Albumin-containing sol–gel glasses: Chemical and biological study

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Glasses incorporating increasing amounts of bovine serum albumin were prepared by sol–gel techniques from a tetra methoxy silane precursor. The surface of the glass samples was studied by X-ray photoelectron spectroscopy, revealing that the protein is present also in the superficial layer of the silica network. Moreover, the protein is distributed in a dose-dependent way, since the N/Si atomic ratio increases linearly with the albumin concentration in the reaction mixture. Angle-dependent measurements show that the protein distribution occurs homogeneously and is the same at different sampling depths. Protein incorporation in the bulk SiO_2 network, with a uniform protein distribution between bulk and surface, is confirmed by infrared spectroscopy measurements, performed both in reflectance and transmittance mode. The reaction with a specific antibody and the adhesivity assay of osteoblastic cells show that embedded albumin present on the glass surface is able to interact with other proteins.

Introduction

The study of biomaterials is a complex area of research that needs a multidisciplinary approach. In this field, the interaction among inorganic, organic and biological chemistry is fundamental for the study of inorganic matrices supporting organic and/or biological molecules (e.g. enzymes) that can be used as biosensors, bioreactors or starting materials for biocompatible implants [1].

The sol–gel technique allows encapsulation of biological molecules in silica-based glasses. The embedded proteins retain their bioactivity and are accessible to external small reactants (such as enzyme substrates) by diffusion through the porous silica; up to now, sol–gel glasses containing many types of encapsulated proteins or enzymes have been prepared [2–4]. Thus, sol–gel glasses can be used to prepare bioactive materials, which should find an useful application as biosensors or implanted devices.

The biocompatibility of a material used for medical applications can be modulated by changes in the chemical composition of the material surface, or the material bulk or both. Due to chemical–physical processes (e.g. adsorption of gas molecules, resulting in surface contamination and/or in surface reactions) taking place at the material surface, the surface properties and chemical composition of a material cannot be simply inferred from the bulk properties. Since the interaction between the biomaterial and the environment takes place at the material surface, a complete surface characterisation is fundamental in biomaterials research and development [5, 6]. In previous papers [7, 8], we studied the formation of an apatite layer and the subsequent adsorption of human albumin on the surface of a biologically active glass (i.e. a glass based on CaO–P_2O_5–SiO_2 composition) and, by comparison, on the surface of a soda lime-glass, both prepared by plasma-spray deposition.

In this paper, we present chemical, physical and biological characterisation of glass samples prepared by sol–gel technique and containing increasing quantities of bovine serum albumin (BSA) embedded in the silica network.

For understanding the physics and chemistry taking place at the surface and interfaces of biomaterials, we performed X-ray photoelectron spectroscopy (XPS), a surface technique yielding information on the first 50–100 Å of the sample surface that has been extensively used in the characterisation of biomaterials and bioglasses [9–10]. We also carried out Fourier-transform infrared (FT-IR) spectroscopy, a bulk technique that can be made surface sensitive (sampling depth few micrometers) by use of reflectance-grazing angle accessories. Finally, we addressed the surface accessibility of

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embedded protein, by reaction with a specific antibody and by adhesivity assay of osteoblastic cells.

Materials and methods

Chemicals

BSA was purchased from Sigma (St. Louis, MO, USA), tetramethoxysilane (TMOS), HCl and Tris (hydroxymethyl)-aminomethane (Tris) were purchased from Fluka (Buchs, Switzerland). All the other reagents, unless otherwise indicated, were from Sigma Chemical.

Sample preparation

Glass samples were prepared by sol–gel technique, i.e. by hydrolysis reaction from TMOS precursor according to the reaction:

$$Si(OCH_3)_4 + 2H_2O \rightarrow SiO_2 + 4CH_3OH$$

BSA was embedded using the sol–gel procedure of Ellerby *et al*. [11], with minor modifications [2]. The sol–gel stock solution was prepared at 4 °C by mixing 5.3 ml TMOS, 1.15 ml distilled water and 0.07 ml of 0.04 M HCl and stirring until the sol phase was formed. At this time 1.8 ml of H_2O (sample A) or 1.8 ml Tris–HCl buffer (sample B) or 1.8 ml of 3.3 mg/ml (sample C) or 8.3 mg/ml (sample D) or 16.7 mg/ml (sample E) of BSA was added to 1.2 ml of the sol stock solution (final concentrations: 2, 5, 10 mg/ml).

