Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 671–677
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Spectrofluorimetric study of the binding of daphnetin to bovine serum albumin

Jiaqin Liu [a,b], Jianniao Tian [b], Wenying He [b], Jianping Xie [b], Zhide Hu [b,*], Xingguo Chen [b]

[a] *Mianyang Teacher's College, Mianyang 621000, PR China*
[b] *Department of Chemistry, Lanzhou University, Lanzhou 730000, PR China*

Received 4 January 2004; received in revised form 9 February 2004; accepted 14 February 2004

Available online 10 April 2004

Abstract

Daphnetin (7,8-dihydroxycoumarin), one of the major bioactive components isolated from Daphne odora (as glucoside), is a chelator, an antioxidant and a protein kinase inhibitor. In this paper, we report for the first time studies on the binding of daphnetin to bovine serum albumin (BSA) under physiological conditions with BSA concentration of 1.5×10^{-6} mol l^{-1} and drug concentration in the range of 6.7×10^{-6} to 2.0×10^{-5} mol l^{-1}. Fluorescence quenching spectra in combination with Fourier transform infrared (FT-IR) spectroscopy and circular dichroism (CD) spectroscopy was used to investigate the drug-binding mode, the binding constant and the protein structure changes in the presence of daphnetin in aqueous solution. The curvature of Scatchard plot indicates that daphnetin binds to more than one class of sites on BSA. The thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) were calculated to be -24.21 kJ mol^{-1} and 19.30 J mol^{-1} K^{-1} according to van't Hoff equation, which indicated that hydrophobic and electrostatic interaction played main role in the binding of daphnetin to BSA.

Keywords: Daphnetin; Bovine serum albumin; Binding; Fluorescence quenching; FT-IR spectroscopy; CD spectroscopy

1. Introduction

Daphnetin (7,8-dihydroxycoumarin, structure shown in Fig. 1), the major bioactive components isolated from Daphne koreane Nakai, is one of coumarin derivatives that has been used in traditional Chinese medicine for the treatment of coagulation disorders. It is also a chelator, an antioxidant and a protein kinase inhibitor [1].

Bovine serum albumin (BSA), one of the major components in the plasma protein, is a single-chain 582 amino acid globular nonglycoprotein cross-linked with 17 cystine residues (8 disulfide bonds and 1 free thiol). It is divided into three linearly arranged, structurally distinct, and evolutionarily related domains (I–III); each domain is composed of two subdomains (A and B) [2–5]. BSA has two tryptophans, embedded in two different domains: Trp-134, located in

* Corresponding author. Tel.: +86-931-8912540; fax: +86-931-8912582.
E-mail address: huzd@lzu.edu.cn (Z. Hu).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.02.010

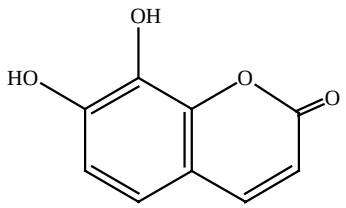

Fig. 1. The chemical structure of daphnetin.

proximity of the protein surface, but buried in a hydrophobic pocket of domain I, and Trp-214, located in an internal part of domain II [6]. Like other serum albumins, BSA has a wide range of physiological functions involving the binding, transport, and delivery of fatty acids, porphyrins, bilirubin, tryptophan, thyroxine, and steroids. It is home to specific binding sites for metals, pharmaceuticals, and dyes [2–5]. It is well known that many of drugs are bound to serum proteins, especially serum albumin. The effectiveness of drugs depends on their binding ability. So the studies on the binding of drugs to BSA may provide information of the structural features that determine the therapeutic effectiveness of drugs, and become an important research field in life sciences, chemistry and clinical medicine.

In a series of study methods concerning the interaction between drugs and protein, fluorescence techniques are great aids in the study of interactions between drugs and plasma proteins in general and serum albumin in particular because of their high sensitivity, rapidity and ease of implementation. FT-IR, a powerful technique for the study of hydrogen bonding, has recently become very popular for structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments. For secondary-structure analysis of proteins, circular dichroism (CD) spectroscopy is a technique used most frequently. So far, many studies have been carried out to investigate the interaction of proteins with drugs by fluorescence technique [7,8], FT-IR [9,10], CD spectroscopy [11], but there is lack of information on the daphnetin–BSA binding mode, the binding constant, and the effects of daphnetin complexation on the protein secondary structure.

