Adsorption 7: 105–116, 2001

Adsorption/Desorption Behavior of Bovine Serum Albumin and Porcine Insulin on Chemically Patterned Porous Gel Networks

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Received May 24, 2000; Revised December 21, 2001; Accepted January 23, 2001

Abstract. Adsorption/desorption of proteins onto a biomaterial surface plays a major role on the biocompatibility of the implanted material. By modifying the biomaterial surface with specially designed functional groups one may achieve the most specific behavior of the developed material used in a biological system. Based on that, porous gel matrixes with functionalized surfaces offer unlimited possibilities to control the protein-substrate interaction behavior. In the present work, we have functionalized the surface of porous glass with several chemical groups during the synthesis of the silica matrix. The porous glass matrixes were obtained using tetraethoxysilane (TEOS)/ethanol and functionalized with 3-mercaptopropyltrimethoxysilane (MPTMS) and 3-aminopropyltriethoxysilane (APTES). In vitro tests of the kinetics of protein adsorption and desorption from the gel matrix were monitored by UV-visible spectroscopy. The bioactivity of the incorporated protein was verified by in vivo experiments with adult male rats, where they presented an acute hypoglycemic peak.

Keywords: protein adsorption, adsorption kinetics, sol-gel process, porous glass, surface engineering

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1. Introduction

Biomaterials have been studied for many decades and, more recently, they have called the attention of many research groups involved on developing a novel class of biomaterials: materials precisely designed for a specific biological application. Instead of preparing a surface, having biomacromolecules, such as proteins, adsorb onto such surfaces and examing the results, the new paradigm requires that we aim to mimic what nature does all the time, that means we should focus on developing a novel class of biomaterials that are precisely designed for a specific biological application. The main strategy to solve this challenge can be based on engineering the surface in such a way that nonspecific reactions and interactions with the biological system are inhibited and only very specific reactions will take place. In the protein-surface interactions, the governing factors are determined both by the material and the intimate solution environment. Factors such as bound ions, surface charges, surface chemical composition, surface roughness and surface energetics, among many others, have to be closely analyzed in defining the solid-solution interface.

The immobilization of proteins onto surface functionalized substrates has been one of the most promising areas in bioengineering field as discussed by several authors (Gill and Ballesteros, 1998; Dave et al., 1994; Ellerby et al., 1992; Hench and West, 1990; Livage et al., 1994; McDonagh et al., 1998). The performance of artificial materials in contact with biological systems is to a large extend determined by surface interactions. Therefore considerable efforts have been made in the last years to improve surface compatibility of materials used for biological and biomedical applications (Lu et al., 1994). It has an enormous potential for application as biomaterial implant, immunological kits, drug delivery systems and biosensors.

In the present work, we have carried out some experiments regarding to the adsorption/desorption behavior of BSA and PI incorporated into porous gels networks of SiO_2 and into gels of SiO_2 with functionalized surface. We chose to study the adsorption of bovine serum albumin because albumin is dominantly present in body fluids among other proteins. Insulin was chosen because its activity on regulating the glucose concentration level found in blood of most mammals. Also, as mentioned by some authors (Yamaguchi, 1992), insulin is the physiologically dominant glucose regulatory factor, and plasma glucose concentration is the major regulator of insulin secretion. Insulin plays a vital role in living organisms by depressing the hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis, stimulates peripheral glucose utilization, and therefore, decreases the plasma concentration of glucose. In response to hypoglycemia, the secretion of glucose counter regulatory hormones increases. They usually involve glucagon, adrenaline, growth hormone and cortisol. Both glucagon and adrenaline are potent stimulators of hepatic glycogenolysis and gluconeogenesis, resulting in a transient increase in hepatic glucose production within minutes. So, as described above, the prevention or correction of hypoglycemia is due to combined effects of dissipation of insulin coupled with glucose activation of its counter regulatory mechanism.

Since its inception a decade ago, sol-gel encapsulation has opened up an intriguing new way to immobilize biological materials. An array of substances, including catalytic antibodies, DNA, RNA, antigens, live bacterial, fungal, plant and animal cells, and whole protozoa, have been encapsulated in silica, metal-oxide, organosiloxane and hybrid sol-gel polymers. The advantages of these "living ceramics" might give them applications as optical and electrochemical sensors, diagnostic devices, catalysts, and even bioartificial organs. With rapid advances in sol-gel precursors, nanoengineered polymers, encapsulation protocols and fabrication methods, this technology promises to revolutionize bioimmobilization.

The high efficiency presented by biological macromolecules in selecting chemical species has motivated the development of devices that combine synthetic materials with biological entities. Proteins can de immobilized in many different ways, but it is crucial that they retain their active conformation after the incorporation procedure (Zusman et al., 1992; Ratner et al., 1996). There are three major methods for immobilizing biomolecules and cells. Two of them are physically based, physical adsorption and physical entrapment. The third method is based on covalent (chemical) attachment (Ratner et al., 1996). Thus, it is important to note that the term immobilization can refer either to a temporary or to a permanent localization.

The relative physical and chemical stability of BSA and PI to remain incorporated into the SiO_2 glass matrixes was evaluated using UV-Vis spectroscopy. We characterized the protein adsorption process onto the porous glass matrixes followed by the kinetics of protein desorption process in PBS and alkaline elution media. Also, we have performed bioactivity assay of the

incorporated PI into SiO_2 glass matrixes using adult mate rats and measuring their plasma glucose concentration with post-implantation time.

