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# Langmuir aggregation of Evans blue on cetyltrimethylammonium bromide and on proteins and its application

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**Abstract**

The microphase adsorption-spectral correction (MPASC) technique is described and applied to the study of the interactions of Evans blue (EB) with cetyltrimethylammonium bromide (CTAB) and with four proteins: bovine serum albumin (BSA), myoglobin (Mb), hemoglobin (Hb) and ovalbumin (OVA). EB can be adsorbed on a cationic surfactant and on protein by electrostatic force and the aggregation obeys the Langmuir isotherm. Results have shown that the products are formed as follows: monomer aggregate EB·CTAB, micellar aggregate $(EB{\cdot}CTAB)_{78}$ and protein aggregates ($EB_{68}$·BSA), ($EB_{14}$·OVA), ($EB_{126}$·Mb) and ($EB_{58}$·Hb). The adsorption constant of the aggregates are calculated to be $K_{EB{\cdot}CTAB} = 2.95 \times 10^6$, $K_{EB68{\cdot}BSA} = 3.40 \times 10^4$, $K_{EB14{\cdot}OVA} = 5.20 \times 10^2$, $K_{EB126{\cdot}Mb} = 6.81 \times 10^2$ and $K_{EB58{\cdot}Hb} = 5.73 \times 10^2$, respectively. The aggregation of EB in proteins is sensitive in the presence of CTAB and selective in the presence of EDTA and it has been applied to the analysis of samples with satisfactory results. 

*Keywords:* Microphase adsorption-spectral correction technique; CTAB; Evans blue (EB); Microelectrostatic field; Langmuir aggregation; Protein

## 1. Introduction

A surfactant is often quite useful for the sensitive determination of trace amounts of components due to its solubilization, stabilization, sensitivity enhancement and so on. Some earlier models were proposed to explain the synergism mechanism, e.g. synergism perturbation [1], hydrogen bond formation [2], micelle catalysis [3], asymmetric microenvmolybdenumment [4] and others. The study of surfactants is always on-going [5–8]. A surfactant molecule (S) has a long chain and various aggregation forms in aqueous solution, e.g. spherical, worm-like, tubules and lamellae [9]. S exists as a monomer when S is less than its critical micelle concentration (CMC) (Fig. 1(1)) and the aggregation of S molecules forms a large electrostatic global micelle when S is above the CMC (Fig. 1(2)). The charged monomer and micelle can both adsorb a dye probe (L) with the opposite charge (Figs. 1(1) and 2). We also found that the adsorption of L obeys the Langmuir isotherm [10]. Because of this, the solubilization of L has been realized in a solution of S.

As organic dyes can serve as effective probes of the structures and functions of biological macromolecules and as study models of some biological processes [11,12], interest is increasing in studies of the interactions of organic dyes with biological macromolecules [13]. Understanding the reaction between a protein and a probe dye is also very helpful to study the interaction of biomacromolecule with a pollutant. However, the interaction of biological macromolecules with dyes has not been elucidated satisfactorily and earlier observations have not been

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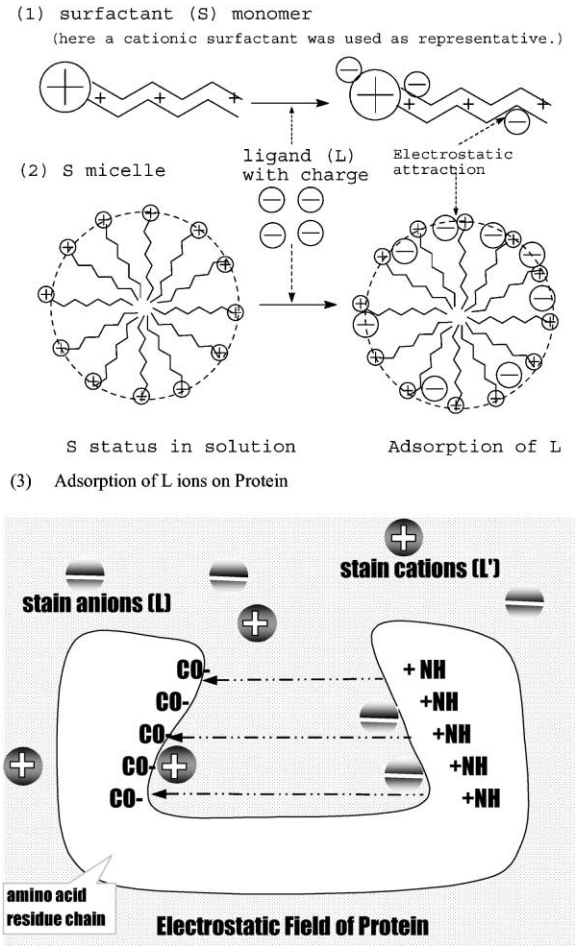



