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A study of the interaction between fluorescein sodium salt and bovine serum albumin by steady-state fluorescence

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ABSTRACT

The binding of fluorescein sodium salt with three kinds of commercially available bovine serum albumin (BSA) of different grades of purity was investigated at 288, 298 and 313 K by fluorescence and absorption measurements at pH 7.50. The association and dissociation constants K_a and K_d were determined by the quenching of BSA fluorescence in the presence of fluorescein sodium salt. The best results were obtained by fitting raw data by non-linear regression and Lineweaver–Burk equations. The modified Stern–Volmer and Scatchard plots gave less reliable data since the fitting was much more difficult.
The agreement of the constants for the three sets of measurements coming from the different BSA was not as good as expected. BSA binding properties differ depending on the different BSA grades of purity. Actually, the binding constants found for the three BSAs used differed in the same set of interactions, even by keeping the experimental conditions constant. These results are a novelty in the field of BSA–ligand binding studies and should be taken into account for future binding studies using BSA. Actually, a large number of aspects should be considered including the grade of purity and the presence of BSA covalent and non-covalent dimers, trimers and oligomers in solution which can affect the goodness of the binding results.

1. Introduction

Serum albumins are the most extensively studied and applied proteins because of their availability, low cost, stability and unusual ligand binding properties. For this reason, a huge number of papers dealing with albumins have been reviewed so far [1,2]. Albumin is the most abundant protein in blood plasma and serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. Albumin is also the principal factor in contributing to the colloid osmotic pressure of the blood and has been suggested as a possible source of amino acids for various tissues. Without question, albumin is the most multifunctional transport protein and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood [3] due to the existence of a limited number of binding regions of very different specificity [2,4].

Albumin is clearly an extraordinary molecule of manifold functions and applications. Perhaps, the most outstanding property of albumin is its capacity to bind reversibly a numerous variety of ligands [2,4]. The physiological importance of albumin, with its properties of transporting protein, and the relative ease with which it can be isolated and purified on a large scale have resulted in a great number of binding studies. Reviews have previously appeared dealing, in relatively general terms, with binding of small molecules to albumin and other proteins such as fatty acids, lysolecithin, bilirubin, warfarin, tryptophan, steroids, anaesthetics and several dyes [2–4].

Most ligands are bound reversibly and typical association constants (K_a) range from 10^4 to 10^6 M^{-1}. Because of the incredible diversity of ligands bound by albumin, early researchers saw ligand binding to serum albumin as non-specific in nature and did not recognize that there were discrete sites *per se*. Instead they envisaged the ligands as randomly attached to the surface, somewhat like a sponge. This view of albumin has changed over the past years, and now it is generally recognized that there are a small number of distinct binding locations [2].

Bovine serum albumin (BSA) is constituted by 582 amino acid residues and on the basis of the distribution of the disulfide bridges and of the amino acid sequence it seems possible to regard BSA as

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Nomenclature

K_d	dissociation constant
K_a	association constant
B_{max}	maximum amount of the complex that can form at saturating the ligand
n	number of binding sites
F_0	steady-state fluorescence intensities in the absence of quencher
F	steady-state fluorescence intensities in the presence of quencher
[Q]	quencher/ligand concentration
[Q_f]	free quencher/ligand concentration
[Q_b]	bound quencher/ligand concentration
[R_t]	total protein concentration

composed of three homologous domains linked together. The domains can all be subdivided into two subdomains. As proposed by Kragh-Hansen [4], there are at least six binding regions and another characteristic feature of albumin–ligand interactions seems to be the presence of one or two high affinity binding sites (primary sites) and a number of sites with lower affinity.

Quenching measurements of albumin fluorescence is an important method to study the interactions of compounds with proteins [5–7]. It can reveal accessibility of quenchers to albumin's fluorophores, help to understand albumin binding mechanisms to compounds and provide clues to the nature of the binding phenomenon.

Dyes are being increasingly used in clinical and medicinal applications [8–10]. The discovery that some dyes would stain certain tissues and not others led to the idea that dyes might be found that would selectively stain, combine with and destroy pathogenic organisms without causing appreciable harm to the host. Actually, some azo, thiazine, triphenylmethine and acridine dyes came into use as antiseptic trypanocides and for other medicinal purposes [11]. It is also known that certain dyes like fluorescein and rose bengal are preferentially adsorbed by cancerous cells [10].

Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components, which then function as carriers. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cell in vivo and in vitro. Consequently, it is important to understand the mechanism of interaction of a bioactive compound with protein.

