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Noncovalent immobilization of collagen on the surface of silanized hydroxyapatite

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The aim of this study was to synthesize and characterize a hybrid composite based on type I hydrated collagen, hydroxyapatite and a silane precursor (amino-propyltriethoxysilane). Through sol-gel approach of the precursor and noncovalent immobilization of collagen on the surface of hydroxyapatite, a new type of hybrid material was obtained in which microspheres comprising silica and hydroxyapatite particles are dispersed in an oriented fibrous network of collagen fibrils. Moreover, the sol-gel reactions between the above-mentioned components were not accompanied by conformational changes of the adsorbed collagen, thus preserving its secondary structure.

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1. Introduction

Hydroxyapatite (HAp: $Ca_{10}(PO_4)_6OH_2$) is widely used for bone implant and cement applications for dental tissue reconstructions due to its compositional and biological similarities to native tissues, as well as to the excellent biocompatibility with hard tissues and high osteoconductivity. Bone is an inorganic–bioorganic composite material consisting mainly of collagen and HAp, and its properties intimately depend on its nanoscale structure, which is dictated specifically by the collagen template [1, 2]. Collagen (Col) is the major component of extracellular matrices, such as tendons, ligaments, skin and scar tissues in vertebrates [3]. However, the biocomposites of Col and HAp alone do not have adequate mechanical properties for various biomedical applications including scaffolding tasks. Also, its brittleness and poor performance of mechanical stability limit its use for the regeneration of nonload-bearing bone defects [4].

The combination of calcium phosphates and natural or synthetic polymers to develop suitable bone substitutes has been intensively investigated and many researches have been performed to obtain the biocompatible, bioactive, biodegradable and osteoconductive properties of the natural bone. These composites are being developed to increase the mechanical scaffold stability and to improve tissue interaction [5-8]. Materials synthesized by sol-gel process are more bioactive than the materials of the same composition prepared by other methods [9, 10]. Only limited attempts have been reported on the sol-gel processing of hydroxyapatite material [11-13].

The general objective of this study was to evaluate the noncovalent immobilization of collagen (Col) on the suface of hydroxyapatite (HAp) in the presence of a silane coupling agent, e.g., amino-propyltriethoxysilane (APS). Col immobilization on APS/HAp surface was investigated by FTIR spectroscopy, SEM analysis and fluorescence measurements.

2. Experimental

2.1. Synthesis of HAp/Col hybrid composite

The Hap/Col hybrid composite was obtained following the reaction between 2 g (9.034 mmol) APS, 0.07 N hydrochloric acid (pH = 3) and stoichiometrical amount of water at room temperature for 2 h. Next, 0.4 g type I hydrated Col and 0.6 g HAp (1.194 mmol) were added to the mixture under vigorous stirring for another 24 h. After solvent evaporation, the product was obtained as a white solid and dried in vacuum at 60ºC for 3 days.

2.2. Measurements

FTIR spectra were performed on a Bruker Vertex 70 instrument, in the 400–4000 cm^{-1} region, 64 scans, at room temperature, using the KBr pellet technique and the Opus 5 FTIR Software. The SEM micrographs were obtained with a Quanta 200 scanning probe microscope, the specimens being fixed with adhesive past on Al conducting supports of cylindrical shape and then sputter-coated with gold. The fluorescence spectra were obtained at room temperature (without correction) with an

equipment containing a double monochromator with diffraction network of the GDM-1000 type, a compensatory printer of the K-201 type and a selective amplifier.

3. Results and discussion

Sol-gel methods offer the possibility of mixing calcium and phosphorus precursors on a molecular scale, which is advantageous in obtaining products of homogeneous composition. The sol-gel reactions allow HAp to be processed into films and coatings on ceramic substrates [14-16]. A bioactive calcium phosphate coating can improve bonding between natural bone and a titanium or titanium alloy implant [17, 18]. The association of type I collagen with minerals forms hybrid materials having unique properties depending on the nature and size of the mineral, its dispersion in the collagen scaffold and the orientation of the fibrils.

