

Collagen-membrane-induced calcium phosphate electrocrystallization

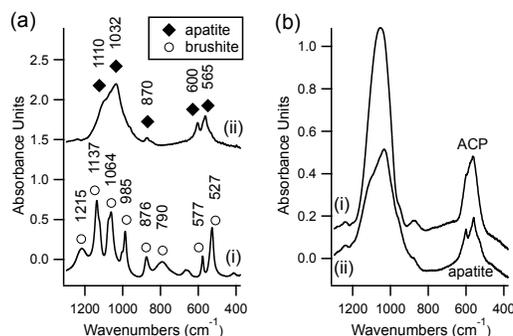
M. Ramesh Kumar,[†] Erika F. Merschrod S.,^{*,†} and Kristin M. Poduska^{*,‡}

Department of Chemistry, and Department of Physics and Physical Oceanography, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X7

E-mail: erika@mun.ca; kris@mun.ca

Abstract

Electrosynthesized type I collagen membranes provide a robust scaffold for phase-controlled nucleation of carbonated hydroxyapatite, which is similar to the mineral composition of natural bone. Phase selectivity persists under a wide range of electrosynthesis conditions that would, in the absence of the membrane, produce other calcium phosphate phases such as brushite or amorphous calcium phosphate. Results also indicate enhanced mineral formation in the presence of collagen.



[†]Department of Chemistry, Phone: 709.864.8765, URL: www.chem.mun.ca/erika

[‡]Dept. of Physics, Phone: 709.864.8890, Fax: 709.864.8739, URL: www.physics.mun.ca/kris

There is an urgent demand for bone healing materials because conventional bone replacement metals and alloys, while mechanically strong, do not usually stimulate cell growth.^{1,2} Bone is a complex composite comprised of mineral and proteinaceous components. Although collagen is the main matrix constituent in bone, there is evidence that non-collagenous proteins also contribute to calcium phosphate mineralization.^{3,4} Rather than attempting to mimic the complex *in vivo* bone regeneration process, there has been considerable scientific effort devoted to the development of *in vitro* methods to produce collagen-calcium phosphate composites that can be used to enhance bone healing.^{5,6} Challenges in the production of such composites include phase selectivity of the mineral component (to control reactivity and solubility) and adhesion between the protein and mineral component (for enhanced mechanical stability).

In this work, we describe an electrochemical isoelectric focusing method that yields collagen-calcium phosphate composites with controlled phase and excellent adhesion between mineral and collagen. Our previous works^{7,8} describe the electrochemical aggregation of pure collagen into a membrane *via* isoelectric focusing. Here, we demonstrate that this electrochemically aggregated collagen can act as a scaffold for calcium phosphate mineralization, and that it also affects the nucleation and growth process of the mineral. Our method offers a distinct advantage over many other electrochemical techniques^{9,10,11,12,13} used to produce calcium phosphate-collagen coatings because it offers the flexibility of producing a scaffolded composite without need of a supporting substrate.

Composites were made by a simple two-step process, shown schematically in Figure 1. First, collagen membranes were prepared from an electrolyte containing type I collagen monomers (final concentration of 0.15 mg/mL from 6.4 mg/mL acidic Nutragen stock solution (Inamed Biomaterials)) in ultrapure water (Barnstead, 18.2 M Ω -cm) with NaOH (EMD Chemicals, ACS reagent grade) to adjust the electrolyte pH to 7. Water electrolysis at the electrodes (8 V between two parallel stainless steel plates) creates a dramatic pH gradient (pH \sim 2 near the anode, pH \sim 12 near the cathode) within tens of seconds. The temporal and spatial evolution of this pH gradient has

