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Hybrid composite scaffolds prepared by sol-gel method for bone regeneration

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Abstract

This study reports the synthesis and characterization of Hydroxyapatite (HA)/Polycaprolactone (PCL) hybrid composite materials synthesized by sol-gel method. The fabrication of scaffolds was performed by salt-leaching technique using NaCl as porogen agent. In the first step, the physico-chemical characterization of composite material was performed to evaluate the composition and the interaction between the organic/inorganic phases. In the second step, optimized scaffolds were bioactivated on the surface. The combined effect of scaffold morphology and surface bioactivity is ideal for bone tissue engineering, supporting bone cells adhesion, proliferation and differentiation. Here, a combined strategy involving biomimetic approach, using a supersaturated Simulated Body Fluid (SBF), and salt-leaching technique has been developed to grow hydroxyapatite in composite scaffolds able to regenerate the natural bone.

Introduction

Calcium phosphates have been successfully used for bone repair and regeneration because of their biocompatibility and osteoconductivity or osteoinductivity which allow to promote the formation of chemical bonds with living tissue by mimicking the apatite phase of the natural bone tissue [1]. In particular, synthetic hydroxyapatite has long been recognized as one of the most important bone substitute materials in orthopaedics and dentistry over past few decades [2,3] because of its ability, e.g. after implantation, to form chemical bonding with surrounding hard tissues [4,5] through the formation of a hydroxyapatite interfacial layer [6]. Moreover, hydroxyapatite particles on nanometric scale (n-HA) has been proved to be an osteoconductive material that also chemically binds to enamel and dentine [7]. However, their not adequate biomechanical properties (i.e high brittleness, low fatigue strength, and low flexibility) do not encounter the mechanical requirements for direct-loading applications, as well as application of dynamic force during the in vitro bone tissue engineering process [8]. This imposes their combination with polymers to form composite systems in order to morphologically and functionally mimic the bone tissue [9]. Recently, different strategies including phase inversion/particulate leaching [10], rapid prototyping [11], phase separation and freeze drying [12] have been developed to realize hydroxyapatite loaded polymer matrices with adverse success in tissue engineering application due to a lack of non-homogeneity distribution of ceramic particles within the polymeric matrix which dramatically reduces the bioactive potential of the composite [13]. In this context, chemically inspired approaches based on the sol-gel transition and colloidal precipitation of calcium phosphates may efficiently improve the particles dispersion by a direct control of precipitated grain sizes though the interaction between calcium and phosphate precursors under controlled temperature and pH conditions [14].

Besides, it is noted that the bone bonding which should occur to artificial material proximity is strictly dependent on the tissue interfaces which have to exhibit a surface topography with features in the same scale-range as that seen on the old bone surface at bony remodelling sites [15]. From this point of view, several approaches also involve the surface modification by surface adsorption and HA grafting onto the substrate [16]. Commonly, the promotion of the apatite crystals deposition on the scaffold surface was provided by using the basic Kokubo treatment, involving the immersion in Simulated Body Fluid (SBF) with an ions concentration, at least, equal to that of the human blood plasma [17]. However, some authors have proposed to accelerate the classical biomimetic process from 7-28 days to few days by immersing substrates into supersaturated SBF, so illustrating the interdependence between ionic strength and pH, as well as the influence of the substrate chemistry on the formation of the resultant apatite [18].

Here, a new strategy of composite manufacturing has been proposed by combining bulk sol-gel transition and surface biomimetic techniques. The sol-gel reaction allows to finely dispersing calcium phosphate nanoparticles into a polycaprolactone (PCL) matrix with an expected improving of the functional features (i.e, mechanical response) as reported in previous works [19]. Meanwhile, the controlled deposition of HA crystals by accelerating simulated body fluid (5xSBF) allows forming a biologically active surface which can drastically improve the bonding to living bone [4]. Proposed integrated approach enables to efficiently exploit the peculiar features of composite material bulk and surfaces by modulating the spatial distribution of bioactive signals, moving toward a more efficient replacement of bone tissue.

