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Bonelike®/PLGA hybrid materials for bone regeneration: Preparation route and physicochemical characterisation

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Bonelike®/PLGA hybrid materials have been developed using γ-MPS as silane-coupling agent between the inorganic and organic phases for controlled drug delivery applications.

Silanization showed to be more effective when cyclohexane was used as a non-polar solvent (nP method) due to a chemical interaction between Bonelike® and the silane film, while by using a 95/5 (V/V) methanol/water as a polar solvent (P method), a much thinner film was achieved.

Functional groups of PLGA, such as the carbonyl group (C=O), were identified using Raman and FTIR-ATR analysis and therefore these groups may be used to link therapeutic molecules. These novel hybrid materials prepared by combining silanization and *post*-hybridisation processes are expected to find use in medical applications of bone regeneration and as drug delivery carrier for therapeutic molecules.

1. Introduction

Hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, is an example of a calcium phosphate ceramic that has been usually applied in bone regeneration surgery, but its use has been limited to low-load applications because of its poor mechanical strength [1]. Apart from that, the human bone mineral is reasonably different from stoichiometric HA regarding its chemical composition, which contains ions, such as K^+, Na^+, Mg^{2+}, CO_3^{2-} and F^- as well as beta-tricalcium phosphate (β-TCP) phase, β-Ca $_3(PO_4)_2$ [2, 3].

Over the past decade, extensive work has been carried out on the development of glass-reinforced hydroxyapatite (GR-HA) recently registered as Bonelike®, which has been prepared with the incorporation of a P_2O_5-based glass in the HA by means of a liquid phase sintering process in order to increase, simultaneously, the mechanical properties of HA and to introduce ions commonly found in bone tissue [4–10]. Bonelike® is known to stimulate osteoblast growth and differentiation [11, 12] and showed much faster osseointegration than commercially available hydroxyapatite [13].

Recently chemically coupled inorganic-organic hybrid materials have attracted a great deal of attention as new materials of significant importance with potential physicochemical and mechanical properties for bone regeneration and drug delivery systems [14, 15]. Several authors have proposed the use of biodegradable polymers to modify the calcium phosphate surface due to their good similarity in terms of mechanical properties compared to that of the bone tissue [16, 17].

By choosing polymeric materials with known biocompatibility and biodegradability, as the poly(D,L-lactide-*co*-glycolide) (PLGA) [18, 19], it becomes possible to prepare biodegradable calcium phosphate/polymer hybrids with some potential for bone regeneration applications [20, 21]. By changing the lactide/glycolide ratio value, the polymer degradation may be controlled [22] and adjusted to new bone formation rate [23], and the release of proteins and therapeutic molecules may also be controlled [24]. However, hybrid materials when exposed to an aqueous physiological environment can loose strength rapidly at the inorganic-organic interface if the two phases are not bound together [25]. The mechanical behaviour of hybrid structures describes the type of interaction between the two phases, and if there is no binding, either physical or chemical, there is a premature failure of the

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hybrid materials [26]. In order to enhance the hybrid materials integrity, studies have been done based on the modification of the surface of inorganic materials by adding a coupling agent, such as organofunctional silanes [27–29], which showed to be effective in the improvement of the adhesion between mineral substrate and polymer matrix [30–32].

Surface modification of the Bonelike® with a silane-coupling agent has been considered as an important attempt to chemically link this inorganic phase and PLGA via the terminal reactive groups in the organofunctional silanes. These novel Bonelike®/PLGA hybrid materials may be used as drug delivery systems with controlled degradation rate and therefore will greatly enlarge the fields of medical applications of Bonelike®.

In this work, Bonelike®/PLGA hybrid materials were prepared and physicochemical characterized using X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD), Fourier transform infra-red attenuated total reflectance (FTIR-ATR), Raman spectroscopy and scanning electron microscopy (SEM- EDX).

2. Materials and methods

2.1. Preparation of inorganic materials

A P_2O_5-based glass with the chemical composition of $65P_2O_5$-15CaO-$10CaF_2$-$10Na_2O$ (mol%) was prepared by taking the appropriate quantities of high purity (>98%) grade phosphorous pentoxide, calcium hydrogen orthophosphate, calcium fluoride and sodium carbonate. Reagents were mixed in a platinum crucible under nitrogen atmosphere using a two-hand AtmosBag^{TM} (Aldrich, USA) and melted following the conventional procedures. After milling the glass, the slip was obtained by wet mixing in methanol 4.0% (wt/wt) of the glass with HA (Plasma Biotal, batch P201 R) as earlier reported by Santos *et al.* [33]. The powders were then dried for 24 h at 60 °C and sieved to less then 75 μm under nitrogen atmosphere.

