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Original Article

INTERACTION OF PROLACTIN HORMONE WITH THE SURFACES OF TWO NEW AZO COMPOUNDS

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ABSTRACT

Objective: Many materials types were used for the biomolecules interaction including insoluble organic compounds to use them in the biosensor or *in vivo*. The aim of the present study is to immobilize the prolactin hormone on the surface of two new synthesized Schiff bases (LH1 &LH2) and to study the different thermodynamic parameters of the adsorption processes.

Methods: Different concentrations of the prolactin solutions were incubated with aliquots of 50mg of LH1 or LH2 and the quantity of the prolactin adsorbed were calculated. The experiments were repeated at different temperatures to calculate the thermodynamic parameters.

Results: There are significant adsorption of prolactin molecules on the surfaces of LH1 & LH2, 97ng/mg and 48ng/mg, respectively. The adsorption process obeyed Freundlich adsorption isotherm indicating heterogeneity of the surface of the compounds or different forces of interaction involved in the adsorption between prolactin and the compounds. Thermodynamic analysis indicated an exothermic and spontaneous adsorption process of the prolactin on the surfaces of LH1 & LH2. The percentage of the quantities of prolactin desorbed from the surface of compound were 7.8% from LH1 and 11.2% from LH2 surfaces indicating a stronger forces of interaction between prolactin molecules and LH1.

Conclusion: It can be concluded that the prolactin molecules can be immobilized on the surface of LH1 with good quantity of adsorption and low desorption ensuring the ability to use LH1 as a surface for *in vivo* or *in vitro* applications.

Keywords: Azo, adsorption, Protein, Prolactin, and Desorption.

INTRODUCTION

Protein interaction with different surfaces is the initial event for the many applications biosensors and many analytical kits. Many studies have been carried out to immobilize different proteins on different surfaces such as albumin [1] and enzymes [2]. Many material types were used for the biomolecules interaction including Schiff bases [3] and azo-compounds [4] The interactions between biomolecules and the surface were occurred mainly by the sorption of a protein on a solid from a water solution as the result of a reversible reaction (sorption–desorption) which reaches a final equilibrium condition between the concentration of the chemical in the two phases [5]. Prolactin (PRL) is a 23 kDa four alpha-helix bundle protein hormone secreted by the anterior pituitary gland. More than 300 different biological functions have been attributed to PRL. The major ones being induction of differentiation and growth in mammary epithelia and stimulation of milk protein secretion [6,7]. Immobilization of this hormone on the surfaces of some organometallic compounds has been studied previously [8, 9]

The aim of the present study is to immobilize the prolactin hormoneon the surface of two newly synthesized organic compounds and study the different thermodynamic parameters of the adsorption processes. Furthermore, the desorption process of the prolactin from the surface of these compounds will be studied quantitatively.

MATERIALS AND METHODS

A- Preparation of LH1 (N,N'-bis(2-hydroxybenzylidene) dithiooxamide)

The compounds was synthesized according to a previously described method [10] by dissolving (10mmol) of dithiooxamide in 20 mL of hot absolute ethanol and then (20 mmol) of 2-hydroxybenzyaldehyde was added, after that 2-3 drops of triethylamine were added to the mixture. The mixture was stirred and refluxed for 6 hours. The precipitate was filtered and washed with cold ethanol several times and then dried at 45ºC for 5 hours. The ligand was partially soluble in dichloromethane, chloroform, toluene, non-soluble in n-hexane and soluble in DMF and DMSO.

B- Preparation of Compound 2(2-((2-phenylhydrazono) methyl) phenol)

A 0.02 mole (2.4 mL) of salicylaldehyde was stirred with 10 mL of ethanol for about 20 minutes and then 0.02 mole (2 mL) of ethanolic solution of phenyl hydrazine was added gradually to the solution. The mixture was refluxed at 60°C for 3 hours. The reaction was visualized by TLC using ethyl acetate as an eluent which shows one spot of the product. The yellow product was dried in an oven at 50°C for 5hours. The product is labeled as LH2 [10].

The prepared compounds were diagnosed using different techniques; UV-Visible Spectrophotometry, melting point apparatus, FTIR spectrophotometry, analysis of the elements (CHN), and 1H-Nuclear Magnetic Resonance (NMR). ELISA reader was used to estimate the prolactin concentration in solution before and after adsorption on the surface of the compounds LH1 and LH2.

