CSIRO Publishing

AUSTRALIAN JOURNAL OF CHEMISTRY

AN INTERNATIONAL JOURNAL FOR CHEMICAL SCIENCE

publishing research papers from all fields of chemical science, including synthesis, structure, new materials, macromolecules, supramolecular chemistry, biological chemistry, nanotechnology, surface chemistry, and analytical techniques.

Volume 55, 2002

All enquiries and manuscripts should be directed to:

Dr Alison Green
Australian Journal of Chemistry – an International Journal for Chemical Science
CSIRO PUBLISHING
PO Box 1139 (150 Oxford St)
Collingwood, Vic. 3066, Australia

Telephone: +61 3 9662 7630
Fax: +61 3 9662 7611
E-mail: publishing.ajc@csiro.au

Published by CSIRO PUBLISHING
for CSIRO and the Australian Academy of Science

www.publish.csiro.au/journals/ajc

Aust. J. Chem. **2002**, *55*, 767–772

Langmuir Aggregation of Eosin-Y in Protein and its Application

Hong-Wen Gao[A,B,C] *and Jian-Fu Zhao*[A,B]

[A] College of Chemical Engineering, Zhejiang University of Technology, Hangzhou 310014, P. R. China.
[B] Current address: State Key Laboratory of Pollution Control and Resource Reuse, Tongji University, Shanghai 200092, P.R. China.
[C] Author to whom correspondence should be addressed (e-mail: gaohongw@mail.hf.ah.cn).

The microsurface adsorption–spectral correction (MSASC) technique is described and applied to the investigation of the interaction of eosin-Y (EOY) with proteins at pH 3.8. The microelectrostatic fields in proteins cause the aggregation of EOY and it obeys Langmuir isothermal adsorption. Results have shown that the binding ratio of EOY to bovine serum albumin (BSA), ovalbumin (OVA), concanavalin A (ConA), and human γ-globulin (γ-G) are 77 : 1, 23 : 1, 4 : 1, and 83 : 1, the adsorption constants of the complexes were calculated to be 2.82×10^6, 1.70×10^5, 1.80×10^5, and 2.49×10^4 M^{-1}, and their molar absorptivities 3.20×10^6, 1.21×10^6, 1.35×10^5, and 5.27×10^6 L mol^{-1} cm^{-1} at 540 nm. The adsorption reaction has been applied to the quantitative detection of proteins in samples with satisfactory results.

Manuscript received: 21 June 2002.
Final version: 9 October 2002.

Introduction

Nowadays, many chemists and biologists are interested in researching supramolecular chemistry. Study of the interaction of spectral probe reagents with proteins is useful for understanding the structure and function of the protein. The quantitative analysis of proteins is also very important in clinical tests and laboratory practice. Classical spectrophotometry is often used, e.g. biuret,[1] Bradford,[2] Lowry,[3] and bromocresol green[4] methods. Recently, the resonance light scattering technique (RLS) has been proposed[5, 6] and applied successfully in the investigation of biomacromolecular complexes with small molecules.[7, 8] The interaction of porphines and porphyrins with macromolecules has been studied.[1–3, 9–13] However, the interaction of a probe reagent with a biomacromolecule has not been elucidated satisfactorily and some earlier observations, e.g. the Pesavento equation[14] and Scatchard model,[15] have not been explained. The present work was undertaken in an attempt to clarify the general principles involved in the protein–stain interaction. On the basis of the formation of microelectrostatic fields in a protein,[16,17] we have described and applied the MSASC technique to the study of the interaction of eosin-Y (EOY) with proteins such as bovine serum albumin (BSA), human γ-globulin (γ-G), ovalbumin (OVA), and concanavalin A (ConA). The structure of EOY is given in Diagram 1.

The reagent EOY was earlier used in the determination of Bi,[18] Pd,[19] and rare earth elements.[20] It forms a bivalent anion in aqueous solution and so can be adsorbed on the protein. The present work has confirmed that the aggregation of EOY on the four above-mentioned proteins obeys

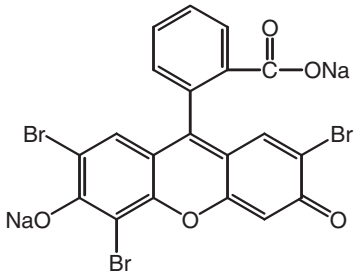

Diagram 1.

