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Spectroscopic analyses on interaction of o-Vanillin-D-Phenylalanine, o-Vanillin-L-Tyrosine and o-Vanillin-L-Levodopa Schiff Bases with bovine serum albumin (BSA)

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ABSTRACT

In this work, three o-Vanillin Schiff Bases (o-VSB: o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL)) with alanine constituent were synthesized by direct reflux method in ethanol solution, and then were used to study the interaction to bovine serum albumin (BSA) molecules by fluorescence spectroscopy. Based on the fluorescence quenching calculation, the bimolecular quenching constant (K_q), apparent quenching constant (K_{sv}), effective binding constant (K_A) and corresponding dissociation constant (K_D) as well as binding site number (n) were obtained. In addition, the binding distance (r) was also calculated according to Foster's non-radioactive energy transfer theory. The results show that these three o-VSB can efficiently bind to BSA molecules, but the binding array order is o-VDP-BSA > o-VLT-BSA > o-VLL-BSA. Synchronous fluorescence spectroscopy indicates that the o-VDP is more accessibility to tryptophan (Trp) residues of BSA molecules than to tyrosine (Tyr) residues. Nevertheless, the o-VLT and o-VLL are more accessibility to Tyr residues than to Trp residues.

1. Introduction

Aromatic Schiff Bases are a large class of organic compounds being from condensation reaction of aromatic aldehyde and amine [1–3]. The –HC═N– group and special plane structure make Aromatic Schiff Bases have the function of antibacterial, antitumour and antivirotic activities [4–6]. Therefore, more and more Aromatic Schiff Bases have already been researched purposively and developed as potential antibacterial, antitumour and antivirotic drugs in recent years [7–14]. 2-Hydroxy-3-methoxybenzaldehyde (o-Vanillin) is a kind of important chemical-industrial raw material [15–17]. Because of many biological activities, such as analgesic, anti-inflammatory, antibacterial, sterilizing and antiviral activities [18–23], o-Vanillin is a useful medicine chemical engineering and organic synthesis midbody [24–27]. In addition, it also can be used as efficient herbicide, pesticide and bactericide [28–31]. Hence, o-Vanillin is a optimal candidate for synthesizing various Aromatic Schiff Bases with important bioactivities.

However, for a long time many researches were only focused on the bioactivities of aromatic aldehyde and –HC═N– group in Aromatic Schiff Bases, and did not pay attention to their targeting to biological tissue [8]. Although the Aromatic Schiff Bases display obvious bioactivities in experimental stage, they are usually unsatisfactory in animal studies or clinical stage due to a lack of targeting. It has been well known that the amino acids are main component element of various proteins. They generally play an important physiological role in life process [32–34]. Because of special three-dimensional geometric configuration, all amino acids also exhibit the perfect identification and selection abilities to biological tissue [35–37]. In order to further investigate the structure, composition and chemical and biologic activities of Aromatic Schiff Bases, it is necessary to adopt some amino acids as amine part for synthesizing new o-Vanillin Aromatic Schiff Bases. Hence, in this work, three amino acids, such as 3-phenyl-D-alanine (D-Phenylalanine), 3-(4-hydroxyphenyl)-L-alanine (L-Tyrosine) and 3-(3,4-dihydroxyphenyl)-L-alanine (L-Levodopa), were used to react with o-Vanillin and three new o-Vanillin Schiff Bases (o-VSB: o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL)) were synthesized. They would display both natural biological activities and identification and selection abilities to biological molecules.

Apart from deoxyribonucleic acid (DNA), many functional proteins could also been considered as a target or carrier in various drug designs. It has been well known that the serum albumins are the most abundant proteins in the plasma [38–40]. It also has many physiological functions. In the present work, the bovine serum albumin (BSA) was chosen as a target protein molecule because of its low cost, ready availability and unusual ligand-binding prop-

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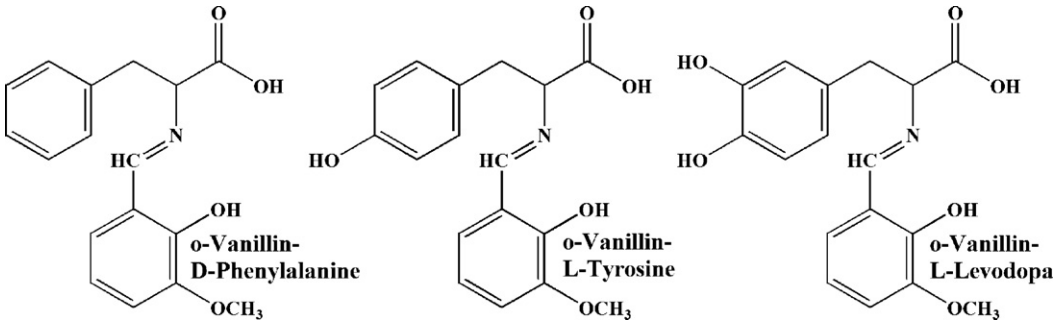

Fig. 1. Molecular structure of o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL).

erties [41–44]. And what's more, the whole structures of BSA molecule are similar to human serum albumin (HSA) molecule in 76%, so the results of all the studies are considered to be consistent with the facts that used HSA as the study model [45]. In this study, the quenching of intrinsic fluorescence of proteins was used to research the interaction between BSA molecules and three synthesized o-VSB (o-VDP, o-VLT and o-VLL). Based on the obtained parameters, the binding degrees were compared. Meanwhile, the influences of substituent group on the interaction were also discussed. Besides, the binding sites of o-VSB (o-VDP, o-VLT and o-VLL) to BSA molecules were also explored by synchronous fluorescence spectroscopy. The molecular structures of three synthesized o-VSB are shown in Fig. 1.

2. Experimental

2.1. Reagents

Commercially prepared bovine serum albumin (BSA, purity > 99.0%) was purchased from Beijing Abxing Biological Technology Company and stored in refrigerator at 4.0 °C. 2-Hydroxy-3-methoxybenzaldehyde (o-Vanillin, analytical reagent grade), 3-phenyl-D-alanine (L-Phenylalanine), 3-(4-hydroxyphenyl)-L-alanine (L-Tyrosine, analytical reagent grade), 3-(3,4-dihydroxyphenyl)-L-alanine (L-Levodopa, analytical reagent grade) were procured from Tianjing Tianhe Chemical Reagent Co., Ltd. o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) Schiff Bases (o-VSB) were synthesized through refluxing, distillation and filtration of mixed solution of o-Vanillin with D-Phenylalanine, L-Tyrosine and L-Levodopa, respectively. All other reagents were commercial products of analytical grade and used as received. The Tris (hydroxyl-methyl) aminomethane (Tris), HCl and NaCl were all of analytical reagent grade, and double distilled water was used for all solution preparation.

