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Stability and activity behavior of human serum albumin doped in silica sol-gel thin film

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ABSTRACT

Transparent sol-gel thin films immobilized with human serum albumin (HSA) were made via the acid catalyzed sol-gel reaction of tetraethylorthosilicate in presence of the human serum albumin. Different surfactants include; cationic cetyl trimethyl ammonium bromide (CTAB), anionic sodium dodecyl sulfate (SDS) and nonionic Triton X-100 (TX-100) were tested for the improvement of the host material mesostructure, increasing its porosity and well accommodation of the HSA protein within the silica matrix. The thin films show similar behavior in presence of surfactants as their free counterparts in aqueous solution with a significant shift in the wavelength of absorption. The activity behavior of the immobilized human serum albumin retained its activity by immobilization. Different parameters include concentration of protein and surfactant, type of surfactant, life time and number of measurements were investigated. The HSA thin film sensor showed stability, repeatability, reproducibility and long life time behavior. Maximum stability of HSA thin films were achieved by drying at 37 °C.

KEYWORDS

Human serum albumin;
Sol-gel;
Biosensors;
Encapsulation;
Immobilization of bioactive substance.

INTRODUCTION

Immobilization of chemically and biologically active molecules onto sol-gel matrices is a promise route to chemical and biological solid-state sensors[1-4]. Over the past decade, many articles have reported the development and characterization of proteins entrapped into inorganic silica sol-gel matrices[5,6]. The sol-gel technique is one of the most promising tools in material science[7]. The porosity of sol-gel glasses allows small analyte molecules to diffuse. The transparency of the matrix enables one to use optical spectroscopic methods to characterize the reactions that occur in the pores of the glass. Porous three-dimensional siloxane (–Si-O-Si–) network of high surface area are generally prepared via sol-gel method by hydrolysis and polycondensation of tetraethoxysilane in presence of water, organic solvent and an acid/base catalyst[3,4,8]. Sol-gel matrices appear as a very important technique for immobilization, entrapment and encapsulation for large variety of materials such as organic, inorganic, and biomolecules as they synthesized at the ambient temperature[9-12].

Due to the inherent low temperature process, the sol-gel technology provides an attractive way for the immobilization of heat sensitive biomolecules (enzyme,

protein, and antibody). In particular, sol-gel silica (SiO_2) possesses chemical inertness, physical rigidity, negligible swelling in aqueous solution, tunable porosity, and thermal stability. These attractive features have led to an intensive research in the electrochemical sensors and biosensors[13].

Sol-gel encapsulation has opened new ways to immobilize biological materials that offer an immense potential for the design of a large variety of applications. Several kinds of biomolecules, including enzymes, antibodies, DNA, RNA, and live animal, plant, bacterial and fungal cells as well as whole protozoa have been encapsulated and then tested and implemented as optical and electrochemical sensors as well as core components of diagnostic, chromatographic, and catalytic devices[14-16].

Proper conditions may be achieved to control the sol-gel process[17]. The sol-gel process involves the addition of a colloidal suspension (sol) of a polymer-forming precursor to a solution of an active material. Further processing of the sol results in the formation of an amorphous glassy like matrix (gel) in which the active material is entrapped[17,18]. The versatility of sol-gel chemistry allows producing a wide range of organic-inorganic hybrid materials with many promising applications. Moreover, the mild conditions associated with sol-gel chemistry allow the successful immobilization of a broad range of enzymes and living cells[19-21].

Entrapped proteins may retain their activities and react with substrates species that diffuse through the pores of sol-gel matrix. The sol-gel entrapment in silica matrix is a rapid technique for immobilizing biomolecules into a chemically stable matrix preserving high activity and long lifetime[22,23].

The proposed work aims to study the entrapment of Human Serum Albumin (HSA) in a silica glass network through silica thin film by using sol gel spin coating technique[23]. Different surfactants include; cationic e.g. CTAB, anionic e.g. SDS, and non-ionic e.g. TX-100 will be added to examine their effect on thin film matrix optimization.

MATERIALS AND METHODS

Chemicals and reagents

Tetraethylorthosilicate $Si(OEt)_4$ (TEOS), human serum albumin (HSA), sodiumdodecylsulfate $C_{12}H_{25}SO_4Na$ (SDS), cetyltrimethylammonium bromide $C_{19}H_{42}BrN$ (CTAB), Triton-X 100 $C_{14}H_{22}O(C_2H_4O)_n$ (TX-100), hydrochloric acid and absolute methanol were purchased from Merck (Darmstadt, Germany). All chemicals were used as received without further purifications.