2. Experimental Procedure

Tetraethoxysilane $Si(OCH_3)_4$ (TEOS > 98%), 3-aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTMS) and bovine serum albumin (BSA—fraction $V > 99.5\%$, $M_r = 67,000$) were supplied by Sigma-Aldrich. Porcine insulin (PI > 99.5%, $M_r = 5,778$) was provided by Biobras SA. PBS solution (phosphate buffered solution) was prepared with final concentration of 10 mM:12 mM:120 mM ($Na_2HPO_4 \cdot 7H_2O/NaH_2PO_4/NaCl$) using Milli-Q water (>18.0 MΩ).

2.1. Porous Matrix

Porous glass matrix was obtained using TEOS in ethanol and PBS solution with $pH = 7.40 \pm 0.05$. The gel surface was chemically patterned by adding the silane reagents 3-mercaptopropyltrimethoxysilane (MPTMS) and 3-aminopropyltriethoxysilane (APT ES) during the process of porous glass network formation. The schematic representation of different functionalized structures obtained for gel networks is shown in Fig. 1(b). The sols were casted into 96-well-plate molds, where gelation occurred. A thermal treatment of aging and drying was conducted to stabilize the porous gel matrix. The glass discs were produced with an average weight of 12 ± 2 mg. All glass discs were accurately weighted before adsorption and desorption experiments, needed for mass balance calculations to quantify the concentration of BSA and PI incorporated into the porous glass matrices. The protein adsorption procedure was carried out with the immersion of the gels of silica in PBS solution containing either PI or BSA, to reach a final protein concentration of 1.0 weight% in SiO_2, i.e. 120 μg of protein/SiO_2 disc. The impregnated gels were subsequently dried for 48h/40°C.

2.2. FTIR Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the presence of specific chemical groups in the materials (Perkin-Elmer, Paragon 1000). Gels were milled and mixed with dried KBr powder.

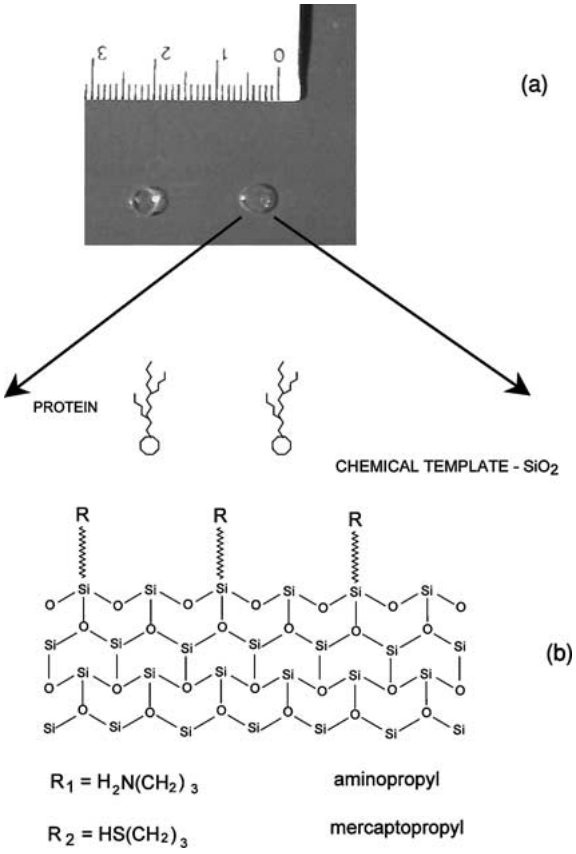

Figure 1. (a) Photograph of SiO_2 porous glass discs. (b) Schematic representation of functionalized gel networks (3-aminopropyltriethoxysilane and 3-mercaptopropyltrimethoxysilane).

FTIR spectra were obtained within the range between 4000 cm^{-1} and 400 cm^{-1} during 64 scans. The incorporation of protein within the gels was also monitored by FTIR spectroscopy. FTIR spectra were used as a qualitative reference of protein incorporation into the gel matrix and were also used to verify the presence of specific chemical groups in the gel network, reflecting the effectiveness of the developed procedure for engineering the porous glass surface.

2.3. Protein Release Procedure

We have used two different solution medium to evaluate the desorption process of incorporated protein into the porous gel network. The PBS solution was used because it is largely used for biological experiments in vitro. The alkaline solution was used as the most aggressive medium where the desorption process is

expected to be extreme. Therefore, we would be able to obtain adsorption/desorption data from a broad range of solution medium. The porous gel chemical stability in alkaline medium was verified by weight loss and by UV-visible spectroscopy. We have observed less than 1% of weight loss from the porous glass and no detectable change on the absorbance for a period of 24 hs. The protein release experiments were performed by incubating gel matrices with BSA and PI incorporated in PBS and alkaline ($8 < \text{pH} < 10$) media. The SiO_2 gels with protein were weighted and then immersed in 1.0 ml of PBS medium for 2 hours and 24 hours at 37°C. After that, PBS solution was collected for absorbance measurement and protein concentration calculation. An aliquot of 1.0 ml of alkaline medium was then added to these same gel matrices previously treated with PBS. After 30 minutes in alkaline solution at room temperature, duplicate samples were collected and absorbance readings were used for BSA and PI desorption evaluation. We have also crushed the SiO_2 gels, with BSA and PI incorporated, until reaching a fine powder. The powder samples were weighted and transferred to a plastic tube. PBS solution (1.0 ml) was added to each tube and let resting for a 24 hours period, at 37°C. Tubes were centrifuged at 1350*g* using International Refrigerated Centrifuge (Model PR-2), the supernatant was carefully collected, filtered using 0.45 μm membrane and stored for latter protein measurement. Then, 1.0 ml of alkaline medium was added to each tube, containing the remaining gel powder. After 30 minutes, at room temperature, each tube was centrifuged, supernatant was collected, filtered in 0.45 μm paper and stored for protein measurement as previously described.

