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Biomaterial with chemically engineered surface for protein immobilization

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The last 3 decades have been a revolution in the area of sol-gel-derived materials. They can be used to encapsulate biomolecules such as enzymes, antibodies, hormones, and proteins retaining their functional state. Proteins can be immobilized in many ways but it is crucial that they retain their native conformational structure and, therefore, bioactivity. Porous silica gel matrixes with modified surfaces offer unlimited possibilities to control the protein-solid interaction behavior. The bioimmobilization process on sol-gel biomaterials with chemically engineered surface has driven applications on solid-phase materials, affinity chromatography, biosensors and many others. In the present work, we have aimed to produce surface-modified silica glass materials obtained via sol-gel route to be used as solid support on drug delivery systems and as solid-phase in immunodiagnostic. The functionalization process was carried out by reacting alkoxysilanes with 5 different silane surface modifying chemical groups: tetraethoxysilane (TEOS), 3-mercaptopropyltrimethoxysilane (MPTMS) and 3-aminopropyltriethoxysilane (APTES), 3-glycidoxypropyltrimethoxysilane (GPTMS) and 3-isocyanatopropyltriethoxysilane (ICPES). The bioactivity assays were based on two main tests: (a) An *in vivo* bioresponse of rats with sol-gel disk implants with insulin protein incorporated. *In vivo* tests with adult male rats were used to verify the immobilized insulin bioactivity after implantation of different biomaterial with functionalized surfaces. All surface modified materials have presented hypoglycemic peak response associated with the insulin bioactivity. (b) The produced solid-phase sol-gel disks with protein substrates were tested through **E**nzyme **L**inked **I**mmuno**S**orbent **A**ssay (ELISA). The immunoassay results have showed that glasses with chemically functionalized surfaces regulated the extent of bioimmobilization of protein. The amine, thiol and hydroxyl terminated porous gels have showed significant interaction with the antibody-antigen, during the coupling process. We believe that it is due to balance of forces associated with Van der Waals interaction, hydrophilic and hydrophobic forces and steric hindrance acting at the surface. Therefore, such novel biomaterial could be advantageously used in drug delivery systems and in immunoassays of diagnostic kits.

1. Introduction

1.1. Porous nanostructured biomaterial

Conventional methods to immobilize biomolecules onto inorganic, organic or polymeric surfaces have usually been based on physical adsorption, covalent binding to surfaces, entrapment in semi-permeable membranes and microencapsulation into polymer microspheres and hydrogels. However, such techniques are not generic, and in most cases can be used only for a limited range of biomolecules or applications [1–4]. Proteins have very particular chain configurations and conformations that promote high levels of specificity during chemical interactions. An emerging route for bioimmobilization involves the entrapment of biological components into inorganic silicate matrixes formed by a low temperature sol-gel processing method [5, 6]. The sol-gel process as a route to form inorganic glasses has been known for well over a century, however, the first report demonstrating the use of silicate materials for the entrapment of a biological moiety did not appear until the mid-1950s. Researches have demonstrated that silicate glasses obtained by sol-gel method can

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provide a host matrix without causing major degradation on the functional characteristics of the immobilized biomolecule [4]. As reported by several authors [2–6], surface chemistry can be used to manipulate protein-surface interaction by controlling the surface density and spatial distribution of chemical groups and ligands. One of the major advances of sol-gel processing that has been seen during the last few years is undoubtedly the synthesis of hybrid organic-inorganic materials. Hybrids have been called ORMOSILS (Organically modified silicates), ORMOCERS (organically modified ceramics) and CERAMERS (ceramic polymers) [7, 8]. The surface may be treated to decrease wettability or increase adhesion of the protein to substrate. Although, proteins that adsorb are naturally found in body, the way they interact with the surfaces can alter their ability to fulfill their roles as biomolecules, yielding deactivated moieties that are rejected by the body. In order to achieve full functionality, proteins have to adsorb specifically, i.e. they cannot (1) have their conformation altered (denaturation); (2) have their active site hidden during adsorption; (3) adsorb in too low or too high densities that would not resemble natural tissues. The main challenge is based on engineering the surface in such a way that nonspecific reactions and interactions with the biological system must be inhibited and only very specific reactions will take place. It has an enormous potential for application as biomaterial implant, immunological kits, drug delivery systems and biosensors [1, 4, 9, 10]. 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, organosiloxane and hybrid sol-gel polymers [11, 12]. The major advantages of sol-gel silicate materials for immobilization of proteins are (a) they can be made to be optically transparent, making them ideal for the development of chemical and biochemical sensors that rely on changes in an absorbance or fluorescence signal, (b) they are open to a wide variety of chemical modifications based on the inclusion of various polymer additives, and organically modified silanes (Ormosils), and (c) they have a controlled pore size and pore distribution, which allows small molecules and ions to diffuse into the matrix while large biomolecules remain trapped in the pores. However, some disadvantages are found in sol-gel based materials such as: (a) they can be brittle, (b) they may undergo cracking owing to hydration stresses, (c) organically modified gels or hybrids are only partially transparent for optical applications, and (d) they have very high surface area and porosity which facilitates protein–silica interactions but may lead to protein denaturation [13]. Therefore, sol-gel based organic–inorganic nanocomposites materials will need to be improved to overcome such challenges.

