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ORIGINAL RESEARCH PAPER

Exploring the interaction of nanocomposite composed of Fe_3O_4, CaAl layered double hydroxide and lamivudine drug with Human serum albumin (HSA): Spectroscopic studies

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ABSTRACT

In the present work, the interaction of Fe_3O_4@CaAl LDH@ Lamivudine with human serum albumin (HSA) was investigated by applying UV–vis and fluorescence spectra. The nanocomposite was quenching the natural fluorescence of HSA, which was indicated the static quenching mechanism. The consequences demonstrated that this nanocomposite can strongly bind to HSA molecules. According to fluorescence quenching computations, the bimolecular quenching constant (k_q), apparent quenching constant (K_{SV}) at various temperatures was calculated (288, 298, 310 k). The binding constants K_b were 12187.09 L mol^{-1}, 62849.24 L mol^{-1} and 350429 L mol^{-1} at 288 K, 298 K and 310 K respectively, and the number of binding sites n is almost >1. Competitive results show that the binding site of nanocomposite placed in subdomain IIIA (site II) of HSA. The thermodynamic parameters defined by the Van't Hoff analysis of the binding constants (ΔH 113.211 kJ mol^{-1} and ΔS 471.4703 J mol^{-1} K^{-1}) clearly illustrated that the hydrophobic force plays a major role in the process. To compare binding behavior and mechanism of the antiviral drug which was loaded on Fe_3O_4@CaAl LDH with HSA, we carried out fluorescence and UV-Visible spectroscopy to investigate the interactions of Fe_3O_4@CaAl LDH@ Lamivudine with HSA.

Keywords: *Double Layered Hydroxide, HAS, Interaction, Lamivudine, Nanocomposite*

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INTRODUCTION

Study of the interaction between small molecules and plasma proteins (HSA) have been the great area of research in pharmacology and chemical biology. Binding of a drug to albumin, results in an increased drug solubility in plasma, decreased toxicity, and protection against oxidation of the bound drug distribution is mainly controlled by HSA, because most drugs circulate in plasma and reach the target tissues by binding to HSA. Therefore, drug binding to proteins such as HSA has become an important determinant of pharmacokinetics, e.g. prolonging in vivo half-life, restricting the unbound concentration and affecting distribution and elimination of the drug. In this regard, HSA is the most extensively studied protein, due to its lack of toxicity and immunogenicity make it an ideal candidate for drug delivery [1]. Serum albumins in specific occupied a majority of the 60% of the blood composition. Also, their binding property with the endogenous and exogenous ligands remains very specific. They are the globular proteins and their complex structure remains quite sensitive to their binding with exogenous materials. The binding of the nanoparticles with serum albumin could disturb the intramolecular forces that are responsible for stabilizing the protein structure. Studies on the interaction of NPs with serum albumins have been reported since long time [2]. Such studies aids in elucidating the impact of NPs on the protein

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structure and also define the binding mechanism behind them. Hence, the analysis on the binding induced conformational changes in the secondary structure of biomolecules in vitro upon interaction with nanoparticles remains very significant in terms of determining their biocompatibility in vivo [3].

In the present study Lamivudine was used as the drug which was loaded on the core-shell structure of Fe_3O_4@LDH. In order to prevent aggregation of magnetic nanoparticles, double layered hydroxides were synthesized and coated on the surface and into the structure of nanocomposite. Magnetic nanoparticles in specific have directed its significance owing to their higher coactivity and magnetic susceptibility, super paramagnetic, lower curie temperature etc. Hence, they have major applications in the diversified fields of catalysis, magnetic fluids, data storage and biological inventions [4]. Lamivudine (3TC; formula: $C_8H_{11}N_3O_3S$) is a nucleoside reverse transcriptase inhibitor (NRTI), which is used clinically to treat the acquired immunodeficiency syndrome (AIDS). As an antiretroviral medication, it is incorporated into the DNA of the virus and acts as a chain terminator, resulting in incomplete DNA that cannot create a new virus [6]. So, it is essential to investigate the binding action of this drug to serum albumin, which may reflect the characteristics of the absorption, transportation, distribution, and excretion [7].