2.4. *Protein Desorption Kinetics—In Vitro Experiments*

The kinetics of protein desorption from the gel matrix was monitored by UV-visible spectroscopy, with the concentration estimated through Lowry method readings at 660 nm wavelength (Lowry et al., 1951). Glass matrices containing protein were weighted and incubated with 1.0 ml of PBS medium at 37°C. After 10 minutes of immersion, an aliquot of approximately 1.0 ml of PBS solution was collected from each tube for protein concentration estimation, and 1.0 ml of fresh PBS medium was then added to the same matrix. This procedure was again repeated for 30, 60, 120, 240, 360, 640 and 1440 minutes of incubation.

2.5. *Colorimetric Determination of Protein Concentration (Lowry Method)*

This study employed a colorimetric method to determine protein concentrations, known as the Lowry assay (Lowry et al., 1951). In this method, a protein solution, which was treated with alkaline copper sulfate, was subsequently treated with another reagent called Folin-Ciocalteau phenol reagent. The former reagent reacts with the nitrogen of the peptide bond, resulting in a faint pink-purple color. The latter reagent intensifies the color by reacting with tyrosine and tryptophan residues in the polypeptide. The addition of these reagents to solutions that have undergone serial dilutions results in a blue-green complex. The intensity of the color complex is directly proportional to the protein concentration of the sample due to the higher the number of tryptophan and tyrosine residues. The absorbance of both BSA and PI solutions were measured using a spectrophotometer Shimadzu UV-160A set at 660 nm.

2.6. *Protein Denaturation Study*

We have used solvent perturbation spectroscopy technique-SPS (Copeland, 1994) to verify denaturation effect of the developed process for incorporation of BSA and PI into the gels of silica and silica with functionalized surface. The SPS analysis was carried out through the UV spectra shift ('blue shift') obtained in the range of 250–300 nm when 4M guanidine hydrochloride (Gd-HCl) was added to BSA and PI solutions. We have used the absorption differences between folded (native) and unfolded (denaturated) states of proteins. The effectiveness of the developed method for incorporation of proteins in porous gel glasses was evaluated by comparing pure gels of silica with gels with chemically functionalized surface.

2.7. *SEM (Scanning Electron Microscopy)/EDX (Energy-Dispersive X-Ray Spectroscopy) Microanalysis*

Surface modification of porous gel was observed by SEM/EDX microanalysis. SEM micrographs were obtained using a JEOL JSM 5410. Gel samples were coated with gold-palladium film to enhance resolution and stabilize the electron beam mainly on protein-rich areas as reported in the literature (Mansur et al., 1999a).

2.8. *Subcutaneous Bioactivity of Insulin Immobilized Gels—In Vivo Assay*

Since the immobilization process may denature or otherwise inactivate the incorporated insulin, the bioactivity was investigated. Male rats were fasted overnight, anesthetized using ethyl ether reflux, weighted and 0.4 ml blood samples were collected by intravenous catheter inserted in the jugular vein. Subcutaneous implants with insulin incorporated of either pure silica gel discs or surface-modified gels were placed under the back skin of each rat. The rat was denied food and blood samples were collected at intervals of 20, 40, 60, 90, 120 and 150 min post-implanting. The heparinized blood samples were centrifuged at 7000g for 10 min, plasma was removed and saved for glucose monitoring.

2.9. *Glucose Analysis*

Plasma glucose levels were obtained by using the Glucose GOD-ANA Colorimetric Assay (Labtest Diagnostica S.A., Minas Gerais, Brazil), following the manufacturer's protocols.

3. Results

We have obtained the SiO_2 porous glass discs via sol-gel route. SiO_2 glasses and SiO_2-functionalized glasses were casted in 96-well-plate molds, where gelation occurred. Figure 1(a) shows a photograph of few glass discs produced.

We have used FTIR technique to characterize the protein incorporation in porous sol-gel matrices. FTIR spectra results showed strong peaks at $v = 1620$–$1680\ cm^{-1}$ and $v = 1480$–$1580\ cm^{-1}$, mainly associated with amide-I and amide-II stretching vibration bands, respectively (Lu et al., 1994; Malmsten, 1998). These results have clearly indicated the presence of both proteins, BSA and PI, incorporated in the gel matrix. Figure 2(a) shows an example of FTIR spectra of porous glass (curve 1) and reference spectrum of BSA. (curve 2). Figure 2(b) shows FTIR spectra of porous glass and porous glass with PI incorporated. (1) reference spectrum of PI; (2) pure silica-gels; (3) the silica-gel modified with 1% MPTMS and 1% porcine insulin incorporated.

The SEM/EDX characterization of the hydrophobic/hydrophilic behavior of porcine insulin onto silica surface has been recently reported in our work (Mansur et al., 1999a, 1999b). SEM micrographs (not shown) of the surface of protein impregnated SiO_2 gels and MPTMS functionalized gels have indicated that the morphology and spatial distribution of PI aggregates onto pure silica-gels were different from the gels functionalized with MPTMS.