Langmuir isothermal adsorption. The maximal binding numbers of EOY in their BSA, OVA, ConA, and γ-G complexes are 77, 23, 4, and 83 at pH 3.8 and the adsorption constants K of the complexes are $K_{BSA–EOY}=2.82\times10^6$, $K_{OVA–EOY}=1.70\times10^5$, $K_{ConA–EOY}=1.80\times10^6$, and $K_{\gamma\text{-}G–EOY}=2.49\times10^4$ M^{-1}. The quantitative detection of proteins in two samples gave satisfactory results.

Theory

A biological macromolecule M contains a complex spatial structure. The winding, folds, coils, and other arrangements lead to many holes, gullies, and helical grooves. Many secondary bonds, such as van der Waals bonds, salt bonds, and hydrogen bonds are close to each other. The charge density becomes very high around the holes, grooves, and gullies. In a protein molecule, the protonization of the amino group in polar amino acids tends to form a positive electrostatic film and the carboxyl group to form a negative electrostatic film. The two films form an electrostatic field (Fig. 1). It can attract cations and anions such as spectral

 10.1071/CH01072 0004-9425/02/120767

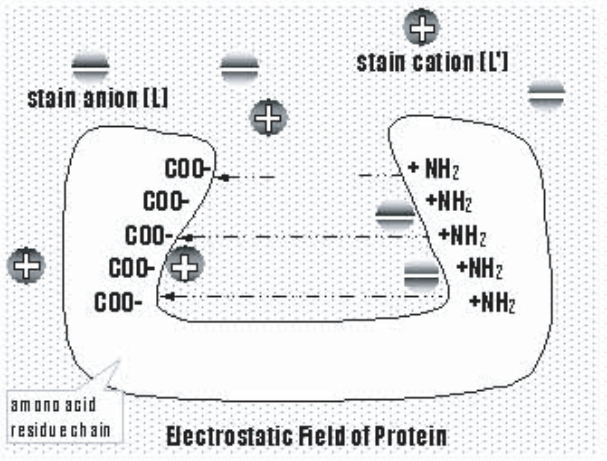

Fig. 1. The formation of microelectrostatic fields in a protein (M), and the adsorption of a stain (L or L′) on its microsurface.

probes or charged stains (L) to form a complex ML_N, where N indicates the maximal binding number of L per M. The existence of the microelectrostatic field is the basis of the binding of L on M. The microelectrostatic field is so narrow that L binds on M in only a monolayer. Therefore, the interaction of L with M obeys the Langmuir isothermal adsorption.[21] The adsorption equilibrium is expressed as:

L (aqueous phase, C_L) $\Leftrightarrow$ ML_N (macromolecular phase, C_M)

The Langmuir equation is expressed as Equation (*1*),[21] where the symbol K is the adsorption constant (units M^{-1}) and C_L indicates the molarity of the free L in solution.

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

The symbol γ is the molar ratio of the binding L to M. Values of C_L and γ are calculated using Equations (*2*)–(*4*).[22]

$$\gamma = \eta \frac{C_{L0}}{C_M} \qquad (2)$$

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

$$\eta = \frac{A_C - \Delta A}{A_0} \qquad (4)$$

C_M and C_{L0} are the initial molarity of M and L, respectively and η indicates the fraction of L binding on M. The symbol A_0 is the absorbance of the reagent blank (solution of L) against water, and ΔA is that of the M–L solution against the reagent blank (ΔA is the absorbance difference between M–L and L solutions), both measured at the peak wavelength λ_2 of the M–L solution against the L solution. With an increase in L, γ approaches the maximum N. Finally, A_c is the absorbance of the M–L complex in the M–L reaction solution which contains free L; it may be calculated by Equation (*5*).[23]

$$A_C = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \qquad (5)$$

The symbol $\Delta A'$ indicates the absorbance of the M–L solution against a reagent blank, measured at the valley wavelength λ_1 of the M–L solution against the L solution. Both α and β are correction coefficients and are calculated by directly measuring the ML_N and L solutions.[22] However, if the self-aggregation of a stain occurs to form a dimer or polymer,[24,25] β will change with L molarity (C_{L0}). In addition, the molar absorptivity ($\varepsilon_r^{\lambda_2}$ but not the apparent $\varepsilon_a^{\lambda_2}$) of the complex ML_N is directly calculated by means of Equation (*6*), where δ is the cell thickness (units cm).

$$\varepsilon_\gamma^{\lambda_2} = \frac{NA_C}{\delta\gamma C_M} \qquad (6)$$

We find that the Pesavento hypothesis[14] is identical with the Langmuir isotherm (Equation (*1*)). Consequently, we believe that the theoretical foundation of the Pesavento hypothesis comes from Langmur isothermal adsorption.

