INTERACTION OF CALCIUM PHOSPHATE NANOPARTICLES WITH HUMAN CHORIONIC GONADOTROPIN MODIFIES SECONDARY AND TERTIARY PROTEIN STRUCTURE

HUSSEIN K AL-HAKEIM[1], RAHMAN S.AL-ZABEBA[1], ERIC GRULKE[2], EMAD A. JAFFAR AL-MULLA[1]

[1]*Department of Chemistry, Faculty of Science, University of Kufa, P.O. Box 21, An-Najaf 54001, Iraq (imad.almulla@uokufa.edu.iq)*
[2]*Department of Chemical Engineering, College of Engineering, Kentucky University, USA*

Abstract: Calcium phosphate nanoparticles (CaPNP) have good biocompatibility and bioactivity inside human body. In this study, the interaction between CaPNP and human chorionic gonadotropin (hCG) was analyzed to determine the changes in the protein structure in the presence of CaPNP and the quantity of protein adsorbed on the CaPNP surface. The results showed a significant adsorption of hCG on the CaPNP nanoparticle surface. The optimal fit was achieved using the Sips isotherm equation with a maximum adsorption capacity of 68.23 μg/mg. The thermodynamic parameters, including ΔH° and ΔG°, of the adsorption process are positive, whereas ΔS° is negative. The circular dichroism results of the adsorption of hCG on CaPNP showed the changes in its secondary structure; such changes include the decomposition of α-helix strand and the increase in β-pleated sheet and random coil percentages. Fluorescence study indicated minimal changes in the tertiary structure near the microenvironment of the aromatic amino acids such as tyrosine and phenyl alanine caused by the interaction forces between the CaPNP and hCG protein. The desorption process showed that the quantity of the hCG desorbed significantly increases as temperature increases, which indicates the weak forces between hCG and the surface.

Key words: Calcium phosphate nanoparticles; hCG; protein; secondary structure; and tertiary structure.

Abbreviations: CaPNP, Calcium phosphate nanoparticles; hCG, Human chorionic gonadotropin; CD, Eircular dichroism; FTIR, Fourier transform infrared spectroscopy; TEM, Transmission electron microscopy; SEM, Scanning electron microscopy; NP, Nanoparticle; Q_e, Quantity adsorbed at equilibrium; C_e, Concentration of hormone at equilibrium; Q_m, Maximum quantity adsorbed; K_s, Sips constant of the energy of adsorption; t, Sips constant of the system heterogeneity; and EDS, Energy-dispersive X-ray spectrometer.

1. Introduction

Hormones are signaling molecules that traffic information from one cell to another and can be classified according to their chemical nature into peptides, proteins, and steroids (GARDNER and SHOBACK, 2011). Human Chorionic gonadotropin (hCG) is a pregnancy hormone that maintains adequate levels of sex steroids synthesized by the corpus luteum until the placenta performs this function (STENMAN *et al.,* 2008). Furthermore, hCG acts as a gonad tropic molecule that functions on the ovaries to promote steroid synthesis (COLE, 2009). This hormone comprises of α-subunit that bound with β-subunit by ionic interactions and non-covalent hydrophobic (COLE,

DOI 10.1515/nbec-2015-0023

2009; ZIN *et al.,* 2010). The interaction of specific proteins with nanoparticles (NPs) significantly improves clinical diagnosis, receptor-targeted delivery, *in vivo* gene delivery, and medical/cancer imaging. The development of biocompatible nanomaterials to enhance or modify bio-properties is the new challenge in the biotechnology field. These applications can be classified into four main categories: biomolecular interactions, application in drug and gene delivery (ZAMANI *et al.,* 2013; JIN *et al.,* 2014), bioimaging (HOLGADO *et al.,* 2014), and biosensors design (CAO *et al.,* 2012). The use of calcium phosphate nanoparticles (CaPNP) as carriers of different molecules into cells depends on their stability in colloidal form by charged molecules or biopolymers (EPPLE *et al.,* 2010). CaPNP has excellent biocompatibility due to its chemical similarity to human bone tissues (EPPLE *et al.,* 2010). Therefore it used in many biological applications including gene delivery (ROY *et al.,* 2003), drug delivery (LIONG *et al.,* 2008), and as fluorescing probes (CHANE-CHING *et al.,* 2007). These uses involve the direct contact between CaPNP and body fluids. Therefore, understanding the possible effect of these NPs on the soluble proteins is very important for the safety of uses of CaPNP.

NPs provide a highly useful tool for studying protein-surface interactions. The high surface area and optical properties of these systems allow the ready application of circular dichroism (CD) methods (FENOGLIO *et al.,* 2011). CD spectroscopy efficiently determines the folding of protein and characterizes its secondary and tertiary structures, as well as the structural family to which it belongs. CD is a valuable technique to monitor the induced conformational changes in the macromolecular structures (RANJBAR and GILL 2009; HOIDY *et al.,* 2010). Moreover, secondary structure can be determined via CD spectroscopy at a wavelength range of 190–250 nm. In this study, a comprehensive investigation was carried out to determine the interaction between hCG hormone and CaPNP and the possible effects of this interaction on the protein structures.

2. Materials and methods

2.1Preparation of NPs

CaPNP: $Ca_3(PO_4)_2$ with a particle size of ~25 was synthesized according to the method of LIU *et al.* (2010). Briefly, 352.8 mg of $CaCl_2.2H_2O$ was added to 20 ml of water and then mixed with 20ml of a solution containing 172.8 mg of NaH_2PO_4. The two solutions were stirred vigorously and heated to 85°C. Subsequently, 40ml of ammonium hydroxide solution (28 %) was added. The mixture was stored at 85°C for 24 hours. The expected yield of $Ca_3(PO_4)_2$ NPs is 248 mg.

