

Nano-composite Carbon Paste Electrode Used for Biophysical Study of Ho³⁺ Ion Interaction with Human Serum Albumin V

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In this study, for the first time the interaction of Ho³⁺ ion and human serum albumin (HSA) was investigated in vitro by nano-composite carbon paste potentiometric sensor, fluorescence, and isothermal titration calorimetry at 27 and 37 °C in biological pH. The resulted data from these methods confirm each other and they reveal that interaction of HSA with Ho³⁺ ion showed a set of two binding sites with negative cooperativity. Thermodynamic parameters for the interaction of holmium ions and HSA indicate that the concentration of this ion causes conformational changes on the structure of HSA which increasing the number of hydrophobic contacts of HSA.

Keywords: holmium(III) ion, human serum albumin, potentiometric sensor, nano-composite carbon paste electrode, isothermal titration calorimetry, fluorescence spectroscopy.

1. INTRODUCTION

Holmium is a member of lanthanoid series, originally known as rare earth metals. The biological properties of the lanthanoid, primarily based on their similarity to calcium, have been the basis for research into potential therapeutic applications of lanthanoid since the early part of the twentieth century. The lanthanoid has similar ionic radii to calcium, but by virtue of possessing a higher charge, they have a high affinity for Ca²⁺ sites on biological molecules [1-3].

The lanthanides (Ln^{3+}) can substitute for calcium in proteins, though it should be noted that the Ln^{3+} can also substitute for other metal ions such as Mg^{2+} , Fe^{3+} , and Mn^{2+} . Calcium dependent enzymes can either be inhibited by lanthanides, or in some cases be activated to a similar or greater extent than by calcium. It has been proposed that the stimulatory or inhibitory effect of the lanthanides may be a function of the role of calcium in the native enzyme [1].

Albumin, comprising nearly 60% of the blood serum protein, is a protein primarily present in the blood serum of humans. Albumin is synthesized in the liver and has a molar mass of approximately 66500 Daltons.

The reference range for albumin concentrations in blood ranges from 30 to 50 g/L. Serum albumins have many physiological functions such as maintaining osmotic pressure, transporting thyroid, as well as other, hormones; particularly fat soluble ones, transporting fatty acids ("free" fatty acids) to the liver, transporting unconjugated bilirubin and transporting many drugs. Serum albumin levels can affect the half-life of drugs and buffers pH. In addition to its presence in the circulatory system, albumin has been located in every tissue and bodily secretion [4].

Many biological molecules, metal ions and a variety of drugs such as anticoagulants, tranquilizers, and general anesthetics (often more than 90% of the drug is bound) can bind to albumin in the blood and then be transported in the circulatory system [5].

Literature survey reveals that there is not any report on the interaction study between holmium ions (Ho^{3+}) with human serum albumin (HSA). In this study, for the first time, interaction between Ho^{3+} ion and HSA is studied by a Ho^{3+} nano-composite carbon paste potentiometric electrode, then the results are compared with fluorescence, and isothermal titration calorimetry at 27 and 37 °C in biological pH=7.4.

In comparison with many complex techniques for pharmaceutical and biological studies, electrochemical methods offer advantages of low cost, portability, selectivity, and simple operatory. They also provide an analysis method without destruction of sample [6-13].

Carbon nanotubes (CNTs) have special physicochemical properties such as ordered structure with high aspect ratio, ultra-light weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semi-metallic behavior and high surface. Recently, multi walled carbon nanotubes (MWCNTs) are used as an electron transfer and signal amplifier in electrochemical sensors and biosensors [14-16].

2. MATERIALS AND METHODS

2.1. Reagents and materials

Holmium chloride was prepared by dissolving holmium oxide (Ho_2O_3) in a hydrochloric acid aqueous solution. Then the products were recrystallized.

Human serum albumin was purchased from Sigma–Aldrich. 8-anilinonaphthalene-1-sulfonic acid ammonium salt was purchased from Fluka Chemie AG. Double distilled water was used during the experiments.

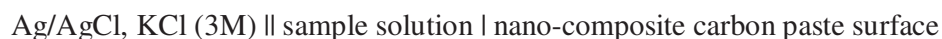
2.2. Stock Solutions

Stock solution of 1×10^{-4} M HSA was prepared in water with the pH value of 7.4. The solutions were stored at 0-4 °C. The Ho^{3+} standard solutions (1.0×10^{-2} M) were also prepared from HoCl_3 .

