Carboxymethylchitosan/Calcium Phosphate Hybrid Materials Prepared by an Innovative Auto-Catalytic co-Precipitation Method

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Abstract. In this study, it is shown that it is possible to prepare carboxymethyl-chitosan/Ca-P hybrids using an innovative “auto-catalytic” co-precipitation method, namely by using an acid and an oxidant bath. The X-ray diffraction (XRD) patterns evidenced the formation of crystalline calcium-phosphate precipitates when using an acid bath, while amorphous ones were obtained for those produced in the oxidant bath. The Fourier Transform Infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM/EDS) studies revealed that the extent of the polymer precipitation and formation of calcium-phosphates is directly dependent on the pH and composition of the baths. Furthermore, by conducting bioactivity tests in a simulated body fluid (SBF) followed by the SEM/EDS analysis it was possible to detect the formation of an apatite layer with a cauliflower-like morphology on the surface of hybrids prepared by the acid bath, after 7 days of immersion. These results are quite promising because they can allow for the production of bioactive and biodegradable 3D porous scaffolds to be used in bone tissue engineering applications.

Introduction

Hydroxyapatite (HA), is a calcium phosphate ceramic that has been widely used as a bone replacement material. However, it has not been possible to apply it in small load applications due to its poor mechanical properties, namely its brittleness. To circumvent this problem, Reis et al. [1] have been giving great attention to the preparation of hybrid materials which typically combines the toughness and flexibility of the polymeric phase, and strength and hardness of the inorganic one. Carboxymethyl-chitosan (CM-chitosan) is a biocompatible and biodegradable polymer. It is negatively charged ether derivative of chitin or chitosan, which can be water-soluble, and has several applications in drug delivery, medicine and pharmacy fields, among others. It has been reported that CM-chitosan may act as a nucleator of metal ions including Ca2+ [2]. Therefore, by attracting the calcium ions it is expected that one can promote the precipitation of calcium phosphates (Ca-P) both in vitro and in vivo.

This work aims at developing and characterising novel biodegradable CM-chitosan/Ca-P hybrids using an innovative co-precipitation method which is based in a previously reported [3] Ca-P “auto-catalytic” deposition route. The developed matrices are aimed at tissue-engineering scaffolding applications.

Materials and Methods

CM-chitosan was prepared by a modification of the reaction process that has been previously described by Chen et al. [4]. Hybrid materials were prepared by a co-precipitation method using two different baths: (i) acid (Calcium chloride 5.6 g.L-1, Sodium hypophosphite 24.0 g.L-1, orthophosphoric acid 3.4 g.L-1, Palladium chloride 0.9 g.L-1, Sodium fluoride 5.0 g.L-1, and pH adjusted to 6), and (ii) oxidant (Calcium chloride 5.6 g.L-1, Sodium pyrophosphate 6.7 g.L-1, Palladium chloride 0.9 g.L-1, Oxygen peroxide 34.0 g.L-1, and pH adjusted to 7). This methodology is based on the auto-catalytic (electroless) route that has been successfully developed in our group to modify the surface of bioinert polymers [3]. Bioactivity tests were performed in a simulated body fluid (SBF)
with the inorganic ion concentrations resembling to those found in human blood plasma, during 7 days. The physicochemical characterization of the hybrid materials before and after immersion in SBF solution was performed using Scanning Electron Microscopy (SEM/EDS), Fourier Transform Infrared spectroscopy (FTIR) and X-ray Diffraction analysis (XRD).

Results and Discussion
The overlaid FTIR spectra revealed that CM-chitosan is present in both co-precipitation baths (Fig.1). Fig.1A shows the more intense absorption bands in the CM-Chitosan and one of those corresponds to the −OH groups (○) located at 3450 cm\(^{-1}\). This band is also detected for the precipitates showing that this methodology is effective in the precipitation of CM-chitosan (Fig. 1B-C). The band observed at 1735 cm\(^{-1}\) is attributed to the −COOH group (−), and is only detected when the acid bath is performed (Fig.1B). This fact is explained by low pH of the acid bath, whereas at the higher pH values it’s absent (Fig.1C). Therefore, proceeding from pH 3 to 7, the band at 1735 cm\(^{-1}\) diminish whereas those arising from substituted amines and imines (1620 cm\(^{-1}\)), and of carboxyl ion (1420 cm\(^{-1}\)) increases. This observation is a consequence of the ionization of −COOH groups to −COO\(^{-}\) [5,6]. The PO\(_4\) band (ν3) detected at 1190-1020 cm\(^{-1}\) (+), and PO\(_4\) (v4) at 566 cm\(^{-1}\)(*) revealed the presence of calcium phosphates in both precipitates. These results demonstrated that CM-Chitosan/Ca-P hybrid materials may be prepared using both acid and oxidising co-precipitation baths.