2.2. Apparatus

The fluorescence measurements were performed with fluorophotometer (Cary 300, Varian Company, USA) and the UV–vis absorption spectra were recorded with UV–vis spectrophotometer (Cary 50, Varian Company, USA). The compositions of o-Vanillin Schiff Bases (o-VSB) were determined by using elementalanalyser (Perkin-Elmer 2400, PerkinElmer Company, USA). And their structures were analyzed by Fourier transform infrared spectrophotometer (Spectrum 100, Perkin-Elmer Company, USA). The solution pH value was measured with pH meter (PHS-3C, Shanghai Leici Instrument Company, Ltd, China).

2.3. Syntheses of o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) Schiff Bases

1.6519 g (0.01 mol) D-Phenylalanine and 0.5611 g (0.01 mol) KOH were added to 50 mL ethanol solution in a 250 mL three-neck flask and stirred for 3.0 h. And then, during reflux at 85 °C in oil bath, 50 mL o-Vanillin ethanol solution (containing 1.5215 g (0.02 mol) o-Vanillin) was dropwise added to above D-Phenylalanine ethanol solution. After 3.0 h reflux, the mixed solution of D-Phenylalanine and o-Vanillin were concentrated to 10 mL through reduced pressure distillation at 65 °C. After naturally cooling to room temperature, the o-Vanillin-D-Phenylalanine (o-VDP) (yellow microcrystals) Schiff Base appeared. The sample was filtrated, washed with ethanol three times and dried at 120 °C to constant weight.

o-Vanillin-L-Tyrosine (o-VLT) (deep yellow microcrystals) and o-Vanillin-L-Levodopa (o-VLL) (yellow brown microcrystals) Schiff Bases were also synthesized according to the similar method above mentioned. Their compositions were analyzed by elementalanalyser and the results were given in Table 1. The infrared spectra of three o-VSB (o-VDP, o-VLT and o-VLL) were also determined by using a Fourier transform infrared spectrophotometer. The corresponding results were offered in Fig. 2.

2.4. Measurement of binding parameters

For each of three synthesized o-VSB (o-VDP, o-VLT and o-VLL), the binding parameters with BSA were measured by using UV–vis and fluorescence spectroscopy. BSA and o-VSB stock solutions were prepared in Tris–HCl–NaCl buffer solution (pH = 7.40 and [Tris–HCl] = [NaCl] = 0.05 mol/L) for keeping the solution acidity and ionic strength and their concentrations were 2.00×10^{-5} mol/L

Table 1
Elementary analyses of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL.

Schiff bases	Molecular formula	Molecular weight	Melting point	C (%)		H (%)		N (%)	
o-VDP	$C_{17}H_{17}NO_4$	299.31	194-204 °C	Calcd.	68.21	Calcd.	5.72	Calcd.	4.68
				Found	68.27	Found	5.77	Found	4.63
o-VLT	$C_{17}H_{17}NO_5$	315.31	Nigger-brown above 250 °C	Calcd.	64.75	Calcd.	5.43	Calcd.	4.44
				Found	64.72	Found	5.46	Found	4.46
o-VLL	$C_{17}H_{17}NO_6$	331.31	Carbonization above 250 °C	Calcd.	61.63	Calcd.	5.17	Calcd.	4.23
				Found	61.65	Found	5.14	Found	4.27

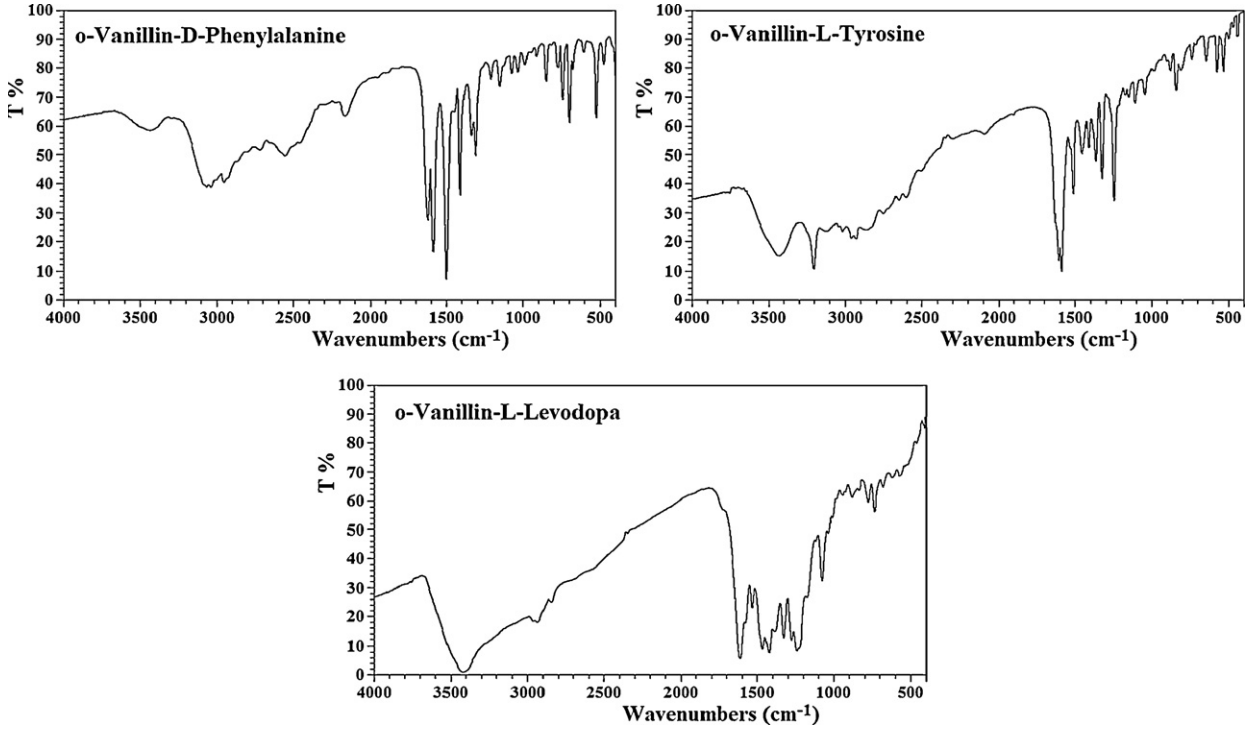

Fig. 2. Infrared spectra of o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) (with potassium bromide (KBr) pellet at 25 °C).