2.3. Apparatus and methods

2.3.1. Potentiometry

Since potentiometric sensors are rapid growing class of electrochemical sensors with low cost, simplicity, high selectivity [17-22], in this work, Ho^{3+} nano-composite carbon paste electrode, which is constructed before [23] was used for interaction study between Ho^{3+} ions and HSA. Potentials were measured with a Corning ion analyzer Model 250-pH/mV meter. The pH of the sample solutions was monitored simultaneously with a conventional glass pH electrode. All emf measurements were carried out with the following cell assembly:



The Ho^{3+} modified carbon paste electrode exhibited a Nernstian response of 19.3 ± 0.3 mV per decade of holmium concentration, and a very wide linear range 1.0×10^{-8} - 1.0×10^{-2} mol L^{-1} . It can work well in the pH range of 3.8-7.5. The lower detection limit (LDL) of the electrode was calculated to be 7.0×10^{-9} mol L^{-1} [10].

2.3.2. Fluorescence Spectroscopy

The fluorescence study was performed using a Perkin-Elmer LS50 spectrofluorimeter. The excitation wavelength was 295 nm in all cases with an excitation and emission band pass (slit) of 7 nm. The solutions were placed in a 1 cm path-length quartz cell for the fluorescence measurements. No explicit correction of our spectra for the instrument response was performed because in our experiments only relative changes in the fluorescence intensity of the lanthanoid as a function of certain experimental conditions were required.

2.3.3. UV-Vis. Spectroscopy

All ultraviolet-visible spectra were recorded in a UV-Vis. Lambda 2 spectrophotometer (Perkin Elmer).

2.3.4. Calorimetry

The isothermal titration calorimetric experiments were carried out on a VP-ITC ultra sensitive titration calorimeter (MicroCal, LLC, Northampton, MA). The micro-calorimeter consists of a

reference cell and a sample cell of 1.8 mL in volume, with both cells insulated by an adiabatic shield. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with HSA solution ($40\ \mu\text{M}$) and the reference cell contained buffer solution. The solution in the cell was stirred at 307 rpm by the syringe (equipped with micro propeller) filled with HoCl_3 solution ($500\ \mu\text{M}$) to ensure rapid mixing. Injections were started after baseline stability had been achieved. The titration of HSA with HoCl_3 solution involved 30 consecutive injections of the ligand solution, the first injection was $5\ \mu\text{L}$ and the remaining ones were $10\ \mu\text{L}$. In all cases, each injection was done in 6 s at 3-min intervals. To correct the thermal effects due to HoCl_3 dilution, control experiments were done in which identical aliquots were injected into the buffer solution with the exception of HSA. In the ITC experiments, the enthalpy changes associated with processes occurring at a constant temperature are measured. The measurements were performed at two constant temperatures of 27.0 and $37.0\pm 0.02\ ^\circ\text{C}$ and the temperature were controlled using a Poly-Science water bath.

3. RESULTS AND DISCUSSIONS

3.1. Potentiometric Study

A simple method for determination of binding isotherm in the protein–ligand interaction was used to study of interaction of HSA with trivalent holmium ion, in Tris buffer solution at $\text{pH}=7.4$. The binding isotherm for Ho–HSA interaction is easily obtained by carrying out potentiometric titration in two different concentrations of HSA as shown in Fig. 1 and Fig. 2.

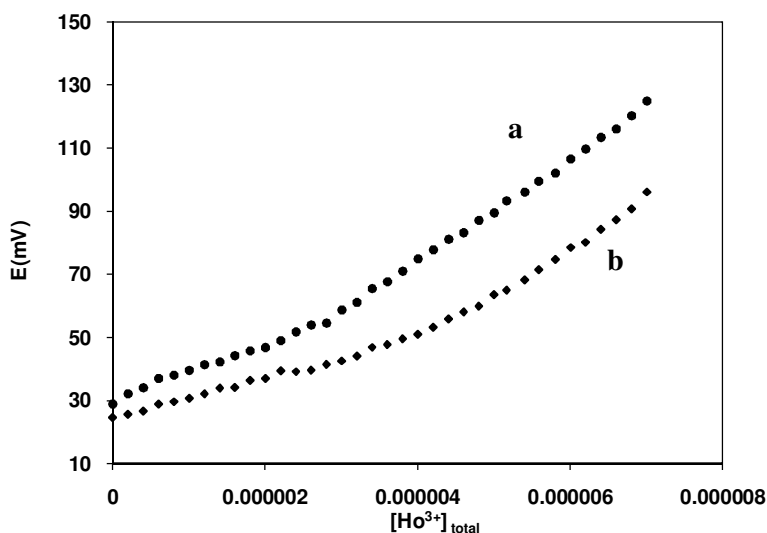


Figure 1. The potential changes of the holmium nano-composite potentiometric electrode during titration of 5.0 mL of a: $1\ \mu\text{M}$ and b: $2\ \mu\text{M}$ of HSA solution with $100\ \mu\text{M}$ of Ho^{3+} ion

