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Interaction of Imidacloprid with Hemoglobin by Fluorescence and Circular Dichroism

Fei Ding · Bin-Yue Han · Wei Liu · Li Zhang · Ying Sun

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Abstract Imidacloprid belongs to a major new class of insecticides, called neonicotinoids, which are accounting for 11–15% of the total insecticide market. The binding characteristics of insecticide imidacloprid with hemoglobin (Hb) have been studied by employing different spectroscopic techniques. The results proved the formation of complex between imidacloprid and Hb. Hydrophobic interaction and hydrogen bond dominated in the association reaction. Hydrophobic probe 8-anilino-1-naphthalenesulfonic acid (ANS) competitive experiments indicated that the binding of imidacloprid to Hb primarily took place in hydrophobic regions. The distance between Hb donor and acceptor imidacloprid was 4.88 nm as derived from Förster's theory. Alternations of Hb secondary structure in the presence of imidacloprid were confirmed by synchronous fluorescence, circular dichroism (CD) and three-dimensional fluorescence spectra. This study enriches our understanding of toxic effect of imidacloprid to the physiologically important protein Hb.

Keywords Imidacloprid · Hemoglobin · Binding site · Fluorescence spectroscopy · Circular dichroism

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Introduction

Imidacloprid, (1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine, structure shown in Fig. 1) is the first commercially available product from the class of neonicotinic insecticides. Since its launch in 1991 by Bayer CropScience, imidacloprid is the most widely used insecticides against numerous sucking and biting pest insects, including aphids, whiteflies, thrips, leaf miners and beetles [1, 2]. Its mechanism of action has been studied extensively, and is relatively known. In essence, imidacloprid acts upon the nervous system, causing blockage of postsynaptic acetylcholine and γ-aminobutyric acid receptors [3–5]. Because of the systemic mode of action and relatively low mammalian toxicity, imidacloprid has become a popular insecticide worldwide for use in field crops, vegetables, ornamentals and animal health applications [6, 7]. However, the current report suggests that acute toxicity of imidacloprid on human beings [8–10] could induce disorientation, drowsiness, dizziness, palpitations, and copious vomiting, which progress to coma, tachycardia, hypertension, apnostic respiration, mydriasis with sluggish reaction to light, fever, leukocytosis, hypokalemia, hypernatremia, and finally bradycardia and cardiopulmonary arrest. Imidacloprid is also a potential groundwater and surface water contaminant, because it can leach and run off from soil and crops. Besides, it may enter water bodies from spray drift or accidental spills, leading to local point source contaminations [11]. Since imidacloprid is used for different applications and has been widely spread around the world indicating potential toxicological risk to humans, it is necessary to investigate the binding of imidacloprid with protein, as it can provide important insight into imidacloprid's toxicity to mammals.

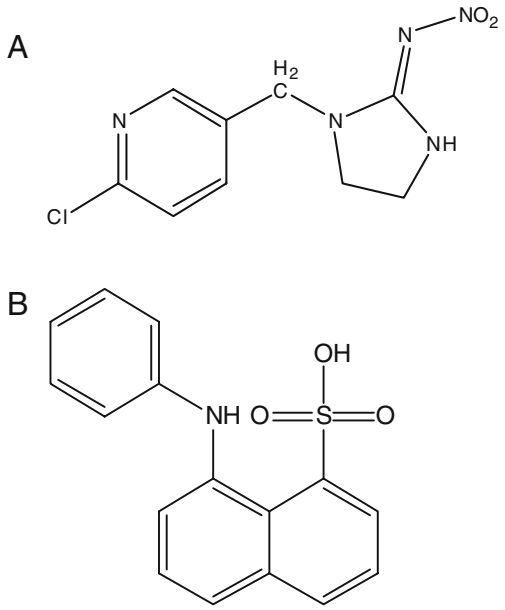

Fig. 1 Chemical structure of imidacloprid **a** and ANS **b**

Hemoglobin (Hb) is a soluble globular tetrameric protein, consists of two identical chains of 141 amino acids, the α-chains, and two identical β-chains, each of 146 residues [12]. The α-chains contain seven and the β-chains eight helical regions. Every chain carries one heme group in a pocket, to which oxygen and several other ligands can bind reversibly [13]. The Hb molecule is a $\alpha_2\beta_2$ type tetramer of molecular mass 64.45 kD and the concentration of Hb in the red blood cells is 330 mg mL^{-1}, which corresponds to a volume fraction of 0.25 [14]. The tetrametric nature of Hb is crucial to its biological functions: (1) transport of O_2 from the respiratory organ to peripheral tissue and (2) transport of CO_2 and protons from peripheral tissues to the respiratory organ for subsequent excretion [15]. In addition, it is also regulate the pH of blood and involved in many clinical diseases such as leukemia, anemia, heart disease, excessive loss of blood, etc [16]. Binding of pesticides to Hb has toxicological importance. Wang et al. [16] have studied the binding of paraquat to Hb in order to investigate that paraquat exhibits a high affinity to Hb. Cui and co-workers [17] have probed the features of two insecticides, chlopyrifos and cypermethrin with Hb in detail. These investigations provided qualitative understanding of the toxic effects of pesticides to Hb. But so far the interaction mechanism of insecticide imidacloprid and Hb has not been quantitative analysis by fluorescence and circular dichroism (CD) spectroscopy in our knowledge.