Results and Discussion

Spectral Analysis

The adsorption of EOY on proteins (BSA as representative) was carried out; the absorption spectra of the EOY and BSA–EOY solution at pH 3.8 are shown in Figure 2. Curves 1 and 2 show the absorption peak of EOY at 520 nm and the absorption peak of the BSA–EOY complex at 530 nm. The spectral red shift of the complex is only 10 nm. This is attributed to the fact that the electrostatic attraction between EOY and the protein is much weaker than a chemical bond

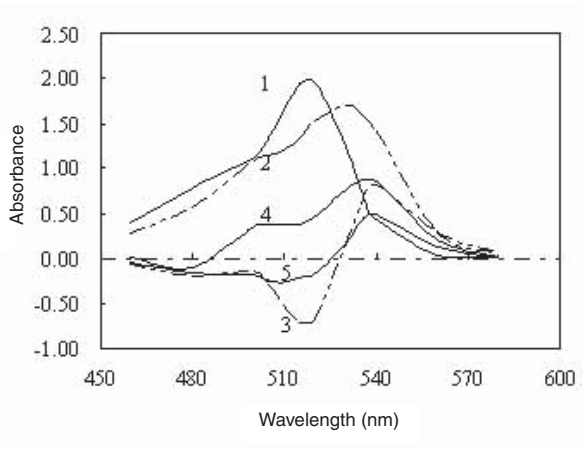

Fig. 2. Absorption spectra of EOY and its BSA solutions. Curve 1: EOY solution (1.00 μmol, pH 3.8); curve 2: EOY–BSA solution (1.0 μmol–3 mg, pH 3.8) no longer containing free EOY; curve 3: EOY–BSA solution (1.00 μmol–1.00 mg, pH 3.8); curve 4: as 3 but at pH 4.56; curve 5: as 3 but at pH 2.5. Both curves 1 and 2 were measured against water; the others against a reagent blank without any protein.

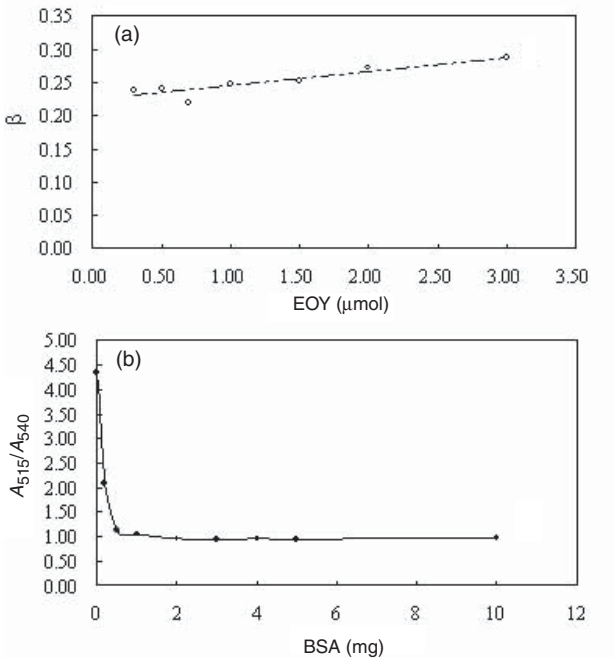

Fig. 3. (*a*) Variation of β for EOY. (*b*) Change of the absorbance ratio A_{515}/A_{540} of the EOY–BSA solutions (1 mmol EOY), both measured at 515 and 540 nm against water.

