C_{12}NE/SDS$ at molar ratios of 1:1, 1:5 and 5:1 are 1.2×10^{-4}, 1.5×10^{-4} and 1.5×10^{-4} M, respectively [24]. Since ASTP systems form at molar ratio range between 1:2 and 2:1, it should be reasonable to take CMC of the mother solution as that of both top and bottom phases. In this way, the total concentrations of mixed surfactants in two phases could be obtained by the product of the dilution factor at the cross point of the surface tension curve and the CMC of the mother solution.

2.6. Protein measurements

The two phases were separated and then diluted. Surfactants were precipitated out when ASTP systems were diluted. The concentrations of BSA and lysozyme were determined by the absorbance at 280 nm, using phases without proteins as reference solutions. For mixtures of BSA and lysozyme, concentration of lysozyme was determined by its activity and concentration of BSA was determined by total absorbance at 280 nm after subtraction of lysozyme contribution to the absorbance at 280 nm. The activity of lysozyme was determined by the lysis rate of *Micrococcus lysodeikticus* cells [25].

2.7. Measurement of surface tension

Surface tension was measured by the drop volume method [26].

2.8. Calculation of partition coefficient and protein recovery

The partitioning of proteins is expressed by partition coefficient K, which is defined as $K=C_t/C_b$ where C_t and C_b are the equilibrium concentration of the partitioned protein in the top phase and the bottom phase, respectively. For ASTP-A systems, since the position of the two phases varies with temperature, K was expressed as the ratio of protein concentration in surfactant-rich phase to that in surfactant-depleted phase. Protein recovery was determined by calculating the total protein amount added to the system and the amounts found in the different phases.

3. Results

3.1. Phase behavior

3.1.1. Formation of ASTP systems

Fig. 1 shows the pseudo ternary phase diagram of $C_{12}NE/SDS$–water mixtures at 20°C. The phase behavior of aqueous mixtures of cationic–anionic surfactants is quite different from that of common aqueous solutions of surfactants and polymers [27,28]. In very low concentration the mixtures form a clear solution, denoted the first homogeneous region. However, the boundary of the first homogeneous region could not be plotted in Fig. 1, since the concentration of solutions in this region is extremely low. For example, for equimolar $C_{12}NE/SDS$, the boundary of the first homogeneous region is 0.0017% SDS, 0.002% $C_{12}NE$, and 99.996% water. Above this region mixtures of cationic surfactants–anionic surfactants–water form precipitate or become turbid at very low concentration, usually slightly above CMC. This phase has been called the heterogeneous region (denoted L+S in Fig. 1). However, with the further increase of either total concentration or in large access of one of the phase components, the mixture forms a homogeneous solution again.

However, mixtures of $C_{12}NE/SDS$–water separate into two phases in two separate regions of the phase diagram. Thus, an aqueous surfactant two-phase

