Biomimetic Calcium Phosphate Synthesis by Using Calcium Metal

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Urbana, IL 61801, USA

ABSTRACT

Metallic calcium was never used before as the sole calcium source in synthesizing biomaterials. Amorphous calcium phosphate (ACP) bioceramic powders were synthesized at room temperature, in synthetic mineralization solutions which contained Na⁺, K⁺, Mg²⁺, Cl⁻, HCO₃⁻ and HPO₄²⁻ ions at concentrations similar to those found in human blood plasma, by using calcium (Ca) metal as the only calcium source. The experimental conditions leading to the formation of ACP or PCA (cryptocrystalline or poorly-crystallized apatite) powders were determined when using metallic Ca in aqueous synthesis in the mineralization solutions. The formation of calcium phosphate (CaP) in synthesis solutions was immediately initiated by the addition of calcium metal granules (or shots), at appropriate amounts, into the solutions while the solutions were being continuously stirred in glass bottles at room temperature (22±1°C). The synthesis reactions were reaching completion in less than 30 minutes with the final solution pH values ranging from 9 to 12, without a necessity for any external pH adjustment in the form of any strong base (such as NH₄OH, LiOH, NaOH or KOH) additions. ACP or PCA powders are useful for dentin and enamel re-mineralization applications or orthopedic (bone) defect-filling applications.

INTRODUCTION

The systematic synthesis and characterization of poorly-crystallized (i.e., cryptocrystalline) apatite (PCA) powders in deionized (i.e., free of Na⁺, K⁺, Mg²⁺, Cl⁻ and HCO₃⁻ ions of blood plasma) water solutions containing dissolved Ca(NO₃)₂·4H₂O and (NH₄)₂HPO₄ were initiated in the early 50's by Hayek and co-workers [1-3]. The work of Hayek et al. [1-3] taught us to raise the pH values of such cryptocrystalline apatite synthesis solutions to around 10.5-11 by the addition of NH₄OH. The method originally developed by Hayek et al. [1-3] to produce cryptocrystalline nanoparticles of calcium phosphate (CaP) was then adopted and popularized by Jarcho et al. [4]. Currently, the above-mentioned Hayek method of synthesizing cryptocrystalline apatitic CaP powders is one of the most preferred.

Posner and co-workers [5-7] were among the first, in the mid 50's, to realize that the mineral of natural hard tissues consisted of non-stoichiometric pseudoapatites. Posner et al. [5] envisaged in 1954 that the carbonate (CO₂)- and foreign cation-free pseudoapatites can be represented by the formula of Ca₁₀₋ₓH₂₂(PO₄)₆(OH)₂, where the value of x would range from zero for stoichiometric hydroxyapatite (HA) to two for an apatite with a Ca/P molar ratio of 1.333. One shall here note the similarity between that pseudoapatite of Ca/P ratio of 1.333 mentioned by Posner [5] and the compound known as octacalcium phosphate (OCP, Ca₁₀H₂O₆(PO₄)₆·5H₂O or more appropriately denoted as Ca₁₀(HPO₄)₂(PO₄)₆·5H₂O). Posner et al. [8] later showed experimentally that the mineral of the rat bones did exhibit a very strong age-dependent (from 1 to 40 days of age) variation in their Ca/P molar ratios, i.e., the younger the rat the lower the Ca/P ratio of its bones, the very young rats having a Ca/P ratio over the range of 1.15 to 1.34, in agreement with the much earlier work of Burns and Henderson [9].

Posner and co-workers [10] have been the first to describe how to prepare synthetic amorphous calcium phosphate (ACP) powders by using CaCl₂- and (NH₄)₂HPO₄-containing
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distilled water solutions (i.e., not containing biologically essential ions such as Na⁺, Mg²⁺, K⁺ or HCO₃⁻) whose pH values were raised to around 11 by NH₄OH additions.

Betts and Posner [11, 12] postulated that ACP actually consisted of roughly spherical clusters (also called as Posner clusters) close to 1 nm in diameter, with a Ca/P molar ratio of 1.5 and the formula of Ca₅(PO₄)₃O which were free of water. Synthetic ACP, according to Posner et al. [11, 12], consisted of roughly spherical Ca₅(PO₄)₃O clusters, which formed in water and were then aggregated randomly to produce the larger spherical particles of ACP with the inter-cluster space being filled with water. A similar aggregation process was however described previously, in 1957, by Glimehe et al. [13] in relation to mineralization of the collagen-hydroxyapatite system.

ACP, when in contact with an aqueous solution, is known to exhibit the unique ability to first nucleate OCP-like nanosize crystallites on the surfaces of its particles, which would then rapidly mature into apatitic calcium phosphate [10, 12]. This property of ACP powders was successfully exploited to prepare injectable orthopedic cements [14, 15]. Posner and his co-workers were also the first to study the interaction of casein micelles of bovine milk with ACP powders [16], and this apparently led to the development of ACP-casein phosphopeptide (CPP) [17] complexes for dental remineralization applications.

Since the early studies of Hayek [1-3] and Posner [5, 10-13], to the best of our knowledge, the ACP and PCA-related literature [18-35] did not contain any novel approaches to the synthesis of ACP powders, i.e., meaning the calcium source employed in the synthesis processes was always selected from the Ca-chloride, Ca-nitrate and Ca-acetate salt group, and the pH values of the synthesis solutions were raised to the basic range (pH ~11) by the addition of strong bases such as NH₄OH, NaOH or KOH.