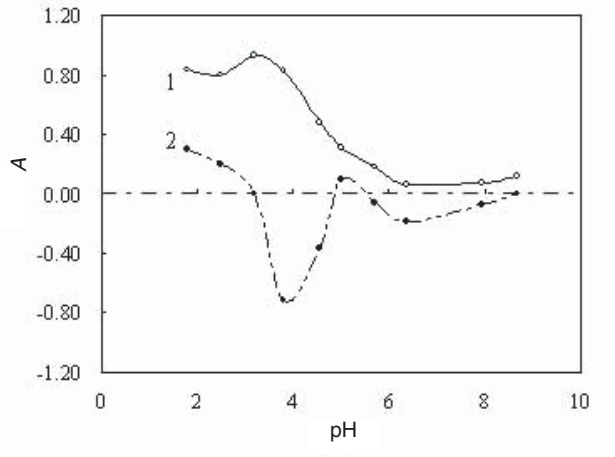

Fig. 4. Effect of pH on the absorbance of solutions containing 1.00 μmol of EOY and 1 mg of BSA at pH 3.8. Curve 1 was measured at 540 nm, curve 2 at 515 nm.

and only slightly affects the electron cloud distribution of EOY. Thus, the free EOY in the mixed solution will affect the measurement of the absorbance of the complex. In the difference spectrum, curve 3, the absorption peak is located at 540 nm and the valley at 515 nm; these are the two wavelengths used in this work. From curve 2, α is calculated to be 0.942. Figure 3(*a*), shows the variation of β with the molarity of EOY. We find that β increases slowly as the EOY concentration increases, especially when the addition of 1.00 mM EOY is greater than 1.2 mL. Therefore self-aggregation[24–25] of EOY occurs. From Figure 3(*b*), we observe that the absorbance ratio A_{515}/A_{540} decreases with an increase in BSA. The binding number of EOY remains almost constant when the addition of BSA is more than

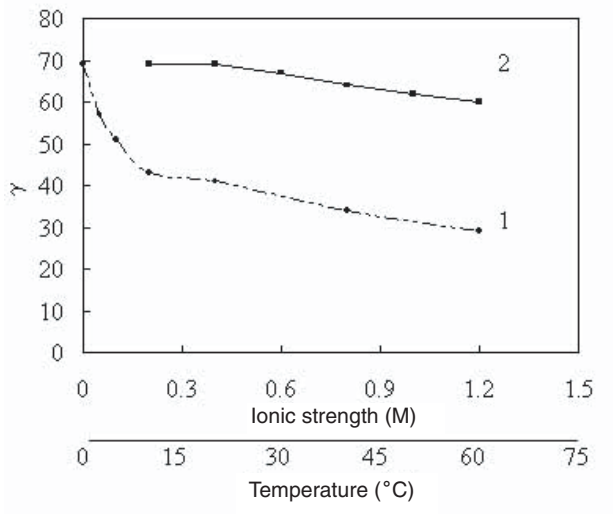

Fig. 5. Effect of ionic strength (curve 1) and temperature (curve 2) on the binding ratio of EOY (1.00 μmol) to BSA (1.00 mg).

1.0 mg. Thus, such solutions no longer contain free EOY and its absorption spectrum is that of the complex only as shown in Figure 2.

Effect of pH

The absorbance of BSA–EOY solutions was measured at varying pH of the solution, and is shown in Figure 4. From curve 1, the use of buffer at pH 1.8–3.8 gives highly positive absorbances; from curve 2, the use of buffer at pH 3.8–4.6 produces negative absorbances. This is attributed to the fact that the amino group of polar amino acids in the protein is protonated easily in acidic solution. In this work, the pH 3.8 buffer solution was selected.

Effect of Ionic Strength, Temperature, and Reaction Time

The influence of ionic strength of the solution on the binding ratio is shown in Figure 5. The binding ratio of EOY to BSA decreases slowly with an increase in ionic strength between 0 and 1.2 M. This is attributed to more chloride being attracted to the positively charged films to occupy the microsurface of protein and thus decrease of the binding number of EOY.

From curve 2, the binding ratio of EOY to BSA decreases slowly with an increase in temperature above 20°C. The electrostatic attraction is weaker than chemical bonding so the adsorption is easily destroyed at high temperatures. This is usual for surface adsorption. At 10°C, the reaction between BSA and EOY is complete in 10 min; the adsorption reaction is often rapid compared with chemical reactions.