3.1. *Kinetics of Protein Desorption Study*

Surface functionalized glass with APTES and MPTMS presented significant less BSA released in PBS when compared to pure gel of silica (Fig. 4(a)). On the contrary, gel functionalized substrates with APTES and MPTMS have clearly showed an increase of PI release in PBS media when compared to pure gel of silica. (Fig. 3(a)).

As we increased the molar fraction of amino terminated groups (R-NH_2) from 1% to 3% we could observe that BSA release in PBS was reduced over 50% (Fig. 4(a)). An opposite trend was verified for thiol-terminated groups (R-SH), where we have noticed a significant increase on BSA release when MPTMS molar fraction was raised from 1% to 3% and then to 10% (Fig. 4(a)). It was also observed that BSA was incorporated and kept immobilized onto the gel powder after crushing when compared to the original gel (Fig. 5(a) solid and dotted).

The gel of silica immersed in PBS for 24 hours was later incubated in alkaline medium. It was observed protein release (PI and BSA) from all three silica substrates, pure glass, thiol-functionalized and amino-functionalized glasses (Fig. 3(a) and (b) Fig. 4(a) and (b)). We have also observed that BSA concentration released in alkaline solution was larger than PI (Fig. 4(a) and (b).

The kinetics of protein released from the gel matrix was monitored by UV-visible spectroscopy, with the concentration estimated through Lowry method at 660 nm wavelength. The results have indicated a different behavior of adsorption and stability of BSA and PI proteins incorporated into the porous glasses. It was observed a significant larger PI release compared to BSA in PBS solutions.

The median time to release 50% of the incorporated protein (T_{50}) is shown on Table 1. Glass matrices incorporated with BSA have presented a minimum of 30 minutes incubation period before any significant detection of protein release. On the other hand, PI delivery study has indicated almost immediately protein release (less than 10 minutes).

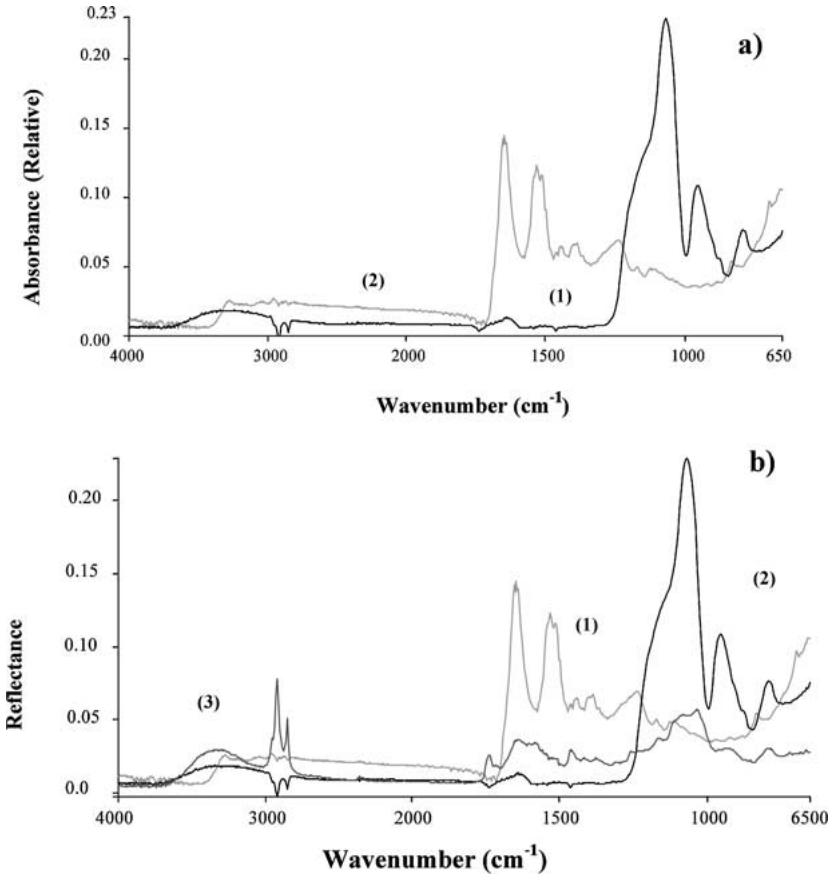

Figure 2. (a) FTIR spectra of porous glass and porous glass with BSA incorporated. (1) PI; (2) pure silica-gels without protein. (b) FTIR spectra of porous glass and porous glass with PI incorporated. (1) reference spectrum of PI; (2) pure silica-gels; (3) the silica-gel modified with 1% MPTMS and 1% porcine insulin incorporated.

Glasses with thiol-functionalized surfaces (R-SH) have presented stronger PI adsorption compared to amino terminated groups (R-NH_2). An opposite trend was verified with thiol-functionalized surface that presented less adsorption with BSA than amino patterned gel of SiO_2.

Table 1. Median release time of bovine serum albumin (BSA) and porcine insulin (PI) in three different porous gel substrates: SiO_2; SiO_2 chemically functionalized with APTES (amino terminated); SiO_2 chemically functionalized with MPTMS (thiol terminated).

	Median release Time/minutes[a] (T_{50})	
Substrate	PI	BSA
SiO_2	30	$120 < T_{50} < 240$
SiO_2-APTES (R-NH_2)	<10	$360 < T_{50} < 640$
SiO_2-MPTMS (R-SH)	<10	30

[a]Time to release 50% (weight) of the incorporated protein.

Figure 6 shows the accumulated percentage of PI release in PBS solution with different functionalized glass substrates. Again, PI presented stronger immobilization behavior with thiol chemically patterned glass than with SiO_2 amino terminated glass.