The current study was originated by the following questions:

Could it ever be possible to synthesize CaP powders (either ACP or PCA) in aqueous solutions totally free of nitrate (NO₃⁻), acetate (CH₃COO⁻) or ammonium (NH₄⁺) ions which are not shown to be present in biological bone or tooth formation processes?

Could it be possible to synthesize ACP or PCA powders by using aqueous solutions having the pH values from 9 to 12 (which was underlined by the early works of Hayek and Posner as a necessity) without even using the smallest aliquot of a strong base such as NH₄OH, NaOH, KOH or LiOH?

Could it be possible to simulate the concentrations of inorganic ions present in human blood plasma in the synthesis solutions while strictly maintaining the conditions set by the above two questions?

In CaP synthesis, nitrate or acetate ions would be introduced into the synthesis solutions by the use of calcium nitrate tetrahydrate or calcium acetate monohydrate as the calcium source. We considered that if we were using Ca metal as the only calcium source, then that would totally eliminate any nitrate or acetate ions. Layrolle et al. [27] used Ca metal shots only to produce calcium dichloride by reacting them with ethanol, and they did not see the Ca metal shots as a possible starting material to be quite useful in ACP or PCA synthesis in aqueous solutions. It is common knowledge [36] that Ca metal is produced by electrolysis of a molten bath of calcium chloride salt, and the produced Ca metal granules react with distilled water to raise its pH under a slow evolution of H₂ gas (i.e., in situ deprotonation). We considered that the use of Ca metal as the calcium source in ACP or PCA synthesis might have eliminated the need for using any strong bases in raising the solution pH to the levels given in the works of Hayek [1-3] and Posner [5, 10-13].

This study, to the best of our knowledge, is the first one to use Ca metal (but not salts such as Ca-chloride, Ca-nitrate or Ca-acetate) in synthesizing calcium phosphates. This study also compared the use of Ca metal (as 25% w/w addition) to the use of ACP or PCA powders (as 25% w/w addition) for the synthesis of calcium phosphates. The preparation of this powder was achieved by mixing the nitrate or acetate salts with ACP or PCA powders, using distilled water as the solvent (pH ~11) in a microprocessor-controlled glass reactor (pH meter) under a constant magnetic stirrer in order to achieve a homogeneous mixture with a pH value of 10.6 ± 0.2. After drying the precipitate prepared in distilled water (pH ~11), the precipitate was dried at 22 ± 1°C for 48 h and then ground using a Teflon®-coated pestle and mortar. This ground powder was washed with distilled water, until its pH value was neutral (pH 7) and then dried again for 48 h.

The experiments were conducted in 500 ml (Cat. No. F8800500) flask bottles, which were first rinsed with an ample amount of doubly-distilled water (pH ~5) and then placed into the experiment bottle. All experiments were carried out under a magnetic stirrer at 250 rpm. The pH value of the solution inside the flask was measured using a digital pH meter (pH 7). A Teflon®-coated pestle and mortar were used to grind the precipitate prepared in distilled water (pH ~11) and then wash it with distilled water to achieve the desired pH value of 7.

Table I represents the chemical composition of each experiment, whereas the third column shows the percentage of each chemical in the solution. The solutions were prepared in 500 ml (Cat. No. F8800500) flask bottles, which were first rinsed with an ample amount of doubly-distilled water, then placed into the experiment bottle. All experiments were carried out under a magnetic stirrer at 250 rpm. The pH value of the solution inside the flask was measured using a digital pH meter (pH 7). A Teflon®-coated pestle and mortar were used to grind the precipitate prepared in distilled water, washed with distilled water to achieve the desired pH value of 7.

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<td>0.1525</td>
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<tr>
<td>NaCl</td>
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<td>NaHCO₃</td>
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<td>1.1341</td>
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<td></td>
</tr>
<tr>
<td>(1) NaHPO₄</td>
<td></td>
<td>0.7098</td>
</tr>
<tr>
<td>(2) Na₂HPO₄</td>
<td></td>
<td>0.3549</td>
</tr>
<tr>
<td>(3) Na₃PO₄</td>
<td></td>
<td>0.0710</td>
</tr>
</tbody>
</table>

The solutions were prepared in 500 ml (Cat. No. F8800500) flask bottles, which were first rinsed with an ample amount of doubly-distilled water (pH ~5) and then placed into the experiment bottle. All experiments were carried out under a magnetic stirrer at 250 rpm. The pH value of the solution inside the flask was measured using a digital pH meter (pH 7). A Teflon®-coated pestle and mortar were used to grind the precipitate prepared in distilled water (pH ~11) and then wash it with distilled water to achieve the desired pH value of 7.
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also compared the use of Ca metal (as the Ca source) to using the above-mentioned calcium salts. This study would also be the first one to synthesize ACP (or PCA) powders in synthetic mineralization solutions developed hereby to mimic the inorganic ion concentrations of human blood plasma. Human body does not use deionized or distilled water alone in synthesizing the mineralized portion of bone and teeth.