Effect of EOY and Characterization of Complexes

The absorbance of various protein solutions was measured at different EOY concentrations and γ and C_L calculated for each solution. Figure 6 shows plots of γ^{-1} against C_L^{-1} for each of the proteins. The relationship is linear in all cases and hence the aggregation of EOY in proteins follows Langmuir

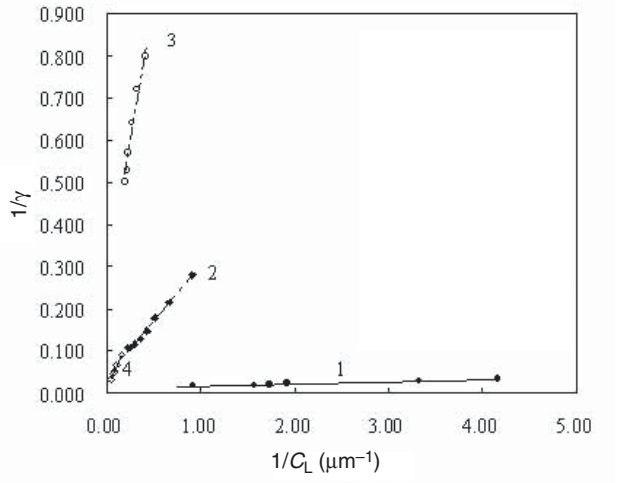

Fig. 6. Plots γ^{-1} versus C_L^{-1}. Curve 1: EOY–BSA solutions (0.06–0.18 μmol – 0.2 mg); curve 2: EOY–OVA solutions (0.06–0.20 μmol – 0.40 mg); curve 3: EOY–γ-G solutions (0.10–0.20 μmol – 0.80 mg); curve 4: EOY–ConA solutions (0.20–1.00 μmol – 0.80 mg).

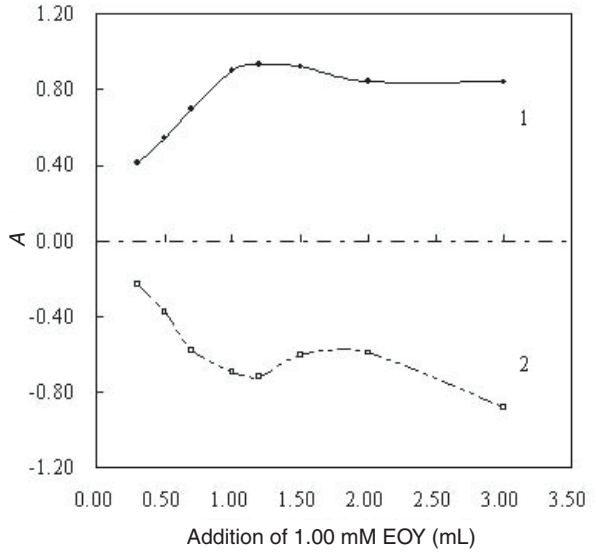

Fig. 7. Effect of addition of 1.00 mM EOY on absorbances of solutions containing 1.00 mg of BSA at pH 3.8. Curve 1 was measured at 540 nm, curve 2 at 515 nm, both against the reagent blank.

isothermal adsorption. The data was fit to linear regressions, from which the binding ratios *N*, adsorption constants *K*, and molar absorptivities ε of the protein–EOY complexes were calculated (see Table 1). Values of *N* decrease from γ-G,

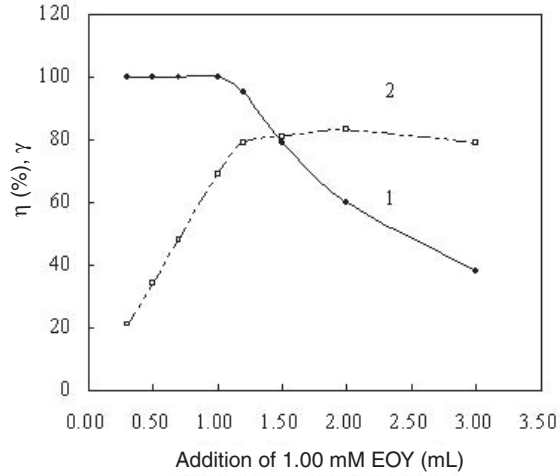

Fig. 8. Effect of EOY on η (curve 1) and γ (curve 2), solutions containing 1 mg of BSA.

BSA, OVA, to ConA. This order is same as the order of their molecular weights. Values of *K* for the complexes decrease from BSA, ConA, OVA, to γ-G. From Table 1, the greater the binding number *N* the higher the molar absorptivity of the complex.