Thin film preparation

(a) Preparation of hydrolyzed TEOS

A volume of 5 mL of TEOS was mixed with 0.5 mL of HCl (0.1M) as a catalyst and 2.5 mL of absolute methanol was added as solvent, then 2.5 mL of deionized water was added. The mixture was stirred for 40-60 min at ambient temperature until a clear, colorless and monophasic solution was obtained. Mthanol was allowed to evaporate in order to obtain a slightly viscous mixture with minor amount of methanol. The solution formed was cooled and stored at room temperature before use. The hydrolyzed TEOS solution (sol) was used as a host matrix for the protein.

(b) Preparation of different pH phosphate buffer solutions

Phosphate buffer solutions were prepared by using calculated volumes of mixture of 0.1M solution of citric acid (19.21g, in 1000 mL) and 0.2M solution of dibasic sodium phosphate hydrated (28.40g. in 1000mL).

(c) Preparation of surfactants solutions

Different concentrations (0.05, 0.01, 0.001, 0.0001 and 0.00001 M) of cationic CTAB, anionic SDS, and non-ionic TX-100 surfactants were prepared in water.

(d) Preparation of stock solution of human serum albumin (HSA)

200µL of HSA (5g/dl) was dissolved in 5mL of phosphate buffer of pH 7.

(e) Sample and blank solutions

Sample solution containing HSA was prepared by mixing 2 mL of hydrolyzed TEOS, 1mL of HSA stock solution and 0.5 mL of CTAB, SDS or TX-100 surfactants. The blank solutions were prepared by mixing the hydrolyzed TEOS (sol), surfactant (CTAB, SDS, or TX-100) and phosphate buffer of pH=7 in 2:1:1 volume ratio respectively.

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(f) Thin films deposition

A glass microscope slides (2.5 cm x 0.8 cm x 1mm) were pretreated before film deposition. The glass slides were treated with concentrated nitric acid for 2 hours, washed with water and ethanol then dried at 120 °C for two hours. All thin films layers prepared were done by spraying a 100 mL of the mention coating solutions onto the clean glass slides. The coating process was performed using the spin coater machine at 1900 rpm spinning speed and 25 seconds time period. The obtained wet coated layers were let to dry gradually at 37 °C for 24 hours followed by drying at 37 °C for 6 days, then stored in the desiccator. The films were washed several times with deionized water then dried at 37 °C. Washings were collected to examine HSA leaching. Characterization was investigated using UV/Vis spectrophotometer and fluorescence spectroscopy.

Activity test for free and encapsulated HSA

The activity behavior for both free and encapsulated HSA was followed spectrophotometrically, by monitoring the formation of *p*-nitrophenol (*p*NPhOH) that produced from catalyzed hydrolysis of *p*-nitrophenyl acetate (*p*NPhOAc). The method was performed as follows:

A 6μM concentration of free and encapsulated HSA were freshly prepared in 2 ml phosphate buffer (0.1M) solution of pH 7. The activity assays were initiated by the addition of 1mL of solution containing 1mM p-nitrophenyl acetate (*p*NPhOAc) dissolved in isopropanol. This reaction mixture was immediately placed in the spectrophotometer set at room temperature. The background substrate hydrolysis was accounted for with blanks containing buffer instead of HSA for all conditions tested. The reaction rate during the activity assay was determined by measuring the increase in absorbance at λ_{max} of *p*-nitrophenol (410 nm) for two minutes. All experiments were performed in duplicate.

UV\Vis spectrophotometer

The optical absorption spectra of the coating solutions and the deposited films were obtained by using a single beam GENESYS 10 UV Scanning Spectrophotometer in the range (190 - 1100 nm) of automatically rotation. Coated slides were placed along the wall of the sample cell and exposed to phosphate buffer solution at pH 7.

Fluorescence spectroscopy

Spectrofluorimetric spectra of the coating films were performed using a Perkin Elmer luminescence (series no. 70412). The spectrometer equipped with a water jacket cuvette holder to maintain the desired temperature. The excitation wavelength was 290 nm, and the emission was recorded in the wavelength range of 350-625 nm. The excitation and emission slits were both 10 nm.

