Three Dimensional Biomimetic Mineralization of Dense Hydrogel Templates

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In biological materials, form and function are intimately related. From nacre to bone, nature achieves unique structural and functional properties through the combination of organic and inorganic phases in complex hierarchical structures.[1] These natural composites are often created through carefully orchestrated biomineralization processes that result in an extremely accurate control of the shape, size and distribution of the inorganic crystals.[2] Recreating natural mineralization in the laboratory to build new bioinspired composites is a very attractive prospect but has been extremely difficult[3-5] This is largely due to the limitations in our basic knowledge of biomineralization and to the technical difficulties associated with ion transport and control of mineral nucleation and growth in dense and nanoporous matrices.[2,4] As a result, most mineralization approaches are confined to the manipulation of crystal formation and growth in two dimensions.[3,6] The problem gets only more complicated when the goal is to control the stoichiometry and crystal morphology of complex oxides or when the objective is to mineralize dense organic matrices.

In this work we describe a technique for the three dimensional mineralization of dense hydrogel scaffolds (**Supporting Information, S-1**) with an apatitic calcium phosphate mineral whose structure is closely related to the inorganic component of bone. Dense hydrogels are very appealing materials for the synthesis of mineralized, bone-like structures. Natural hydrogels, such as collagen, are the structural scaffolds in various connective tissues including bone, and it is possible to formulate synthetic hydrogels with similar intrinsic elasticity and water retention ability.[7] These hydrogels can be easily assembled in three dimensions while displaying multiple functional domains and their polymerization chemistry allows the incorporation of polar ligands that mimic the acidic matrix proteins regulating mineral growth.[8] In nature, microscopic vesicles often act as vehicles for ion transport and provide the microenvironment needed to promote controlled mineral nucleation and assembly. It is believed that matrix vesicles released by the osteoblast plasma membrane play an important role in the mineralization of the extracellular matrix and the formation of bone.[2] In this work, phase separation is used to create liquid vesicles inside a dense hydrogel matrix formulated to provide specific sites for the attachment of Ca-ions and template the crystallization of nano-apatite. The final result is a bone-like composite that could be used for the engineering and regeneration of calcified tissue. Because the experimental conditions can be easily controlled, the process can help to clarify some poorly understood aspects in the crystallization of apatite and the origins of the transition between amorphous and crystalline inorganic phases during bone formation.

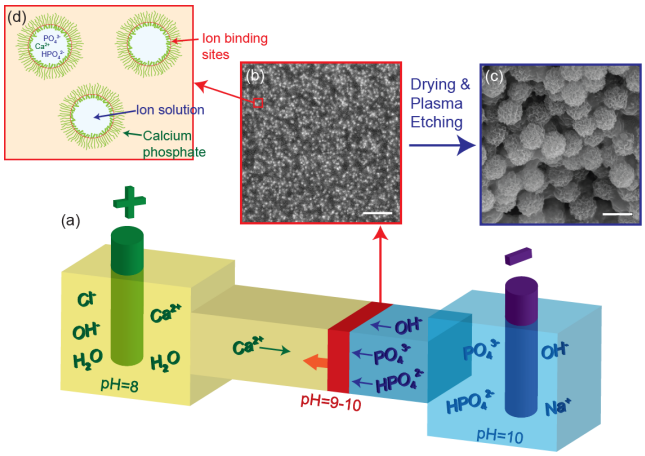

Figure 1. Schematic of the direct current-assisted ion diffusion process. (a) An electrical current is established and the ions travel through the hydrogel to meet at the reaction front. (b) The in situ SEM analysis of a fully wet hydrogel. (c) The interconnected spherical mineral domains are clearly visible after removing the organic matrix using a plasma etching treatment. (d) The amide groups in polyacrylamide hydrogel interact with the Ca-ions in the solution and the interface between the aqueous domain and the gel act as a center for the nucleation of an apatitic calcium phosphate mineral. (Scale bars: (b) 10 μm; (c) 2 μm.).