Fluorescence spectroscopy is a powerful method to study protein-ligand interaction because it is exceptional sensitivity, selectivity, convenience and abundant theoretical foundation. It can also reveal the accessibility of ligands to fluorophores, help to understand the binding mechanism of ligand to protein. In this paper, bovine hemoglobin was selected as the protein model because of its low cost, ready availability and the results of all the studies are consistent with the fact that bovine and human hemoglobin are homologous proteins. The binding of imidacloprid to Hb was studied by spectroscopy including fluorescence, UV/vis, CD and three-dimensional fluorescence spectra. The binding mechanism between imidacloprid and Hb regarding the binding constants, the thermodynamic functions, the binding site and the alternations of Hb secondary structure in the presence of imidacloprid were investigated in our work.

Experimental

Materials

Bovine hemoglobin (product number: H2500, lyophilized powder), imidacloprid (product number: 37894) and 8-anilino-1-naphthalenesulfonic acid (ANS, product number: A1028) were purchased from Sigma-Aldrich Chemical Company. All other reagents were of analytical reagent grade. The Milli-Q ultrapure water was used throughout the experiments. NaCl (1.0 mol L^{-1}) solution was used to maintain the ionic strength at 0.1. Tris (0.2 mol L^{-1})-HCl (0.1 mol L^{-1}) buffer solution containing NaCl (0.1 mol L^{-1}) was used to keep the pH of the solution at 7.4. Dilutions of the Hb stock solution (3.0×10^{-5} mol L^{-1}) in Tris-HCl buffer solution were prepared immediately before use. The concentration of Hb was determined spectrophotometrically using millimolar extinction coefficient of $mE_{555}=50$ [18]. The stock solution (1.5×10^{-4} mol L^{-1}) of imidacloprid was prepared in absolute ethanol. All pH measurements were performed with an Orion-868 digital pH meter combined glass electrode (Orion, USA).

Apparatus and methods

Fluorescence spectra were recorded on a F-4500 spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cell and a thermostat bath. Fluorescence quenching spectra were recorded at 298, 304 and 310 K in the range of 250–500 nm. The width of excitation and emission slit was set to 5.0 nm, respectively. An excitation of 280 nm was chosen and very dilute solution were used (Hb: 3.0×10^{-6} mol L^{-1}, imidacloprid in the range of 0–27.0×10^{-6} mol L^{-1}). The quenching effect of ethanol was evaluated and the result indicated that there was almost no affection of ethanol on the imidacloprid-Hb interaction. The fluorescence intensities were corrected for absorption of exciting light and

reabsorption of the emitted light to decrease the inner filter effect using the relationship [19]:

$$F_{cor} = F_{obs} \times e^{\frac{A_{ex}+A_{em}}{2}} \quad (1)$$

where F_{cor} and F_{obs} are the fluorescence intensities corrected and observed, respectively, and A_{ex} and A_{em} are the absorption of the systems at the excitation and the emission wavelength, respectively. The intensity of fluorescence used in this paper is the corrected fluorescence intensity.

ANS competitive experiments: In a first series of experiments, imidacloprid/ANS were added to solution of Hb. Hb concentration was kept fixed at 3.0×10^{-6} mol L^{-1} and imidacloprid/ANS concentration was varied from 3.0 to 27.0×10^{-6} mol L^{-1}. Fluorescence emission spectra of Hb was performed under identical conditions (λ_{ex}=280 nm, λ_{em}=330 nm). In a second series of experiments, imidacloprid was added to solutions of Hb and ANS held in equimolar concentrations (3.0×10^{-6} mol L^{-1}), the concentration of imidacloprid was also varied from 3.0 to 27.0×10^{-6} mol L^{-1} and the fluorescence emission spectra of ANS was recorded (λ_{ex}=370 nm, λ_{em}=470 nm).

The three-dimensional fluorescence spectra were performed under the following conditions: the emission wavelength was recorded between 200 and 400 nm, the initial excitation wavelength was set to 200 nm with increment of 10 nm, the number of scanning curves was 16, and other scanning parameters were just the same as those of the fluorescence emission spectra.

The UV/vis absorption spectra was performed at room temperature on a Cary-100 spectrophotometer (Varian, USA) equipped with 1.0 cm quartz cuvette.

CD spectra were performed between 200 and 250 nm with a Jasco-810 spectropolarimeter (Jasco, Japan) using a 0.1 cm path length quartz cuvette. All spectra were accumulated in triplicate with 0.2 nm step resolution at a scanning speed of 50 nm min^{-1}. Each CD spectrum represented the average of three successive scans. All observed spectra were baseline subtracted for buffer and the results were taken as molar ellipticity ([e]) in deg cm^2 $dmol^{-1}$, the α-helical content of Hb was calculated from the [e] value at 222 nm using the following equation [20]:

$$\%\alpha - helix = \frac{-[e]_{222} - 2,340}{30,300} \quad (2)$$

Results and discussion

Interactions between imidacloprid and Hb

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions, such as excited state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching [19]. Such decrease in intensity is called fluorescence quenching. Hb contains three tryptophan (Trp) residues in each $\alpha\beta$ dimer, for a total of six in the tetramer: two α-14 Trp, two β-15 Trp and two β-37 Trp [21]. Hb intrinsic fluorescence is primarily due to the fluorescence of β-37 Trp [22] at the $\alpha_1\beta_2$ interface, though it may contain some contribution by the surface Trp residues, α-14 and β-15 Trp. Furthermore, Hb intrinsic fluorescence serves as a reporter for R-T transition. The R form is the oxy/ligand bound form while the T form is the deoxy form [23] and R-T forms show significant changes in relative fluorescence intensity. Figure 2 shows the fluorescence emission spectra of Hb with various amounts of imidacloprid following an excitation at 280 nm. The working concentration of Hb used was 3.0×10^{-6} mol L^{-1} so as to prevent quenching of Trp fluorescence by the neighboring heme group [24, 25]. Hb shows a weak fluorescence emission with a peak at 330 nm. Its intensity decreased gradually with the addition of imidacloprid. It was also observed that the maximum wavelength of Hb shifted from 330 to 334 nm upon increasing the concentration of imidacloprid. Under the same condition, no fluorescence of imidacloprid was observed. These phenomena indicated that imidacloprid could interact with Hb and the conformation changes induced by the interaction leading to a further exposure of Trp residue to the polar solvent [26].