1.2. Bioactivity of protein immobilization

Porcine Insulin (PI) was chosen to be studied because it is an extremely important peptide hormone. Insulin is the physiologically dominant glucose regulatory factor [14]. It plays a vital role in living organisms with depressing the hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis, stimulates peripheral glucose utilization, and therefore, decreases the plasma concentration of glucose [14]. Therefore, the bioactivity response of the immobilized porcine insulin molecule in substrates would be detectable by glucose concentration analysis.

1.3. ELISA immunoassay background

ELISA assays are methods for determination of substances such as peptides, proteins, antibodies and hormones, in which a crucial element of the detection is an antigen-antibody interaction. ELISA assay depends on the reaction of a predominant protein with specific antibody to form a complex. Antibodies are host proteins produced in response to the presence of foreign molecules in the body (antigens). Because the antigen-antibody reaction is specific, antibodies are an important reagent for immunological research and clinical diagnostics [16]. The ELISA method has very high sensitivity and specificity. In recent years there has been an increasing interest in the development of a simple immunoassay technique like ELISA for several diseases [17–20]. Most commonly, ELISAs are performed in microtiter plates, which are also called microwell plates, usually made of polystyrene or poly (vinyl chloride), which will bind antibodies spontaneously [20].

In the present work, the main goal was to create surface functionalized biomaterials via sol-gel route that could be recognized by biomolecules, allowing the development of novel processes on drug delivery systems (insulin), biorecognition and protein adsorption kinetics (ELISA assays).

2. Experimental procedure

Tetraethoxysilane $Si(OC_2H_5)_4$ (TEOS > 98%), 3-aminopropyltriethoxysilane (APTES), 3-mercaptopropyltrimethoxysilane (MPTMS) were supplied by Sigma-Aldrich. 3-glycidoxypropyltrimethoxysilane (GPTMS) and 3-(triethoxysilyl)propylisocianate (TESPI) were supplied by Merck. Porcine insulin (PI > 99.5%, $M_r = 5{,}778$) was provided by Biobras SA, Brazil. PBS solution (phosphate buffered solution) was prepared using the reagents: Na_2HPO_4 (>99.0%), NaH_2PO_4 (>99.0%), Na_2CO_3 (>99.5%) and NaCl (>99.0%) supplied by Sigma-Aldrich.

2.1. Porous solid-phase synthesis via sol-gel

Porous glass matrix was obtained using TEOS and PBS solution with pH = 7.40 ± 0.05. The gel surface was chemically patterned by adding the silane reagents 3-mercaptopropyltrimethoxysilane (MPTMS), 3-aminopropyltriethoxysilane (APTES), 3-glycidoxypropyltrimethoxysilane (GPTMS) and 3-(triethoxysilyl)propylisocianate (TESPI) during the process of porous glass network formation (silane