EXPERIMENTAL

Sodium chloride (NaCl; Catalog No: 1.06404, Merck KGaA, Darmstadt, Germany), potassium chloride (KCl; No: 1.210517, Merck), magnesium chloride hexahydrate (MgCl₂·6H₂O; No: 459331, Carlo Erba Reagenti, Milano, Italy), sodium bicarbonate (NaHCO₃; No: 1.06329, Merck), and disodium hydrogen phosphate anhydrous (Na₂HPO₄; No: 1.06586, Merck) were used in solution preparation. Calcium metal (Ca; spherical granular, 2.5 mm in diameter; No: 1.02053, Merck), calcium chloride dihydrate (CaCl₂·2H₂O; No: 1.02382, Merck), calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O; No: 1.02121, Merck), calcium acetate monohydrate (Ca(CH₃CO₂)₂·H₂O; No: 402850, Sigma-Aldrich), and calcium hydroxide (Ca(OH)₂; No: 1.02110, Merck) were separately tested as the sources of calcium. In some experiments diammonium hydrogen phosphate ((NH₄)₂HPO₄; No: 1.01207, Merck) was tested as the source of phosphorus instead of Na₂HPO₄. Finally, ammonium hydrogen carbonate (NH₄HCO₃; No: 1.01131, Merck) was also tested to replace NaHCO₃ in some experiments.

The solutions were prepared in 500 mL-capacity Pyrex glass bottles (Fisher Scientific, Cat. No: FB800500). The bottles were first cleaned by washing with 5 vol% HCl, followed by rinsing with an ample amount of doubly-distilled water, and overnight drying at 90°C. Five hundred mL of doubly-distilled water was first placed into the bottles at room temperature (RT, 22±1°C). A Teflon®-coated (25 mm long, 5 mm in diameter) rod-shaped magnetic stirrer was then placed into the experiment bottle. All of the synthesis experiments were performed on a magnetic stir-plate and the stirring rate was constant at 750 rpm. The carefully weighed chemicals were added, one by one, to the bottle, under constant stirring of the solution inside. The next chemical was not added prior to the complete dissolution of the previous one. Table I shows the procedure of preparing the synthesis / mineralization solutions (MS) in 500 mL distilled water (not boiled prior to use to remove any possible HCO₃⁻). The chemicals were added to water in the order given. Table I offered three choices of solution preparation to the reader; the first one would lead to preparing a solution with 10 mM HPO₄²⁻, whereas the third would result in a solution with 1 mM HPO₄²⁻. All three solutions of Table I were transparent and precipitate-free at the time of preparation, and thus they were ready for the addition of the pre-weighed amount of Ca metal (or calcium chloride, calcium acetate monohydrate or calcium nitrate tetrahydrate in a limited number of experiments).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Preparation of mineralization solutions (MS)</th>
<th>500 mL H₂O basis</th>
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</thead>
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<tr>
<td></td>
<td>mg/L cation</td>
<td>mg/L anion</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1865</td>
<td>5 K⁺</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.1525</td>
<td>1.5 Mg²⁺</td>
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<tr>
<td>NaCl</td>
<td>2.7760</td>
<td>95 Na⁺</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.3411</td>
<td>27 Na⁺</td>
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<tr>
<td>(NH₄)₂HPO₄</td>
<td>0.7098</td>
<td>20 Na⁺</td>
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<td>Na₂HPO₄</td>
<td>0.3549</td>
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</tr>
<tr>
<td>Na₂PO₄</td>
<td>0.0710</td>
<td>2 Na⁺</td>
</tr>
</tbody>
</table>

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To further clarify the solution preparation technique described in Table 1; one first adds KCl to 500 mL of water, dissolves it, then performs the respective additions of MgCl₂·6H₂O, NaCl and NaHCO₃. At that moment, the solutions contain 5 mM K⁺, 1.5 mM Mg²⁺, 103 mM Cl⁻, and 27 mM HCO₃⁻. These ion concentrations are identical with those of the blood plasma. If one were then adding 0.7098 g of NaHPO₄, the solution would have a total Na⁺ ion concentration equal to 142 mM. This concentration of Na⁺ is exactly that of the blood plasma. The solution thus obtained according to the choice-1 of Table 1 was able to match the Na⁺, K⁺, Mg²⁺, HCO₃⁻ Cl⁻ concentrations of the blood plasma, but will possess 10 times the HPO₄²⁻ concentration of plasma. However, the solution of choice-3 (of Table 1) will have the identical HPO₄²⁻ concentration with that of blood plasma.

If one were using CaCl₂·2H₂O as the calcium source (instead of Ca metal), it would not be possible to maintain the proper Cl⁻ ion concentration in the solution, i.e., it would have been in excess of 103 mM. Blood plasma contains exactly 103 mM Cl⁻. If one were using Ca(NO₃)₂·4H₂O as the calcium source, then the synthesis medium would have contained nitrate ions, which are not present in the blood plasma. The same applies to the use of Ca-aceate, as well.

Powder synthesis began instantly by the addition of prescribed amount of calcium metal granules into the mineralization solutions stirred at 750 rpm. Reactions were continued for 25 minutes at RT (22±1°C), pH values were recorded (pH meter, Model: S40, Mettler-Toledo, with combined pH-temperature electrode), at every 30 seconds, starting from the moment of adding Ca metal into the solutions. At the end of 25 minutes of stirring, the formed solids were immediately and quickly filtered out of their mother liquors by using a Whatman No. 2 filter paper via a Buchner funnel apparatus, backed up with a mechanical vacuum pump. The solid residues were washed with 750 mL of distilled water and then dried on watch-glasses at RT for 40 hours in an air-ventilated drying cabinet. In the duplicate experiments, samples were synthesized once more as described above, but then left in the solutions overnight (i.e., at least 17 h), in the bottles, at RT. The pH values of the solutions were measured once again after that long period of RT ageing and exactly the same values were found with those measured after only 25 minutes of reaction.