Many drugs, including anti-coagulants, tranquillizers, and general anaesthetics, are transported in the blood while bound to albumin [12]. Drug–protein interactions are important since most of the drugs and other bioactive small molecules are extensively and reversibly bound to serum albumin and they are transported mainly as a complex with protein. The nature and magnitude of drug–protein interaction influences the biological activity (efficacy and rate of delivery) of the drug [13]. It is then important to study the binding parameters in order to know and try to control the pharmacological response of drugs and design of dosage forms. This kind of studies may provide salient information on the structural features that determine the therapeutic effectiveness of drugs/dyes, and hence become an important research field in chemistry, life science and clinical medicine [13–15]. Serum albumin is considered as a model for studying drug–protein interaction in vitro since it is the major binding protein for drugs and other physiological substances.

The use of dyes for protein determination is well established [16,17]. However, other parameters such as mode of interaction, association constant and number of binding sites are important, when dyes are used as drugs. Several spectrophotometric methods such as fluorescence, UV–vis, circular dichroism, light scattering, FT-IR, nuclear magnetic resonance have been used to study the interaction of small molecules and proteins and clarify the conformational change of protein [7,18,19]. Some techniques such as electrochemical technique [20] and capillary electrophoresis [21] have also been utilized for the evaluation of binding mode and binding constants. Among them, fluorescence spectroscopy has been widely used due to its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation. Critical literature survey reveals that attempts have not been made so far to investigate the mechanism of interaction of fluorescein sodium salt (Fig. 1) with BSA.

The present paper deals with the mechanism of binding of fluorescein sodium salt as a ligand with different BSAs by fluorescence steady-state measurements. This study highlights for the first time how binding properties can change for different BSAs. The three commercially available BSAs taken into account differ for the grade of purity and for the purification method (Table 1).

2. Results and discussion

2.1. Fluorescence intensity

Fluorescence-quenching measurements have been widely used to study the interactions of organic compounds with proteins [5–7]. This method can reveal accessibility of quenchers to protein fluorophores, help to understand protein binding mechanisms to compounds and provide clues to the nature of the binding phenomenon.

In principle both native protein fluorescence or ligand (if any) fluorescence can be exploited to monitor the complex formation. However, most of the studies rely on the quenching of protein fluorescence. Only few studies were performed by titrating the dye with the protein but they were mainly related to the use of absorbance instead of fluorescence measurements [22,23].

In this case, in order to investigate the binding of fluorescein sodium salt to BSA, protein concentration is held constant and increasing concentrations of ligand are added. Fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 295 nm. This excitation wavelength avoids excitation of tyrosines and selectively excites tryptophans. Monitoring quenching of tryptophan fluorescence yields much better signal to noise ratio than monitoring increases in ligand fluorescence. As shown in Fig. 2, by increasing the fluorescein sodium salt concentration, there is a decrease of the BSA fluorescence intensity but the emission maximum does not move to shorter or longer wavelength. These results indicated that interaction between fluorescein sodium salt and BSA occurs and the fluorophore quenches the intrinsic fluorescence emission of BSA.

As a preliminary study, some BSA/fluorescein sodium salt concentration ratios were investigated and a binding curve was constructed in order to check which range of concentration has to

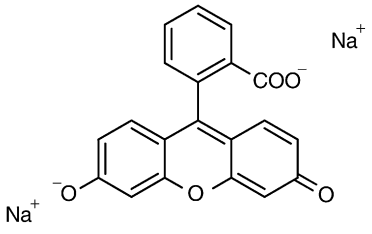

Fig. 1. Structure of fluorescein sodium salt.

Table 1
Different BSAs used

BSA type	Purity	Purification
A	>96%	(1) Heat shock (2) Charcoal (3) Extensive dialysis
B	>92%	(1) Heat shock
C	>98%	(1) Heat shock (2) Charcoal

be taken for the interaction study. The chosen concentrations have been reported in Section 3.

On the basis of these previous results, binding studies were performed and the obtained steady-state maximum fluorescence intensity was recorded and data were treated by several methods. The analysis of quenching titration data allowed the evaluation of the equilibrium association (K_a) and dissociation (K_d) constants (which are reciprocals of each other). Five different methods were taken into account and are discussed in detail even if some of them gave poor results. In the following figures the graphs reported refer to different data treatments for selected experiments.