The FTIR spectra of HAp/Col hybrid composite and type I hydrated Col are shown in Figure 1. The spectrum corresponding to HAp/Col hybrid composite is characterized by the presence of absorption bands arising from HAp, Col, as well as by the ones (vibrational) from the silicate. A characteristic broad band appears around 3437 cm^{-1}, and corresponds to O–H stretching vibrations of the hydroxyl groups due the strong hydrogen bonds of intra(inter) molecular type [19] and hydrogen-bonded N-H units from collagen. The sample presents a larger amount of structural or adsorbed water molecules: the lower the pH during the synthesis (pH = 3), the larger the presence of water in material structure. The C-H alkyl stretching bands can be observed also around 2928-2980 cm^{-1}. The FTIR spectrum of Col evidences, among other signals, the presence of amide I peak at 1651 cm^{-1} (C=O stretching), amide II peak at 1543 cm^{-1} (N-H deformation), and amide III peak around 1243 cm^{-1} (N-H deformation). The amide I, II and III band regions from collagen molecules are directly related to polypeptide conformation, while the amide I one in particular is a sensitive marker of polypeptide secondary structure [20]. Following sol-gel reaction, the amide I, II and III positions from Col in HAp/Col hybrid composite were shifted to smaller wave numbers (1648, 1516 and 1231 cm^{-1}). Results of the amide I band analysis (a small shift of only 3 cm^{-1}) show that protein secondary structure is not affected by Col/HAp sol-gel interactions and this type of reactions are not accompanied by conformational changes of the adsorbed protein.

In FTIR spectrum corresponding to commercially available, reagent-grade HAp, the most intense bands, in the 1110–1050, 978, 874, 606, 573, 476 cm^{-1} domains, can be assigned to the main absorption modes of the phosphate groups of apatite [21, 22]. For HAp/Col hybrid composite, the absorption bands from 1125-1030 (overlapping with Si-O-Si vibrational spectra), 940, 900, 603, 563, 483 cm^{-1} are associated with the PO_4^{3-} groups of HAp. Furthermore, all these signals are shifted in comparison with the ones belonging to HAp. This behavior can be related to the interactions between the OH groups from HAp, Col and APS, respectively.

In the case of hydrated collagen, the most tightly bound water fraction consists of two water molecules for every three aminoacid residues, and provides water bridges between the three strands of the collagen molecules, linking backbone carbonyl groups [23]. Therefore, the absence of –COOH end groups (normally appearing at 1716 cm^{-1} for type I collagen) precludes the possibility of covalent bonding of collagen to HAp/silane coupling agent.

The vibrational spectra of the silicates can be divided into two regions. The first region covers the frequency range 4000-1600 cm^{-1}, where stretching and bending vibrations of water molecules appear. The second region (below 1300 cm^{-1}) includes the vibrations due to the silicate layers. In general, the γ_{as}(Si-O-Si) modes appear in the 1200-1000 cm^{-1} region, whereas γ_s(Si-O-Si) becomes observable in the 700-400 cm^{-1} region. With increasing silica contents of the hybrid composites there is an intensity increase of some characteristic peaks – such as Si–O–Si stretching – of the silica phase, the presence of such type of bonds confirming the existence of covalent linkages between the organic groups and the silica. These bonds lead to a better compatibility and to the formation of crosslinks between organic and inorganic components. For HAp/Col hybrid composite, asymmetric Si-O-Si bands can be observed at 1125 and 1030 cm^{-1}. Additional peaks at around 698-750 cm^{-1} (for symmetric Si-O-Si stretching vibration) can be also seen.

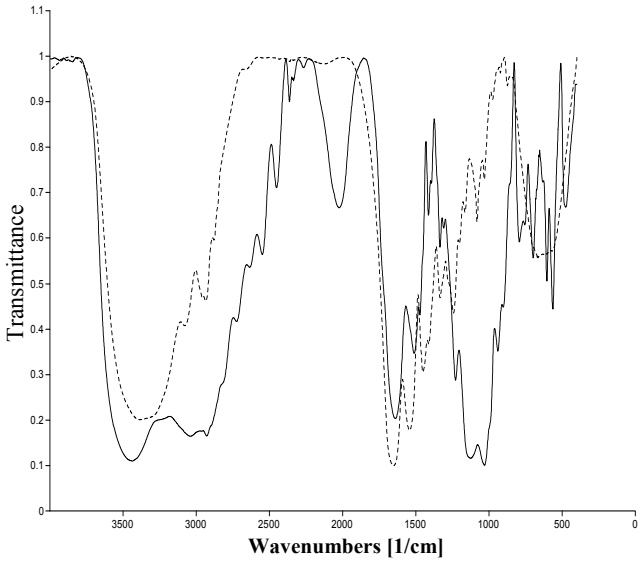

Fig. 1. FTIR spectra of HAp/Col hybrid composite (—) and type I hydrated Col (---).