The samples were aged at room temperature and ambient pressure for 14 days, during which time the gelification process and the formation of the silica network took place. Samples were obtained in the form of 1 mm thick glass discs, having a diameter of 1.45 cm. After that, samples were dried in vacuum (10^{-1} torr) before introduction in the XPS spectrometer.

XPS measurements

XPS analysis of the glass samples containing embedded BSA (2, 5, 10 mg/ml, samples C–E) and of the reference glasses without BSA (samples A and B) were performed directly on the glass discs in an instrument of our own construction and design. The spectra of pristine BSA, deposited as thin powder films onto polished stainless steel substrates, were also recorded for comparison.

The instrument consists of preparation and analysis chamber separated by a gate valve, a sample transfer and a multi sample parking systems, plus the possibility for sputtering, heating and cooling and a manipulator having six degrees of freedom. In a typical experiment, samples were first introduced in the preparation chamber and left outgassing overnight at a base pressure of about 10^{-8} torr, before introduction in the analysis chamber. Typical vacuum in the analysis chamber during the measurements was in the 10^{-9}–10^{-10} torr range.

The analysis chamber is equipped with a 150 mm mean radius hemispherical electron analyser with a five lens input system combined with a 16-channel detector. Al K α non-monochromatised X-ray radiation ($h\nu = 1486.6$ eV) was used during the measurements; the resolution is 1.0 eV as measured for the $Ag3d_{5/2}$ line. Spectra were recorded at normal (90°) and grazing (40°) emission take off angles; a higher surface-sensitivity is expected at grazing emission.

Data derive from the measurements carried out on at least three different samples for all the investigated materials; values reproducibility is within $\pm$ 0.2 eV. Si2p, O1s and C1s core-level spectra were recorded for sample A, Si2p, O1s, C1s and N1s spectra for samples B–E; the spectra were calibrated in energy with respect to the Si2p signal taken at a binding energy (BE) of 103.3 eV, as expected for glassy silica [12]. C1s, N1s, O1s and S2p spectra were recorded for BSA; in this case, the spectra were energy referenced to the N1s signal of the peptide nitrogen located at 400.0 eV BE, according to other literature reports [12–14]. Atomic ratios were calculated from peak intensities by using Scofield's cross-section values [15] and by experimentally determined sensitivity factors. The C1s spectra of BSA and of samples C–E were analysed by curve-fitting analysis using gaussian curves as fitting functions.

IR measurements

IR spectra were run on a VECTOR 22 (Bruker) FT-IR interferometer operating in the wavenumber range 400–4000 cm^{-1} equipped with a DTGS detector. Transmittance/absorbance spectra were recorded from KBr pressed pellets: glass samples were thinly grinded (approximate particle size in the 10^{-2} mm range) and the glass powder was mixed with KBr; the powders were then pressed into pellets (1 mm thick, diameter 13 mm) under vacuum (10^{-1} torr) by means of a Silfradent hydraulic press equipped with a Specac 13 mm evacuable pellet dye. Reflectance IR spectra measurements were carried out directly on the glass discs (see sample preparation) by means of a Specac P/N 19650 series monolayer/grazing angle accessory, operating at 50° reflection angle.

Cell cultures

The osteoblastic SAOS cell line was grown in a 1 : 1 mixture of minimal essential medium and Ham's F-12 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; HyClone, Oud-Beijerland, Holland), 1.2 g/l sodium bicarbonate, 1% (v/v) non-essential amino acids and 15 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) at 37 °C with 5% CO_2 in a humidified atmosphere. No antibiotics were used.

ELISA assay

The presence of serum albumin onto the disc surface was determined by ELISA. Glasses containing increasing amounts of embedded BSA were synthesised in 96-wells plates, as described above. As control, different concentrations of BSA (1, 2, 6 and 10 µg/ml) were linked to plastic surface, by using 50 mM carbonate buffer pH 9.6, as coating buffer, and incubating samples at 4 °C for 16 h.