PI and BSA concentration in solution and immobilized into porous glass network were estimated by the adsorption spectra obtained for the respective protein standard. Based on the UV-Vis absorbance change at 275 nm we were able to calculate the mass balance and the extension of each protein adsorption process. The calculation was done using Beer-Lambert law where the absorbance is proportional to the concentration of the protein in the solution. One typical result for the kinetics of PI adsorption in three different porous glass substrates (pure glass,

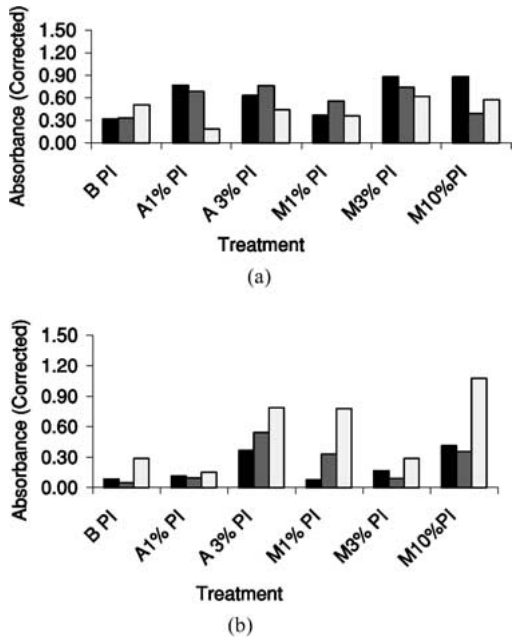

Figure 3. Histogram of porcine insulin release (a) PBS solution: Solid bar—2 hours immersion; dashed bar—24 hours immersion; Dotted bar—crushed powder—24 hours immersion; (b) immersion in alkaline solution: Solid bar—after pre-treated for 2 hours in PBS solution; dashed bar—after pre-treated for 24 hours in PBS solution; Dotted bar—crushed powder after pre-treated for 24 hours in PBS solution.

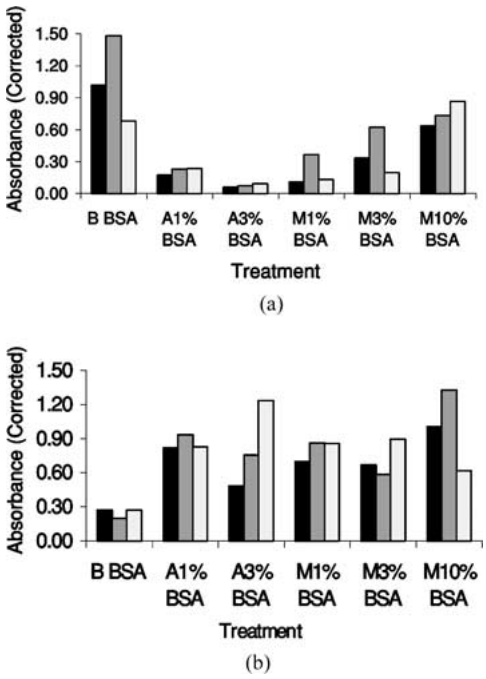

Figure 4. Histogram of bovine serum albumin release (a) PBS solution: Solid bar—2 hours immersion; dashed bar—24 hours immersion; Dotted bar—crushed powder—24 hours immersion; (b) immersion in alkaline solution: Solid bar—after pre-treated for 2 hours in PBS solution; dashed bar—after pre-treated for 24 hours in PBS solution; Dotted bar—crushed powder after pre-treated for 24 hours in PBS solution.

thiol-functionalized and amino-functionalized glasses) is showed in Fig. 7.

3.2. Incorporated Protein Native Structure Stability

We have used solvent perturbation spectroscopy to evaluate if the incorporation process of BSA and PI into the gels of SiO_2 and SiO_2 with chemically functionalized surface has denaturated these proteins. We have used the absorption differences between the folded (native) and unfolded (denaturated) states of proteins to investigate their stability. After adding 4 M guanidine hydrochloride to BSA and PI solutions, released from the porous SiO_2 matrices, the UV spectra (not shown) have indicated "blue shift" on the maximum absorbance peaks. These results have given strong evidence that no denaturation of BSA or PI has occurred in the immobilization procedure developed in this work.

4. Discussion

FTIR spectra have permitted to verify the incorporation BSA and PI into SiO_2 porous glass network due to the observed peaks at $v = 1620$–$1680\ cm^{-1}$ and $v = 1480$–$1580\ cm^{-1}$, clearly associated with amide-I and amide-II stretching vibration bands, respectively (Lu et al., 1994; Malmsten, 1998). These vibration bands are typically related to the presence of primary and secondary amides generally found in biomolecular structures. Also an increase in the peak intensity with raising the immobilized protein concentration in the porous glass matrix was observed.