Many recent researches demonstrated that nanomaterials based drug delivery systems enhance cell permeation capacity and eventually improve the therapeutic efficacy of drugs. In particular, inorganic anionic nanoclays, layered double hydroxides (LDHs), have attracted much attention as delivery carriers for bioactive molecules, because they exhibit low toxicity and possess high reserving capacity. Moreover, LDH can be easily taken up by cells via clathrin-mediated endocytic process. Efficient drug delivery and enhanced drug efficacy could be, therefore, obtained by using LDH-drug hybrid systems [5]. Additionally, LDH is easily degraded in acidic environments. 8 While smart LDH nanohybrids show even more superior. Among which magnetic LDH nano-composites display promising properties as drug vehicles due to their targeted drug delivery by magnetic manipulation.

So the nanoparticles must be coated to prevent dissolution and aggregation of magnetic nanoparticles under physiological conditions (i.e. neutral pH and high salt concentration), to protect against protein adsorption. So improvement of the stability of magnetic LDHs in aqueous solution by surface modification with organic species is essential.

HSA comprises three homologous domains (I, II, and III) that assemble to form a heart-shaped molecule. Each domain contains two subdomains (A and B) that possess common structural motifs.11,13 The main regions of ligand interaction are located in hydrophobic pockets at subdomains IIA and IIIA, IIIA having the highest affinity. Many ligands bind specifically to HSA, either in site I (located at subdomain IIA) or in site II (located at subdomain IIIA). The binding of the ligands with active sites of the proteins can change their structure and function and cause toxic effects [8].

The aim of this work was to investigate the interaction of Fe_3O_4@LDH@Lamivudine with HSA under simulated physiological conditions (ionic strength = 0.1 mol L^{-1}; pH 7.40) by multiple spectroscopy methods, to determine the binding constant, number of binding sites and quenching constant

EXPERIMENTAL AND METHODS

Materials and methods

HSA (fatty acid-free, 99%), lamivudine, warfarin, ibuprofen, were purchased from Sigma Chemical Company. Other chemicals were of reagent grade. All reagents were used as supplied without further purification. The stock solution of nanocomposite was prepared in 0.1M phosphate (NaH_2PO_4) buffer (pH 7.4) containing 0.15M NaCl. The solution of Human Serum Albumin 3×10^{-5} M was prepared by dissolving HSA in phosphate buffer solution at pH 7.4. The concentration of HSA was defined by spectrophotometrically was used an extinction coefficient of 35219 M^{-1} cm^{-1} at 280 nm.

Preparation of lamivudine@LDH@Fe3O4

Synthesis of Fe_3O_4

Fe_3O_4 magnetic nanoparticles were synthesis trough a co-precipitation method. In order to achieve this purpose, ferric chloride hexahydrate $FeCl_3.6H_2O$ (3.25 g) and ferrous chloride tetrahydrate $FeCl_2.4H_2O$ (1.6 g) with the molar ratio 2Fe(III):1Fe(II) were dissolved in 250 mL of distilled water under N_2 atmosphere by vigorous stirring (60 °C). Then pH value of the solution was adjusted to 10 by subjoining 15 mL of 25% NaOH solution quickly

and reaction was performed under continuous stirring (500 rpm) for 60 min at 60 °C. The black precipitation was cooled down to the temperature of room and washed 3 times with double distilled water and ethanol to remove unreacted chemicals. The magnetite precipitates were dried at room temperature for 24 h under vacuum [9].

Synthesis of Fe_3O_4@LDH

First of all, the slick suspension got ready by using ultrasonic dispersion of Fe_3O_4 (0.15 g) into 75 ml solvent (Vmethanol/Vwater=1/1) for 20 min. In the following 50 ml alkaline solution of Na_2CO_3 and sodium hydroxide (NaOH) (0.16 g and 0.24 g respectively) was added dropwise into the ready suspension until pH ca. 10.0 and hold for 10 min. Then 50 ml of the salt solution of 0.25 g of Iron(III) nitrate nonahydrate ($Al(NO_3)_3{\cdot}9H_2O$), (1.2 mmol) and 0.425 g of calcium (II) nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) (3.6 mmol) was added to the former suspension and applying the alkaline solution of Na_2CO_3 and NaOH, the pH of above mixture was adjusted to 10.0. The obtained slurry dispersion was stirred vigorously for 5 min, then collected by a magnet and washing three times with deionized water and ethanol. At last, Fe_3O_4@CaAl LDH was dried 24 hat 60°C giving the desired product.