RESULTS AND DISCUSSION

Human serum albumin (HSA) has been entrapped within silica gel thin films through spin coating process. The glass substrate was spinned at a controlled speed where the sol was spread on to the substrate. The preparation method is summarized in the following steps:

a) Hydrolysis of tetraethylorthosilicate by water at room temperature.
b) Addition of HSA of different concentrations in the presence or absence of surfactant.
c) Deposition of transparent thin film using spin coating technique.

The deposited HSA thin films were studied and characterized under different factors.

UV/Vis Spectra of the free and immobilized HSA

The absorption spectra for the free HSA, and encapsulated HSA/SDS in the range of 250-400 nm, in buffer phosphate at pH=7 are given in Figure 1. The spectrum of the free HSA in phosphate buffer solution shows an absorption band at 280 nm (Figure 1 a) while the spectrum of the immobilized HSA/SDS thin film exhibits an absorption band at 290 nm (Figure 1 b). The red shift of 10 nm of the encapsulated HSA compared with the free HSA is attributed to change in protein conformation[24] due to the physical interactions of HSA molecules with the silica network. The interactions essentially involve van der waals force and hydrogen bonding, with an influence of the diversity of the environmental changes that may affected the protein conformations during sol-gel process[25]. The presence of surfactant affects the interaction of protein which may lead to the formation of protein-surfactant com-

plexes[26,27]. Due to the existence of nonpolar and ionic amino acid side-chains in protein molecule, the formation of these complexes is driven by electrostatic interactions between the charged head groups of the surfactant and the oppositely charged units of the protein and also by hydrophobic interactions[28,29]. The effect of SDS on the HSA interaction with matrix surface will be discussed later.

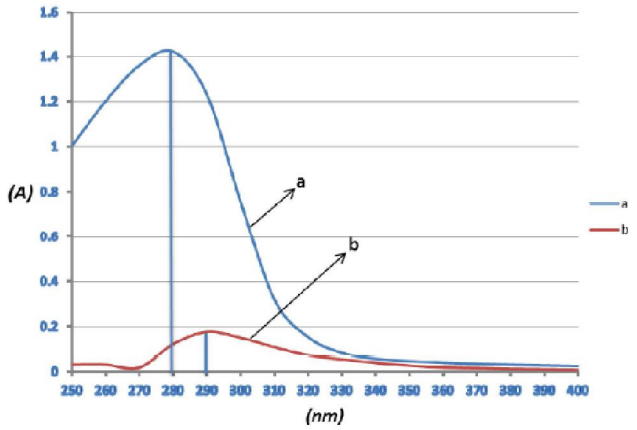

Figure 1: Absorption spectra of (a) free HSA, (b) immobilized HSA/SDS, in phosphate buffer (pH=7), at 25ºC.

Effect of concentration of HSA

Different concentrations of HSA (5, 7.5, 10, 12.5, 15μM) in presence of SDS were used to investigate the effect of amount of HSA trapped within the thin film on absorption capacity. The sol mixture by volume ratio was 1:1:0.5 of HSA: hydrolyzed TEOS: surfactant, respectively. The trapped HSA thin films were examined versus the absorbance at λ_{max} = 290 nm (Figure 2).

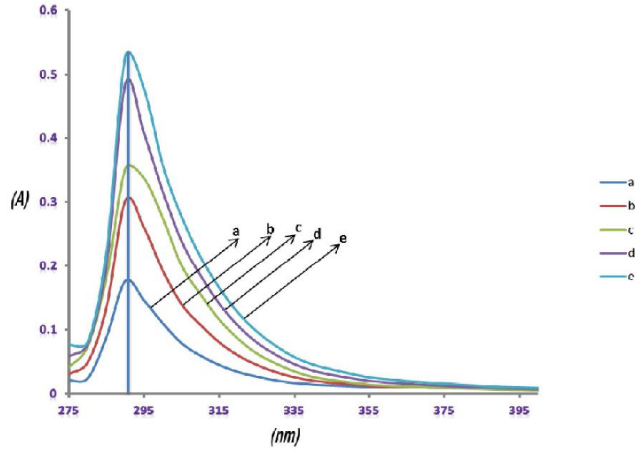

Figure 2: Absorption spectra of immobilized HSA/SDS at different concentrations of HSA. (a) 5 μM, (b) 7.5 μM, (c) 10 μM (d) 12.5 μM, and (e) 15 μM, in phosphate buffer (pH=7), at25ºC.

The absorbance increases with increasing concentration of added HSA and reach its maximum at 15μM. At high concentration of HSA (>15 μM) leaching occurred.