been visualized and quantified in previous work.⁷ When the amphoteric collagen monomer is exposed to this pH gradient, a collagen film forms parallel to and approximately mid-way between the electrodes, where the pH matches the isoelectric point of collagen (pH \sim 6). This collagen-rich interfacial region is approximately 2 mm in width and maintains the separation of the acidic and basic regions of the electrolyte. After sufficient collagen film accumulation (30 minutes), or after the insertion of a cellulose membrane (Spectra/Por Biotech RC Membrane, MWCO: 8000, Spectrum Laboratories Inc.), solutions of calcium (either CaCl_2 or $\text{Ca}(\text{NO}_3)_2$, Aldrich Chemicals, ACS reagent grade) and phosphate (K_2HPO_4 or NaH_2PO_4 or $\text{NH}_4\text{H}_2\text{PO}_4$, Aldrich Chemicals, ACS reagent grade) were added to the acidic and basic sides, respectively, inducing calcium phosphate formation on the alkaline side of the membrane. Ca^{2+} and PO_4^{3-} concentrations less than 0.025 M lead to an immeasurably low quantity of calcium phosphate on the membrane; higher concentrations (investigated up to 0.1 M) did not generally impact mineral phase selectivity. For subsequent analyses, the membrane-based composite was air dried on a glass slide. Dried composites have thicknesses \sim 1 μm .

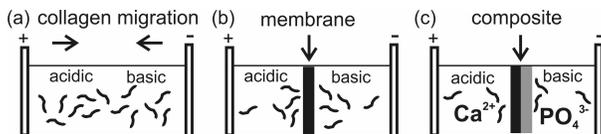


Figure 1: A schematic illustration of the two-step process for preparing the membrane-based electro-synthesized collagen-mineral composite. An applied voltage is required both to form the collagen membrane (Step 1, shown in panels (a,b)) and to stimulate crystallization of the calcium phosphate on the membrane (Step 2, shown in panel (c)). The acidic (pH \sim 2) and basic (pH \sim 12) regions are produced by water electrolysis in the electrolyte (initial pH 7). Once established, the pH gradient is maintained throughout the composite formation process.

For comparison, electrochemical precipitation experiments followed the same procedure as with the membrane, but using an electrolyte without collagen. In addition, simple solution mixing methods were investigated, wherein calcium and phosphate salt solutions (0.1 M, 1000 μL each) were combined to form calcium phosphate precipitates that were subsequently rinsed with ultrapure water and air-dried.

Mineral phases were identified with infrared spectroscopy (FTIR, Bruker Alpha, transmission mode with KBr matrix, 0.9 cm^{-1} resolution). Each spectrum was collected from a single and entire composite membrane, prepared under comparable conditions, so it is reasonable to make qualitative comparisons of relative peak heights between protein and mineral components among different specimens. Energy dispersive X-ray (EDX, Rontec Quantax with software from JKTech, University of Queensland, Australia) data further confirmed the phase assignments. Phase identification from X-ray diffraction data was not conclusive due to small sample volumes. In general, amorphous materials (such as ACP) and poorly crystalline materials (such as CHAp) cannot be readily differentiated *via* XRD because of their non-existent or broad diffraction peaks, respectively.¹³ Microstructural information came from scanning electron microscopy (SEM, FEI Quanta 400 environmental) on samples dried onto metal pucks and then carbon-coated. Elastic modulus values were obtained using an atomic force microscope (Asylum Research MFP-3D) using a method described elsewhere.⁸

Our results show that the presence of the collagen membrane, in addition to the use of electrochemically controlled precipitation, impacts mineral phase selectivity. For example, infrared (IR) spectra indicate brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) formation from a simple mixing of a phosphate solution ($0.1\text{ M K}_2\text{HPO}_4$) with either 0.1 M CaCl_2 or CaNO_3 , while electrochemical methods applied to the same starting solutions yield amorphous calcium phosphate (ACP). Comparisons between collagen-membrane-based, electrochemical, and solution production using a variety of starting salts are summarized in Table 1. Carbonated hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ with carbonate ions substituting for either phosphate or hydroxide,⁶ CHAp) dominates in all protein-mineral composite membranes, with one exception.