Materials and Methods

Preparation of porous and non-porous composite scaffold

Hybrid composite of HA/PCL (25/75w/w) was prepared at room temperature by using calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 99.0%, Sigma-Aldrich) and di-phosphorous pentoxide (P_2O_5 , 99.99%, Sigma-Aldrich) as precursors and using ethanol as the solvent. Poly(ϵ -caprolactone) ($M_w=65\text{kDa}$) dissolved in chloroform was used as biodegradable polymer, while ammonium hydroxide (NH_4OH , Assay 28.0-30.0%, Sigma-Aldrich) was utilized to adjust the pH at 11. In the first part of the preparation, it was synthesized HA solution. In particular, the procedure (Fig.1) consists of adding phosphorous pentoxide (P_2O_5) in ethanol and, after 30min, ammonium hydroxide (NH_4OH) up to a pH in excess of pH>10 is achieved (P solution). Calcium nitrate tetrahydrated was dissolved in ethanol at room temperature (Ca solution). After mixing for 20min, P solution was added drop wise to Ca solution in proper proportion in order to achieve a Ca/P ratio of. 1.67.

As second step, the solution was mixed with PCL/chloroform solution by stirring at 37°C up to the gelification (around 3 days) occurs. Finally, the aging of solution was provided for 2 days.

The preparation of scaffold was further provided by the addition of NaCl crystals with predefined size range (212-300 μm), as porogen agent, which was homogeneously mixed to the HA/PCL system with a weight ratio of about 9/1 (NaCl/PCL). The mixture was poured into a Teflon mould and daily rinsing in bi-distilled H_2O (Carlo Erba, Italy) for 7 days, to allow the dissolution of NaCl and the removal of any post-reaction ammonium nitrate traces.

Biomimetic surface treatment

To produce the apatite coating, scaffold substrates were submitted to a bio-inspired procedure, namely biomimetic treatment, inspired by the method described by Kokubo and co-workers [20,21].

In particular, the treatment combines the preliminary use of a supersaturated SBF solution ($5xSBF_1$) to stimulate the nuclei formation, while a fresh chemically-modified solution ($5xSBF_2$) is further used, in order to promote the growing of apatite nuclei, once formed. Meanwhile, $5xSBF_1$ was prepared by sequentially dissolving $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$ and $K_2HPO_4 \cdot 3H_2O$ in distilled water. Solution pH was lowered to 6 by adding hydrochloric acid HCl (1M) to increase the solubility. Na_2SO_4 , KCl and NaCl were added until the previous solution become clear. The final pH was adjusted with 1M NaOH to reach a final pH equal to 6.5 ($5xSBF_1$).

Therefore, Mg^{2+} and HCO_3^- free $5xSBF$ was prepared by adding $CaCl_2$, $K_2HPO_4 \cdot 3H_2O$ and NaCl subsequently to distilled water up to obtain a fresh solution ($5xSBF_2$). In this case, the solution pH was lowered to 6.0 with hydrochloric acid to increase the solubility. Both solutions were buffered at pH 6.5 and 6 respectively, by using Trishydroxymethylaminomethane-chloric acid (Trizma-HCl). In table 1, the ionic concentration of blood plasma, SBF, $5xSBF_1$ and $5xSBF_2$ were reported. All solutions were prepared freshly before the use.

The biomimetic treatment consists in two steps in pH-controlled environment: during the first step, samples with pre-ordered size were soaked into $5xSBF_1$ at pH=6.5 where the $5xSBF$ solution volumes have been calculated respect to the total scaffold material surface by using an exposed surface to SBF volume ratio equal to $10 \text{ mm}^2/\text{ml}$, as reported in literature [22]. The solution temperature was fixed at 37°C during the treatment. After the

sequential immersion in 5xSBF₁ (3 days) and in 5xSBF₂ (4 days), all scaffolds were gently rinsed in distilled water to remove excess ions and, then, dried overnight under laminar hood. To examine the distribution of HA particles in the scaffolds and a presence of coating after treatment in 5xSBF, the materials were stained with a hydrophilic dye (0.5% w/v trypan blue, Sigma) as reported elsewhere [13]. The residual dye was removed by washing in 100% ethanol.

Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was performed to evaluate the effective weight percentage of HA in the composite scaffold using a TA instrument TGA model Q500. Measurement was performed under nitrogen atmosphere by increasing the temperature from 20 up to 600°C through a 10°C/min scan.

Morphological Investigations

The morphological analyses were performed on HA/PCL composite and HA/PCL scaffold before and after treatment in 5xSBF by using a Quanta200 (FEI, the Netherland) Scanning Electron Microscope. For SEM analysis, the materials were deposited on side of a double adhesive tape and then stuck to an aluminium stub. The stub was then gold coated in an automatic sputter coater (Emitech Sk500, 20KV) up to a thickness of around 20nm.

Transmission Electron Microscopy (TEM) analysis was conducted using a Philips CM-10 instrument, at 100kV, in order to evaluate the dispersion of HA nano-particles in the polymer. The composite was embedded in resin and the block was cut by a diamond saw and ground up to 30 µm in thickness. X-ray energy dispersive spectroscopy (EDAX, Genesis 2000i) analysis was used for a qualitative estimation of the Ca/P ratio.

X-Ray diffraction and Infrared Spectroscopy FTIR analyses

Phase analysis was conducted using X-ray diffraction (XRD) to detect phase composition and crystallinity of HA powders and composite materials. An X-ray diffractometer (Philips) was used; on powders taken from specimens were scanned from $2\Theta = 10^\circ$ to 60° using $\text{CuK}\alpha$ radiation. FTIR spectroscopy (Nexus FT-IR, Nicolet) was used to identify the functional groups of hydroxyapatite powders, while an ATR support (Smart ARK, Nicolet) was utilized in order to evaluate a chemical composition of both phases in the composite material. FTIR spectra was recorded in the $500\text{-}4000\text{ cm}^{-1}$ region (4 cm^{-1} resolution, average 64 scans), while for ATR analysis a spectra was recorded in the $650\text{-}4000\text{ cm}^{-1}$ region.

Results*Composite scaffold characterization*

A sol-gel method was used in order to obtain a composite material with a ceramic phase homogeneously dispersed in polymeric phase. In order to evaluate the dispersion of organic and inorganic phases, a qualitative analysis by FESEM on the HA/PCL composite material was performed. The SEM image, in Fig. 2A, shows material which has been washed several times in water to remove ammonium nitrate residues. It is notable that the image shows a good dispersion of HA particles in the polymer matrix and the presence of microporosity due to solvent evaporation. Furthermore, the second SEM image of this sample (Fig.2B) shows the presence of large pores, due to the removal by water dissolution of the salt particles, which had previously been introduced during its preparation. Pore sizes ranges from 200 to 300 μm are in agreement with particle sizes (212-300 μm size range) used. Fig.2C shows a TEM analysis of HA/PCL composite. A homogeneous distribution of

n-HA crystals with 10–30 nm as diameter and 40–50 nm as length was detected into the composite. Element distribution analysis (EDAX) (Fig.2D) was executed in order to evaluate the presence of chemical elements like Calcium and Phosphorous typical of ceramic phase, Carbon and Oxygen typical of polymeric compound.

The representative FTIR spectrum (Fig.3A) shows all characteristic absorption peaks of stoichiometrically pure hydroxyapatite. The first indication of the hydroxyapatite formation is in the form of broad FTIR band centered at about 1000–1100 cm^{-1} [23]. The bands at 960–965 cm^{-1} and at 565–601 cm^{-1} correspond to ν_1 and ν_4 symmetric P–O stretching vibration of the PO_4^{3-} ion, respectively [24,25].

As a major peak of phosphate group, the ν_3 vibration peak could be identified in the region between 1100 cm^{-1} and 960 cm^{-1} , which is the most intensified peak among the phosphate vibration modes. The band between 565 cm^{-1} and 601 cm^{-1} belongs to ν_4 vibration mode of phosphate group which occupies two sites in the crystal lattice (at 601 cm^{-1} and 567 cm^{-1}).