Samples containing the protein either embedded into glasses or linked to plastic surface were incubated in

blocking buffer composed of 10% chicken serum in phosphate buffered saline (PBS; ICN Biomedicals, Aurora, OH, USA) at room temperature for 1 h. Then wells were washed four times in washing buffer (0.1% Tween-20 in PBS) and incubated with anti-BSA, horseradish peroxidase (HRP)-conjugated antibody (ICN Biomedicals), diluted 1 : 6000, at room temperature for 1 h. After four washes with 0.1% Tween 20 in PBS, samples were incubated with 0.5% *o*-phenylenediamine (OPD); ICN Biomedicals) in 1 M citrate buffer pH 4.5 and 8.3 mM H_2O_2 for 5 min. Finally, the reaction was stopped by addition of 2.5 M H_2SO_4 and the absorbance was monitored at 490 nm. The obtained data are means ± standard error (SE) of three independent experiments, each one performed in duplicate.

Adhesivity assay

SAOS cells were employed to evaluate the adhesivity onto glasses. Glass discs were synthesised as described above; at the end of the gelification process, the disc diameter matched the 24-wells plates diameter (14.5 mm). Thus, glasses were put in 24-wells plates; cells were plated at 3×10^4 density on empty or glass-containing plates and incubated at 37 °C to allow adhesion to the support. During incubation, the adhesion was monitored with an optical microscope: after 5 h, all cells were attached to the plastic support (control cells). The medium was then removed and cells were lysed by incubating them on ice for 20 min with 1% Triton X-100. In order to ensure a complete extraction, glasses were washed two more times with 1% Triton X-100 and all washes were collected together. To remove Triton X-100, the DNA in the cell extracts was precipitated overnight at − 20 °C in 95% ethanol; then, DNA was resuspended in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) buffer and absorbance was measured at 260 nm. The reported data are means ± SE of three independent experiments, each one performed in duplicate.

Results and discussion

XPS results

XPS measurements were carried out on the sol–gel glass disc samples without Tris buffer (A), with Tris buffer (B), with increasing quantities of embedded BSA (samples C–E) and, by comparison on pure BSA; results are collected in Table I.

For all the samples, the BE of the O1s signal is in agreement with the literature value [12]; the O/Si atomic ratio is 2 for samples A–B, as expected for silica. A weak carbon contamination is detected on the surfaces of the glasses without albumin (A and B), evidenced by the C1s signal occurring at a BE typical of hydrocarbons. For sample B, a weak and broad N1s signal due to the Tris buffer is detected at 400.5 eV BE, resulting from superimposition of peaks corresponding to ammine-type (∼ 399 eV) and ammonium-type (401–402 eV) nitrogens [12].

The BEs of the C1s, N1s, O1s and S2p signals of pure BSA are consistent with literature values [13, 14]; the atomic ratios are in agreement with the expected values as well, as it is shown in Table I. The C1s signal appears considerably broad (full width at half maximum FWHM = 4.4 eV) and is built up by several contributions from carbon atoms in different chemical environments. The curve-fitting analysis of the C1s spectrum of pure albumin is shown in Fig. 1 (bottom curve). The overall C1s spectrum results from three major contributions, labeled a, b and c in the figure: the main peak at 285.0 eV (a) corresponds to aliphatic carbons of the amino acid pending groups R; peak b at 286.5 eV corresponds to NH–CHR–CO carbons of the protein backbone, peak c at 288.0 eV is assigned to –CO–NH–peptidic carbons

TABLE I XPS analysis of the investigated samples (A–E) and of pure albumin (BSA)