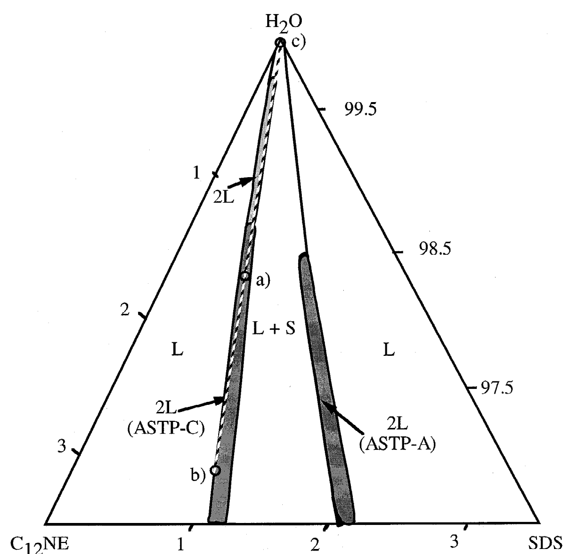

Fig. 1. Pseudo ternary phase diagram of $C_{12}NE$/SDS mixtures at 20°C. These mixtures separates into two phases in two different regions in the phase diagram, either in excess of SDS or $C_{12}NE$ (denoted ASTP-A and ASTP-C, respectively). The dark gray-marked two-phase region follows the definition of ASTP used in this paper (see Section 2.3). The light gray-marked two-phase region falls outside this definition. L, homogeneous solution; S, solid; 2L, aqueous two-phase systems (ASTP); (a (o)) ASTP-C system with a total concentration of 0.05 *M* and a molar ratio of 1.8:1; (b (o)) the surfactant-rich phase of the ASTP-C system shown in point (a); (c (o)), the surfactant-depleted phase of the ASTP-C system shown in point (a).

system (ASTP) is formed. These two regions of ASTP represent two different kinds of phase system formed when either cationic or anionic surfactants are in excess (denoted ASTP-C and ASTP-A, respectively). The ASTP systems are located on the boundary between the second homogeneous region (L) and the heterogeneous region (L+S) containing precipitate. The boundary of ASTP systems expressed by the solid line in Fig. 1 was obtained by the definition of ASTP given in this paper (Section 2.3). The dashed line is the compositions of the two phases of ASTP system by concentration measurements. Therefore, there exist at least five different regions in the dilute regime of the pseudo ternary phase diagram of $C_{12}NE$/SDS–water mixtures: two two-phase regions; two homogeneous regions at very low and relatively high concentrations, which are called the first and the second homogeneous regions, respectively, and a precipitate-containing heterogeneous region at intermediate concentrations.

3.1.2. Phase composition

Table 1 shows the total concentrations of mixed surfactants of each phase in different ASTP systems. It can be seen from Table 1 and Fig. 1 that the phase separation is associative, that is, one phase is rich, and another is depleted in surfactants. For ASTP-C, the top phase is surfactant-rich, and the bottom phase is surfactant-depleted. For ASTP-A, the position of two phases varies with temperature [2]. For simplicity, the two phases are denoted as surfactant-rich and surfactant-depleted phases, respectively.

With increasing total concentration in the ASTP-C system, at constant molar ratio, the concentration difference between the two phases increases. At the same total concentration, with increasing molar ratio, the concentration difference between the surfactant-rich phase and the surfactant-depleted phase decreases.

3.1.3. Phase separation time

In general, phase separation of ASTP systems is a fast process. Some systems can separate within 10 s without being centrifuged. It was shown that phase separation time varies with total concentration and molar ratio of surfactants. A typical set of data is shown in Fig. 2a–c and the following conclusions can be summarized:

Table 1
Concentration of surfactants in two phases of ASTP systems formed in mixtures between $C_{12}NE$ and SDS (20°C)[a]

ASTP ($C_{12}NE$/SDS)		C_{sr} (*M*)	C_{sd} (*M*)
C_{Total} (*M*)	Molar ratio $C_{12}NE$/SDS		
0.2	1.7:1	0.348	0.007
0.1	1.7:1	0.22	0.004
0.05	1.7:1	0.17	0.002
0.05	1.75:1	0.117	0.003
0.05	1.8:1	0.087	0.005
0.1	1:1.9	0.191	0.018

[a] C_{sr} and C_{sd} are equivalent to the total surfactant concentrations in the surfactant-rich and the surfactant-depleted phases, respectively.