Fig. 1. The aggregation of L on S monomer (1) and micelle (2) and the formation of microelectrostatic field of protein and adsorption of stain ions (3).

explained clearly and reasonably, e.g. the Pesavento equation [14] and the Scatchard model [15]. In a protein molecule, the many similar protonated imino (NH) groups trend to be arrayed on the same side to form a weak positively charged electrostatic film, and the negative carbonyl (CO) dipolar bonds trend to array on the opposite side to form an negative electrostatic film. Protein contains complex spatial structures, e.g. winding, folding, coil and helix and these cause the double electrostatic films to cross to form many microelectrostatic fields. They can attract ions until kinetic equilibrium is achieved [16] (Fig. 1(3)). The microelectrostatic field is so narrow that dye molecules are adsorbed in only a monolayer.

Because the two adsorptions described above depend on the electrostatic force, the adsorption is easily destroyed by operational conditions, e.g. high ionic strength and high temperature. In L solutions, the following equilibrium occurs: L (aqueous phase, $C_L$) $\Leftrightarrow$ $SL_N$ (surfactant or protein phase, $C_S$). The Langmuir isotherm equation [17] is used:

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_L} \quad (1)$$

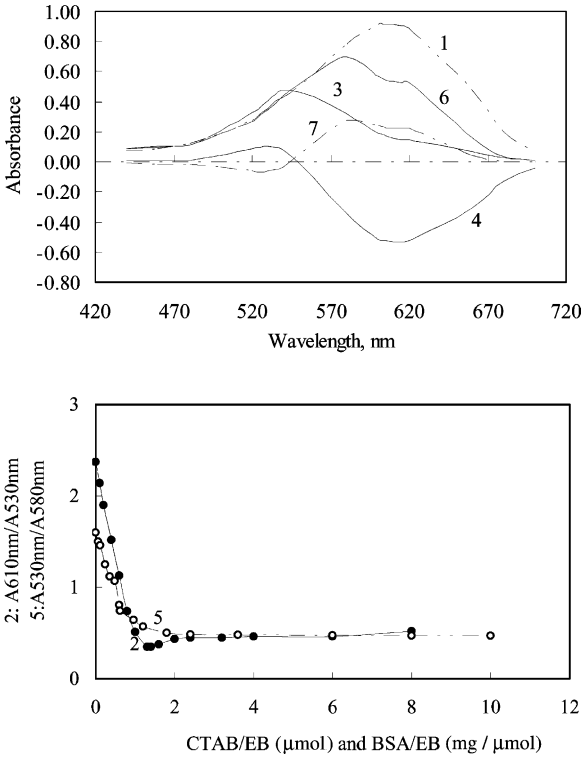



Fig. 2. Absorption spectra of EB, EB–CTAB and BSA–EB–CTAB solutions at pH 6.8: 1—EB (0.250 μmol), 2—variation of two absorbances ratio (A610 nm/A530 nm) of solution containing 0.250 μmol of EB with CTAB molarity, 3—EB (0.250 μmol)–CTAB (0.35 μmol) solution containing no free EB at equilibrium, 4—EB (0.250 μmol)–CTAB (0.20 μmol) solution, 5—variation of two absorbances ratio (A530 nm/A580 nm) of solution containing 0.250 μmol of EB and 0.35 μmol of CTAB with addition of BSA, 6—EB (0.250 μmol)–BSA (1.50 mg)–CTAB (0.35 μmol) solution containing no free EB–CTAB aggregate at equilibrium, 7—EB (0.250 μmol)–BSA (0.30 mg)–CTAB (0.35 μmol) solution. 1, 2, 3, 5 and 6 against water, the others against a reagent blank.

