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The synergic role of collagen and citrate in stabilizing amorphous calcium phosphate precursors with platy morphology

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ABSTRACT

Bioinspired *in vitro* collagen mineralization experiments have been performed in the presence of citrate and the combined role of the two bone organic matrix components in controlling mineral formation was investigated for the first time. Mineralized and non-mineralized collagen fibrils have been in depth characterized by combining small- and wide-angle X-ray scattering (SAXS/WAXS) techniques with Atomic Force Microscopy (AFM) imaging. A synergic effect of collagen and citrate in driving the formation of long-term stable amorphous calcium phosphate (ACP) nanoparticles with platy morphology was found. AFM images on mineralized collagen fibrils revealed that some of the ACP nanoparticles were deposited on the intramolecular nanoscopic holes of collagen fibrils.

Statement of Significance

Citrate is an important component of the bone organic matrix but its specific role in bone mineralization is presently unclear. In this work, bioinspired *in vitro* collagen mineralization experiments in the presence of citrate have been carried out and the combined role of collagen and citrate in controlling mineral formation has been addressed for the first time. Through X-ray scattering and Atomic Force Microscopy characterizations on mineralized and non-mineralized collagen fibrils, we have found that citrate in synergy with collagen stabilizes an amorphous calcium phosphate (ACP) phase with platy morphology over one week and controls its deposition on collagen fibrils.

1. Introduction

Bone is a composite material with an extremely complex structure exhibiting many levels of organization [1–6]. Mineralized collagen fibrils are the building blocks of such a complex architecture. Collagen triple-helix molecules self-assemble in a periodic axially staggered array forming the so-called fibrils (100–300 nm in diameter), which exhibit a characteristic banding pattern of 67 nm, where a densely packed 27 nm-long region (the so-called overlap zone) alternates with the less dense 40 nm-long gap zone [1,5,7,8]. This organic matrix is further reinforced by an array of plate-shaped apatite nanocrystals, both through intra- (within the fibrils) and inter- or extra-fibrillar (on the fibrils surface) mineralization. In the intrafibrillar mineralization, the platelets pile up [3] on their largest {01–10} hexagonal facets and elongate with their crystallographic *c*-axis parallel to the collagen fibrils axis, resulting in an organic/inorganic composite with unique biomechanical properties. Gaining a better understanding of the mechanisms of this biologically controlled mineral formation at the molecular level is of paramount importance to design new therapeutic engineered scaffolds for repairing or regenerating hard tissues [2,9].

Thanks to the intensive investigation on biological samples complemented by *in vivo* and *in vitro* studies, great advances have been reached over the years on the role of the organic matrix (*i.e.* collagen fibrils, non-collagenous proteins (NCPs) and small molecules), exerting a high level of control over the composition,

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structure, size and morphology and organization of the mineral phase [1,4,5,7,10–15]. However, an overall exhaustive comprehension of the mechanism is still to be attained and many issues either on the specific action or the interplay between the different organic players, and on the mineral component as well, need to be elucidated [7,12,14].

Two different mineralization pathways are nowadays explored through *in vitro* experiments. In line with the mechanism relying on classical nucleation, Nassif and co-workers [12] were able to mineralize collagen fibrils *in vitro* in the absence of any organic/polymeric additives, with side chains of polypeptide charged amino acids of collagen likely providing the binding sites for calcium and phosphate ions of the (supersaturated) mineralizing solution [16]. On the other hand, many *in vitro* experiments carried out in the presence of either selected NCPs or polypeptides rich in poly-carboxylic acids (*e.g.*, poly-aspartic acid), mimicking the poly-anionic character of the NCPs [1,7,10,11,13,14], demonstrated their role in controlling the mineral nucleation and growth. The rationale behind such an approach is that mineral deposition occurs *via* a non-classical nucleation mechanism, involving the precipitation of an amorphous precursor, which infiltrates into the nanoscopic gaps of the assembled collagen fibrils and further transforms into platy nanocrystalline apatite [1,13,14,17,18]. Nano-spheres of amorphous calcium phosphate (ACP) have been observed at the early stages of mineralization [19,20], whereas thin apatite platelets are observed at the final crystalline phase. However, the mechanism underlying the origin of this platy morphology still remains unclear [4]. Making this situation even more complex, recent solid-state NMR studies have shown that citrate, a small molecule of the organic bone components somewhat neglected so far, occurring in a relatively large amount (~5.5 wt %, which accounts for ~80% of all citrate in the body [21]), is strongly bound to bone apatite and controls the platelet thickness [22]. This finding opened new scenarios in bone mineralization, where citrate might play a broader role than has been thought to date [23]. Inspired by this study, in a previous work [24] we have investigated the role of citrate in a simplified system model (without collagen) providing experimental evidence of its role in controlling the size and morphology of apatite nanoparticles (NPs) likely inherited from an amorphous precursor of platy morphology.