Prior to powder X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) analyses, the dried samples were ground, manually, in an agate mortar by using an agate pestle. XRD runs were performed (Advance D8, Bruker, Karlsruhe, Germany) in the step scan mode, with the step size of 0.02° and preset time of 5 seconds. The powder diffractometer was equipped with a Cu tube and operated at 40 kV and 40 mA. XRD samples were prepared by gently packing the powders into the sample holder cavity of around 1 mm-deep. FTIR samples were mixed with KBr powders at the ratio of 1 mg sample-to-250 mg KBr in an agate mortar. FTIR pellets of 13 mm diameter were pressed at 10 tons. FTIR data were collected (Spectrum One, PerkinElmer, Waltham, MA) by using 256 scans. Scanning electron microscopy (Vega-2, Tescan, A.S., Brno, Czech Republic) samples were not ground and the small sample chunks were sputter-coated with a thin gold layer before imaging.

RESULTS AND DISCUSSION

Until this study, the researchers working in CaP-based biomaterial synthesis have chosen either one of the following as their calcium source: calcium chloride (anhydrous or dihydrate), calcium nitrate (tetrahydrate), calcium acetate (monohydrate), calcium carbonate or calcium hydroxide. The former three of these have significant solubility even in cold water, but the latter two had much lower solubilities in comparison to the former. One of the novelties of this study is that it used metallic calcium as the calcium source. Metallic calcium presented clear advantages: (i) it did not bring into the synthesis solutions any foreign or spectator anions, such as nitrate or deprotonation of the aqueous synthesis media shown below, totally eliminating the need for additions to maintain the synthesis pH above of this study. The necessity of maintaining synthesis of either ACP (amorphous calcium phosphate) or hydroxyapatite apatite calcium phosphate (Hayek [3,7] and Posner [5,14,15]).

The below reactions would be helpful when using Ca metal.

\[ \text{Ca}(s) + \text{H}_2\text{O}(l) \rightarrow \text{Ca}^{2+}(aq) + 2\text{OH}^- \]  
\[ \text{Ca}(s) + 2\text{H}_2\text{O}(l) \rightarrow \text{Ca(OH)}_2(s) + \text{H}_2(g) \]  
\[ \text{Ca}^{2+}(aq) + \text{HCO}_3^-(aq) \rightarrow \text{CaCO}_3(s) + \text{H}^+(aq) + \text{Cl}^- \]

Equation-1 explains the evolution of the CaO granules into the solutions. Equation-2 dissolve in doubly-distilled water. Equation-3 dissolved in blood plasma-like, mineralization (i.e., 103 mM) of Cl⁻ ions, in such a short time as a good strong similarity between the behavior of mineralization in biomineralization.

Ca metal shots/granules were not experiments. In other words, in the absence of Cl⁻ hydroxide and/or Ca-carbonate and water, experiment 6 (Table 2), 25 mM of calcium Na₂HPO₄ did not dissolve completely, but then the small amount of precipitates formed were comprised of biphasic mixtures of hydroxide.

Experiments 7 and 8 were performed and 2.50, in reacting Ca granules with the experiments produced cryptocrystalline apatite with final pH values greater than 12, without experiment 8 had 115 mM Na⁺, 103 mM Cl⁻, 5 mM K⁺, experiments one would be able to form the concentration of any other ion in the solution metal in CaP synthesis. This would not be possible as the calcium source. Experiments 7 and 8: cryptocrystalline (some call it poorly-crystalline apatite CaP powders at RT, in a very short technique, such as drop-wise addition of (OH) at an in situ solution pH of 12. Experiments 8 which was equal to that of blood plasma. But definitely can if the synthesis solutions were at using Ca metal in PCA synthesis.

Experiments 9 and 10 (Table 2) were run with (NH₄)₂HPO₄, while keeping all the other presence of NH₄⁺ ions in a synthesis system listed.
foreign or spectator anions, such as nitrates, chlorides or acetates, and (ii) it caused in situ deprotonation of the aqueous synthesis media resulting in a smooth and rapid pH increase (as shown below), totally eliminating the need for base (NaOH, KOH, LiOH, NH₂OH, etc.) additions to maintain the synthesis pH above neutral. These two points further define the novelty of this study. The necessity of maintaining the solution pH much above the neutral during the synthesis of either ACP (amorphous calcium phosphate) or PCA (poorly-crystallized, eutectocrystalline apatitic calcium phosphate) was well-proven throughout the previous work of Hayek [1-3], Posner [5-7] and Rey [14, 15].

The below reactions would be helpful in explaining the powder synthesis mechanism when using Ca metal.

\[
\begin{align*}
\text{(1)} & & \text{Ca(s) + H}_2\text{O(l)} & \rightarrow \text{Ca}^{2+}(aq) + 2\text{OH}^- (aq) + \text{H}_2(g) \\
\text{(2a)} & & \text{Ca(s)} & + 2\text{H}_2\text{O(l)} & \rightarrow \text{Ca(OH)}_2(s) + \text{H}_2(g) \\
\text{(2b)} & & \text{Ca}^{2+}(aq) & + \text{HCO}_3^-(aq) & \rightarrow \text{CaCO}_3(s) + \text{H}^+(aq) \\
\text{(3)} & & \text{Ca(OH)}_2(s) & + \text{H}^+(aq) + \text{Cl}^- (aq) & \rightarrow \text{Ca}^{2+}(aq) + \text{HCl} + 2\text{OH}^- (aq) 
\end{align*}
\]

Equation-1 explains the evolution of H₂ gas and the observed rise in pH upon adding the calcium granules into the solutions. Equations (2a) and (2b) explain why the Ca granules did not dissolve in doubly-distilled water. Equation 3 explains why the Ca-metal granules readily dissolved in blood plasma-like, mineralization solutions (MS), containing significant amounts (i.e., 103 mM) of Cl⁻ ions, in such a short time by causing such a strong rise in pH. There is a strong similarity between the behavior of magnesium metal and calcium metal in this respect.

