Urease Functionalized Silica: A Biohybrid Substrate To Drive Self-Mineralization

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Calcium phosphate based biomaterials plays a key role in bone replacement and/or reconstruction procedures,[1] either as the main component of cements, minor component of bioglasses, or simply dispersed in the form of surface coatings onto hard metallic pieces.[2] In the latter case, the films are mainly deposited under energetic conditions, with physically driven procedures such as plasma-spray or magnetron sputtering.[3] Alternative solution mediated methods, based in the controlled precipitation of HAP-like coatings, require hydrothermal conditions.[4] Then, both aforementioned routes do not allow the incorporation of labile bioactive molecules within the matrix. Moreover, the use of thermolabile scaffolds as biopolymers or gels is also limited. If labile substrates are employed, the soft chemistry procedures, mainly based on the immersion in simulated body fluids (SBF), are required, demanding long periods of time to achieve HAP-like film deposition. Nowadays, the deeper knowledge of the biologically driven mineralization process inspires novel biomimetic approaches for the preparation of composite biomaterials, under mild or even physiological conditions.[5] In this sense, a successful approach consists in the emulation of the active site of certain specific biomineralizing enzyme,[6] by means of related enzyme-mimetic small molecules, allowing the formation of silica phases in the neutral pH typically employed by living entities.[7] Another approach is based in the proper coupling of a known enzymatic driven reaction with the proper reagents, in order to develop a certain mineral phase, naturally developed by living organisms. In this scenario, the in situ generation of phosphate ions in the presence of Ca(II) ions, driven by alkaline phosphatase, has proved to be a suitable strategy for the generation of HAP-like phases.[8] However, this enzyme remains active in a narrow pH window (8.0−9.0), limiting its application.[9] As an alternative, Ureases catalyze urea hydrolysis into ammonium carbonate (eq 1) in a wider pH range (4.0−9.5); that reaction can act both as a source of carbonate or hydroxyls. In the first case, it can be properly coupled to drive the homogeneous precipitation of biomineral phases such as calcium and/or magnesium carbonates (eq 2).[10] In the second case, when acid solutions containing phosphate and Ca(II) ions, different calcium phosphates phases (eq 3), can be obtained, depending on the initial Ca(II) to P(V) ratio as well as the aging conditions.[11] If an excess of urea is hydrolyzed, the already precipitated solid evolves into carbonate containing calcium deficient apatites (see Supporting Information, eq 4). Then, this family of urea−urease driven precipitations arises as a versatile route for the formation of several biomineral phases.[12]

$$OC(NH_2)_2 + 2H_2O \rightarrow 2NH_4^+ + HCO_3^- + OH^- \quad (1)$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad (2)$$

$$10Ca^{2+} + 6H_2PO_4^- + 14OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6H_2O \quad (3)$$

Previous reports indicate that once aged in the presence of urea, free urease, Ca(II), and phosphate ions, certain metallic substrates developed HAP-like particulated coatings.[13] More recently, the chemical attachment of urease onto the surface of polymeric fiber substrates was proposed as a way to localize the mineralization process onto those substrates. Notwithstanding, the high Ca(II) concentrations employed in both cases resulted in nonhomogeneous coat-