In this work we focus on *in vitro* collagen mineralization experiments, aimed at gaining a better understanding of the role of citrate in bone mineral formation. Here, we address particular attention to the nature and morphology of the first formed mineral phase when both collagen and citrate are at work. To this aim, collagen fibrils were mineralized in the presence of citrate under physiological conditions (T = 37 °C and pH = 7.4) and in-depth characterized through a combination of X-ray scattering (both in the small (SAXS) and the wide angle (WAXS) regions) and imaging (Atomic Force Microscopy, AFM) techniques. Wide−angle synchrotron X-ray scattering enabled us to measure simultaneously the scattering originating from the mineral precipitated at the early stages and from the collagen molecular arrangement within the fibrils (the so-called equatorial lateral packing). The morphology of the mineral phase was investigated by SAXS and AFM, the latter providing also the direct observation of both mineral deposition on collagen fibrils and mineral precipitation outside the fibrils and trapped within the composite.

2. Materials and methods

2.1. Reagents and solutions

2.1.1. Collagen stock solutions

Type I collagen gel, extracted from equine tendon using the standardized manufacturing method of Opocrin S.p.A. (Corlo di Formigine, Modena, Italy) as described elsewhere [25], was kindly provided by Dr. Michele Iafisco (Institute of Science and Technology for Ceramics, ISTEC-CNR, Italy). Then, 1 g of type I collagen gel was diluted in 10 mL of acetic acid (0.1 M) and stirred overnight. The collagen solution was centrifuged at 5000 rpm for 15 min and the supernatant was collected and stored at 4 °C. The pH of the supernatant was 2.7. The concentration of collagen in this stock solution was 0.5 mg mL^{-1}, as measured by UV spectroscopy.

2.1.2. Mineralizing solutions

High purity $CaCl_2{\cdot}2H_2O$, Na_2HPO_4, K_2HPO_4, $Na_3Cit{\cdot}2H_2O$ (where Cit is $C_6H_5O_7^{3-}$) and Hepes (4- [2-hydroxyethyl]-1-piperazineethane sulfonic acid) buffer were purchased from Sigma-Aldrich. All the solutions were prepared using ultrapure MilliQ water (ρ = 18.2 MΩ cm at 25 °C).

2.1.3. Mineralization experiments

The collagen-containing solution (pH 2.7, 0.5 mg mL^{-1}) was mixed to a Ca- and citrate-containing solution. Then, a phosphate-containing solution was added to the latter mixture. All the solutions were prepared in Hepes buffer (10 mM, pH 7.4). Taking inspiration, from our previous work on citrate-controlled apatite nanoparticles [24] on one hand, and from the study on collagen mineralization in the presence of poly-aspartic acid (p-Asp) by Nudelman et al. [7], on the other hand, two series of experiments were performed, employing two different Ca:Cit ratios (1:4 and 1:2, respectively). The following concentrations were used: (1) 5.0 mM $CaCl_2$, 20.0 mM sodium citrate and 6.0 mM NaH_2PO_4 (as in Ref. [24]); these samples are referred to as MinCol_1_4; (2) 2.7 mM $CaCl_2$, 5.4 mM sodium citrate and 1.35 mM NaH_2PO_4 (as in Ref. [7], but with citrate replacing pAsp); these samples are referred to as MinCol_1_2. The mixtures were then maturated at 37 °C for 5 min, 96 h or 168 h in a thermostated oven. A blank Ref. sample containing only collagen in Hepes (pH 7.4, referred to as Col) was also prepared. Using the same concentrations as in (1), parallel blank mineralization experiments, either without citrate (MinCol_1_0) or without collagen (Min_1_4), were also prepared (maturation times: 24 h). Table 1 summarizes the concentrations used in the mineralization experiments. After maturation, the samples were repeatedly washed with MilliQ water by centrifugation (9000 rpm, 10 min, Centrifuge 5810 R, eppendorf,) and the wet precipitates were freeze-dried over night at −50 °C (LyoQuest, Telstar). Then, they were stored at room temperature until further characterization.

2.1.4. Synchrotron X-ray total scattering measurements

Dry samples were loaded in glass capillaries of 1.0 mm diameter and measured at the X04SA-MS Beamline of the Swiss Light Source (SLS) of the Paul Scherrer Institut (PSI, Villigen, Switzerland). The beam energy was set at 16 keV and the operational wavelength (λ = 0.77449 Å) precisely determined by collecting, under the same experimental conditions, a silicon powder standard [NIST 640c, a_0 = 0.54311946(92) nm at 22.5 °C]. Data were collected in the 2–120° 2θ range with the aid of the position sensitive single-photon counting MYTHEN II detector [26]. Independent He/air and capillary scattering curves, as well as sample-loaded capillary transmission coefficients, were also measured and used for data subtraction of all extra-sample scattering effects and absorption corrections [27]. The linear absorption coefficient for the empty capillary was calculated on the basis of glass composition. Additionally, an X-ray powder diffraction pattern of the Min_1_4 sample was recorded using a Rigaku Miniflex 300 diffractometer with Bragg-Brentano geometry in the θ:2θ mode (Ni-filtered Cu $K_{\alpha 1,2}$ radiation, λ = 1.5418 Å).