In this paper, we report the fluorescence, CD and FT-IR spectroscopic results on the binding of daphnetin to BSA, in aqueous solutions at physiological pH, using drug concentration of 6.7×10^{-6} to 2.0×10^{-5} mol l^{-1} with final BSA concentration of 1.5×10^{-6} mol l^{-1}. Spectroscopic evidence regarding the drug binding mode, the association constant and the change of protein secondary structure are provided here.

2. Materials and methods

2.1. Materials

Bovine serum albumin was purchased from Sino-American Biotechnology Company (China). All BSA solution were prepared in the pH 7.40 buffer solution, and BSA stock solution was kept in the dark at 4 °C. Daphnetin was purchased from the National Institute for Control of Pharmaceutical and Bioproducts (China), and daphnetin stock solution was prepared in methanol. NaCl (analytical grade, 1.0 mol l^{-1}) solution was used to maintain the ion strength at 0.1. Buffer (pH 7.40) consists of Tris (0.2 mol l^{-1}) and HCl (0.1 mol l^{-1}), and the pH was adjusted to 7.40 by adding 0.5 mol l^{-1} NaOH when the experiment temperature was higher than 296 K. The pH was checked with a suitably standardized pH meter.

2.2. Apparatus and methods

Fluorescence spectra were measured with a RF-5301PC spectrofluorophotometer (Shimadzu), using 5 nm/5 nm slit widths. The excitation wavelength was 285 nm, and the emission was read at 300–500 nm.

Fluorometric experiments: 3.0 ml solution containing appropriate concentration of BSA was titrated by successive additions of a 1.0×10^{-3} mol l^{-1} methanol stock solution of daphnetin (to give final concentration of 6.7×10^{-6} to 2.0×10^{-5} mol l^{-1}). Titrations were done manually by using trace syringes, and the fluorescence intensity was measured (excitation at 285 nm and emission at 345 nm). All experiments were measured at three temperatures (296, 303, 310 K). The temperature of sample was kept by recycle water throughout experiment. The data herein obtained

were analyzed by the modified Stern–Volmer equation and Scatchard equation to calculate the binding constants.

Circular dichroism measurements were made on a Jasco-20c automatic recording spectropolarimeter (Japan) in cell of pathlength 2 mm at room temperature. CD spectra (200–350 nm) were taken at a BSA concentration of 1.5×10^{-6} mol l^{-1}, and the results are expressed as molar ellipticity ([θ]) in deg cm^2 dmol^{-1}. The α-helical content of BSA was calculated from the [θ] value at 208 nm using the equation % helix = $\{(-[\theta]_{208}-4000)/(33\,000-4000)\}\times100$ as described by Lu et al. [12].

FT-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (America) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the attenuated total reflection (ATR) method with resolution of 4 cm^{-1} and 60 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition. Then, subtract the absorbance of buffer solution from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm^{-1} was featureless [13].

3. Results and discussion

3.1. Binding of daphnetin to BSA

To investigate whether daphnetin binds to BSA, fluorescence measurements are carried out. The conformational changes of BSA were evaluated by the measurement of intrinsic fluorescence intensity of protein before and after addition of drug. Fluorescence measurements give information about the molecular environment in a vicinity of the chromophore molecules. Fig. 2 shows the typical fluorescence spectra of BSA in the absence and presence of daphnetin at pH 7.40. Daphnetin causes a concentration-dependent quenching of the intrinsic fluorescence of BSA, without changing the emission maximum, indicating that daphnetin binds to BSA without altering the local dielectric environment.