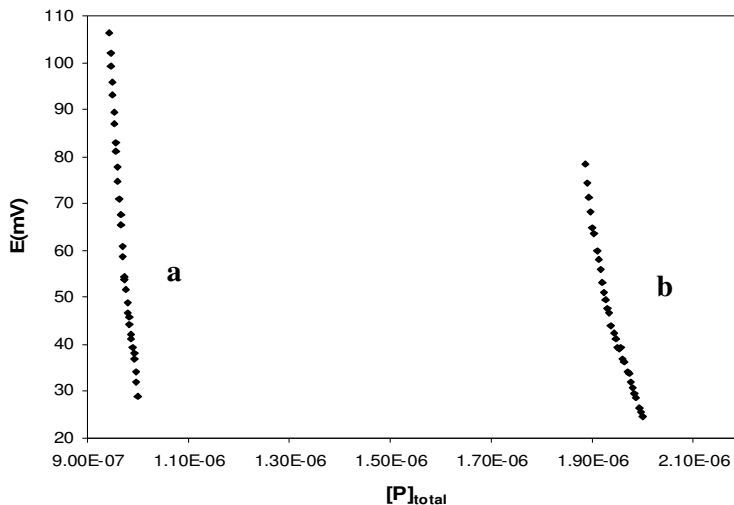


Figure 2. The potential changes of the titration of 5.0 mL of a: 1 μM and b: 2 μM of HSA solution with 1.0×10^{-4} M of Ho^{3+} vs. total concentration of HAS

The potentiometric titration experiments were performed by the holmium potentiometric nano-composite sensor, which is used as an indicator electrode [23].

In general, there will be "g" sites for binding of holmium ions per protein macromolecule and "v" is defined as the average moles of bound holmium ions per mole of total HSA. At any constant value of potential (E), the free concentration of holmium ion (L_{free}) and "v" are also constant at equilibrium on both two curves in Fig.1. Holmium ions exist in two forms of free and bound. Hence, $L_{\text{free}} = L_{\text{total}} - L_{\text{bound}}$, where L_{total} and L_{bound} are the total and bound concentration of holmium ion, respectively.

$$L_a^{\text{free}} = L_b^{\text{free}} \tag{1}$$

$$L_a^{\text{total}} - L_a^{\text{bound}} = L_b^{\text{total}} - L_b^{\text{bound}} \tag{2}$$

By applying $v = L_{\text{bound}} / P^{\text{total}}$, which P^{total} is the total concentration of the protein, and equality of v at any constant value of potential on both curves in Fig. 1, it can be deduced equation (3) from equation (2):

$$L_a^{\text{total}} - vP_a^{\text{total}} = L_b^{\text{total}} - vP_b^{\text{total}} \tag{3}$$

This equation can be rearranged to give equation (4):

$$v = \frac{L_a^{\text{total}} - L_b^{\text{total}}}{P_a^{\text{total}} - P_b^{\text{total}}} \tag{4}$$