Preparation of Fe_3O_4@LDH@ lamivudine

Loading the Lamivudine into the structure and on the surface of Fe_3O_4@CaAl LDH was accomplished by appending 0.01 g of Fe_3O_4@CaAl LDH to 30.00 mL of 2.5 mM Lamivudine solution in distilled water and stirred for 24 h at 25 C. In this level, the pH value of the solution adjusted to 7.4. The resulting solid was separated by applying a magnet, then washed three times with distilled water and dried under vacuum condition at 25 C and giving product Fe_3O_4@CaAl LDH@Lamivudine.

Characterization

Powder X-ray diffraction (XRD, Rigaku, D/Max-RA, Cu Ka) was applied to recognize the crystal structure of the samples. Scanning electron microscopy (SEM) has been investigated using SU3500 microscope with a scanning range from 0 to 20 keV.

Apparatus

Uv-visible spectroscopy was analyzed by an HP Uv–vis spectrophotometer (Agilent 8453) which was equipped with thermostat bath (Huber polysat ccl). Emission spectra were recorded with a JASCO (FP6200) fluorescence spectrophotometer.

HSA-binding experiments

Absorption spectroscopy

The UV absorbance spectra of the nanocomposite with HSA were recorded on a HP Uv–vis spectrophotometer (Agilent 8453) from 200 to 350 nm at 298 K using a slit of 5 nm and a scan speed of 250 nm min^{-1}. Quartz cuvettes of 1 cm were used for measurements. Absorption titration experiments were done by keeping the concentration of HSA constant 3.0×10^{-5} M and varying the concentration of nanocomposite from 0 to 98 µM.

Fluorescence quenching

Fluorescence emission spectra were carried out by a JASCO (FP6200) fluorescence spectrometer in the wavelength of 300 to 550 nm (excited at 295 nm) and the pH value was 7.4 at three different temperatures (288 K, 298 K, 310 K). Phosphate Buffer solution was applicate as a reference when measuring the fluorescence spectra of HSA–nanocomposite. In order to use fluorescence emission spectroscopy, the concentration of HSA keep constant 3.0×10^{-5} mol L^{-1} and the concentration of nanocomposite varies from 0 to 98 µM. The fluorescence quenching of HSA at different temperatures were defined using the Stern-Volmer equation [10].

$$\frac{F_0}{F} = 1 + K_q\,\tau_0\,[Q] = 1 + K_{sv}\,[Q] \qquad (1)$$

Where F_o and F are the fluorescence intensities in absence and presence of quencher, respectively, [Q] is the quencher concentration, and K_{sv} is Stern-Volmer quenching constant.

Displacement experiments

The displacement experiments were done at 298 K by using two kinds of site markers (warfarin and ibuprofen). The concentration of HSA (3×10^{-5} M), warfarin and ibuprofen (9×10^{-5} M) were constant during the experiments. In order to investigate the binding site of nanocomposite with HSA, warfarin and ibuprofen were applied.

Calculation of binding constant

The binding constant (K_b) and the number of binding sites to HSA (n) were defined by plotting the double log graph of the fluorescence data by applying (Eq. (2)) [11].

$$\log[\frac{F_0 - F}{F}] = \log K_b + n \log[Q] \quad (2)$$

The K_b was calculated from the intercept and slope of plots of the log[F_o-F/F] vs. log[Q].

Determination of thermodynamic parameters

The forces of molecule contribute to the interaction of the protein with small molecules and these non-covalent interaction forces, mainly include hydrophobic force, electrostatic interactions, van der Waals interactions, and multiple hydrogen bonds. According to the data of enthalpy variation (ΔH) and entropy change (ΔS), the interaction between nanocomposite and biomacromolecules may be deduced: (1) ΔH<0 and ΔS<0, van der Waals interactions and hydrogen bonds; (2) ΔH>0 and ΔS>0, hydrophobic forces; (3) ΔH<0 and ΔS>0, electrostatic interactions. The thermodynamic parameters were computed from the van't-Hoff equation (Eq.3)

$$\ln K_b = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

$$\Delta G = \Delta H - T\,\Delta S \quad (4)$$

Where K_b is the binding constant at the related temperature and R is gas constant. ΔH and ΔS were computed from the slope and intercept of the linear plot (Eq. (3)) based on ln K_b versus 1/T. The free energy change (ΔG) was calculated by using (Eq. (4)) [12].

