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# Investigation of biomacromolecular assembly: replacement occurring on proteins

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**Abstract**

The non-chemical bond interaction between small molecule and macromolecule coming from the electrostatic attraction obeys the Langmuir assembly. The interaction of 1,5-di(2-hydroxyl-5-sulfophenyl-)-3-cyanoformazan (DSPCF) and three kinds of proteins: bovine serum albumin (BSA), α-globulins (Gb) and ovalbumin (OVA) at pH 1.83 has been investigated and then sodium dodecyl benzene sulfonate (SDBS) was added to replace the DSPCF binding in protein. The microsurface adsorption-spectral correction (MSASC) technique and the break point approach were both used to characterize the aggregates. Results showed that the products: $SDBS_{99}BSA$, $SDBS_{50}OVA$ and $SDBS_{25}Gb$ at 30 °C and $SDBS_{90}BSA$, $SDBS_{40}OVA$ and $SDBS_{20}Gb$ at 40 °C are formed.


## 1. Introduction

Understanding the chemical, physical, biological and pharmacological activity of a complex system requires knowledge of the state of molecular assembly of the system's components. The recent flurry of research activity on biomacromolecular assembly and its application [1–4] in the construction of nanodevices has encouraged the development of experimental techniques capable of characterizing and detecting the assembly. The interaction among molecules has many kinds such as covalent bond, ionic bond, coordinate bond and other physical forces e.g., electrostatic attraction, hydrogen bond, van der Waals force, hydrophobic bond and insertion. Among them, the electrostatic attraction is original and primary because it shortens the distance between active molecules to induce the formation of the other physical forces. It is well known that a chemical bond is much stronger than a physical force. The physical interaction often exists in a cell e.g., vitamin, glucose with protein and anticarcinogen with DNA. Molecular spectrometry is one conventional and classical method [5–9] in study of the biomacromolecular assembly. Pesavento [10] and Scatchard models [11–14] are often

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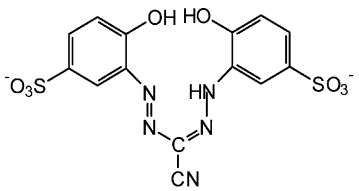
applied to characterize the assembly. Recently, some examples of assembly of strong-spectral stains on biomacromolecules were investigated by microsurface adsorption-spectral correction (MSASC) technique [15–18]. However, how to characterize the interaction of a non- or weaklyspectral compound e.g., drug, surfactant, pesticide and toxicant with biomacromolecule by spectrometry, a replacement of a spectral probe by a non- or weaklyspectral compound was proposed for this purpose. The MSASC technique and the break point approach are used in characterization of the assembly. As an example, the interaction of sodium dodecyl benzene sulfonate (SDBS) with proteins: bovine serum albumin (BSA), ovalbumin (OVA) and α-globulins (Gb) have been investigated with 1,5-di(hydroxyl-5-sulfophenyl-)-3-cyanoformazan (DSPCF) as the spectral probe. The structure of DSPCF is given below.




1,5-di(2-hydroxyl-5-sulfophenyl)-3- cyanoformazan (DSPCF)

It forms bivalent anion in aqueous solution and protein often carries large amounts of positive charge because of protonation of $-NH_2$ in basic amino residues in acidic solution. Certainly, both DSPCF and SDBS anions can be adsorbed in protein. The assembly of DSPCF in protein was determined and the replacement of DSPCF with SDBS investigated. The assembly reactions both obey the Langmuir isothermal adsorption. The characterization of the aggregates has been made by the MSASC and break point approach. The aggregates: $SDBS_{99}BSA$, $SDBS_{50}OVA$ and $SDBS_{25}Gb$ at 30 °C and $SDBS_{90}BSA$, SDB $S_{40}OVA$ and $SDBS_{20}Gb$ at 40 °C are formed at pH 1.83. The assembly of DSPCF in protein was tried to apply to the quantitative analysis of protein samples.