C-Adsorption study

To study the adsorption isotherms of the adsorption of prolactin on the compounds (LH1 & LH2), 100µl of prolactin solution at concentrations 0, 5, 10, 25, 50 and 100ng/ml was added to 50mg of each compounds in an Eppindroff® tubes. The tubes were incubated with shaking for one hour which is more than the equilibrium time obtained from previous experiments. The mixture then centrifuged

at 4000rpm for 10 minutes. The prolactin concentration in the aspirated supernatant was estimated by the Enzyme Linked Immunosorbent Assay (ELISA) using Kits supplied by Monobind Co. USA. The amount of prolactin adsorbed on the surfaces was calculated using the following equation:

$Q_e = X/m = [V(C_o - C_e)]/m$ .....(1)

X: The quantity adsorbed (ng), V: Volume of solution (ml), C_o: Initial concentration (ng/ml), C_e: Equilibrium concentration (ng/ml), m: Weight of adsorbent (compound 1 or 2). The Q_e values were plotted versus the equilibrium concentrations (C_e) and the resulting figures representingthe adsorption isotherms that required for understanding and interpreting the systems under investigation.

D-Thermodynamics of the adsorption process:

In order to obtain a thermodynamic parameters of the adsorption process, the adsorption experiments were repeated at different temperatures (10, 20, 30, and 40°C) to measure the thermodynamic parameters (ΔHº, ΔGº, ΔSº). The equilibrium constant (K) for the adsorption process at each temperature is calculated from division of the quantity of prolactin adsorbed on the surface of LH1 and LH2 compoundson the quantity of prolactin still dissolved in solution:-

$$K = \frac{Qe * 0.05}{Ce * 0.01} \quad \text{----------(3)}$$

Where (0.050g) represent the weight of the compounds LH1 and LH2 that has been used and (0.010ml) represents the volume of theprolactin solution used in the adsorption process.

The change in free energy (ΔGº) could be determined form the equation:-

$$\Delta G^\circ = -RT \ln K \quad \text{----------(4)}$$

Where R is the gas constant (8.314 J.mole^{-1}.ºK^{-1}) and T is the absolute temperature.

The heat of adsorption (ΔHº)was obtained from the vant Hoff's equation:-

$$\ln K = \frac{-\Delta H^\circ}{RT} + cons \tan t \quad \text{----------(5)}$$

Where K representsthe equilibrium constant when Ce approaches to zero at certain temperature. It wasobtained from plotting (LnK) of each concentration againstthecorresponding Ce. Plotting (InK) versus (1/T) should produce a straight line with a slope =(-ΔHº/R) from which the enthalpy (ΔHº) of the adsorption process is calculated.

The change in entropy (ΔSº) was calculated from Gibbs equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad \text{----------(6)}$$

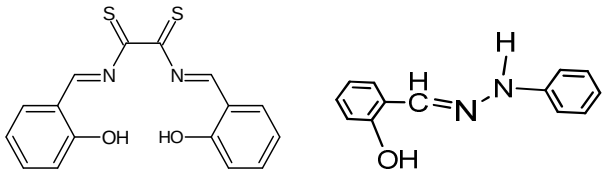
E-Desorption process:

To study the strength of the interaction of prolactin with compounds 1and 2, the desorption experiments were carried out. In each case, PRLwas adsorbed from solution onto the compounds LH1 and LH2 using a protocol similar to that for the adsorption using one initial prolactin concentration (100µl) for each compound. After incubation and centrifugation as describedabove, the supernatant was removed and replaced by 100µl of normal saline solution (0.9%NaCl) to prevent PRL denaturation. The samples were further incubated at the room temperature to allow desorption to occur. Before measurement, the tubes were centrifuged at 3000rpm for 5 minutes to precipitate the compoundsLH1and LH2) and the PRL level was estimated in the aspirated supernatant by ELISA technique. The desorption percentages were calculated from the ratio between the PRL quantity released into the solution on the PRL quantity desorbed initially on the compounds 1& 2 i.e., weight of PRL in 100µl/weight of PRL in 50mg:

$\%Desorbed = [(C_e/10) / (Q_e/20)] * 100\%$ ..... (7)

RESULTS AND DISCUSSION

The identification of the compounds were confirmed using the all techniques mentioned in the materials and methods part. The chemical formula for each of these compounds were



LH1 LH2

Fig. (1): Chemical structures of LH1 & LH2 compounds

Each of these compounds was insoluble in water. The properties are comparable with that obtained from the work of [10]. These compounds were used as surfaces for the interaction with the prolactin protein.