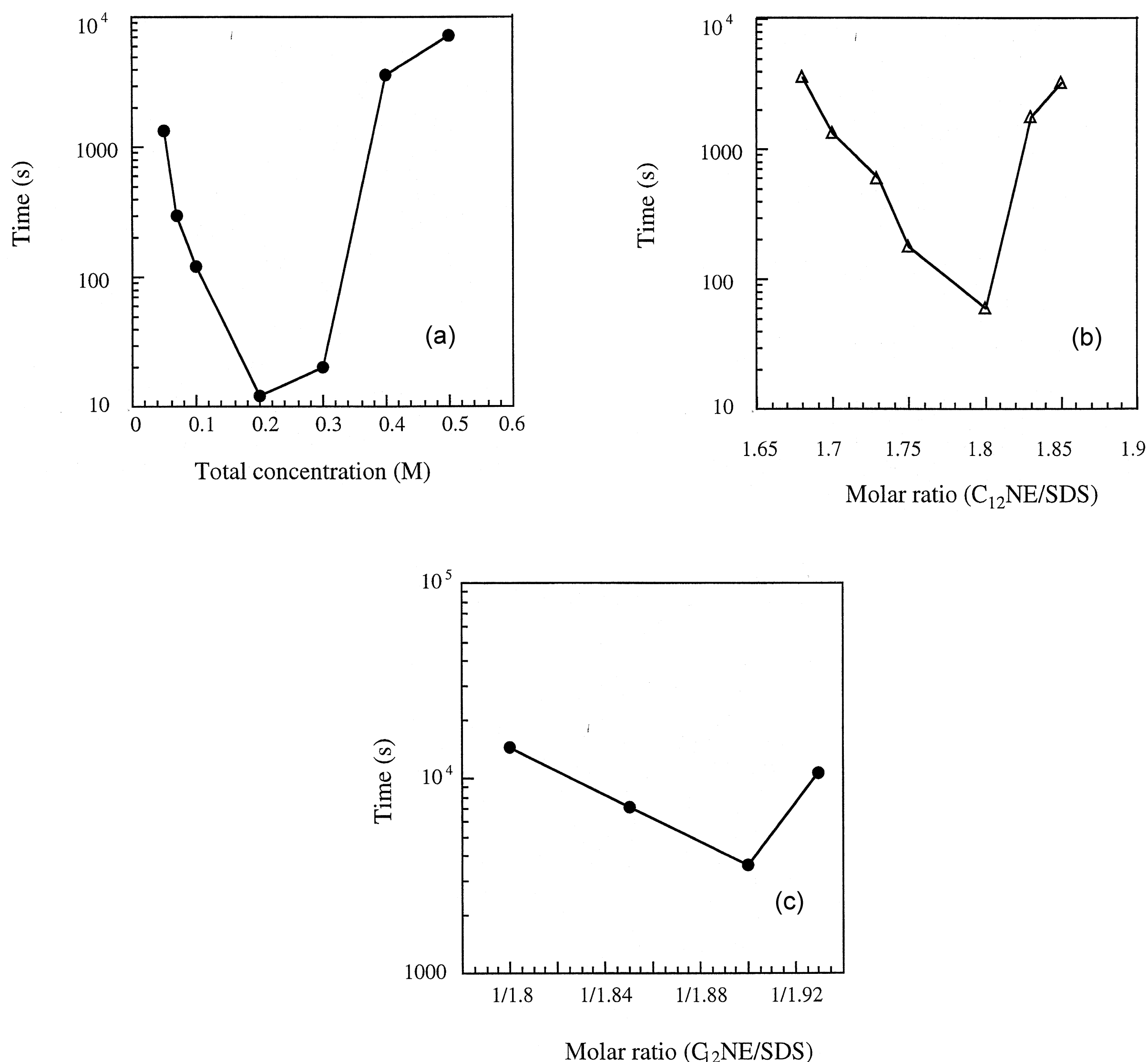

Fig. 2. Phase separation time of ASTP systems of C_{12}NE/SDS (20°C). (a) ASTP-C, phase separation time as function of total concentration of surfactants. Molar ratio (C_{12}NE/SDS), 1.7:1. (b) ASTP-C, phase separation time as function of molar ratio of surfactants. Total concentration of surfactants, 0.05 *M*. (c) ASTP-A, phase separation time as function of molar ratio of surfactants. Total concentration of surfactants, 0.05 *M*.