## 2. Principle

### 2.1. MSASC technique

To see Figs. 1a, b, the stain probe (L) can be attracted on an oppositely charged biomacromolecule (M) (1) to form an aggregate, **ML** and the assembly obeys the Langmuir isotherm adsorption [19]. The M–L solution equilibrium occurs as follows (m.s. means microsurface phase):

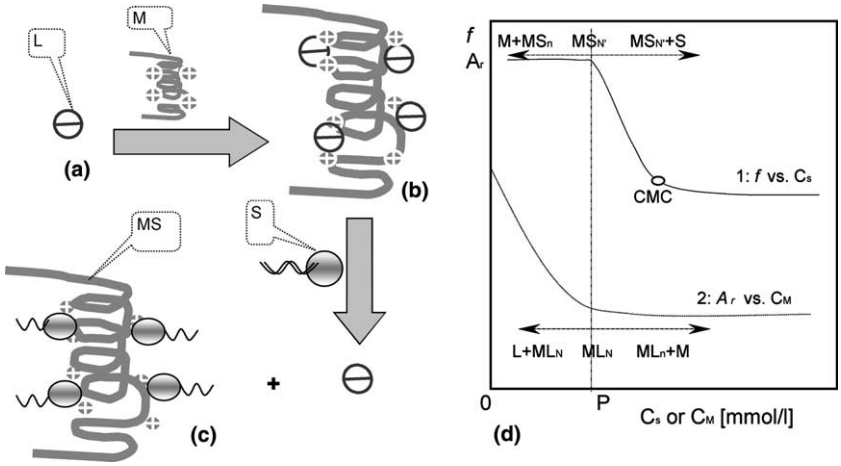



Fig. 1. The diagrammatic sketch for assembly (b) of spectral probe L (a) in protein (M), replacement of L and assembly (c) of S in M. Break point sketch (d): (1) variation of surface tension ($f$) with S molarity in the S–M binding reaction and (2) variation of absorbance ratio ($A_r$) with M molarity in the L–M binding reaction.

| | **L** | + | **M**(m.s.) | → | **ML**$_n$(m.s.) |
|---|---|---|---|---|---|
| Initial state | $C_{Lo}$ | $(A_0, A_0')$ | $C_{M0}$ | | 0 |
| Equilibrium | $C_L$ | | | | $C_{M0}(A_c)$ |

The Langmuir isotherm equation is obeyed

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_L}, \tag{1}$$

Fig. 1c. The replacement reaction is expressed as followed:

| | **ML**$_n$ | + | **S**(m.s.) | → | **MS**$_m$(m.s.) | + | **L** |
|---|---|---|---|---|---|---|---|
| Initial state | $C_{M0}(A_c)$ | | $C_S$ | | 0 | | |
| Equilibrium | $C_M(A_c')$ | | | | $C_S$ | | $C_L + n(C_{M0} - C_M)$ |

where $K$ is the equilibrium constant and $C_L$ the molarity of the excess L. $\gamma$ is the molar ratio of the effective L adsorbed to M. Within increase in L concentration, $\gamma$ approaches a maximal binding number, $N$. Both $N$ and $K$ may be calculated by plotting line $\gamma^{-1}$ vs. $C_L^{-1}$. Both $C_L$ and $\gamma$ are calculated by the relations [16]:

$$\gamma = n = \eta \frac{C_{L0}}{C_{M0}}, \tag{2}$$

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

where

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

where both $C_{M0}$ and $C_{L0}$ are the molarities of M and L in initial solution and $\eta$ indicates the effective fraction of L. $A_c$, $A_0$ and $\Delta A$ are the real absorbance of the M–L product, the measurement absorbance of L against water and that of the M–L solution against reagent blank directly measured at the peak wavelength $\lambda_2$, respectively. $A_c$ is calculated by means of [20–23]

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

where $\Delta A'$ indicates the absorbance of the M–L solution measured at the valley wavelength $\lambda_1$. In common, $\alpha$ and $\beta$ are the correction constants and they are calculated by measuring directly $ML_N$ and L solution.

In the reaction above, the addition of SDBS will result in a replacement of DSPCF and formation of a novel aggregate (**MS**) as shown in

$$\gamma = m = \frac{C_{M0} - C_M}{C_S} = \frac{C_{M0} - C_M}{C_{M0}} \frac{C_{M0}}{C_S} = \eta \frac{C_{M0}}{C_S}, \tag{6}$$

$$C_M = C_{ML} = (1 - \eta)C_{M0}, \tag{7}$$

where

$$\eta = \frac{A_c - A_C'}{A_c} = \frac{\Delta A_c}{A_c}. \tag{8}$$

Within increase in ML concentration, $\gamma$ approaches a maximal binding number, $N'$. The assembly of S on M obeys the Langmuir isotherm adsorption. Thus, the characterization of $MS_{N'}$ can be made by analyzing variation of the ML aggregate.