Ca metal shots/granules were not expected to fully react in water only containing HPO₄²⁻ ions. In other words, in the absence of Cl⁻ ions, the granules would be easily covered with Ca-hydroxide and/or Ca-carbonate and would stop reacting. This expectation was tested in experiment 6 (Table 2). 25 mM of calcium granules stirred in water only having 10 mM Na₂HPO₄ did not dissolve completely, but the pH of the solution was able to rise above 12 and the small amount of precipitates formed were found, by XRD (data not shown [37, 38]), to be comprised of biphasic mixtures of eutectocrystalline apatitic CaP (PCA) and calcite.

Experiments 7 and 8 were performed to study the effect of Ca/P molar ratio, i.e., 1.677 and 2.50, in reacting Ca granules with the MS solutions free of HCO₃⁻ ions. Both of these experiments produced eutectocrystalline apatitic calcium phosphate (PCA) samples in solutions with final pH values greater than 12, without any calcite. The MS solutions of experiments 7 and 8 had 115 mM Na⁺, 103 mM Cl⁻, 5 mM K⁺, 1.5 mM Mg²⁺ and 10 mM HPO₄²⁻, and in both experiments one would be able to freely change the Ca content without disturbing the concentration of any other ion in the solution; i.e., another significant advantage of using Ca metal in CaP synthesis. This would not be possible if one were using, for instance, CaCl₂·2H₂O as the calcium source. Experiments 7 and 8, therefore, showed a simple way of producing eutectocrystalline (some call it poorly-crystalline or poorly-crystallized or nanocrystalline) apatitic CaP powders at RT, in a very short 25 minutes, without employing any external pH control technique (such as drop-wise addition of a strong base such as NH₄OH, NaOH, KOH, or LiOH) at an in situ solution pH of 12. Exp-8 had the nominal solution Ca/P molar ratio of 2.5, which was equal to that of blood plasma. Bacteria cannot grow at a solution pH of 12, but they definitely can if the synthesis solutions were at neutral pH (6.8 to 7.6). This is another advantage of using Ca metal in PCA synthesis.

Experiments 9 and 10 (Table 2) were replacing the Na₂HPO₄ used in experiments 7 and 8 with (NH₄)₂HPO₄, while keeping all the other synthesis parameters unchanged. Although the presence of NH₄ ions in a synthesis system claiming to mimic the ions and ion concentrations in...
Table II  Sample preparation details

<table>
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<th>Experiment</th>
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<th>Ca source</th>
<th>CO(_3) source</th>
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<th>Ca (mM)</th>
<th>CO(_3) (mM)</th>
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Biomimetic Calcium Phosphate Synthesis by using Calcium Metal

Blood plasma would not be acceptable. Experiment 9 produced amorphous calcium phosphate (ACP) at the Ca/P molar ratio of 1.667 and the final pH value of 11.3.

It was quite easy to distinguish between the ACP and PCA phases by using their FTIR data, as exemplified by the IR traces of experiments 9 and 7 in Figure 1a, respectively. In the IR data of ACP samples the phosphate bands over the range of 660 to 490 cm⁻¹ do not show that splitting, which was otherwise observed in PCA samples. When the Ca/P molar ratio was increased to 2.5 in experiment 10, the produced powders were not ACP but PCA. The solution pH in this experiment was 12. Upon repeating the experiment 9, but ageing the formed precipitates in the mother solution for 5 days at RT (solution pH dropping to 10.7 from 11.3, in 5 days), followed by filtering and drying, the obtained powders were consisted of PCA, not ACP, as shown in Figure 1b. This was quite an expected result since ACP was not a stable phase (even in its mother liquor over a period of 5 days) and it acted as a precursor to PCA.

Experiments 16 through 18 tested the synthesis conditions closest to the ionic concentrations of the human blood plasma, by using the metallic Ca granules or shots. In experiment 16; calcium, phosphate (HPO₄²⁻), bicarbonate (HCO₃⁻), potassium, chloride, and magnesium ion concentrations were made identical with that of blood plasma, but in that experiment the sodium concentration was equal to 124 mM. In experiment 18, on the other hand; bicarbonate (27 mM), sodium (142 mM), magnesium (1.5 mM), potassium (5 mM) and chloride (103 mM) ion concentrations were identical with that of blood plasma. In other words, experiments 16 through 18 tested the MS solutions given in Table 1 under three different choices. The combined XRD and FTIR data of the resultant ACP samples were given in Figure 2. The second inset of Figure 2 confirmed the absence of the octacalcium phosphate (OCP, Ca₈(HPO₄)₂(PO₄)₂·5H₂O) phase in the samples of experiments 16 to 18. At such high solution pH values it would be very difficult, if not impossible at all, to observe acidic OCP. The sample of experiment 18 showed the presence of a small amount of calcite (CaCO₃) phase in its XRD data. However, when we duplicated experiments 16 through 18, and left the precipitate-containing solutions overnight without stirring, followed by filtering and drying, the resultant XRD data of especially experiment 18 did not show that second phase of calcite. All three samples (16 through 18) depicted the characteristic XRD pattern of ACP. The Ca metal granules in experiments 16 through 18 all dissolved/disappeared at around the 11th minute. When
experiment 18 is performed (i.e., experiment 19) in doubly-distilled water (containing 10 mM HPO$_4^{2-}$, 27 mM HCO$_3^-$, and 47 mM Na$^+$), instead of the MS solution, Ca metal granules did not dissolve and no precipitates were obtained. This again proved the role of Cl$^-$ ions, as explained by equations (1) through (3) above. Figure 3 showed the pH-time curves for experiments 16 through 19.