Effect of concentration of surfactants

Different concentrations of SDS (0.00001M-0.05M) which trapped with HSA (0.05M), were prepared and examined versus the absorbance at λ_{max} = 290 nm. Figure 3 depicted the effect of SDS concentration on the absorbance of HSA thin films. It is found that the absorbance increased with increasing concentration of surfactant and reaches its maximum at 0.05 M. Increasing SDS concentration lead to leaching of HSA from silica matrix as the excess of SDS surfactant removed the adsorbed HSA from the solid matrix[30].

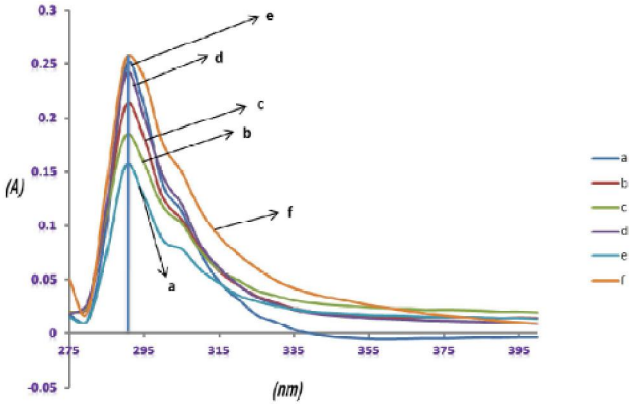

Figure 3: Absorption spectra of immobilized HSA/SDS at different concentrations of SDS. a) 0.00001M, (b) 0.0001M, (c) 0.001M, (d) 0.005M, (e) 0.01M and (f) 0.05M, in phosphate buffer (pH=7), at 25ºC.

Comparison between surfactants

The absorption spectra of the immobilized HSA within the sol-gel matrix thin films with and without surfactants are given in Figure 4. Three different surfactants (SDS, CTAB and TX- 100) were used to examine their effect on efficiency to form a uniform film. It was observed that HSA/SDS gave the best results in absorption spectra. The spectra show an absorption band at 290 nm for HSA/SDS, while in case of HSA/CTAB, HSA/Triton X-100, HSA/without surfactant, the spectra are nihilistic. The reason of this behavior is due to the nature of interaction of doped HSA with silica matrix in the presence of SDS in comparison with the presence of CTAB or Triton X-100 or in case of absence of surfactant.

Generally the presence of surfactant modifies the structure of gel matrix and therefore the surface area increases, so more HSA molecules can penetrate this matrix which make a cage around these molecules. In addition, the presence of surfactant layer affects the adsorption of protein molecules within mineral matrices[30]. The presence of SDS surfactant of low concentrations within hydrophilic matrix might lead to higher affinity of protein to this matrix. On the other hand, at high amount of SDS the adsorption of protein decreases within solid matrices. Increasing amount of surfactant within the hydrophilic matrix leads to increase of its hydrophobicity and the interaction between protein and SDS is most probably of hydrophobic character[29].

Many studies have revealed that the interaction between SDS and HSA is a complex process according to the dual property of sodium dodecyl sulphate (SDS) which consists of a hydrophobic tail and a negatively charged head. In low concentrations, SDS induces conformational changes in HSA via electrostatic interactions, with a net stabilizing effect on the structure. This implies that HSA structure becomes more stabilized and compact, presumably as a result of the interaction of negatively charged SDS heads with several positively charged residues on HSA surface, and its associated cations[31].

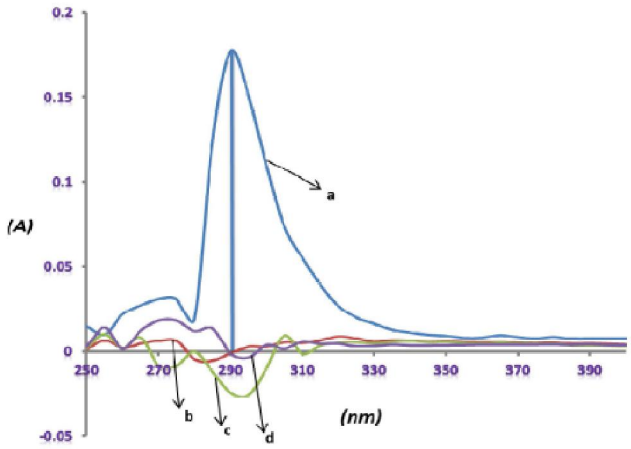

Figure 4: Absorption spectra of HSA with and without surfactant, in phosphate buffer (pH=7), at 25°C (a)HSA/SDS, (b)HSA/CTAB, (c)HSA/TX-100, (d)HSA/without surfactant.