Solutions containing 1.00 mg of BSA and various EOY concentration were prepared to select a suitable amount of 1.00 mM EOY to add when detecting the protein. The variation of absorbance with the amount of EOY is shown in Figure 7. The positive and negative absorbances both reach maxima at the addition of 1.2 mL of 1.00 mM EOY; therefore this was the quantity of EOY added in the analysis of protein samples. Both η of EOY and γ of EOY–BSA are shown in Figure 8. The value of γ approaches a constant value of 80 which is very close to the binding ratio of EOY to BSA shown in Table 1. From Figure 8, η reaches 95% and free EOY is only 5% in the solution containing 1.0 mg of BSA at the addition of 2.0 mL of 1.00 mM EOY. However, the free EOY reaches 90% in the solution containing only 0.1 mg of BSA. Consequently this large amount of free EOY will affect the measurement of the absorbance of the complex. Hence usual spectrophotometry methods are unsuitable for the stain–protein interaction system and the spectral correction technique[27–29] was used. Also this novel approach has some merits in the characterization of the stain–protein complex in contrast to the classical methods of the Scatchard model,[15] molar ratios,[30] continuous variations,[31] and equilibrium movements.[32]

Table 1. Calculation of the binding ratio, adsorption constant and molar absorptivity of the protein–EOY complexes at pH 3.8

Reaction	*N* Protein:EOY	*K* (M^{-1})	ε_r (M^{-1} cm^{-1}) at 540 nm
BSA (0.20 mg)–EOY (0.06–0.18 μmol)	1:77	2.82×10^6	3.20×10^6
OVA (0.40 mg)–EOY (0.06–0.20 μmol)	1:23	1.70×10^5	1.21×10^6
ConA (0.80 mg)–EOY (0.10–0.20 μmol)	1:4	1.80×10^5	1.35×10^5
g-G (0.80 mg)–EOY (0.20–1.00 μmol)	1:83	2.49×10^4	5.27×10^6

Table 2. The linear regression equations for the determination of proteins with EOY as reactant at pH 3.8 in the presence of EDTA

Determination of protein	Linear scope mg/25 mL	Regression equation at 540 nm (x = mg protein)	Correlation coefficient
BSA	0.0–1.0	$A_c = 1.536x+0.012$	0.9980
		$A = 0.886x+0.062$	0.9941
OVA	0.0–1.0	$A_c = 0.740x-0.001$	0.9988
		$A = 0.443x+0.050$	0.9819
ConA	0.0–1.0	$A_c = 0.440x+0.003$	0.9950
		$A = 0.238x+0.038$	0.9709
γ-G	0.0–1.0	$A_c = 0.368x+0.012$	0.9961
		$A = 0225x-0.001$	0.8882

Calibration Graph and Precision

The standard series of proteins were prepared and measured at pH 3.8 with 1.2 mL of the EOY solution added. Both ΔA and $\Delta A'$ of each solution were measured at 540 and 518 nm and their A_c were calculated using Equation (*5*). The regression equations are given in Table 2. They are all linear in the recommended protein range. The slopes of lines A_c versus x, the amount of protein (mg) in a 25 mL flask, are always higher than the slopes of the lines ΔA versus x; the linearity of the former lines is always better than that of the latter. Therefore, the spectral correction technique is more sensitive and more accurate than the usual spectrophotometry.

The detection limit of protein was calculated to be 0.02 mg of BSA using three times the standard deviation of 15 replicated determinations of the reagent blanks. Seven replicated determinations of 0.100 mg of BSA were made and the mean was 0.098±0.006 mg.

Effect of Foreign Ions

The effect of foreign substances such as cations, anions, sugars, and surfactants on the determination of proteins was tested by adding a masking reagent. None of the following ions affected the direct determination of 0.50 mg of BSA (less than 10% error): 1 mg of K^+, NH_4^+, or SO_4^{2-}; 0.5 mg of Cl^-, F^-, PO_4^{3-}, $C_2O_4^{2-}$, I^-, Ac^-, glucose, or amino acid; 0.2 mg of Ca(II), Mg(II), acetone, or ethanol; 0.05 mg of Al(III); 0.02 mg of Pb(II), Zn(II), Fe(III), Mn(II), Cu(II), Ni(II), Co(II), or Cd(II); and 0.01 mg of Hg(II).