2.2 Interaction of hCG hormone with CaPNP

Briefly, 10 mg/ml CaPNP were dispersed in 40 ml of PBS buffer to obtain a concentration of 0.25 mg/ml. The mixture was mixed and ultrasonicated for 10 min twice. The following hCG concentrations were prepared in phosphate buffer: 36, 32,

28, 24, 20, 16, 12, and 8 µg/ml. Subsequently, 500 µl of the $Ca_3(PO_4)_2$ nanoparticle suspension was mixed with 0.5ml of the different concentrations of hCG solution supplied by Sigma-Aldrich Co. The mixture was stirred for 30 min at different temperatures (15, 25, 35, and 45°C). The mixture was ultracentrifuged at 20 000 rpm. The supernatant was separated, and the hCG concentration in the solution was determined using a ready-to-use ELISA kit supplied by Monobind® Co. USA. Isotherms were constructed between the hCG concentration in the solution at equilibrium (C_e) and the amount of the hCG adsorbed on the NPs (Q_e). The amount of the hCG hormone adsorbed was calculated using the following equation:

$$Q_e=V(C_o-C_e)/m$$

where Q_e: amount of adsorption (µg/g), C_o: initial concentration (µg/ml), C_e: concentration at equilibrium (µg/ml), m: weight of adsorbent (mg), and V: volume of solution (ml).

Isotherms were analyzed to determine the optimal equation applicable for practical results. Thermodynamic parameters (ΔH°, ΔS°, and ΔG°) were initially calculated using the Vant–Hoff's equation (JACKSON, 2006):

$$ln\ K_e = -\Delta H°/RT + constant$$

where ΔH°: enthalpy in reaction, K_e: maximum amount adsorbed, R: gas constant = 8.314 J/mol.K. By plotting ln K_e against $1/T$, a straight line should be formed with a slope of $-\Delta H°/R$.

To calculate the free energy change (ΔG°) of the adsorption process at a specific temperature, the equilibrium constant of the adsorption–desorption process of hCG on the CaPNP was calculated:

$$K_e=Q_e \times M / C_e \times V$$

where Keq: equilibrium constant, Q_e: adsorption amount (µg/mg), M: mass of nanoparticle (mg), C_e: concentration of hCG at equilibrium (µg/ml), and V: volume of solution (ml). Gibbs free energy change can be calculated from the following equation (JACKSON 2006; ZHANG *et al.,* 2008):

$$\Delta G° = -RT \ln K_e.$$

Entropy (ΔS°) can be calculated from the following formula:

$$\Delta S° = (\Delta G° - \Delta H°)/T.$$

All experiments were repeated three times and the mean of the results were taken.

2.3 Preparation of nanoparticle–hCG for TEM imaging

The nanoparticle–hCG sample for TEM was prepared by adding 0.3 mg/ml hCG to 1 mg/ml NPs to form a monolayer of the hormone on the NPs. After adsorption, 5 µL of the solution was placed on a copper grid and then left to dry for 2 minutes.

Subsequently, 5µl of the negative stain (2% uranyl acetate in dry weight) was added and left to dry for one hour. The images were then obtained.

2.4 Dynamic light scattering (DLS) method

The particle size distribution of the CaPNP powders was determined using DLS (90 Plus particle size, Brookhaven Instruments, USA) at 25°C.

2.5 Fluorescence method

Fluorescence spectra were scanned between 250 and 450 nm after excitation at 262 nm at room temperature. Excitation and emission slits were set to 5 nm.

2.6 Preparation of samples for CD study

The CD spectra of the 30 µM free or immobilized hCG in 50mM phosphate buffer (pH = 7.4) were obtained using a 1-mm Spectrosil quartz cuvette (Starna Cells) and a Jasco CD spectrometer at 20°C. Three scans were averaged using Chirascan Pro-Data Viewer software. Thermal stability was examined by monitoring the changes in ellipticity at 220 nm as a function of temperature.

CD spectra were explained by the online project called K2D2, which was developed by PEREZ–IRATXETA *et al.,* (2008). K2D2 can be accessed at K2D2 web site (*http://www.ogic.ca/projects/k2d2/*). K2D2 inputs a CD spectrum outputs the estimated α-helix and β-strand content of the corresponding protein and the estimated measure of error.

2.7 Desorption process

The percentages of the quantities of the hCG desorbed on the surface of the CaPNP surface were measured, as described previously AL-HAKEIM *et al.* (2014), by dividing the quantity released into 1 mL of solvent = (C_e/1) on the adsorbed quantity of hCG on 0.125 mg of the CaPNP surfaces = (Q_e/0.125); thus:

$$\% \text{ Desorbed } = [(C_e/1)/(Q_e/0.125)] \times 100\%$$

Desorption processes were carried out at different temperatures (288, 298, 308, and 318°K) by adding 1 ml of the buffer to the hCG–CaPNP precipitate. Desorption (%) was then calculated.

3. Results and discussion

3.1 Preparation and identification of CaPNP

CaPNP was prepared in an aqueous environment. Most NPs are prepared in an aqueous solution to complete the nucleation and crystal growth processes (FINNEY

and FINKE, 2008; BONEVICH, 2010). The particle morphology and the microstructure of the synthesized CaPNP were examined via TEM by using the highly diluted samples prepared by dispersing and sonicating small amounts of powder in 100% ethanol. The images of CaPNP were obtained using an accelerating voltage of 200-keV field-emission analytical TEM equipped with an Oxford energy-dispersive X-ray spectrometer (EDS) and a charge-coupled camera (Gatan 794CCD). The dimensions and the shape of NPs were estimated using a program incorporated in the TEM device. The shape of the particle was sliced with dimensions equal to 65 nm × 24 nm), as shown in Fig. 1.

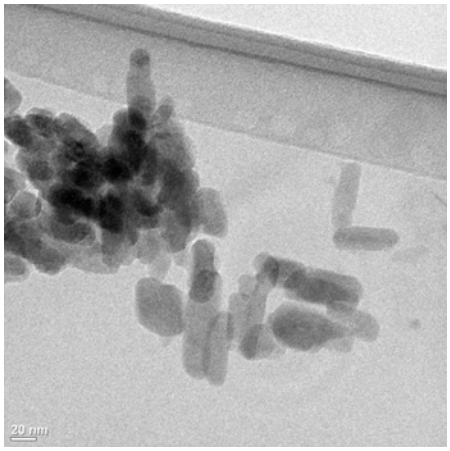

Fig. 1. TEM images of the prepared CaPNP.