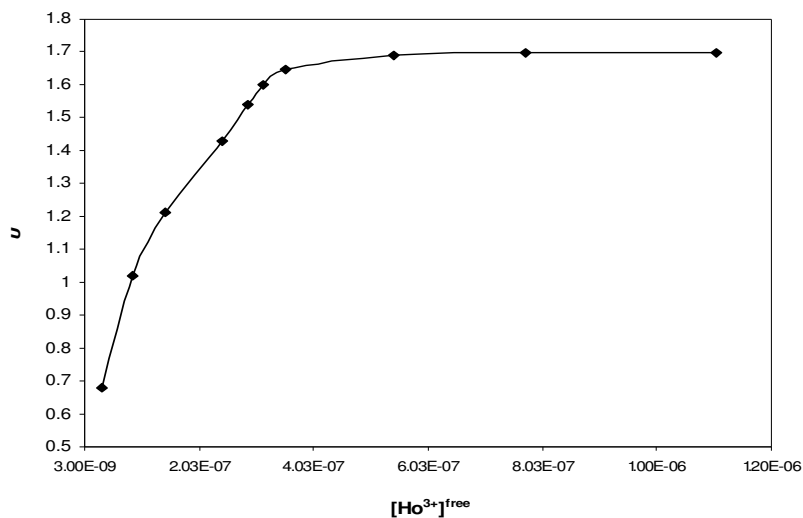


Figure 3. The binding isotherm of binding holmium ion to HSA

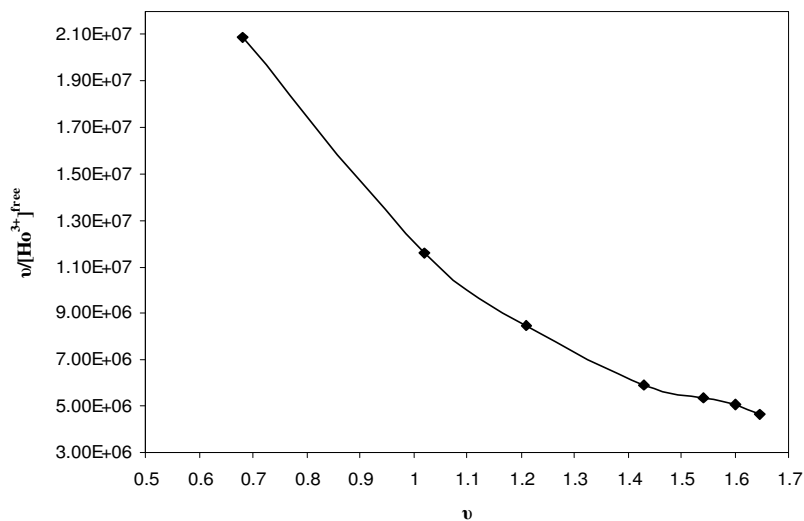


Figure 4. The Scatchard plot ($\frac{\nu}{[Ho^{3+}]_{free}}$ vs. ν) of binding holmium ion to HSA

By using the calibration curve of the proposed nano-composite electrode, the L^{free} can be calculated easily. Then, the binding isotherm (Fig. 3) and the Scatchard plot (Fig. 4) can be plotted [24]:

$$\frac{\nu}{[Ho^{3+}]_{free}} = gK - \nu K \tag{5}$$

Which K is the binding constant of metal ion to HSA. It is sometimes the case that binding data does not form a straight line when plotted in a Scatchard plot. In this case, ligand binding at one site affects binding at the other. In fact, the data does not support the independent, equivalent site. The shapes of the Scatchard plots are clearly characteristic of different types of cooperativity [25,26]. A concave downward curve, as shown in Fig. 4, describes a system with negative cooperativity.

For obtaining approximated values of binding parameters, it might be possible to fit the binding data to the Hill equation [27]:

$$v = \frac{g(K([Ho^{3+}]^{free})^{n_{Hill}})}{1 + (K([Ho^{3+}]^{free})^{n_{Hill}})} \quad (6)$$

where K and n_{Hill} are the binding constant and Hill coefficient, respectively. The binding data for the binding of holmium ions to HSA can be fitted to the Hill equation using a computer program for nonlinear least-square fitting [28]. A quick and easy alternate to this method is to rearrange the binding function to make it possible to plot a linearized version of the equation. The eye and a straightedge can readily measure the values of n_{Hill} and K and also can detect if the data deviates from the theoretical curve.

$$\log\left(\frac{v}{g-v}\right) = n_{Hill} \log[Ho^{3+}]^{free} + n_{Hill} \log K \quad (7)$$

Eq. (7) is in the form of $y=mx+b$, i.e. a straight line, with slope n_{Hill} and vertical axis intercept $n_{Hill} \log K$ if $\log(v/g-v)$ versus $\log[Ho^{3+}]^{free}$ is plotted as shown in Fig. 5.

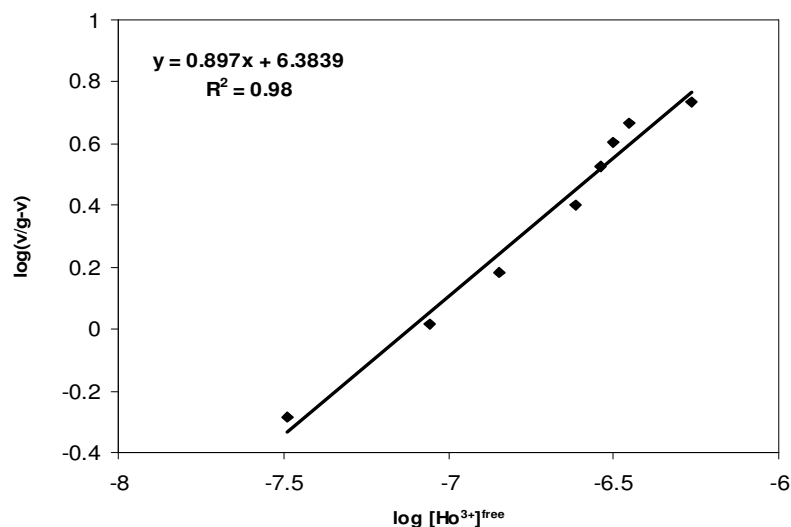


Figure 5. The Hill plot of binding holmium ion to HSA

Therefore, converting binding data to this form and plotting it will produce the value of the Hill coefficient and the constant K , which is the artificial binding constant for the simultaneous binding of two holmium ions to HSA. The results are: $g=2$, $K=2.4 \times 10^8 \text{ moMI}^{-1}$ and $n_{\text{Hill}}=0.89$. The best-fit curve of the experimental binding data was then transformed to a Scatchard plot as shown in Fig. 4. A simple method for calculating intrinsic association equilibrium constants for system with two cooperative sites (K_1 and K_2) has been introduced from the Scatchard plot. It has been shown that, in the limit as v approaches 0, $v/[\text{Ho}^{3+}]^{\text{free}}=2K_1$ and when $v=1$, or at half-saturation, $v/[\text{Ho}^{3+}]^{\text{free}}=(K_1K_2)^{1/2}$. Thus, K_1 can be obtained from the ordinate intercept of a Scatchard plot and K_2 is derived from the value of $v/[\text{Ho}^{3+}]^{\text{free}}$ at half-saturation. The results obtained from Fig. 4 are $K_1=2.88 \times 10^8 \text{ M}^{-1}$ and $K_2=8.77 \times 10^7 \text{ M}^{-1}$. Thus, occupation of the first site has decreased the binding affinity of the second site.