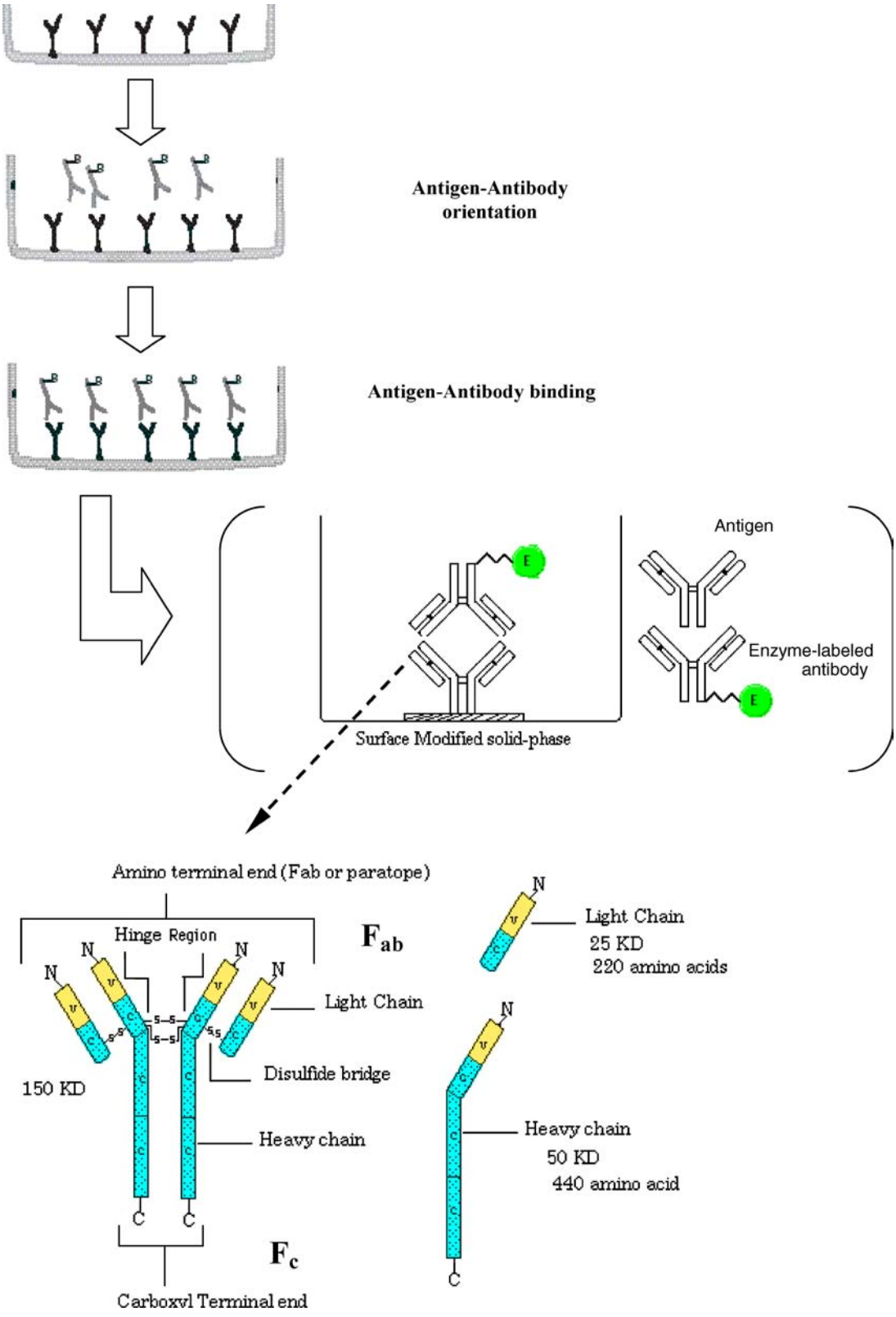

Figure 1 Schematic representation ELISA immunoassay with surface engineered solid-phase.

reagents shown in Fig. 2). The sols were cast into 96-well polystyrene microplates, where gelation (24–72 h) occurred at room temperature (25 °C ± 2 °C). After thermal treatment of aging and drying (60 °C for 7 days), the gels of silica were immersed in PBS solution containing PI, to reach a final protein concentration of 1.0 wt % in SiO_2. The impregnated gels were subsequently dried for 48 h at 40 °C. These glass disks were produced with an average weight of 12 ± 2 mg (Fig. 2(a)). All glass disks were accurately weighed before adsorption and desorption experiments, needed for mass balance calculations to quantify the concentration of PI immobilized into the porous glass matrices. The surface area of the porous network was characterized as previously reported in our work [12].

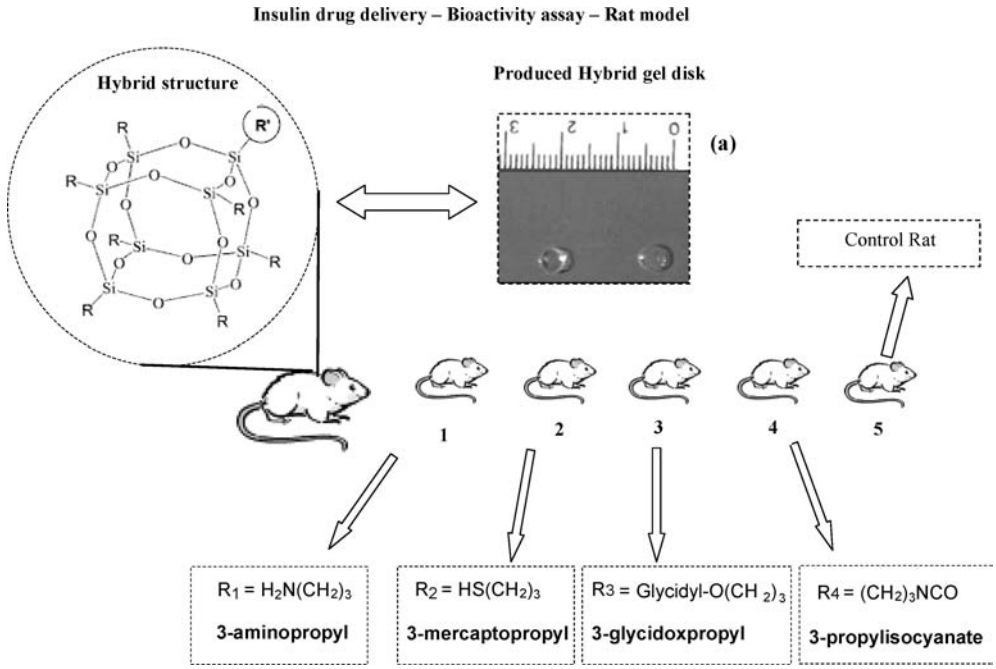

Figure 2 Schematic representation of 5 different chemical groups of functionalized gel disks implanted in the rats.

