Short Term Mobility (STM) Program: Self-assembly and mineralization of collagen fibers in the presence of citrate by vapor diffusion method

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Introduction

Bone is a hierarchically organized organic-inorganic material whose building blocks, the mineralized collagen fibrils, form a scaffold into which the hydroxyapatite nucleate and grow.[1] This intrafibrillar mineralization has been repeatedly proposed to occur from a transient amorphous calcium phosphate (ACP) precursor that infiltrates into the 40 nm-long gap regions of self-assembled collagen fibrils through electrostatic interactions being further transformed into nanocrystalline platy-shaped apatites (Ap).[2] The mechanism underlying the spherical ACP to platy apatite transformation and clarifying the origin of this platy morphology has never been directly detected in neither *in vivo* nor *in vitro* experiments. Over the past years, new insights into these mechanisms have been reported; however, open debates still persist concerning the chemical nature of the first mineral formed; the factors controlling the initial deposition and growth of crystals; the role of the organic matrix (*i.e.* collagen, non-collageneous proteins (NCPs) and small molecules).[3] Numerous studies were focused on exploring the role of collagen and NCPs suggesting that they may be involved in different steps of bone mineralization, such as the formation of the ACP precursor phase, its further transformation into and organization of apatite crystals.[1a,2,4] However, most of the above-mentioned questions still remain unanswered or at least under discussion.[2] Recent solid-state NMR studies have evidenced the relatively large amount of citrate in bone, where it accounts for ~5.5% wt of the total organic component[5b] (~2% wt of the total amount[5a]), and found to be strongly bound to apatite platelets, controlling their size and morphology (Fig. 1c).[5] Therefore, citrate might have a broader role to play than it has been thought to date and even directly intervene in bone mineralization.

Objectives

According to the above described issues, this program was mainly aimed at studying simultaneously the self-assembly and mineralization of collagen fibers in the presence of citrate by using the vapor diffusion method.

Working plan

Vapor diffusion method: The self-assembly of collagen fibrils were carried out by vapour diffusion in the “crystallization mushroom” (Fig. 1). This technique allows for controlling the pH increasing rate as well as the final pH value by the concentration of ammonium carbonate (Fig. 1). In addition, collagen mineralization in the absence and in the presence of citrate was

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[1] a) W. Landis, R. Jacquet, *Calcif. Tissue Int.* **2013**, *93*, 329.; b) S. Weiner, H.D. Wagner, *Annu. Rev. Mater. Sci.* **1998**, *28*, 271.; c) M.J. Glimcher, H. Muir, *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **1984**, *304*, 479.; d) M.J. Olszta, X. Cheng, S.S. Jee, et al., *Mater. Sci. Eng. R* **2007**, *58*, 77.; e) J.Y. Rho, L. Kuhn-Spearing, P. Zioupos, *Med. Eng. Phys.* **1998**, *20*, 92.

[2] a) F. Nudelman, K. Pieterse, A. George, et al., *Nat. Mater.* **2010**, *9*, 1004.; b) Y. Wang, T. Azaïs, M. Robin, et al., *Nat. Mater.* **2012**, *11*, 724.

[3] A.L. Boskey, *J. Cell. Biochem. Suppl.* **1998**, *30-31*, 83.

[4] A. George, A. Veis, *Chem. Rev.* **2008**, *108*, 4670.

[5] a) E. Davies, K.H. Müller, W.C. Wong, et al., *Proc. Natl. Acad. Sci. USA* **2014**.; b) Y.Y. Hu, A. Rawal, K. Schmidt-Rohr, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22425.