Dense Bonelike® and HA disc samples, with 25 mm diameter, were obtained by uniaxially pressing at 288 MPa and sintering at 1300 °C using a ramp rate of 4 °C/min with 1 h dwelling time at this temperature followed by natural cooling inside the furnace. After cooling, specimens were mechanically polished down to 1 μm finish using silicon carbide paper, ultrasonically degreased with ethanol and cleaned with deionised water. Bonelike® and HA granules were prepared with particle size ranging from 150 to 500 μm for the present study using standard milling and sieving techniques.

2.2. Silanization procedure

The silanization solutions were prepared by two different methods, using a non-polar solvent (nP) and a polar solvent (P). A 10 wt% γ-methacryloxypropyltrimethoxy-silane (γ-MPS from SIGMA, USA) and 2 wt% n-propylamine in cyclohexane solution was used for the nP method. For the P method, 10 wt% γ-MPS was dissolved in a 95/5 (V/V) methanol/water solution as previously reported by Davy *et al.* [32].

A modification to the previous P method was also performed using a 0.01 M chloridic acid solution to adjust the pH of silanization solution to 3.5.

Bonelike® and HA specimens were respectively immersed in the silanization solutions and ultrasonically treated for 45 min followed by increasing solution pH to 10.5 using a 25% ammonia solution [25].

The silane-treated specimens were firstly dried at room temperature to improve the stability of the coating, and then subsequently heated under vacuum at 120 °C for 24 h to obtain the final condensed silane on the surface and washed with dichloromethane to remove weakly attached silane molecules.

2.3. Preparation of hybrid materials

Hybrid materials were prepared using poly(D,L-lactide-*co*-glycolide) (PLGA) with ratio of D,L-lactide to glycolide of 85:15 (Aldrich, USA) and molecular weight ranging from 50,000 to 75,000.

Silanized and non-silanized disc samples of Bonelike® and sintered HA were soaked in 1–20 wt% of PLGA/ethyl lactate solution under ultrasonication, followed by solvent evaporation at room temperature and heat treated at 120 °C in vacuum for about 8 h. To obtain a uniform coating, post-hybridisation was repeated three times for both dense and granular samples.

2.4. Materials characterisation

X-ray diffraction analysis was performed on powder samples of Bonelike® and HA before and after sintering, using X-ray diffractometer with Cu-K_α radiation at 40 mA and 40 kV (MAC Science Co., Ltd., MXP3V). Using flat plate geometry, data were collected for 2θ values ranging from 10 to 70° in increments of 0.05° and a counting time of 2 s/step. Quantitative phase analysis was performed by the Rietveld method using General Structure Analysis Software (GSAS; Los Alamos National Laboratory). The Chi-squared test was used to validate the fitting and the null hypothesis (there is no difference between the fitting and the experimental curves) was rejected for squared-chi values higher than the critical value for $P = 0.05$.

A scanning electron microscope (Hitachi, S-3500N) was used to analyse the microstructure of the prepared materials surfaces. Specimens were coated with gold using a Quick Coater (UPS-020) before examination. The current was set at 5 mA with a coating time of 180 s. The phosphorus, calcium, silicon, fluoride, sodium, carbon and oxygen elemental analysis was achieved by using an X-ray detector (Horiba, Ltd., EMAX ENERGY EX-400) attached to the S-3500N and 10 keV was used.

FTIR-ATR analysis was performed using a Perkin Elmer spectroscope (System 2000 NIR FT-RAMAN) with InGaAs detector. All spectra were recorded in the region of 4000–400 cm^{-1}using a 4 cm^{-1}resolution.

Laser Raman spectroscopy (NRS-2100, Jasco Co., Tokyo, Japan) measurements were performed at 180° reflection configuration with 514.5 nm line of an Ar^+ laser at 200 mW.