3.2. Fluorescence Study

The fluorescence of proteins is caused by three intrinsic fluorophores present in the protein, tryptophan, tyrosine and phenylalanine residues. Because of very low quantum yield of phenylalanine and tyrosine, normally the fluorescence of tryptophan residue is investigated. Previous studies reveal that HSA has only one tryptophan residue, Trp-214 [29,30]. Generally, when the excitation wavelength of serum albumins was selected at 280 nm, the fluorescence was produced from the tryptophan and tyrosine residues. At 295 nm, the emission is only due to the tryptophan residue [29,30]. In this work, tryptophan fluorescence emission spectra over the 300–400 nm wavelength range were recorded with the excitation set at 295 nm. Fig. 6 shows the fluorescence emission spectra (at $\lambda_{\text{ex}}=295 \text{ nm}$, 300 K) obtained for HSA titrated by holmium ion.

The Stern–Volmer plot of the titrations of HSA by holmium ion is shown in Fig. 7. These plots show a small downward curvature towards the x-axis at holmium concentration higher than 2.0 μM . At different temperatures, the Stern–Volmer plots have the same trends. According to Fig. 7, it is indicated that the quenching was not initiated by collision and it is a static quenching.

The quenching data were analyzed according to the modified Stern-Volmer equation [29-31]:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K_a} \times \frac{1}{[Q]} \quad (8)$$

Where f_a is the fraction of the initial fluorescence, which is accessible to quencher. The value of f_a refers to the fraction of fluorescence accessible to quenching, which need not be the same as the fraction of tryptophan residue, accessible to quenching [31,32]. The dependence of $F_0/(F_0-F)$ on the reciprocal value of the quencher concentration ($1/[\text{Ho}^{3+}]$) is linear as shown in Fig. 8. The values of f_a and K_a were obtained from the values of intercept and slope, respectively. The values of f_a for 300 and 310 K were found to be 0.29 and 0.28 indicating that 29% and 28% of the total fluorescence of tryptophan is accessible to holmium ion. The value of K_a is calculated $3.47 \times 10^5 \text{ M}^{-1}$ for 300 K and $1.82 \times 10^5 \text{ M}^{-1}$ for 310 K. The values of K_a decrease with the temperature rising, which was in accordance with K_{SV} dependence on temperature, a confirmation for a static quenching.

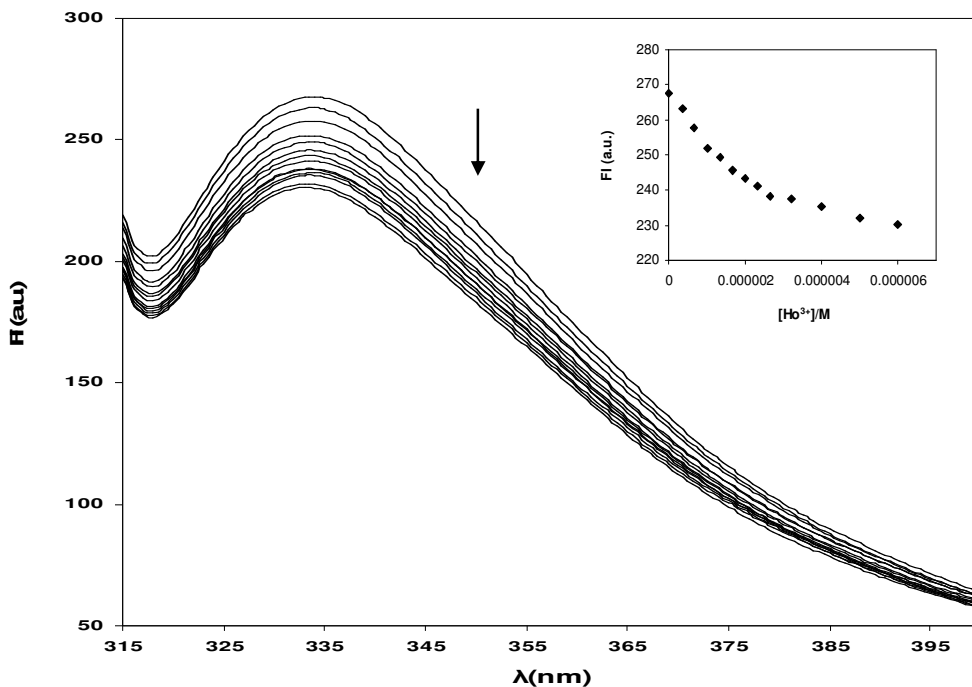


Figure 6. Effect of holmium ion on fluorescence spectra of HSA ($T=300\text{ K}$, $\text{pH}=7.4$, $\lambda_{\text{ex}}=295\text{ nm}$). $C_{\text{HSA}}: 1\ \mu\text{M}$; $C_{\text{holmium}} \times 10^{-7}\text{ M}$: 3.3 to 40.0, respectively. Insert figure: Fluorescence intensity of HSA vs. holmium concentration