To further elaborate the fluorescence quenching mechanism, the fluorescence data was usually analyzed using the Stern-Volmer equation [19]:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (3)$$

where F_0 and F denote the fluorescence intensities in the absence and presence of imidacloprid, K_{SV} is the Stern-

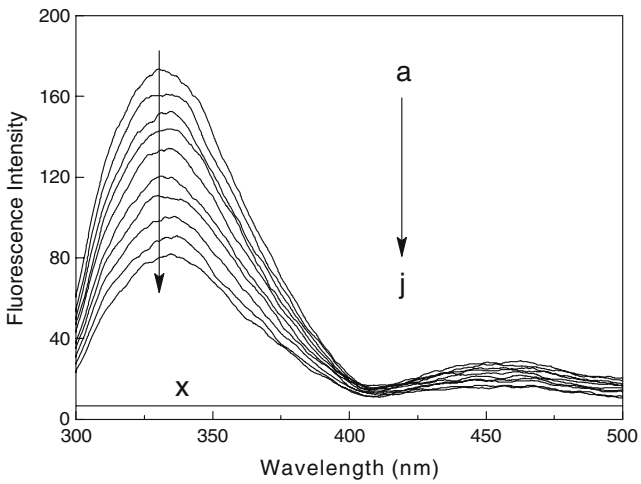

Fig. 2 The fluorescence spectra of Hb-imidacloprid system. (**a**) 3.0×10^{-6} mol L^{-1} Hb; (b→j) 3.0×10^{-6} mol L^{-1} Hb in the presence of 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0, 27.0×10^{-6} mol L^{-1} imidacloprid; (x) 27.0×10^{-6} mol L^{-1} imidacloprid. pH=7.4, T=298 K

Volmer quenching constant, k_q is the quenching rate constant of Hb, τ_0 is the average lifetime of the Hb without imidacloprid and fluorescence lifetime of the biopolymer is 10^{-8} s [27], and [Q] is the concentration of imidacloprid. Figure 3 displays the Stern-Volmer plots of F_0/F versus [Q] at three temperatures and calculated K_{SV} and k_q values were presented in Table 1. The results showed that Stern-Volmer quenching constant K_{SV} is inversely correlated with temperature and the values of k_q are much larger than the maximum scattering collision quenching constant (2.0×10^{10} L mol^{-1} s^{-1} [28]), which indicated that the probable quenching mechanism of fluorescence of Hb by imidacloprid was not initiated by dynamic collision but compound formation [29].

UV/vis absorption spectra measurement is a very simple method and applicable to explore the structural change and know the complex formation. Figure 4 shows the the UV/vis absorption spectra of Hb in Tris-HCl buffer solution in the presence of different imidacloprid concentrations. The absorption spectra of Hb are characterized by a peak at 270 nm due to Trp absorption and a sharp peak at 405 nm due to the Soret absorption by the heme system [30]. The absorbance of Hb at 405 nm decreased with the addition of imidacloprid, while the absorption wavelength remains unchanged. A reasonable explanation may come from the interaction between imidacloprid and Hb and ground state complex formed [31]. It also implied that the heme is not exposed from the crevices at the exterior of the subunit and imidacloprid is easily integrated into the hydrophobic cavity of Hb.

Therefore, the fluorescence quenching data were further examined using modified Stern-Volmer equation [19]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a f_a [Q]} \quad (4)$$

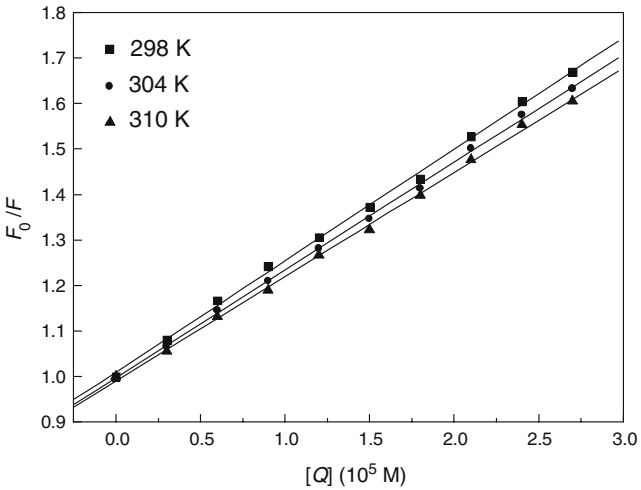

Fig. 3 Stern-Volmer plots for the quenching of Hb by imidacloprid at different temperatures. c(Hb)=3.0×10^{-6} mol L^{-1}; pH=7.4

Table 1 Stern-Volmer quenching constants for the interaction of imidacloprid with Hb at three different temperatures

T (K)	K_{SV} ($\times10^4$Lmol^{-1})	k_q ($\times10^{12}$Lmol^{-1}s^{-1})	R^a	S.D.[b]
298	2.451	2.451	0.9992	0.011
304	2.366	2.366	0.9996	0.007
310	2.291	2.291	0.9993	0.008

[a] R is the correlation coefficient

[b] S.D. is the standard deviation

where f_a is the fraction of accessible fluorescence, and K_a is the effective quenching constant for the accessible fluorophores. The modified Stern-Volmer plots are shown in Fig. 5 and the corresponding quenching constants K_a at different temperatures are listed in Table 2. The decreasing trend of K_a with increasing temperature was in accordance with K_{SV}'s dependence on temperature as mentioned above. The K_a values show that binding between imidacloprid and Hb was moderate which indicates that a reversible imidacloprid-Hb complex came into being and Hb serves as a depot [26]. This result also demonstrated that toxic effect of imidacloprid to Hb.