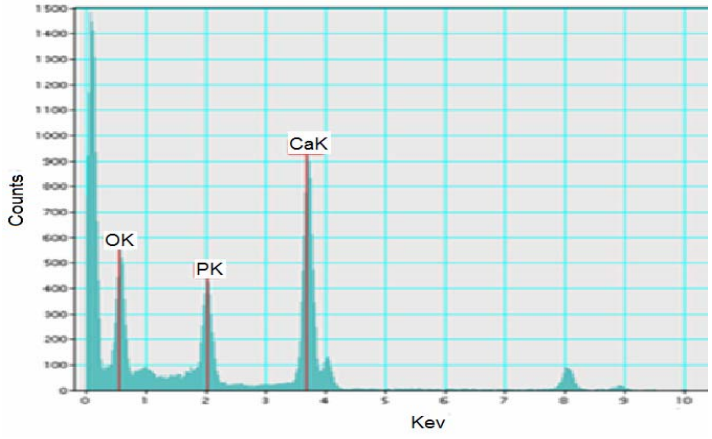

Fig. 2. EDS of CaPNP.

EDS can be used to determine the chemical composition of the materials and to plot the elements of composition maps to provide the fundamental compositional

information for various materials (MOFFET *et al.,* 2010). A typical EDS spectrum is represented as a plot of energy (in keV) versus X-ray counts. The interaction of an electron beam with NPs produces various emissions, including X-rays. Energy peaks correspond to the various elements in the sample. The EDS results of CaPNP are shown in Fig. 2.

The data from EDS indicates that the percentage of Ca is equal to 72.59% ± 2.02%, whereas that of P is 27.41% ± 1.53%. This result confirms the conformation of CaPNP.

3.2 hCG interaction with CaPNP

The incubation of hCG with CaPNP decreases the hCG concentration in the solution, which indicates that some hormones are bound to the nanoparticle surface with different surface phenomena, namely, adsorption processes. Adsorption refers to the accumulation of a dissolved solute onto the surface of a solid-adsorbing material. Adsorption is achieved by establishing a contact between the solution and the adsorbing material (adsorbent). The adsorbed substance is called adsorbate, whereas the surface that adsorbs this substance is called adsorbent. Adsorption is classified into chemical (chemisorption) and physical (Van der Waals adsorption) (UMOREN *et al.,* 2013).

The energy distribution of the adsorption of protein on the NPs shows a heterogeneous behavior. This behavior results from the surface heterogeneity and the lateral effect between the adsorbed molecules. The Sips isotherm model is a combination of the Freundlich and Langmuir isotherm-type models and describes the heterogeneous surface more accurately (WANG *et al.,* 2012).

$$Q_e = Q_m \times K_s \times C_e^t/(1 + K_s \times C_e^t)$$

The Sips isotherm approaches the Freundlich isotherm at low adsorbate concentrations, whereas approaches the Langmuir isotherm at high concentrations. K_s is a Sips constant related to the energy of adsorption, and parameter (t) is the parameter characterizing the system heterogeneity (AHMED and DHEDAN, 2012; MONEMTABARY *et al.,* 2013). The adsorption isotherms of the different hCG concentrations from aqueous solutions on a constant weight of CaPNP at 25 °C and pH 7.4 are plotted in Fig. 3.

This figure showed the successful prediction of the hCG hormone adsorption isotherm data using the Sips isotherm. The fitting of the Sips equation for the adsorption isotherm of the hCG hormone adsorption on CaPNP is consistent with the general finding in the ability of sips equation to explain the adsorption of proteins on different NPs (LIU *et al.,* 2010). Other studies confirmed the ability of the Sips isotherm to predict the adsorption of large molecules and proteins on the surface of NPs (AHMED and DHEDAN 2012; KUMAR *et al.,* 2011). The adsorption of protein on NPs is the first step of a complex series of biophysical and biochemical processes that determine the biocompatibility of the material. Adsorption is affected by many factors, including shape, energy, and functional group of the NPs surface and protein

properties (charge distribution, net charge and hydrogen bonding) (WANG *et al.,* 2012). The present findings demonstrated the heterogeneous adsorption behavior of hCG on CaPNP. Heterogeneity is a general feature of the surface properties caused by different unsaturation of a sorption on the sites leading to their different energetic characteristics. Surface imperfection and the presence of impurities are thus important; different types of adsorption forces on different active sites resulting in the creation of clusters or assemblies of adsorption molecules on the surface (WANG *et al.,* 2012).

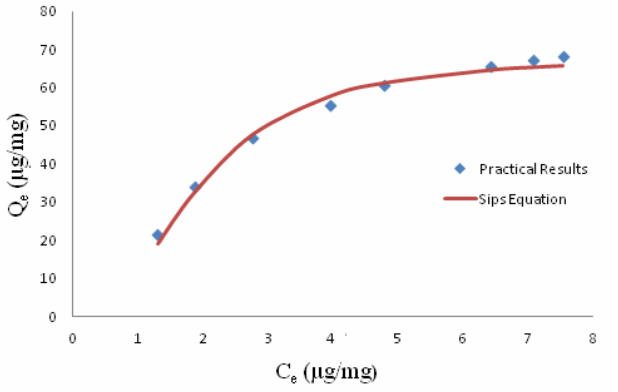

Fig. 3. Sips adsorption isotherm of hCG hormone on CaPNP at 25 °C.

The Sips model for heterogeneous adsorption produces three important constants, which describe the maximum adsorption amount (Q_m), K_s, and t. These constants can be estimated as part of the fitting exercise but can also be used to determine the parameters affected by the general shape of the molecule (TOTH, 2002). The three constants extracted from the adsorption isotherm of hCG on CaPNP are Q_m = 68.2 µg/mg, t = 2.4, and K_s = 0.2.