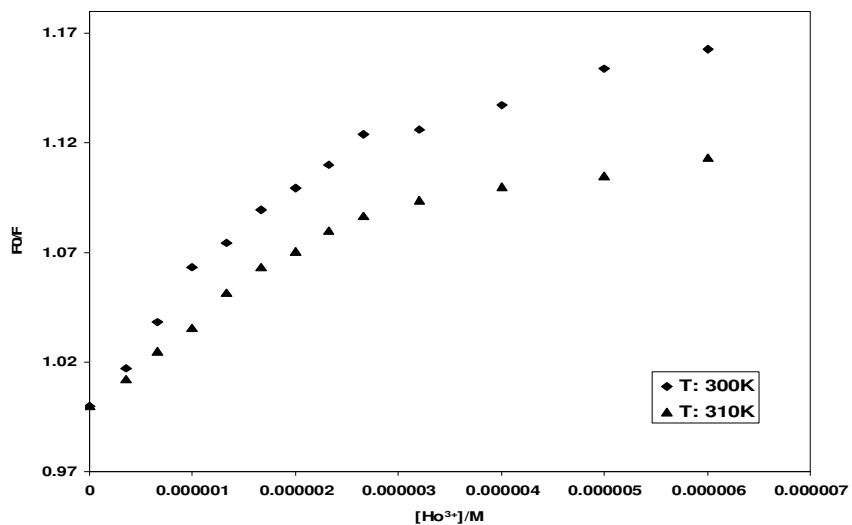


Figure 7. The Stern-Volmer plots of titration of HSA with holmium ion at different temperatures (300 and 310 K). $\lambda_{\text{ex}}=295\text{ nm}$; $\lambda_{\text{em}}=331\text{ nm}$; $C_{\text{HSA}}: 1\ \mu\text{M}$

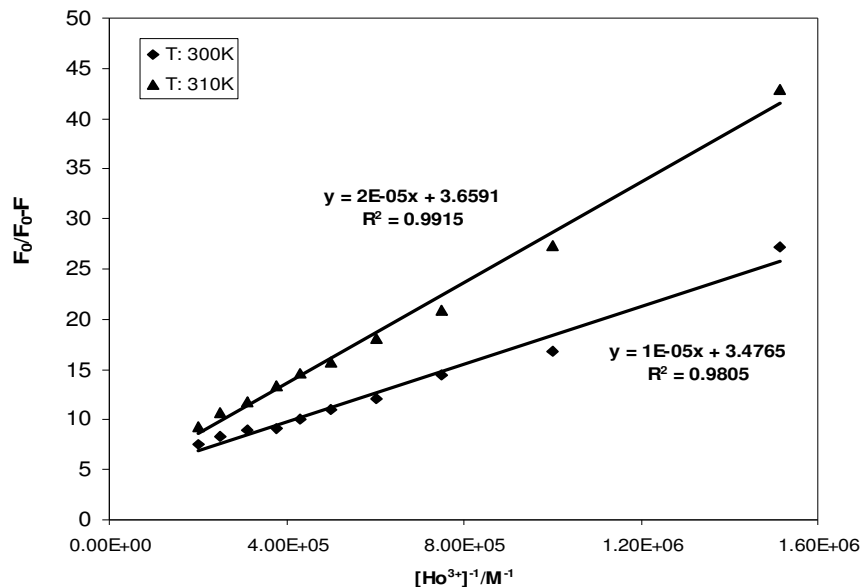


Figure 8. Modified Stern-Volmer plots of titration of HSA with holmium ion at different temperatures (300 and 310 K). λ_{ex} =295 nm; λ_{em} =331 nm; C_{HSA} : 1 μ M

3.2.1. Distance between donor-acceptor

According to Förster's non-radiation energy transfer theory, the energy transfer can occur only when the fluorescence emission spectra of the donor and the absorption spectra of the acceptor have enough overlap and the distance between donor and acceptor is not longer than 7 nm. In detail, it can be described by the following three equations [34, 35]:

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

$$R_0 = 8.8 \times 10^{-25} K^2 N^{-4} \phi J \quad (10)$$

$$J = \frac{\sum F(\lambda) \varepsilon(\lambda_0) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda} \quad (11)$$

where E is the efficiency of transfer between the donor and the acceptor, R_0 the critical distance when the efficiency of transfer is 50%, r the distance between the acceptor and the donor, K^2 the space factor of orientation, N the refracted index of medium, ϕ the fluorescence quantum yield of the donor, J the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, $F(\lambda)$ the corrected fluorescence intensity of the donor in the wavelength range λ_0 to λ and $\varepsilon(\lambda_0)$ the extinction coefficient of the acceptor at λ_0 .