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). FTIR spectra were obtained within the range between 4000 and 400 cm^{-1} (Perkin-Elmer, Paragon 1000), using diffuse reflectance spectroscopy method (DRIFTS-FTIR). Gels were milled and mixed with dried KBr powder (1.0 wt %). Samples were placed in a sampling cup and 64 scans were acquired at 2 cm^{-1} resolution with the subtraction of KBr background. The incorporation of protein within the gels was also monitored by FTIR spectroscopy. We would like to point out that FTIR spectra were used as a qualitative reference of protein incorporation into the gel matrix. FTIR was also used to characterize the presence of specific chemical groups in the gel network, reflecting the effectiveness of the developed procedure for functionalization of porous glass.

2.3. 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, weighed and 0.4 ml blood samples were collected by intravenous catheter inserted in the jugular vein. Subcutaneous implants, with insulin incorporated in either pure silica gel disks or surface-modified gels, were placed under the back skin of each rat. A schematic representation of the different gel implants in the rats is shown in Fig. 2. 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 7000 g for 10 min, plasma was removed and saved for glucose monitoring. 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 with UV-Vis spectroscopy absorbance reading at 505 nm.

2.4. Biochemical assays (ELISA)—Organically modified gel solid-phase

2.4.1. Immunoassay ELISA for IgG-Bov/Anti-IgG-Bov

Reagents. Purified bovine IgG, Anti-Bovine IgG (whole molecule)-Peroxidase Conjugate, produced in rabbit (Anti-Bov-IgG-HRP) (Sigma Cat# A5295), TMB Substrate (Sigma Cat# S5814), Tween-20 (Sigma Cat# P7949) were supplied by Sigma (St. Louis, MO, USA); solution Phosphate buffered saline + Tween20 (PBS-T) and Phosphate buffered saline + casein (milk) 3% w/v (PBS-M) were prepared just before use.

ELISA procedure-1. The chemically tailored silica gels prepared with TEOS and organic modifiers MPTMS, APTES, GPTMS and TESPI, were weighed and put in the respective ELISA 96-well microplates (Nunc Maxisorp Prod# 4420404). All samples were prepared in duplicate. Microtiter plates were coated with purified bovine IgG at a concentration of 20 μg/mL in PBS buffer (pH = 7.4). The remaining sites for protein binding on the microtiter plate were saturated by incubating with blocking buffer (PBS-M). Approximately 100 μl of the labeled antibody (Anti-Bov-IgG-HRP) solution was added to each well. Microplates were incubated for 1 hour at 37 °C to allow complete binding. Then, 100 μl/well TMB substrate was added as ELISA stop reagent. After the incubation time of 15 min has elapsed, 50 μl/well of H_2SO_4 (2.0 N) was added to each well. After that, the absorbance was read at 450 nm (BIORAD ELISA spectrophotometer).

2.4.2. Immunoassay ELISA for scorpion venom (Tityus serrulatus)

Protein. The toxic fraction from *Tityus serrulatus* scorpion venom (protein TsTxFG50) was supplied by FUNED, Minas Gerais, Brazil [21].

ELISA procedure-2. The chemically tailored silica gels prepared with TEOS and organic modifiers MPTMS, APTES, GPTMS and TESPI, were weighed and put in the respective ELISA 96-well microplates (Nunc Maxisorp Prod# 4420404). Subsequently, the microplates were coated for 2 h at 37 °C, shaking with 100 μl of a 5-μg/ml solution *of Tityus serrulatus* venom (TsTxFG50), in 0.02 M of sodium bicarbonate buffer (pH = 9.6). The detailed protocol for ELISA technique used in this study has been reported in the literature [21, 22]. Absorbance at 492 nm was determined with an ELISA reader (BIORAD ELISA spectrophotometer). Duplicate readings were taken for all samples.

3. Results and discussion

3.1. Gel synthesis, surface functionalization and protein immobilization

We have produced the SiO_2 porous glass and SiO_2-functionalized glasses disks via chemical reaction of hydrolysis and policondensation of alcoxides. They were found to be optically transparent to visible light and mechanically stable to be handled. We have used FTIR spectroscopy for the characterization of the gel matrices synthesis, their surface functionalization and protein bioimmobilization. In Fig. 3, FTIR spectra of reference protein (PI), pure silica gels and silica gels modified with 3-mercaptopropyltrimethoxysilane (MPTMS) are shown. In Fig. 3(a) spectrum, it can be noted the strong peaks at ν = 1620–1680 cm^{-1} and ν = 1480–1580 cm^{-1}, mainly associated with amide-I and amide-II stretching vibration bands of porcine insulin (PI), respectively [16, 17]. The spectrum of pure silica-gels (Fig. 3(b)) clearly reveals the peaks due to Si–O–Si bonds (1080 and 450 cm^{-1}) and Si–OH bonds (3500 and 950 cm^{-1}) [5, 12, 13]. The spectrum in Fig. 3(c) is associated with the silica-gel modified with 1% molar with MPTMS and 1% porcine insulin (PI) incorporated. This spectrum (Fig. 3(c)) shows peaks at 2550 cm^{-1} that are characteristic of the thiol (–SH) and the presence of –CH stretching vibration bands (2850–3000 cm^{-1}) mainly associated with the propyl group introduced by the functionalization of the gel surface with MPTMS [5, 12, 13]. These results have clearly indicated the presence of proteins incorporated in the porous gel matrix. Based on the literature [15], the peptide group, the structural repeat unit of proteins, has 9 characteristic bands named amide (A, B, I, II ... VII). Amide I and amide II bands are two major bands of the protein infrared spectrum. The amide I band (ranging from 1600 to 1700 cm^{-1}) is mainly associated with the C=O stretching vibration (70–85%) and is directly related to the backbone conformation. Amide II results from the N–H bending vibration (40–60%) and from the C–N stretching vibration (18–40%). Therefore, FTIR data shown in Fig. 3 gave strong evidence that the experimental procedure used in this work was successful in altering the chemistry of silica based gels. The presence of thiol (–SH) and other chemical groups 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. Different chemical species well dispersed within a silica matrix yield patterns of chemical activity that can be useful in tailoring the inorganic substrate towards interacting with a specific protein. Similar FTIR spectra were obtained for glass surface modifying groups, GPTMS and TESPI.