Analysis of Samples

The quantitative determination of proteins in the two samples and the recovery is given in Table 3. An average of 0.688 mg of protein was contained in 0.0400 mL of a commercial children's drink. The content of protein is 1.72% in the drink and this agrees with the 'approximately 2% protein' marked on the container. By adding the standard BSA to the first sample, the recovery of BSA is 98.5% and the relative standard deviation (RSD) is 2.9%. From Table 3, the recovery of various proteins is between 93.0 and 108%. In addition, the experimental analysis of the samples was carried out using bromocresol green[4] as indicator by spectrophotometry. The recommended method gives a similar content of protein to the conventional method. The protein concentration in a sample from the novel method is only an average of various proteins equivalent to BSA or OVA or γ-G. Certainly, this method is accurate and specific if a sample contains only one kind of protein such as OVA in the drink.

Table 3. Determination of proteins in two kinds of samples with EOY as reactant at pH 3.8 in the presence of EDTA

Sample	Added	Found (mg)
Drink	0.040 mL of sample	0.688±0.020 (0.669[A]) RSD 2.9%
	0.200 mg of BSA in 0.040 mL of sample	0.885±0.018 rec. 98.5%
Synthetic sample with drinking water background	0.200 mg of BSA	0.186±0.007 (0.194 [A]) rec. 93.0%
	0.500 mg of OVA	0.529±0.022 (0.519 [A]) rec. 106%
	0.500 mg of γ-G	0.542±0.026 (0.533 [A]) rec. 108%
	0.500 mg of ConA	0.472±0.029 (0.475 [A]) rec. 94.4%

[A] Average of two determinations with bromocresol green[4] as indicator using spectrophotometry.

Conclusion

The investigation of the aggregation of EOY in proteins supports the monolayer aggregation of a stain on the biomacromolecule due to microelectrostatic attraction. The incorporation of both Langmuir isothermal adsorption and the spectral correction technique provides a useful experimental strategy for study of the chromophore or its metallic complex's adsorption in a surfactant solution. This method provides an experimental strategy for the study of the aggregation of small molecules on a macromolecule. Though it lacks the higher sensitivity of other methods such as RLS,[6] it meets the precision and accuracy criteria and offers the benefits of simplicity and versatility. The classical method remains important for the characterization of the interaction of a macromolecule with small molecules.

Experimental

Apparatus and Materials

Absorption spectra were recorded with a TU1901 spectrophotometer (PGeneral, Beijing) and an independent absorbance was measured on a Model 722 Spectrophotometer (Third Instruments, Shanghai). A DDS-11A conductivity meter (Second Analytical Instruments, Tianjin) was used to measure conductivity together with a DJS-1 conductivity immersion electrode (electrode constant 0.98; Tienkuang Devices, Shanghai) in the production of deionised water of less than 1 $\mu\Omega^{-1}$ cm^{-1}. The pH of the solution was measured with a pHS-2C acidity meter (Leici Instruments, Shanghai) and model 620D pH Pen (Ren's Electronics, Shanghai). The temperature was adjusted and remained constant in a Model 116R electronic heated thermostatic bath (Changjiang Test Instruments, Tongjiang).

Preparation of Solutions

Stock solutions of proteins were prepared by dissolving the commercial BSA (Beitai Biochem., Beijing), γ-G (Serva, Heidelberg), ConA or OVA (both Shanghai Chemical Reagents, Shanghai) in deionised water.

The protein content w (mg mL^{-1}) in the above solutions was determined by measuring the absorbances (A_{260}, A_{280}) at 260 and 280 nm and applying the relation $w = 1.45A_{280} - 74A_{260}$.[26] The 1.00 mM EOY solution was prepared by dissolving 0.8140 g of purified EOY (85%; Third Reagents, Shanghai) in 1000 mL of deionised water. Sodium chloride (2 M) was used to adjust the ionic strength of the aqueous solutions. Britton–Robinson buffer solutions (pH 1.8–8.7) were prepared. The masking reagent was prepared by mixing 100 mL of 5% Na_2EDTA, 100 mL of 2% ethylenediamine, and 100 mL of 5% potassium sodium tartrate; this solution was used to mask foreign metal ions in the samples.

Measurements

Aggregation of EOY with Proteins. The working solution of a protein, 2.5 mL of Britton–Robinson buffer solution, and the appropriate EOY solution were placed in a 25 mL calibrated flask. The mixture was then diluted with deionized water to 25 mL and mixed thoroughly. Absorbances were measured at 540 and 515 nm against the blank which was treated in the same way but without proteins.