Sample	Signal	BE (eV)	FWHM (eV)	Atomic ratios (calculated)	Atomic ratios (expected)
Albumin (BSA)	C1s	285.0	4.4	C/N = 4.0	C/N = 3.68
	N1s	400.0	2.7		
	O1s	531.4	3.0	O/N = 1.18	O/N = 1.14
	S2p	163.4	4.5	S/N = 0.06	S/N = 0.05
Sol–gel glass (sample A)	Si2p	103.3	2.8		
	O1s	532.8	2.8	O/Si = 2.0	O/Si = 2.0
	C1s	284.6	2.5	C/Si = 0.4	
Sol–gel glass + Tris buffer (sample B)	Si2p	103.3	2.6		
	O1s	532.8	2.4	O/Si = 1.9	O/Si = 2.0
	C1s	284.6	2.7	C/Si = 0.45	
	N1s	400.2	3.3	N/Si = 0.011	
Sol–gel glass + Tris buffer + 2 mg/ml BSA (sample C)	Si2p	103.3	2.5		
	O1s	532.7	2.6	O/Si = 2.05	O/Si = 2.0
	C1s	285.1	4.8	C/N = 4.8	C/N = 3.68
	N1s	400.0	2.6	N/Si = 0.06	
Sol–gel glass + Tris buffer + 5 mg/ml BSA (sample D)	Si2p	103.3	2.5		
	O1s	532.8	2.5	O/Si = 2.1	O/Si = 2.0
	C1s	285.2	4.8	C/N = 4.5	C/N = 3.68
	N1s	400.1	2.5	N/Si = 0.15	
Sol–gel glass + Tris buffer + 10 mg/ml BSA (sample E)	Si2p	103.3	2.5		
	O1s	532.8	2.5	O/Si = 2.1	O/Si = 2.0
	C1s	285.2	4.6	C/N = 4.4	C/N = 3.68
	N1s	400.1	2.5	N/Si = 0.22	

BE = binding energy; FWHM = full width at half maximum.

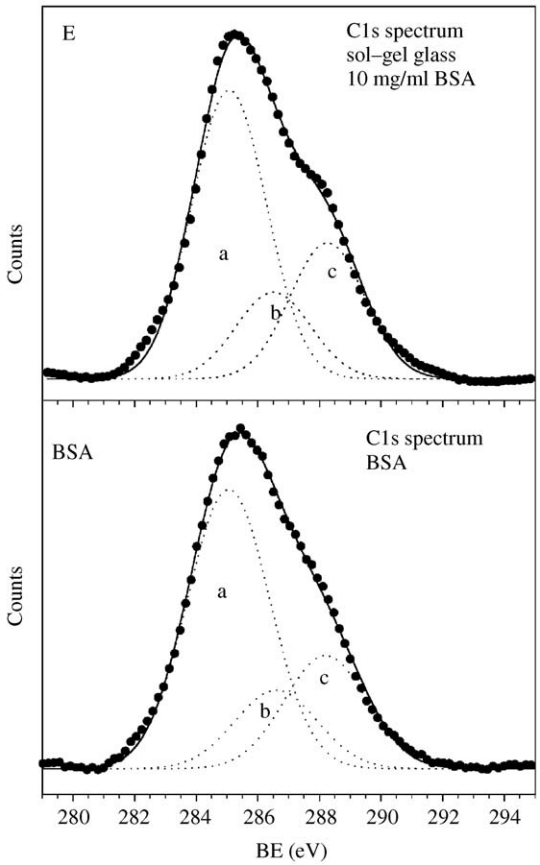

Figure 1 Curve-fitting analysis of the C1s of pure BSA (bottom spectrum) and of the sol–gel glass prepared from reaction mixture containing 10 mg/ml BSA (E, top spectrum); dots represent the experimental points, continuous lines the calculated spectra, dashed lines the fitting components.

[13, 14]. The pending groups of some aminoacids of BSA contain also hydroxyl, amino or carboxylic groups that contribute to the intensity of peaks b and c.

For samples C–E containing BSA incorporated in the silica network, the C1s and N1s signal are found approximately at the same BE as for pure BSA. The C/N ratio is in fairly good agreement (particularly for samples B and C having a higher BSA content) with the value found for pure BSA, revealing a low carbon contamination of these samples. The C1s spectra of samples C–E are extremely similar to the spectra of pure BSA (and show the same large FWHM); curve-fitting analysis of the C1s spectrum of sample E (10 mg/ml BSA content) is shown in Fig. 1 (top curve). Therefore, XPS data confirm that, as expected, the protein structure is not modified upon encapsulation.

