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Enhanced apatite precipitation on a biopolymer-coated bioactive glass

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Abstract: In this work, sintered pellets of a silica-based bioactive glass were dip-coated with a biocompatible natural-derived polymer in order to investigate the influence of the organic coating on the glass bioactivity. After the sintering process optimization, uncoated and coated pellets have been characterized by means of scanning electron microscopy with energy dispersive spectroscopy (SEM, EDS), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and pH measurements, after the immersion in a simulated body fluid (SBF). An increased apatite forming ability and a better control of the pH during soaking of the samples in SBF were observed in the presence of the biopolymer. This result opens a new insight on the simple fabrication of highly bioactive hybrid inorganic-organic materials for medical applications.

Keywords: Bioactive glass; Bioactivity; Melanin coating

1 Introduction

The characteristic lack of interaction between metallic/ceramic implants and the bone often guides to a poor adhesion of the prosthesis, resulting in interfacial displacements or, in most of the cases, in clinical failure [1]. Owing to their excellent biocompatibility and remarkable ability to interact with bone and promote bone repair, bioactive glasses have been regarded as promising materials for bone replacement [2–4]. The integration of these materials with bone is mediated by the formation of an apatite layer on their surface, in contact with body fluids. This apatite layer has high chemical similarity to the mineral constituent of bone and promotes a firm bond with living bone. This layer is usually formed by a complex ion-exchange mechanism between the glass surface and the surrounding fluids [5].

A wide range of bioactive glasses compositions has been investigated and some them reached commercial diffusion. For example, among them, apatite-wollastonite (A/W) glass-ceramics have been extensively used in oral, maxillofacial and orthopedic applications [6]. A good example is the commercial glass-ceramic Cerabone®, which combines high mechanical strength and fracture toughness, mostly provided by its apatite-wollastonite crystalline phase. This glass-ceramic presents good mechanical properties and a moderate bioactivity index, which may be resultant from its glass-ceramic nature [3], so this material is suggested for clinical applications where a long-term stability is preferred to a very fast dissolution rate. The influence of crystallization on the bioactivity have been already investigated for various glass compositions [7].

Hybrid inorganic-organic materials have been proposed as an alternative (or a complement) to high temperature treatments, by combining the toughness of an organic phase (usually a biodegradable polymer) with the stiffness of an inorganic phase (glass/glass-ceramics, other bioceramics) [8]. However, most biodegradable polymers lack a bioactive function, not allowing bone tissue apposition of bonding on the polymer surface. Furthermore, the release of acidic degradation products often leads to undesired inflammatory responses [9].

The coating approach, to obtain hybrid inorganic-organic materials, has been also investigated. Several attempts to coat a bioceramic substrate with biodegradable polymers, such as Poly(3-hydroxybutyrate) (P(3HB)), Poly(DL-lacticde) (PDLLA) or poly(ε-caprolactone) (PCL) often resulted in a decay of substrate's bioactivity, arising from the decrease of ionic exchanges between the bioac-

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tive ceramic and the surrounding fluids [10–12]. Recently, a coating of genipin cross-linked gelatin on 45S5 Bioglass® scaffolds has been proposed [13]; this coating improved the mechanical properties but caused a delay in the *in vitro* bioactivity. Other recent studies included the use of cellulose nanowiskers or Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) coatings on 45S5 Bioglass® scaffolds, but in both cases the formation of calcium-phosphate crystals was mainly observed on the uncoated zones of the scaffolds [14, 15]. Usually, the strategy involves the substrate coating with a hybrid organic-inorganic material composed by a natural derived or synthetic polymer and a bioactive ceramic or, in most of the cases, by infiltration of the polymer into the substrate [16, 17]. These processes often involve vacuum conditions, complex procedures and are often expensive. In these kinds of hybrid-organic inorganic materials, apart from the control over the degradation rate of the organic material, highly demanded properties, such as tunable drug delivery ability, may also be achieved [18, 19].