Preparation of Samples and Determination of Proteins. Two types of samples were used. One was a children's drink. The other was a synthetic mixture of a protein solution and 0.3 mg of each Ca(II), ethanol, acetate, DNA, and glucose, 0.1 mg of each Mg(II) and F^-, 0.02 mg of each Cu(II), Al(III), Mn(II), Zn(II), and Pb(II); the background was drinking water. The first sample was diluted to 100 times by volume with deionised water for use. In the analysis of the samples, 1 mL of 5% Na_2EDTA was added to complex the metal ions. Aggregation of the samples with EOY followed the procedure given above.

Acknowledgments

Financial support from the Natural Science Foundation of Anhui Province (No.01045301) is acknowledged. The authors wish to thank colleagues Nai-Liang Hu and Jia-Xiang Yang for technical assistance.

References

[1] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 245.
[2] N. Li, S. Y. Tong, *Anal. Lett.* **1995**, *28*, 1763.
[3] N. Li, S. Y. Tong, *Talanta* **1994**, *41*, 1657.
[4] R. F. Pasternack, E. J. Gibbs, J. J. Villafranca, *Biochemistry* **1983**, *22*, 2406.
[5] H. F. Arlinghau, M. N. Kwoka, *Anal. Chem.* **1997**, *69*, 3747.
[6] R. F. Pasternack, P. J. Collings, *Science* **1995**, *269*, 935.
[7] C. Z. Huang, K. A. Li, S. Y. Tong, *Anal. Chem.* **1997**, *69*, 514.
[8] C. Z. Huang, K. A. Li, S. Y. Tong, *Bull. Chem. Soc. Jpn.* **1997**, *70*, 1843.
[9] R. F. Pasternack, C. Bustamante, P. J. Collings, *J. Am. Chem. Soc.* **1993**, *115*, 5393.
[10] R. F. Pasternack, E. J. Gibbs, J. J. Villafranca, *Biochemistry* **1983**, *22*, 5409.
[11] C. Z. Huang, Y. F. Li, J. G. Mao, *Analyst* **1998**, *123*, 1401.
[12] C. Z. Huang, Y. F. Li, H. Q. Luo, *Anal. Lett.* **1998**, *31*, 1149.
[13] C. Z. Huang, K. A. Li, S. Y. Tong, *Anal. Chem.* **1996**, *68*, 2259.
[14] M. Pesavento, A. Profumo, *Talanta* **1991**, *38*, 1099.
[15] G. Scatchard, I. H. Scheinerg, S. H. Armstrong, *J. Am. Chem. Soc.* **1950**, *72*, 535.
[16] H. W. Gao, J. Jiang, L. Q. Yu, *Analyst* **2001**, *126*, 528.
[17] H. W. Gao, J. X. Yang, J. Jiang, *Supramol. Chem.* **2001**, *14*, 315.
[18] D. L. Yang, H. W. Wang, X. M. Yu, *Chin. J. Anal. Chem.* **1986**, *14*, 725.
[19] C. E. Wang, Z. Y. Kou, X. L. Liu, *Chin. J. Anal. Lab.* **1999**, *18*, 71.
[20] P. X. Kuang, L. R. Xu, *J. Sci. Technol. Univ. Chin.* **1985**, *15*, 299.
[21] I. Langmuir, *J. Am. Chem. Soc.* **1918**, *40*, 1361.
[22] H. W. Gao, *Chem. Anal.* (*Warsaw*) **2001**, *46*, 249.
[23] H. W. Gao, S. Y. Zhang, S. M. Ye, *J. AOAC Int.* **2000**, *83*, 231.
[24] X. F. Zhang, J. S. Ma, P. J. Xia, *Sci. China*, *Ser. B* **1992**, *3*, 225.
[25] Y. Cao, X. W. He, *Chem. J. Chin. Univ.* **1998**, *19*, 714.
[26] J. B. Murphy, M. W. Kies, *Biochim. Biophys. Acta* **1960**, *45*, 382.
[27] H. W. Gao, J. X. Yang, *Colloids Surf., A* **2002**, *205*, 283.
[28] H. W. Gao, Q. S. Ye, W. G. Liu, *Anal. Sci.* **2002**, *18*, 455.
[29] H. W. Gao, N. L. Hu, *J. Solution Chem.* **2002**, *31*, 165.
[30] V. N. Tikhonov, *Zh. Anal. Khim.* **1975**, *30*, 1501.
[31] W. Likussar, *Anal. Chem.* **1973**, *45*, 1926.
[32] A. I. Laearev, *Zavod. Lab.* **1975**, *41*, 534.