Table 2
Quenching constants (K_{SV} and K_q), binding constants, stable constants and binding site numbers calculated according to Stern–Volmer plots, Lineweaver–Burk plots and Double logarithm plots of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions with o-VDP, o-VLT and o-VLL concentrations (from 0.00×10^{-5} mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals) ([BSA] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL).

System	Stern–Volmer plot	R^2	K_{SV} (L/mol)	K_q (L/mol s)
BSA + o-VDP	$F/F_0 = 0.2471$ [o-VSB] + 1	0.9986	2.47×10^4	2.47×10^{12}
BSA + o-VLT	$F/F_0 = 0.1689$ [o-VSB] + 1	0.9958	1.69×10^4	1.69×10^{12}
BSA + o-VLL	$F/F_0 = 0.1332$ [o-VSB] + 1	0.9991	1.33×10^4	1.33×10^{12}

System	Lineweaver–Burk plot	R^2	f	K_{LB} (L/mol)	K_D (mol/L)
BSA + o-VDP	$1/[(F_0 - F)/F_0] = 4.5071/$[o-VSB] + 0.7149	0.9982	0.7149	1.59×10^4	6.31×10^{-5}
BSA + o-VLT	$1/[(F_0 - F)/F_0] = 6.5776/$[o-VSB] + 0.7279	0.9994	0.7279	1.11×10^4	9.04×10^{-5}
BSA + o-VLL	$1/[(F_0 - F)/F_0] = 7.9683/$[o-VSB] + 0.7609	0.9994	0.7609	0.96×10^4	10.47×10^{-5}

System	Double logarithm plot	R^2	K_A (L/mol)	n	ΔG_0 (kJ/mol)
BSA + o-VDP	$\log[(F_0 - F)/F] = 1.0509 \log$[o-VSB] + 4.6344	0.9983	4.31×10^4	1.0509	−27.50
BSA + o-VLT	$\log[(F_0 - F)/F] = 1.0587 \log$[o-VSB] + 4.4945	0.9985	3.12×10^4	1.0587	−26.67
BSA + o-VLL	$\log[(F_0 - F)/F] = 1.0352 \log$[o-VSB] + 4.2905	0.9989	1.95×10^4	1.0352	−25.46

and 5.00×10^{-5} mol/L, respectively. In a 25.00 mL volumetric flask, 12.50 mL BSA stock solution and appropriate volume of o-VSB stock solutions were added and diluted to the mark with the same Tris–HCl–NaCl buffer solution. The final BSA concentration was 1.00×10^{-5} mol/L, and the o-VSB concentrations were varied from 0.00×10^{-5} mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals. The fluorescence spectra of BSA solutions along with the increase of o-VSB concentrations were recorded in the wavelength of 250–550 nm with excited wavelength at 280 nm and 5.0 nm/5.0 nm slit widths. All test solutions were incubated for 10 min before measurement. The curves of fluorescence quenching spectra were got at 37.00 ± 0.02 °C and given in Fig. 3. The maximal intrinsic fluorescence intensities of BSA were recorded at 348 nm for the calculation of quenching parameters. The corresponding results were shown in Fig. 4 and Table 2. The binding distances were determined according to Föster's nonradiative energy transfer theory (FRET). The spectral overlaps of fluorescence emission of BSA solution and UV–vis absorption of o-VSB solutions were all given in Fig. 5, and the corresponding parameters were offered in Table 3.

2.5. Determination of synchronous fluorescence spectra

In order to confirm the binding sites of o-VSB (o-VDP, o-VLT and o-VLL) to BSA molecules, the synchronous fluorescence spec-

Table 3
Energy transfer efficiency (E), critical binding distance (R), overlap integral (J) and binding distance (r) calculated according to Foster's non-radioactive energy transfer theory ([BSA] = [o-VDP] = [o-VLT] = [o-VLL] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL).

System	E (%)	R (nm)	J (cm^3 L/mol)	r (nm)
BSA + o-VDP	24.34	3.3083	7.12×10^{-15}	3.9967
BSA + o-VLT	11.88	3.3131	7.18×10^{-15}	4.6266
BSA + o-VLL	9.79	3.3407	7.55×10^{-15}	4.8366