As shown in Table 1 and in earlier work,^{13,14} different mineral phases can be obtained from electrochemically assisted deposition by selecting appropriate precursor salts. In addition to the effects from using either monobasic or dibasic phosphate salts, counterions can also play a significant role, most likely from their effects on buffering (the addition of ammonium counterions)

Table 1: Comparison of mineral products resulting from an electrochemically (EC) produced collagen membrane composite, electrochemically assisted precipitation or solution precipitation using different starting electrolytes. Control experiments performed with a cellulose membrane did not yield a measurable amount of mineral on the membrane.

Ca ²⁺ source	PO ₄ ³⁻ source	collagen membrane EC product	EC product	solution product
CaCl ₂	K ₂ HPO ₄	CHAp	ACP	brushite
CaCl ₂	KH ₂ PO ₄	CHAp	ACP	brushite
CaCl ₂	NaH ₂ PO ₄	CHAp	CHAp	none
CaCl ₂	NH ₄ H ₂ PO ₄	brushite	ACP	none
Ca(NO ₃) ₂	K ₂ HPO ₄	CHAp	CHAp	brushite
Ca(NO ₃) ₂	KH ₂ PO ₄	CHAp	CHAp	brushite
Ca(NO ₃) ₂	NaH ₂ PO ₄	CHAp	ACP	none
Ca(NO ₃) ₂	NH ₄ H ₂ PO ₄	CHAp	ACP	none

or their electrochemical activity (either nitrate or chloride counterions). For example, chloride incorporation has been observed by others,¹⁴ and it has also been reported that nitrate reduction can contribute to enhanced electrolyte alkalinity.¹³ In our studies, we find that there is surprisingly little difference in the phase selectivity when using either chloride- or nitrate-based electrolytes.

Others have shown that the formation of ACP, a precursor to the bone-like apatitic phase, requires a rapid reaction between calcium and phosphate ions, and stabilizing agents (such as Mg²⁺ or HCO₃⁻) are needed to maintain ACP under ambient conditions.¹⁵ We find that select precursors yield stable amorphous calcium phosphate from electrochemically assisted syntheses, but only in the absence of collagen.

Figure 2 shows representative IR spectra that demonstrate collagen's influence on phase selectivity. Absorption peaks with wavenumbers above 1300 cm⁻¹ are due to collagen, while the phosphate peaks below 1300 cm⁻¹ can be assigned to the mineral component.^{11,16} When there is no collagen present in the electrolyte, amorphous calcium phosphate (ACP) forms (PO₄³⁻ peak at 1051), but when collagen is present this peak shifts to 1035 cm⁻¹ which is characteristic of CHAp's ν_3 (PO₄³⁻) mode.¹⁷ In these and all other samples, IR spectra show a peak near 870 cm⁻¹ that is indicative of carbonate incorporation, presumed due to dissolution of ambient CO₂(g).^{12,14}

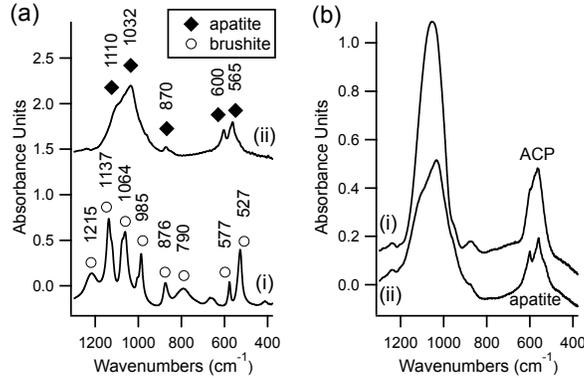


Figure 2: (a) Representative IR spectra for collagen-calcium phosphate composites prepared with collagen monomers plus 0.1 M CaCl_2 and either 0.1 M (i) K_2HPO_4 or (ii) $\text{NH}_4\text{H}_2\text{PO}_4$ show that precursor salts affect phase selectivity. The dominant peaks in spectrum (i) are from carbonated hydroxyapatite (CHAp) while those in (ii) indicate brushite. (b) Representative IR Spectra for specimens prepared using CaCl_2 and K_2HPO_4 , either without (i) or with (ii) collagen, highlight the importance of the presence of collagen for phase selectivity. CHAp forms in the presence of collagen; otherwise, amorphous calcium phosphate (ACP) dominates. In both panels, spectra are offset along the absorbance axis for clarity.