RESULTS AND DISCUSSIONS

Structural characterization

XRD

The XRD patterns of Fe_3O_4, Fe_3O_4@CaAl LDH, and Fe_3O_4@CaAl LDH@Lamivudine are illustrated in Fig. 1. The XRD pattern of Fe_3O_4 (curve a), clearly indicated the diffraction peaks of cubic-phase Fe_3O_4 crystalline structure at 2θ= 30.2°, 35.7°, 43.1°, 53.3°, 56.8° and 62.8° which are attributed to the (220), (311), (400), (422), (511) and (440) reflections of typical Fe_3O_4 nanoparticles [JCPDS-019-0629]. The XRD pattern of Fe_3O_4@ CaAl LDH obviously shows the formation of CaAl LDH shell around Fe_3O_4 nanoparticles (curve b). The reflections at 10.1°, 20.2°, 30.6°, 37.3°, 48.2°, 62.6°, 63.1° are attributed to (003), (006), (009), (015), (018), (110) and (113) plane of CaAl LDH structures. It is notable that, the shift and broadening of (003) basal reflection of CaAl LDH illustrate that the Lamivudine molecules are entered into the layers of the Fe_3O_4@CaAl LDH@ Lamivudine structure (curve c).

SEM

In order to more study of the morphology of Fe_3O_4@CaAl LDH@Lamivudine, the surface structure has been studied using SEM technique (Fig. 2). It is notable that, SEM image of Fe_3O_4@ CaAl LDH@Lamivudine indicates the presence of Lamivudine on the surface of LDH structures (Fig. 2a). As it is depicted from the SEM images, the core-shell structure and layered surface of Fe_3O_4@ CaAl LDH are obviously verified (Fig. 2b).

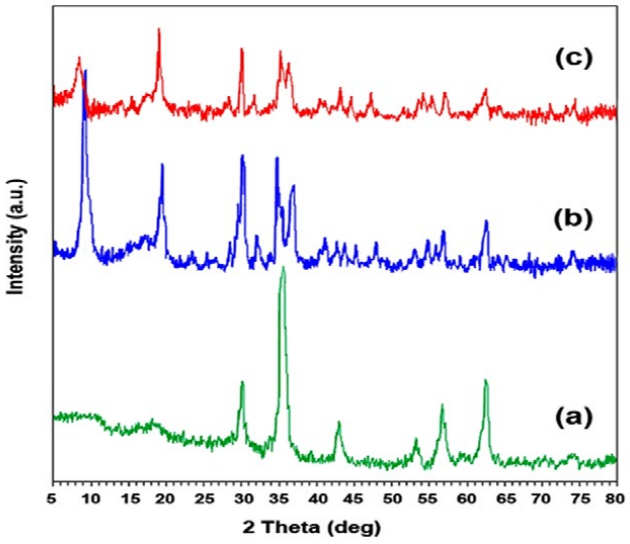

Fig. 1. XRD patterns of a) Fe_3O_4, b) Fe_3O_4@CaAl LDH and c) Fe_3O_4@CaAl LDH@Lamivudine.

Fluorescence quenching and binding constant of HSA in the presence of drugs

Tryptophan residue at position 214 (TRP214) in HSA takes mainly responsible for the fluorescence of this macromolecule. The emission spectra of HSA with different amount of Fe_3O_4@CaAl LDH@ Lamivudine are recorded and displayed in Fig. 3. The results showed that HSA solution gave strong fluorescence when excited by ultraviolet radiation with the wavelength at 344 nm. Its emission intensity decreased when increasing amounts of 3TC were added, which was known as quenching.

The fluorescence emission spectra of HSA in the presence of various concentrations of nanocomposite decreased remarkably Fig. 3.

This results indicated that the nanocomposite interacted with HSA and quenched its intrinsic fluorescence [13]. To study the quenching mechanism of fluorescence the Stern-Volmer equation was used (Eq. (1)) [1]. In this study the K_{sv} value was decreased with the increasing the temperature, that was illustrated the static quenching mechanism which was shown in (Table 1).