2.1.1. Non-linear least squares

The most straightforward way of analyzing data is to use a non-linear least-squares fit procedure which has been discussed in detail elsewhere [24]. Many of the commercially available fitting programs (Sigma-Plot, TableCurve and GraphPad Prism) have a non-linear least-squares fit program as part of the package based on Eq. (1) [7]:

$$y = \frac{B_{max}[Q]}{K_d + [Q]} \quad (1)$$

where [Q] is the fluorescein sodium salt concentration in solution, acting as a quencher, y is the specific binding derived by measuring fluorescence intensity, B_{max} is the maximum amount of the complex BSA/fluorescein sodium salt formed at saturation of the ligand and K_d is the equilibrium dissociation constant. In Fig. 3, a binding curve is reported as an example where the percentage of bound BSA, i.e. y, derived from the fluorescence intensity emission maximum, is plotted against the fluorescein sodium salt concentration. The corresponding K_d and B_{max} are shown in Table 2 and were used to generate the reported binding curve.

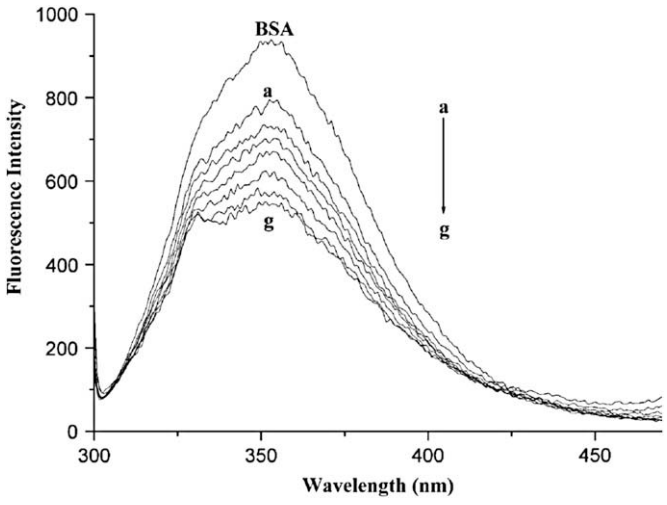

Fig. 2. Fluorescence spectra of the interaction between BSA (1 μM) and fluorescein sodium salt at different concentrations: 0.12 μM (a), 0.24 μM (b), 0.35 μM (c), 0.65 μM (d), 1.00 μM (e), 1.50 μM (f) and 2.20 μM (g).

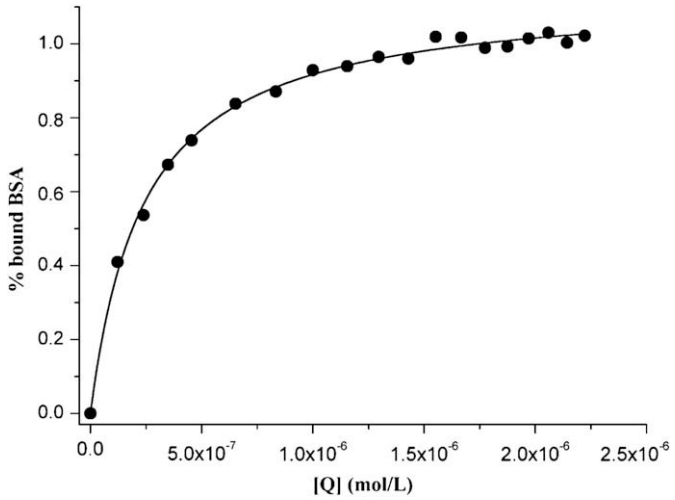

Fig. 3. Non-linear least square analysis of the A5 experiment.

2.1.2. Stern–Volmer

The fluorescence-quenching data were also analyzed by the Stern–Volmer equation:

$$\frac{F}{F_0} = 1 + K_{SV}[Q] \quad (2)$$

where F_0 is the steady-state fluorescence intensity of the BSA alone while F is the steady-state fluorescence intensity at the increasing of the quencher concentration, K_{SV} is the Stern–Volmer quenching