The N/Si ratio increases, as expected, with the albumin concentration in the reaction mixture; a plot of the N/Si atomic ratio as a function of the albumin concentration is shown in Fig. 2. The N/Si ratio measured at 0 mg/ml BSA content is due to the N1s signal of the Tris buffer. The relationship between the N/Si ratio on the material surface and the albumin concentration in the reaction mixture is approximately linear, with a slight saturation effect at 10 mg/ml concentration.

Angular dependent XPS measurements have also been performed in order to achieve information on the in-depth distribution of the embedded BSA. Notice that the C/N, N/Si, O/Si atomic ratios shown in Table I are independent on the emission angle of the photoemitted

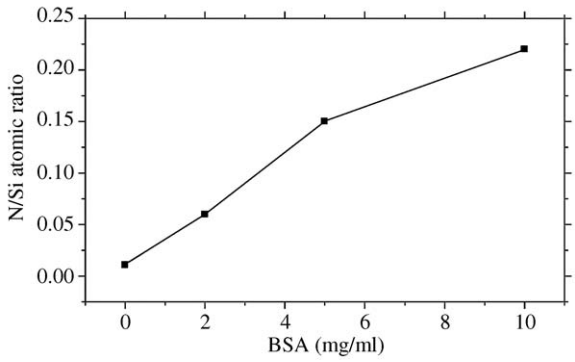

Figure 2 Plot of the N/Si atomic ratio in the sol–gel glasses as a function of BSA concentration in the reaction mixture.

electrons, since these measurements have been performed in the same geometry (90° take-off). Angular measurements were performed at normal (90°) and grazing (40°) take-off angles, corresponding to a sampling depth (SD) of approximately 100 and 50 Å respectively. The sampling depth is related to the inelastic mean free path (λ) of the photoemitted electrons travelling through the material; 95% of the measured signal intensity is produced by a surface layer of a thickness corresponding to 3λ. Therefore, at normal emission the sampling depth can be calculated by the equation $SD = 3\lambda$; for grazing emission, the take-off angle α must be taken into account and the sampling depth becomes to $SD = 3\lambda \operatorname{sen} \alpha$.

According to Seah and Dench [16], the inelastic mean free path can be calculated from the equation:

$$\lambda = B \cdot \mathrm{KE}^{1/2}$$

where KE is the kinetic energy of the photoemitted electrons ($\mathrm{KE} = h\nu - \mathrm{BE}$), and B depends on the investigated material; for inorganic materials $B = 0.096\,\mathrm{nm\,(eV)}^{-1/2}$.

The calculated sampling depths for the analysed core-level electrons, shown in Table II, are in the range 90–107 Å at normal take-off angle, while in the range 46–55 Å at grazing take-off angle. We can, therefore, assume a uniform concentration of albumin in the silica network within the sampling ranges under investigation.

Infrared results

The infrared spectra (absorbance, transmission mode from KBr pressed pellets) of pure BSA, of glass B (with Tris buffer, without BSA), D and E (BSA concentration 5 and 10 mg/ml, respectively) in the wave number range 2000–400 cm^{-1} are shown in Fig. 3.

In the spectrum of pure BSA (bottom curve), the most intense peaks are the amide-I band (C=O stretching vibration) localised at 1650 cm^{-1}, and the amide-II band (N–H bending vibration) at 1540 cm^{-1}. The main peaks in the spectrum of glass B, localised at 1083 and 463 cm^{-1}, are related respectively to stretching and bending vibration modes from Si–O–Si bridging oxygens [17–19]. Other small peaks are found at 944 and 798 cm^{-1}; bands in the 950–750 cm^{-1} range in the spectra of glasses have been usually attributed to Si–O vibrational modes of non-bridging oxygens [17–19]; in our case, the most likely hypothesis is terminal Si–OH.

TABLE II Calculated sampling depths for the analysed core-level signals at normal and grazing emission

Signal	BE (eV)	KE (eV)	λ (nm)	Sampling depth (nm)	
				Normal (3λ)	Grazing (3λ sen α)
C1s	285	1202	3.32	9.96	6.60
N1s	400	1087	3.15	9.45	6.05
O1s	533	954	2.96	8.88	5.67
Si2p	103	1384	3.56	10.68	6.83

The peak at 1645 cm^{-1} can be related to residual H_2O (symmetric bending) in the bulk of the sol–gel glasses; an O–H stretching band is also observed at 3480 cm^{-1} (data not shown).