The binding constant (K_b) of Fe_3O_4@CaAl LDH@ Lamivudine with HSA was increased by increasing the temperatures and the value of n approximately equal to 1 indicated the existence of, only a single binding in HSA for this nanocomposite [14].

Thermodynamic parameters

The thermodynamic parameters were calculated by (Eq. (3)) and (Eq. (4)) and summarized in Table 1. The plot of log K_b versus 1/T (Fig. 5.) allows to calculate thermodynamic parameters. The negative value of ΔG was indicated that the interaction process is spontaneous [15]. The positive values of ΔS and ΔH demonstrated that

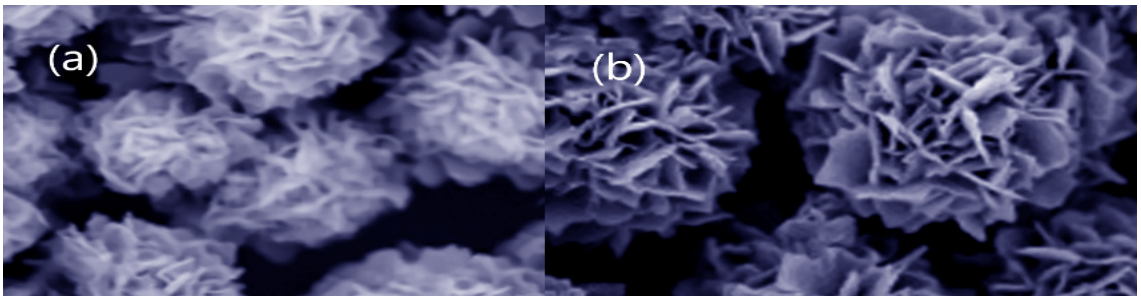

Fig. 2. SEM images of a) Fe_3O_4@CaAl LDH@Lamivudine, b) Fe_3O_4@CaAl LDH.

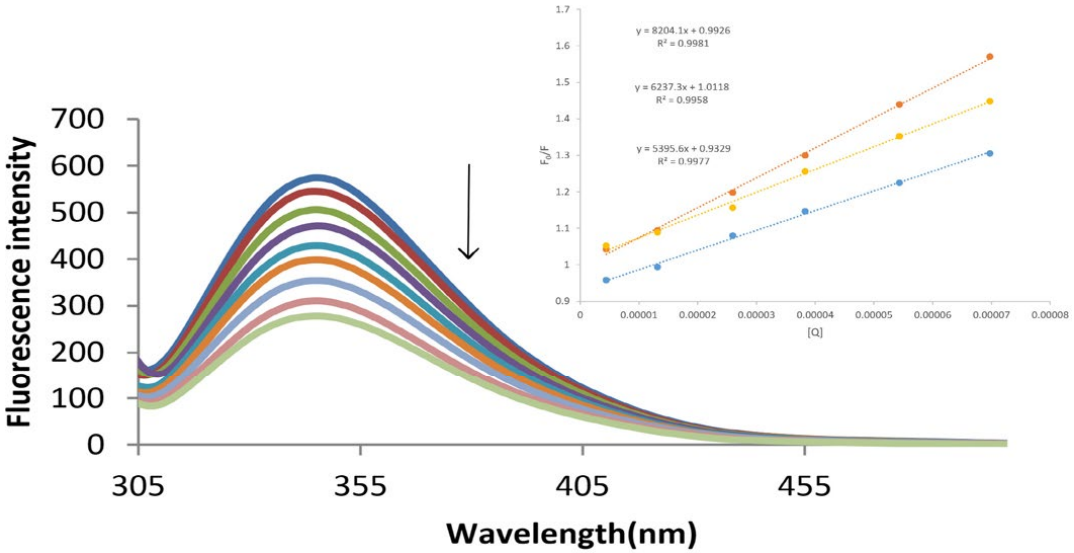

Fig. 3. Emission spectra of HSA in the absence and presence of Fe_3O_4@CaAl LDH@ Lamivudine at 298 K and pH 7.4. [HSA] = 3×10^{-5} M and Fe_3O_4@CaAl LDH@ Lamivudine concentrations from 0 to 98 μM . the Stern-volmer plots of F_0/F versus quencher concentration at three different temperatures (288, 298 and 310 K).