Aiming to investigate the role of surface modifications of bioactive glasses with biopolymers, in this work a new hybrid organic-inorganic material, composed by a bioactive glass (AP40) coated with a natural derived polymer (melanin), was developed. AP40 is a well-known bioactive glass, based on a similar oxide system as the commercial Cerabone®, whose bioactivity has been already investigated in literature [20, 21]. The use of natural derived polymers typically presents common advantages such as availability, biocompatibility, biodegradability and low toxicity [22]. Melanin is a natural-derived polymer resultant from the oxidative polymerization of phenolic and/or indolic compounds [23]. Besides its wide abundance in nature, proven biocompatibility and absence of cytotoxicity, this biopolymer presents attractive properties such as photo-protection and heat storage capacity, antioxidant behavior and tunable drug delivery ability [24–26]. Moreover, the chelating ability of melanin and its structural characteristics can be exploited as preferential site for the nucleation and growth of hydroxyapatite. In fact, the ability of the polymer to accelerate the first steps of bioactivity process was verified in a previous paper [27]; in this paper a coating of melanin was applied on 45S5 (Bioglass®) glass pellets using a melanin concentration of 0,15 and 1,5 (%w/v) and it was observed an increasing of the kinetics of bioactivity. In the present paper, the influence of the melanin coating on the bioactivity was estimated using a different glass composition (AP40 sintered pellets) and the reproducibility of the coating process was investigated.

2 Methods

2.1 Glass synthesis

Technical grade powders of reactants were weighted and mixed in the required proportions to achieve a glass with nominal composition: (wt%) 44.30% SiO_2, 24.50% β-$Ca_3(PO_4)_2$, 18.60% CaO, 0.19 K_2O, 4.60 Na_2O, 2.82 MgO, 4.99 CaF_2 (AP40), herein named B1. The glass was synthesized by melting the reactants in a platinum crucible at 1400°C for 2 h (Nannetti electric furnace) and quenching the melt in water, obtaining a frit. The glass-frit was subsequently wet-milled in a high speed porcelain mill, using water and Al_2O_3 spheres, dried and sieved to obtain fine glass powders with grain size < 64 μm.

2.2 Thermal characterization and sintering of glass pellets

Simultaneous differential scanning calorimetry thermogravimetric analysis (DSC-TG) of the glass was recorded in a Netzsch STA 449C in air atmosphere using Pt-Rh crucibles to determine its characteristic temperatures. For this experiment, 50 mg of the glass powders were weighted and heated from room temperature to 1350°C ($\beta = 3°\text{Cmin}^{-1}$).

The sintering behaviour of the glass was studied on 3 mm height cylinders of green compacted powders, prepared by cold pressing. The samples, placed on an alumina support, were subsequently heated in a hot stage microscope (side-view HSM Misura equipped with an image analysis system and an electrical furnace 1750/15 Leica) up to 1350°C ($\beta = 10°\text{C min}^{-1}$) and their linear shrinkage was determined by analysing the images (i.e. by measuring the samples height) recorded every 10°C, from 400°C to 1350°C. The temperature was measured with a Pt/Rh (6/30) thermocouple in contact with the alumina support.

Cylindrical glass pellets of 1 cm in diameter and 0.5 cm height have been prepared by viscous flow sintering. The green compacts were prepared by cold pressing of 750 mg of glass powders (< 63 μm) at 9 bars for 30 seconds. On the basis of DSC/HSM studies, the green compacts were then sintered at 710°C for 20 minutes ($\beta = 20°\text{ C min}^{-1}$) and subsequently washed for 5 minutes in acetone under sonication before being used.