where $K$ is the equilibrium constant, $C_L$ the concentration of the excess L and $\gamma$ is the mole ratio of the effective L adsorbed by S. With increase in L concentration, $\gamma$ will approach a maximum, called the adsorption ratio N. We calculated $N$ and $K$ by plotting $\gamma^{-1}$ versus $C_L^{-1}$. Both $C_L$ and $\gamma$ are calculated by the relations [18]:

$$\gamma = \eta \frac{C_{L0}}{C_S} \tag{2}$$

$$C_L = (1 - \eta) C_{L0} \tag{3}$$

where

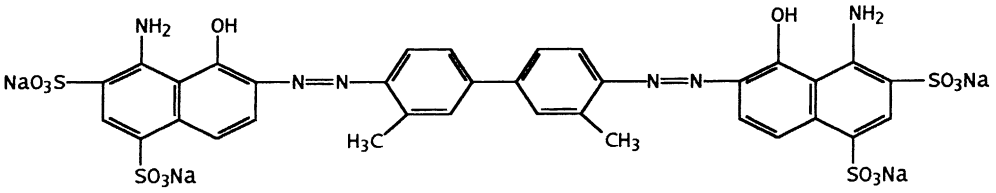
$$\eta = \frac{A_c - \Delta A}{A_0} \tag{4}$$

$C_S$ and $C_{L0}$ are the concentrations of S and L added initially and $\eta$ indicates the effective fraction of L. $A_c$, $A_0$ and $\Delta A$ are the real absorbance of the S–L product, the measured absorbance of the reagent blank against water and that of the S–L solution against reagent blank measured directly at the peak wavelength $\lambda_2$, respectively. $A_c$ is calculated by means of [19]:

$$A_c = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \tag{5}$$

where $\Delta A'$ indicates the absorbance of the S–L solution measured at the valley absorption wavelength $\lambda_1$. In general, $\alpha$ and $\beta$ are the correction constants and they are calculated by measuring directly $SL_N$ and L solutions [18,19].

The cooperation of both the Langmuir adsorption and microphase adsorption-spectral correction (MPASC) technique provides a very helpful experimental strategy for study of the aggregation of stains on a surfactant and on biomacromolecules. We have studied in detail the aggregation of Evans blue (EB) on CTAB and on four kinds of proteins. The structure of EB:




Evans Blue (EB)

It forms negative ions $EB^{4-}$ in neutral medium and it can be adsorbed on a cationic surfactant, e.g. CTAB and biomacromolecules, e.g. proteins. We found that the EB–protein aggregate gave the same peak wavelength as EB itself. So the determination of the formation constants of the EB–protein aggregate must be carried out in the presence of CTAB. CTAB was used to adsorb the excess EB in the EB–protein solution but it cannot substitute the adsorbed EB in the EB–protein aggregate. We have found that the aggregation of EB on CTAB monomer, on its micelle and on proteins obeys the Langmuir adsorption isotherm. The aggregates formed are as follows: $(EB{\cdot}CTAB)_{78}$, $(EB_{68}{\cdot}BSA)$, $(EB_{14}{\cdot}OVA)$, $(EB_{126}{\cdot}Mb)$ and $(EB_{58}{\cdot}Hb)$, respectively. The adsorption constant of the aggregates was calculated to be EB·CTAB: $2.95 \times 10^6$, $EB_{68}{\cdot}BSA$: $3.40 \times 10^4$, $EB_{14}{\cdot}OVA$: $5.20 \times 10^2$, $EB_{126}{\cdot}Mb$: $6.81 \times 10^2$ and $EB_{58}{\cdot}Hb$: $5.73 \times 10^2$ at pH 6.8, respectively. The aggregation of EB on CTAB and proteins has been applied to the quantitation of protein in samples, with satisfactory results.

## 2. Experimental

### 2.1. Materials

Absorption spectra were recorded on a TU1901 Spectrophotometer (PGeneral, Beijing) with a 1 cm cell and individual absorbances were measured on a Model 722 spectrophotometer (Shanghai 3rd Analytical Instruments) with 2 cm cells. A conductivity meter, Model DDS-11A (Tianjin Second Analytical Instruments) was used to measure conductivity together with a Model DJS-1 conductivity immersion electrode (electrode constant 0.98, Shanghai Tienkuang Devices) for production of deionized water between 0.5 and 1 $(\mu\Omega\,cm)^{-1}$. The pH of the

solutions was measured on a pHS-2C acidity meter (Leici Instruments, Shanghai) and Model 630D pH Pen (Shanghai Ren's Electronic). The temperature was adjusted and remained constant in an electrically heated thermostat bath, Model 116R (Changjiang Test Instruments of Tongjiang).