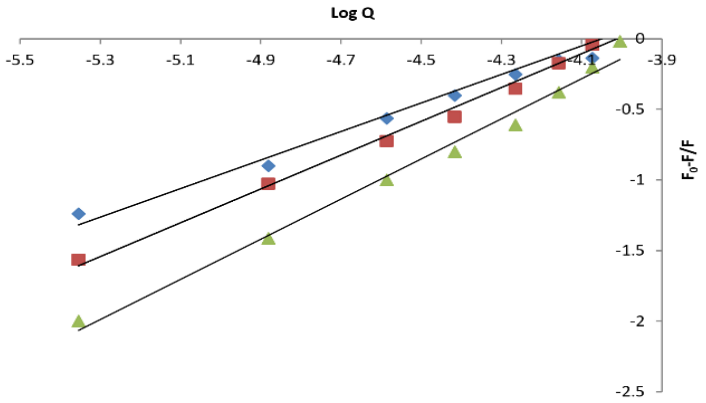

Fig. 4. The plot of log(F_0-F/F) versus log[Q].

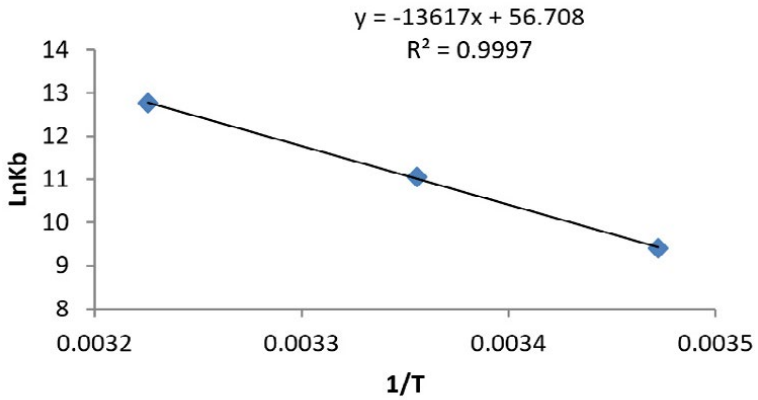

Fig. 5. the plot of lnK_b versus 1/T for HSA and nanocomposite.

Table 1. The binding constants, number of binding sites (n), thermodynamic parameters and Stern-Volmer dynamic quenching constant of HSA-nanocomposite system at different temperatures (288, 298 and 310 K) at pH 7.4.

T	K_{sv}	k_b	R^2	n	ΔG	ΔH	ΔS
288	8265.26	12187.09	94.83	1.0091	-22.527	-	-
298	6164.55	62849.24	99.15	1.1962	-27.373	113.211	471.4703
310	5783.04	350429	98.76	1.421	-32.904	-	-

the interaction binding of nanocomposite and HSA was hydrophobic force and this kind of interaction plays an important role in the binding of small molecules to biomacromolecules [16].

Location binding site

In competitive experiment for Fe_3O_4@CaAl LDH@Lamivudine after adding nanocomposite regularly, the fluorescence intensity of HSA was decreased and was shown in Fig. 6.

The binding constant (K_b) of nanocomposite-HSA-Warfarin and nanocomposite-HSA-Ibuprofen system were calculated and the binding constant (K_b) of them, was 4.0634×10^4 and 1.59×10^2 $Lmol^{-1}$ respectively. The K_b decreased by adding ibuprofen, provided binding constant increased by adding warfarin. The consequences meant that the binding site of nanocomposite and ibuprofen was similar with HSA which was located in subdomain IIIA (Sudlow's site II) [17].