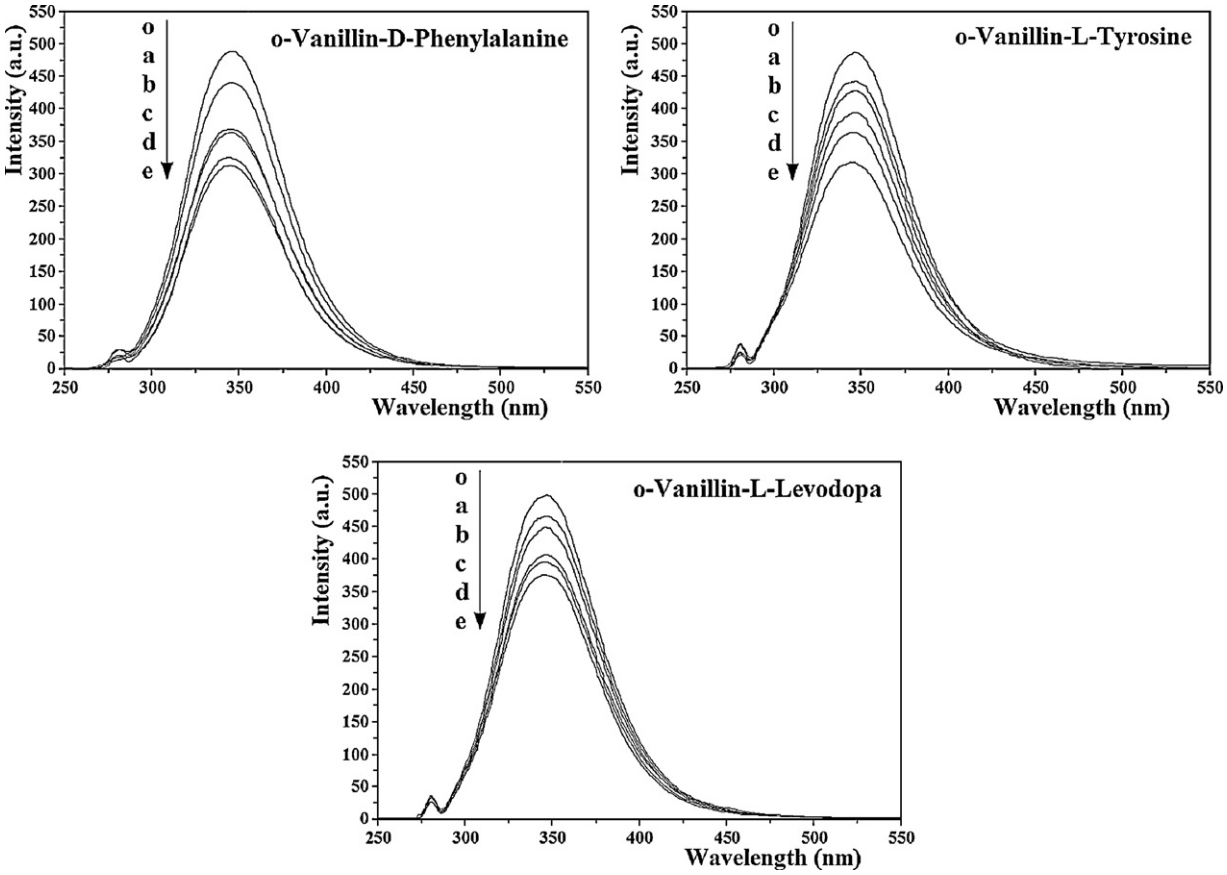

Fig. 3. Fluorescence spectra of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions with o-VDP, o-VLT and o-VLL concentrations (from 0.00×10^{-5} mol/L (o) to 2.50×10^{-5} mol/L (e) at 0.50×10^{-5} mol/L intervals) ([BSA] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL).

tra of BSA solutions along with the increase of o-VSB concentration from 250 to 350 nm for $\Delta\lambda$ = 15 nm and from 225 to 325 nm for $\Delta\lambda$ = 60 nm were determined. The corresponding results were all given in Fig. 6. And the ratios of synchronous fluorescence quenching were shown in Fig. 7.

3. Results and discussion

3.1. The infrared spectra of o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) Schiff Bases

The comparison of infrared spectra reveals the reaction between o-Vanillin and D-Phenylalanine [46,47]. From Fig. 2 it can be seen that the ν(C=O) of o-Vanillin at 1639 cm^{-1} and the $\nu_{as}(NH_3^+)$ at 3065 cm^{-1} and $\nu_s(NH_3^+)$ at 2546 cm^{-1} of D-Phenylalanine disappears, while a new peak appears at 1618 cm^{-1}. It indicates that the C=N bond of o-Vanillin-D-Phenylalanine (o-VDP) Schiff Base was formed. Similarly, the $\nu_{as}(NH_3^+)$ at 3066 cm^{-1} and $\nu_s(NH_3^+)$ at 2554 cm^{-1} for L-Tyrosine and at 3067 cm^{-1} and 2551 cm^{-1} for L-Levodopa all disappear. And that at 1634 cm^{-1} and 1638 cm^{-1} new peak occur for o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) samples, respectively. It also indicates the formation of o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) Schiff Bases. For all three o-Vanillin Schiff Bases (o-VSB) there are broad ν(OH) absorption bands around 3200 cm^{-1}, revealing the existence of OH bonds.

3.2. Fluorescence spectra of BSA + o-Vanillin-D-Phenylalanine (o-VDP), BSA + o-Vanillin-L-Tyrosine (o-VLT) and BSA + o-Vanillin-L-Levodopa (o-VLL) solutions

In general, most of protein molecules in aqueous solution intrinsically fluoresce around 348 nm (with 278 nm excitation), which is mainly attributed to the tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues [48,49]. From Fig. 3 it can be seen that the intrinsic fluorescence of bovine serum albumin (BSA) molecules also appears around 348 nm. However, for all three courses along with the addition of o-VSB (o-VDP, o-VLT and o-VLL) the intrinsic fluorescences of (BSA) are gradually quenched compared with that of pure BSA solution. Apparently, this is owing to the interaction of o-VSB with BSA molecules in aqueous solution.

3.3. Binding constant and binding site number

Being similar to many reported studies on the interaction of small molecules with BSA [50–55], the interaction of o-VSB with BSA molecules can also be investigated by measuring the change of the intrinsic fluorescence along with the increase of o-VSB concentrations. Firstly, as a hypothetical dynamic quenching process, the data of fluorescence intensities were analyzed by using the Stern–Volmer equation (1) [50]:

$$\frac{F_0}{F} = 1 + K_{SV}[\text{o-VSB}] = 1 + K_q\tau_0[\text{o-VSB}] \quad (1)$$

where the F_0 and F are the fluorescence intensities of BSA solutions at 348 nm, respectively, in the absence and presence of o-VSB with