The relative atomic percentage of each element on the silane treated materials surface was estimated from the X-ray photoelectron spectra peak areas using a VG-SCIENTIFIC ESCALAB 200A spectrometer. An

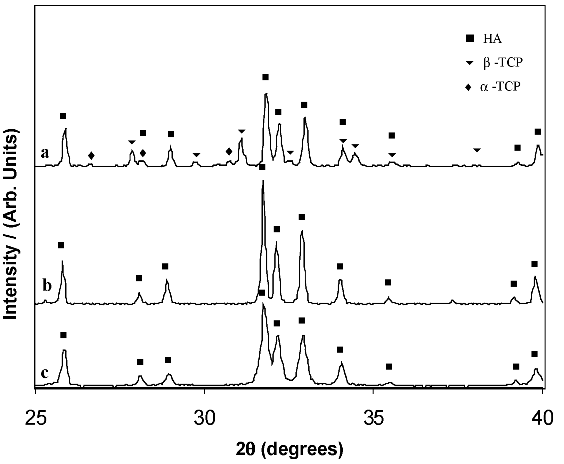

Figure 1 XRD patterns for: (a) Bonelike®, (b) sintered HA and (c) HA as received.

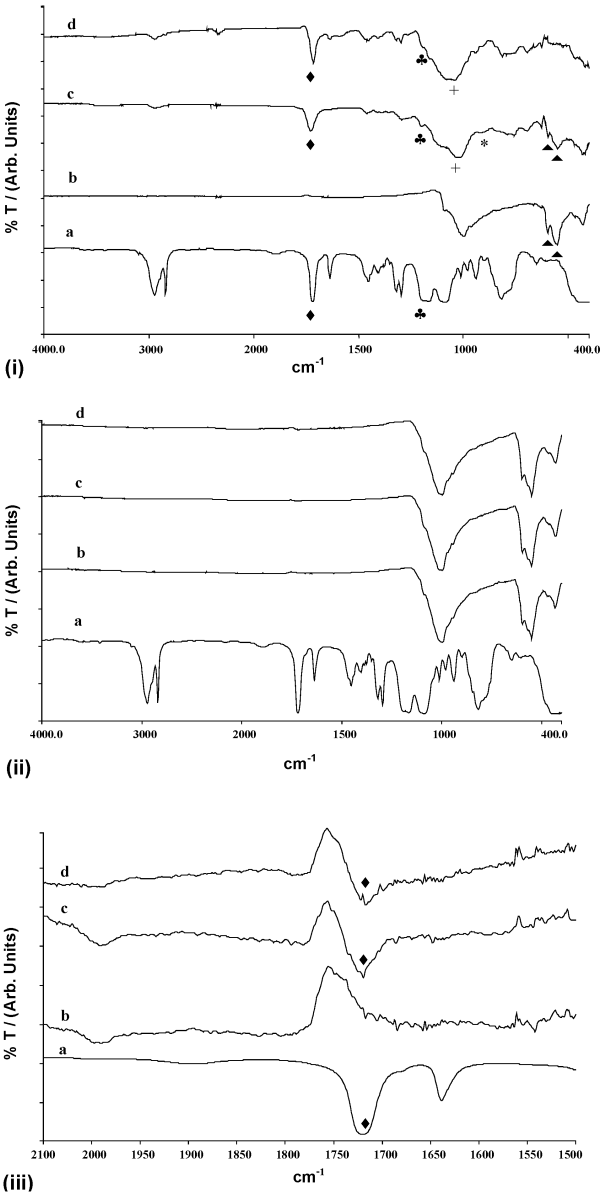

Figure 2 FTIR spectra of silanization. (i) sintered HA, (a) γ-MPS, (b) non-treated sintered HA, (c) silanized HA (P method) and (d) silanized HA (nP method). (ii): Bonelike®, (a) γ-MPS, (b) non-treated Bonelike®, (c) silanized Bonelike® (P method), (d) silanized Bonelike® (nP method). (iii): Zoom of Fig. 2(ii).

MgAlα radiation operating at 300 W and 15 keV was used. Survey spectra (2–1002 eV) were taken at pass energy of 50 eV. All spectra were referenced to C1s peak adventitious carbon fixed at 285.0 eV. High-resolution spectra of C1s peak, O1s peak, Si2p peak, Ca2p peak, P2p peak and N1s peak at pass energy of 20 eV were taken. The peak deconvolution was performed using the XPS PEAK 41 software. The Chi-squared test was used to validate the fitting and the null hypothesis (there is no difference between the fitting and the experimental curves) was rejected for squared-chi values higher than the critical value for $P = 0.05$.