![Figure 2. Combined XRD and FTIR traces for the samples of experiments 16, 17, and 18 (CaCO$_3$ peaks were indicated by + in the XRD trace of experiment 18)](image)

Figure 2. Combined XRD and FTIR traces for the samples of experiments 16, 17, and 18 (CaCO$_3$ peaks were indicated by + in the XRD trace of experiment 18)

![Figure 3. pH-time curves for experiments 16, 17, 18, and 19 (the dissolution time of Ca granules was indicated by the straight dashed line)](image)

Figure 3. pH-time curves for experiments 16, 17, 18, and 19 (the dissolution time of Ca granules was indicated by the straight dashed line)

The SEM photomicrographs of the samples of experiments 16 and 18 are given in Figures 4a and 4b, respectively. It should be noted that these are filtered and dried samples, they were not even lyophilized following separation from their mother liquors. Regular drying causes agglomeration of individual particles or moieties.
It was apparent from Figures 4a and 4b that the average particle diameter in these x-ray amorphous, carbonated and mesoporous CaP powders was pretty much less than 70 nm. This is the particle size directly observed by the SEM, not the crystallite size. Crystallite sizes cannot be determined by using the Scherrer equation while using the XRD data of x-ray amorphous samples.

The concentration of Ca metal added into the MS solutions (starting from 2.5 mM in experiment 16 and going up to 25 mM in experiment 18) was found to be quite influential on the final pH values attained in syntheses. When the Ca concentration was kept equal to that of the blood plasma (i.e., 2.5 mM in exp. 16), the pH of the solution has risen only to 9.2 and stabilized at that value. By increasing it to 12.5 mM (i.e., 5 times that of plasma in exp. 17), the pH rose to 10.3, and the pH increased to 12 when the Ca concentration in the MS solution was increased to ten times that of the blood plasma (exp. 18).

The conditions of Exp-16 was of pivotal significance for this study, since the Ca\(^2\), HPO\(_4\)^{2-}, HCO\(_3\), Mg\(^2\), K\(^+\), Cl\(^-\) concentrations of this experiment were identical with those of human blood, and moreover, no foreign ions such as nitrate, ammonium and acetate were introduced to the synthesis process. As shown by the data of Fig. 3, maintaining a literally constant pH in CaP synthesis, without employing any pH control (such as adding bases or acids to keep the pH constant), was never shown before to be possible. These define the novelty and practicability of the approach of using Ca metal as the sole calcium source in CaP synthesis.

The influence of the use of (NH\(_4\))\(_2\)HPO\(_4\) and NH\(_4\)HCO\(_3\) salts, instead of Na\(_2\)HPO\(_4\) and NaHCO\(_3\) was also tested in synthesizing CaP powders by using Ca metal granules. Such a direct comparison was necessary. Experiments 20 through 24 (of Table 2 [37, 38]) all produced ACP powders in MS solutions. The use of Na-phosphate or Na-bicarbonate (as shown in experiments 20 and 21) raised the solution pH to above 10, but when both of Na\(_2\)HPO\(_4\) and NaHCO\(_3\) were replaced by (NH\(_4\))\(_2\)HPO\(_4\) and NH\(_4\)HCO\(_3\) the solution pH values dropped to about 9.3 to 9.5 (experiments 22 through 24). Of course, the solutions used in these experiments could not mimic the physiological solutions, since they contained significant amounts of ammonium ions which are not found in blood plasma.

Experiments 25 through 30 of Table 2 studied the synthesis of CaP in MS solutions, without using Ca metal. These experiments were planned to show what difference the use of Ca metal would really cause in comparison to the more commonly preferred calcium ion sources, such as CaCl\(_2\), 2H\(_2\)O, calcium acetate monohydrate (Ca(CH\(_3\)CO\(_2\))\(_2\)-H\(_2\)O), Ca(NO\(_3\))\(_2\)-4H\(_2\)O, and Ca(OH)\(_2\). Figure 5 showed the XRD traces of samples obtained in experiments 25 through 29, all indicating PCA. The inset in Figure 5, on the other hand, exhibited the IR traces of the samples
of experiments 25 through 27. The IR traces of experiments 26, 28 and 29 were very similar to one another, and they all exhibited much less carbonate ion presence (according to the qualitative IR data) in comparison to, for instance, the sample of experiment 27.

Figure 5. Combined XRD and FTIR data traces of the samples of experiments 25 through 29

MS solutions were working perfectly well, at the stated ion concentrations, in providing a reaction pH of exactly 7 for Ca-chloride, Ca-acetate, or Ca-nitrate; without a need for any external pH adjustments by acids or bases of any kind. This is another significant finding of this study. Ca metal granules, on the other hand, made it possible to synthesize ACP or PCA powders at pH values higher than 7, without needing any base additions for pH control, in the biomimetic MS solutions.