The overlap of the absorption spectrum of holmium ion and the fluorescence emission spectrum of HSA is shown in Fig. 9. The overlap integral, J , can be evaluated by integrating the spectra in Fig. 6 according to the equation (11). Under our experimental conditions, $K^2=2/3$, $N=1.336$ and $\phi=0.13$, J can be evaluated to be $2.21 \times 10^{-11} \text{ cm}^3\text{M}^{-1}$. Under these experimental conditions, R_0 is 1.40 nm and energy transfer effect (E) is 0.125 from equation (9). Thus, the maximum distance between holmium ion and amino acid residue in HSA, r is 1.91 nm. This confirms that the energy transfer between holmium ion and HSA contributes to a static quenching.

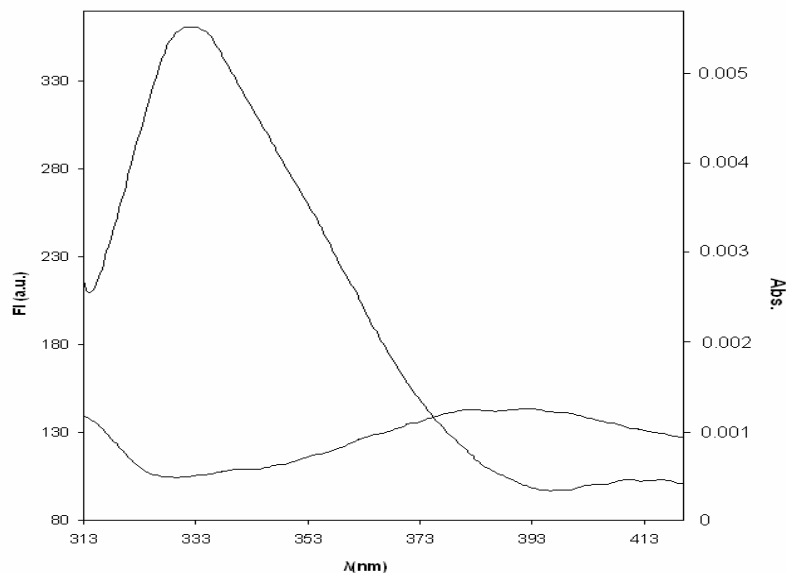


Figure 9. Overlap of the absorption spectrum of holmium ion ($C_{\text{holmium}}=1 \mu\text{M}$) and the fluorescence emission spectrum of HSA ($C_{\text{HSA}}=1 \mu\text{M}$) in 1:1 mol ratio

3.2.2. Thermodynamic parameters

Generally, the force between organic micromolecule and biological macromolecule may include hydrogen bond, van der Waals force, electrostatic force and hydrogen bond interaction force and so on [36]. Because the effect of the temperature is pretty small, the enthalpy change of the interaction can be regarded as a constant if the temperature range is not too wide. Therefore, from the following equations (12-14):

$$\Delta G^\circ = -RT \ln K_a \quad (12)$$

$$\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \quad (13)$$

$$\ln K_a = -\Delta H^\circ / RT + \Delta S^\circ / R \quad (14)$$

where K_a is the binding constant obtained from equation (8) at the corresponding temperature, and R is the gas constant. The temperatures used were 300 K and 310 K. Thus, to explain the interaction of holmium ion with HSA, the thermodynamic parameters were calculated.

Table 1. The binding efficiency of micromolecules to biological macromolecules (K_a) and thermodynamic parameters (ΔH° , ΔG° and ΔS°) for holmium ion-HSA complex

| Temperature (K) | $K_a \times 10^5$ (M^{-1}) | ΔG° ($kJ\ mol^{-1}$) | ΔH° ($kJ\ mol^{-1}$) | ΔS° ($J\ mol^{-1}K^{-1}$) |
|-----------------|--------------------------------|-------------------------------------|-------------------------------------|--|
| 300 | 3.97 | -32.154 | -22.614 | 32.58 |
| 310 | 3.02 | -31.621 | | |

Table 1 shows the values of ΔH° , ΔG° and ΔS° . The negative value of ΔG° shows that the interaction process is spontaneous. Electrostatic interaction plays major role in the HSA-Ho³⁺ interaction. The negative values of enthalpy (ΔH) and positive value of entropy (ΔS) in the interaction of holmium and HSA indicate that the binding is mainly enthalpy and entropy stabilized. Desolvation of polar groups on HSA and desolvation of holmium ions are specific and directed, which may be the best identified through their negative enthalpy and positive entropy of complex formation.