### 2.2. Break point approach

In the S–M binding solution where M is being held constant, from curve 1 in Fig. 1d, S is completely bound on M when S concentration ($C_S$) is less than $C_P$ (P-break point) and the binding number ($n$) of S increases with increase in S concentration. Such a solution contains both MS and excess of M and its surface tension ($f$) remains always high or changes little. In the contrary, S is always excessive when $C_S$ is over $C_P$. Such a solution contains both MS and excess of S. The free S results in a rapid decrease of $f$ down to the critical micelle concentration (CMC). Similarly, in the M–L binding solution where L is being held constant, from curve 2, L is always excessive when M concentration ($C_M$) is less than $C_P$ and the binding number of L on M always remains a maximum, $N$. Such a solution contains both $ML_N$ and excess of L. The free L results in

a rapid increase of the absorbance ratio ($A_r = A_{\lambda 1}/A_{\lambda 2}$). On the contrary, L is completely bound on M when $C_M$ is over $C_P$. Such a solution contains both $ML_n$ ($n < N$) and excess of M. Therefore, the break point may indicate the maximal assembly number ($N'$) of S binding on M and that ($N$) of L binding on M.

We can often observe the formation of sludge or suspending substance in the M–L or M–S reaction at P nearby. This is attributed to the formation of neutral $MS_{N'}$ or $ML_N$ molecule, which cannot often dissolves in aqueous solution. $MS_n^{(N'-n)+}$ ($n < N'$) is formed when $C_S$ is much less than $C_P$ and $ML_n^{(N-n)+}$ ($n < N$) formed when $C_M$ is much more than $C_P$. On the contrary, the ionized colloid particle $\{(MS_{N'})S_m, (m-p)Na\}^{p+}$ is formed when $C_S$ is much more than $C_P$ and $\{(ML_N)L_m, (m-p)Na\}^{p+}$ formed when $C_M$ is much less than $C_P$. They can dissolve in aqueous solution.

## 3. Experimental

### 3.1. Materials and methods

Absorption spectra were recorded on a TU1901 Spectrophotometer (PGeneral, Beijing) with 1-cm and the individual absorbance was measured on a Model 722 spectrophotometer (Shanghai third Analytical Instruments). The Drop Shape Analysis System (DSA 10 MK 2, KRUSS GmbH, Germany) was used to measure the surface tension of solutions. pH of solution was measured on pHS-2C acidity meter (Leici Instruments, Shanghai). The temperature was adjusted and remained constant in an electronic heated thermostat bath, Model 116R (Changjiang Test Instruments of Tongjiang, China).

Protein standard solutions were prepared by dissolving the commercial BSA (Dongfeng Biological Technological, Shanghai), α-globulins horse cohn fraction IV (Gb) (Koch–Light Lab., England) and OVA (Shanghai Chemical Reagents of Chinese Medicine Group) in deionized water. The protein content ($w$, mg/ml) in the above solutions was determined and calculated by the relation: $w = 1.45A_{280\,nm} - 0.74A_{260\,nm}$ [24] by measuring their absorbances ($A_{260\,nm}$ and $A_{280\,nm}$) at 260 and 280 nm by UV spectrophotometry. The contents were examined with coormassie brill blue G250 by spectrophotometry [25]. The standard stock solution of SDBS (1.00 mmol/l) was prepared by dissolving SDBS (A.R., Shanghai Chemical Reagents) in deionized water. DSPCF solution (1.00 mmol/l) was prepared by dissolving 0.2420 g of DSPCF (A.R., $C_{16}H_{18}N_3S \cdot Cl$, Sigma Chemicals) in 250 ml of deionized water. The Britton–Robinson buffer solutions between pH 1.83 and 8.27 were prepared to control the acidity of the interaction solution. 2.0 mol/l NaCl was used to adjust the ionic strength of the aqueous solutions. The masking reagent solution, 2% $Na_2EDTA$ was prepared for masking metals in the determination of proteins.