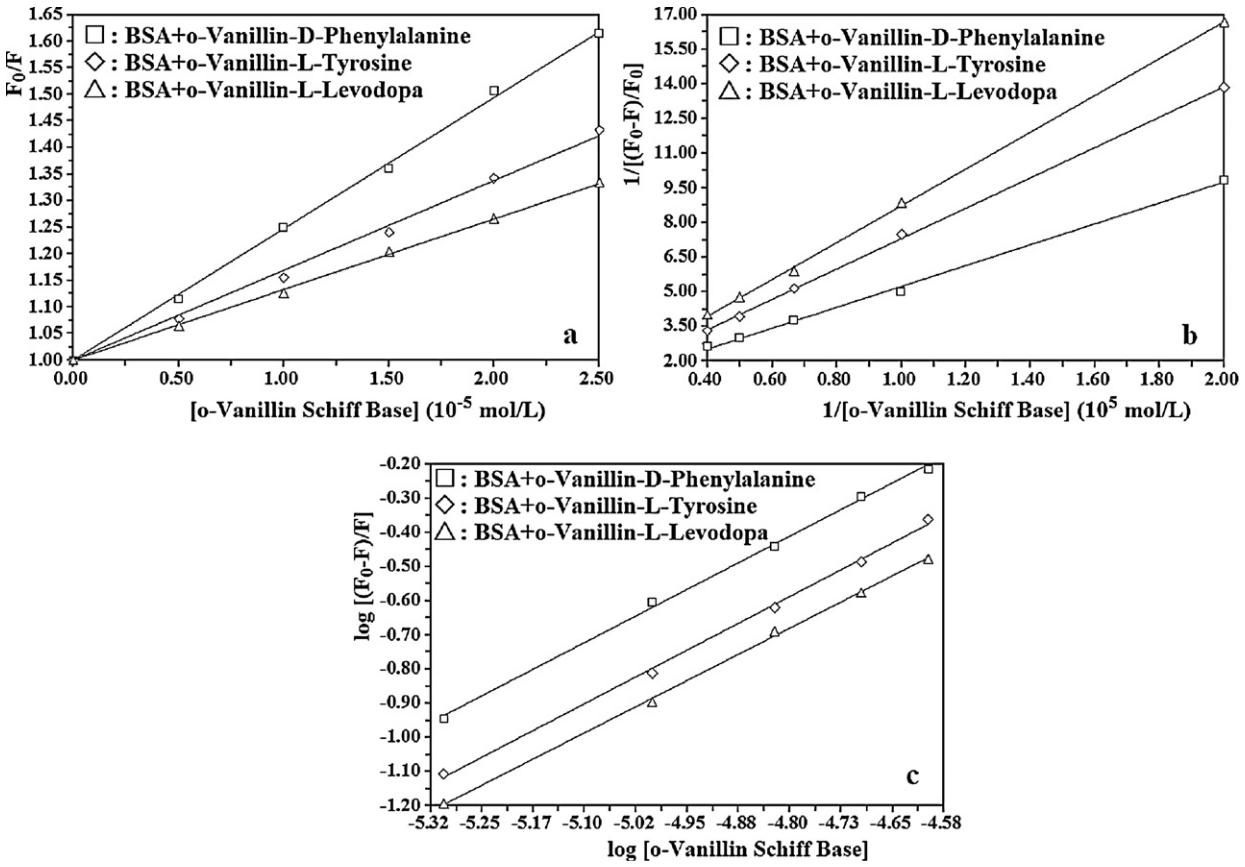

Fig. 4. Stern–Volmer plot (a), Lineweaver–Burk plot (b) and Double logarithm plot (c) of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions with o-VDP, o-VLT and o-VLL concentrations (from 0.00×10^{-5} mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals) ([BSA] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, $T_{solu} = 37.00 \pm 0.02$ °C and $V_{total} = 25.00$ mL).

various concentrations. K_q is the apparent quenching constant of bimolecular fluorescence, τ_0 is the life time of the fluorophore, K_{SV} is the Stern–Volmer fluorescence quenching constant and [o-VSB] is the concentration of o-VSB (o-VDP, o-VLT and o-VLL).

Fig. 4(a) displays the Stern–Volmer plots of BSA + o-VSB solutions. It can be found that three plots all exhibit a comparatively good linear relationship ($R^2 = 0.9986$ for BSA + o-VDP, $R^2 = 0.9958$ for BSA + o-VLT and $R^2 = 0.9991$ for BSA + o-VLL). The corresponding plot expressions were shown in Table 2. According to Stern–Volmer equation (1), for these three systems their K_{sv} could be obtained ($K_{sv} = 2.47 \times 10^4$ L/mol for BSA + o-VDP, $K_{sv} = 1.69 \times 10^4$ L/mol for BSA + o-VLT and $K_{sv} = 1.33 \times 10^4$ L/mol for BSA + o-VLL). In general, for the most of protein molecules, the τ_0 is known to be approximately 10^{-8} s. Therefore, based on the $K_q = K_{sv}/\tau_0$, the K_q could be calculated ($K_q = 2.47 \times 10^{12}$ L/mol s for BSA + o-VDP, $K_q = 1.69 \times 10^{12}$ L/mol s for BSA + o-VLT and $K_q = 1.33 \times 10^{12}$ L/mol s for BSA + o-VLL). It can be seen that all of the above K_q values were greater than the maximum value (2.00×10^{10} L/mol s) of the diffusion controlled quenching process of biological macromolecules. It provides the preliminary evidences that the dominating quenching mechanism is not dynamic but static. The array order is $K_{q(BSA+o\text{-}VDP)} > K_{q(BSA+o\text{-}VLT)} > K_{q(BSA+o\text{-}VLL)}$, which indicates that the interaction degree of o-VSB to BSA relates to the amino acid parts in o-VSB Schiff Bases.

For reconfirming the static fluorescence quenching mechanisms of o-VSB (o-VDP, o-VLT and o-VLL) to BSA, the data of fluorescence quenching are analyzed again according to the Lineweaver–Burk (modified Stern–Volmer or double reciprocal) equation (2).

$$\frac{1}{(F_0 - F)/F_0} = \frac{1}{fK_{LB}[\text{o-VSB}]} + \frac{1}{f} \quad (2)$$

The F_0 and F are also the fluorescence intensities of BSA solutions at 348 nm in the absence and presence of o-VSB (o-VDP, o-VLT and o-VLL) with various concentrations. The f is the fraction of accessible fluorescence, and the K_{LB} is the static fluorescence quenching association constant. From the corresponding plots in Fig. 4(b), the f and K_{LB} could be obtained ($f = 0.7149$ and $K_{LB} = 1.59 \times 10^4$ L/mol for BSA + o-VDP, $f = 0.7279$ and $K_{LB} = 1.11 \times 10^4$ L/mol for BSA + o-VLT and $f = 0.7609$ and $K_{LB} = 0.96 \times 10^4$ L/mol for BSA + o-VLL). Correspondingly, the dissociation constants (K_D) are 6.31×10^{-5} mol/L for BSA + o-VDP, 9.04×10^{-5} mol/L for BSA + o-VLT and 10.47×10^{-5} mol/L for BSA + o-VLL. In addition, from Table 2, it could be found that all of these Lineweaver–Burk plots have a better linear relationship ($R^2 = 0.9982$ for BSA + o-VDP, $R^2 = 0.9994$ for BSA + o-VLT and $R^2 = 0.9994$ for BSA + o-VLL) than the corresponding Stern–Volmer plots. Thus, it could be confirmed again that the fluorescence quenching mechanism of o-VSB (o-VDP, o-VLT and o-VLL) to BSA is mainly a static quenching procedure indeed. Just like the results calculated by Stern–Volmer equation, the K_{LB} array order is $K_{LB(BSA+o\text{-}VDP)} > K_{LB(BSA+o\text{-}VLT)} > K_{LB(BSA+o\text{-}VLL)}$. And that the K_D array order is $K_{D(BSA+o\text{-}VDP)} < K_{D(BSA+o\text{-}VLT)} < K_{D(BSA+o\text{-}VLL)}$.