The presence of weak bands of the CO_3^{2-} group (1415, 1450 and very weak 1540 cm^{-1}) indicates that it is slightly carbonated hydroxyapatite, which would be expected to be present since the sol–gel process involves organic solvent. The peak at 1040 cm^{-1} is the triply degenerated vibration, ν_3 , while at 946 cm^{-1} is the non-degenerated symmetric stretching mode, ν_1 , of the P–O bond of the phosphate group. The peaks at 603 and 565 cm^{-1} are assigned to a triply degenerated bending mode, ν_4 , of the O–P–O bond. The bands assigned to the stretching modes of hydroxyl groups (OH) in the hydroxyapatite at 3660 cm^{-1} [26] can be clearly observed in the spectra. It is possible to distinguish a broad envelope about 3400 cm^{-1} due to the O–H stretch of water; these O–H groups are hydrogen bonded.

The FTIR spectra (Fig.3B) of composite material (HA/PCL) prepared at 37°C shows the presence of both compounds. In addition to the presence of hydroxyapatite with a typical stretching and bending of PO_4^{3-} groups ($1027\text{-}1031\text{ cm}^{-1}$), it is possible to distinguish the presence of PCL with typical peaks at 1723 cm^{-1} , due to carboxyl group, and at 2800 and 2900 cm^{-1} , due to CH_2 and CH_3 groups

Figures 4(a-c) show the XRD patterns of HA, HA/PCL composite and PCL, respectively. The XRD pattern (fig.4a) of hydroxyapatite nanopowder as synthesized indicates the poor crystalline nature of typical apatite crystal structure with broad diffracted peaks. In addition in the pattern appears a presence of CaCO_3 derivated from organic solvents. The XRD pattern shows crystalline phases for both components. In particular, it can be inferred that pure PCL shows a high crystallinity (fig.4c), which decreases in the HA/PCL composite (fig.4b). Here, the peak positions of HA do not exhibit a significant difference, in compared with the pattern for HA (Fig.4a).

Figure 5 shows the thermogram curve reporting the weight loss (%) as a function of temperature. It clearly shows a residue at 400°C equal to 21.4%, slightly lower than the theoretical weight fraction used during the preparation. It was probably due to the leaching of some HA particles during the elimination of porogen.

Surface bioactivation

The SEM/Energy Dispersive X-ray Spectroscopy (EDAX) image of composite material after treatment in supersaturated solution 5xSBF is shown in Fig. 6. The image shows that after 3 days of soaking in 5xSBF_1 , followed by immersion for 4 days in 5xSBF_2 , the composite material was uniformly covered with a calcium phosphate crystals layer with a thickness of few microns. The analyses show that the rose petal-like apatite crystallites are

composed mainly of hydroxyapatite, with a Ca/P molar ratio of 1.67. The EDAX spectra clearly shows Ca and P peaks which are typical of hydroxyapatite, and smaller peaks of sodium, chlorine and magnesium due to the presence of impurities such as sodium, chlorine and magnesium commonly present in biomimetic apatite. It was also possible to elucidate the presence of HA deposition on the internal pore walls, confirming that the high concentration of SBF solution induced a fast and uniform deposition of hydroxyapatite on the scaffold. This result was confirmed by staining with a hydrophilic dye, 0.5% trypan blue (Fig. 7) which shows the progressive increase in blue intensity moving from PCL (A) and untreated HA/PCL (B) to treated HA/PCL scaffold, respectively, after 3 days and 7 days in 5xSBF solutions.