Promoting ion transport into the organic matrix is one of the key issues in the mineralization of dense polymer networks. Often, this is done through immersion in simulated body fluid or a similar solution.[9-11] As a result, mineralization is slow and difficult to control. In addition, the process works better with porous materials or with "soft" polymers that have very large equilibrium water contents.[12,13] Here we use a direct electric current-assisted diffusion approach to promote the transport of Ca^{2+}, PO_4^{3-}, HPO_4^{2-} and OH ions into the hydrogel matrix[14] (**Fig. 1a**). Two chambers, one containing a $CaCl_2$ aqueous solution with a Tris-HCl buffer solution (pH=8) and a positive electrode and the other with a Na_2HPO_4 aqueous solution (pH=10) and a negative electrode are connected through the hydrogel. A voltage drop maintains a constant current between the electrodes such that the Ca^{2+} ions migrate toward the negative electrode, while the HPO_4^{2-} and PO_4^{3-} ions move toward the positive one. The only pathway for the ions is through the hydrogel and they meet at the reaction front inside the organic matrix. By controlling the pH of both electrode chambers it is possible to promote the precipitation of calcium phosphate mineral when the two counter ions meet. Compared to

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concentration or gravitational diffusion, this current-assisted approach allows careful control over the mineral concentration and precipitation rates, as ion-transport can be easily adjusted by changing the current density. As a result it is possible to form previously unattainable mineral morphologies at much higher ion concentrations.

Following nature's approach, the control of ion transport should be combined with the right organic matrix to template mineral formation. It has been shown that synthetic biocompatible hydrogels such as polyacrylamide and polyhydroxyethylmethacrylate (pHEMA) provide a versatile template for the surface mineralization of calcium phosphate films.[15] In our current-assisted diffusion set-up the use of pure pHEMA hydrogels at all concentrations tested resulted in the nucleation of octacalcium phosphate (OCP, $Ca_8(HPO_4)_2(PO_4)_4\square 5H_2O$) crystals that precipitate inside the hydrogel and grow into micron-sized particles. These particles push away the pHEMA network as they grow (**Supporting Information, S-2**). Incorporating a few percent of sodium methacrylate into the pHEMA structure significantly alters the morphology of the growing mineral phase (**Supporting Information, S-3**) due to strong interaction of the carboxyl with the calcium ions.

Organic-inorganic integration at the nanoscale was achieved using a polyacrylamide hydrogel. Scanning Electron Microscopy (SEM) shows a structure formed by microspherical mineral aggregates distributed regularly in the organic network (**Figs. 1 b, c**). Fig.1b is an SEM image of a fully hydrated sample, whereas Fig. 1c is a image of a dried and plasma etched sample. In Fig 1b, only top potion of the microsphere is captured in the image as the electron conductive window is pressed against the sample surface, compared Fig. 1c, where full surface structure of the microsphere are visible. These results demonstrate the microspheres are formed during precipitation process rather than during the hydrogel drying and processing. Fig. 2 is TEM images of the micro and nano composition of the aggregates. The microspheres are hollow; their size is very uniform and changes inversely with the hydrogel concentration. Mineral nanofibers (5-20 nm wide, 200 nm long) can be observed growing from the surface of these spheres into the hydrogel while short and broader lamellar nanoparticles (up to 50 nm wide) grow towards the interior. The fibers and lamellae are formed by the assembly of amorphous and crystalline nanodomains (5-20 nm in size). The crystal lattice parameter based on electron diffraction agrees with a semicrystalline hydroxyapatite (HA). In the fibers growing into the hydrogel, the (100) plane measured at 8.4 Å is parallel to the crystal growth direction. The measured (100) plain indexes range from 8.5 Å to 7.7 Å, as can be expected from the semi-crystalline nature of the mineral particles.[16]