(1) At the same molar ratio, phase separation time decreases with increasing total concentration of surfactants. However, if concentration is very high, phase separation time increases with concentration. Thus, a minimum separation time of 10 s was achieved at 0.2 *M* for a system with molar ratio of 1.7:1, C_{12}NE/SDS (Fig. 2a).

(2) At the same total concentration of surfactant (0.05 *M*), phase separation time initially decreases with increasing molar ratio, passes through a minimum at 1.8:1 C_{12}NE/SDS, and then increases again (Fig. 2b).

(3) In general, phase separation of ASTP-C systems is faster than that of ASTP-A (Fig. 2a–c).

3.1.4. Phase volume ratio

Phase volume ratio varies with the molar ratio and total concentration of surfactants. It can be seen from Fig. 3a,b that: (i) at the same total concentration, phase volume ratio increases with increasing molar ratio; and (ii) at the same molar ratio, phase volume ratio increases with increasing total concentration.

Thus, ASTP systems are labile and adjustable, phase behavior is strongly influenced not only by total concentration but also molar ratio of mixed surfactants. Effect of molar ratio is strong, which enables one to get desired phase systems even at very low total surfactant concentration by tuning the molar ratio of surfactants.

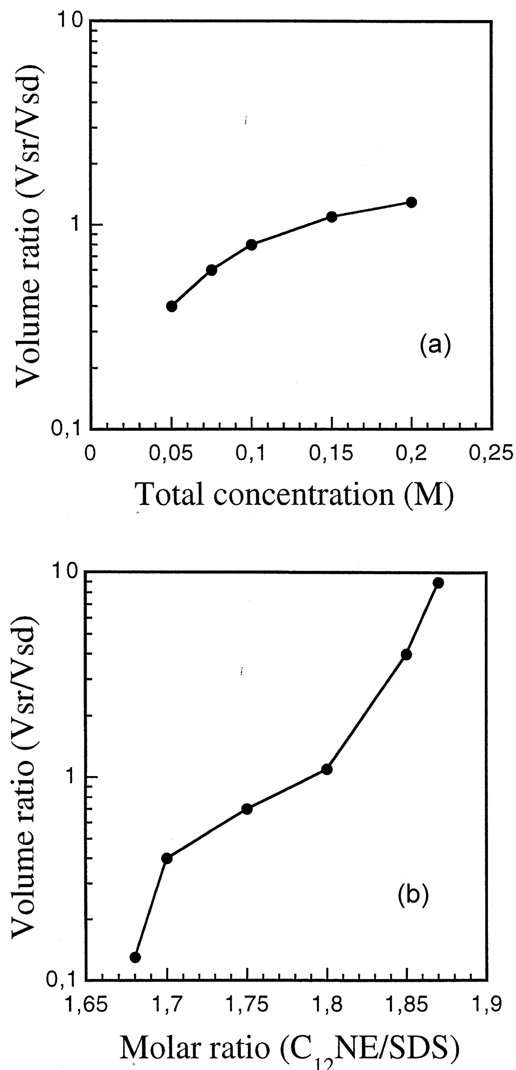

Fig. 3. Phase volume ratio of ASTP systems of $C_{12}NE/SDS$ (20°C). (a) Phase volume ratio as function of total surfactant concentration. Molar ratio ($C_{12}NE/SDS$), 1.7:1; (b) phase volume ratio as function of molar ratio of $C_{12}NE/SDS$. Total concentration of surfactants, 0.05 *M*. V_{sr} and V_{sd} are volumes of surfactant-rich phase and surfactant depleted phases, respectively.