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ings, with micrometric globular structures.[14] Despite the growing interest on bioglass based materials and/or coatings, no data concerning the application of urease based routes over silica substrates can be found in literature.[15] The aim of the present work is to explore the use of urease to drive HAP-like film deposition over the silica surface, after short times and mild conditions. In order to simplify mass transport boundary conditions, synthetic monodispersed submicrometric silica spheres were employed as a model substrate. Our first attempt to achieve silica functionalization was based on direct adsorption of urease onto the bare silica surface. However, after washing the treated particles, no significant ureolitic activity nor mineralization capacity was retained on the substrate. This result is consistent with previous reports, which indicate that the intrinsic affinity of urease for silica surface is not strong enough to achieve active functionalization. The texturation of support combined with the stabilization of the loaded enzymes with polyelectrolyte shells is required to preserve useful activities.[16] Then, in order to achieve proper active enzyme loadings onto the silica surface without shielding it with polyelectrolytes, a first step based on covalent anchorage was employed. To this aim, amino-capped silica particles were synthesized and bio-functionalized with urease, by direct covalent attachment using glutaraldehyde as the coupling agent.[17] Once the excess of nonlinked enzyme was washed away, the bioactive particles were aged in the presence of the mineralizing solution (Ca(II) 6×10^{-3} M; P(V) 3.6×10^{-3} M; $[\text{urea}]_0 =$ 0.5 M). The initial pH = 4.00 was chosen in order to maximize the initial soluble Ca(II) and P(V) while preserving urease from irreversible acid denaturation.[18] In parallel to the aforementioned experience, a reference solid was prepared aging the same starting solution using free urease in the absence of silica substrate, in a homogeneous fashion. The enzyme was added in a proper amount, in order to achieve a similar alkalinization/precipitation rate in both experiments. Concerning the matrix effects over the urease activity, it was reported that Ca(II) does not affect this enzyme and only transition metal cations are able to inactivate it.[19] However, effects related with the presence of phosphate anions cannot be disregarded.[20] Preliminary kinetic runs indicated an aging period of 6 h to achieve mineralization (see Supporting Information). After that period, both the mineralized silica particles and the reference material were filtered and washed for further analysis; Figure 1 shows TEM images of a typical enzyme capped silica particles before and after the biomineralization process.

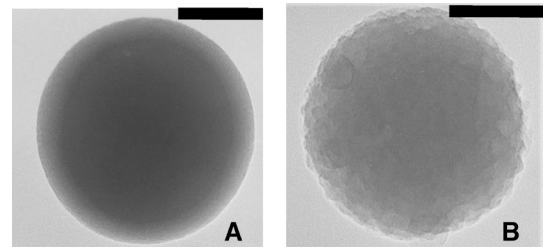

Figure 1. TEM images of a typical enzyme capped silica sphere before (A) and after (B) the mineral shell growth. Scale bar represents 100 nm in both images.

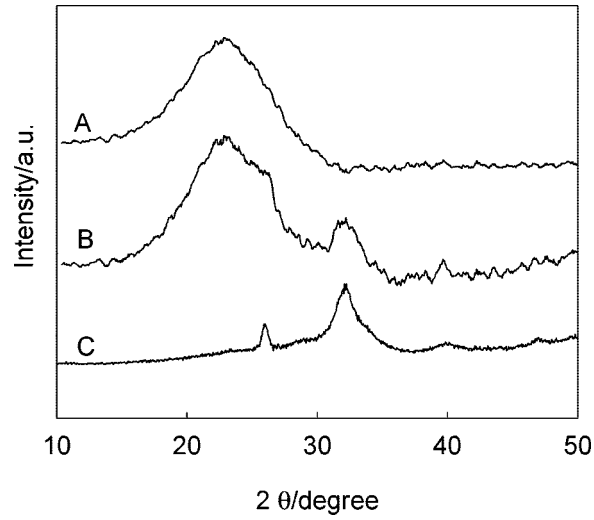

Figure 2. PXRD patterns of a typical enzyme capped silica sphere before (A) and after (B) the mineralization process. The pattern of pure HAP-like particles obtained by homogeneous precipitation in the absence of silica spheres is also presented (C).