Further experiments were carried out with CD and FT-IR technique to verify the binding of daphnetin to BSA. As Fig. 3 showed, CD spectra of BSA exhibit two negative bands in the ultraviolet region at 208 and 219 nm, characteristic of an α-helical structure of protein. The binding of daphnetin to BSA decreases both of these bands, clearly indicating the decrease of the α-helical content in protein. However, the CD spectra of BSA in the presence and absence of daphnetin

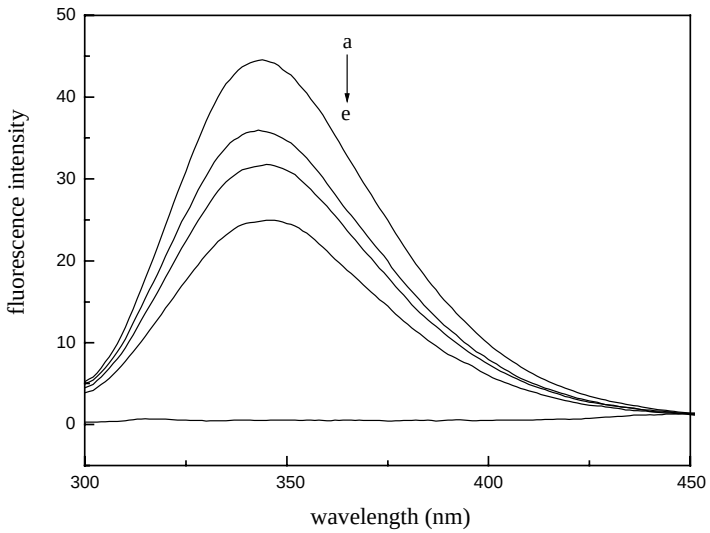

Fig. 2. Fluorescence emission spectra excited at 285 nm (pH = 7.40) (a) 1.0×10^{-5} mol l^{-1} BSA; (b–d) 1.0×10^{-5} mol l^{-1} BSA in the presence of 3.0×10^{-6}, 9.0×10^{-6}, 1.5×10^{-5} mol l^{-1} daphnetin, respectively; (e) 9.0×10^{-6} mol l^{-1} daphnetin.

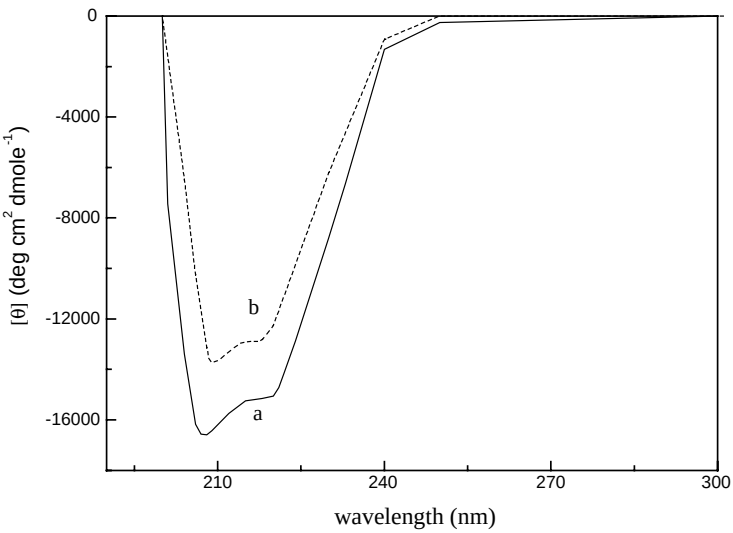

Fig. 3. CD Spectra of the BSA–daphnetin system. (a) 1.5×10^{-6} mol l^{-1} BSA; (b) 1.5×10^{-6} mol l^{-1} BSA + 4.5×10^{-6} mol l^{-1} daphnetin.

are similar in shape, indicating that the structure of BSA after daphnetin binding to BSA is also predominantly α-helical. The loss in protein helicity (71% content in native BSA and 59% content after daphnetin binding to BSA) is evidence that the binding of daphnetin to BSA may induce some conformational changes.

Fig. 4 is the FT-IR spectra of the daphnetin-free and daphnetin-bound form of BSA with its difference absorption spectrum. The spectrum in Fig. 4a was obtained by subtracting the absorption of Tris buffer from the spectrum of protein solution. Difference spectrum in present paper (Fig. 4b) was obtained by subtracting the spectrum of the daphnetin-free form from that of the daphnetin-bound form. The protein amide bands have a relationship with the secondary structure of protein. The evident peak shift of amide band from 1558.23 to 1538.94 cm^{-1} in Fig. 4b indicate that the secondary structure of BSA is changed when daphnetin was added. That is, there is an interaction between daphnetin and BSA.