The Sips equation is also used to model the cooperative adsorption among the adsorbed macromolecules. The t value for CaPNP is higher than 1 that represents a favorable adsorption condition; thus, the adsorbed hCG affects the adsorption of other hCG molecules. The magnitude of t has been linked to the factors affecting heterogeneous adsorption. At $0 < t < 1$, heterogeneity is linked to the variations in the solid surface. When the adsorbed molecule has a strong affinity to other adsorbent molecules (a positive cooperative effect), t is higher than 1 (TOTH, 2002). This mechanism is consistent with the low hCG adsorption at low solution concentrations followed by a rapid increase. This lateral effect (positive cooperative effect) is also consistent with the energy site distribution analysis in many studies (TOTH, 2002; AHMED and DHEDAN 2012).

3.3 Morphology of the immobilized hCG on CaPNP

The morphologies of the hCG adsorbed on the surface of the CaPNP are presented in Fig. 4. The figure shows several interesting conclusions, particularly in the

formation of the hCG layers on the surface of CaPNP. Furthermore, CaPNP aggregate with each other after hCG adsorption, which indicates the cement-like action of the adsorbed layer of hCG and the cooperative interaction among the adsorbed hCG molecules. This finding indicates the high possibility of forming more the one adsorbed layer of hCG on the surface of CaPNP.

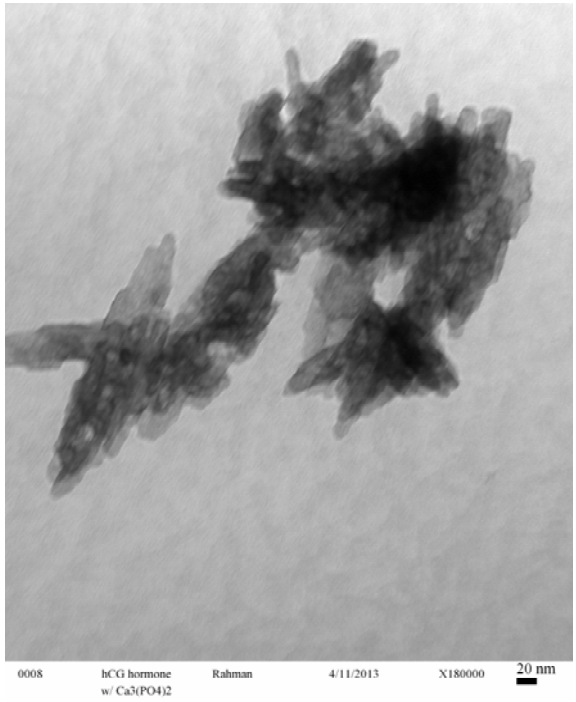

Fig. 4. TEM morphology of the hCG–NP conjugates in addition to the aggregation of the NP-hCG flakes caused by the hCG outer layer.

The interaction of the adsorbed hCG molecules with each other indicates that more adsorbed solutes results in easier accumulation of the additional amounts. This finding suggests a lateral association among the adsorbed molecules that holds them on the surface. This process is called "cooperative adsorption" (SHEMETOV *et al.,* 2012). The adsorption of proteins onto CaPNP is interesting because the interaction between proteins and CaPNP is fundamental to understand biomineralization. A higher concentration of protein on the surface indicates that more proteins are adsorbed on the NPs surface and make the protein molecules easy to interact laterally (FEI and PERRETT, 2009). The effective forces among the adsorbed protein molecules are stronger and more long-ranged than that in the solution; thus, the protein molecules aggregate on the surface but in the bulk at similar conditions (NEOGI and WANG, 2011). These explanations may be applied for the adsorption process of hCG on the surfaces of CaPNP.

3.4 Thermodynamics of the interaction between hCG and NP

The thermodynamic data (changes in enthalpy (ΔH), entropy (ΔS), and free energy (ΔG)) obtained from all protein NPs interactions can be used to detect the nature of the interaction due the fact that thermodynamics regulates most aspects of biomolecular interactions. The heat of adsorption ($\Delta H°$) provides a direct measure of the strength of the binding forces between the adsorbate molecules and the surface of the adsorbent. To analyze the thermodynamics of the adsorption of hCG on CaPNP, adsorption processes were performed at different temperatures (288, 298, 308, and 318°K) by using 18 µg/ml hCG hormone and 0.125 mg/mL CaPNP. The results of adsorption at different temperatures are demonstrated in Table 1.

Table 1. Amount of the hCG hormone adsorbed on CaPNP at different temperatures.

T (°K)	C_0 (µg/ml)	C_e (µg/ml)	Q_e (µg/g)
288	8	12.05	47.63
298	8	11.82	49.37
308	8	11.20	54.36
318	8	10.91	56.81

The results showed that the amount of adsorption slightly increases with the increase in temperature. The plotting of Vant–Hoff's equation for the adsorption process is presented in Fig. 5.

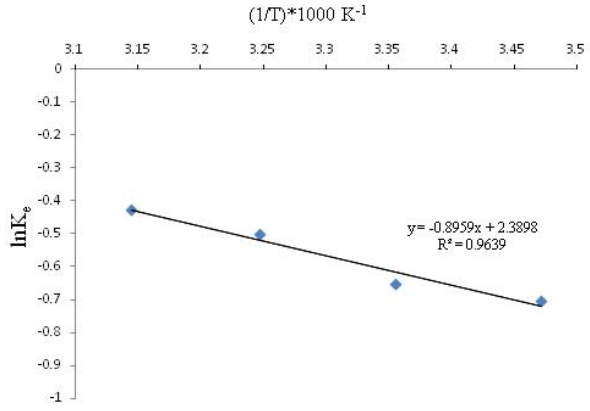

Fig. 5. Vant–Hoff's lines of the adsorption of hCG on CaPNP at different temperatures (288, 298, 308, and 318 °K).

The positive value of $\Delta H°$ (7.45 kJ/mol) indicates that the adsorption process is endothermic. The adsorption of protein to a surface may induce conformational changes in the protein. The degree of conformational changes is determined with the combination of the native stability of a protein, the hydrophobicity and charges of the

protein, and the sorbent surface. Protein adsorption can be driven by conformational entropy changes, particularly if the adsorption is endothermic (CZESLIK *et al.,* 2002).