3.2. Bioactivity of insulin immobilized gel implants—*in vivo*

We have conducted *in vivo* assay using insulin (PI) incorporated into gel network because of the high

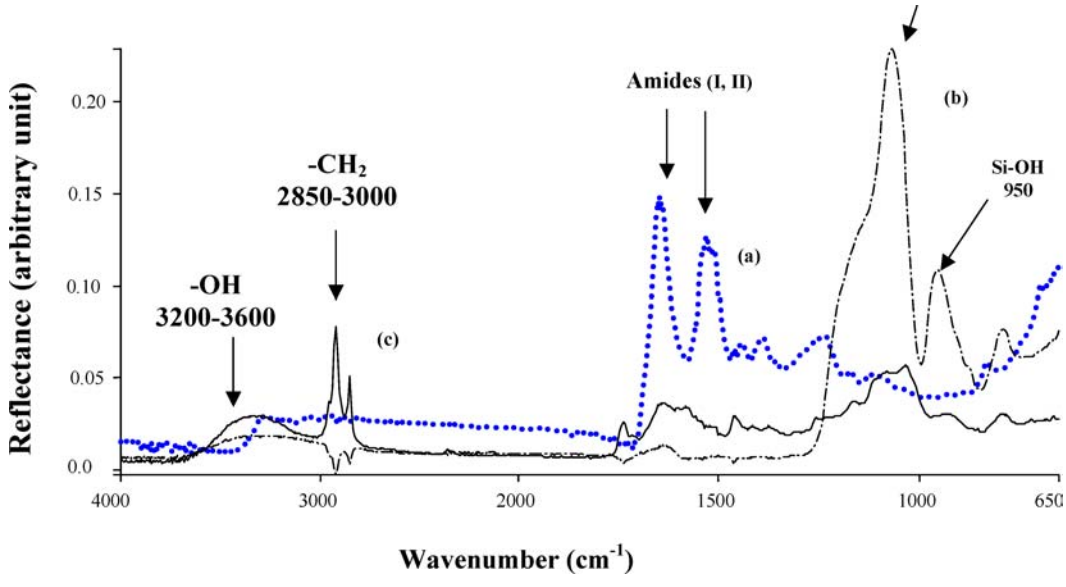

Figure 3 FTIR spectra of porous glass and porous glass with PI incorporated. (a) Reference spectrum of PI (dotted line); (b) pure silica-gel (dashed line); (c) the silica-gel modified with 1% MPTMS and 1% porcine insulin incorporated (solid line).

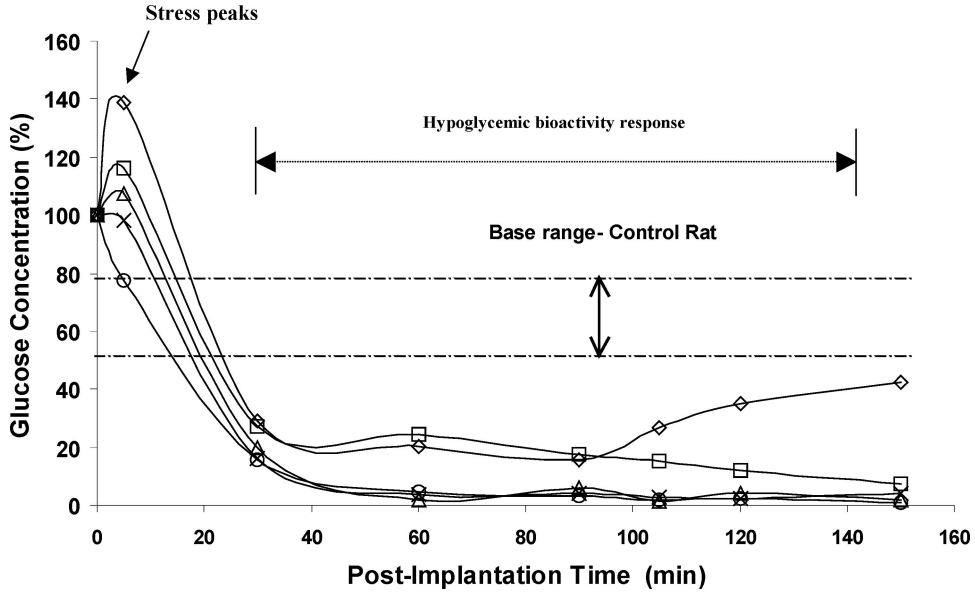

Figure 4 Plasma glucose concentration in response to insulin administration time; 5 different silica-gel surface; Central band represents range of blood glucose level of the control rat with implant of pure SiO_2 glass (without PI).