3.2. Quenching mechanism and binding constant

Data from the fluorescence experiments can be analyzed using modified Stern–Volmer equation [14]:

$$\frac{F_0}{F_0 - F} = \frac{1}{fK[Q]} + \frac{1}{f} \quad (1)$$

where F and F_0 are current and initial fluorescence, respectively; K is the Stern–Volmer quenching constant and $[Q]$ is quencher concentration; f is the fraction of the initial fluorescence which is accessible to quencher. The plots of $F_0/(F_0 - F)$ versus $1/[Q]$ (Fig. 5) yields f^{-1} as the intercept, and $(fK)^{-1}$ as the slope. The results are shown in Table 1. The intercept on y-axis (f^{-1}) is 1.3, indicating that 77% of the total fluorescence of BSA is accessible to quencher. When

Table 1
Binding constants, numbers of binding site per one BSA molecule (n) and thermodynamic parameters for the binding of daphnetin to BSA

T (K)	K ($\times 10^3$ l mol^{-1})	K' ($\times 10^3$ l mol^{-1})		n		f^{-1}	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔH (kJ mol^{-1})
		K_1	K_2	n_1	n_2				
296	192.53	225.48	6.69	0.75	0.37	1.2	−29.89		
303	148.48	156.91	2.12	0.76	0.46	1.2	−30.06	19.30	−24.21
310	123.54	131.57	1.79	0.78	0.62	1.2	−30.19		

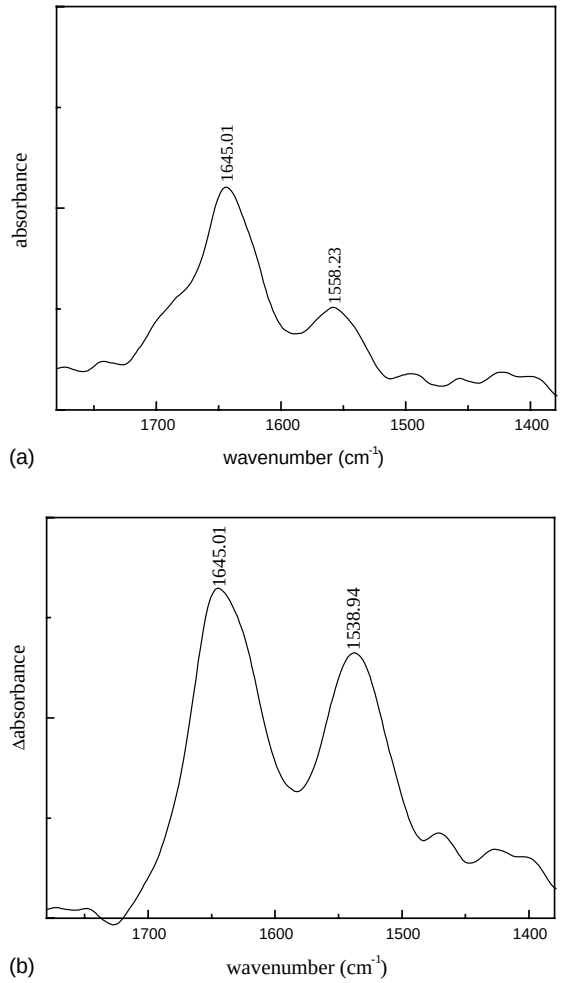

Fig. 4. FT-IR spectra and difference spectra of BSA in aqueous solution (a) FT-IR spectrum of BSA; (b) FT-IR difference spectrum of BSA obtained by subtracting the spectrum of the daphnetin-free form from that of the daphnetin-bound form in the region of 1780–1380 cm^{-1} at physiological pH (BSA: 3.0×10^{-5} mol l^{-1}; daphnetin: 9.0×10^{-5} mol l^{-1}).

$f = 1$, all the tryptophan residues are accessible to quencher. As Table 1 showed, the quenching constant decreases with increasing temperature, a characteristic that coincides with the static type of quenching mechanism. The quenching constant can be interpreted as the association constant or binding constant of the complexation reaction because static quenching arises from the formation of a dark complex between fluorophore and quencher [15].