From Fig. 4(c), the equilibrium constants (K_A) and the binding site numbers (n) could be calculated by using the Double logarithm equation (3):

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_A + n \log[\text{o-VSB}] \quad (3)$$

Apparently, the K_A and n could be measured, respectively, from the intercept and slope obtained through plotting $\log[(F_0 - F)/F]$ against log[o-VSB]. It could be seen that the plots also exhibited a good linear relationship ($R^2 = 0.9983$ for BSA + o-

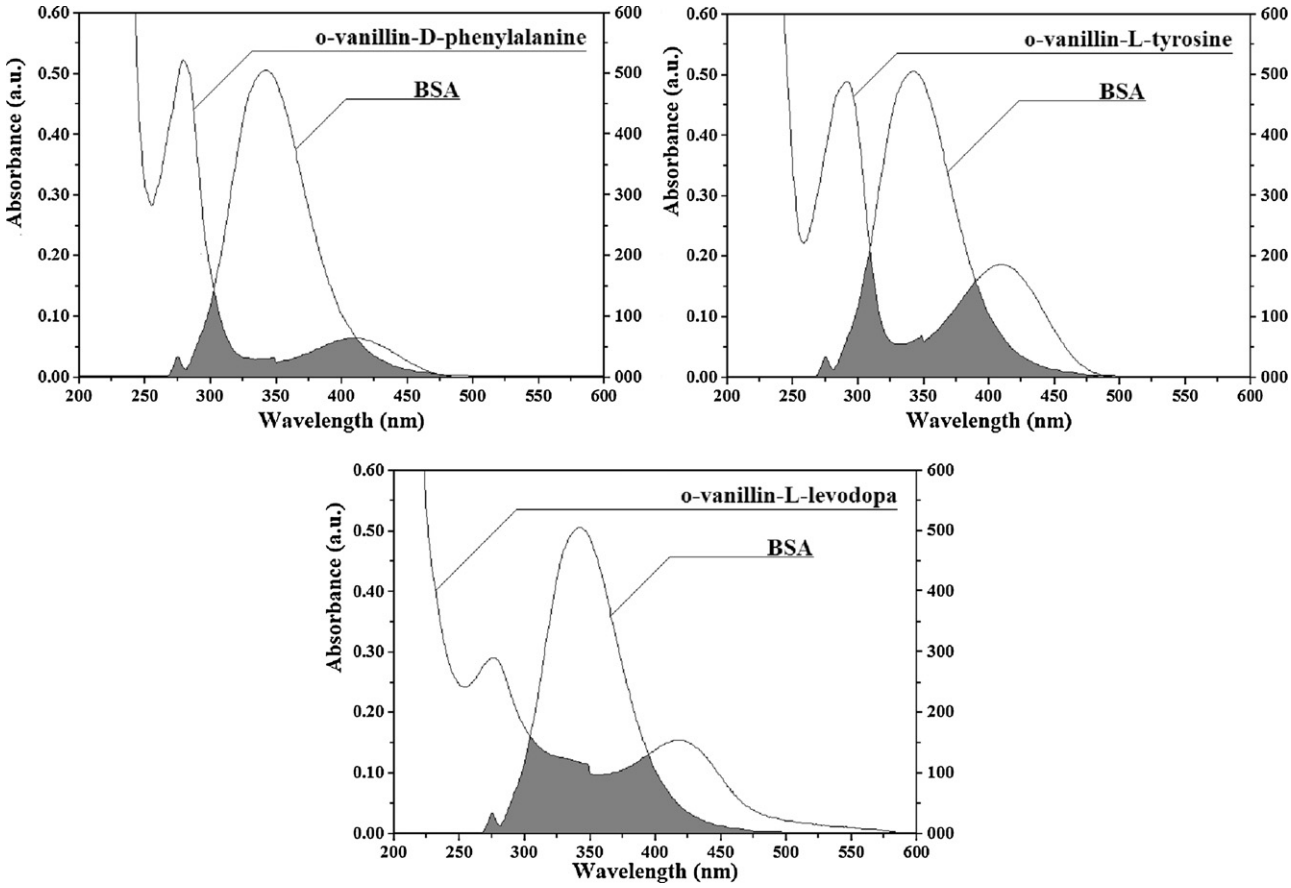

Fig. 5. Spectral overlap of fluorescence (λ_{ex} = 278 nm) of BSA solution and absorption of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions ([BSA] = [o-VDP] = [o-VLT] = [o-VLL] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, $T_{solu} = 37.00 \pm 0.02$ °C and V_{total} = 25.00 mL).

VDP, $R^2 = 0.9985$ for BSA + o-VLT and $R^2 = 0.9989$ for BSA + o-VLL). Further, $K_A = 4.31 \times 10^4$ L/mol and $n = 1.0509$ for BSA + o-VDP, $K_A = 3.12 \times 10^4$ L/mol and $n = 1.0587$ for BSA + o-VLT and $K_A = 1.95 \times 10^4$ L/mol and $n = 1.0352$ for BSA + o-VLL were obtained. Obviously, being similar to the K_{LB}, the same array order is $K_{A(BSA+o\text{-}VDP)} > K_{A(BSA+o\text{-}VLT)} > K_{A(BSA+o\text{-}VLL)}$. It illustrates that the increase of hydroxyl group number on the benzene ring weakens the interaction of o-VLT and o-VLL with BSA molecules. Maybe, it is because that the hydroxyl group not only hinders the aromatic ring stacking but also decreases the electrostatic bonding between o-VLT and o-VLL with BSA molecules. Even though, the three K_A indicate that the bindings can be formed more easily between BSA and o-VSB (o-VDP, o-VLT and o-VLL). The three n values are equal to about 1, indicating that there is one class of binding site for these three o-VSB to BSA molecule.

Utilizing K_A, the free energy change (ΔG_0) can be calculated from the relationship (4):

$$\Delta G_0 = -RT \ln K_A \tag{4}$$

Here, $R = 8.314$ J/mol K and $T = 310$ K were fixed, and the ΔG_0 are −27.50 kJ/mol for BSA + o-VDP, −26.67 kJ/mol for BSA + o-VLT and −25.46 kJ/mol for BSA + o-VLL could calculated. The negative sign for ΔG_0 indicates the binding spontaneity of three o-VSB (o-VDP, o-VLT and o-VLL) to BSA molecules. Obviously, the same array order is found to be $\Delta G_{0(BSA+o\text{-}VDP)} < \Delta G_{0(BSA+o\text{-}VLT)} < \Delta G_{0(BSA+o\text{-}VLL)}$. It demonstrates that the o-VDP binds more easily with BSA molecules.