specificity and sensibility of this characterization procedure. PI was incorporated into sol-gel silica matrices by introducing freshly formed gels into solutions containing the PI. Fig. 4 shows a typical *in vivo* post-implantation curve of the five different functionalized glass substrates with insulin incorporated. The central band represents range of blood glucose level of the control rat with implant of pure SiO_2 glass (without PI). It can be clearly observed the bioactivity of the insulin incorporated in the silica glass disk by decreasing the plasma concentration of glucose compared to the control rat without PI incorporated (central strip). After 90 min. the glucose level is less than 40 μg/ml indicating strong response to the PI implanted. It could be observed hyperglycemic peaks for all surface modified gels on the first 10 min. post-implantation. Such fact is generally caused by the experiment stress over the rats. After that, pure silica-gels and surface modified gel implants have presented a similar trend on blood sugar level, with an acute hypoglycemic effect observed in these implanted animals compared to glucose level of the control group. A significant reduction from about 120 μg/ml to less than 40 μg/ml within 30 min. was verified due to strong insulin response [14]. 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 believe 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. Also, no major effect on the PI response was observed among the surface modified substrates when comparing the blood glucose concentration curves. Based on these results of insulin bioactivity, we had clear evidence that no major denaturation of insulin has occurred associated with the developed protein-gel immobilization procedure.

3.3. Biochemical activity of silica gels—immunoassay (ELISA)

3.3.1. Biochemical activity—immunoassay (ELISA) for IgG-Bov/Anti-IgG-Bov

ELISA immunoassay results for three different gels of silica with surface modified by amino, thiol and hydroxyl chemical groups are showed in Fig. 5. The ELISA assay was carried out following the protocol (Section 2.4.1) with absorbance readings at wavelength of 450 nm, indicating the sensitivity related to the protein detection by each solid-phase used. It can be noted a better response, as far as absorbance is concerned, for the amino-terminated biomaterial surface (Fig. 5(a)). The results presented here, despite the complexity associated with protein-solid interactions, is believed to be due to the higher affinity of the amine-propyl group (APTES) presented by the biomaterial functionalized surface with the carboxyl terminal IgG chemical group (F_c portion) when compared to the other chemical groups, that is thiol (Fig. 5(c)) and hydroxyl (Fig. 5(b)). The specificity of the molecular recognition of antigens by antibodies to form a stable complex is the basis of the immunoassay. Such assumption is based on the fact that all antibodies or immunoglobulins (IgG) exhibit a typical Y-shaped structure made of four chains based on pairs of identical heavy and light chains. IgG is the major immunoglobulin in normal human serum (Fig. 1). These antibodies have two fixation sites that can bind antigens with high level of specificity. The amino terminated site called F_{ab} and the carboxyl terminated group called F_c. Consequently, the amino groups present in the silica-gel modified substrate (APTES), would preferably bind to the carboxyl end (–COOH) found in all immunoglobulin molecules (IgG, F_c) through the formation of amide bonds between the surface amino groups and the carboxylic groups present in the biomolecules [23]. The schematic representation of amino-terminated surface