To synthesize PCA by using Ca metal granules, we found that one needed to eliminate HCO₃⁻ from the MS solutions. Using CaCl₂·2H₂O in doubly-distilled water or HCO₃⁻-free MS solutions containing phosphate ions, without any pH adjustments, would never allow the synthesis of PCA, since the pH of the solutions was lower than neutral (i.e., 7) and would thus only be suitable for the crystallization of brushite (CaHPO₄·2H₂O) phase, as also shown in this study.

Human blood, which provides the necessary nutrients to the trabecular/cancellous bones and the dentine of teeth, does not contain Tris (or Hpes), nitrate, acetate and/or ammonium ions. Therefore, it would be difficult to classify the synthesis (or coating) processes using Tris-HCl (or Hpes-NaOH) buffered solutions and especially the synthesis methods using one or more of the starting chemicals of Ca-nitrate tetrahydrate, Ca-acetate monohydrate, ammonium hydroxide, diammonium hydrogen phosphate or ammonium dihydrogen phosphate as properly mimicking the physiological processes [39-43].

Ammonium-, nitrate- and acetate-free synthesis recipes (especially those of experiments 7, 8, 16, 17 and 18) given in Table 2 of this study provided easy-to-reproduce and quite simple procedures to synthesize PCA (cryptocrystalline apatitic CaP) and ACP (x-ray amorphous CaP) powders at RT in glass media bottles, without requiring any special reactor designs and pH adjustment/control measures. It would be naïve to assume that the PCA or ACP synthesized in such blood plasma-like solutions would be completely free of ionic substitutions of Na⁺, K⁺, Mg²⁺, CO₃²⁻ and Cl⁻ ions at the crystallographic Ca, PO₄ and OH sites of hydroxyapatite structure. In a follow up study, we will study plasma atomic emission spectroscopy analyses in synthesized in synthesis media free of K⁺, Na⁺.

The ionic strength of the synthesis media of experiments 7, 8, 16, 17 and 8 of this study were 211.5 mM, respectively. If one was to prepare 142 mM Na⁺, 5 mM K⁺, 1.5 mM NaH₂PO₄·H₂O exact ion concentrations of human blood plasma, it would have been 148.5 mM. The ionic strength of this study to facilitate the synthesis of larger particles needed attention in the previous literature studies to mention the basicity of apatitic CaP.

The current study obtained pH values from the Ca-nitrate/(NH₄)₂HPO₄ route and studies 11, whereas the high pH values in that study et al. [44] study was not designed to make previous studies on the basicity of apatitic CaP apatite which is basically a hydroxyapatite compound with a significantly basic surface. et al. [45] deliberately and quantitatively studied the Ca-nitrate/(NH₄)₂HPO₄ route of experiments 26 and found that (i) the solution pH had the produced and (ii) the basic site density in apatite. Therefore, the current study using Ca metal granules at the high pH values (from 10 to 12) study [45].

Although the size of the Mg²⁺ ion (0.78 nm), magnesium ions can substitute for Ca²⁺ (1.17 nm). The incorporation of Mg into human Tris-HCl buffered SBF (synthetic body fluid) solutions (MS) contained Mg²⁺ at a concentration of 0.1 M. In the author's lab has been the first to use Tris-buffered SBF (synthetic body fluid) solutions (MS) contains Mg²⁺ at a concentration of 0.1 M.

The incorporation of Mg into the human Tris-buffered SBF (synthetic body fluid) solutions (MS) containing Mg²⁺ at a concentration of 0.1 M. The incorporation of Mg into the human Tris-buffered SBF (synthetic body fluid) solutions (MS) containing Mg²⁺ at a concentration of 0.1 M. It was found that Mg ions are required in order to produce CaP or ACP at concentrations of Mg²⁺ in human Tris-buffered solutions (MS) containing Mg²⁺ at a concentration of 0.1 M.
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structure. In a follow up study, we will publish the results of ICP-AES (inductively-coupled plasma atomic emission spectroscopy) analyses on such samples in comparison to PCA or ACP synthesized in synthesis media free of K	extsuperscript{+}, Mg	extsuperscript{2+} and Cl	extsuperscript{−} ions.

The ionic strength of the synthesis solutions (after the addition of Ca metal granules) of experiments 7, 8, 16, 17 and 18 of this study was adjusted to be 167.83, 184.5, 139.5, 171.5 and 211.5 mM, respectively. If one were to prepare an aqueous solution comprising 2.5 mM Ca	extsuperscript{2+}, 1 mM HPO	extsubscript{4}²⁻, 142 mM Na⁺, 5 mM K⁺, 1.5 mM Mg	extsuperscript{2+}, 27 mM HCO	extsubscript{3}⁻ and 103 mM Cl⁻ (i.e., the exact ion concentrations of human blood plasma) then the ionic strength of that solution would have been 148.5 mM. The ionic strengths higher than 148.5 mM were intentionally chosen in this study to facilitate the synthesis of larger amounts of PCA or ACP powders.