1-Equilibrium Time

Figure (2) showed that the time required to obtain the equilibrium state between the adsorbed PRL molecules and the released PRL molecules from compoundsLH1 and LH2is between 30-60 minutes. Therefore all experiments were carried out in on hour to insure the occurance of the equilibrium state. The equilibrium times in the present study are, in general, in accordance with different previous adsorption studies of various compounds on different surfaces [11]. However, some studiesshowed very long [12] or very short equilibrium time [13].

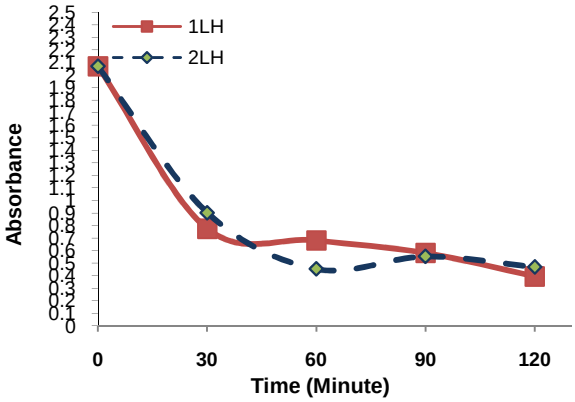


Fig. 2: Equilibrium time of the interaction between the prolactin and compounds LH1 & LH2.

2- Adsorption Process

The absorbance of the PRL hormone standards before and after incubation with the compounds LH1 & LH2 are presented in Figure (2). The figure showed a decrease in the absorbencies (i.e., concentration of prolactin solution) after incubation with the LH1 and LH2 indicating the occurrence of the adsorption process of PRL from solution on the surface of the Te compounds significantly.
The protein adsorption occurs in two steps. The first is reversible adsorption and the second is irreversible conformational modification [14]. The second step includes an orientation change on the surface, or even unfolding or uncoiling and the protein adsorption turns out to be irreversible. It can also be noticed that in most protein adsorption the adsorption is reversible if the protein concentration is low, which is highly important in applications *in vivo*.Because of this, several authors have considered changes in

orientation, conformation and formation of two-dimensional structures to explain observed adsorption phenomena. Most proteins have a tertiary structure that is spheroidal with a major and minor axis, rather than spherical. Hence, initial adsorption to a surface can occur in more than one orientation with respect to the surface. Subsequent transition in time from one orientation to the other is likely if a favorable higher contact area is created. When the concentration of bulk protein is high, overshoots in the adsorbed amount, have been reported in the literature [15]. Up to date, no conclusive a priori knowledge exists to predict which of the discussed processes is dominant in a given system. The adsorption of protein on surfaces may involve a formation of certain covalent chemical bonds as seen in another work [16].

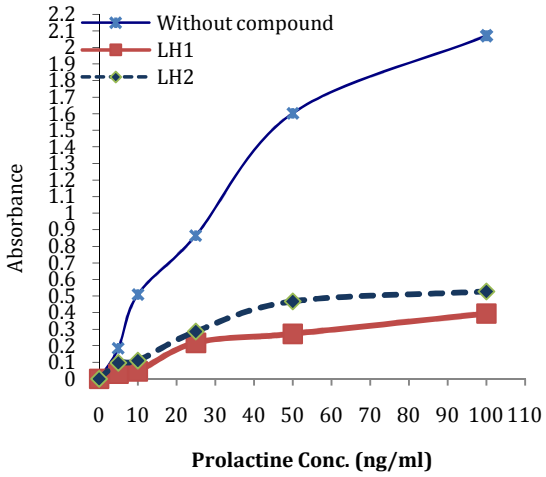


Fig. 3: Calibration curve of prolactin before and after incubation with compounds LH1 & LH2.

Figure (4) showed the adsorption isotherms of the adsorption of PRL on the LH1 & LH2. From these figures its obvious that there is an increase in the quantity adsorbed as the initial concentration increases until reaching a pseudo-flat area at the highest concentration used. The behavior of the changes of the quantities adsorbed as the concentration changes indicated two types of the adsorption isotherms according to Giles classification (Figure 5) [17]. The first type is H1 for the compound LH1 and S1 type for the LH2 compounds.