3. Results

Fig. 1 shows the XRD patterns of HA as received (Plasma Biotal), sintered HA and Bonelike®. No phase transformation was observed for the commercial HA after sintering (Fig. 1(a)–(b)) while Bonelike® showed the presence of three phases, HA, beta-tricalcium phosphate (β-TCP), $Ca_3(PO_4)_2$, and also a small amount of α-TCP (Fig. 1(c)). Rietveld refinement was performed to determine the proportion of each phase, and contents of 67.7% HA, 25.8% β-TCP and 6.5% α-TCP were observed.

The silane treatment was successfully performed on sintered HA samples, as it was clearly identified by silane-carbonyl (C═O groups) bands at 1726–1728 cm^{-1} for non-polar (nP) and polar (P) methods (Fig. 2(i)). The Si─O─Si stretching band at 1039 cm^{-1} and Si─OH band at 913 cm^{-1} also revealed the presence of the silane coating. When sintered HA samples were silanized using the nP method, an increase in the silane-carbonyl group was observed at 1728 cm^{-1}. The presence of PO_4^{3-} bands at 598 and 559 cm^{-1} that belongs to the HA substrate were detected when using the P method.

After silane treatment optimisation in terms of immersion time and concentration of silane solution, a silane thin-film was obtained on the Bonelike® surface using both the P and nP methods (Fig. 2(ii)). The presence of thin-film was confirmed by the existence of the silane-carbonyl band. This band appeared in the spectrum at a slightly lower wavelength number compared to the characteristic band for the pure silane-coupling agent (Fig. 2(iii)).

The XPS survey scan spectra revealed the presence of Si atoms on the Bonelike® surface both for P and nP methods (Figs. 3(a)–(b)), which were identified by Si2p band in all spectra with a binding energy of ~102 eV. Peak deconvolution was performed and revealed that the Si2p band was in fact composed of two different peaks (Figs. 3(c)–(d)).

For the silanization using the P method (Fig. 3(c)) one peak was observed at ~102.6 eV (Si2p-I), which is attributed to the Si-O group, and another peak at 101.9 eV (Si2p-II), attributed to the Si─O─CH_3 and Si─O─Si groups. When the nP method was employed a new component with higher binding energy (Si2p-III) at 103.0 eV was observed, due to the O─SiO─O group (Fig. 3(d)) in addition to the Si─O─CH_3 and Si─O─Si peaks.