SEM/EDX microanalysis results (Mansur et al. 1999a, 1999b) have clearly indicated a difference on the surface adsorption behavior of BSA and PI onto the porous gel substrate. SEM micrographs (not shown) of SiO_2 gels and MPTMS functionalized gels have given strong evidence that the morphology and spatial distribution of PI aggregates onto pure silica gels were different from the gels functionalized with MPTMS. The adsorption procedure proposed in these experiments of incorporation of PI and BSA into the porous gel has been used as an indication of what we called the "wettability" of the glass modified surface. That

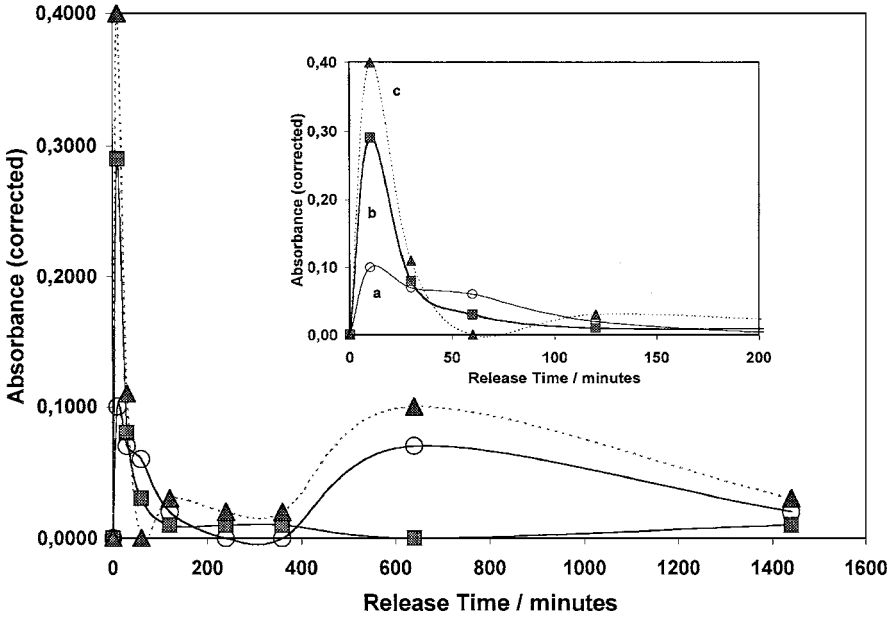

Figure 5. Kinetics of PI release in PBS solution with different functionalized glass substrates; (O) SiO_2; (■) R-NH_2 terminated/APTES; (σ) R-SH terminated/MPTMS; Inset detail: Initial stage of protein release.

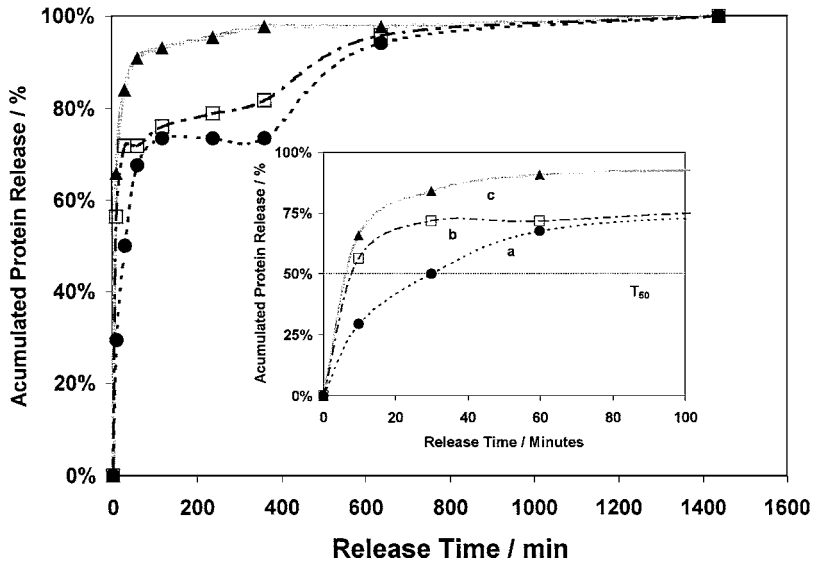

Figure 6. Accumulated percentage of PI release in PBS solution with different functionalized glass substrates; (●) SiO_2; (□) R-SH terminated/MPTMS; (▲) R-NH_2 terminated/APTES; Inset detail: Initial stage of protein release with T_{50} evaluation.

means, when the protein solution in PBS medium was added to the porous gel matrix and left for a period of time until fully dried out, the hydrophobic/hydrophilic behavior of the glass surface would likely to be responsible for either the complete percolation in the porous matrix or the formation of protein agglomerates. Therefore, it was observed a "de-wetting" characteristic of the surface of the functionalized gel, usually forming PI agglomerates. This difference would be attributed to the fact that gels with modified surface will present a more hydrophobic behavior concerning to the adsorption of protein macromolecules when compared to the behavior of pure silica gel. The first one would have a thiol terminated propyl chain (Si-CH_2-CH_2-CH_2-SH)

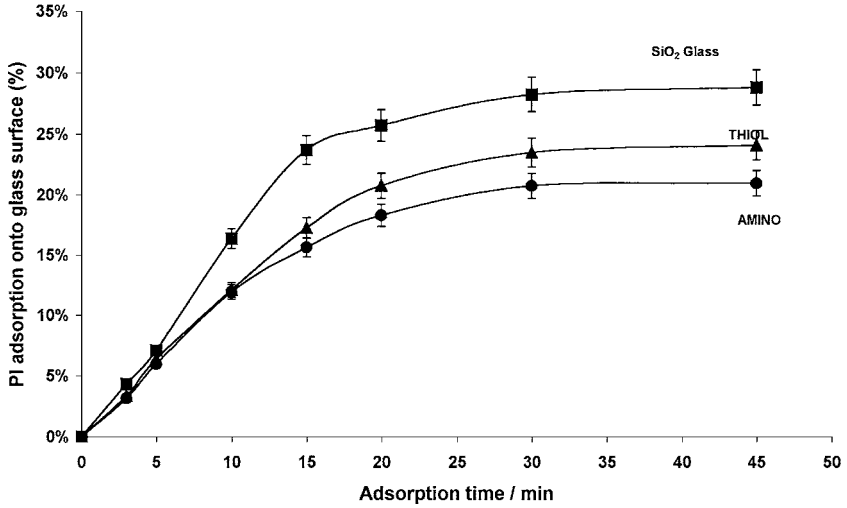

Figure 7. Calculation of PI adsorption in three different porous glass substrates obtained by UV-Vis spectroscopy absorbance at wavelength of 275 nm; (■) SiO_2; (▲) R-SH terminated glass; (●) R-NH_2 terminated glass.

and the second would present a hydrophilic silanol (Si-OH) group on the outer surface.