Silica has a complex morphology that spans nanometer to micron size-scale. Manipulation of the morphology at each structural level determines the properties of the product. The physical properties of silica, e.g., the specific surface, the dimensions of the primary particles and aggregates, as well as the porosity,

essentially depend on the synthesis procedure. The differences between these physical characteristics are correlated with the organization form of the silica particles (aggregates or agglomerates). SEM characterization was conducted in order to evaluate the morphology of HAp/Col hybrid composite. SEM micrographs (Figure 2) show microspheres comprised of silica and HAp particles dispersed in an oriented fibrous network of collagen fibrils. Previously, it was reported that I type collagen fibrils act as a template for the structuration of silica. Moreover, it seems that pH controls not only the initial collagen aggregation state, but also defines the silica particle size and morphology within the hybrid materials [24].

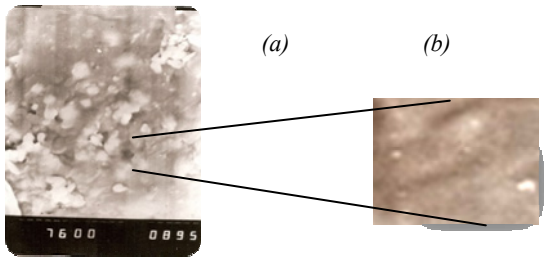

Fig. 2. SEM images of HAp/Col hybrid composite at different resolutions: (a) 13 μm, (b) 1.8 μm.

The fluorescence measurements provide useful information for better understanding the relationship between the environment of Col in a hybrid composite and its spectroscopic properties. Fluorescence spectra of Col/HAp hybrid composite in buffer solution (pH = 7.2), at different excitation wavelengths, e.g., 280, 320 and 370 nm (Figure 3), show three major emission peaks at 400, 412 and 440 nm. The first region has an emission maximum at 400 nm, consistent with the fluorescence of pyridinoline [25]. The second region (412 nm) is an indication of closely spaced overlapping peaks attributed to the fluorescence of Col crosslinks [26, 27]. The crosslinks, consisting of many self-assembled collagen molecules with intra- and intermolecular crosslinks, are believed to be responsible for the observed strong auto-fluorescence of collagen. The final structure of the crosslinks from Col fibers has been identified for hydroxylysyl pyridinoline and lysyl pyridinoline [28], since both compounds have excitation/emission maxima at 325/400 nm. The red-shift of the peak emission (from 400 nm – free pyridinoline in water – to 412 nm – Hap/Col hybrid composite in buffer solution) is likely due to hydrogen bonding between pyridinoline carbonyl and hydroxyl silane groups.

The fluorescence at 370 nm excitation (440 nm emission) is predominantly determined by the fluorescence properties of Col crosslinks due to maturation of the collagen fibers. The formation of Col crosslinks usually requires either an enzyme, lysyl oxydase, or the presence of carbohydrates or lipids [29-31]. None of these substances were added in the synthesis of Hap/Col hybrid composite. However, residual crosslinking enzymes could be present in the Col sample, thus resulting in the formation of Col crosslinks.

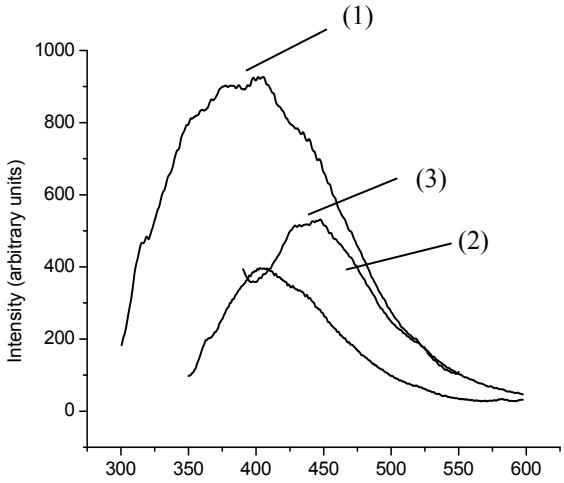

Fig. 3. Fluorescence spectra of the HAp/Col hybrid composite at different excitation wavelengths: (1)λ_{ex} = 280 nm; (2) λ_{ex} = 320 nm; (3) λ_{ex} = 370 nm.

4. Conclusions

The technique based on sol-gel polymerization described in this paper represented a versatile synthetic approach yielding hybrid composites with tailor-made composition, of organic core and silica or organo-silica shells. Type I hydrated Col fibrils acted as a template for the structuration of silica. Thus, microspheres comprising silica and HAp particles dispersed in an oriented fibrous network of Col fibrils were obtained. FTIR measurements highlighted the ability to control protein conformation and orientation upon noncovalent immobilization onto the surface of silanized HAp while preserving the secondary structure of Col, whereas the fluorescence ones provided useful information on the behaviour of Col fibrils in a hybrid composite and its spectroscopic properties.

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