2.3 Dip-coating of sintered glass pellets

The biopolymer (melanin) was extracted from *Sepia Officinalis* and purified following a previously reported pro-

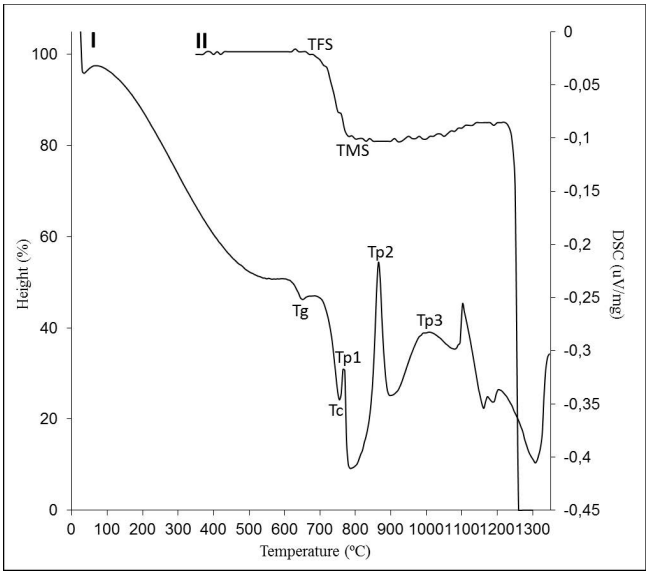

Figure 1: DSC (I) and HSM (II) curves of B1 (A) A – Tg: glass transition temperature; Tc: onset of crystallization; Tp: Maximum temperature of crystallization.

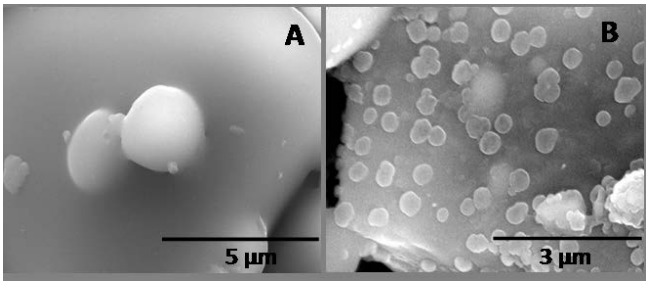

Figure 2: SEM analysis of bioactive glass B1 uncoated (A) and coated with 0.15 (%m/v) polymer suspension (B). Magnifications are 10000 × (A) and 18000 × (B).

cedure [26]. After several washings and centrifugations, the polymer was suspended in water-propylene glycol and dried using spray drying (Buchi, B-290). Cylindrical B1 sintered pellets were dip-coated into a melanin suspensions (water/monopropylene glycol) with concentration of 0.15 (% w/v). The coating procedure was repeated three times.

2.4 Soaking in SBF

The apatite forming ability of the coated (B1C) and uncoated (B1U) glass pellets were investigated by soaking them in 30 mL of simulated body fluid (SBF) at 37°C, pH = 7.4. The SBF had an ionic concentration nearly equivalent to human plasma (Na^+ 142.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.5, Cl^- 148.8, HPO_4^- 1.0, HCO_3^{2-} 4.2, SO_4^{2-} 0.5 mmol l^{-1}) [28] and was filtered through sterilized filters (cameo 25 AS-MSI, pore size 0,22 µm) before pellets immersion. B1C and B1U pellets soaked in SBF were sealed into sterilized polyurethane containers and placed in an oven at 37°C (± 0.5°C) for 1, 3, 7, 14 and 21 days. The experiments were performed in triplicate to assure the accuracy of results. Changes occurring in the pH of the solutions were also recorded. After each time point, the glass pellets were extracted from SBF, rinsed extensively with water and dried at 100°C for 1 h.

2.5 Samples characterization after soaking in SBF

Surface modifications occurred on the glass samples during soaking in SBF were investigated by Fourier transform infrared spectroscopy (FTIR). For FTIR analyses, the pellets surface (coated and uncoated) was scratched and the obtained powders were mixed with KBr; compressed pellets were prepared by weighing 200 mg of powders (KBr and 2% of scratched powders) and by pressing them at 5 tons for 10 seconds in an automatic press (Graseby-Specac T-40). The FTIR spectra were acquired in a Hyperion 2000 FTIR (Tensor 27, Bruker S.p.A) from 4000 to 400 cm^{-1} and with 2 cm^{-1} resolution.

