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Incorporation of proteins and enzymes at different stages of the preparation of calcium phosphate coatings on a degradable substrate by a biomimetic methodology

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Abstract

In this work, the possibility of incorporating proteins into calcium phosphate (Ca-P) coatings, prepared on the surface of starch polymeric biomaterials by means of a biomimetic route, was investigated. The morphology, chemical composition and crystallinity of Ca-P coatings was assessed and related to the incorporation of the studied biomolecules. For that, bovine serum albumin (BSA) and α-amylase were added in concentrations of 1 mg/ml to simulated body fluid (SBF) solutions, being both added at the nucleation or growth stages of the biomimetic coating process. A biodegradable blend of corn starch/ethylene vinyl alcohol (SEVA-C) was used as substrate and bioactive glass (45S5 Bioglass®) was used as the nucleating agent. The obtained Ca-P coatings were characterised by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), Fourier transform infrared spectroscopy using an attenuated reflectance device (FTIR-ATR) and thin-film X-ray diffraction (TF-XRD). Additionally, to evaluate the activity of the incorporated enzyme and the stability of the Ca-P films, coated samples were immersed in an SBF solution for different periods of time. The enzyme activity was measured and the morphology of the coating examined by SEM. The results obtained showed that the presence of protein molecules, at the nucleation or growth stages, lead to the formation of a dense Ca-P film presenting different morphologies that were different of the selected coating conditions. FTIR-ATR analysis detected the presence of carbonate and phosphate groups on the Ca-P layer, indicating the formation of a coating similar to the mineral component of vertebrates bone tissue. When proteins were added, amide I and amide II bands, characteristic groups of protein molecules, were also detected, revealing the efficient incorporation of these biomolecules into the Ca-P coatings.

Ca-P coatings, with α-amylase incorporated at the nucleation stage, showed no degradation of the film after incubation in SBF for 28 days. The release of increasing concentration of reducing sugars with degradation time revealed that α-amylase was efficiently incorporated in the coating remaining active throughout the coating preparation. This can be a strategy that will allow, in addition of conferring osteoconductive properties to biodegradable polymers, also simultaneously tailoring their degradation kinetics.

Keywords: Biodegradable polymers; Starch; Biomaterials; Biomimetic coatings; Calcium phosphate; Enzymes and proteins incorporation; Degradation; Bone

1. Introduction

Hydroxyapatite (HA) has been often designated as an osteoconductive material [1]. However, one common problem of ceramics, intended for load-bearing clinical applications, is their poor mechanical properties, such as low bending strength and brittle nature combined with low values of fracture toughness [2,3]. On the basis of these findings, adaptative interface functionalities were set as a target, keeping in mind that they should not compromise either chemical or physical bulk properties, and must simultaneously improve the tissue integration. This would open up the possibility of using non-bioactive materials including metals and non-metals that have the necessary strength and toughness to act as bone-bonding implant

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materials on load-bearing sites. HA can be effectively used as a coating material on a substrate that will ensure the biofunctionality.

In the last few years, coating orthopaedic implants with calcium phosphates (Ca-P) have been investigated, being proposed as a rather successful approach [4–7]. However, most of the available and common methods for producing adequate Ca-P coatings, such as the plasma-spraying technique, are difficult to control on what concerns to the calcium phosphate (Ca-P) layer composition, degree of crystallinity and capability for strong adhesion to the substrates [4,6,8,9]. Generally, these techniques need temperatures of more than 500 °C, which result in the degradation of HA to various calcium phosphate phases [10]. HA properties will tend to differ in chemical composition, in crystal structure as number of defects of apatite crystals, and/or in the crystallite sizes [11]. Furthermore, techniques that involve high temperatures are unsuitable for coating polymeric biomaterials and do not allow for the incorporation of bioactive agents into the coating.

To overcome the above-mentioned disadvantages, a biomimetic methodology, adapted from Kokubo et al. [11], has been used in our research group to produce Ca-P coatings on the surface of starch-based biomaterials [12–19] without requiring the use of high processing temperatures.

Bone growth into HA-coated implants is not always satisfactory. In fact, there is still a need for improvements regarding the induction of bone formation at the implantation site. There are indications [20] that the ion release from Ca-P coatings, as well as the composition and topography of the Ca-P layer, may indirectly affect cellular pathways like attachment and proliferation. These bioprocesses are involved in bone integration through altered ligand-cell receptor affinities, varied calcium and pH-dependent enzyme kinetics, and a compositionally or structurally altered extracellular matrix protein environment [21,22]. For the successful application of these methodologies, there is the need to understand the integration process of newly formed bone as well as the calcification at HA–bone interface, so far could not be fully elucidated.

Mineralisation of bone is a highly organised process controlled by many kinds of modulators. These modulators may affect the nucleation and growth of the mineral phases. Several proteins have been identified to have the ability to induce bone formation, including the well-known bone morphogenetic proteins (BMPs) [23–27]. The functional groups of these biomolecules are thought to play a major role in inducing bone formation and binding to bone minerals, thus affecting a uniquely strong interface.

The use of nature inspired methods, such as biomimetic coatings, presents the advantage of using physiological conditions, like mild temperature and pH. This in turn allows for incorporating bioactive species without compromising the performance of the coatings and to improve the functionality of the inorganic layer at the implantation interface. Several authors [28–41] have proposed models to explain the nucleation and formation of Ca-P layers in the presence of biomolecules. Proteins, enzymes, bone related proteins, mixtures and serum protein models, as other biologically important molecules, have been investigated for this purpose.

In this work, the possibility of incorporating proteins and enzymes at different stages of the preparation of Ca-P coatings by a biomimetic methodology was studied. Serum albumin was used as a model protein due to its concentration in the blood plasma, binding and diffusion properties. On the other hand, α-amylase was exploited in terms of enzymatic properties, ability to interact with starch-based materials and tailor their degradation kinetics for a specific application. The influence of protein supplementation on the morphology, chemical composition, crystallinity and stability of the Ca-P coatings was investigated for both the nucleation and the growth stages. The adequacy of the selected procedure on the incorporation efficiency and activity of the biomolecules was also evaluated. In this case, the aim was to investigate the carrier potential of the Ca-P coatings for sustained release applications.

2. Experimental part

2.1. Materials

The material used in this study was a biodegradable polymeric blend of corn starch with poly(ethylene-vinyl alcohol) copolymer (50/50 wt.%), designated by SEVA-C. Using conventional injection moulding technology (Klockner-Ferromatik Desma FM20), 2×4 mm^2 cross-section dumb-bell ASTM tensile samples were produced. Further details on the material processing and respective mechanical properties can be found elsewhere [42,43].

A bioactive glass (45S5 Bioglass®) with the following composition: 45 SiO_2, 24.5 CaO, 24.5 Na_2O and 6.0 P_2O_5 in wt.%, was supplied by US Biomaterials Corp. (Florida, USA). This glass powder was used as precursor for calcium phosphate film deposition and, by laser scattering analysis (Coulter LS 100 particle size analyser), particle size was found to be lower than 20 μm.

Bovine serum albumin (BSA, Fraction V) was obtained from Sigma (St. Louis, USA) and α-amylase, from *Bacillus amyloliquefaciens*, was gently supplied by Genencor International (Rochester, NY, USA).

2.2. Preparation of Ca-P coatings—adapted biomimetic route

The procedure used to produce Ca-P coating was based on the methodology previously developed by Abe et al. [11] and further adapted by Reis et al. [12,19]. Prior to the coating process, SEVA-C samples were sterilized by ethylene oxide in optimized conditions previously described [44].