The mineralization patterns are determined by the characteristics of the electric current-assisted diffusion process and the chemistry of the hydrogel scaffold. Ion migration at high concentration into the hydrogel promotes phase separation (a salt-out effect[17]). Spherical aqueous domains (~0.5-1 μm in diameter) with relative large ionic content form inside the organic matrix as the polyacrylamide chains aggregate to open up the micron-sized pores while retaining the bulk gel structure(**Fig. 1d**). Micro-phase separation results in the formation of aqueous domains with a very narrow size distribution whose diameter changes inversely with the hydrogel concentration (**Supporting Information, S-4**). These domains play a role similar to the vesicles that mediate biomineralization.[2] Like the vesicles, the domains contain an aqueous solution with high ionic concentrations providing the right environment for mineral nucleation. The strong interaction between the amide group in the hydrogel and the Ca-ions promotes the heterogeneous nucleation of nanocrystalline structures at the interface between these "artificial vesicles" formed by the phase separation process in the hydrogel.[18,19] The nanocrystals further assembly at the vesicle surface to form hollow microspheres (**Fig. 1c,d**). Further growth occurs on both sides of the interface as the electric current continuously carry ions to the nucleation sites. Because of the different environments inside and outside of the synthetic vesicles (The crystals growing in the gel are confined by the polymer), the morphology of the crystals growing into the hydrogel and towards the center of the vesicles is different (nanofiber and lamellar respectively).

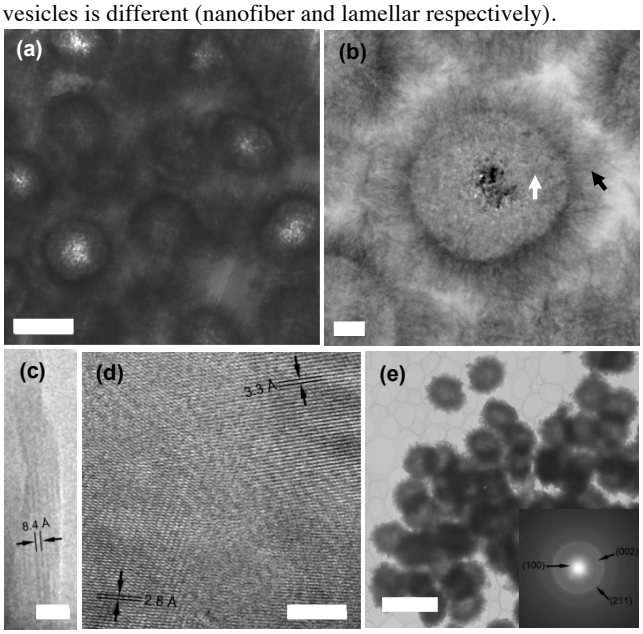

Figure 2. TEM images of the mineralized polyacrylamide hydrogel. FIB samples (a-d). (a) A low magnification image reveals a homogeneous distribution of HA mineral microspheres, ~ 1 μm in diameter. (b) The dark field image of the microsphere shows its hollow structure. (c) The high resolution image of a mineral nanofiber growing in the organic matrix (black arrow in b) shows a mixture of amorphous and crystalline nanodomains. (d) The lamellar grains grown inside the microsphere (white arrow in b) also show a similar mixture of crystalline and amorphous nanodomains (e) After stripping away the hydrogel of the bulk sample by oxygen plasma, hollow mineral aggregates with uniform size distribution can be observed. The corresponding electron diffraction pattern shown in the inset has the characteristic ring features of the HA (100) plain at 8.0 Å, (002) plain at 3.4 Å and (211) plain at 2.9 Å with (211) being the strongest. (Scale bar: (a) 1 μm; (b) 200 nm; (c) 5 nm; (d) 5 nm; (e) 2 μm.).

The polyacrylamide network acts in two ways, first it limits the size of the aqueous domains (the vesicles) and second it provides nucleation sites for the formation of apatite.[20] In addition, the polymer also limits crystal growth inside the organic network resulting in the assembly of amorphous and crystalline (apatite) nanosized domains into a fiber arrangement. The critical role of the scaffold chemistry is illustrated by the mineralization behavior of the pHEMA gels. The hydroxyl groups on the pHEMA do not provide a binding place for the Ca-ions.[15] Therefore octacalcium phosphate nucleates in the aqueous phase and grows uncontrollably into larger crystals that push the hydrogel away (**Supporting Information, S-2**). This is not surprising as OCP, that consists of intercalated apatite and hydrated (water molecules) layers, is often found as an intermediate phase during the precipitation of thermodynamically more stable calcium phosphates such as HA.[21,22] This result is consistent with previous

experiments that have shown how the surface of pHEMA does not template the growth of calcium phosphate films during urea-based mineralization.[14]