### 3.2. Procedures

#### 3.2.1. Assembly of DSPCF on proteins

Into a 10-ml calibrated flask were added an appropriate working solution of BSA, 1.0 ml of buffer solution (pH 1.83) and a known volume of 1.00 mmol/l DSPCF. The mixture was diluted to 10 ml with deionized water and mixed thoroughly. After 5 min, absorbances were measured at 452 and 580 nm, respectively, against the blank treated in the same way without any protein and then $A_c$, $\eta$, $C_L$ and $\gamma$ were calculated by Eqs. (2)–(5).

#### 3.2.2. Assembly of SDBS on proteins

In a 10-ml volumetric flask, added 1.0 ml of pH 1.83 buffer solution and a known volume of DSPCF and same times protein solutions. Diluted to about 7 ml and mixed. After 5 min, added a known volume of 1.00 mmol/l SDBS. Diluted to 10 ml and mixed well. After 10 min, measured the absorbances at 452 and 580 nm against the blank and then $\Delta A_c$, $\eta$, $C_{ML}$ and $\gamma$ were calculated by Eqs. (6)–(8).

## 4. Results and discussion

### 4.1. Effect of pH and spectral analysis

The absorption spectra of the DSPCF, DSPCF–BSA and DSPCF–BSA–SDBS solutions at pH 1.83 are shown in Fig. 2a. Effect of pH

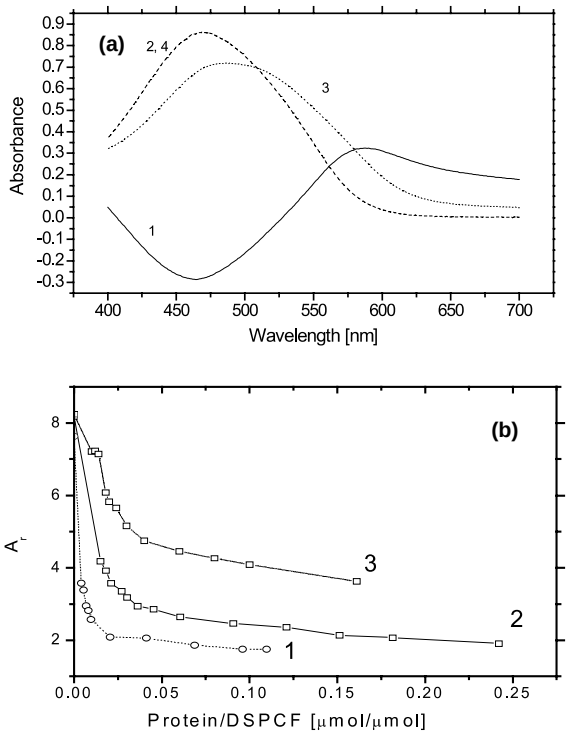



Fig. 2. (a) Absorption spectra of DSPCF, DSPCF–SDBS and DSPCF–SDBS–BSA solutions at pH 1.83 containing: (1) 0.100 mmol/l DSPCF and 0.04445 mg/ml BSA; (2) 0.100 mmol/l DSPCF; (3) 0.050 mmol/l DSPCF and 0.32 mg/ml BSA and (4) 0.050 mmol/l DSPCF, 0.0445 mg/ml BSA and 0.200 mmol/l SDBS. 1 is measured against the reagent blank without any protein and the others against water. (b) Effect of BSA (1), OVA (2) and Gb (3) on $A_r$ ($=A_{452\,nm}/A_{580\,nm}$). All the solutions contained 0.050 mmol/l DSPCF and were measured against water.

indicated the DSPCF–BSA reaction is most sensitive in acidic solution. This is attributed to the fact that protein molecule will carry much more positive charges because of protonation of $-NH_2$. In this work, pH 1.83 was selected. The maximal peak of DSPCF is located at 469 nm from curve 2 and that of the DSPCF–BSA aggregate is located at 487 nm from curve 3. The spectral red shift of the aggregate is only 18 nm. This is attributed to the fact that the electrostatic attraction is often much weaker than a chemical bond. The former causes only a little spectrum variation but the latter can bring an obvious spectral shift. However, from curve 1, we observe that its peak and valley are located at 580 and 452 nm, so such two wavelengths were used. In the BSA–DSPCF solution, a great amount of SDBS was added and the replacement of DSPCF was observed. Curve 4 shows the absorption spectrum of the replacement solution in the presence of a great amount of SDBS. It is coincident with curve 2 so the BSA–DSPCF aggregate was destroyed by SDBS and the DSPCF binding on BSA was separated away. From curves 2 and 3, the correction coefficients were calculated to be $\beta_{DSPCF} = 0.150$ and $\alpha_{BSA-DSPCF} = 0.745$. The real absorbance of the BSA–DSPCF aggregate was calculated by $A_c = 1.13\ (\Delta A - 0.150\Delta A')$.