The amorphous/crystalline nature of the glass surface, as well as qualitative analysis of crystalline phases, were determined by X-ray diffraction (XRD) analysis using a conventional Bragg-Brentano diffractometer (X'Pert Philips) with Cu-K_α radiation. Data were recorded in 2θ angle range 3°–90° (step size 0.02° and 19.7 seconds of counting time for each step) and the pattern analysis was carried out using X'Pert High Score software and the PCPDF data bank.

Morphological and compositional modifications occurring on the surface of the pellets were investigated by scanning electron microscopy (SEM, model Philips XL20) with energy dispersive spectroscopy (EDS, model EDAX PV9900). The pictures were acquired at 15 kV and using a work distance of 10 mm, and the EDS analysis performed at a work distance of 37 mm. The analysis were performed on Au-sputtered samples.

3 Results

The thermal behaviour of glass B1 was studied by DSC-TG and HSM, as shown in Fig. 1. The glass exhibits three main crystallization peaks and a shrinkage beginning at about

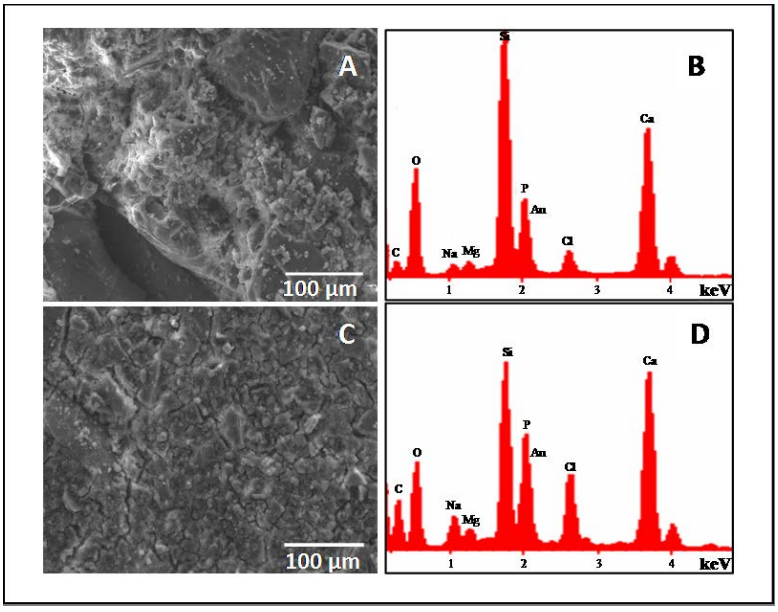

Figure 3: SEM analysis bioactive glass B1 uncoated (A) and coated with 0.15 (%m/v) polymer suspension (B) after 1 day soaking in SBF solution. Magnifications are 500 ×. EDS spectra are resultant from a general analysis of the images A and C.