Other research found that the adsorption capacity of protein increases with increasing temperature, which indicates an endothermic process. The values of the changes in free energy and entropy are presented in Table 2.

Table 2. Thermodynamic parameters of hCG adsorption on CaPNP at four different temperatures.

Temperature (°K)	$\ln K_e$	ΔH^o (kJ/mol)	ΔG^o (kJ/mol)	ΔS^o (J/mol.K)
288	-0.71	7.45	1.69	-20.27
298	-0.65		1.61	-19.59
308	-0.50		1.28	-20.03
318	-0.43		1.13	-19.86

Thermodynamic quantities reveal that hCG-NPs complexes involve electrostatic interaction and other non-covalent forces, including hydrophobic, hydrogen bonding, and π–π interaction obtained from the surface functional groups of the NPs (DE, 2009). Adsorption energy is consistent with the adsorption energy derived as the sum of the electrostatic and Van der Waals forces as seen previously (ADAMCZYK *et al.,* 2011). The stability of the adsorption system (hCG–NP) depends on the equilibrium between the entropy and enthalpy change on adsorption.

3.5 CD spectroscopy

The CD spectrum of hCG was analyzed to obtain the secondary structural topographies, such as the percentage of α-helical, β-sheet, or random coil regions. At the CD conditions the most important chromophore in the protein molecules is the peptide bond. The spectra of the hCG molecules in the far-UV regions are dominated by the transitions of the amide groups (π → π* and n/π*) and are affected by the morphology and the environment of the polypeptide skeleton which reflects the different types of secondary structures (ZHANG *et al.,* 2008).

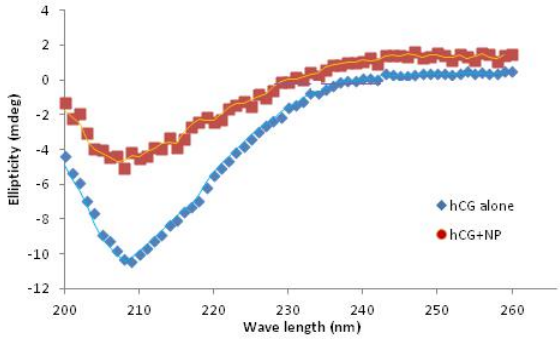

Fig. 6. CD spectra of the free hCG and hCG–CaPNP.

The CD signal is strong for α-helix but weak for β-sheets. CD is very useful for estimation the changes in the conformation of proteins at different environments. The CD spectra for the free hCG hormone and the hCG bound with CaPNP are provided in Fig. 6.

After the CD spectra of the free and nanoparticle–immobilized hCG were analyzed by the K2D2 server, the obtained results are listed in Table 3.

Table 3. Percentages of the secondary structures of the free and immobilized hCG obtained from the CD spectra.

Secondary structure	**Free hCG (%)**	**CaPNP-hCG (%)**	**Change in the Structure after adsorption (%)**
α-helix%	84.27	67.45	-16.82
β-sheet%	1.24	3.24	+2.00
Random Coiled%	14.49	29.31	+14.82

The secondary structure of the free hCG consist of 84.27 % α-helix and 1.24 % β-sheet; after binding to CaPNP, the secondary structure of the immobilized hCG consists of 67 % α-helix and 3.24 % β-sheet. These results indicate that β-sheet (+2 %) and random coils (+14.82 %) increase, whereas α-helix decreases by −16.82 %, which estimated a high interference between the hCG molecules and the surface of CaPNP. Moreover, these changes indicate the presence of more than one interaction site between the hCG molecule and the surface; the sites interact with α domains, whereas the other site with β-domains. Other research showed similar conclusion regarding the decrease in α-helix percentage, which affects the bioactivity of the adsorbed molecules (SHIRAZI *et al.,* 2013).

The change in the protein structure after the interaction of the protein molecule with the NPs surface depends mainly on the type and properties of the NPs surface. A study showed that the secondary structure of glucose oxide enzyme (protein) changes after adsorption on the nanoparticle surface, whereas maintains its native structure when adsorbed on other type of NP (ZHAOA *et al.,* 2013). Another factor that affects the interaction between the hCG and NPs is the differences in the particle curvature, which significantly affects the amount of hCG adsorption and subsequent changes in its secondary structure. As the size of the nanostructure decreases, the curvature of NP increases. Therefore, in the smaller nanostructures, only a small part of the approaching molecule are attached with the absorbent surface and thus results in less interaction with the protein. Larger NPs allow the formation of larger contact surfaces and thus cause larger changes in the secondary structure of the protein upon binding (LUNDQVIST *et al.,* 2004).

The CD spectra in Fig. 6 and Table 3 is due mainly to the α-helix structure, which shows a large peak at 208 nm, whereas the β-sheet percentage of hCG is very low with unrecognized peak around the common wavelength of β-sheet (216–220 nm). The amide chromophore of the peptide bond dominates the CD spectra of the proteins at lower than 250 nm. The transition n/π* is electrically prohibited but magnetically permitted; this transition is primarily responsible for the negative bands at 222 nm,

which is the characteristic of the α-helix spectrum, and at 216–218 nm, which is the characteristics of the β-sheet spectrum. The transitions n/π* and π → π* are primarily responsible for the negative band at 208 nm and the positive band around 190 nm, which are the characteristics of the α-helix spectrum, as well as for the positive band at 198 nm, which is the characteristic of the β-sheet spectrum (KHAN *et al.,* 2012). CD provides very good information about the residue fractions in the secondary structure involved in α-helix, β-sheet, or random coils (CHAUDHURI *et al.,* 2011). CD also provides important additional information on the effect of temperature on the secondary structure of the proteins (CORRÊA and RAMOS, 2009). Another useful function of CD is molar ellipticity, which is plotted in Fig. 7. Molar ellipticity is useful for comparing the results of the present study with those of different studies; thus, molarity should be considered in this case.