Uv-visible absorption spectroscopy

The Uv-vis absorption measurement is a convenient but effective method to confirm the

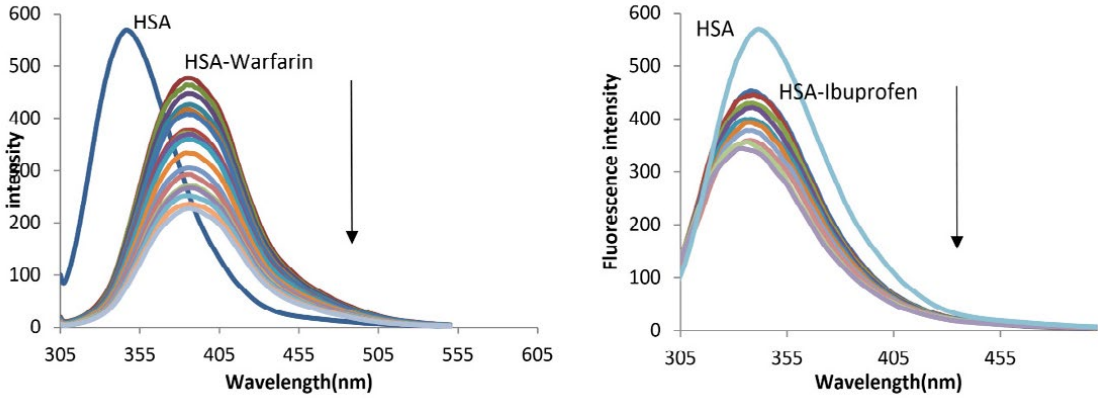

Fig. 6. Effect of site marker; (a) warfarin and (b) ibuprofen to Fe_3O_4@CaAl LDH@Lamivudine and HSA system; [site marker] = [HSA] = 3×10^{-5} M.

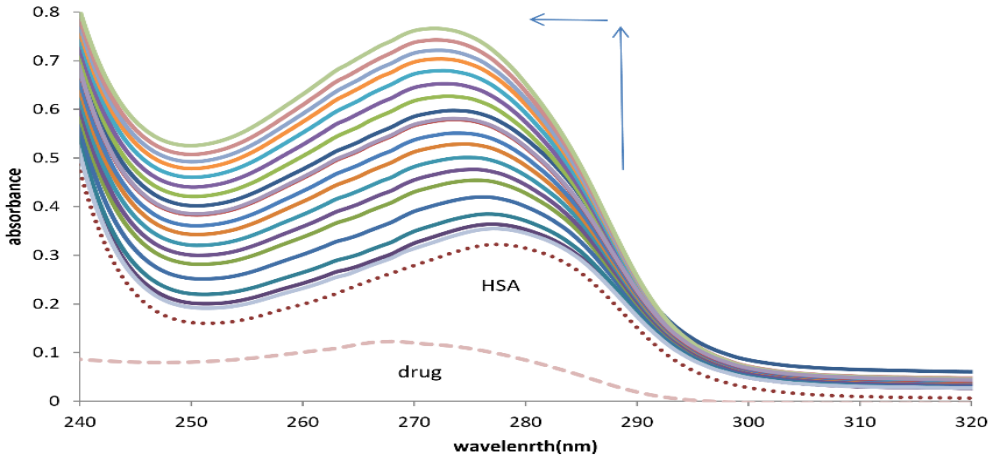

Fig. 7. Uv-vis absorption of HSA in the presence of different concentration of nanocomposite at pH 7.4 and room temperature. The concentration of HSA was 3×10^{-5} M and the concentration of nanocomposite varies from 0 to 98 μM.

formation of complexes. The complex which was formed between nanocomposite and HSA was resulted from the data of Uv-vis absorption spectra (Fig. 7.). The Uv-vis absorption of HSA was increased by adding nanocomposite and it may be due to formation of complex between HSA and nanocomposite. In some cases, complex formation may lead to change the conformation of protein[18].

CONCLUSION

The experimental results indicated that the nanocomposite binds to HSA with moderate affinity and the intrinsic fluorescence of HSA was quenched through static quenching mechanism. The binding parameters were calculated using the modified Stern–Volmer equation. The thermodynamic parameters, positive value of ΔH, positive value of ΔS and the negative value of ΔG indicate that, hydrophobic forces play an important role in interaction binding of nanocomposite to HSA. Competitive results show that the binding site of nanocomposite placed in subdomain IIIA (site II) of HSA. The Uv-vis absorption of HSA was increased by adding nanocomposite and it can be due to the formation of complex between HSA and nanocomposite. Our results may provide valuable information to understand the mechanistic pathway of drug delivery and to pharmacological behavior of drug.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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