### 4.2. Analysis to interaction of DSPCF with proteins

Fig. 2b shows change of $A_r$ ($=A_{452\,nm}/A_{580\,nm}$) of the solutions. From curve 1, $A_r$ approaches a minimum when the molar ratio of BSA to DSPCF over 0.02. No free DSPCF exists in the solutions and curve 3 in Fig. 2a shows the spectrum of such a solution containing only the protein–DSPCF aggregate. From curves 1, 2 and 3 in Fig. 2b, their break points always are located at 0.01, 0.025 and 0.04, respectively. Therefore, the maximal binding number of DSPCF on BSA, OVA and Gb was estimated to be about 100, 40 and 25 and they will be again examined below.

### 4.3. Effect of ionic strength and temperature

In order to investigate the effect of ionic strength of solution on the assembly of DSPCF, NaCl was added. From Fig. 3a, $\gamma$ increases rapidly down to about 0 with increase in ionic strength. Though the assembly of $Cl^-$ on protein is possibly weaker than that of two sulfonyl groups in DSPCF on protein, much more concentrated $Cl^-$ than DSPCF will occupy the electrostatic points of protein in place of DSPCF.

From Fig. 3b, we observe the effect of temperature on $\gamma$. $\gamma$ begins to decrease slowly over 40 °C. At high temperatures, it is possible that the protein molecular chain unfolds slowly from the multifolding. This makes the electrostatic field's intensity weaken. However, a DSPCF molecule binds on protein by two sulfonyl groups to own a strong

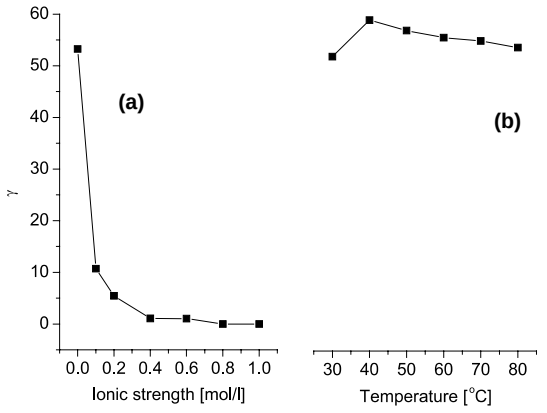



Fig. 3. Effect of ionic strength (a) and temperature (b) on $\gamma$ of DSPCF to BSA in the solutions containing 0.050 mmol/l DSPCF and 0.0467 mg/ml BSA.

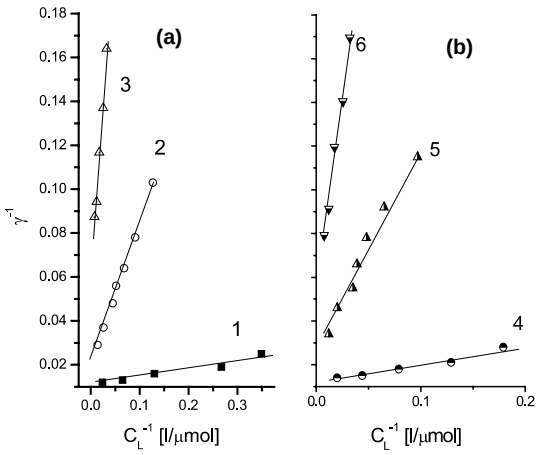



Fig. 4. Plots of $\gamma^{-1}$ vs. $C_L^{-1}$ at 30 °C (a) and 40 °C (b) where the solutions contained 0.0467 mg/ml BSA ((1) $\gamma^{-1} = 0.088C_L^{-1} + 0.0113$, $R^2 = 0.9701$ and (4) $\gamma^{-1} = 0.0387C_L^{-1} + 0.0105$, $R^2 = 0.949$), 0.0651 mg/ml OVA ((2) $\gamma^{-1} = 0.9485C_L^{-1} + 0.0268$, $R^2 = 0.9784$ and (5) $\gamma^{-1} = 0.6508C_L^{-1} + 0.0199$, $R^2 = 0.998$) and 0.0859 mg/ml Gb ((3) $\gamma^{-1} = 3.668C_L^{-1} + 0.0506$, $R^2 = 0.9908$ and (6) $\gamma^{-1} = 3.0988C_L^{-1} + 0.061$, $R^2 = 0.9894$) and DSPCF between 0.030 and 0.150 mmol/l.

binding force. Therefore, the simultaneous interaction of such two cases results in a weak desorption of DSPCF from the DSPCF–protein aggregate at higher temperatures.