performed in the “crystallization mushroom”. This device consists in two cylindrical glass chambers connected through a hole of 6 mm diameter (Fig. 2). The lower chamber is the gas generation chamber, which was filled with 2 mL of NH_4HCO_3 solution at different concentrations (50 mM and 2 M). Drops of 20 mL of the collagen solution (see caption of Fig. 1 for further details) were placed on the upper chamber (crystallization chamber). The decomposition of NH_4HCO_3 released CO_2 and NH_3, which flowed through the crystallization chamber. The ammonia diffusion into the collagen drops caused a gradual increase of the pH until it reached an asymptotic value. Simultaneous assembly and mineralization of collagen fibrils was also performed in the same device. To this aim, twelve drops (1:1 v/v, 60 mL) containing (i) 3 mM $CaCl_2$ + diluted collagen (1:8) + 6 mM Na_3(Citrate) and (ii) 3 mM K_2HPO_4 were placed in the crystallization chamber while 2 mL of NH_4HCO_3 solution (2 M) were deposited in the gas generation chamber. The setup was carefully sealed and kept at 37 ºC.

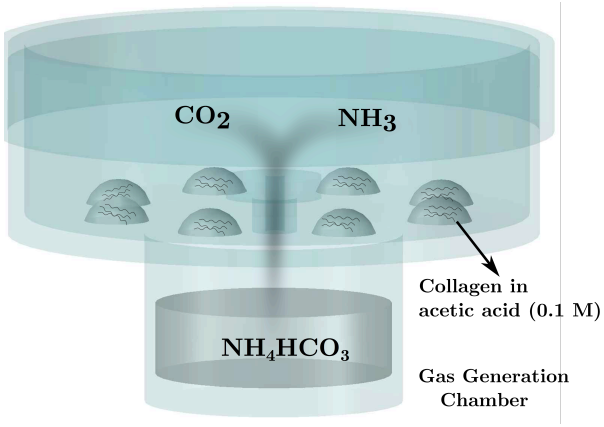

Figure 1. *Schematic view of the “crystallization mushroom” used for vapour diffusion experiments. To obtain pure collagen solutions, 1 gr of collagen gel (Collagen type I extracted from equine Achilles tendon using the manufacturing method of Opocrin S.p.A., Corlo di Formigine, Italy) was diluted in 10 mL of acetic acid (0.1M) and stirred overnight. Subsequently, collagen solution was centrifuged at 5000 rpm for 15 minutes and the supernatantwas collected and stored at 4 C. The pH of this supernatant was close to 2.7. Under these conditions, collagen fibrils are non-assembled.*

After 48 hours, the drops were collected and deposited over copper microgrids and/or freshly cleaved mica surfaces for the characterization by TEM and atomic force microscopy (AFM), respectively. These characterizations will be further performed by Dr. Delgado-López at IACT (Granada, Spain).

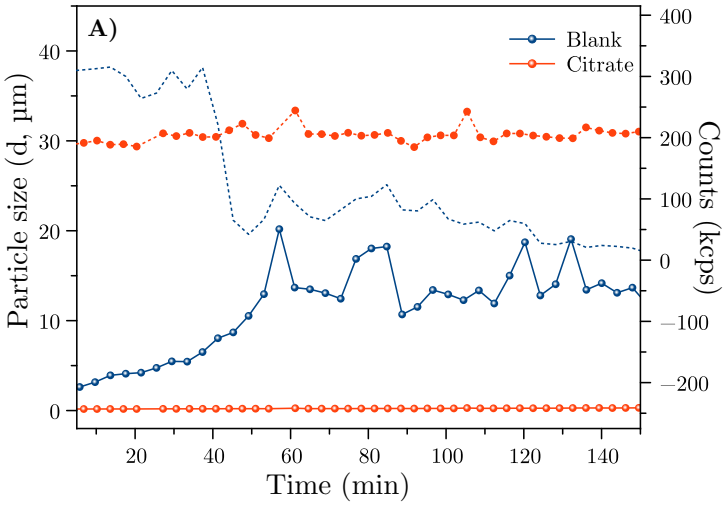

Figure 2. Dynamic light scattering (DLS) measurements of the mineralizing solutions in the presence (red lines) and absence (blue lines) of citrate. Solid lines represent the average particle size (R_h) whereas dotted lines are related to the mean count rate (kcps).