The final mineral contents are around 60% of the weight of the organics (**Supporting Information, S-5a**). These large inorganic contents can not be attained in bulk hydrogels using conventional methods. Other unique features of this process are the formation of nanocrystals and their assembly into uniform hollow microspheres. The close packing of the spheres in the organic matrix provides the mechanical support needed to form a three dimensionally connected microporous structure after removing the hydrogel through thermal treatment. The elimination of the polyacrylamide network using thermal treatments was monitored using differential thermal analysis and thermogravimetry (DTA-TG). Prior to the DTA, the hydrogel was dried at 37ºC for a week to remove water. After drying, the starting mineral phase is HA (**Supporting Information, S-5b**). The broad diffraction peaks are in agreement with the semi-crystalline and nanocrystalline nature observed in TEM experiments. The weight loss observed at temperatures up to 560°C was due to the loss of residual water and decomposition of hydrogel. The heat transition and weight loss observed above 600°C are attributed to the phase transformation from HA to β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$) as shown in the X-ray diffraction pattern (**Supporting Information, S-5b**). The sharp and narrow diffraction peaks indicates the formation of a well defined crystalline phase after firing. The microstructural evolution of the mineralized polyacrylamide hydrogel during heating is characterized first by the removal of the organic phase to leave the polycrystalline inorganic hollow spheres (**Fig. 3a**) followed by their densification to form β-TCP microspheres with a very uniform particle size distribution (**Fig. 3b**). The final step upon heating is the sintering of the tricalcium phosphate to create a microporous inorganic structure with a very uniformly distributed porosity, with a Brunauer-Emmett-Teller (BET) surface area of 0.68 m^2/g for the sintered structure (**Fig. 3c**). The final pore sizes and specific surfaces may be controlled by manipulating the hydrogel concentration, the mineral content and the heating rate (**Supporting Information, S-6**). The detailed relationship is under investigation. This porous structure is much more homogeneous than the ones obtained after firing mixtures of synthetic nanosized HA particles with hydrogels. These exhibit a much more irregular microstructure due to the random aggregation of the mineral nanoparticles (**Supporting Information, S-7**).

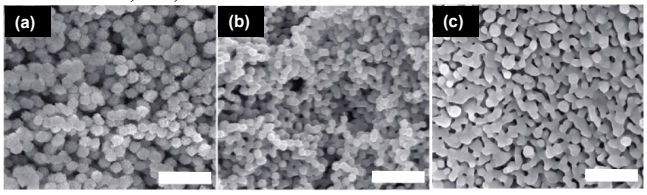

Figure 3. SEM micrographs of the mineralized polyacrylamide hydrogel heated at (a) 750°C, (b) 950°C and (c) 1050°C. (Scale bar: 5 μm)

In conclusion, we have shown how current-assisted diffusion can be used to mimic vesicle-mediated mineralization in dense hydrogels. The mineralization technique and the chemistry of the organic matrix control the microstructure of the final material. Here we combine both aspects into a seamless process to develop hierarchical structures in which the organic and inorganic phases are integrated at the nanoscale while the mineral particles assemble into well-defined microscopic structures leading to high mineral concentrations. Some other parallels can be drawn between this process and bone development. The biomineralization of bone is believed to occur through the formation of intermediate phases and there is some controversy regarding the first mineral phase to precipitate, either OCP or an amorphous calcium phosphate.[23] In our system octacalcium phosphate nucleates when the matrix lacks specific sites to interact with the Ca-ions while templated mineral nucleation promotes the formation of a nanostructured materials with crystalline and amorphous domains that will be closer to the amorphous calcium phosphate.[24] By controlling the microstructure and the mineral contents we hope to develop hybrid materials with optimal mechanical and biological properties for the treatment of bone defects in a wide range of situations.

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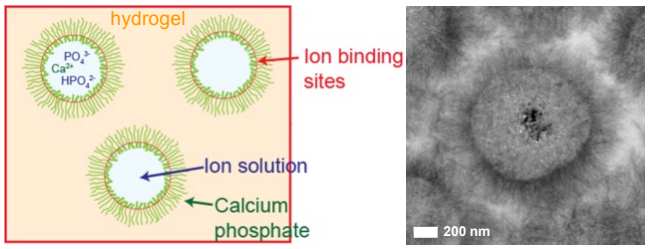
Supporting Information Available: Experimental procedures, Figures S-1, S-2, S-3, S-4, S-5, S-6, S-7 are available free of charge via the Internet at http://pubs.acs.org.