3.2. Protein partitioning

Fig. 4 shows *K* values of BSA and lysozyme in ASTP-C and ASTP-A systems. The effect on partitioning of molar ratio of mixed surfactants in ASTP-C systems is shown in Fig. 5 and of the total concentration of mixed surfactants is shown in Fig. 6. All systems contained 10 m*M* sodium phosphate buffer, pH 7.1, at this pH BSA and lysozyme have net charges of −18 [29] and +7 [30], respectively.

(1) In ASTP-C systems, negatively charged BSA was partitioned to the top surfactant-rich phase, while positively charged lysozyme was partitioned to the bottom phase. In ASTP-A systems lysozyme was partitioned to the surfactant-rich phase, while BSA was partitioned to the surfactant-depleted phase (Fig. 4). Thus, the partitioning behavior is the opposite in the two systems, a fact that can be related to

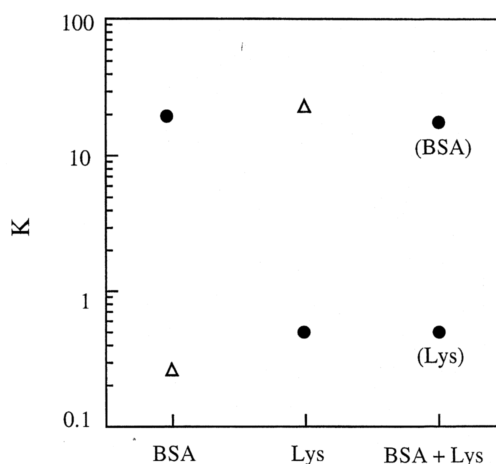

Fig. 4. Partition coefficient of proteins: (●) in ASTP-C, $C_{12}NE/SDS$, 0.05 *M*, 1.7:1; (△) in ASTP-A, $C_{12}NE/SDS$, 0.075 *M*, 1:1.95. In 10 m*M* sodium phosphate buffer, pH 7.1, at 20°C. Protein concentrations were: BSA, 1 mg/ml, and lysozyme, 0.5 mg/ml.

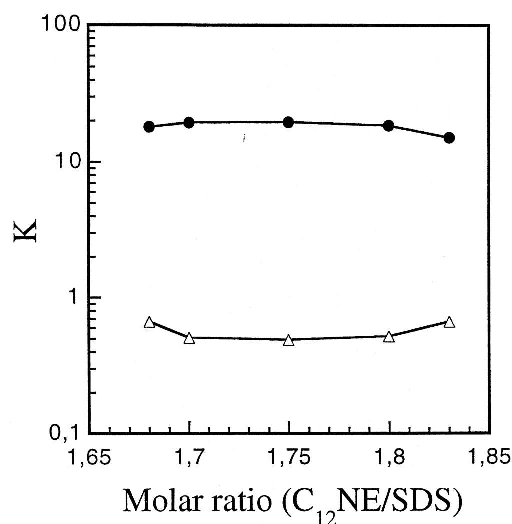

Fig. 5. Effect of molar ratio of surfactants in ASTP-C system on partition coefficient of (●) BSA and (△) lysozyme. Total concentration of surfactants, 0.05 *M*. In 10 m*M* sodium phosphate buffer, pH 7.1, at 20°C. Protein concentrations were: BSA, 1 mg/ml, and lysozyme, 0.5 mg/ml.

electrostatic effects between charged mixed micelles of the surfactant phase and the net charge of protein.

(2) At the same total surfactant concentration in ASTP-C systems, with increase of molar ratio (C_{12}NE/SDS) of surfactants, protein partitioning at first becomes more extreme, then passes through a maximum, after which it is reduced (Fig. 5a,b).

(3) With increase of total concentration of surfactants in the ASTP-C system, for BSA, a small increase in the partitioning was observed. For lysozyme, no change was observed (Fig. 6a,b).