Discussion

Tissue engineering represents a new field, which aims to grow complex, three-dimensional tissues or organs to replace damaged tissues [27]. For this purpose, investigation of novel biomaterials for bone engineering represents an essential area for planning tissue engineering approaches. Future biomaterials should combine bioactive and bioresorbable properties to activate *in vivo* mechanisms of tissue regeneration, stimulating the body to heal itself and facilitating replacement of the scaffold by the regenerating tissue. A variety of biomaterials, including synthetic polymers [28-30], ceramics [31] and natural polymers [32] are being used to fabricate synthetic scaffolds which act as guides and stimuli for three-dimensional tissue growth [33,34]. Either biodegradable polymer or ceramic scaffolds, commonly being considered for bone tissue engineering, show different deficiencies in mechanical properties required for this application. Synthetic bioresorbable polymers are easily fabricated into complex structures, but are too weak to meet the rigours

of surgery and the *in vivo* physiological environment. Conversely, ceramic scaffolds have low toughness and strength. Efforts have been made in developing composites of polymers and ceramics with the aim of increasing the mechanical stability and to improving tissue interaction [35]. Such materials might possess favourable mechanical properties and strength due to the ceramic phase together with a toughness and plasticity attributable to the polymer phase. Thus, hybrid materials derived from the integration of biodegradable polymers with bioactive inorganic material might provide promising scaffolds to support bone regeneration.

In this context, a biodegradable hydroxyapatite/polymer (HA/PCL) composite material has been successfully produced by the sol-gel process, which enables the preparation of such hybrid organic-inorganic composite materials [36-38]. In this study, hydroxyapatite was synthesized by using non-alkoxide precursors and gel formation was achieved without the need for any refluxing steps. The presence of HA particles in the composite material beneficially offsets the acidic release from the polymer through the alkaline calcium phosphate [39] and mitigates erosion problems associated with the release of acidic degradation products. *In vivo* and *in vitro* measurements of pH in bone chambers have shown that the pH drop is 0.2 units near the eroding polyesters [40].

Molecular-level mixing of calcium and phosphorous precursors with the polymer chains derived from the sol-gel process resulted in composites having enhanced dispersion and exhibiting good interaction between the inorganic phase and the polymer matrix. The resulting morphology is apparent in the SEM/TEM micrographs of such a non-porous composite, material which shows a good dispersion of bioceramic particles (Fig.2). Here, common limitations of composites by melt processes such as particles agglomeration and undesired phase separation have been overcome by the use of a “cold” process with the use

of a reduced amount of ceramic phase. In addition, chemical analysis by EDAX identifies the typical peaks of HA and PCL respectively. This result was confirmed by FTIR spectra (Fig.3) with the peak due to carboxyl group of PCL and the presence of the stretching and bending mode of PO_4^{3-} . The XRD spectra (Fig.4) showed low crystallinity of a composite material, with any changes of the HA crystal structure during the process of composite preparation. Moreover, from the composite material spectra it is noticeable that the PCL crystallinity significantly decreased, probably due to chemical interaction with HA particles. Adaptation of scaffold manufacturing techniques based on salt leaching allows to obtain highly porous scaffolds with suitable pore size and morphology for cell colonization. Porosity is a key factor in the design of bioresorbable scaffolds for tissue engineering, essential for promotion of tissue growth and transportation of nutrients into the scaffold. The minimum pore diameter required for bone ingrowth and angiogenesis into a scaffold is considered to be at least 100 μm [41]. Previous *in vitro* studies have suggested that the ideal pore diameter for bone ingrowth is between 300-400 μm [42,43]. Here, macropores with hundred microns has been assured by introducing NaCl crystals with tailored sizes (Fig.2B). The presence of micropores, 0.1 to 5 μm in size, ascribable to solvent extraction by solvent evaporation, may concur to enhance the surface area for cell attachment and protein adsorption [44–47].

Both chemical and morphological cues are synergistically necessary to ensure an adequate biological response following implantation into bone tissue. Osteoconductivity is essential for the recruitment of cells capable of forming bone matrices and ultimately for successful bone in growth into scaffold [48]. Bone invasion in scaffolds can potentially be improved and accelerated by chemical surface modifications, or by the addition of an osteoconductive component in the polymer [48]. To modify the inner pore wall surfaces efficiently, without

altering the bulk structure and properties of the scaffolds, a biomimetic approach has been developed as a 3D surface modification technique to grow hydroxyapatite (the bonelike mineral) in prefabricated porous polymer scaffolds using a Simulated Body Fluid (SBF) [49]. Because the apatite particles are generated from aqueous solution, this technique can be used for various complex porous scaffolds, unlike many other surface modification methods that are limited to flat surfaces or very thin porous layers. This technique uses a biocompatible solution and very mild conditions (body temperature reaction), allowing a variety of polymers or composite materials to be used. The process is easy to carry out and does not require specialized equipment. Also, the hydroxyapatite generated is more similar to natural bone mineral in its low crystallinity and nanocrystal size, which is beneficial to its degradation and remodelling properties.