Table 2
K_d and K_a obtained for the three different BSA at 298 K

	Non-linear			Lineweaver–Burk	
	$K_d \times 10^{-7}$ (M)	B_{max}	$K_a \times 10^6$ (M^{-1})	$K_d \times 10^{-7}$ (M)	$K_a \times 10^6$ (M^{-1})
A1	1.00	1.07	9.97	1.00	10.1
A2	1.11	1.08	9.01	1.04	9.58
A3	2.67	1.15	3.74	3.26	3.07
A4	4.97	1.22	2.01	3.98	2.51
A5	2.41	1.14	4.14	2.13	4.70
A6	0.81	1.06	12.4	0.76	13.1
A7[a]	0.78	1.06	12.7	0.95	10.5
A8[a]	1.60	1.10	6.25	1.64	6.11
A9[a]	1.80	1.12	5.54	1.41	7.08
A10[b]	4.68	1.23	2.14	6.38	1.57
A11[b]	3.97	1.21	2.52	3.61	2.77
B1	0.97	1.07	10.4	1.00	10.0
B2	6.78	1.29	1.45	7.48	1.34
B3	3.12	1.16	3.21	2.52	3.97
B4	1.11	1.08	9.01	1.27	8.03
B5	1.96	1.11	5.10	2.19	4.57
B6	1.48	1.10	5.05	1.34	7.44
C1	3.20	1.17	3.13	3.46	2.89
C2	1.91	1.12	5.24	1.94	5.16
C3	1.07	1.07	9.35	1.21	8.25
C4	2.22	1.13	4.50	2.21	4.53
C5	1.00	1.07	10.0	1.23	8.15
C6	0.66	1.05	15.2	0.69	14.3
C7	0.16	1.01	62.5	0.30	33.3
C8	0.26	1.02	38.5	0.35	28.2
C9	0.35	1.03	28.6	0.37	27.3
C10	0.26	1.04	38.5	0.37	27.0
C11	0.26	1.02	38.5	0.36	27.8
C12	0.37	1.03	27.0	0.53	18.8
C13	2.71	1.15	3.69	2.95	33.9
C14	2.30	1.21	4.35	2.07	4.84
C15	0.20	1.03	50.0	0.30	33.5

[a] Performed at 313 K.
[b] Performed at 288 K.

constant (i.e. the association constant K_a for a collisional quenching) and [Q] is the quencher concentration (fluorescein sodium salt). In Fig. 4 a plot of F/F_0 vs. fluorescein concentration is reported, from the slope of the straight line the K_{SV} can be easily calculated. Sometimes, for example as reported in Fig. 4, at high quencher concentration, there is a deviation from linearity indicating the presence of both dynamic and static quenching [25]. The dynamic portion of the observed quenching should be determined by lifetime measurements which are not available in our laboratory. Alternatively, Stern–Volmer plots can deviate from linearity toward the *x*-axis (for example in this case) when two fluorophore populations are present and one class is not accessible to the quencher. This result is frequently found for the quenching of tryptophan fluorescence in proteins by polar or charged quenchers. These molecules do not readily penetrate the hydrophobic interior of proteins and only those tryptophan residues on the surface of the protein are quenched [25]. The evaluation of the apparent bimolecular quenching constant (k_q) could be useful since it reflects the efficiency of quenching or the accessibility of the fluorophores to the quencher. Diffusion-controlled quenching typically results in values of k_q near $1 \times 10^{10}\ M^{-1}\ s^{-1}$. The value of k_q smaller than the diffusion-controlled value can result from steric shielding of the fluorophore or a low quenching efficiency. Apparent values of k_q larger than the diffusion-controlled limit usually indicate some type of binding reaction [25]. In the present case, the Stern–Volmer plots show a mean value of K_{SV} around $2 \times 10^2\ M^{-1}$. The apparent value of k_q is around $4 \times 10^{10}\ M^{-1}\ s^{-1}$ considering that the fluorescein lifetime is nearly 4.6 ns [26]. This value is larger than the diffusion-controlled limit. Hence, fluorescein must be bound to BSA but this kind of analysis should not be taken into consideration for the evaluation of the binding constants. Moreover, it is important in this study because it indicates that a binding process has to be taken into account.

2.1.3. Modified Stern–Volmer

Eq. (3) is the so-called "modified" Stern–Volmer [6] from which it is possible to get the K_a from the antilog of the intercept and the *n* (i.e. the number of binding sites) value from the slope of the straight regression line as shown in Fig. 5; the parameters are the same as that of the Stern–Volmer Eq. (2):

$$\log\frac{F_0 - F}{F} = \log K_a + n \log[Q] \tag{3}$$

The foundations of this equation are the same as the Stern–Volmer and it suffers from the same problems.