By means of performing potentiometric titration of the CM-chitosan it was possible to verify that the precipitation of this polymer occurred preferentially in the range of pH’s comprised from ~2-6 (data not shown). Additionally, SEM studies of the CM-chitosan/Ca-P hybrids have shown the existence of two phases, the organic and the inorganic one (Fig. 2A-B). The EDS analysis revealed that the inorganic phase consisted of calcium-phosphates (Fig. 2C-D). Furthermore, the elemental analysis evidenced a higher content of C, and lower of Ca and P for the CM-chitosan/Ca-P hybrids prepared from the acid solution when compared with those of oxidising bath. These results have shown that the polymer precipitation occurred at a higher extent when using the acid bath, as expected. This observation is extremely important because by changing the pH of the precipitation baths we are able to tailor the chemical composition of the hybrids as well as its mechanical properties.

One of the main obstacles to date in the development of certain biomedical applications of chitosan is the impossibility to incorporate pH-sensitive proteins due to inability to maintain chitosan solution up to physiological pH [7]. Therefore, by preparing CM-Chitosan by means of chemical modification of chitin/chitosan will make possible to circumvent this limitation. Besides the possibility of controlling the chemical and mechanical properties of the hybrid materials by varying
the extent of the polymer/Ca-P precipitation, these innovative co-precipitation baths also allow to adjust the pH of the medium to incorporate a larger variety of molecules, in a wider range of pH’s. In this context, we believe that this innovative method is a reliable and feasible route to incorporate different pH-sensitive proteins in the bulk of 3D scaffolds without any detriment for its activity.

The XRD pattern of the CM-chitosan/Ca-P hybrids and precipitates obtained through the acid bath, clearly demonstrated that the calcium phosphates peaks perfectly matched with the standard file for HA (JCPDS 9-432; Fig. 3D-E). As seen the Ca-P, obtained from the oxidant bath are poorly crystallized (exhibited broad diffraction peaks) when comparing with those of the acid bath (Fig. 3B-C). As shown using these co-precipitation baths it is possible to tailor and control the Ca and P composition of hybrids and the crystallinity of the inorganic phase, and consequently the respective absorption rates.

Kokubo et al. [8] has shown that the formation of an apatite layer occurred on the surface of several materials (e.g. ceramics, metals and polymers) in a simulated body fluid. On the base of this finding, one is now able to understand in a greater extent how an artificial material bonds to living bone. In this context, in vitro studies in SBF were conducted to assess the capacity of these novel CM-chitosan/Ca-P hybrids to induce the formation of an apatite layer on its surface. The SEM analysis revealed the presence of a dense and uniform apatite layer with a cauliflower-like
morphology, recovering all the surface of the CM-chitosan/Ca-P hybrids prepared using the acid bath, after 7 days of immersion in SBF (Fig.4A-B). The EDS analysis revealed that these precipitates consisted of calcium-phosphates (Fig. 4C). Further studies will be carried out to clarify, in detail, the nature of the apatite layer on the surface of the CM-chitosan/Ca-P hybrids, namely performing TF-XRD analysis.

Fig. 4. SEM images of: (A-B) CM-chitosan/Ca-P hybrids prepared through the acid co-precipitation bath, after 7 days of immersion in SBF solution, and (C) respective EDS analysis.

**Conclusions**
The obtained results shown that it is possible to prepare CM-chitosan/calcium-phosphate hybrids by applying a novel auto-catalytic co-precipitation method both using a novel oxidant and acid baths. Additionally, it was possible to conclude that the extent of the polymer precipitation and formation/crystallinity of calcium-phosphates are directly dependent on the pH and content of the bath. The developed materials are very promising whenever degradability and bioactivity are simultaneously desired. Furthermore, this method can be easily adapted in order to produce bioactive and biodegradable 3-D porous scaffolds which combine both organic and inorganic matrices finding applications in tissue engineering scaffolding.

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**References**