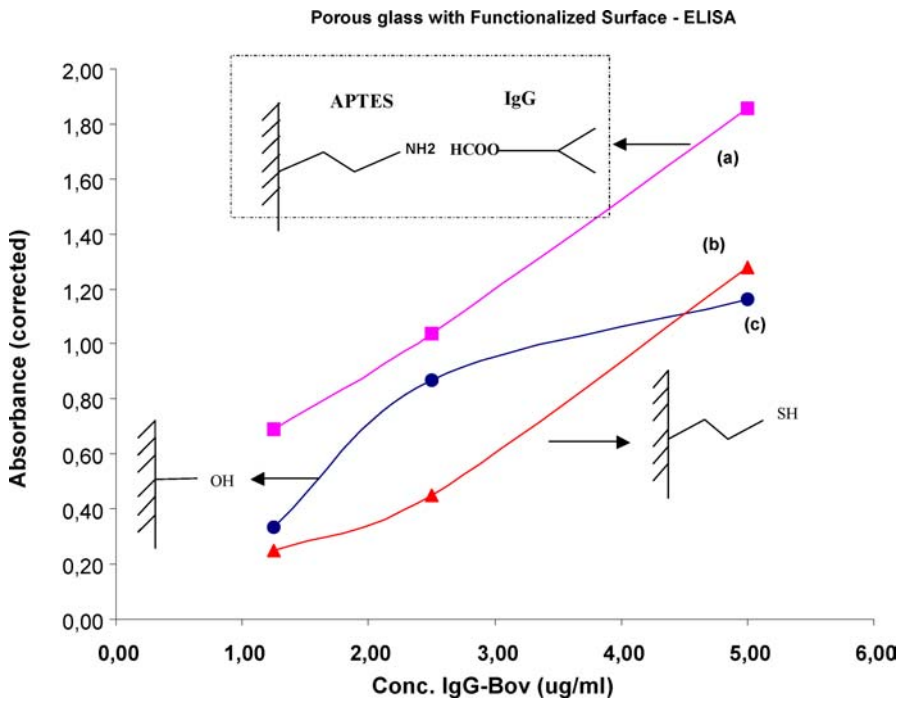

Figure 5 ELISA immunoassay response for 3 different chemically modified silanes; Amine (squares), Thiol (triangle) and Hydroxyl (disks), using IgG-Bov/anti-bov-IgG-HRP, obtained by UV-Vis spectroscopy absorbance at $\lambda = 450$ nm.

interaction with carboxylic site (F_c) of IgG is showed in Fig. 5 (insert).

3.3.2. Biochemical activity—immunoassay (ELISA) for scorpion venom

Tityus serrulatus is the most dangerous scorpion species accounting for fatal stings, especially among children, in Brazil. Approximately 10,000 human cases of scorpion stings are treated at hospital centers and recorded annually. 50% of these cases occur in the state of Minas Gerais, with mortality rates as high as 1.1% [21, 22]. Apart from this important public health problem, scorpion venoms are rich sources of different classes of peptides that represent useful tools for biological research. The ELISA technique has been widely used in clinical and experimental studies for the quantification of venoms and antivenoms [21, 22]. Because of its low cost and easy laboratory execution this biochemical assay has a wide potential application in animal and human health.

ELISA immunoassay results are presented in Fig. 6, for three different gels of silica with surface modified by amino (APTES), thiol (MPTMS) and hydroxyl chemical groups, using the protocol for scorpion venom protein (TsTFG50) [21]. It can be observed on the

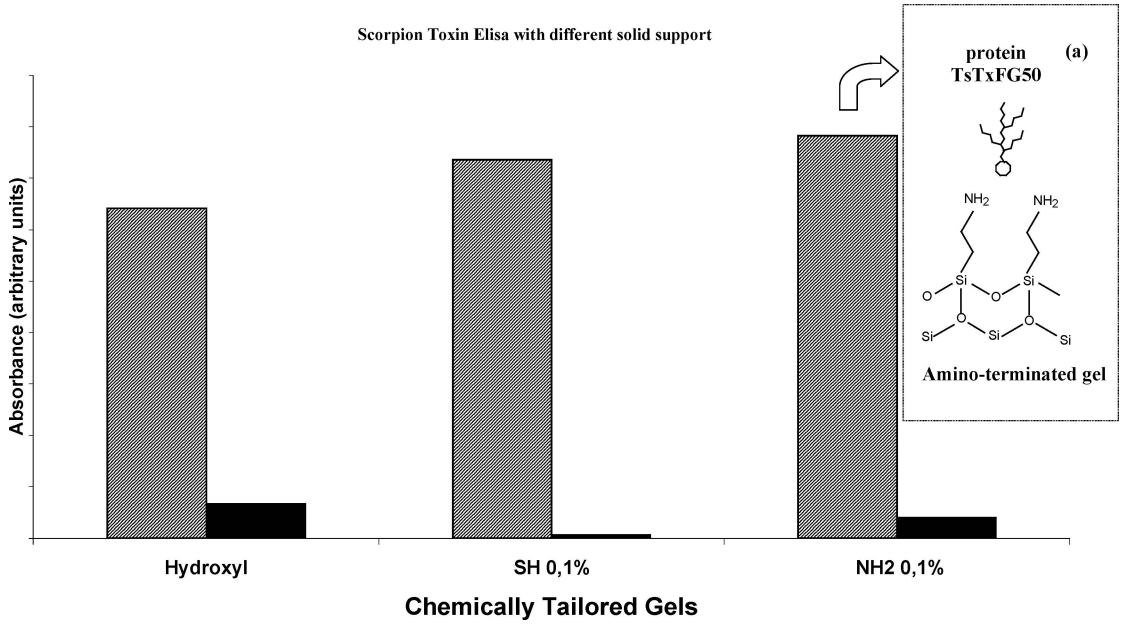

Figure 6 Histogram of ELISA immunoassay in 3 different chemically modified silanes; Amine (■), Thiol (▲) And Hydroxyl (●),using scorpion venom protein system, obtained by UV−Vis spectroscopy absorbance at $\lambda = 452$ nm; gel sample (dashed bar), control (solid bar).