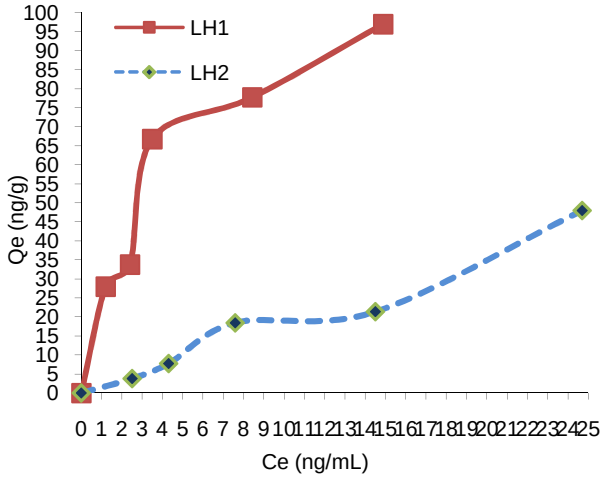


Fig. 4: Adsorption isotherms of the adsorption of prolactin on the compounds LH1 & LH2 at 20°C..

S-Type refers to the fact that; in the initial part of the *S* curves, however, the opposite condition applies, and the more solute that is already adsorbed, the easier it is for additional amounts to become fixed. This implies a side-by-side association between adsorbed molecules, helping to hold them to the surface. This has been called "co-operative adsorption" [18]. Adsorption process of protein was assumed to consist of two phases. First, a monolayer of irreversibly bound protein under the side-on orientation, which is independent of the bulk protein concentration and the second is theformation of the reversibly bound, end-on monolayer, whose coverage was dependent on the bulk concentration. The adsorption energy was in accordance with adsorption energy derived as the sum of the van der Waals and electrostatic interactions [19]. Neogi *et al* [20] found that the adsorption of proteins from the bulk is at times accompanied by a rearrangement which leads to the formation of closed packed bodies that may or may not be crystalline. Forced diffusion by van der Waals and electrostatic forces leads to segregation, which is eventually a different phase that is assumed to be thermodynamically favored.

3-Applicability of Langmuir and Freundlisch Adsorption Isotherms in the Compound-Prolactin system

In order to clarify the surface forces homogeneity and the interaction behavior of the compounds 1 & 2 surface with prolactin, the adsorption isotherms were constructed using Langmuir (Figure 5) and Freundlisch models (Figure 6). Langmuir and Freundlisch isothermswere used to interpret the adsorption data of the investigated systems. When the adsorption data obey the linear form of any equation, this means that the adsorption process obeys the best-fit model i.e., the adsorption process tends to obey the higher correlation coefficient value (r).The adsorption of prolactin from solution on surface of the compounds LH1 & LH2 obeyed the Freundlisch adsorption isotherm indicated the heterogeneity of the surfaces of these compounds. This phenomenon needs more investigation about the microscopic properties of the surface and calculations to estimate the surface energy, which should be, according to the results of the present study, different and heterogeneous.

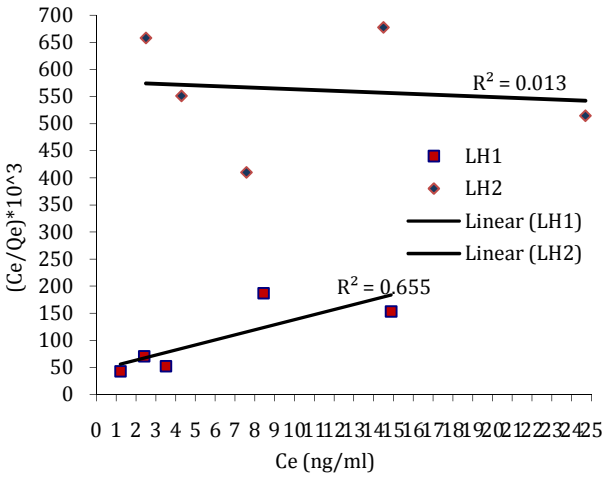


Fig. 5: Langmuir lines of the adsorption of the prolactin on the surface of the Compounds LH1 & LH2.

***4*-Thermodynamics**

The thermodynamic parameter, Gibb's free energy change ($\Delta G°$) is calculated using K obtained from Langmuir equationas shown in Table 1. The enthalpy change ($\Delta H°$)and the entropy change ($\Delta S°$) for the adsorption processes were obtained from the intercept and the slope of the equation. The negative values of $\Delta G°$ confirm the feasibility of the processand the spontaneous nature of adsorption. The decrease inthe negative value of $\Delta G°$ with an increase in temperature indicates that the adsorption process becomes more favorable at higher temperatures [21].