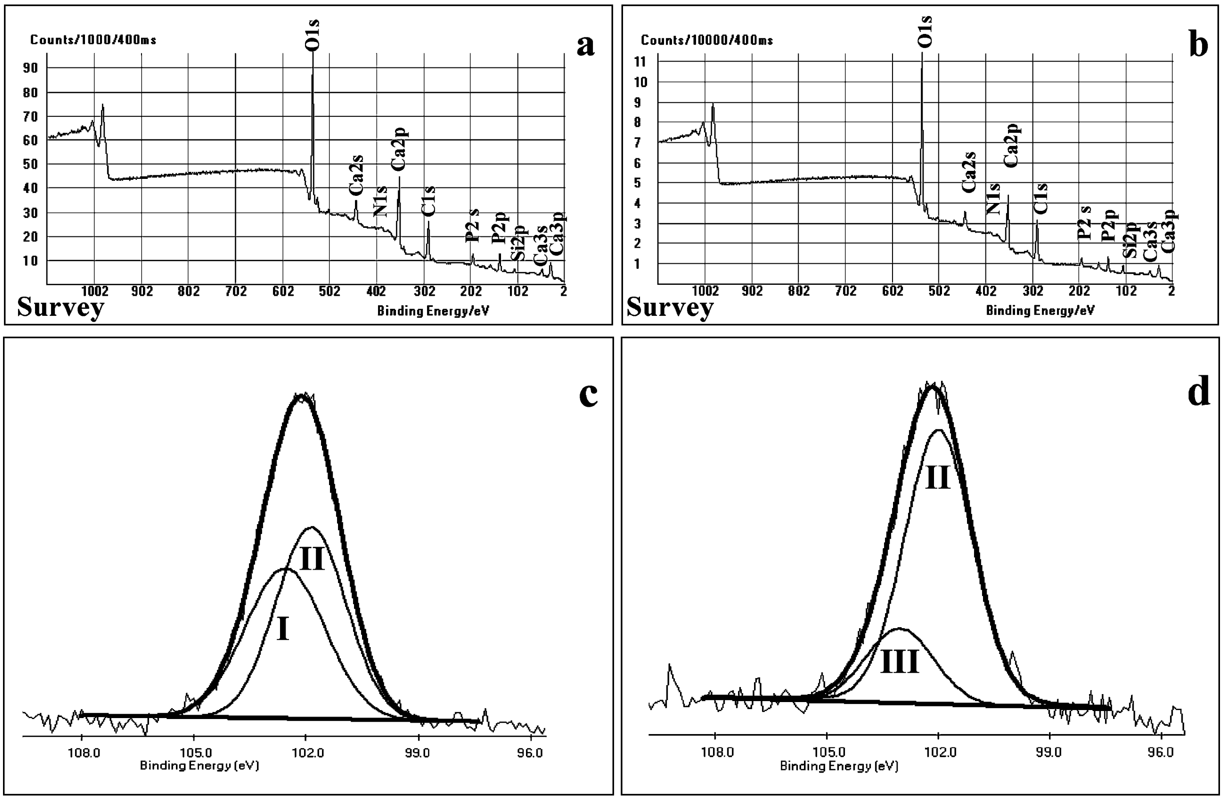

Figure 3 XPS spectra of Bonelike® silanized by solvent evaporation method using a 10 wt% γ-MPS solution showing the appearance of most significant peaks Si2p, C1s, O1s, Ca2p (2p3 and 2p1), P2p, N1s: (a) silanization by P method, (b) silanization by nP method, (c) Si2p in P method after peak deconvolution and (d) Si2p in nP method after peak deconvolution.

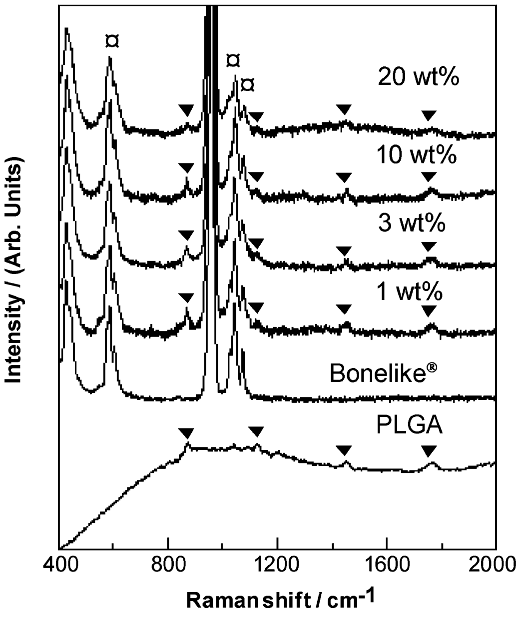

Figure 4 Raman spectra of Bonelike® surface treated with various concentrations of PLGA/ethyl lactate solution using the solvent evaporation method.

The results from Raman Spectroscopy for Bonelike®/PLGA hybrid materials are shown in Fig. 4. The presence of bands at ~1740 cm^{-1} and 1100 cm^{-1}, due to carbonyl group (C=O bond) and to ether group (C—O—C, stretching vibration) respectively, clearly indicates that PLGA coating can be applied to non-silanized Bonelike® materials by *post*-hybridisation.

Fig. 5(i) showed that a uniform silane and PLGA coatings were achieved on the granules of Bonelike® for all studied surface treatments. Elemental EDX analysis of silanized Bonelike® samples showed that besides the peaks corresponding to the Bonelike® composition, i.e. Ca, P, F, O and Na, additional peaks of Si, C and O were also detected, which should be assigned to the silane-coupling agent (Fig. 5(ii) (a)–(b)). The increasing C content in Fig. 5(ii) (c)–(d) is related to the presence of PLGA coating on Bonelike® surface.

Fig. 6 shows the infrared spectra of Bonelike® before and after treatment with silane-coupling agent followed by post-hybridization with the PLGA. The presence of one band at 1748 cm^{-1} due to the carbonyl group (C=O) indicates the presence of the PLGA coating on the silanized and non-silanized Bonelike® surface (Fig. 6(e)–(f)). The band at 1603 cm^{-1} should be attributed to C=C and was only observed for Bonelike® samples that underwent silane treatment followed by PLGA coating (Fig. 6(e)). This aspect will be addressed in the discussion section.