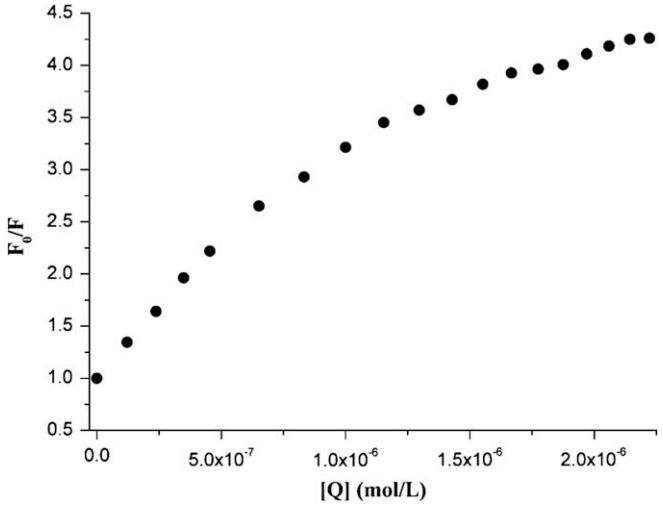

Fig. 4. Stern–Volmer plot of the C13 experiment.

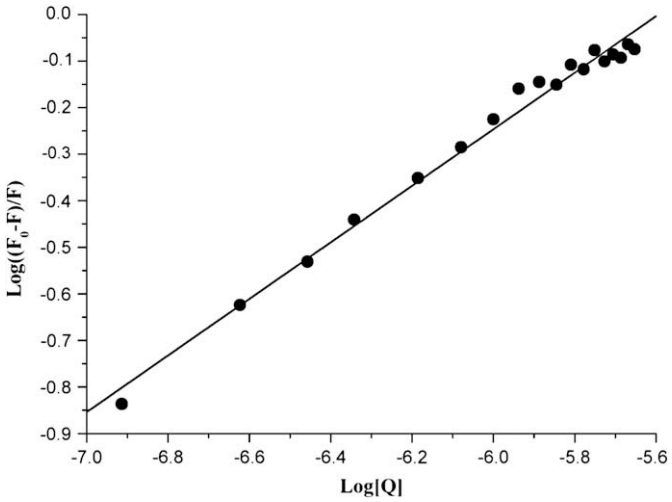

Fig. 5. Modified Stern–Volmer plot of the A4 experiment.

2.1.4. Lineweaver–Burk

Another method reported in literature is the Lineweaver–Burk [27] based on Eq. (4):

$$\frac{1}{F_0 - F} = \frac{1}{F_0} + \frac{K_d}{F_0[Q]} \tag{4}$$

As shown in Fig. 6, the slope of the line is the K_d/F_0 ratio while the intercept is the reverse of F_0. In this way, the equilibrium dissociation (K_d) constant is easily calculated.

2.1.5. Scatchard plot

Scatchard method [28] has been the traditional method for analysis of binding data until the introduction of non-linear fitting software and is based on Eq. (5):

$$\frac{[Q_b]}{[Q_f]} = \frac{-[Q_b]}{K_d} + \frac{n[R_t]}{K_d} \tag{5}$$

where $[Q_f]$ is the free quencher/ligand concentration, $[Q_b]$ is the bound quencher/ligand concentration, $[R_t]$ is the total protein concentration and K_d is the dissociation constant. In this equation $n[R_t]/K_d$ is the *y*-intercept, $n[R_t]$ is the *x*-intercept and $-1/K_d$ is the slope, as shown in Fig. 7.

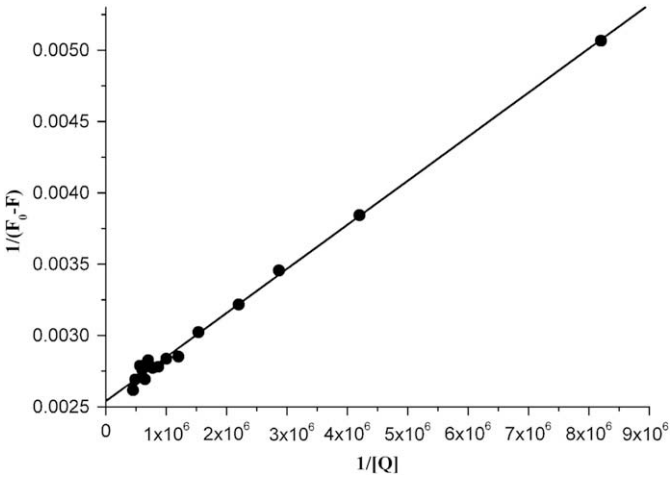

Fig. 6. Lineweaver–Burk graph of B4 experiment.