The IR spectra of samples D and E (glasses containing BSA) closely resemble the spectrum of sample B (glass sample not containing BSA), the only difference being the appearance at about 1540 cm^{-1} of the amide-II band (the amide-I band unfortunately is superimposed to the H_2O bending band, present also in glass B). The band appears obviously more intense in the spectrum of sample E (10 mg/ml) than for sample D (5 mg/ml).

IR spectra were recorded also in reflectance mode, by means of a grazing angle accessory; transmittance IR spectroscopy is a bulk analysis technique, while reflectance IR spectroscopy yields information on the first few μm of the sample surface.

Reflectance IR spectra recorded on the pristine glass discs in the wave number range 1400–1800 cm^{-1} (the spectral region of the amide-I and amide-II bands) for glass samples B, D and E are shown in Fig. 4. The intensity increase in the amide-II band at 1540 cm^{-1} as a function of BSA concentration is evident also in the reflectance IR spectra. The comparison between the IR spectra recorded in reflectance and transmittance mode suggests a uniform distribution of BSA concentration between the bulk and the surface of the glass sample, supporting the already discussed XPS results.

Biological results

In order to assess from a biological point of view if the protein is accessible on the glass surface or embedded in the bulk, an ELISA assay was carried out. To this end, glasses synthesised into 96-wells plates were reacted with a specific antibody raised against BSA.

Although the antibody could adhere to empty glasses in a non-specific manner, nonetheless the immunoglobulin specifically reacted with BSA, thus indicating that the protein is accessible on the surface. Indeed, as shown in Fig. 5(a), BSA-containing glass discs showed a two-fold increase in absorbance values over empty glasses. The antigen–antibody interaction appeared to be complete, as no significant differences were seen with increasing (2–10 mg/ml) BSA concentrations; this could indicate that all the freely accessible sites on the surface layer were already covered at 2 mg/ml BSA. The saturation of reaction was further confirmed by control experiments, where increasing amounts (1–20 μg/ml) of BSA have been directly linked to the plastic surface of 96-wells plates. In this case, the binding curve showed a clear dose-dependent trend reaching a plateau with 10 μg/ml BSA solution (data not shown).

To evaluate if the embedded protein exerted some biological effects, we investigated the ability of osteoblasts to adhere to glasses. SAOS cells (3×10^4) were plated on either plastic or glass surface and allowed to adhere for 5 h; as index of cell number, we extracted and quantified DNA from adherent cells. As shown in Fig. 5(b), glasses allowed cell adhesion, although plastic

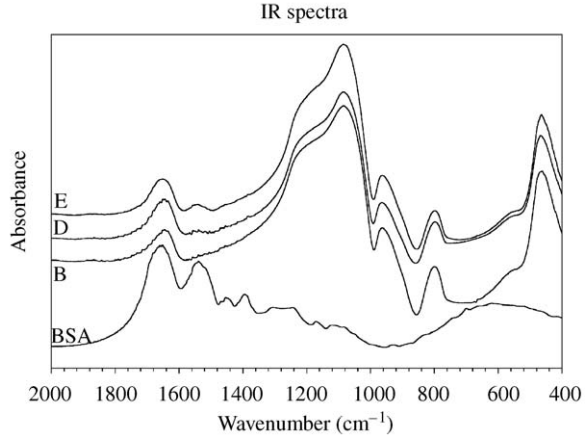

Figure 3 FT-IR spectra (transmission from KBr pellets) of pure BSA, sol–gel glass with Tris buffer and without albumin (B), sol–gel glass with 5 mg/ml BSA (D), sol–gel glass with 10 mg/ml BSA (E).