Results

Dynamic light scattering (DLS) was firstly used to study the colloidal stability of the mineralizing solutions. Figure 2A represents the time evolution of the hydrodynamic diameter (R_h) of the

particles precipitated in the absence (solid blue line) and in the presence (solid red line) of citrate. In the absence of citrate, micrometric-sized particles of calcium phosphate were grown (R_h>30 um). On the other hand, nanosized particles (R_h of *ca.* 100 nm) remained stable in the presence of citrate. These results clearly demonstrated that citrate induces the formation of nanoparticles stabilizing them likely by specific adsorption.

The pH evolution during vapor diffusion from two NH_4HCO_3 concentrations (50 mM and 2 M) was then monitored. To this aim, a drop was deposited over a pH probe, which was inserted within the crystallization mushroom through a hole. Figure 3 represents the pH evolution of a drop containing initially pure collagen solution (0.1 M acetic acid, pH 2.7). The pH evolution recorded for the 50 mM NH_4HCO_3 solutions (the lowest concentration) showed a gradual pH increase as well as a lower final pH than that measured for the highest concentration (2M). Additionally, the pH increase was clearly more pronounced in this latter case. After 24 hours of vapor diffusion, collagen fibrils were then analyzed by Transmission Electron Microscopy (TEM). Only when the highest concentration of NH_4HCO_3 was used, most of the fibrils showed the characteristic periodic 67 nm banding pattern (inset of Figure 4). For this reason, this concentration was selected for the following mineralization experiments.

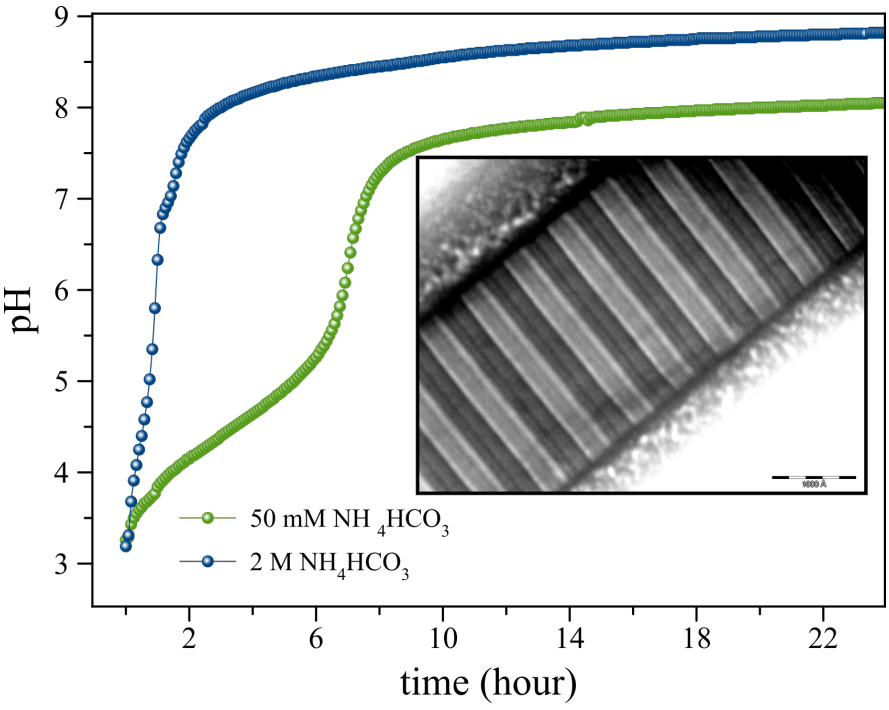

Figure 3. pH evolution of a drop containing initially pure collagen solution (0.1 M acetic acid, pH 2.7) under vapor diffusion from 50 mM (green line) and 2 M NH_4HCO_3 solutions. The inset shows a TEM micrograph of a assembled collagen fibril showing the characteristic 67 nm band pattern.

Mineralizing experiments were also performed according to the concentrations described in the working plan section. The results of such experiments are now being analyzed by Dr. Delgado-López. In conclusion, this short-term mobility program allowed for setting up a protocol, based on vapor diffusion, to perform simultaneously the self-assembly and mineralization of collagen fibers in the presence of citrate.