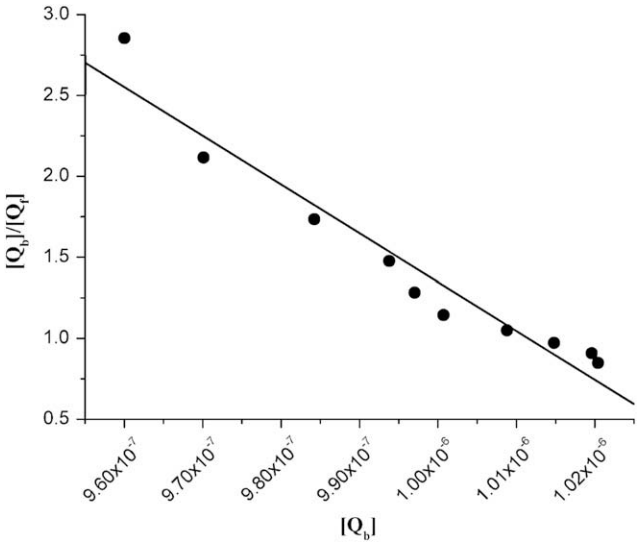

Fig. 7. Scatchard plot of the experiment C13.

While Scatchard plots are very useful for visualizing data, they are not the most accurate way to analyze data. The problem is that the linear transformation distorts the experimental error. Linear regression assumes that the scatter of points around the line follows a Gaussian distribution and that the standard deviation is the same at every value of *X*. These assumptions are not true with the transformed data. A second limit of this approach is that the Scatchard transformation alters the relationship between *X* and *Y*. The value of *X* (bound) is used to calculate *Y* (bound/free), and this violates the assumptions of linear regression for which *X* is the independent variable and *Y* is the dependent one. Since the assumptions of linear regression are violated, the B_{max} and K_d which are determined by linear regression of Scatchard transformed data are likely to be farther from their true values than the B_{max} and K_d determined by non-linear regression. Non-linear regression produces the most accurate results while Scatchard plots produce approximate results.

In Table 2 a complete view of all results obtained by non-linear least squares and Lineweaver–Burk analysis is reported.

The results obtained from the fitting by Scatchard method and the modified Stern–Volmer are not reported since the goodness of the data was poor and the fitting to the reported equations was impossible to apply (see Figs. 4 and 7). On the contrary, the agreement of the results from non-linear regression and Lineweaver–Burk is good and provides comparable values. The Stern–Volmer and the modified Stern–Volmer equations gave less reliable data since the fitting was much more difficult, as evident from Figs. 4 and 5, due to the non-perfect linear behaviour of the log $(F_0 - F)/F$ vs. log[Q] data showing the presence of both static and dynamic quenching. As can be seen from Table 2, the agreement of the constants is good for the two sets of measurements of BSA type A and B. The K_d and K_a obtained for the BSA type C differ in the same set of experiments. Actually, the K_d can vary for example from 2.0×10^{-8} M up to 3.20×10^{-7} M, which means one order of magnitude. Surprisingly, this variation was recorded for the most purified among the studied BSAs. The BSA type C has 98% purity after heat shock and charcoal treatments which remove globulins and fatty acids.

2.2. Thermodynamic parameters

Small molecules are bound to macromolecules by four binding modes: H-bonding, Van der Waals, electrostatic, and hydrophobic interactions. The thermodynamic parameters, enthalpy (ΔH^0) and entropy (ΔS^0) of reaction, are important for confirming binding modes. For this purpose, the temperature-dependence of the binding constant was studied at 288, 298, and 313 K so that BSA does not undergo any structural degradation. A plot of ln K vs. $1/T$ gives a straight line according to the Van't Hoff equation:

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \tag{6}$$

obtained by substituting Eq. (7) into Eq. (8):

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{7}$$

$$\ln K = -\frac{\Delta G^0}{RT} \tag{8}$$

The analysis of the temperature effect was carried out only for the BSA type A in order to elucidate the interaction of fluorescein sodium salt with BSA. In order to calculate the thermodynamic constants of the interaction, a Van't Hoff plot of the fluorescein–BSA system was built. Table 3 shows the values of ΔH^0 and ΔS^0 obtained for the binding site of the slopes and the ordinates of the origin of the fitted lines. From Table 3 it can be seen that both ΔH^0 and ΔS^0 have a positive value: 35.8 kJ/mol and 247.94 J/mol K, respectively. The negative sign for ΔG^0 means that the binding process is spontaneous. For drug–protein interaction, positive entropy is frequently taken as evidence for hydrophobic interaction, but it has been pointed out that positive entropy may also be a manifestation of electrostatic interaction [29].