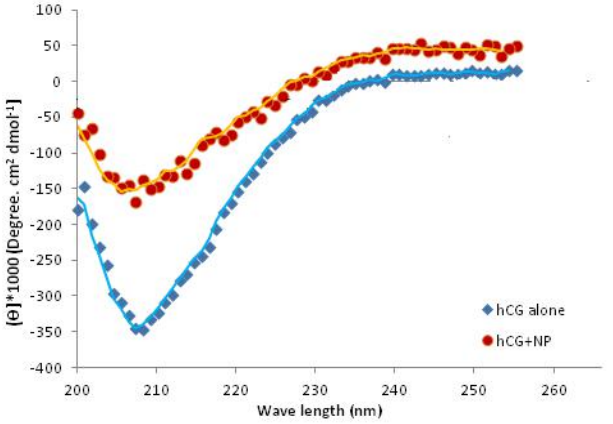

Fig. 7. Molar ellipticity of the free hCG and hCG–CaPNP.

The molar ellipticity of hCG showed the same general conclusion regarding the changes in the secondary structure of hCG when adsorbed on the surface of CaPNP. The affected secondary structures depend on the changes in their orientation and indicate the structural changes within a protein molecule. Structural changes, in which the secondary and/or tertiary structure of the protein changes because of adsorption or desorption, have been observed. These changes could be a substantial driving force for adsorption. The three dimensional structure of hCG is well determined by LAPTHORN *et al.,* (1994) and WU *et al.,* (1994). The disulphide bonds and all covalent bonds, determined by these studies, are not changed after adsorption on the surface of NPs. These results extracted from the thermodynamic values that showed relatively low free energy change and from desorption ratios.

3.6 Fluorescence spectroscopy

As shown in Fig. 8, the intrinsic fluorescence spectrum was different between the free hCG and hCG–CaPNP.

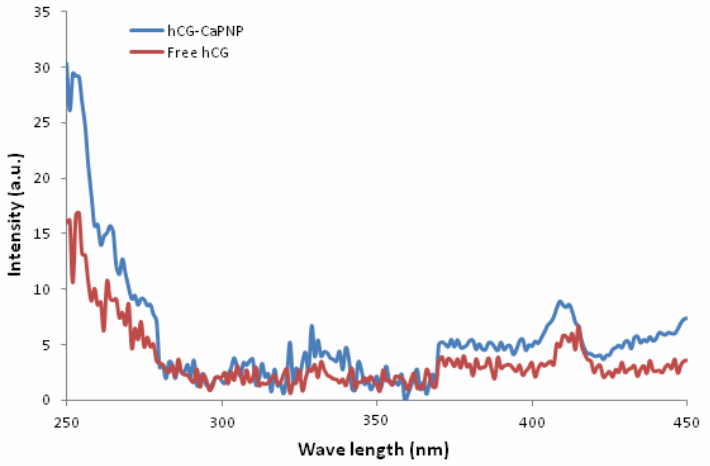

Fig. 8. Fluorescence spectra of the free hCG and hCG–CaPNP.

Valuable information regarding the local environment and the tertiary conformational variations can be achieved via fluorescence spectroscopy. The fluorescence emission spectra of the protein is produced from the contribution of the aromatic amino acids, including tryptophan, tyrosine, and phenylalanine, and the intrinsic fluorescence of the proteins are highly sensitive to their surrounding environment. This difference can be explained through the significant changes in the microenvironment of aromatic residues after adsorption on the surface of CaPNP through different forces. The hCG used in the present experiment is a tryptophan-free protein, hence the decrease in the fluorescence signal in the fluorescent spectrum is due mainly to tyrosine and phenyl alanine. In a protein containing the three fluorescent amino acids, detecting tyrosine and phenylalanine fluorescence is complicated because of the interference by tryptophan with resonance energy transfer and the weak signal of phenylalanine (CORRÊA and RAMOS, 2009). Therefore, the application of tyrosine and phenylalanine fluorescence is limited to tryptophan-free proteins, such as the hCG in the present work.

3.7 Desorption of protein on the surface

Desorption refers to the dissociation of the surface-molecule bonds, and the molecule leaves the surface to the bulk. The quantity desorbed with a suitable solvent may refer to the type of interaction between the adsorbent and adsorbate. The values of desorption (%) of hCG from CaPNP at four different temperatures are provided in Fig. 9.

Fig. 9 shows that the quantity desorbed depends on temperature; thus, the quantity increases as the temperature increases. Generally, when the quantity desorbed is high, the adsorption forces are weak and thus the linkage between the adsorbent and adsorbate can be easily broken. The adsorption of protein on the hydrophobic surfaces

is mostly irreversible (MILLER *et al.,* 2005). Hence, when a layer of the adsorbed protein is in contact with a solvent or buffer, no (or only small) desorption can be observed over experimental time scales. This finding is a direct consequence of the large number of interactions that a protein can arrange with the functional groups of a given surface. All bonds between the adsorbed protein and the surface should be damaged to allow desorption. The activation energy for such process may be very high, rendering a constant but low desorption rate that depends strongly on the protein, surface, and buffer used (CAMARERO *et al.,* 2004). Desorption is high when the adsorbed quantity is high because of the heterogeneous part of the adsorbent surface (the less favorable protein adsorption sites are desorbed first) (MILLER *et al.,* 2005).

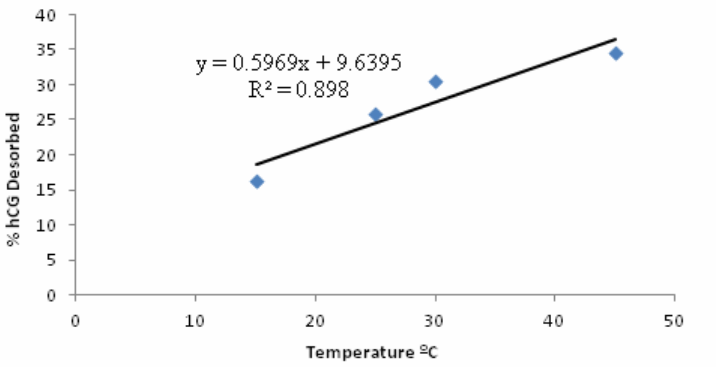

Fig. 9. Desorption percentages of hCG as a function of temperature.