Binding mode

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for this interaction. Therefore, the thermodynamic parameters dependent on temperatures were analyzed in order to further characterize the acting force between imidacloprid-Hb complex. The acting forces

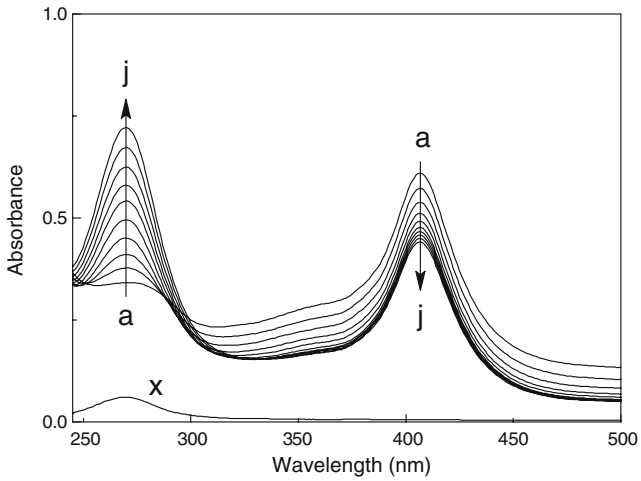

Fig. 4 UV/vis absorption spectra of Hb. Hb concentration was at 3.0×10^{-6} mol L^{-1} (**a**) and imidacloprid concentration for Hb-imidacloprid system was at 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0, 27.0$\times10^{-6}$ mol L^{-1} (b→j). A concentration of 3.0×10^{-6} mol L^{-1} imidacloprid (x) was used for imidacloprid only. pH=7.4, T=298 K

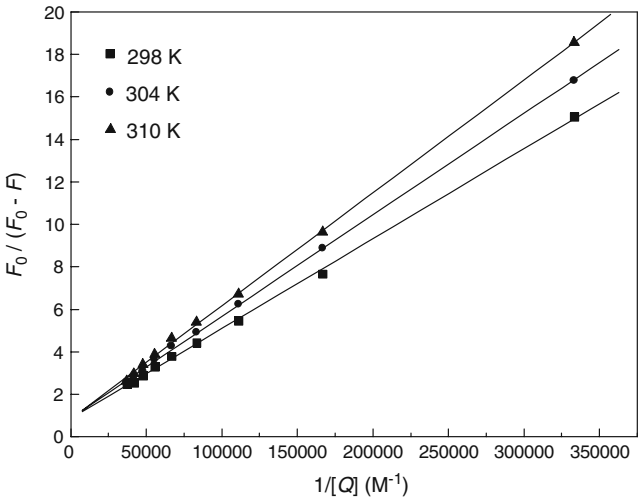

Fig. 5 Modified Stern-Volmer plots for the Hb-imidacloprid at different temperatures. c(Hb)=3.0×10^{-6} mol L^{-1}; pH=7.4

between a small molecule and macromolecule mainly include hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces. The thermodynamic parameters, enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) are the main evidence to determine the binding mode [32]. If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then its value can be calculated from the van't Hoff equation:

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{5}$$

K is analogous to the effective quenching constants K_a and R is gas constant. The value of ΔH and ΔS were obtained from linear van't Hoff plot. The value of ΔG was calculated from the relation:

$$\Delta G = \Delta H - T\Delta S \tag{6}$$

From the linear relationship between ln$K$ and the reciprocal absolute temperature (Fig. 6), the value of ΔH and ΔS can be obtained and the results were presented in Table 2. As shown in Table 2, the negative sign for ΔG means that the binding process was spontaneous and the formation of imidacloprid-Hb complex was an exothermic reaction accompanied by positive ΔS value. The positive

Table 2 Modified Stern-Volmer association constants K_a and relative thermodynamic parameters of the imidacloprid-Hb system

T (K)	K_a (×10^{4}Lmol^{-1})	R^a	ΔH (kJmol^{-1})	ΔG (kJmol^{-1})	ΔS (Jmol^{-1}K^{-1})
298	2.054	0.9996	−14.85	−24.61	32.83
304	1.889	0.9997		−24.89	
310	1.628	0.9997		−24.99	

[a] R is the correlation coefficient for the K_a values

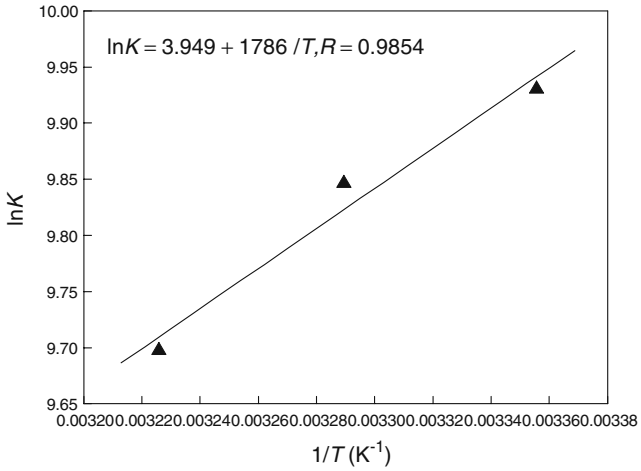

Fig. 6 Van't Hoff plot for the interaction of Hb and imidacloprid in Tris-HCl buffer solution, pH=7.4