2.3. Energy transfer between fluorescein sodium salt and BSA

The overlap of the UV absorption spectra of fluorescein sodium salt with the fluorescence emission spectra of BSA is shown in Fig. 8. The importance of the Förster resonance energy transfer in biochemistry is that the efficiency of transfer can be used to evaluate the distance between the ligand and the tryptophan residues responsible of the natural intrinsic fluorescence of the protein. According to Förster's non-radiative energy transfer theory [30], the rate of energy transfer depends on: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, and (iii) the distance between the donor and the acceptor. The energy transfer effect is related not only to the distance between the acceptor and donor, but also to the critical energy transfer distance, R_0, as described in Eq. (9):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{9}$$

where *F* and F_0 are the fluorescence intensities of BSA in the presence and absence of fluorescein sodium salt, *r* is the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50%. R_0^6 is calculated using the equation:

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{10}$$

Table 3
Thermodynamic parameters of fluorescein–BSA interaction

ΔH^0 (kJ/mol)	ΔS^0 (J/mol K)	Temperature (K)	ΔG^0 (kJ/mol)
35.8	247.94	288	−35.624
		298	−38.104
		313	−41.824

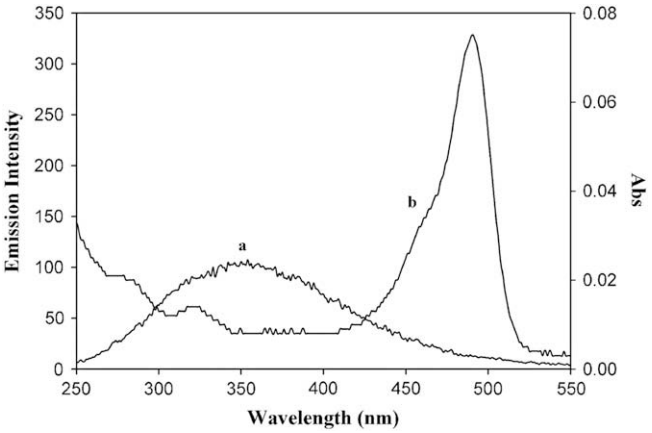

Fig. 8. The overlap of the fluorescence spectrum of BSA (a) and the absorbance spectrum of fluorescein sodium salt (b). $\lambda_{ex} = 295$ nm, $\lambda_{em} = 350$ nm, [BSA]:[FluoNa] = 1:1.

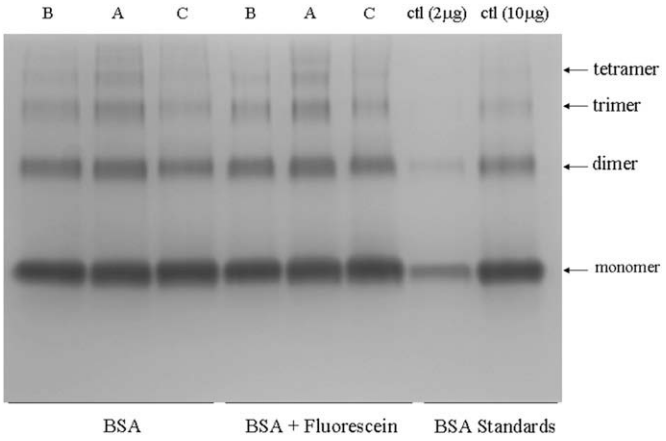

Fig. 9. Gel electrophoresis to test all the different BSAs in the presence and in the absence of fluorescein sodium salt.

where k^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of the fluorescence emission spectrum of the donor (BSA) and the absorption spectrum of the acceptor (fluorescein sodium salt). J is given by:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{11}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, $K^2 = 2/3$, $N = 1.334$ and $\Phi = 0.15$ [31,32]. From Eqs. (7)–(9), we would be able to calculate that $J = 2.38 \times 10^{13}$ mol^{-1} cm^{-1} nm^4, $R_0 = 23$ Å (2.3 nm), $E = 0.51$ and $r = 22.1$ Å (2.2 nm). The donor-to-acceptor distance, $r < 7$ nm [19,33] indicated that the energy transfer from BSA to fluorescein sodium salt occurs with high possibility. Further the value of r obtained this way agrees very well with literature value of substrate binding to serum albumin at site IIA [27,34]. The only aspect that should be noticed is that the BSA has two Trp residues, so that the donor-to-acceptor distance found is in fact an average distance of the fluorescein sodium salt from the two Trp residues.