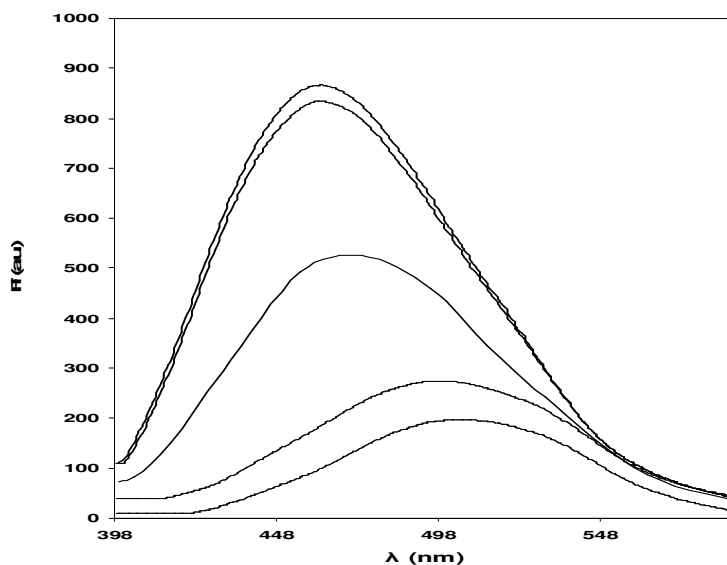


Figure 10. Fluorescence emission spectra of 1-anilino-8-naphthalene-6-sulfonic acid (ANS); λ_{ex} =385 nm; λ_{em} =505 nm; A: ANS free (100 μ M); B: HSA (3 μ M) added to ANS; C: HSA+Ho (1:1) added to ANS; D: HSA+Ho (1:2) added to ANS; E: HSA+Ho (1:3) added to ANS

3.2.3. Binding property

There are a number of dyes that can be used to non-covalently label proteins. These are typically naphthylamine sulfonic acids, of which 1-anilino-8-naphthalene-6-sulfonic acid (ANS) and 2-(p-toluidinyl)naphthalene-6-sulfonic acid (TNS) are most commonly used [29]. Dyes of this class are frequently weakly or non-fluorescent in water, but fluoresce strongly when bound to proteins or membranes. ANS has valuable property that in aqueous solution it fluoresces very weakly. However, in a non-polar environment fluorescent quantum yield increases markedly and the spectrum shifts toward shorter wavelengths [37]. The extrinsic fluorophore ANS has no detectable fluorescence in aqueous solution, but it fluoresces when added to the solution of HSA which is indicating the binding of ANS to the hydrophobic region of the HSA (Fig.10).

As it can be seen from Fig. 10, the addition of holmium ions to this system increases the fluorescence of ANS and shifts the fluorescence peak to a shorter wavelength by about 30 nm. This indicates that ANS-binding site does not overlap with the binding site of holmium ions. The increase in fluorescence intensity of the ANS can be the result of increase in binding of ANS or decrease polarity of the ANS binding site by the formation of HSA-holmium complex. However, the shift of the emission peak to shorter wavelength is suggestive of the change of polarity of the ANS-binding site. The change in polarity may be result from polar groups moving away from the binding site or from covering of the polar group, in either case a conformational change is associated with the binding of the holmium ions.

3.3. Isothermal Titration Calorimetry Study

The above results are in good agreement with our results obtained by calorimetry. The calorimetry study reports the thermodynamic parameters that govern HSA- Ho^{3+} interactions. The extended solvation theory [38,39] was used to reproduce the enthalpies of HSA- Ho^{3+} interactions over the whole range of holmium ion concentrations. The binding parameters recovered from the new model were attributed to the structural change of HSA and its biological activity. The results obtained indicate that there is a set of two identical binding sites for Ho^{3+} ions with negative cooperativity. The enhancement of complex formation by holmium ion and concomitant increase in ΔS suggest that the metal ion plays a role in increasing the number of hydrophobic contacts. The binding parameters discovered from the extended solvation model indicate that the stability of HSA molecule is increased as a result of its interaction with holmium ions.

4. CONCLUSION

The interaction between holmium ion and human serum albumin (HSA) was studied at biologic pH=7.4, 27 and 37 °C by nano-composite Ho^{3+} carbon paste electrode. The results were compared to fluorescence spectroscopy and isothermal titration calorimetry. The obtained results by these methods

confirm and complete each other very well. As a result of these studies, there is a set of two identical binding sites for Ho^{3+} ions with negative cooperativity. The binding parameters for the interaction of holmium ions and HSA indicate that the concentration of this ion causes conformational changes on the structure of HSA. Also, the enhancement of complex formation by Ho^{3+} ion and increase in ΔS , and ANS check suggest that the metal ion plays a role in increasing the number of hydrophobic contacts of HSA.

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