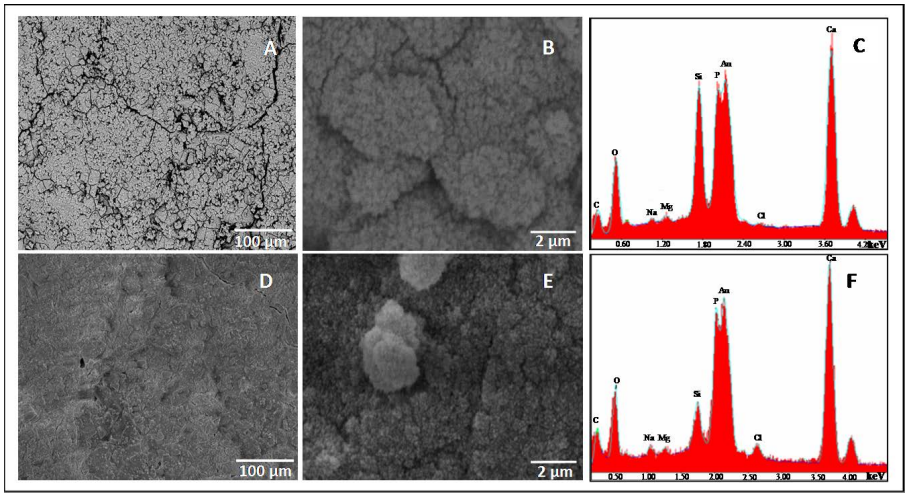

Figure 4: SEM-EDS of bioactive glass B1 uncoated (A–C) and coated with 0.15 (%m/v) polymer suspension (D–E) after 14 days immersion in SBF solution. Magnifications are 500 × (A;D) and 20000 × (B,E). EDS spectra are resultant from a general analysis of the images B and E.

690°C; the maximum linear shrinkage (18%) was reached at about 770°C prior to the onset of crystallization (Tc). The DSC curve shows three main crystallization peaks above 770°C, that should correspond to the formation of multiple phases, in agreement with literature [21]. As reported in the previous section, the green compacts were then sintered at 710°C for 20 minutes (β = 20°C min^{-1}), obtaining amorphous glass pellets.

The resultant sintered glass pellets were dip-coated in a 0.15 (%w/v) melanin suspension and their morphology compared with the one of an uncoated samples by SEM observation (Fig. 2). While a very smooth surface was observed for uncoated samples (B1U, Fig. 2A), the coated samples (B1C, Fig. 2B) presented a rough surface exhibiting spherical particles of dimension between 200–300 nm entrapped on the glass surface.

The coated and uncoated samples were soaked in a SBF solution up to 21 days in order to assess their bioactivity. After 1 day soaking in SBF solution, relevant modifications occurred on the surface of the glass (Fig. 3). The higher amount of cracks observed on the glass surface of sample B1C (after drying at 100°C) in comparison to the uncoated one (B1U) is characteristic of the formation of a thick silica-gel layer, suggesting a higher bioactivity of

Table 1: Si/P and Ca/P weight ratio (wt.%) of samples B1U and B1C after 1 day (B1U_1D and B1C_1D) and 14 days (B1U_14D and B1C_14D) immersion in SBF solution.

Sample	Si/P	Ca/P
B1U_1D	2.53	2.38
B1C_1D	1.28	2.01
B1U_14D	0.99	1.40
B1C_14D	0.28	1.43

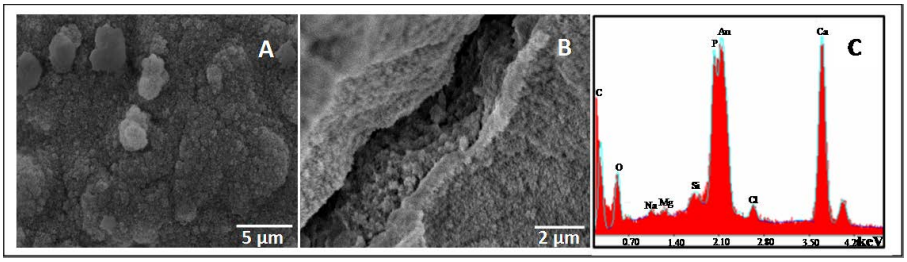

Figure 5: SEM-EDS of bioactive glass B1 with 0.15 (%m/v) polymer suspension (A–C) after 14 days immersion in SBF solution. Magnifications are 10000 × (A) and 20000 × (B). EDS spectrum is resultant from a general analysis of image A.