The relative intensities of the phosphate absorption peaks in specimens prepared with collagen are consistently higher than those prepared without collagen. Since this trend holds for comparisons among dozens of samples, it suggests that collagen also triggers increased calcium phosphate formation. This could be due to either enhanced precipitation (the membrane provides favorable nucleation sites) or trapping of the calcium phosphate particles as the collagen continues to aggregate. Scanning electron microscopy (SEM) images show evidence for crystallites forming on and within the membrane (Figure 3), and corresponding energy-dispersive X-ray analyses confirm that the crystallites contain calcium and phosphorous.

There are also interesting spatial differences that occur in the presence of collagen. When no collagen is present in the electrolyte, calcium phosphate precipitates form only in very alkaline environments ($\text{pH} \geq 8$, near and on the cathode). In contrast, the presence of collagen allows crystals to grow also at the more acidic pH values present in the electrolyte near the membrane (~ 6.5). The alkaline side of the collagen membrane appears to act as a nucleation site, and the majority of the mineralization occurs directly at the collagen membrane rather than as precipitation in the

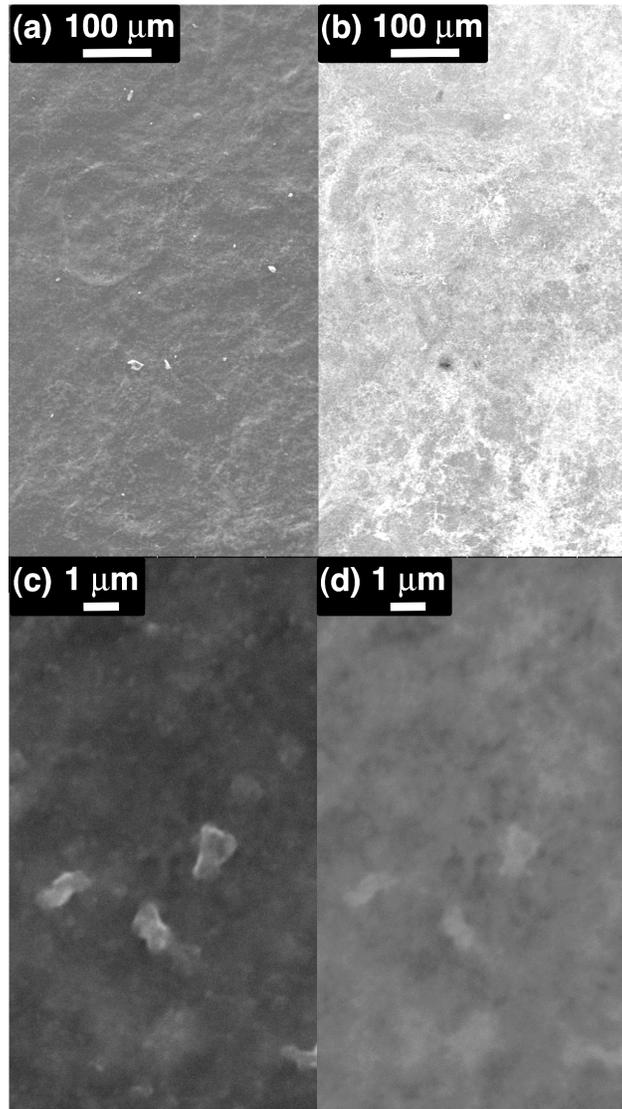


Figure 3: Representative SEM micrographs, at low (a, b) and high (c, d) magnifications, of a carbonated hydroxyapatite-collagen composite (here, from $\text{Ca}(\text{NO}_3)_2$ and $\text{NH}_4\text{H}_2\text{PO}_4$ precursors). The secondary electron images (a, c) show that composite topography is dominated by micron and sub-micron crystallites that appear to be embedded in the protein scaffold. The companion backscattered electron images of the identical areas (b, d) confirm that the mineral, indicated by bright regions, is spread rather uniformly throughout the scaffold.