Stock standard solutions of proteins were prepared by dissolving the commercial bovine serum albumin (BSA) (Dongfeng Biological Technological, Shanghai), myoglobin (Mb) (Serva, Heidelberg, Germany), hemoglobin (Hb) (Dongfeng) and ovalbumin (OVA) (Shanghai Chemical Reagents of Chinese Medicine Group) in deionized water. The protein content ($w$, mg ml$^{-1}$) in the above solutions was determined and calculated by the relation: $w = 1.45A_{280\text{nm}} - 0.74A_{260\text{nm}}$ [20] by measuring their absorbances ($A_{260\text{nm}}$ and $A_{280\text{nm}}$) at 260 and 280 nm by UV spectrophotometry. The standard stock solution of CTAB (10.00 mmol l$^{-1}$) was prepared by dissolving cetyltrimethylammonium bromide (CTAB) (Shanghai Chemical Reagents Center) in deionized water, and 0.100 and 1.00 mmol l$^{-1}$ CTAB were prepared daily by diluting the stock solution. EB solution (0.500 mmol l$^{-1}$) was prepared by dissolving 0.2830 g of EB (content 85%, Shanghai Xinzhong Chemicals) in 500 ml of deionized water. Britton–Robinson buffer solutions between pH 2.5 and 9.6 were prepared to control the acidity of the interaction solution. NaCl (2.0 mol l$^{-1}$) was used to adjust the ionic strength of the aqueous solutions. The masking reagent solution, 1% $Na_2$EDTA, was prepared for masking metals in the determination of proteins.

### 2.2. Methods

#### 2.2.1. Aggregation of EB on CTAB

Into a 10 ml calibrated flask were added an appropriate working solution of CTAB, 1 ml of buffer solution (pH 6.8) and 0.500 ml of 0.500 mmol l$^{-1}$ EB. The mixture was diluted to 10 ml with deionized water and mixed thoroughly. After 5 min, absorbances were measured at 530 and 610 nm in a 1 cm cell, against the blank treated in the same way without CTAB.

#### 2.2.2. Aggregation of EB on proteins, and determination of proteins

A 0.3 mg of standard BSA as representative was taken in a 10 ml volumetric flask. The 1 ml of pH 6.8 buffer solution and 0.50 ml of 0.500 mmol l$^{-1}$ EB were added, and the solution diluted to 5.0 ml and mixed. After 5 min, 0.35 ml of 1.00 mmol l$^{-1}$ CTAB was added and diluted to 10 ml. The solution was mixed well. After 10 min, the absorbances at 530 and 580 nm were measured in a 2 cm cell against the blank treated in the same way without any protein. Three samples were prepared: sample 1—0.1% children's milk powder solution, sample 2—5% children's drink solution and sample 3—drinking water. The 0.50 ml of sample 1, 0.50 ml of sample 2 and 1 ml of sample 3 were placed in 10 ml volumetric tubes in place of standard BSA. To them, the following possibly interfereneing ions: were added 0.5 mg of Ca(II), $Cl^-$, glucose on $PO_4^{3-}$, 0.1 mg of Fe(II), Mn(II), Zn(II) on Pb(II) and 0.02 mg of Hg(II) and some drops of BSA solution. Next, 0.5 ml of 1% EDTA solution was added, and the next operations were the same as above.

## 3. Results and discussion

### 3.1. Spectral analysis

The absorption spectra of the EB, CTAB–EB and BSA–EB–CTAB solutions are shown in Fig. 2, where BSA is used as a typical protein. From curve 1, the spectral peak of EB is located at 610 nm. Curve 2 gives the change of the two absorbances ratio of solutions of various CTAB concentrations, respectively measured at 610 and 530 nm. We observe that the ratio reaches a minimum and remains almost constant when the CTAB concentration is >1.2 times that of EB. No free EB exists in the solution containing 0.25 μmol of EB and 0.35 μmol of CTAB and curve 3 shows its absorption spectrum. The spectral peak of the EB–CTAB aggregate is located at 540 nm. The spectral violet shift of the aggregate is 70 nm. However, from the relative spectrum 4, we observe that its peak and valley are located at 530 and 610 nm, so the two wavelengths were used in a study of the aggregation of EB on CTAB. The EB–BSA reaction forms a blue aggregate similar in color to EB at pH 6.8. The direct interaction of EB with proteins is unsuitable for study by spectrophotometry because of the too small difference of EB color and its BSA aggregate color. However, CTAB can bind completely the free EB present in the EB–BSA solution but also it will not substitute