### 4.4. Characterization of the aggregates

By varying DSPCF concentration, absorbance of the protein–DSPCF solutions at 30 and 40 °C was measured and $\gamma$ of DSPCF to protein and $C_L$ of DSPCF at equilibrium were calculated by Eqs. (2)–(4). Plots of $\gamma^{-1}$ vs. $C_L^{-1}$ is shown in Figs. 4a, b. We observe that all curves are linear. Therefore, the assembly of DSPCF in protein obeys the Langmuir isotherm adsorption. From the intercepts, the maximal binding number of DSPCF in proteins was calculated to be DSPCF:BSA = 88:1 at 30 °C and 95:1 at 40 °C, DSPCF:OVA = 37:1 at 30 °C and 50:1 at 40 °C and DSPCF:Gb = 20:1 at 30 °C and 16:1 at 40 °C. These binding numbers are similar to those obtained previously by the absorbance ratio method. Besides, from the line slopes, the binding constants of the aggregates were calculated to be $K_{30\,^\circ C}^{\text{BSA–DSPCF}} = 1.28 \times 10^5$ and $K_{40\,^\circ C}^{\text{BSA–DSPCF}} = 2.71 \times 10^5$, $K_{30\,^\circ C}^{\text{OVA–DSPCF}} = 2.83 \times 10^4$ and $K_{40\,^\circ C}^{\text{OVA–DSPCF}} = 3.06 \times 10^4$ and $K_{30\,^\circ C}^{\text{Gb–DSPCF}} = 1.38 \times 10^4$ and $K_{40\,^\circ C}^{\text{Gb–DSPCF}} = 1.97 \times 10^4$.

By varying the addition of the DSPCF and same times protein solutions in SDBS solutions, the absorbance of the SDDBS–DSPCF–protein solutions at 30 and 40 °C was measured. Both $\gamma$ of SDBS to protein and $C_M$ of the protein–DSPCF aggregate at equilibrium were calculated by Eqs. (6)–(8). Plots of $\gamma^{-1}$ vs. $C_M^{-1}$ are shown in Figs. 5a, b. The assembly of SDBS on BSA, OVA and Gb all obey the Langmuir isotherm adsorption, too. From the intercepts, the maximal binding numbers of the aggregates were calculated to be SDBS: BSA = 99:1 at 30 °C and 90:1 at 40 °C, SDBS: OVA = 50:1 at 30 °C and 40:1 at 40 °C and SDBS:Gb = 60:1 at 30 °C and 50:1 at 40 °C. From the line slopes, the binding constants of the SDBS–protein aggregates is calculated to be $K_{30\,^\circ C}^{\text{SDBS–BSA}} = 4.12 \times 10^6$ and $K_{40\,^\circ C}^{\text{SDBS–BSA}} = 9.00 \times 10^6$, $K_{30\,^\circ C}^{\text{SDBS–OVA}} = 2.34 \times 10^5$ and $K_{40\,^\circ C}^{\text{SDBS–OVA}} = 6.15 \times 10^5$, $K_{30\,^\circ C}^{\text{SDBS–Gb}} = 8.78 \times 10^4$ and $K_{40\,^\circ C}^{\text{SDBS–Gb}} = 1.55 \times 10^5$. From both $K$ of each, $K$ at 40 °C is always more than that at 30 °C. This is attributed to the fact that each SDBS is distributed to more electrostatic points of protein and the electrostatic attraction force becomes strong.