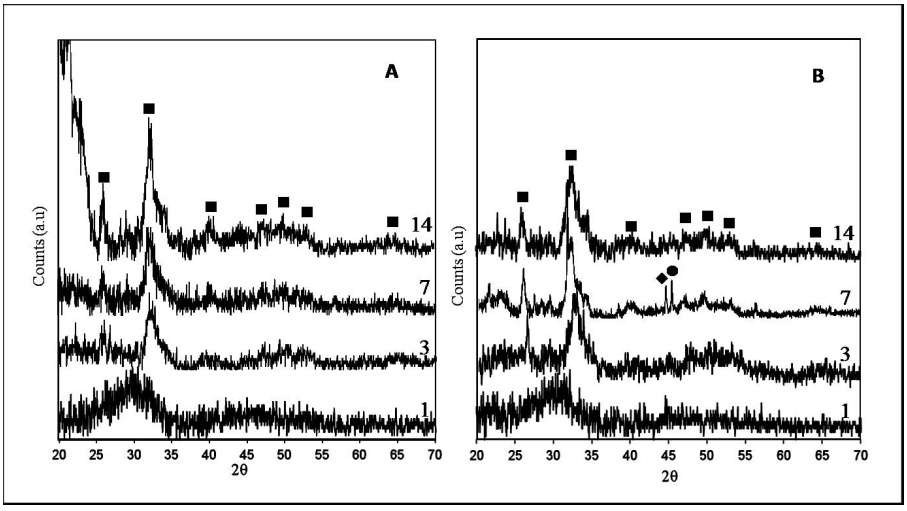

Figure 6: XRD Bioglass B1 uncoated (A) and coated with 0.15 (%m/v) polymer suspension (B) after 1, 3, 7 and 14 days immersion in SBF solution. XRD: hydroxypatite (■), NaCl (●), CrFeNi (♦).

B1C vs B1U. After 14 days of immersion in SBF, this trend was confirmed by the lower Si/P and Si/Ca ratios observed in EDS spectrum of the coated sample (Fig. 4F; Table 1). The uncoated sample exhibits a high amount of cracks (Fig. 4A and B) and a much higher Si/P ratio (Fig. 4C), although the presence of Ca-P crystals has been clearly observed. The accentuated decrease on the amount of visible cracks at the surface of sample B1C (Fig. 4D) is also coherent with the formation of a calcium-phosphate layer (Fig. 4D and F) with high thickness on the surface of this sample. In Fig. 5A, it is clearly evident the difference of morphology between the polymer, composed of spherical particles (bottom layer), and the calcium-phosphate layer, composed of agglomerates of particles with a morphology similar to the one of hydroxyapatite (HAp - upper layer). Figure 5B–C reports the formation of Ca-P agglomerates rich in Ca and P; the EDS spectrum reveals also a high amount of C and O, probably ascribable to the polymer presence.

The identification of the Ca-P surface layer as apatite (PCPDF 98-007-7967) was achieved by performing XRD analysis on samples soaked for 3 days in SBF solution. This crystalline layer is characterized by the presence of two characteristic peaks around $2\theta = 26°$ and 32°, and was observed in both coated and uncoated samples (Fig. 6A–B). In the XRD pattern corresponding to the coated sample af-

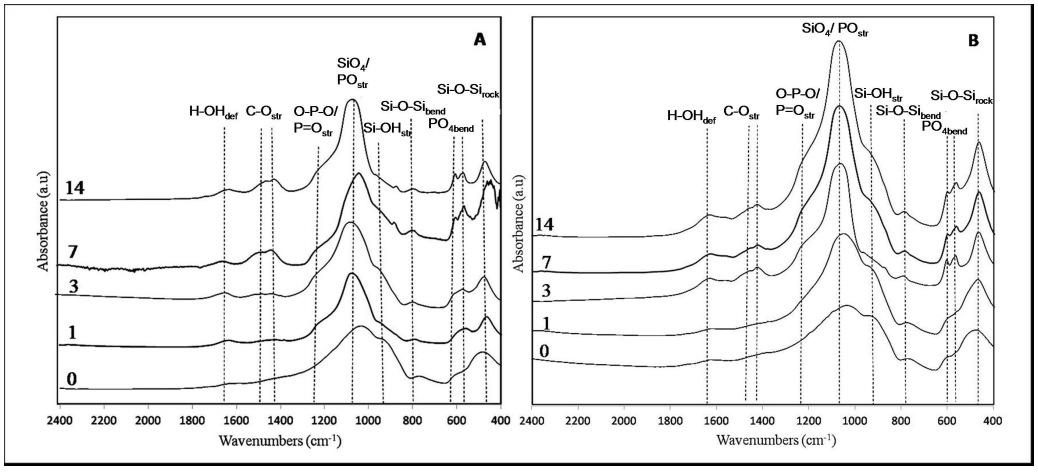

Figure 7: FTIR of bioactive glass B1 uncoated (A) and coated with 0.15 (%m/v) polymer suspension (B) after 1, 3, 7 and 14 days immersion in SBF solution.