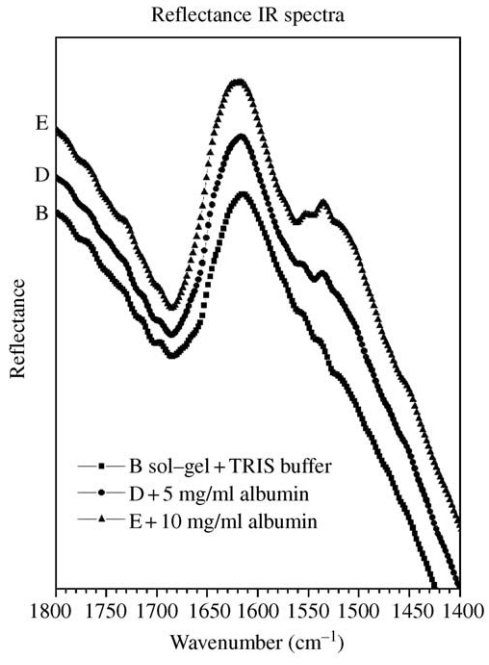

Figure 4 Reflectance FT-IR spectra (expansion of the 1400–1800 cm^{-1} region) of sol–gel glass with Tris buffer and without BSA (B), sol–gel glass with 5 mg/ml BSA (D), sol–gel glass with 10 mg/ml BSA (E).

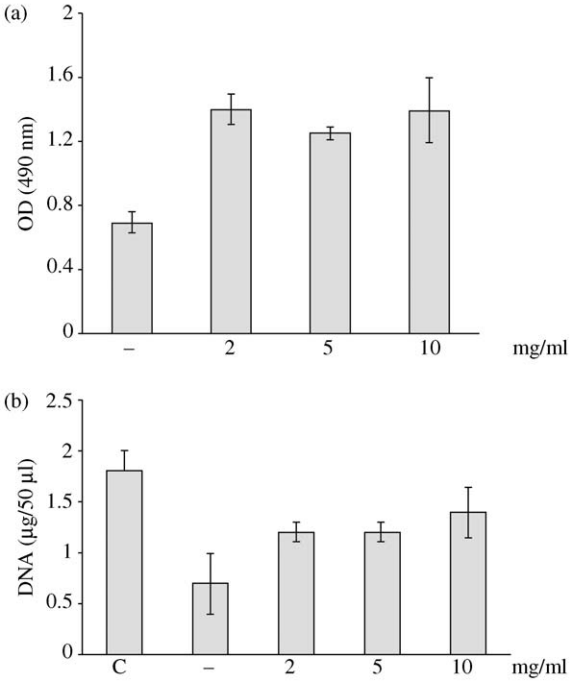

Figure 5 Biological evaluation of embedded BSA. (a) ELISA assay for presence of BSA on the glass surface. Glasses with (2, 5, 10 mg/ml) or without (–) the protein were incubated with anti-BSA, HRP-conjugated antibody for 1 h. (b) Adhesivity assay of SAOS cells. Cells were plated on either plastic or glasses and incubated at 37 °C for 5 h.

remained the best support for growth (compare ''C'' that stands for control and ''–'' that corresponds to 0 mg/ml BSA bars). It is noteworthy that embedded BSA exerted a positive effect on adhesion: indeed protein-containing glasses showed a little increase in the amount of DNA recovered after incubation. This may be due to non-specific interactions between albumin and membrane proteins.

Conclusions

In this paper, we investigated the chemical and biological surface properties of glass samples containing increasing amounts of embedded BSA.

Chemical analysis of the sol–gel glasses carried out by means of surface-sensitive (XPS) and bulk (FT-IR) analysis techniques indicates that the protein is homogeneously distributed in the silica network retaining, as expected, its chemical structure. Moreover, the surface concentration of BSA increases linearly with the protein dose added to the reaction mixture during the sol–gel process. In addition, BSA seems to be exposed to the glass surface with a correct folding, as a commercial antibody can recognise the specific epitope on the molecule. Finally, BSA-containing glasses appear to be a valid support for cell growth.

It is well demonstrated that chemical composition (i.e. enrichment in calcium phosphate) influences the bioactive behaviour of glass. Here, we demonstrated that embedded proteins can also modulate biological responses to glass. Thus, it should be interesting to go further insight by using phospho-silicate glasses containing specific adhesion molecules, in order to improve the material biocompatibility allowing medical applications.

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