The desorption of hCG from CaPNP depends on temperature, that is, the quantity of the hCG desorbed increases as temperature increases. This phenomenon may be due to the weak forces between hCG molecules and the nanoparticle surface; such forces can be broken by increasing the temperature. Another phenomenon is due to the increase in the kinetic energy of the adsorbed molecule until the energy is higher than the forces of interaction with the surface of the NPs. Other authors showed a similar desorption behavior (Mansur *et al.,* 2001; Van der Veen *et al.,* 2007).

4. Conclusion

The interaction between hCG and CaPNP is mainly caused by the adsorption phenomenon that obeyed the Sips isotherm. The adsorption of hCG changes the secondary and tertiary protein structures and subsequently changes the biological activity of the protein. Hence, the interaction between other NPs used *in vivo* with different human proteins should be evaluated prior to use.

References

ADAMCZYK, Z., BARBASZ, J.: CIEŚLA M. Mechanisms of fibrinogen adsorption at solid substrates. Langmuir, 27, 2011, 6868 - 6878.

AHMED, M.J., DHEDAN, S.K.: Equilibrium isotherms and kinetics modeling of methylene blue adsorption on agricultural wastes-based activated carbons. Fluid Phase Equilib., 317, 2012, 317: 9 -14.

AL-HAKEIM, H.K., AL-DAHAN, I.M., AL-HILLAWI, Z.H., BUSTAN, R.S.: Interaction of prolactin hormone with the surfaces of two new azo compounds. Int. J. Pharm. Pharm. Sci., 6, 2014, 383-387.

APTHORN, A.J., HARRIS, D.C., LITTLEJOHN, A., LUSTBADER, J.W., CANFIELD, R.E., MACHIN, K.J., MORGAN, F.J., ISAACS, N.W.: Crystal structure of human chorionic gonadotropin. Nature, 369, 1994, 455-61.

BONEVICH, J.E.: Preparation and properties of nanoparticles of calcium phosphates with various Ca/P ratios. J. Res. Nat. Inst. Stand. Technol., 115, 2010, 243-255.

CAMARERO, J., KWON, Y., COLEMAN, M.: Chemoselective attachment of biologically active proteins to surfaces by expressed protein ligation and its application for "protein chip" fabrication. J. Am. Chem. Soc., 126, 2004, 14730-14731.

CAOL, S.T., WANG, X., LUO, G.P., LIU, J.H., SAHU, S., LIU, Y., SUN, Y.P.: Competitive performance of carbon “Quantum” dots in optical bioimaging. Theranostics, 2, 2012, 295-301.

CHANE-CHING, J.Y., LEBUGLE, A., ROUSSELOT, I., POURPOINT, A., PELLE, F.: Colloidal synthesis and characterization of monocrystalline apatite nanophosphors. J. Mater. Chem., 17, 2007, 2904-2913.

CHAUDHURI, S., CHAKRABORTY, S., SENGUPTA, P.K.: Probing the interactions of hemoglobin with antioxidant flavonoids via fluorescence spectroscopy and molecular modeling studies. Biophys. Chem., 154, 2011, 26-34.

COLE, L.A.: New discoveries on the biology and detection of human chorionic gonadotropin. Reprod. Biol. Endocrinol., 7, 2009, 8-37.

CORRÊA, D.H., Ramos, C.H.: The use of circular dichroism spectroscopy to study protein folding form and function. Afr. J. Biochem. Res., 3, 2009, 164-173.

CZESLIK, C., JACKLER, G., ROYER, C.: Driving forces for the adsorption of enzymes at the water/silica interface studied by total internal reflection fluorescence spectroscopy and optical reflectometry. Spectroscopy, 16, 2002, 139-145.

DE, M.: Engineering the Nanoparticle Surface for Protein Recognition and Applications. A PhD Dissertation in Chemistry, University of Massachusetts Amherst, 2009, 46-47.

EMRE, C., MEHMET, S., FATIH, A.: Immobilization of urease on copper chelated EC-Tribeads and reversible adsorption. Afr. J. Biotechnol., 10, 2011, 6590-6597.

EPPLE, M., GANESAN, K., HEUMANN, R., KLESING, J., KOVTUN, A., NEUMANN, S., SOKOLOVAA, V.: Application of calcium phosphate nanoparticles in biomedicine. J. Mater. Chem., 20, 2010, 18-23.

FEI, L., PERRETT, S.: Effect of nanoparticles on protein folding and fibrillogenesis. Int. J. Mol. Sci., 10, 2009, 646-655.

FENOGLIO, I., FUBINI, B., GHIBAUDI, E.M., TURCI, F.: Multiple aspects of the interaction of biomacromolecules with inorganic surfaces. Adv. Drug. Deliv. Rev., 63, 2011, 1186-1209.

FINNEY, E.E., FINKE, R.G.: Nanocluster nucleation and growth kinetic and mechanistic studies: A review emphasizing transition-metal nanoclusters. J. Coll. Interface Sci., 317, 2008, 351-374.

GARDNER, D., SHOBACK, D.: Greenspan's basic and clinical endocrinology. Ninth Edition, Mc Graw Hill Lange, USA, 2011, 2-5.

GILIOLI, A., CAVEJON, M., QUADRI, M.: *C. hildmannianus* peel for Protein Adsorption. Chem. Eng. Transac., 32, 2013, 1099-1104.

HOIDY, W.H., AHMAD, M.B., YUNUS, W.M.Z.W., IBRAHIM, N.A.B.: Chemical synthesis and characterization of palm oil-based difatty acyl thiourea. J. Oleo Sci., 59, 229-233.

HOLGADO, M., SANZA, F.J., LÓPEZ, A., LAVÍN, Á., CASQUEL, R., LAGUNA, M.F.: Description of an advantageous optical label-free biosensing interferometric read-out method to measure biological species. Sensors, 14, 2014, 3675-3689.

JACKSON, M.: Molecular and Cellular Biophysics. Cambridge University Press: New York, Humana Press Inc., Totowa, NJ., 2006, 261-275.

JIN, L., ZENG, X., LIU, M., DENG, Y., HE, N.: Current progress in gene delivery technology based on chemical methods and nano-carriers. Theranostics, 4, 2014, 240-255.