ter 7 days of soaking in SBF (Fig. 6B7), peaks evidencing the presence of NaCl (01-077-2064) arising from the SBF solution, or from the source of the biopolymer (sea water), are also visible at $2\theta = 31.8^\circ$ and 45.5°, while the other peak appearing at $2\theta = 44.8^\circ$ is characteristic of the CeFeNi sample support (PCPDF 00-035-1375).

FTIR analyses evidenced the presence of peaks characteristic of a crystalline apatite layer, between 570–610 cm^{-1} (P-O bending vibrations), already after 3 days soaking in SBF solution, both on coated and uncoated samples (Fig. 7) [29]. The presence of peaks characteristic of C-O stretching vibrations at 1450 and 1410 cm^{-1} allows the classification of this layer as a B-substituted hycroxycarbonate apatite [30]. It is also worthy of mention that the peak around 930 cm^{-1}, characteristic of Si-OH stretching in Q^2 and Q^1 units is maintained in sample B1C even after 14 days of bioactivity (Fig. 7B). Figure 8 shows the trend on the pH for the uncoated and coated samples. As it can be observed, the trend is similar for both samples, with more acidic values, during the whole soaking time, for B1C.

4 Discussion

In this work the influence of a melanin coating on the bioactivity of silica based glass (AP40, herein named B1) sintered pellets was investigated. First of all, a thermal characterization of the glass (Fig. 1) was carried out, in order to find the adequate temperature for the sintering process, in order to obtain amorphous glass pellets. Viscous flow sintering (i.e. a sintering schedule carried out in the absence of crystallization phenomena) is commonly used to obtain samples with high density and good mechanical properties [21, 31]. Moreover, the absence of crystalline phases in the glass pellets was useful to avoid interferences with the XRD signal of the apatite layers formed on the glass surface during soaking in SBF.

SEM analysis of coated samples (Fig. 2B) evidenced the presence of spherical particles. These particles were not observed on uncoated samples and showed the typical morphology of the biopolymer used for coating the bioactive glass [24]; so they can be ascribed to the biopolymer presence on the glass surface.

The *in vitro* test in SBF solution confirmed that the presence of the biopolymer didn't have a negative influence on the crystallization kinetics of the apatite layer; moreover a faster apatite forming ability induced by the biopolymer can be presumed. Even if no significant difference in the hydroxyapatite nucleation was observed by means of XRD analyses, a faster silica-gel formation and HAp precipitation was observed by means of SEM-EDS analyses. In fact, after 1 day of immersion in SBF (Fig. 3) a higher amount of cracks, characteristic of the silica-gel, was observed on the glass surface of sample B1C in respect with B1U; moreover the EDS analysis of B1C showed higher peaks of Ca and P in comparison with B1U. After 14 days of immersion in SBF some cracks are still visible on B1U surface, while only agglomerate with morphology close to the HAp one are observable on B1C samples. This could be due to the formation of a thicker HAp layer on coated samples, as confirmed also by EDS analyses: the EDS spectrum of B1U after 14 days of treatment (Fig. 4C) shows a higher Si peak than B1C spectrum (Fig. 4F).