ΔS arised from water molecules arranged more random around Hb and imidacloprid, caused by hydrophobic interaction forces between imidacloprid and Hb. The negative ΔH value (−14.85 kJ mol^{-1}) observed can't be mainly attributed to electrostatic forces since for electrostatic forces ΔH is very small, almost zero [32]. Negative ΔH is observed whenever there is hydrogen bond in the binding. Therefore, from the thermodynamic characteristics summarized above, the negative ΔH and positive ΔS values indicated that hydrophobic interaction and hydrogen bonds played major roles in the imidacloprid-Hb binding reaction and contributed to the stability of the complex.

Analysis of binding equilibria

When ligand molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the numbers of binding sites (n) can be determined by the following equation [33]:

$$\log\frac{F_0 - F}{F} = n\log K_b + n\log\left(\frac{1}{[Q_t] - \frac{F_0 - F}{F_0}[P_t]}\right) \tag{7}$$

where F_0 and F are the fluorescence intensities before and after the addition of imidacloprid, $[Q_t]$ and $[P_t]$ are the total imidacloprid concentration and the total Hb concentration, respectively. By the plot of log(F_0-F)/F versus log(1/([Q_t]-(F_0-F)[P_t]/F_0)), the binding constant K_b and the number of binding sites n can be obtained (Table 3). As can be seen from Table 3, the K_b value decreased with the increasing of temperature, which implied the forming of an unstable imidacloprid-Hb complex in the binding reaction. The complex would be partly decomposed when the temperature increased. The values of n at the experimental temperatures are approximately to 1, which shows that there was one high affinity binding site to imidacloprid in Hb [34].

Table 3 The binding constants K_b and binding sites n at different temperatures

T (K)	K_b ($\times 10^4 Lmol^{-1}$)	n	R^a	S.D.[b]
298	2.443	1.01	0.9989	0.016
304	1.644	0.97	0.9993	0.012
310	1.084	0.94	0.9986	0.017

[a] R is the correlation coefficient

[b] S.D. is the standard deviation

The intrinsic fluorescence of Hb primarily originates from β-37 Trp, which hint that imidacloprid binding site on Hb was located closer to the β-37 Trp.

To further verify the binding region of Hb when exposed to imidacloprid, hydrophobic probe ANS competitive experiments are carried out. The relative fluorescence intensity (F/F_0) *versus* quencher concentration (c_{ligand}) plot is shown in Fig. 7. In a first series of experiments, imidacloprid/ANS were added to solution of Hb, both imidacloprid and ANS quench the fluorescence of Hb. However, the extent of fluorescence quenching effect by imidacloprid and ANS were different. In a second series of experiments, imidacloprid was added to solutions of Hb and ANS held in equimolar concentrations (3.0×10^{-6} mol L^{-1}) and ANS fluorescence was measured (λ_{ex}= 370 nm, λ_{em}=470 nm). As can be seen from Fig. 7, with the addition of imidacloprid to Hb-ANS system, it can compete with ANS for hydrophobic sites on the interface. In that case, imidacloprid would displace ANS from its binding site and fluorescence intensities decreased. The above experimental results and analysis indicated that the binding of imidacloprid to Hb mainly located in hydrophobic patches.

Conformation investigations

Synchronous fluorescence of Hb was studied to evaluate the change in the environment of tyrosine (Tyr) and Trp residues as a result of imidacloprid binding. Synchronous fluorescence spectroscopy introduced by Lloyd has been used to characterize complex mixtures providing fingerprints of complex samples [35]. It provides several advantages like spectral simplification, reduction in the spectral noise and spectral area, over other modes of fluorescence study. According to the theory of Miller [36], when the D-value ($\Delta\lambda$) between excitation and emission wavelength are stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of Tyr or Trp residues. Figure 8 showed that the effect of the addition of imidacloprid on the synchronous fluorescence spectra of Hb when $\Delta\lambda$=15 or 60 nm. It was apparent from Fig. 8 that the maximum emission wavelength of Tyr and Trp residues did significant red shift (Tyr: 290→293 nm, Trp: 280→285 nm) which indicated that the conformation of Hb was changed, the polarity around the Tyr and Trp residues was increased and the hydrophobicity was decreased [37, 38]. This may be due to the changes of residue microenvironment with the insertion of imidacloprid. In Hb, aromatic residues such as α-42 Tyr, α-140 Tyr, β-37 Trp and β-145 Tyr are located at the $\alpha_1\beta_2$ subunit interface and undergo changes of environment with the quaternary structure transition from T to R. The Hb central cavity contains functional significantly center for binding several class of allosteric effectors [39], which modulate Hb affinity to oxygen. The red shift observed in Fig. 8 implies that imidacloprid has ability to bind into Hb central cavity and induces the structural and functional changes of Hb.