the adsorbed EB in the EB–BSA aggregate. This can help us to reach a wide spectral shift of the aggregate compared to the reactant solution. Thus we can determine accurately and sensitively the aggregation of EB on proteins by spectrophotometry. From curve 5, the absorbance ratios approach a minimum and remain almost constant when BSA is >1.5 mg in a solution containing 0.50 ml of 0.500 mmol $l^{-1}$ EB and 0.35 ml of 1.00 mmol $l^{-1}$ CTAB. So no free EB–CTAB aggregate exists in such a solution and curve 6 gives its absorption spectrum. Its peak is located at 580 nm. The spectral red shift is 50 nm by comparing curves 6 and 3. Curve 7 shows the relative absorption spectrum of a BSA–EB–CTAB solution against that without BSA. Its peak and valley are located at 580 and 530 nm so these two wavelengths were used in the study of the interaction of EB with proteins. From curves 1, 3 and 6, the correction coefficients were calculated to be $\beta_{EB} = 0.391$, $\alpha_{CTAB-EB} = 0.348$, $\beta_{EB-CTAB} = 0.630$ and $\alpha_{CTAB-EB-BSA} = 0.496$. The equations: $A_c = 1.16(\Delta A - 0.391\Delta A')$ and $A_c = 1.45(\Delta A - 0.630\Delta A')$ were used, respectively, in calculation of the real absorbance of the CTAB–EB aggregate and the EB-protein aggregate.

### 3.2. Effect of pH on aggregation of EB

The effect of pH on the adsorption ratio of EB to CTAB is shown in Fig. 3. We observe that the aggregation of EB on CTAB is almost constantly sensitive over a wide pH range between 2.5 and 9.6. This is attributed to the fact that $EB^{4-}$ was easy to form and was attracted closely by the CTAB monomer or micellar surface.

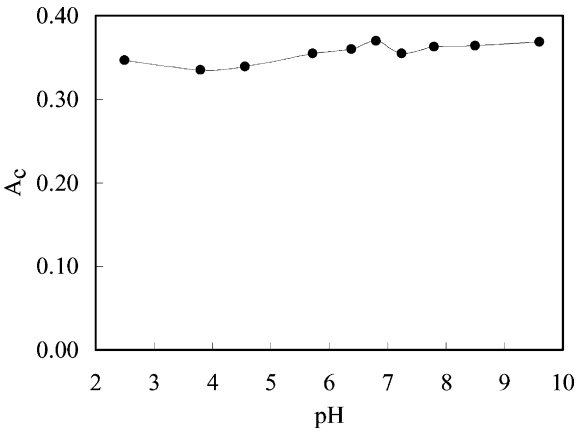



Fig. 3. Effect of pH on the adsorption ratio ($\gamma$) of EB to CTAB (solution initially containing 0.250 μmol of EB and 0.200 μmol of CTAB).

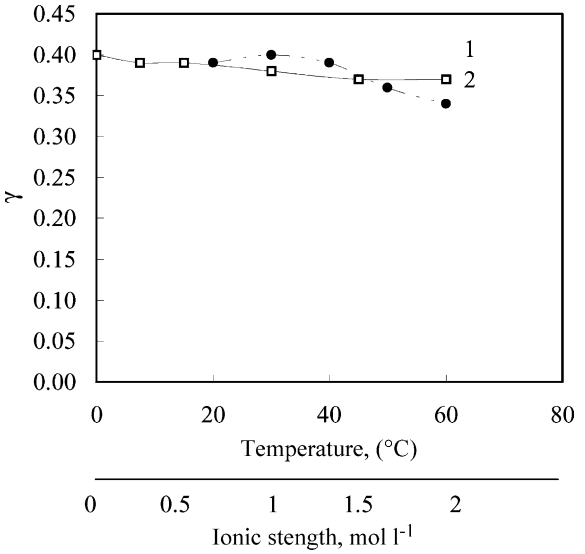



Fig. 4. Effect of ionic strength (1) and temperature (2) on the adsorption ratio ($\gamma$) of EB to CTAB (solution initially containing 0.250 μmol of EB and 0.200 μmol of CTAB).