3.4. Binding distances between BSA with o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL) Schiff Bases

In the preceding paragraph, the interaction of three o-VSB (o-VDP, o-VLT and o-VLL) with BSA molecules could be speculated through the regular fluorescence quenching of BSA solutions along with the increase of o-VSB concentration. The binding distances between BSA and o-VSB (o-VDP, o-VLT and o-VLL) can be determined according to Foster's non-radioactive energy transfer theory [51,52]. Based on this theory, the efficiency (E) of energy transfer between donor (BSA) and acceptor (o-VDP, o-VLT and o-VLL) can be calculated by Eq. (5):

$$E = \frac{1}{1 + (r/R_0)^6} \tag{5}$$

where r is the binding distance between donor and acceptor, and R_0 is the critical binding distance when the efficiency (E) of energy transfer is 50%, which can be calculated by Eq. (6):

$$R_0^6 = 8.8 \times 10^{-25}(K^2 n^{-4} \varphi_D J) \tag{6}$$

where the K^2 is the spatial orientation factor of the dipole, n is the refractive index of medium, φ_D is the quantum yield of the donor in the absence of acceptor and J is the overlap integral of the emission spectrum of the donor and the absorption spectrum of the acceptor. In the present case, K^2, n and φ_D are 2/3, 1.336 and 0.15, respectively, for BSA [53]. And then, the J can be calculated by Eq.

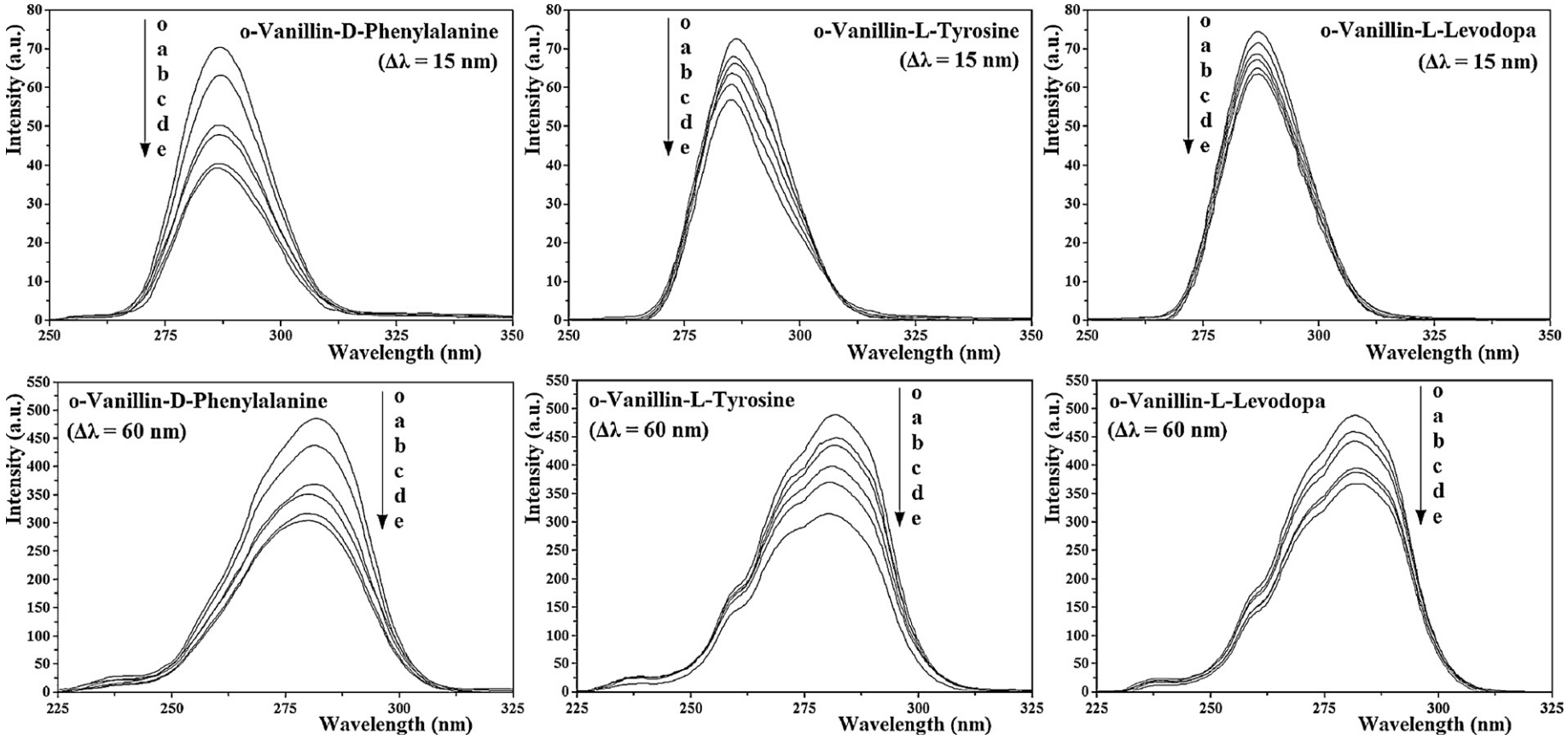

Fig. 6. Synchronous fluorescence spectra of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions with o-VDP, o-VLT and o-VLL concentrations (from 0.00×10^{-5} mol/L (o) to 2.50×10^{-5} mol/L (e) at 0.50×10^{-5} mol/L intervals) ([BSA] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, $T_{solu} = 37.00 \pm 0.02\,^{\circ}C$ and $V_{total} = 25.00$ mL).

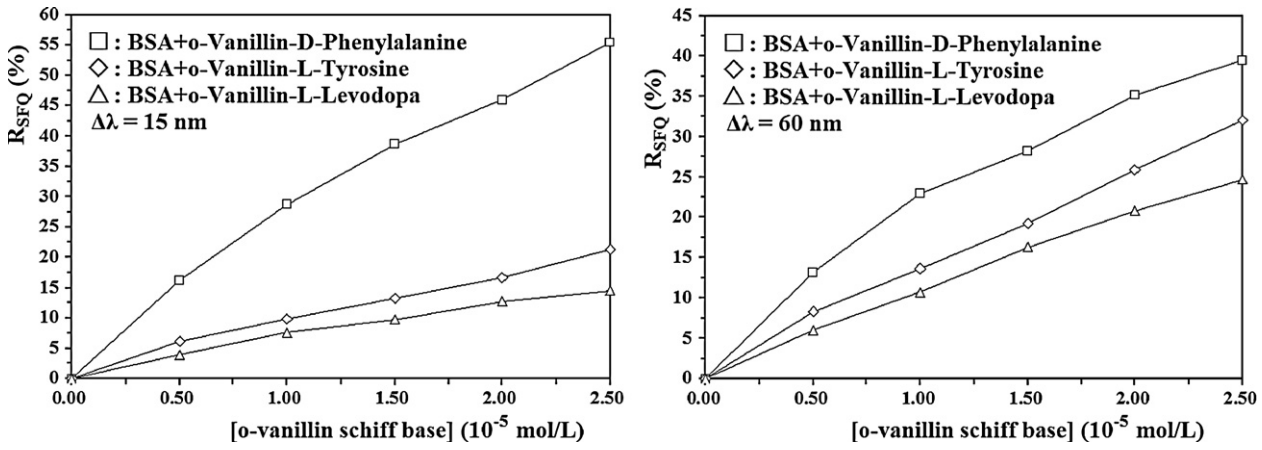

Fig. 7. Ratios of synchronous fluorescence quenching (R_{SFQ}) of BSA + o-VDP, BSA + o-VLT and BSA + o-VLL solutions with o-VDP, o-VLT and o-VLL concentrations (from 0.00×10^{-5} mol/L to 2.50×10^{-5} mol/L at 0.50×10^{-5} mol/L intervals) ([BSA] = 1.00×10^{-5} mol/L, [Tris–HCl] = [NaCl] = 50 mmol/L, pH = 7.40, T_{solu} = 37.00 ± 0.02 °C and V_{total} = 25.00 mL).