FTIR analyses (Fig. 7) confirmed the results obtained from SEM-EDS: after 3 days of immersion in SBF more intense peaks between 570–610 cm^{-1}, ascribable to P-O

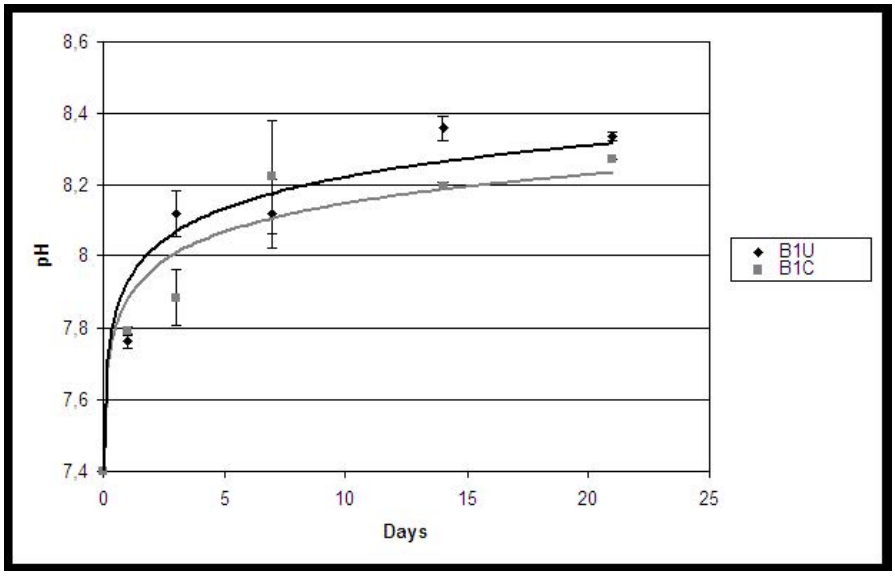

Figure 8: pH changes of SBF solutions after immersion of samples B1U(■) and B1C (●) for 1, 3, 7, 14 and 21 days.

bending vibrations, were observed for B1C sample than B1U one. Thus, it cannot be ruled out that a Ca/P layer with higher thickness is formed in the coated sample, which would be in accordance with the SEM-EDS results. Moreover, the peak around 930 cm^{-1}, characteristic of $Si\text{-}O^-$ stretching in Q^2 and Q^1 units is maintained in sample B1C even after 14 days of bioactivity (Fig. 7B), suggesting that the re-polymerization of the surface glass network is not significant in the coated sample [32]. In this case, the -COOH and -OH surface groups of the biopolymer would provide an environment similar to a silica-gel/polymerized glass network, inducing the nucleation of the apatite layer, as usually happens during the last stages of bioactivity mechanism [33, 34].

The different behaviour of the coated glass samples was also confirmed by the pH evaluation (Fig. 8). It is well known that the first stages of bioactivity give rise to a depletion of the hydrogen ions in the solution, thus producing a slight enhancement of pH [5]. This process should be carefully controlled in view of biological *in vitro* test, in order to avoid pH values not compatible with cell viability. In this contest, the lower pH values observed for B1C can be considered a positive issue and may be resultant from the deprotonation of surface carboxylic groups present in the biopolymer [24]. The functional groups present on the biopolymer may have also an important role on the increase of the bioactivity of the B1 glass. The phenolic and carboxylic groups of the monomeric units (dihydroxyindole and dihydroxyindole carboxylic acid) that compose the biopolymer may have a similar nature as the surface network resultant from the re-polymerization of the glass network in the bioactivity mechanism (stage 3) [35]. As a result, the biopolymer may act as nucleation sites for the formation of the Ca-P layer, introducing an acceleration in the bioactivity mechanism and a consequent increased apatite forming ability.

5 Conclusions

A hybrid organic-inorganic material composed by a bioactive glass (B1) and a natural-derived polymeric coating (melanin) was successfully developed. The melanin coating enhanced the apatite forming ability of the glass, by accelerating the kinetics of the bioactivity process, and induced a lower enhancement of the pH during immersion in SBF. Further analyses need to be performed to investigate the influence of melanin on the bioactivity mechanism, for example by evidencing the presence of nucleation sites for the formation of the Ca-P layer. The biocompatibility and biodegradability of melanin [21], as well as its high availability in nature, together with its drug delivery ability and possibility to be used without modifications are key factors to suggest this biopolymer in the production of economic and multifunctional hybrid organic-inorganic materials for biomedical applications.

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