2.4. Further considerations

Native polyacrylamide gel electrophoresis (PAGE) has been performed in order to check all the BSA used. BSA type A, B and C have been studied both in the presence and in the absence of fluorescein sodium salt and compared to other standard BSAs. As can be seen from Fig. 9, all the BSAs are present as monomers and can also form dimers, trimers and tetramers. The test, even if not quantitative, can show the presence of a higher trimer concentration for BSA type A. For the previous reasons, the BSA used for binding studies should be purified by chromatography because of the presence of dimers and oligomers. Lyophilized albumin usually has from 10 to 15% of a covalent dimer (formed between sulhydryl residues during lyophilization). Also there is evidence in the literature [35,36] that BSA can form non-covalent dimers and higher order oligomers as a function of concentration. So, it could be that the state of association changes as the BSA concentration is changed. All these aspects have to be taken into account because they affect binding results.

Another aspect to take into account is the verification of the oligomerization state of the BSA as a function of BSA concentration. Also one would have to verify the effect of fluorescein binding on the oligomerization state of the BSA. It was not detected in this case but many cases are known in which binding of a ligand influences the aggregation state of the protein [37].

3. Experimental

3.1. Materials

Different kinds of Bovine Serum Albumin (BSA) with different grades of purity (Table 1) were obtained from Sigma–Aldrich and Fluka and were used as received. BSA type A and B were purchased from Fluka (code: 05488 and 05479, respectively) while BSA type C was from Aldrich (code: A 7030).

The grade of purity of the three BSAs is reported in Table 1; it is important to recall that heat shock purification removes globulins while charcoal or organic solvent (i.e. iso-octane) precipitation removes fatty acids.

Fluorescein sodium salt for fluorescence was purchased from Fluka and used without any further purification. The solutions of Fluorescein sodium salt and BSA were prepared in 0.1 M HEPES (from Sigma–Aldrich) buffer of pH 7.5.

All reagents were of analytical reagent grade and double distilled water was used throughout. BSA solutions were prepared based on its molecular weight of 66,000.

3.2. Spectral measurements

Fluorescence measurements were recorded using a LS55 Perkin Elmer spectrofluorimeter equipped with a xenon lampsource, a 5 mm path lenght quarz cell and a thermostat bath. As a preliminary approach, in order to investigate the binding of fluorescein sodium salt to BSA, fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 295 nm. Then, fluorescence experiments were performed in a time drive mode in order to check whether and when the solution had reached the stability. Samples were excited at 295 nm and monitored at 353 nm. Slits widths were 2.5/5 nm.

UV–vis measurements were recorded using a Shimadzu UV-1700 Pharma Spec Spectrophotometer equipped with 1.0 cm path length quartz cells. UV–vis measurements were performed in order to check for Inner filter effect. IFE was negligible since BSA absorbance value was very low (Abs = 0.020).

3.3. Fluorescein–BSA interactions

Based on preliminary experiments, BSA concentration was kept fixed at 1 μM and fluorescein sodium salt concentration was varied

from 0.1 to 2 μM. Actually, to obtain accurate binding constants from fluorescence measurements, the protein concentration to be titrated should be near or less than the dissociation constant [38]. Monitoring quenching of tryptophan fluorescence was preferred since it yielded much better signal to noise than monitoring increases in ligand fluorescence.

After pre-equilibration, the appropriate amount of protein stock was added and the fluorescence signal monitored until stable. The sample was then titrated with aliquots (10 or 20 μl) of fluorescein sodium salt solution. Fluorescence spectra were recorded at 25 °C.

3.4. Native PAGE characterization of the different BSA

Discontinuous non-denaturing PAGE analysis was performed by loading native BSA (unheated samples) and run at 150 V constant voltage per gel. The stacking gel, the separatory gel (8% acrylamide), and the running buffer were prepared in the same way as the conventional SDS-PAGE, except that no SDS or DTT was used. Proteins were visualized by Coomassie Blue staining (Bio-Safe Coomassie from BIORAD).

4. Conclusions

This paper provided evidences of new aspects dealing with the binding of BSA with a ligand, fluorescein sodium salt in this case. The K_d and K_a obtained for one of the three BSA used differed in the same set of experiments and from the other constants evaluated for the other two BSAs. These results are a novelty in the field of BSA binding studies and show these experiments involving BSA should be undertaken in a very careful way since a large number of aspects have to be taken into account: the grade of purity, the presence of BSA covalent and non-covalent dimers, trimers and oligomers in solution which can affect the goodness of the binding results.

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