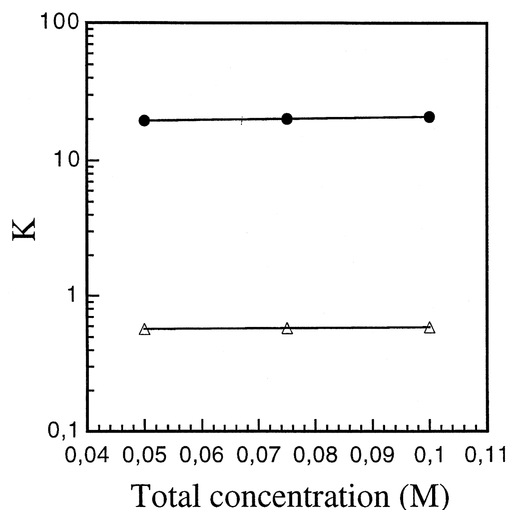

Fig. 6. Effect of total concentration of surfactants in ASTP-C system on partition coefficient of (●) BSA and (△) lysozyme. In 10 m*M* sodium phosphate buffer, pH 7.1 at 20°C. Protein concentrations were: BSA 1 mg/ml and lysozyme 0.5 mg/ml. Molar ratio (C_{12}NE/SDS): 1.7:1.

Table 2
Total yield of lysozyme activity after partitioning in the ASTP-C system consisting of a mixture of C_{12}NE and SDS[a]

Time (h)	Yield (%)
4	98.4
24	96.8

[a] Total surfactant concentration, 0.075 *M*; and molar ratio, C_{12}NE/SDS, 1.7:1. In 10 m*M* sodium phosphate buffer, pH 7.1, at 20°C. Lysozyme concentration was 0.5 mg/ml.

3.3. Enzyme activity

Table 2 shows the data of activity of lysozyme in the ASTP-C system. It was found that the activity of lysozyme was retained even after 24 h in the ASTP system.

4. Discussion

4.1. Phase behavior

Mixtures of cationic–anionic surfactants form a homogeneous solution at very low and relatively high concentrations and a heterogeneous solution at intermediate concentration. Such phase behavior has been explained in terms of variation in composition of micelles with concentration [31,32]. It was shown that for non-equimolar mixtures, at very low concentration, nearly equimolar mixed micelles are formed because of strong electrostatic attraction between opposite charged polar groups, even though the mother solution is non-equimolar [32]. The equimolar mixed micelles are uncharged, grow to a large size and precipitate out. With increase of concentration, the composition of micelles in non-equimolar mixtures will deviate from 1:1 and approach that in bulk solution gradually [31,32]. As a result, the precipitate will be solubilized and mixed

micelles will gradually increase their surface net charge. Thus, on the boundary between the intermediate concentration region and the second homogeneous region, the micelles have very large sizes [31,32]. The increase of charge density on the micellar surface will, after a time, decrease the size of the micelle structure because of the electrostatic repulsion between the polar groups of similar charge. As a result, the second homogeneous region appears in the phase diagram. Herrington et al. [33] noticed formation of very large aggregates of mixed micelles in DTAB–SDS–water mixtures compared to single SDS micelles. They explained this formation by a partial micellar surface neutralization, which will reduce head group interaction and lead to formation of larger micelles. They also found cryo-TEM micrograph evidence for formation of multilamellar vesicles (MLVs) in mixtures of DTAB–SDS in a corresponding region of the phase diagram where C_{12}NE/SDS starts to phase separate (ASTP-A region). A slow two-phase separation with time was noticed at a mixing ratio of 35:65 DTAB to SDS.

Based on the above, we can explain the formation of the ASTP system. The precipitate of equimolar mixed cationic–anionic surfactants formed at low total surfactant concentration will be solubilized with increasing molar ratios. At the phase boundary of the two-phase area, all precipitate is solubilized and the large micelles can associate and separate from the bulk solution and form a surfactant-rich phase. With further increase of molar ratio the micelle surface net charge increases, and thus breaks down the associated surfactant micelles by head-to-head repulsive interactions, which leads to the formation of a homogeneous mixed micelle solution. The two-phase area will thus disappear.