histogram (Fig. 6) that the amine-functionalized surface gel has higher absorbance, therefore higher sensitivity, than the other chemically engineered biomaterials (Fig. 6(a)). These results have endorsed the previously obtained results on ELISA with IgG-Bov system. As mentioned before, protein TsTxFG50 used as antigen, behaved quite similar to the antibody IgG-Bov. Despite of being a smaller protein molecule than IgG-Bov, the origins of the interactions between protein-substrate are found to be the same, such as Coulomb forces, van der Waals forces, Lewis acid-base forces, and more entropically based effects such as hydrophobic interactions, conformational entropy and restricted mobility. It is difficult to determine exactly the extent of these interactions and even more difficult to predict them. At the molecular level, proteins are chemically and physically extremely complex molecules, and the nature of the physical-chemical interactions taking place is implicit widespread [1–3]. However, based on the ELISA results, we may assume that the amino-terminated gel matrix has favored the orientation of protein TsTFG50 (Fig. 6(a)) when compared to other surface modified gels (Fig. 6, thiol and hydroxyl). Despite the vast array of potential interactions that usually exist between proteins and surfaces, a general rule has been formulated to predict the outcome. Proteins will have a higher affinity for a surface with amino groups due to amino-acid strong interactions [23]. This simplified view often works well for well-defined model proteins and surfaces. Nevertheless, for more complex systems where the heterogeneous nature of surfaces complicates interpretation, some other important aspects have to taken into account.

4. Conclusions

FTIR spectroscopy results have shown that the experimental procedure developed in this work was successful in altering the chemistry of silica based gels. The synthesis of the gels with surface modifying groups can satisfactorily be used to produce materials containing specifically designed chemical functionalities.

The bioactivity of incorporated proteins was confirmed through *in vivo* evaluation experiments, where an acute hypoglycemic effect observed in the implanted animals caused by the insulin bioresponse. Also, no major effect on the PI bioactivity difference was observed among the surface modified solid substrates when comparing the blood glucose concentration curves.

The ELISA immunoassays have clearly indicated the activity and stability of proteins, IgG-Bov and TsTFG50, on binding to novel developed solid phases. The sensitivity of the ELISA was enhanced by the surface amine-terminated group when compared to conventional polystyrene plates and other silane functionalized gels.

Acknowledgments

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References

1. V. HLADY and J. BUIJS, *Curr. Opin. Biotechn.* **7** (1996) 72.
2. I. GILL and A. BALLESTEROS, *J. Amer. Chem. Soc.* **120** (1998) 8587.
3. W. JIN and J. D. BRENNAN, *Analy. Chimica Acta* **461** (2002) 1.
4. B. C. DAVE, B. DUNN, J. S. VALENTINE and J. I. ZINK, *Analyt. Chem.* **66** (1994) 1120.
5. H. S. MANSUR, L. J. C. MACHADO, W. L. VASCONCELOS and Z. I. P. LOBATO, *Spectr.-An Intern. J.* **16** (2002) 351.
6. L. M. ELLERBY, C. R. NISHIDA, F. NISHIDA, S. A. DUNN, B. YAMANAKA, J. S. VALENTINE and J. I. ZINK, *Science* **255** (1992) 1113.
7. J. LIVAGE, *Curr. Opin. Colloid State Mater.* **2** (1997) 132.
8. J. LIVAGE, J. Y. BARREAU, J. M. DA COSTA and I. DEPORTES, SPIE, *Sol-Gel Optics III* **2288** (1994) 493.
9. R. ZUSMAN, D. A. BECKMAN, I. ZUSMA and R. BRENT, *Analyt. Biochem.* **201** (1992) 103.
10. B. D. RATNER, A. S. HOFFMAN, F. J. SCHOEN and J. E. LEMONS, in "Biomaterials Science: An Introduction to Materials in Medicine" (Academic Press, New York, 1996).
11. H. S. MANSUR, Z. P. LOBATO, R. OREFICE, W. L. VASCONCELOS and L. J. C. MACHADO, *Biomacromolecules* **1** (2000) 789.
12. H. S. MANSUR, Z. P. LOBATO, R. L. OREFICE, W. L. VASCONCELOS, L. J. C. MACHADO and E. MANSUR, *Adsorption* **7** (2001) 105.
13. K. FLORA and J. D. BRENNAN, *Chem. Mater.* **13** (2001) 4170.
14. N. YAMAGUCHI, *Can J. Physiol. Pharmacol.* **70** (1992) 167.
15. K. K. CHITTUR, *Biomaterials* **19** (1998) 357.
16. B. S. BELTZ and G. D. BURD, "Immunocytochemical Techniques, Principles and Practice" (Blackwell Scientific Publications, Inc. Brookline Village, 1989) p. 26.
17. E. ENGVALL and P. O. PERLMANN, *Immunochemistry* **8** (1971) 871.
18. P. PALOMÄKI, *J. Immunol. Methods* **145** (1991) 55.
19. P. N. RAO, and I. B. TARAPOREWALA, *Steroids* **57** (1992) 154.
20. K. SUZUKI, N. OKAMOTO, S. WATANABE, and T. KANO, *J. Virol. Methods* **38** (1992) 113.
21. C. CHAVEZ-OLÓRTEGUI, C. M. FERREIRA, M. RICHARDSON and C. R. DINIZ, *Toxicon* **32** (1994) 1649.
22. R. A. M. DE ÁVILA, L.M. ALVARENGA and C. CHAVEZ-OLÓRTEGUI, *Toxicon* **44** (2004), 233.
23. W. KUSNEZOW and J. D. HOHEISEL, *J. Mol. Recognit.*, **16** (2003) 165.

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