4. Discussion

The XRD analysis in Fig. 1 of the HA samples shows that they do not decompose to tricalcium phosphate (TCP) phases after sintering while Bonelike® showed phase transformation of the HA to β- and α-TCP due to the glass addition. This result indicates that HA becomes unstable when sintered in the presence of CaO-P_2O_5 glassy phase, in agreement with previous studies [33]. Due to the presence of TCP phases,

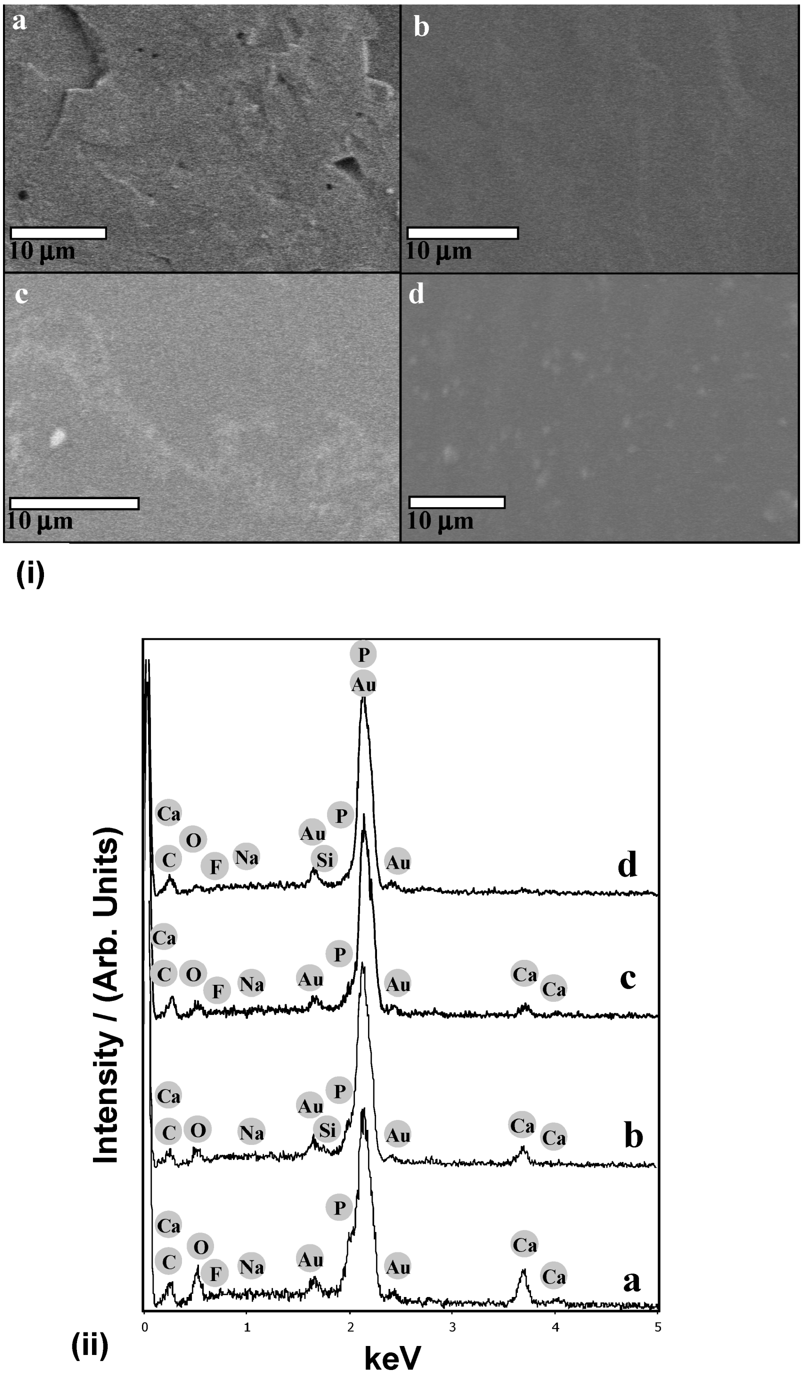

Figure 5 Bonelike® granules surface treatment using the solvent evaporation method. (i): SEM images, (a) Bonelike® surface, (b) silanized Bonelike® (modified P method) and (c) Bonelike®/PLGA hybrid *post*-hybridised with 1 wt% PLGA/ethyl lactate solution and (d) silanized Bonelike®/PLGA hybrid (modified P method). (ii): Respective EDX spectra.