One interesting property in relation to the phase behavior of ASTP systems is that a dilution of the surfactant-rich phase induces a formation of a new two-phase system. When this system is further diluted, mixed surfactants will be precipitated out. This behavior can be utilized for a multi-step partitioning procedure simply by addition of solvent. After the partitioning of proteins, phase components can be removed from the protein solution by precipitation and recycled [21].

As mentioned above, the charge of the micellar surface increases with increasing molar ratio and concentration of mixed surfactants. Based on this, we might explain the variation of phase volume ratio with total concentration and molar ratio of surfactants. The volume of the surfactant-rich phase should be decided mainly by the amount of water bound to the surfactant micelles, which in turn should increase with the micelle net charge. Therefore, the phase volume ratio increases with the increase of total surfactant concentration and molar ratio.

The time of phase separation in a normal polymer–polymer two-phase system [7] depends on several factors, such as the density difference between the two phases, the phase viscosity, the time for small droplets to coalesce into larger droplets, as well as phase volume ratio. If the phases have different viscosity, this will result in a longer settling time if the largest phase has the highest viscosity. This is the case for cationic–anionic surfactant mixtures, where the more viscous surfactant-rich phase forms the larger phase with increasing total concentration and molar ratio. Presumably, this explains the increase in settling time at high total surfactant concentrations and molar ratio. In the lower concentration range the separation time decreases with the same parameters. This is probably due to a larger surfactant concentration difference between the phases with increasing total concentration and molar ratio. Thus, the settling time of ASTP systems is due to a fine balance between increased viscosity and density difference between phases, which is counteracted by the effect from increased volume of the surfactant phase. This is the reason for the minimum point of phase separation time with concentration and molar ratio seen in Fig. 2a–c.

4.2. Partitioning of proteins

For the mixtures of cationic–anionic surfactants, almost all surfactants exist in micellar form because of their very low CMC. It is known [2] that micelles in ASTP systems are charged. Electrostatic interactions between micelles and proteins play an important role in the partitioning behavior of proteins. It should be pointed out that there is a very large concentration difference of micelles between the two phases, which might be another important factor that affects protein partitioning due to excluded volume

effects. This has been reported to be the major driving force for the partitioning of water-soluble macromolecules, proteins and viruses, in cloud point extraction in non-ionic surfactant systems [34], and also been noted to have importance in detergent/polymer ATPS [19].

Based on the above, we can explain the partitioning behavior of BSA and lysozyme in ASTP systems. In ASTP-C systems, the micelles are positively charged, and the top phase has much higher concentration of micelles than the bottom phase. So BSA, which has a negative net charge at pH 7.1, is partitioned to the surfactant-rich phase while positively charged lysozyme is partitioned to the surfactant-depleted phase. For ASTP-A systems, the situation is the opposite, BSA is partitioned to the surfactant-depleted phase while lysozyme is partitioned to the surfactant-rich phase, because the micelles are negatively charged. The partitioning is clearly related to electrostatic interactions between charged micelles and proteins. This effect on partitioning from charged micelles has also been observed in nonionic detergent–polymer systems with addition of either SDS or DTAC [35].The effect on partitioning of water-soluble proteins of total surfactant concentration and molar ratio is relatively small (see Figs. 5 and 6) compared to the electrostatic effects. The small effects can be explained in the following way. At the same total concentration, the charge density on the micellar surface increases with increasing molar ratio, which should make the partitioning more extreme. However, with increased molar ratio the concentration difference of surfactants in two phases decreases, which should make the partitioning more even. In addition, increased surfactant molar ratio also decreases the micelle size, as mentioned above, due to repulsive interactions. Smaller size of one phase component generally favors partitioning into that phase, due to reduced excluded volume effect [23]. This favors partitioning into the micelle phase with increased molar ratio in ASTP. Thus, partitioning of water-soluble proteins with increased molar ratio seems to be a balance between two opposite effects. At low molar ratios, the increase of charge density on the micellar surface is dominant, which makes partitioning more extreme, while at high molar ratios, the decrease of concentration difference is dominant, thus the partitioning becomes more even.