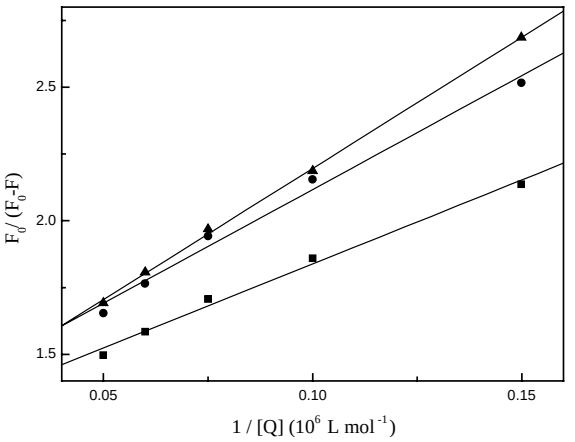

Fig. 5. The binding constant, K, of the BSA–daphnetin. (λ_{ex} = 285 nm, λ_{em} = 345 nm, pH = 7.40, $C_{BSA} = 1.5 \times 10^{-6}$ mol l^{-1}, $C_{daphnetin} = 6.7 \times 10^{-6}$ to 2.0×10^{-5} mol l^{-1}) at 296 K (squares); 303 K (circles); 310 K (triangles).

The binding constants were also calculated according to Scatchard equation. Fig. 6 shows the Scatchard plots for the daphnetin–BSA system. The curvature of the Scatchard plot indicates that daphnetin binds to more than one class of sites and that the simple form of the Scatchard equation is no longer valid. The data were analyzed according to the methods in Ref. [16]. The results (K', n) were summarized in Table 1. In Table 1, the binding constants obtained by two kinds of method are large; indicating the binding reaction of daphnetin to BSA is strong. Thus, daphnetin can be stored and removed by protein. In this paper, the binding constants obtained with modified Stern–Volmer equation were applied in the discussion of binding mode.

3.3. Binding modes

Small molecules are bound to macromolecule by four binding modes: H-bonds, van der Waals, electrostatic and hydrophobic interactions. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS) of reaction, are important for confirming binding mode. For this purpose, the temperature-dependence of the binding constant was studied. The temperatures chosen were 296, 303 and 310 K at which BSA does not undergo any structural degradation. By plotting the binding constants (K values in Table 1) according

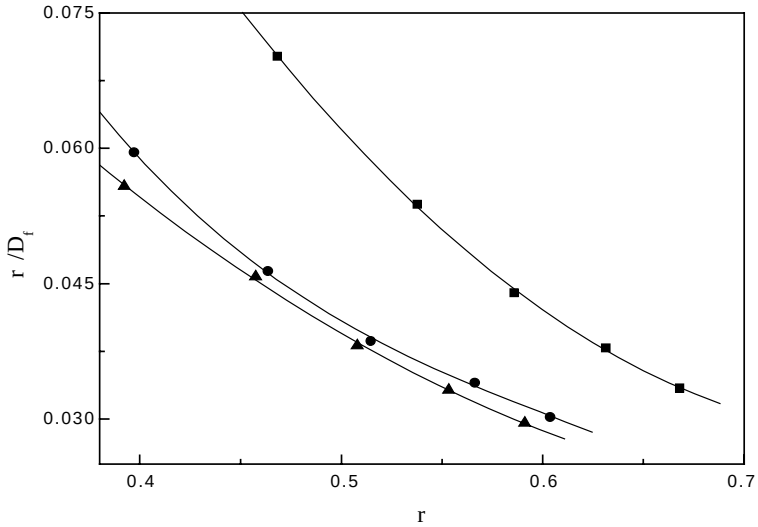

Fig. 6. The Scatchard plots of the BSA–daphnetin. (λ_{ex} = 285 nm, λ_{em} = 345 nm, pH = 7.40, $C_{BSA} = 1.5 \times 10^{-6}$ mol l^{-1}, $C_{daphnetin} = 6.7 \times 10^{-6}$ to 2.0×10^{-5} mol l^{-1}) at 296 K (squares); 303 K (circles); 310 K (triangles).