The influence of synthesis pH on the CaP formation seemed to be not receiving the required attention in the previous literature. To the best of our knowledge, there are very few studies to mention the basicity of apatitic CaP forming in solutions with pH values around 11. The current study obtained pH values from 9 to 12 without adding any base. Liu et al. [44] used the Ca-nitrate/(NH₄)₂HPO₄ route and studied the ACP and apatite CaP precipitation at pH 10 to 11, whereas the high pH values in that study were apparently obtained by NH₄OH additions. Liu et al. [44] study was not designed to measure the basicity of the CaP formed. The lack of previous studies on the basicity of apatitic CaP may even force the field researchers to think that apatite (which is basically a hydroxyl-containing phosphate in its formula and structure) is not a compound with a significantly basic surface, which is not true. However, the work of Tsuchida et al. [45] deliberately and quantitatively studied the surface basicity of apatitic CaP, by again using the Ca-nitrate/(NH₄)₂HPO₄ route of synthesis (with ammonia additions during synthesis) and found that (i) the solution pH had the greatest influence on the Ca/P ratio of apatitic CaP produced and (ii) the basic site density in apatite depended only on the Ca/P ratio of the sample. Therefore, the current study using Ca metal provided a very simple method of synthesizing CaP at the high pH values (from 10 to 12) studied separately by Liu et al. [44] and Tsuchida et al. [45].

Although the size of the Mg	extsuperscript{2+} ion (0.066 nm) is quite smaller than that of Ca	extsuperscript{2+} (0.101 nm), magnesium ions can substitute for Ca in a number of CaP phases, including whitlockite (Ca₅(PO₄)₃OH). The incorporation of Mg into amorphous CaP has been relatively well studied. Termine et al. [46] found that the elapsped time between the precipitation of ACP and its solution-mediated transformation into cryptocrystalline apatitic CaP (PCA) may be increased considerably with the addition of small amounts of Mg	extsuperscript{2+} ions. The current study was not focused on the hydrothermal transformation of ACP into PCA or vice versa, however our synthesis solutions (MS) contained Mg	extsuperscript{2+} at a concentration equal to that of blood plasma.

The author's lab has been the first to synthesize cryptocrystalline apatitic CaP powders in Tris-buffered SBF (synthetic body fluid) solutions (by using Ca-nitrate) at 37°C and to show (via ICP analyses) that Mg and Na were indeed incorporated into the obtained powders [46]. Such biomimetic apatitic powders were also shown to possess unprecedented high stability against thermal decomposition [46].

For readers who may ask the question of why one would need a solution pH as high as 9.2 (as in Exp-16) to synthesize CaP mimicking the physiological processes, it is a well-known fact that alkaline phosphatase (ALP) enzyme is secreted in bones by the osteoblast cells while depositing nanosize apatitic CaP crystals, and the optimum pH of ALP secretion is between 9.5 and 10.5 [47-49]. Synthesis procedures described for Experiments 16 and 17 in Table 2 were both able to produce the ACP phase at or around this biomimetic pH value of ALP secretion.
CONCLUSIONS

Metallic calcium was used for the first time in synthesizing CaCO₃, poorly-crystalline (cryptocrystalline) apatite (PCA) or x-ray amorphous calcium phosphate (ACP) powders. Calcium phosphate synthesis with metallic Ca was tested both in doubly-distilled water and in water containing ions found in human blood.

The use of metallic Ca eliminated the need for external pH control in calcium phosphate synthesis solutions in the form of adding strong bases such as NaOH, KOH, LiOH or NH₂OH.

The use of metallic Ca made it possible to synthesize PCA or ACP powders in solutions completely free of foreign ions such as ammonium, nitrate or acetate, which are not encountered in human blood.

ACKNOWLEDGEMENT

This study was performed, between 2009 and 2010, at the Department of Biomedical Engineering of Yeditepe University (Istanbul, Turkey), when the author was working there as a Professor.

REFERENCES

Biomimetic Calcium Phosphate Synthesis by using Calcium Metal


Biomimetic Calcium Phosphate Synthesis by using Calcium Metal


SURFACE MODIFICATION OF SOL-GELED INGREDIENTS FOR INCORPORATION IN POLYLACTIC ACID BIOMATERIALS

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ABSTRACT

Submicron bioactive glass particles were prepared and used in comparison with larger particles. Surface modifications allow for better dispersion and improved adhesion to the bone. The modified surface subsequently promotes bioactivity and enhances the fixation capacity of the implant framework. In the current study, submicron glass particles were modified using sol-gel route and subsequent crosslinking process using methacryloxypropyltriethoxysilane. The surface modification improves the bioactivity of the biomaterials at the interface with bone tissue.

INTRODUCTION

In general, (bioactive) glasses and powders are used to form scaffolds that are used as bone regenerating agents. These materials are characterized by a high surface area to volume ratio which allows for faster rate of biological fixation compared to solid biomaterials. The surface modification of these materials is an important aspect of their applications. Surface modifications can be performed to improve the bioactivity of the material as well as sintering and densification rates. The current study focuses on the improvement of the bioactivity of the material by using sol-gel route and subsequent crosslinking process using methacryloxypropyltriethoxysilane. The surface modification process is performed in two steps. The surface modification makes the material more bioactive and enhances the fixation capacity of the implant framework.

EXPERIMENTAL

Materials

The following chemicals were used for the preparation of the scaffold: calcium hydroxide, Aldrich, ≥99.9%, triethyolphosphate (TEP; Aldrich, ≥99.9%), sodium DL-lactate aqueous solution (Fisher, ≥98%), sodium DL-lactate solution (Fisher, ≥99.0%), and methacryloxypropyltriethoxysilane (MPTS; Sigma-Aldrich).