In *in vitro* experiments, mineral crystals were observed to grow on composite materials (Fig.6) after 7days in supersaturated biological solution (5xSBF). It was not surprising that higher concentration facilitated apatite formation, since it was reported [49] that increasing the ionic concentrations of a SBF upper the saturation limit is a practical way to facilitate apatite nucleation and growth. The crystals grew on the specimen surface to form a dense mineral layer, the blue staining with trypan blue showed a presence of ceramic phase in the composite material (blue region). More highly coloured (dark blue) regions have been detected on the surfaces after treatment in 5xSBF where HA crystals are completely formed.

In summary, results indicated that the nucleation of the mineral phase is highly enhanced by the presence of HA nanoparticles. The mechanism of apatite formation in SBF has been described by many researchers [50,51]. It has been reported that the formation of apatite on artificial materials is induced by functional groups which could reveal negative charge and

further induce apatite *via* the formation of amorphous calcium phosphate. In this work, the copious apatite formation on the composite scaffolds may directly correlate to the powerful contribution of nanoscale HA particles which act as more active nucleation initiation sites than microscale particles.

Conclusion

This paper has proposed a new strategy for composite manufacturing, combining bulk sol-gel transition and surface biomimetic techniques. The presence of a bioactive component such as the nanoscale HA particles in the composite materials obtained by the sol-gel process improves the development of an apatite coating that may enhance cell-seeding subsequent tissue growth in tissue-engineered products. This proposed integrated approach enables the efficient exploitation of the distinctive features of composite material bulk and surface regions by modulating the spatial distribution of bioactive signals, contributing to a more efficient replacement of bone tissue.

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Figure legend

Fig.1: Scheme of HA/PCL hybrid composite preparation by sol-gel technique.

Fig.2: SEM of HA/PCL composite material: (A) micro, (B) macroporosity. (C) TEM and EDAX analyses (D) of composite material.

Fig.3: FTIR of (a) HA as synthesized and (b) HA/PCL hybrid composite material.

Fig.4: XRD of a) HA as synthesized; b) HA/PCL hybrid composite material; c) PCL.

[■ PCL; * HA; ° CaCO_3].

Fig.5: Thermogravimetric analysis of HA/PCL composite scaffold performed in N_2 atmosphere at $10^\circ\text{C}/\text{min}$. Evaluation of average residual weight of HA.

Fig.6: SEM images and EDAX spectrum of HA/PCL scaffold after 7 days immersion in 5xSBF solutions.

Fig.7: Image of scaffolds after trypan blue treatment: (A) PCL; (B) HA/PCL hybrid composite material; (C) HA/PCL after 3 days of treatment in 5xSBF₁; (D) HA/PCL after 7 days of treatment in 5xSBF solutions.

| Ion Concentration (mM) | Na⁺ | K⁺ | Ca²⁺ | Mg²⁺ | HCO₃⁻ | Cl⁻ | HPO₄⁻ | SO₄²⁻ |
|---------------------------------------|-----------------------|----------------------|------------------------|------------------------|------------------------------------|-----------------------|------------------------------------|------------------------------------|
| Blood plasma | 142 | 5 | 2.5 | 1.5 | 27 | 103 | 1 | 0.5 |
| SBF | 142 | 5 | 2.5 | 1.5 | 4.2 | 148 | 1 | 0.5 |
| 5xSBF₁ | 710 | 25 | 12.5 | 7.5 | 21 | 740 | 5 | 2.5 |
| 5xSBF₂ | 710 | 10 | 12.5 | - | - | 735 | 5 | - |

Table1: The ionic concentration of human blood plasma, SBF, 5xSBF₁ and 5xSBF₂