Catalytic behavior of encapsulated HSA

The most important issue in immobilization of a biomolecule is the retention of its biological activity upon immobilization. The immobilization or encapsulation process has to be mild enough to retain most of the activity of an encapsulated molecule. Figure 5 shows the variation of absorbance of the produced *p*-nitrophenol (*p*NPhOH) at λ_{max} = 410 nm versus time by the hydrolysis of *p*-nitrophenyl acetate (*p*NPhOAc) for both free and encapsulated HSA, in which the slope ($\Delta A/t$) for free HSA is slightly higher than that of encapsulated HSA.

The reaction scheme proposed by Means and coworkers[32,33] for the *p*-nitrophenyl acetate - HSA activity assay is depicted in equation 1.

$$\mathrm{HSA} + p\mathrm{NPhOAc} \rightleftharpoons p\mathrm{NPhOAc} \cdot$$
$$\mathrm{HSA} \rightarrow \mathrm{Ac} - \mathrm{HSA} + p\mathrm{PhOH} \quad \textbf{(1)}$$

The activity units for both free and encapsulated HSA were calculated according to equation 2.

$$\mathrm{U} = \mathrm{Units/mg} = \frac{\Delta A/t}{\varepsilon \times m_{(HSA)} / V_{(\text{reaction mixture})}} \quad \textbf{(2)}$$

Where U: activity in μmol/min/mg; $\Delta A/t$: change of absorbance with time; ε: extinction coefficient for p-nitrophenol at 25°C =$1.8 \times 10^4 M^{-1}cm^{-1}$; $m_{(HAS)}$: mass of human serum albumin in mg unit; $V_{(\text{reaction mixture})}$: volume of reaction mixture in mL unit.

By applying equation 2, it is found that the activities are 0.458 and 0.394 μmol/min/mg for free and encapsulated HSA respectively. It is shown that HSA will retain 86% of its activity when kept encapsulated in the studied films. It's suggested that, silica gel matrix does not lead to denaturation of HSA during the formation of silica gel matrix. The silica matrix (network) protects the encapsulated HSA conformation against leaching and denaturation by the small pore size in a well hydrated cage matrix.

HSA thin film repeatability

Repeatability of HSA thin film was studied by conducting ten cycles measurement using HSA/SDS thin film. The cycles were repeated within 24 hours to check stability of the HSA thin film on repeating measurements. Figure 6 shows the absorbance versus number of measurements at pH=7. It is found that the first measurement is higher in absorbance than the other measurements; this is due to loss a little amount of HSA molecules after the first washing, while the absorbance after each measurement remains almost unchanged, Absorbance of the repeated measurements was performed at 290 nm.

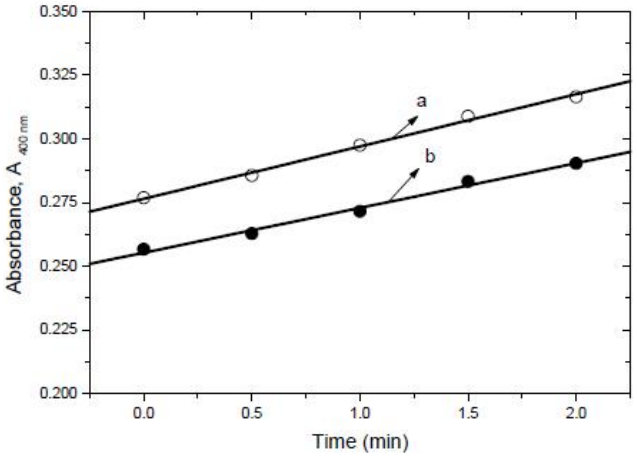

Figure 5: Variation of absorbance of the produced *p*-nitrophenol at λ_{max} = 410 nm, in case of (a) Free HSA and (b) encapsulated HSA both in phosphate buffer (pH=7), and T = 25ºC.