(7).

$$J = \frac{\Sigma F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda}{\Sigma F(\lambda)\Delta\lambda} \quad (7)$$

where the λ is the common wavelength of corresponding fluorescence spectrum of BSA and absorption spectrum of o-VSB (o-VDP, o-VLT and o-VLL). The $F(\lambda)$ and $\varepsilon(\lambda)$ are the fluorescence intensity of BSA solution and the absorbance of o-VSB (o-VDP, o-VLT and o-VLL) solution, respectively. Fig. 5 shows the spectral overlap of fluorescence emission of BSA solution and UV–vis absorption of o-VSB (o-VDP, o-VLT and o-VLL) solutions in the wavelength range of 260–500 nm, respectively. The efficiency (E) of energy transfer can be determined by Eq. (8):

$$E = 1 - \frac{F}{F_0} \quad (8)$$

where F_0 and F are the fluorescence intensities of BSA solutions with 1.0×10^{-5} mol/L at 345 nm in the absence and presence of o-VSB (o-VDP, o-VLT and o-VLL) with 1.0×10^{-5} mol/L, respectively. According to the above Eqs. (4)–(8), E, R_0 and J could all be calculated and the corresponding results were given in Table 3. At the same time, the binding distance (r) between BSA and o-VSB (o-VDP, o-VLT and o-VLL) are obtained and the corresponding results are 3.9967 nm for BSA-o-VDP, 4.6266 nm for BSA-o-VLT and 4.8366 nm for BSA-o-VLL, respectively. Being similar to the results of fluorescence quenching, the array order of the binding distances (r) is also $r_{(BSA-o\text{-}VDP)} < r_{(BSA-o\text{-}VLT)} < r_{(BSA-o\text{-}VLL)}$. Apparently, they are all less than 7.0 nm, which indicates that the energy transfer from BSA to o-VSB (o-VDP, o-VLT and o-VLL) occurs with high possibility [54]. It also suggested that the bindings of these three o-VSB Schiff Bases to BSA molecules were formed through energy transfer, which quenched the fluorescence of BSA molecules.

3.5. Synchronous fluorescence spectra of BSA + o-Vanillin-D-Phenylalanine (o-VDP), BSA + o-Vanillin-L-Tyrosine (o-VLT) and BSA + o-Vanillin-L-Levodopa (o-VLL) solutions

Synchronous fluorescence spectra can provide much valuable information about the microenvironment around fluorogens in biomolecules [55]. In general, for protein molecules, when the Δλ = 15 nm is fixed, the spectrum characteristic of tyrosine (Tyr) residues could be observed, and when Δλ = 60 nm is done, the spectrum characteristic of tryptophan (Trp) residues could be obtained [53,54,56]. In this work, the ratios of synchronous fluorescence quenching (R_{SFQ}) were used to determine the binding sites of o-VSB (o-VDP, o-VLT and o-VLL) to BSA molecules. The R_{SFQ} were calculated by using $R_{SFQ} = 1 - F/F_0$ equation, in which F and F_0 are the synchronous fluorescence intensities of BSA in the presence and absence of o-VSB (o-VDP, o-VLT and o-VLL), respectively.

From Fig. 6 it can be seen that for three courses the fluorescence intensities for Δλ = 15 nm (upper) and Δλ = 60 nm (nether) are both quenched more and more seriously along with increasing o-VSB (o-VDP, o-VLT and o-VLL) concentration. Fig. 7 markedly reveals that, for both Δλ = 15 nm and Δλ = 60 nm, the array order of R_{SFQ} is BSA + o-VDP > BSA + o-VLT > BSA + o-VLL. It proves again that the existence of hydroxyl group somewhat weaken the interaction of o-VLT and o-VLL with BSA molecules. In addition, for BSA + o-VDP at any o-VDP concentration, the R_{SFQ} for Δλ = 15 nm are slightly bigger than corresponding ones for Δλ = 60 nm. By contraries, for BSA + o-VLT and BSA + o-VLL the R_{SFQ} for Δλ = 15 nm are slightly smaller than the corresponding ones for Δλ = 60 nm. It indicates that the existence of hydroxyl group changes the interaction degree and mode of o-VLT and o-VLL with BSA molecules. That is, these three o-VSB Schiff Bases (o-VDP, o-VLT and o-VLL) affect the microenvironment of Tyr and Trp residues upon binding to BSA molecules with different accessibility [57]. To sum up, the o-VDP may be more accessibility to Tyr residues, whereas the o-VLT and o-VLL do more accessibility to the Trp residues. It illuminates that these three o-VSB Schiff Bases can bind to BSA molecules with different opportunities to Tyr and Trp residues.

4. Conclusions

The interaction of three synthesized o-Vanillin Schiff Bases (o-VSB: o-Vanillin-D-Phenylalanine (o-VDP), o-Vanillin-L-Tyrosine (o-VLT) and o-Vanillin-L-Levodopa (o-VLL)) with Bovine serum albumin (BSA) molecules in aqueous solution were investigated by fluorescence spectroscopy. The experimental results showed that these o-VSB Schiff Bases can obviously bind to BSA molecules, and then quench their intrinsic fluorescence efficiently. According to the fluorescence quenching calculation, the bimolecular quenching constant (K_q), apparent quenching constant (K_{sv}), effective binding constant (K_A) and corresponding dissociation constant (K_D), binding site number (n) and binding distance (r) were obtained. In comparison, the interaction strength of these three o-VSB Schiff Bases with BSA molecules decreases as the array order o-VDP, o-VLT and o-VLL. The synchronous fluorescence spectroscopy indicates that the o-VDP is more accessibility to tryptophan (Trp) residues of BSA molecules than to tyrosine (Tyr) residues, but the o-VLT and

o-VLL are more accessibility to Tyr residues of BSA molecules than to Trp residues.

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