### 3.3. Effect of ionic strength and temperature on aggregation of EB

In order to investigate the effect of ionic strength of the solution on the aggregation of EB, NaCl was added. From curve 1 in Fig. 4, the adsorption ratio of EB to CTAB remains almost constant between ionic strength 0 and 2 mol $l^{-1}$. This is attributed to the fact that the aggregation of $EB^{4-}$ on CTAB is much stronger and closer than $Cl^-$.

From curve 2, we observe the effect of temperature on the adsorption ratio of EB to CTAB. The $\gamma$ value decreases with increase in temperature especially above 30 °C. This is attributed to the higher temperature causing the desorption of EB from the CTAB surface. Experiments indicated that the adsorption of EB is complete on CTAB in 5 and in 10 min on BSA at 20 °C.

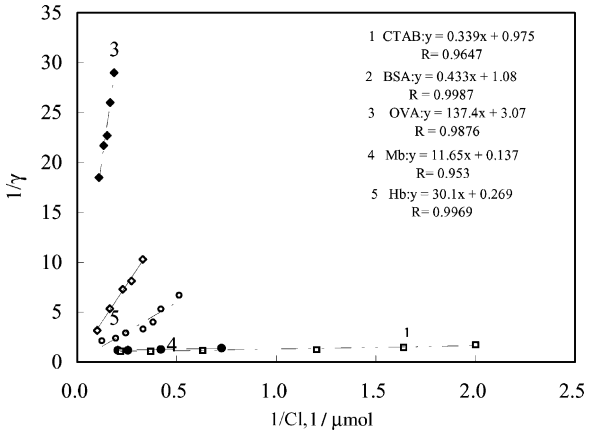



Fig. 5. Relationship between the adsorption ratio ($\gamma$) and free EB molarity ($C_L$, $\mu mol\,l^{-1}$): $\gamma^{-1}$ vs. $C_L^{-1}$. 1—CTAB, 2—BSA, 3—OVA, 4—Mb and 5—Hb.

### 3.4. Aggregation of EB on CTAB and on proteins

By varying the CTAB concentration of the solution initially containing 0.250 μmol of EB, the absorbances were measured and $\gamma$ of EB to CTAB and $C_L$ of EB calculated. Curve $\gamma^{-1}$ versus $C_L^{-1}$ is shown in Fig. 5. Similarly, $\gamma^{-1}$ versus $C_L^{-1}$ for the protein-EB–CTAB reaction is shown in Fig. 5, too. We observe that all plots are quite linear. Therefore, the aggregations of EB on CTAB and on proteins obey the Langmuir isotherm adsorption. The regression equations are expressed as: CTAB–EB, $\gamma^{-1} = 0.975 + 0.339C_L^{-1}$; BSA–EB, $\gamma^{-1} = 1.08 + 0.433C_L^{-1}$; OVA–EB, $\gamma^{-1} = 3.07 + 137.4C_L^{-1}$; Mb–EB, $\gamma^{-1} = 0.137 + 11.65C_L^{-1}$; Hb–EB, $\gamma^{-1} = 30.1 + 0.269C_L^{-1}$ where the $\gamma$ unit is EB–CTAB μmol/μmol and the others μmol/mg. From the intercepts, the adsorption ratio of each aggregate was calculated to be: EB:CTAB = 1:1, EB:BSA = 68:1, EB:OVA = 14:1:1, EB:Mb = 126:1 and EB:Hb = 58:1 at pH 6.8. From the slopes, the adsorption constants of the aggregates are calculated and the results are listed as $K_{EB\cdot CTAB} = 2.95 \times 10^6$, $K_{EB68\cdot BSA} = 3.40 \times 10^4$, $K_{EB14\cdot OVA} = 5.20 \times 10^2$, $K_{EB126\cdot Mb} = 6.81 \times 10^2$ and $K_{EB58\cdot Hb} = 5.73 \times 10^2$. By means of the conventional Scatchard model [15], the adsorption ratios of EB to CTAB and to various proteins were regressed and calculated to be: EB:CTAB = 1:1, EB:BSA = 64:1, EB:OVA = 16:1, EB:Mb = 121:1 and EB:Hb = 59:1 and the adsorption constants of their aggregates are $3.11 \times 10^6$, $2.99 \times 10^4$, $5.09 \times 10^2$, $6.62 \times 10^2$ and $5.51 \times 10^2$, respectively. Therefore, the recommended method is suitable to study of the aggregation of a stain probe on a surfactant monomer and micelle and proteins. If CTAB is present at more than its CMC of $0.97\,mmol\,l^{-1}$, the large aggregate $(EB\cdot CTAB)_{78}$ was formed and its precipitation was observed after a long time. In the determination of the formation constants of an aggregate, the spectral correction method is more suitable and simpler than the classical methods, such as the mole ratios method [21].