KHAN, T., AMANI, S., NAEEM, A.: Glycation promotes the formation of genotoxic aggregates in glucose oxidase. Amino Acid., 43, 2012, 1311.

KUMAR, P., RAMALINGAM, S., SATHISHKUMAR, K., KOREAN, J.: Adsorption characteristics of methylene blue onto the N-succinyl-chitosan-g-polyacrylamide/attapulgite composite. Chem. Eng., 28, 2011, 149-155.

LIONG, M., LU, J., KOVOCHICH, M., XIA, T., RUEHM, S.G., NEL, A.E., TAMANOI, F., ZINK, J.I.: Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery. ACS Nano, 2, 2008, 889-896.

LIU, X., QIN, D., CUI, Y., CHEN, L., LI, H., CHEN, Z., GAO, L., LI, Y., LIU, J.: The effect of calcium phosphate nanoparticles on hormone production and apoptosis in human granulosa cells. Reprod. Biol. Endocrinol., 8, 2010, 32.

LUNDQVIST, M., SETHSON, I., JONSSON, B.H.: Protein adsorption onto silica nanoparticles: Conformational changes depend on the particle's curvature and the protein stability. Langmuir, 20, 2004, 10639-10647.

MANSUR, H.S., RODRIGO, L.O., LOBATO, Z.P., VASCONCELOS, W.L., MANSUR, E.S., MACHADO, L.J.: Adsorption/Desorption behavior of bovine serum albumin and porcine insulin on chemically patterned porous gel networks. Adsorption, 7, 2001, 105-116.

MILLER, R., GRIGORIEV, D.O., KRAGEL, J., MAKIEVSKI, A.V., MALDONADO-VALDERRAMA, J., LESERC, M.E., MICHEL, A., FAINERMAND, V.B., Experimental studies on the desorption of adsorbed proteins from liquid interfaces. Food Hydrocolloid, 19, 2005, 479-483.

MOFFET, R.C., HENN, T., LASKIN, A., GILLES, M.K.: Automated chemical analysis of internally mixed aerosol particles using x-ray spectromicroscopy at the Carbon K-Edge. Anal. Chem., 82, 2010, 7906-7914.

MONEMTABARY, S., SHARIATI, M., JAHANSHAHI, N.M., GHOREYSHI, A.A.: Equilibrium and thermodynamic studies of methane adsorption on multi-walled carbon nanotube. Iran. J. Energ. Environ. Nanotech., 4, 2013, 17-23.

NEOGI, P., WANG, J.C.: Stability of two-dimensional growth of a packed body of proteins on a solid surface. Langmuir. 9, 2011, 5347-53.

PEREZ-IRATXETA, C., ANDRADE-NAVARRO, M.A.: K2D2: estimation of protein secondary structure from circular dichroism spectra. BMC Struct. Biol., 8, 2008, 25.

RANJBAR, B., GILL, P.: Circular dichroism techniques: biomolecular and nanostructural analyses- A review. Chem. Biol. Drug. Des., 74, 2009, 101-120.

ROY, I., MITRA, S., MAITRA, A., MOZUMDAR, S.: Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. Int. J. Pharm., 250, 2003, 25-33.

SHEMETOV, A.A., NABIEV, I., SUKHANOVA, A.: Molecular interaction of proteins and peptides with nanoparticles. ACS Nano, 6, 2012, 4585-4602.

SHIRAZI, N., RANJBAR, B., KHAJEH, K., TAHEREH, T.: Structure–function analysis of a new bacterial lipase: Effect of local structure reorganization on lipase activity. Int. J. Biol. Macromol., 54, 2013, 180-185.

STENMAN, U.H., HOTAKAINEN, K., ALFTHAN, H.: Gonadotropins in doping: pharmacological basis and detection of illicit use. J. Pharmacol., 154, 2008, 569-583.

TOK, A.B., DONG, Z.S., MERTENS, S.F.L., PITA, M., FERNANDEZ, V.M., SCHIFFRIN, D.J.: Functionalization of thioctic acid capped gold nanoparticles for specific immobilization of histidine- tagged proteins. J. Am. Chem. Soc., 127, 2005, 5689-5694.

UMOREN, S., ETIM, U., ISRAEL, A.: Adsorption of methylene blue from industrial effluent using poly (vinyl alcohol). Mater. J. Environ. Sci., 4, 2013, 75-86.

VAN DER VEEN, M., STUART, M.C., NORDE, W.: Spreading of proteins and its effect on adsorption and desorption kinetics. Coll. Surfaces B: Biointerfaces, 54, 2007, 136-142.

WANG, B., WUA, P., YOKELB, R.A., GRULKE, E.A.: Influence of surface charge on lysozyme adsorption to ceria nanoparticles. Appl. Surf. Sci., 258, 2012, 5332-5341.

WU, H., LUSTBADER, J.W., LIU, Y., CANFIELD, R.E., HENDRICKSON, W.A.: Structure of human chorionic gonadotropin at 2.6A resolution from MAD analysis of the selenomethionyl protein. Structure, 2, 1994, 545-558.

ZAMANI, M., PRABHAKARAN, M.P., RAMAKRISHNA, S.: Advances in drug delivery via electrospun and electrosprayed nanomaterials. Int. J. Nanomed., 8, 2013, 2997-3017.

ZHANG, Y.Z., ZHOU, B., LIU, Y.X., ZHOU, C.X., DING, X.L., LIU, Y.: Fluorescence study on the interaction of bovine serum albumin with p-aminoazobenzene. J. Fluoresc., 18, 2008, 109-118.

ZIN, W.Y., IBRAHIM, N.A.B., Rahman, M.Z.A.: Difatty acyl urea from corn oil: Synthesis and characterization. J. Oleo Sci., 59, 157-160

ZHAO, W., FANG, Y., ZHU, Q., WANG, Q., LIU, M., HUANG, X., SHEN, J.: A novel glucose biosensor based on phosphonic acid-functionalized silica nanoparticles for sensitive detection of glucose in real samples. Electrochim. Acta., 89, 2013, 278-283.