Previous SEM coupled to EDX microprobe results (Mansur et al., 1999a) have indicated an important difference on the surface adsorption behavior of BSA and PI into the porous glass network. SEM micrographs of SiO_2 gels, MPTMS gels and APTES gels have given important evidence that the morphology and spatial distribution of PI aggregates onto pure silica gels were different from the gels with chemically modified surfaces. It was also noted a hydrophobic characteristic of the surface of the functionalized gel, usually forming aggregates of PI. Gels modified with MPTMS have more spherical protein clusters than amino-modified gels and pure silica gels, suggesting that the former has surfaces with poor levels of wettability associated with proteins. This difference is attributed to the fact that gels with modified surface will present a more hydrophobic behavior concerning to the adsorption of protein molecules when compared to the behavior of pure silica gel. The first one presents a thiol terminated propyl chain (Si-CH_2-CH_2-CH_2-SH) and the second presents a hydrophilic silanol (Si-OH) group on the outer surface. These assumptions are also valid comparing amine-terminated gel with pure silica network. One would have expected such trend on the hydrophilic property of the modified glass surface once the -SH groups is known to be more hydrophobic than either NH_2 or -OH groups. Also, the relatively high content of hydrophobic amino acid residues such as Val, Leu, Tyr, Phe and Ile generally found in mammals' insulin would certainly decrease its wettability property. On the other hand, the high number of polar amino acid residues found in BSA would enhance its hydrophilic behavior. These characterization techniques have been able to verify that the wettability of the SiO_2 surface can be dramatically altered by the addition of chemical modifying groups such as thiol and amine. The presence of functional chemical groups (thiol and amine) in the modified gels proves that the combination of different silane agents during synthesis of the gels can satisfactorily be used to produce materials containing specifically designed chemical functionalities.

The behavior of two different proteins, BSA and PI, regarding to their adsorption stability and their release kinetics was studied in order to define the relationship between the chemistry of modified gel and the pure silica-gel network. On the first approach, the primary structure, related to the type and sequence of aminoacids found in Bovine Serum Albumin (BSA) and Porcine Insulin (PI), are strictly different and therefore, will certainly influence their adsorption and release behavior on solid supports. In addition to that, the relative small size and the large number of hydrophobic amino acid residues of PI compared to BSA will play a very important role on the chemical affinities between the biomacromolecule and the gel surface.

We have observed less BSA release from the gel powder after crushing than the original pure silica glass. It could be attributed to the fact that many previously closed porous of SiO_2 glasses were exposed to the BSA

after crushing. Therefore, the encapsulated excess of protein was promptly adsorbed onto the freshly exposed gel surface (Fig. 4(a) solid, dotted). This assumption is validated by surface area and contact angle measurements of the porous gel network reported in our previous work (Mansur et al., 1999b) with average value of 500 ± 100 g/m^2. The connectivity of the porous nanostructured glass is limited and, after crushing, there would be an increase on the exposed surface for further protein adsorption.

It was observed protein release (PI and BSA) from all three substrates (gel of silica, thiol functionalized gel, amino functionalized gel) when gels were immersed in PBS for 2 or 24 hours and later incubated in alkaline medium. We have noted in Fig. 4(b) that BSA concentration released in alkaline solution was larger than PI (Fig. 3(b)). One may assume that in fact the excess of PI onto the surface, released in PBS medium at the former stage of the kinetics study, has reduced the amount of available PI inside the porous gel matrix. An opposite behavior was observed with BSA that has percolated into the gel network, resulting in a more uniform distribution of the protein throughout the glass matrix. Therefore, BSA has been released only with alkaline medium, a more aggressive medium than PBS. These results have been confirmed by mass balance calculations where the total amount of protein incorporated into the glass matrix was constant (1.0 weight% in SiO_2) for both BSA and PI.

It was observed a significant larger PI release compared to BSA in PBS solutions (Fig. 3(a) and Fig. 4(a)). That is believed to be associated with the difference in protein interactions with the chemically functionalized surface. It is also valid to assume that the size difference would play an important role on the release kinetics, where the small PI molecules would percolate through the porous glass network much more easily than the large BSA molecules. These results can be considered to be related to two different mechanisms of protein release. The first one would be mainly associated to the excess of PI onto the glass surface being released by the elution media. The thiol-terminated surface has presented a stronger affinity with the PI, retaining the protein adsorbed for longer period of time, than amino-functionalized substrate. The second mechanism would be basically associated with the diffusion behavior of BSA protein throughout the porous glass network. The thiol patterned surface has shown a much more open structure than the amino functionalized one, according to N_2 adsorption results using B.E.T. model (Mansur et al., 1999a). That would facilitate the percolation process of BSA macromolecule with an average molecular weight of 67,000 g/mol compared to PI with molecular weight of 5,778 g/mol. That would represent a difference of approximately 250% on the average molecule size between BSA and PI, assuming an estimative of protein size dependence on the cubic root of molecular weight.