### 3.5. Effect of EB concentration on the determination of proteins

Fig. 6 shows the effect of the addition of $0.500\,mmol\,l^{-1}$ EB on the absorbance of the BSA–EB–CTAB solution and the effective fraction ($\eta$) of EB where BSA a typical proteins. Despite unstable change of the measured absorbances at 610 and

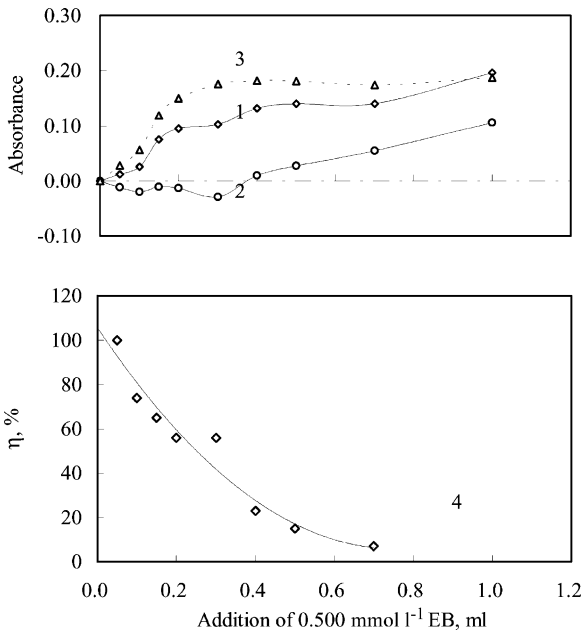



Fig. 6. Effect of addition of 0.500 mM EB on absorbance and $\eta$ of solutions initially containing 0.35 μmol of CTAB and 0.15 mg of BSA. 1—absorbance at 580 nm against a blank, 2—absorbance at 530 nm against a blank, 3—real absorbance at 580 nm, 4—variation of $\eta$.

530 nm from curves 1 and 2, the real absorbance of the aggregate approaches a maximum and remains almost constant from curve 3 when the addition of 0.500 mmol $l^{-1}$ EB is >0.20 ml. So, 0.50 ml of 0.500 mmol $l^{-1}$ EB was added for the determination of protein. From curve 4, the effective fraction of EB is only 18% when 0.50 ml of 0.500 mmol $l^{-1}$ EB is added. The excess of EB is >80% in the solution containing 0.300 mg of BSA. Excessive EB interferes the measurement of absorbance of the BSA–EB aggregate. Therefore, the spectral correction method was used instead of normal spectrophotometry in this study.

### 3.6. Calibration graph for determination of proteins

The adsorption of EB on proteins can be used for the determination of protein in the presence of CTAB. Standard series of various protein solutions were prepared and measured at pH 6.8 according to the procedure in Section 2.2. $A_c$ of the aggregate in each solution was calculated and their curves are shown in Fig. 7. We see that only plot 1 is linear and the others are logarithmic. Among them, BSA is most sensitive. Their regression equations are expressed as follows: $A_c^{BSA} = 2.12x - 0.017$, $A_c^{OVA} = 0.172\ln(x) + 0.225$, $A_c^{Mb} = 0.170\ln(x) + 0.571$ and $A_c^{Hb} = 0.168\ln(x) + 0.428$ ($x$ = mg protein). For $A_c = 0.010$, the detection limit of protein was 0.005 mg of BSA, 0.3 mg of OVA, 0.04 mg of Mb and 0.1 mg of Hb in a 10 ml volumetric tube. Six replicate determinations of 0.300 mg of BSA were carried out and the mean ± S.D. was 0.304 ± 0.006 mg.