Further evidence of conformational changes of Hb upon addition of imidacloprid was provided by CD technique. The raw CD spectra of Hb in the absence and presence of imidacloprid are shown in Fig. 9. As evident from Fig. 9, the CD spectra of Hb exhibited two negative bands in the UV region at 208 and 222 nm, which are characteristic features of α-helical structure of proteins [40]. The reasonable explanation is that the negative peaks between 208 and 209 nm and 222 and 223 nm are both contributed to $n\rightarrow\pi^*$ transition for the peptide bond of α-helix [41]. The band intensities of Hb at 208 and 222 nm decreased with negative Cotton effect by the addition of imidacloprid without causing any significant shift of the peaks, illustrating the change of the protein secondary structure on Hb-imidacloprid interaction. The α-helical content from 47.06% to 43.10% at a molar ratio of Hb to imidacloprid of 1:1. From the above analysis, the percentage of α-helical structure decreased implied that imidacloprid bound with

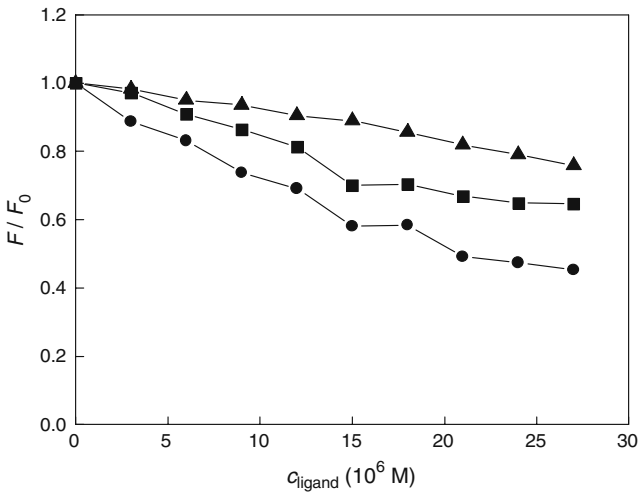

Fig. 7 Fluorescence quenching pattern of Hb and Hb-ANS system. Binding isotherm of imidacloprid (*filled square*) and ANS (*filled circle*) induced quenching of Hb fluorescence and quenching of Hb-ANS system fluorescence by imidacloprid (*filled triangle*). pH=7.4, T=298 K

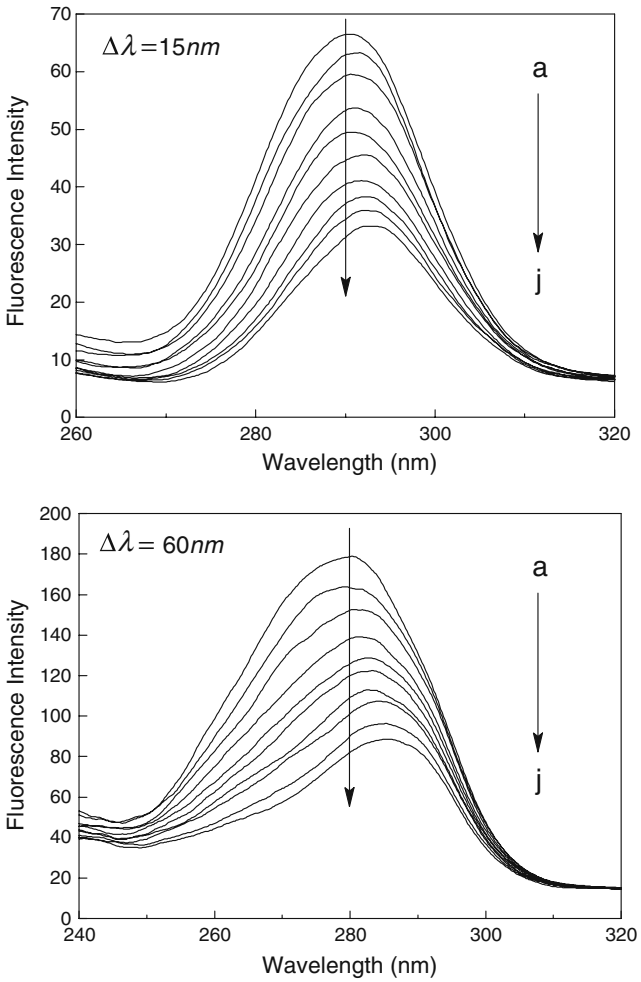

Fig. 8 Synchronous fluorescence spectra of Hb in the absence and presence of imidacloprid (pH=7.4, T=298 K). (**a**) 3.0×10^{-6} mol L^{-1} Hb; (b→j) 3.0×10^{-6} mol L^{-1} Hb in the presence of 3.0, 6.0, 9.0, 12.0, 15.0, 18.0, 21.0, 24.0, 27.0×10^{-6} mol L^{-1} imidacloprid, respectively

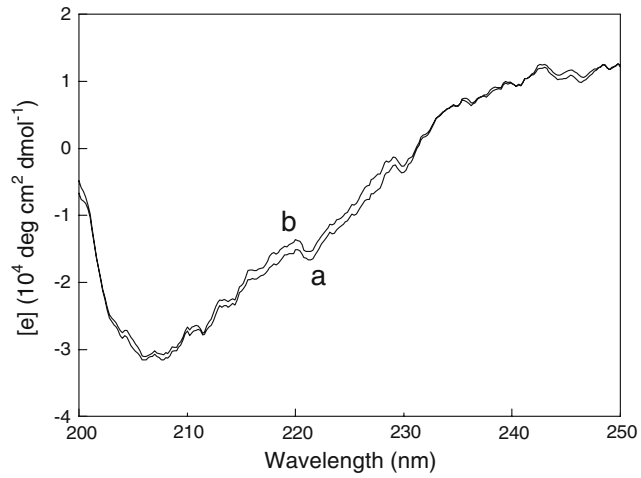

Fig. 9 The far-UV CD spectra of the Hb-imidacloprid system. (**a**) 3.0×10^{-6} mol L^{-1} Hb; (**b**) 3.0×10^{-6} mol L^{-1} Hb in the presence of 3.0×10^{-6} mol L^{-1} imidacloprid. pH=7.4, T=298 K

amino acid residue of the main polypeptide chain of Hb and destroyed their hydrogen bonding network.