Bonelike® dissolves faster than sintered HA and therefore this novel biomaterial has the potential to degrade *in vivo* whilst encouraging simultaneous new bone formation [34].

This work reports the preparation route of Bonelike® combined with biodegradable PLGA polymer being these two phases grafted together by a silane-coupling agent. The biodegradability of the polymeric phase of Bonelike®/PLGA hybrid may be controlled as reported in literature [18], so this hybrid system has a great potential to be applied as a carrier for controlled drug delivery.

The silanization process that was conducted in this study developed a continuous silane layer on both the HA and Bonelike® surface. Using FTIR-ATR analysis, the silane-coupling agent was detected on sintered HA by the presence of a band at 1726–1728 cm^{-1} for P and nP methods, which are attributed to the silane-carbonyl group (C═O) (Fig 2(i)). The Si─O─Si stretching was detected by the presence of a band at 1039 cm^{-1}, as confirmed by Fransen *et al.* [35], and also one band at 913 cm^{-1} that indicates the presence of silanols groups (Si─OH), as has been previously reported by Rider *et al.* [36]. The band at 1199 cm^{-1} is assigned to the silane Si─O─CH_3 group.

Comparing the silanization P and nP methods, an increase in the concentration of silane-carbonyl group (C═O) at 1728 cm^{-1} was observed on HA surface

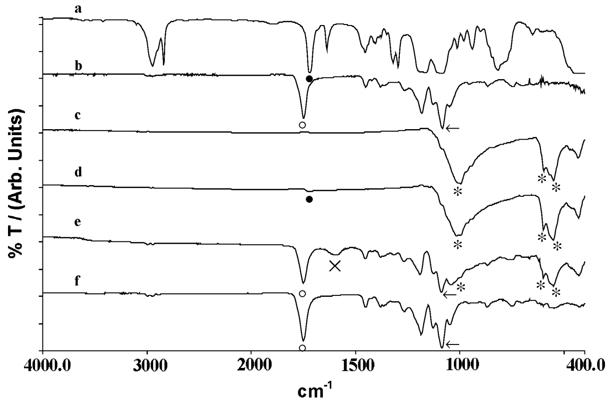

Figure 6 FTIR-ATR spectroscopic results of Bonelike® surface treatment: (a) γ-MPS, (b) PLGA, (c) Bonelike® surface, (d) silanized Bonelike® (modified P method), (e) silanized Bonelike®/PLGA hybrid using the modified P method and *post*-hybridised with 1 wt% PLGA/ethyl lactate solution and (f) Bonelike®/PLGA hybrid *post*-hybridised with 1 wt% PLGA/ethyl lactate solution.

silanized by the nP method. Furthermore, the presence of the phosphate (PO_4^{3-}) bands belonging to the HA substrate at 598 and 559 cm^{-1} indicates that the silane-film obtained for samples silanized by the P method is thinner than that obtained by the nP method (Fig. 2(i)(b)–(d)). This observation corroborates previously reported results, which indicated that the silanization is more effective when non-polar solvents are used [32]. This phenomenon accounts for the chemical interaction between the silane-coupling agent and calcium phosphate substrate that is confirmed by the O—SiO—O groups that were identified by XPS results.

The FTIR-ATR spectra (Fig. 2(ii)) of silanized Bonelike® recorded by the P and nP, methods showed no significant differences between the two. The silane-thin film was confirmed by the presence of weak silane-carbonyl band at 1716 cm^{-1} (Fig. 2(iii)(c) and (d)).

The chemical composition and bonding state of the silane-thin film on Bonelike® treated with nP and P methods was investigated by XPS. The Si2p peak (Fig. 3(c)–(d)) was found to be asymmetric and the deconvolution performed, using Gaussian peak shapes with Lorentzian tails and Shirley background, in fact revealed the presence of two peaks. Separation values of 0.7 and 1.1 eV for P and nP methods were respectively obtained between these two peaks. For P method the deconvoluted Si2p peak at 102.6 eV [Si2p-I] with a full-width-at-half maximum (FWHM) = 2.5 eV is attributed to Si—O groups, as in the Si—OH case. The existence of the silanols (Si—OH) groups was corroborated by the intense FTIR-ATR band at 914 cm^{-1} as previously shown in Fig. 2(i). The peak at 101.9 eV [Si2p-II] with FWHM = 2.2 eV corresponds to the Si present in $Si{-}O{-}CH_3$ and Si—O—Si chemical states, as previously reported by van Ooij *et al.* [37], confirming the FTIR-ATR data.