4.3. Activity of enzyme during partitioning

Usually ionic surfactants are strong denaturing agents of proteins [36–38]. However, in ASTP systems, lysozyme can maintain the activity. This is probably due to the very low CMC of mixed cationic–anionic surfactants, which are listed in Table 3.

It has been shown that for single surfactants, the denaturing of proteins occurs at concentrations around their CMC. For example, the co-operative binding of SDS denatures lysozyme at SDS concentration of 5×10^{-3} *M*, and the binding of SDS to BSA occurs at an SDS concentration about 1×10^{-4} *M* [36–38]. From Table 3 it can be seen that for cationic–anionic surfactant mixtures, the concentrations of surfactant monomers are much lower than those needed for denaturing proteins. Almost all surfactants are in micellar state. It is a generally accepted notion that surfactant binding onto proteins, and indeed onto other substrates, involves only surfactant monomers, with micelles essentially acting as a reservoir of monomers. Therefore, for ASTP systems, micelle formation in the bulk, which actually competes with the binding process, is a more favorable process than interaction with proteins. Thus, in the case of cationic–anionic surfactants, binding of surfactant monomer to protein is limited by the micelle, which explains the retained activity of lysozyme.

In order to prove this, the interactions between proteins (BSA and lysozyme) and mixed cationic–anionic surfactants were investigated by surface tension measurements. We could not detect any shifts in the surface tension when 0.1wt% proteins were added to different concentrations of surfactant mix-

Table 3
CMC of SDS, $C_{12}NE$ and catanionic mixtures at different molar ratios at 20°C

Surfactant	CMC_{Total} (*M*)	$CMC_{C_{12}NE}$ (*M*)	CMC_{SDS} (*M*)
$C_{12}NE$		1.3×10^{-2}	
SDS			8.1×10^{-3}
$C_{12}NE$/SDS 1:1	1.2×10^{-4}	6×10^{-5}	6×10^{-5}
$C_{12}NE$/SDS 1:2	1.3×10^{-4}	4.3×10^{-5}	8.7×10^{-5}
$C_{12}NE$/SDS 2:1	1.3×10^{-4}	8.7×10^{-5}	4.3×10^{-5}

tures, which shows that there is no surfactant-binding to proteins (the details of this work is to be published).

5. Conclusion

ASTP systems may provide a new, useful partitioning system for separation and purification of biomaterials. ASTP systems are labile and adjustable. Phase behavior, volume ratio and settling time, is strongly influenced by total concentration and molar ratio of mixed surfactants. Since phase behavior of ASTP systems is effected by molar ratio of surfactants, desired phase systems can be obtained by tuning molar ratio even at very low total concentration of surfactants.

The marked difference of surfactant concentration between the two phases makes proteins distribute between the phases. The charges on the micellar surface, which can be adjusted by tuning the molar ratio of cationic to anionic surfactants, enhance the selectivity of protein partitioning. The partitioning behavior of proteins can be controlled and optimized by tuning micellar characteristics, such as micellar size and electric net charge. In other words, ASTP systems offer a method for separation of proteins based on the net charges of proteins and charges of the mixed micelles. Proteins can maintain activity in ASTP systems because of the extremely low CMC of cationic–anionic surfactant mixtures that makes concentration of surfactant monomers much lower than that needed for denaturing of proteins. Large shift in phase behavior with small changes in system composition due to a small two-phase area might be a problem in biomolecule separation with ASTP systems, since a variation in composition of the protein mixture might change the phase behavior. However, such shifts in phase behavior had small effect on protein partitioning and the phase behavior can be adjusted by titration of the ASTP systems.

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