References

[1] G. Mayer, *Science* **2005**, *310*, 1144-1147.
[2] S. Mann, *Biomineralization : principles and concepts in bioinorganic materials chemistry*, Oxford University Press, Oxford ; New York **2001**.
[3] A. W. Xu, Y. R. Ma, H. Colfen, *Journal of Materials Chemistry* **2007**, *17*, 415-449.
[4] E. Pouget, E. Dujardin, A. Cavalier, A. Moreac, C. Valery, V. Marchi-Artzner, T. Weiss, A. Renault, M. Paternostre, F. Artzner, *Nature Materials* **2007**, *6*, 434-439.
[5] J. D. Hartgerink, E. Beniash, S. I. Stupp, *Science* **2001**, *294*, 1684-1688.
[6] C. Sanchez, H. Arribart, M. M. G. Guille, *Nature Materials* **2005**, *4*, 277-288.
[7] N. A. Peppas, *Hydrogels in Medicine and Pharmacy*, CRC Press, Boca Raton **1986.**
[8] D. A. Barrera, E. Zylstra, P. T. Lansbury, R. Langer, *Journal of the American Chemical Society* **1993**, *115*, 11010-11011.
[9] W. L. Murphy, D. J. Mooney, *Journal of the American Chemical Society* **2002**, *124*, 1910-1917.
[10] O. Grassmann, P. Lobmann, *Biomaterials* **2004**, *25*, 277-282.
[11] T. Taguchi, A. Kishida, M. Akashi, *Chemistry Letters* **1998**, 711-712.
[12] S. A. Hutchens, R. S. Benson, B. R. Evans, H. M. O'Neill, C. J. Rawn, *Biomaterials* **2006**, *27*, 4661-4670.
[13] H. B. Qu, Z. M. Xia, D. A. Knecht, M. Wei, *Journal of the American Ceramic Society* **2008**, *91*, 3211-3215.
[14] Junji Watanabe, Mitsuru Akash, *Biomacromolecules* **2006,** *7*, 3008-3011.
[15] J. Song, E. Saiz, C. R. Bertozzi, *Journal of the American Chemical Society* **2003**, *125*, 1236-1243.
[16] Sudarsan.K, R. A. Young, *Acta Crystallographica Section B-Structural Crystallography and Crystal Chemistry* **1969**, *B 25*, 1534-1543.
[17] Q. Liu, E. L. Hedberg, Z. W. Liu, R. Bahulekar, R. K. Meszlenyi, A. G. Mikos, *Biomaterials* **2000**, *21*, 2163-2169.
[18] A. Masuyama, A. Shindoh, D. Ono, M. Okahara, *Journal of the American Oil Chemists Society* **1989**, *66*, 834-837.
[19] J. Song, V. Malathong, C. R. Bertozzi, *Journal of the American Chemical Society* **2005**, *127*, 3366-3372.
[20] J. Watanabe, M. Akashi, *Biomacromolecules* **2007**, *8*, 2288-2293.
[21] P. Koutsoukos, Z. Amjad, M. B. Tomson, G. H. Nancollas, *Journal of the American Chemical Society* **1980**, *102*, 1553-1557.
[22] S. V. Dorozhkin, M. Epple, *Angewandte Chemie-International Edition* **2002**, *41*, 3130-3146.
[23] W. E. Brown, *Clinical Orthopaedics and Related Research* **1966**, *44*, 205.
[24] J. D. Termine, A. S. Posner, *Nature* **1966**, *211*, 268-270.



An electric-current assisted method was used to mineralize dense hydrogels and create hydroxyapatite/hydrogel composites with unique hierarchical structures. The microstructure of the final material can be controlled by the mineralization technique and the chemistry of the organic matrix. A hydroxyapatite/hydrogel composite was obtained with large inorganic content (around 60% of the weight of the organics). After been heated to 1050 ºC, the sintered inorganic phase has a very uniformly distributed porosity and its Brunauer-Emmett-Teller (BET) surface area is 0.68 m^2/g.