For the nP method, deconvolution of Si2p showed the existence of a broad peak at 102.0 eV (Si2p-II with FWHM = 2.2 eV), attributed to $Si{-}O{-}CH_3$ and Si—O—Si groups, and one second peak observed at 103.0 eV (Si2p-III with FWHM = 2.1 eV) that is characteristic of O—SiO—O groups. These results seem to confirm that when the nP method is used, there is a chemical interaction between the Si—O groups of silane-coupling agent and the –OH groups of the Bonelike® substrate.

XPS quantification of the total relative atomic ratio of Si on the outermost layer was based on peak areas and results of 6.18% for nP method and 3.46% for P method were obtained. These clearly indicated that the nP method was more effective in grafting Si-based groups on the surface of Bonelike® thus providing a method to prepare a more stable interface to link an organic phase or/and to control the degradation rate of the hydrid.

The aim of the present study was to use a silane-coupling agent to chemically adhere a coating of PLGA to the surface of Bonelike®. The modification proposed in silanization process consisted in controlling the rate of silane condensation by adjusting the pH of silanization solution and therefore eliminating the $Si{-}O{-}CH_3$ groups.

PLGA coating was performed on Bonelike® surface to develop the hybrid structures using post-hybridisation methods (Fig. 4). Raman analysis revealed the presence of one band at ~1740 cm^{-1} attributed to C=O stretching vibration of the carbonyl group and another at 1100 cm^{-1} due to C—O—C ether group, which clearly indicate that the PLGA coating was covering the Bonelike® surface. A sharp and dominant band at 960 cm^{-1} was observed, which accounts for the presence of PO_4^{3-} groups of the Bonelike® substrate. Additionally, PO_4^{3-} symmetric and antisymetric stretching modes were also observed at 1080 and 1040 cm^{-1}. The P—O peak intensity decreases when Bonelike® was immersed in several PLGA/ethyl lactate solutions ranging from 1 to 20 wt%, indicating that the PLGA coating thickness increases with PLGA concentration as expected (Fig. 4).

The SEM images showed that when Bonelike® surface was silanized and/or post-hybridised, the surface was covered by total and uniformly coated, as shown in Fig. 5(i), with an increase in carbon from PLGA and a correspondent decrease in Ca and P from Bonelike® substrate. A Silicon peak was also observed due to the silane-coupling agent (Fig. 5(ii)).

FTIR-ATR studies also indicated that the PLGA coating could be grafted to silanized and non-silanized Bonelike® surface as observed by the presence of bands at 1748 cm^{-1}, attributed to carbonyl group (C=O) stretching vibration, and to ether (C—O—C) group at 1082 cm^{-1} (Fig. 6). The decrease in the intensity of PO_4^{3-} bands at 998, 599 and 551 cm^{-1} is also indicative of the availability of both the PLGA and the silane-coupling agent on Bonelike® surface. For silanized Bonelike® the decrease in the intensity of C=O band at 1748 cm^{-1} and the appearance of the PO_4^{3-} bands (Fig. 6(e)) demonstrated that a thinner PLGA coating was obtained compared to that of non-silanized Bonelike®. The observation of a band near 1603 cm^{-1} should reflect the linking between PLGA and the silane coating through the alcoholic oxygen atom of the ester group.

The work that has been performed clearly demonstrated that carbonyl functional groups from PLGA are present after the hybridisation of silanized and non-silanized Bonelike®, which indicates that these groups are available to link therapeutic molecules and therefore Bonelike®/PLGA hybrid materials have good potential to be used as drug delivery carrier.

5. Conclusions

Silanization of HA and Bonelike® materials has been successfully obtained using γ-MPS as a coupling agent. In the present work, we concluded that a chemical interaction between the silane-coupling agent and Bonelike® surface has been achieved more effectively by means of silanization using a non-polar method. Chemical interactions between the silane-coupling agent and PLGA phase seems to occur, as confirmed by the presence of C=C groups conjugated with C=C or in a C=O ambient detected by FTIR-ATR analysis.

These novel Bonelike®/PLGA hybrid materials may find use for bone regeneration applications with simultaneous local delivery of therapeutic molecules.

Acknowledgments

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