Three-dimensional fluorescence spectra have become a popular technique in recent years. It can comprehensively exhibit the fluorescence information of the sample, which makes the investigation of the characteristic conformational change of Hb be more scientific and credible [42]. The three-dimensional fluorescence contour map of Hb and imidacloprid-Hb complex is shown in Fig. 10, and the corresponding parameters are listed in Table 4. Peak *a* is the Rayleigh scattering peak ($\lambda_{ex}=\lambda_{em}$) [43] and the fluorescence intensity of peak *a* increased with the addition of

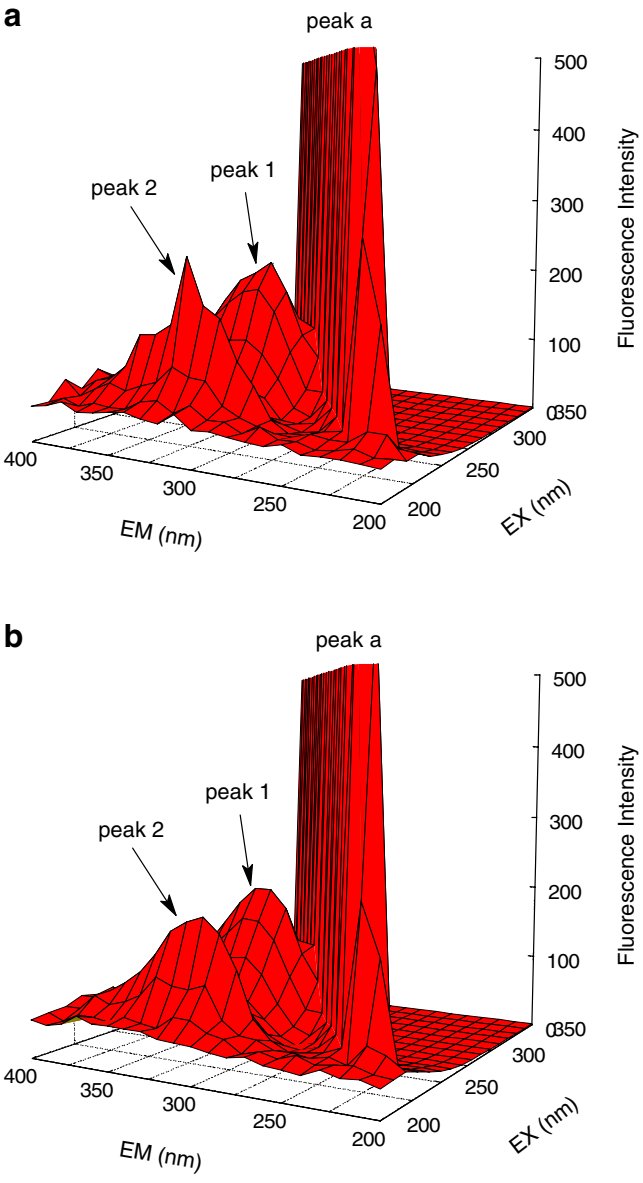

Fig. 10 Three-dimensional fluorescence contour map of Hb (**a**) and the Hb-imidacloprid system (**b**). (**a**): c(Hb)=7.5×10^{-6} mol L^{-1}, c(imidacloprid) = 0; (**b**): c(Hb)=7.5×10^{-6} mol L^{-1}, c(imidacloprid)= 7.5×10^{-6} mol L^{-1}. pH=7.4, T=298 K

Table 4 Three-dimensional fluorescence spectral characteristics of Hb and Hb-imidacloprid system

Peaks	Hb			Hb-imidacloprid		
	Peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	Stokes $\Delta\lambda$ (nm)	Intensity F	Peak position $\lambda_{ex}/\lambda_{em}$ (nm/nm)	Stokes $\Delta\lambda$ (nm)	Intensity F
Rayleigh scattering peaks	235/235→350/350	0	178.5→1640	235/235→350/350	0	188.6→1675
Fluorescence peak 1	280.0/323.0	43.0	133.3	284.0/327.0	43.0	109.3
Fluorescence peak 2	245.0/326.0	81.0	43.16	245.0/329.0	84.0	42.87

imidacloprid. The reasonable explanation is that an imidacloprid-Hb complex came into being after the addition of imidacloprid, making the diameter of the macromolecule increased, which in turn resulted in the scattering effect enhanced. Peak *1* (λ_{ex}=280.0 nm, λ_{em}=323.0 nm) which mainly reveals the spectral behavior of Trp and Tyr residues is the primary fluorescence peak we studied. The reason is that when protein is excited at 280 nm, it mainly reveals the intrinsic fluorescence of Trp and Tyr residues, and the fluorescence of phenylalanine (Phe) residue can be negligible [19]. Compared with UV/vis absorption spectra of Hb, there is an absorption peak around 270 nm, which is mainly caused by transition of $\pi\rightarrow\pi^*$ of aromatic amino acids in Hb. The Trp, Tyr and Phe in the binding cavity of Hb have conjugated π-electron and easily from charge transfer compound with other electron deficient species or π-electron system [44]. The binding of imidacloprid to Hb included the hydrophobic interaction forces between the aromatic heterocyclic ring and the hydrophobic amino acid residues. Besides peak *1*, there is another fluorescence peak *2* (λ_{ex}=245.0 nm, λ_{em}=326.0 nm), which mainly caused by the transition of $n\rightarrow\pi^*$ of characteristic polypeptide backbone structure C=O of Hb. Analyzing from the fluorescence intensities of peak *1* and *2*, they decreased obviously but to different degrees: in the absence and presence of imidacloprid, the intensity ratios of peak *1* and peak *2* are 1.22:1 and 1.01:1, respectively. The decrease of fluorescence intensity of peak *1* and *2* in combination with the synchronous fluorescence and CD results demonstrated that the interaction of imidacloprid with Hb induced the slight unfolding of the polypeptide of Hb, which resulted in a conformational change of Hb that increased the exposure of some hydrophobic regions which were previously buried. All these phenomena and analysis revealed that the binding of imidacloprid to Hb induced some conformational and microenvironmental changes in Hb.

Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a nondestructive spectroscopic method that can monitor the proximity and relative angular orientation of fluorophores. FRET has been used as a "spectroscopic ruler" for measuring molecular distance in the biological system [45]. According to the Förster theory [46], the efficiency of FRET depends mainly on the following factors: (1) the extent of overlap between the donor emission and the acceptor absorption spectrum, (2) the orientation of the transition dipole of donor and acceptor, and (3) the distance between the donor and the acceptor. Here the donor and acceptor are Hb and imidacloprid, respectively. The overlap of the absorption spectrum of imidacloprid with the fluorescence emission spectrum of Hb is shown in Fig. 11. The efficiency of energy transfer between the donor and acceptor, E, could be calculated by the following equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{8}$$

where r is the binding distance between donor and acceptor, and R_0 is the critical distance at 50% transfer efficiency

$$R_0^6 = 8.79 \times 10^{-25} k^2 \cdot n^{-4} \cdot \varphi \cdot J \tag{9}$$

where k^2 is the orientation factor between the emission dipole of the donor and the absorption dipole of the acceptor, n is the average refractive index of medium in the wavelength range where spectral overlap is significant, φ is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of

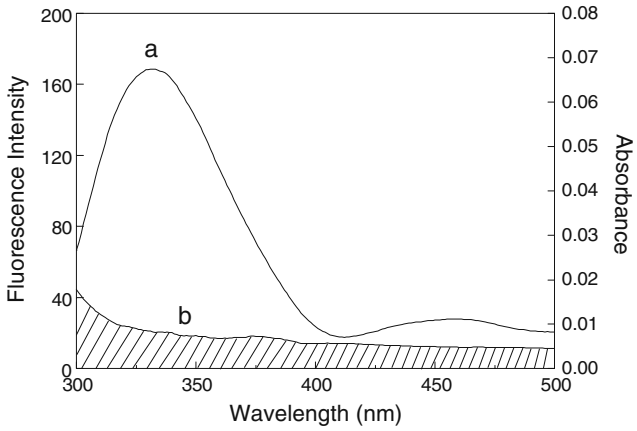

Fig. 11 Overlapping between the fluorescence emission spectrum of Hb (**a**) and UV/vis spectrum of imidacloprid (**b**), c(Hb) = c(imidacloprid)=3.0×10^{-6} mol L^{-1}; pH=7.4, T=298 K

the donor and the absorption spectrum of the acceptor. Therefore,

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (10)$$

where $F(\lambda)$ is the fluorescence intensity of the donor in the wavelength range λ to $\lambda+\Delta\lambda$, and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ. In the present case, $k^2=2/3$, $n=1.36$ and $\phi=0.062$ for Hb [47]. From equations (8)–(10), the values of the parameters were found to be: $J=6.164\times 10^{-14}$ cm^3 L mol^{-1}, $R_0=2.95$ nm, $E=0.046$ and $r=4.88$ nm. The donor-to-acceptor distance, $r<7$ nm is in accordance with Förster's nonradiative energy transfer with high probability and since, r was greater than R_0 in this study suggested that imidacloprid could strongly quench the intrinsic fluorescence of Hb by static quenching mechanism [48].

Conclusions

In summary, the interaction of imidacloprid with Hb has been investigated in vitro (pH 7.4, ionic strength 0.1) using fluorescence emission, UV/vis, CD, synchronous fluorescence and three-dimensional fluorescence spectroscopy. Fluorescence experiment results revealed that the intrinsic fluorescence of Hb was quenched through static quenching process. The enthalpy change (ΔH) and entropy change (ΔS) for the reaction were calculated to be −14.85 kJ mol^{-1} and 32.83 J mol^{-1} K^{-1}, which indicated that hydrophobic interaction and hydrogen bonds were the dominant intermolecular force in stabilizing the complex. Competitive experiments exhibited that hydrophobic region are the major sites for imidacloprid binding on Hb. The distance between imidacloprid and Hb was close enough (r= 4.88 nm) to arose nonradiative energy transfer from Hb to imidacloprid. The results of synchronous fluorescence, CD and three-dimensional fluorescence spectra indicated that the protein secondary structure altered and the physiological state of Hb was affected by imidacloprid. The binding study of imidacloprid with Hb is of biological and toxicological importance. This study is expected to provide important insight into the interactions of the physiologically important protein Hb with neonicotinoid insecticides, which may be a useful guideline for further toxicology investigation. Information is also obtained in the construction of pesticide biosensors. Biosensor assays can offer measures of the toxic effects of chemicals on the target organism and of the molecular mechanisms that underlie toxicity. These measures are of particular interest in ecotoxicology and environmental risk assessment.

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