The initially smooth surface gives birth to a rough shell with a flake-like texture; those units of 10 nm long flakes were not observed as free entities. In addition, no uncoated silica spheres and/or surface regions were observed (see Supporting Information), indicating a good attachment and homogeneous deposition of the biomineral coating. The chemical nature of the shell was estimated by means of TEM-EDS probe applied over isolated particles; in all the cases, the Ca/P ratio was ca. 1.6 ± 0.1, practically that of HAP. The SEM-EDS probe exploration of large clusters of coated spheres confirmed the same composition. PXRD inspection of the mineralized spheres revealed a minor shoulder centered at $2\theta = 26°$ mounted on the main (broad) peak of silica substrate, and a more intense one centered at $2\theta = 32°$ (see Figure 2). The latter asymmetric peak can be ascribed to overlapped 211, 112, 300, and 202 reflections of HAP while the former shoulder corresponds to the 002 one, exclusively. Then, the mineral layer will be referred in the following as a HAP-like phase. The control precipitation experiment with free urease (homogeneous precipitation reaction) resulted in larger micrometric polycrystalline rose-like aggregates, suggesting the occurrence of less nucleating events (see Supporting Information). The PXRD pattern of this phase also denotes a more defined cristallinity HAP-like structure (see Figure 2).

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Electrokinetic measurements of the nonmineralized spheres and the mineralized ones revealed a partial decrease of surface charge of the latter with respect to the former, toward the values observed for pure HAP-like particles (reference material) precipitated in the absence of silica substrate. It is worth mentioning that, in both cases, single Gaussian profiles of mobility distribution were observed in each measurement, indicating that both the initial functionalization and the final HAP-like shells were homogeneously distributed over the entire silica particles population.

Concerning to the growth mechanism of the HAP-like shell, two extreme cases can be envisaged. In the first one, the urease capped silica particles just act as a source of base, triggering the homogeneous precipitation of HAP-like nanoparticles in the bulk solution. Once those newly born particles reach a critical size, they can self-assemble onto the silica surface due to an electrostatic driven heterocoagulation process.[21] In the second one, the HAP-like nanoparticles are nucleated and grown onto the silica surface in a strictly heterogeneous process, arresting the possible growth of larger crystals or aggregates in the bulk solution. If the first mechanism rules the precipitation process, no differences must be observed when the precipitation reaction proceeds with the free urease in solution or attached to silica, which is not the present case. A strictly homogeneous nucleation followed by the heterocoagulation of HAP-like particles onto the silica surface is unprobable, since electrokinetic measurements revealed that both the silica and the HAP-like particles exhibit negative charges along the pH range of precipitation.[22] Then, direct growth of the HAP-like particles onto the silica surface, seems to be the ruling mechanism. This is in line with several studies that documented the nucleation and growth of HAP-like deposits onto silanol rich phases, as is the case soda-based glasses, once exposed to simulated body fluids (SBF).[23] In the present scenario, the role of silica is two-fold: it provides a region in which the base concentration is maximized while it offers reactive silanol groups, minimizing nucleation energy barriers and stabilizing the nuclei toward redispersion in solution.

In order to illustrate the potential applications of the present biomineralization method, the nature of the HAP-like phase obtained under different conditions was explored. Carbonate containing and calcium deficient apatites are well-known constituent materials of natural hard tissues; the final Ca/P ratio can be tuned by means of both the initial urea concentration and/or the aging time, as can be anticipated from eqs 1 and eq 4. (see Supporting Information). In addition, other forms of apatites, such as the fluoride containing ones, are also relevant; if NaF is also added to the starting solution, stoichiometric fluoroapatites, as well as partially substituted ones, can be obtained, depending on the initial Ca(II) to F^- ratio (see Supporting Information). In summary, urease functionalized silica provides a useful interface for nucleation and growth of HAP-like coatings, under mild conditions and short aging times. This enzymatic route arises as a general procedure to prepare a wide range of biomimetic coatings of tuned composition (see Table 1, Supporting Information). Taking into account the ability of silica to develop shells over a wide range of materials, the current procedure opens the gate for the preparation of novel biphasic biocompatible coatings.[24] This method can be applied over different geometries, allowing the design of novel biocompatible films, scaffolds, core shell nanoparticles, and so forth. Moreover, this reaction can be developed in the presence of labile macromolecules or gels, allowing the preparation of novel biocomposites.[25] In addition, since silica templates can be readily dissolved in mild conditions, this procedure can act as a first step in the achievement of novel HAP hollow structures.[26]

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Supporting Information Available: Details on experimental-procedures for samples preparation and further sample characterization (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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