to van't Hoff equation, the thermodynamic parameters were determined from linear van't Hoff plot (not shown) and listed in Table 1. As shown in Table 1, the formation of daphnetin–BSA coordination compound is an exothermic reaction accompanied by positive ΔS value. Nemethy and Scheraga [17], Timasheff [18] and Ross and Subramanian [19] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes, as described below. From the point of view of water structure, a positive ΔS value is frequently taken as evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS and a negative ΔH value. Accordingly, it is not possible to account for the thermodynamic parameters of daphnetin–BSA coordination compound on the basis of a single intermolecular force model. It is more likely that hydrophobic, electrostatic interactions are involved in its binding process. For daphnetin–BSA binding system, daphnetin is half-ionized under the experiment conditions (pH 7.40) in view of its pK_a value, 7.36 [20]; so electrostatic interaction cannot be excluded from the binding. That is, daphnetin bound to BSA was mainly based on the hydrophobic and electrostatic interactions.

4. Conclusions

Studies on a BSA fluorescence quenching reaction have been presented. The results show that daphnetin is a strong quencher and binds to BSA with high affinity. This study shows that daphnetin quenches the intrinsic fluorescence of BSA through static quenching mode and the binding of daphnetin to BSA is predominantly owing to hydrophobic and electrostatic interactions. In addition, the results of CD, FT-IR also show that the secondary structure of BSA is changed after daphnetin bound to BSA.

References

[1] E.B. Yang, Y.N. Zhao, K. Zhang, P. Mark, Biochem. Biophys. Res. Commun. 260 (1999) 682–685.
[2] K.F. Brown, M.J. Crooks, Biochem. Pharmacol. 25 (1976) 1175–1178.
[3] V.M. Rosenoer, M. Oratz, M.A. Rothschild (Eds.), Albumin Structure, Function and Uses, Pergamon Press, New York, 1977.
[4] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[5] T. Peters, All About Albumin: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego, CA, 1995.
[6] Y. Moriyama, D. Ohta, K. Hachiya, Y. Mitsui, K.J. Takeda, Protein Chem. 15 (1996) 265–271.
[7] J.Q. Liu, J.N. Tian, J.Y. Zhang, Z.D. Hu, X.G. Chen, Anal. Bioanal. Chem. 376 (2003) 864–867.

[8] J.N. Tian, J.Q. Liu, J.Y. Zhang, Z.D. Hu, X.G. Chen, Chem. Pharm. Bull. 51 (2003) 579–582.
[9] J.F. Neault, H.A. Tajmir-Riahi, Biochim. Biophys. Acta 1384 (1998) 153–159.
[10] J.F. Neault, A. Novetta-Delen, H.A. Tajmir-Riahi, J. Biomol. Struct. Dyn. 17 (1999) 101–106.
[11] J.M. Chamouard, J. Barre, S. Urien, G. Houin, J.P. Tillement, Biochem. Pharmacol. 34 (1985) 1695–1700.
[12] Z.X. Lu, T. Cui, Q.L. Shi, Applications of Circular Dichroism (CD) and Optical Rotatory Dispersion (ORD) in Molecular Biology, 1st ed., Science Press, Beijing, 1987.
[13] A. Dong, P. Huang, W.S. Caughey, Biochemistry 29 (1990) 3303–3305.
[14] J.R. Lakowica, Principles of Fluorescence Spectroscopy, 2nd ed., Kluwer Academic Publishers/Plenum Press, New York, 1999.
[15] M.R. Eftink, C.A. Ghiron, J. Phys. Chem. 80 (1976) 486–493.
[16] A. Suarez Varela, M.I. Sandez Macho, J. Minones, J. Pharm. Sci. 81 (1992) 842–844.
[17] G. Nemethy, H.A. Scheraga, J. Phys. Chem. 66 (1962) 1773–1789.
[18] S.N. Timasheff, in: H. Peeters (Ed.), Proteins of Biological Fluids, Pergamon Press, Oxford, 1972, pp. 511–519.
[19] P.D. Ross, S. Subramanian, Biochemistry 20 (1981) 3096–3102.
[20] N. Sauerwald, M. Schwenk, J. Polster, E. Bengsch, Z. Naturforsch. B 53 (1998) 315–321.