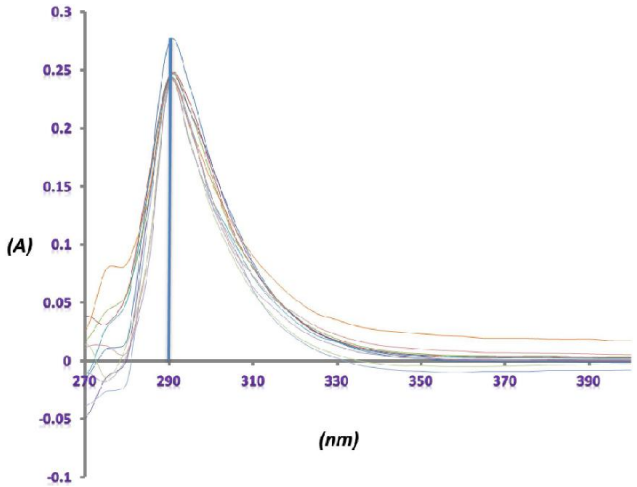

Figure 6: Absorption spectra of HSA/SDS for ten measurements in phosphate buffer (pH=7), at 25ºC.

HSA thin film reproducibility

The reproducibility was also studied by performing eight independent preparations of HSA/SDS thin films using the same method of preparation. Figure 7 shows the absorbance of eight different prepared films at λ_{max}= 290 nm. Results given in Figure 7 showed good agreement and stability for all the prepared thin ilms.

Effect of life time on HSA /SDS thin film stability

The life time of HSA/SDS thin films was studied at six-month time period. The films were stored at ambient temperature in dried conditions. Almost, no leaching or change in absorbance response was shown for these films (Figure 8). So these films are considered to be very stable at long time periods.

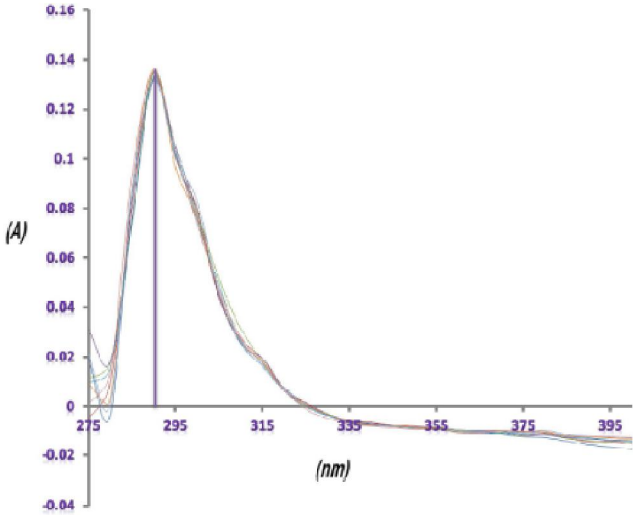

Figure 7: Absorption spectra of eight different HSA/SDS thin films in phosphate buffer (pH=7), at 25ºC.

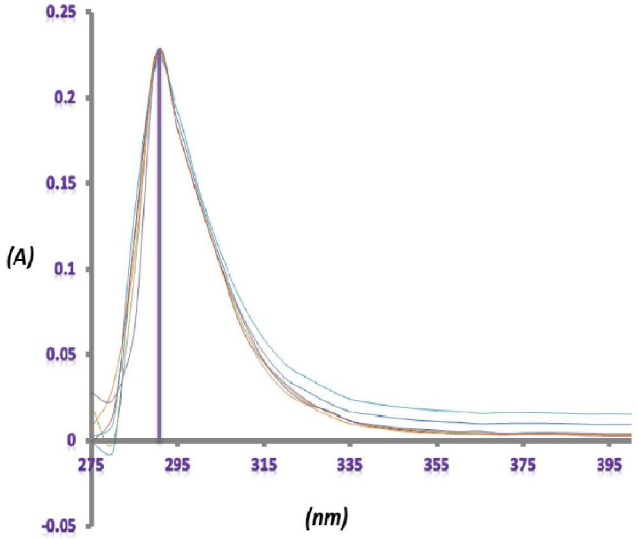

Figure 8: Absorption spectra of immobilized HSA/SDS within six-month time period of measurements in phosphate buffer (pH=7), at 25ºC.

CONCLUSION

A monolithic HSA immobilized thin films were prepared by spin coating technique in the presence of cationic CTAB, anionic SDS, and non-ionic TX-100 surfactants. The sol gel process was used to prepare encapsulated HSA thin films deposited on glass slides, which involves hydrolysis and polycondensation of tetraethylorthosilicate in presence of surfactant. HSA molecules were properly accommodated within silica network pores when the thin films were dried at 37 ºC and well interacted when SDS surfactant was used. The

immobilized HSA retains its activity towards *p*-nitrophenol acetate as a substrate. The HSA thin film shows good reproducibility, high stability at long time intervals and repeating measurements.

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