### 3.7. Effect of foreign ions on determination of proteins

After adding 1% EDTA to the protein samples, the influence of foreign substances including ions, organic compounds and other surfactants, on the determination of BSA (as representative) was tested at pH 6.8. None of the following substances affected the determination of 0.300 mg of BSA (<10% error): 1 mg of $Cl^-$, $SO_4^{2-}$, $Ac^-$, 0.5 mg of Triton X-100, sodium dodecyl sulfate (SDS), glucose, humic acid, amino acids, Ca(II), Mg(II); 0.2 mg of DNA, RNA, $F^-$; 0.1 mg of Mn(II), Ni(II), Zn(II), Pb(II); 0.05 mg of Cu(II), Co(II), Cd(II), Fe(III) and Hg(II).

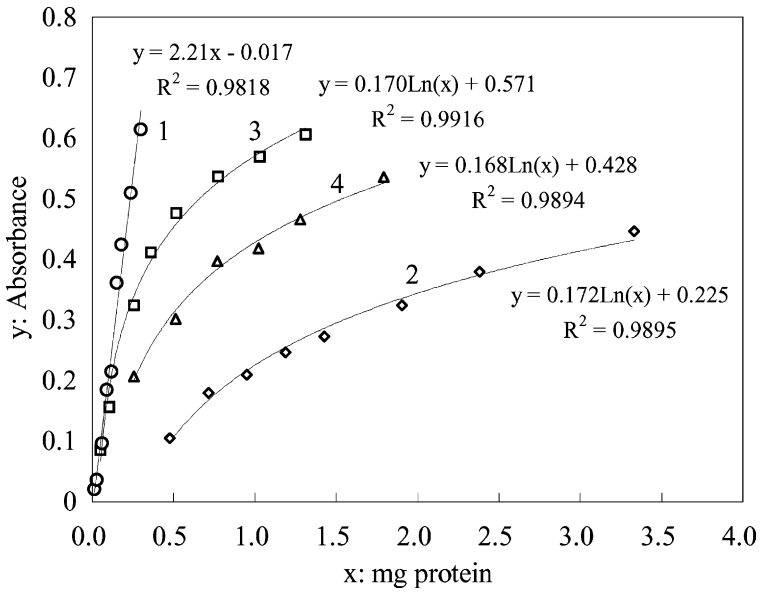



Fig. 7. Standard curves for the determination of protein at pH 6.8: 1—BSA, 2—OVA, 3—Mb and 4—Hb. All are real absorbances at 580 nm.

Table 1
Five replicate determinations of protein content in a 10 ml volumetric flask with EB as adsorbate at pH 6.8 in the presence of CTAB and EDTA

| Sample | BSA added ($\mu g\,ml^{-1}$) | Found ($\mu g\,ml^{-1}$) |
|---|---|---|
| 1 | 0.000 | 5.70 ± 0.2, R.S.D. = 3.5% |
| | 5.00 | 10.4 ± 0.3, recovery = 94.0% |
| 2 | 0.000 | 1.90 ± 0.08, R.S.D. = 4.2% |
| | 6.00 | 7.30 ± 0.5, recovery = 90.0% |
| 3 | 0.000 | 2.90 ± 0.1, R.S.D. = 3.4% |
| | 3.00 | 6.30 ± 0.4, recovery = 113% |

### *3.8. Determination of proteins in samples*

According to the procedure in Section 2.2, three samples were color-developed and measured. The analytical results are given in Table 1. The recovery of BSA is between 90.0 and 113% and the R.S.D. <4.2%.

## 4. Conclusions

The investigation of the interactions of EB with CTAB and with proteins supports the Langmuir monolayer aggregation of EB. Though the MPASC technique has not given higher sensitivity than other methods, such as RLS [22,23], it may meet precision and accuracy criteria and offers the additional benefits of simplicity and versatility. We also understand that classical spectrophotometry can still play an important role in studying the synergic mechanism of the surfactant and the interaction of dyes with macromolecules.

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