Glass matrices incorporated with BSA have presented a minimum of 30 minutes incubation period before any significant detection of protein release. On the other hand, PI release study has indicated a very short time for protein desorption, usually less than 10 minutes, as shown in Fig. 5. Based on these results we can assume that PI release was related to the excess on the surface of the porous matrix and BSA was homogeneously incorporated in the glass network. We were unable to observe any segregation of BSA onto the glass surface indicating that the protein was evenly distributed in the SiO_2 matrix.

The aromatic amino acids tyrosine and tryptophan are commonly found in proteins, and both give rise to π-π^* absorption bands in the vicinity of 280 nm (Zheng et al., 1997; Lowry et al., 1951). For this reason, most proteins, including BSA and PI, show a strong absorption band in this region. This property can be used to estimate the concentration of protein in solution and also distinguish the native (folded) and denaturated (unfolded) forms. A typical UV absorption spectrum of native BSA in phosphate buffered saline (PBS) would present a peak absorbance occurring at 278 nm. This reflects the combined contributions of aromatic amino acids tyrosine and tryptophan in solution used as elution medium. The immersion of native protein in 4M guanidine hydrochloride (Gd-HCl) would denaturate them, resulting in a "blue shift" in the UV-Vis absorbance spectrum. This procedure is generally used as a probe technique to identify possible denaturation of protein in polar solution (Zheng et al., 1997; Lowry et al., 1951). The SPS essays were conducted with absorbance measurements in the UV range from 250 nm to 300 nm. Based on these spectra (not shown) we had strong evidence that no denaturation occurred in either BSA and PI incorporated into the glass network. This observation is of major importance considering biological activity of proteins in living organisms. The denaturation process of proteins is mainly related to modification of the three-dimensional conformation structure. According to these results, we can assume that the proposed procedure for BSA and PI

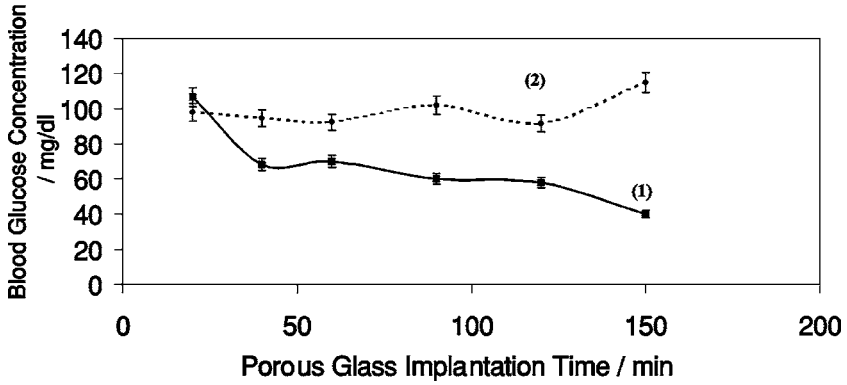

Figure 8. Plasma glucose concentration in response to insulin administration time; (1) rat with implant of porous SiO_2 containing 1% weight of PI; (2) control rat with implant of pure SiO_2 glass (without PI).

incorporation in functionalized glass has not caused any denaturation of these proteins.

These SPS findings were confirmed through in vivo evaluation experiments. We have conducted in vivo bioassay using PI incorporated into gel network to due to the high specificity and sensibility of this characterization procedure. The bioactivity of the gels with PI incorporated into the matrix was demonstrated by the acute hypoglycemic effect observed in these implanted animals compared to glucose level of the control group. Figure 8 shows a typical in vivo response of the glass implanted material. It can be clearly observed the bioactivity of the insulin incorporated in the glass disc (curve 1) by decreasing the plasma concentration of glucose compared to the control rat (curve 2). In few cases we have even observed convulsive response in rats, mainly due to dramatically reduction of rat blood sugar level induced by PI administration through the subcutaneous gel implant. Therefore, we assume that the incorporation process of protein into SiO_2 glass network has retained the bioactivity and has not caused any detectable loss of its bioactivity. These results obtained through in vivo experiments have confirmed the previous spectroscopy (SPS) observations where no major denaturation of the proteins has been verified. From the current in vitro and in vivo study, we have verified that the process of incorporation of proteins into porous gel network could be useful for the preparation of controlled delivery system for proteins.

5. Conclusions

BSA and PI were incorporated into sol-gel silica matrices by introducing freshly-formed gels into solutions containing the BSA or PI. The effectiveness of the process was verified by FTIR spectroscopy.

The UV-Vis absorbance spectroscopy results have indicated that we have developed a successful process for patterning the SiO_2 gel with different chemically terminated groups. That has attributed a different hydrophilic behavior of the functionalized surfaces. Also, we have identified two major protein release systems. The first one with almost no incubation period of protein release found in PI immobilized glass matrices. The second one, with an incubation time of over 30 minutes, that we believe is mainly associated to desorption and percolation process of BSA through the porous glass network.

The solvent perturbation spectroscopy has indicated that the proposed procedure of BSA and PI incorporation in chemically functionalized glasses has not caused the denaturation of the proteins. In vivo experiments results have also confirmed the bioactivity response of incorporated PI into SiO_2 porous glass. Therefore, one might expect no major loss of bioactivity and functionality of such proteins in biological systems.

Acknowledgments

The authors acknowledge the important contribution of CDTn/CNEN for the facilities and UV-Vis spectroscopy measurements.

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