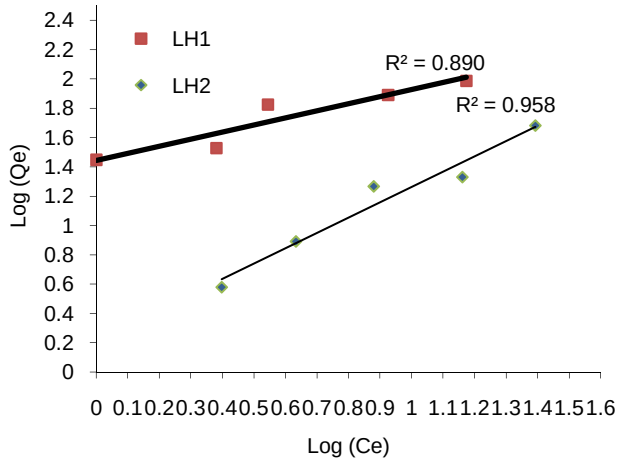


Fig. 6: Frundlich lines of the adsorption of the prolactin on the surface of the LH1 & LH2.

However, a positive value for Δ*S*° indicated that the adsorption is not homogeneous and there are different confirmations of the prolactin molecules on the surface of LH1 &LH2. Entropy has been definedas the degree of chaos of a system. The positive value of Δ*S*°suggests that some structural changes occur on the adsorbentand the randomness at the solid/liquid interface in the adsorptionsystem increases during the adsorption process.

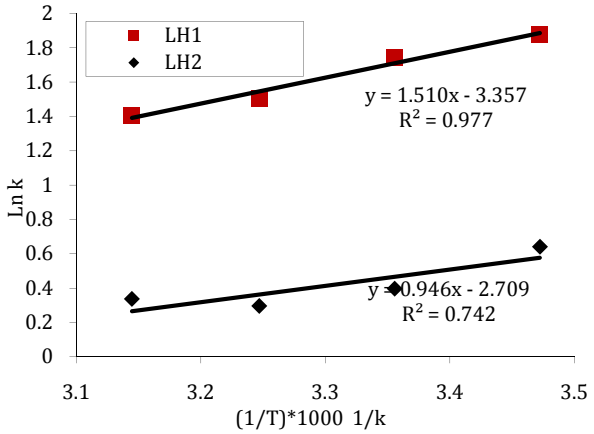


Fig. 7: Vant Hoff's equation for the adsorption of prolactin hormone on the surfaces of LH1& LH2 at (10, 20, 30, and 40°C).

Table (1): Thermodynamic parameters of the adsorption process of prolactin on LH1 and LH2.

K	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol)
5.73	-4648.13	-12.56	15.56
1.90	-1588.66	-7.87	5.33

4-Desorption Processes

The percentage of the quantities of prolactin desorbed from the surface of compound LH1 & LH2 were measured from division of the quantity released into the 100µl of solvent (Ce/10) on the adsorbed quantity of prolactin on the 50mg of complex surfaces (Qe/20) i.e.

%Desorbed=[(Ce/10)/(Qe/20)] *100%.

The results of the desorption process are presented in Figure (7). In general, when the quantity desorbed increases, the adsorption forces are weak leading to easily breaking of the linkage between the adsorbent and the adsorbate. The results of desorption in the Figure (7) revealed that the LH1 has the lowest desorption percentage (7.8%) leading to the conclusion that the adsorption forces between prolactin and LH1 are strongerthan LH2. LH2showed the highest desorption percentage due to the weak forces with prolactin.

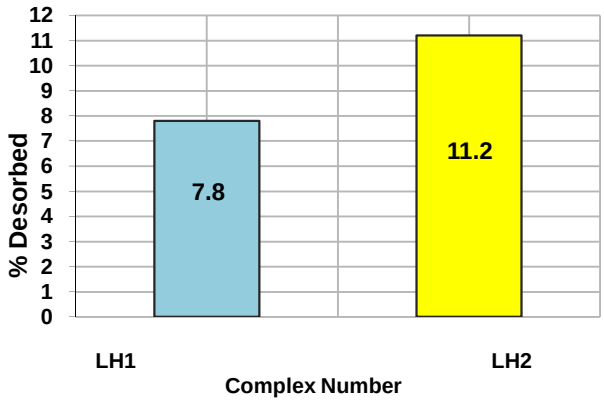


Fig. 8: The percentages of prolactin desorbed from the surface

The highest the desorption percentage, the lowest energy of adsorption and lowest attraction forces. The adsorption on solid surfaces is often irreversible especially on hydrophobic surfaces

CONCLUSION

In conclusion, prolactin molecules can be immobilized on the surface of LH1 with good quantity of adsorption and low desorption ensuring the ability to use LH1 as a surface for *in vivo* or *in vitro* applications. The adsorption process is spontaneous, exothermic and associated with an increase in entropy indicating different prolactin- active site interaction.

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