From curves 7, 8 and 9 in Fig. 5c, their break points at 30 °C are located at about 100, 50 and 50. The results are anastomotic with those in the last

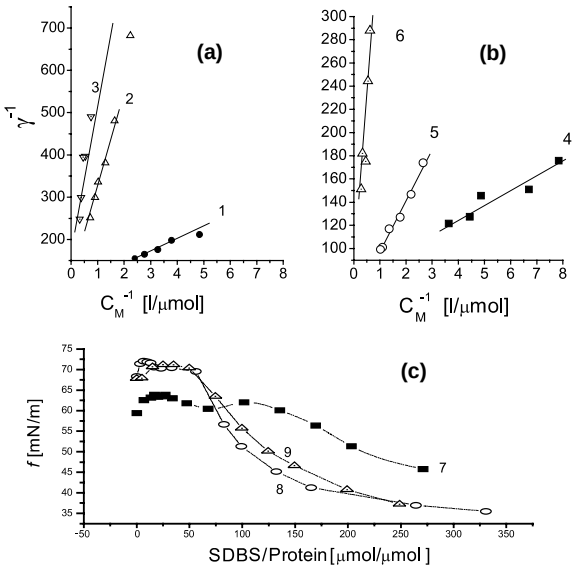



Fig. 5. Plots of $\gamma^{-1}$ vs. $C_m^{-1}$ at 30 °C (a) and (2) 40 °C (b) where the SDBS–protein solutions contained DSPCF between 0.030 and 0.100 mmol/l, BSA being 0.0074 times DSPCF and 0.050 mmol/l SDBS ((1) $\gamma^{-1} = 24.074C_L^{-1} + 99$, $R^2 = 0.9633$ and (4) $\gamma^{-1} = 10.02C_L^{-1} + 90$, $R^2 = 0.885$), DSPCF between 0.040 and 0.090 mmol/l, OVA being 0.0030 times DSPCF and 0.100 mmol/l SDBS ((2) $\gamma^{-1} = 214.12C_L^{-1} + 50$, $R^2 = 0.9392$ and (5) $\gamma^{-1} = 65.051C_L^{-1} + 40$, $R^2 = 0.9009$) and DSPCF between 0.060 and 0.150 mmol/l, Gb being 0.0034 times DSPCF and 0.200 mmol/l SDBS ((3) $\gamma^{-1} = 683.26C_L^{-1} + 60$, $R^2 = 0.9506$ and (6) $\gamma^{-1} = 322.55C_L^{-1} + 50$, $R^2 = 0.92$). (c) Variation of $f$ of the solution containing: (7) 0.100 mg/ml BSA and SDBS between 0 and 1.0 mmol/l, (8) 0.130 mg/ml OVA and SDBS between 0 and 1.0 mmol/l and (9) 0.114 mg/ml Gb and SDBS between 0 and 1.5 mmol/l.

paragraph. So the maximal binding number of SDBS above in proteins is accurate.

### 4.5. Application

The adsorption of DSPCF in proteins at pH 1.83 has been applied to the quantitative detection of protein in the presence of $Na_2EDTA$. The standard series of various protein solutions were prepared and measured at 452 and 580 nm and all $A_c$ are linear. The linear equations are followed: $A_c = 2.74x$ ($R^2 = 0.9959$, $x$-mg/ml) between 0 and 0.120 mg/ml BSA, $A_c = 1.12x$ ($R^2 = 0.9902$) between 0 and 0.135 mg/ml OVA and $A_c = 1.46x$ ($R^2 = 0.9958$) between 0 and 0.15 mg/ml Gb.

By adding 0.5 ml of 2% $Na_2EDTA$ in protein detection to mask metals possibly existed in samples, none of the following substances affected the direct determination of 0.023 mg/ml BSA (less than 10% error): 0.1 mg/ml $Cl^-$, $SO_4^{2-}$; 0.05 mg/ml glucose, Ca(II), Fe(II), Pb(II), Zn(II) and Mg(II); 0.01 mg/ml calf thymus DNA, tyrosine, glutamic acid, lysine and vitamin C and 0.005 mg/ml Cu(II).

Two samples, milk powder and children drinking were prepared into aqueous solutions and then analyzed. The determination results of the protein contents accord with the index mark signed on the containers. The recoveries of OVA added were between 93.2% and 106% and the relative standard deviations less than 4.8%.

## 5. Conclusion

The investigation of the interactions of DSPCF and SDBS with proteins supports the Langmuir monolayer assembly of small molecules on biomacromolecule. The combination of both the MSASC technique and the break point approach applied to characterization of a macromolecular assembly is different from the other methods, e.g., Scatchard [11], continuous variations [26] in principle and operation because the background interference of a strongly spectral probe is eliminated.

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