electrolyte. There is no evidence of mineral formation on the glass surfaces of the electrochemical cell, but a thin calcium phosphate film does form on the stainless steel cathode, similar to earlier studies.¹³ Given the complexity of the calcium phosphate formation environment (including pH gradients and counterions), we find it quite surprising that there is no evidence of mixed phases, and that the mineralization occurs only on the alkaline side of the membrane. The composite membranes are mechanically robust when dried, and the mineral coating is intimately incorporated into the collagen scaffold. Figure 4 shows that composite membranes are stiffer (higher elastic modulus values) than for non-mineralized membranes.⁸ Rinsing in water or ethanol does not degrade the composites in any way.

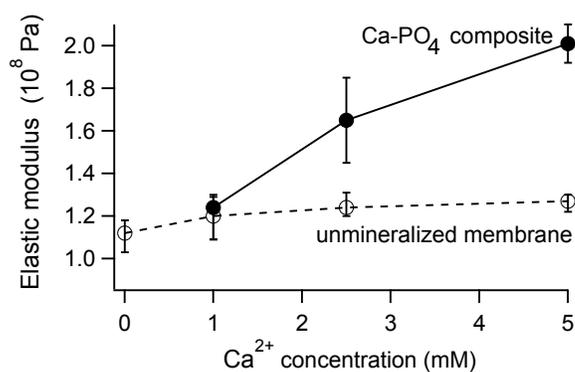


Figure 4: Comparison of Young’s elastic modulus values for composite mineralized collagen membranes (solid line) and unmineralized collagen membranes (dashed line). Preparing an unmineralized membrane in the presence of higher amounts of Ca^{2+} will lead to a slightly stiffer scaffold, but composite membranes are consistently more stiff. The lines connecting data points serve merely to guide the eye.

To confirm that the presence of collagen is important – and not merely the membrane-based pH partitioning that occurs during membrane formation – we replicated our experiments with a commercially available cellulose membrane. Although a small amount of calcium phosphate adhered to the cellulose, most formed as precipitates in the alkaline region of the electrolyte, similar to the case with no membrane present. In contrast, virtually no precipitates form elsewhere in the cell when collagenous membranes are present. We note that other membranes have been shown to support calcium phosphate crystallization, including a recent report of the mineralization of a

polymeric membrane in the presence of an alternating (AC) electric current.¹⁸

The issues of specific chemical functionalities and local pH values undoubtedly play an important role in the calcium phosphate crystallization process in these composite membranes. Our experiments do not substantiate a probable mechanism, but recent theoretical and experimental investigations have begun to address aspects of this question.^{6,12,14,15,19}

In conclusion, electrochemically assisted synthesis, based on isoelectric focusing, offers an expedient way to make robust collagen-calcium phosphate composites with controlled mineral phase. Collagen not only provides a scaffold on which the mineral phase can nucleate, but its presence also triggers a strong preference for one specific mineral phase (carbonated hydroxyapatite) relative to other phases (such as brushite) that would otherwise form during electrochemical synthesis. This offers two distinct advantages for the electrosynthesized composite over many other existing biocomposite materials. First, the composite is produced as a suspended membrane that can be removed and applied to other surfaces. Second, the selectivity of the carbonated hydroxyapatite phase is fortuitous, since this is the mineral phase that is most similar to that found in natural bone. These two features make this protein-mineral composite material promising for future biocompatibility studies.

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