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TITLE:

Calcium Carbonate Formation in the Presence of Biopolymeric Additives

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SUMMARY:

We describe a protocol for the precipitation and characterization of calcium carbonate crystals that form in the presence of biopolymers.

ABSTRACT:

Biomineralization is the formation of minerals in the presence of organic molecules, often related with functional and/or structural roles in living organisms. It is a complex process and therefore a simple, in vitro, system is required to understand the effect of isolated molecules on the biomineralization process. In many cases, biomineralization is directed by biopolymers in the extracellular matrix. In order to evaluate the effect of isolated biopolymers on the morphology and structure of calcite in vitro, we have used the vapor diffusion method for the precipitation of calcium carbonate, scanning electron microscopy and micro Raman for the characterization, and ultraviolet-visible (UV/Vis) absorbance for measuring the quantity of a biopolymer in the crystals. In this method, we expose the isolated biopolymers, dissolved in a calcium chloride solution, to gaseous ammonia and carbon dioxide that originate from the decomposition of solid ammonium carbonate. Under the conditions where the solubility product of calcium carbonate is reached, calcium carbonate precipitates and crystals are formed. Calcium carbonate has different polymorphs that differ in their thermodynamic stability: amorphous calcium carbonate, vaterite, aragonite, and calcite. In the absence of biopolymers, under clean conditions, calcium carbonate is mostly present in the calcite form, which is the most thermodynamically stable polymorph of calcium carbonate. This method examines the effect of the biopolymeric additives on the morphology and structure of calcium carbonate crystals. Here, we demonstrate the protocol through the study of an extracellular bacterial protein, TapA, on the formation of

calcium carbonate crystals. Specifically, we focus on the experimental set up, and characterization methods, such as optical and electron microscopy as well as Raman spectroscopy.

INTRODUCTION:

Biomineralization is the formation of minerals in the presence of organic molecules, often related with functional and/or structural roles in living organisms. Biomineralization may be intracellular, as in the formation of magnetite inside magnetotactic bacteria[1], or extracellular, as in the formation of calcium carbonate in sea urchin spikes[2], of hydroxyapatite that is related with collagen in bones[3] and of enamel that is associated with amelogenin in teeth[4]. Biomineralization is a complex process that depends on many parameters in the living organism. Therefore, in order to simplify the system under study, it is necessary to evaluate the effect of separate components on the process. In many cases, biomineralization is induced by the presence of extracellular biopolymers. The purpose of the method presented here are as follows: (1) To form calcium carbonate crystals in the presence of isolated biopolymers in vitro, using a vapour diffusion method. (2) To study the effect of the biopolymers on the morphology and structure of calcium carbonate.

Three principal methods to precipitate calcium carbonate in vitro in the presence of organic additives are used[5,6]. The first method, which we will refer to as the solution method, is based on mixing a soluble salt of calcium (e.g., $CaCl_2$) with a soluble salt of carbonate (e.g., sodium carbonate). The mixing process may be performed in several ways: inside a reactor with three cells that are separated by porous membranes[7]. Here, each of the outer cells contains a soluble salt and the central cell contains a solution with the additive to be tested. Calcium and carbonate diffuse from the outer to the middle cell, resulting in the precipitation of the less soluble calcium carbonate when the concentrations of calcium and carbonate exceed their solubility product, $K_{sp} = [Ca^{2+}][CO_3^{2-}]$. An additional mixing method is the double-jet procedure[8]. In this method, each soluble salt is injected from a separate syringe to a stirred solution containing the additive, where calcium carbonate precipitates. Here, the injection and therefore the mixing rate is well controlled, in contrast with the previous method where mixing is controlled by diffusion.

The second method used to crystallize $CaCO_3$ is the Kitano method[9]. This method is based on the carbonate/hydrogen carbonate equilibrium ($2HCO_{3\,(aq)}^- + Ca_{(aq)}^{2+} \rightleftharpoons CaCO_{3\,(s)} + CO_{2\,(g)} + H_2O_{(l)}$). Here, CO_2 is bubbled into a solution containing $CaCO_3$ in a solid form, shifting the equilibrium to the left and therefore dissolving the calcium carbonate. The undissolved calcium carbonate is filtered and the desired additives are added to the bicarbonate-rich solution. CO_2 is then allowed to evaporate, thereby shifting the reaction to the right, forming calcium carbonate in the presence of the additives.

The third method of calcium carbonate crystallization, which we will describe here, is the vapor diffusion method[10]. In this set-up, the organic additive, dissolved in a solution of calcium chloride, is placed in a closed chamber near ammonium carbonate in a powder form. When ammonium carbonate powder decomposes into carbon dioxide and ammonia, they diffuse into the solution

containing calcium ions (e.g., $CaCl_2$), and calcium carbonate is precipitated (see **Figure 1** for illustration). The calcium carbonate crystals can grow by slow precipitation or by fast precipitation. For the slow precipitation, a solution containing the additive in $CaCl_2$ solution is placed in a desiccator next to the ammonium carbonate powder. In the fast precipitation, described in length in the protocol, both the additive solution and the ammonium carbonate are placed closer together in a multi-well plate. The slow precipitation method will produce fewer nucleation centers and larger crystals, and the fast precipitation will result in more nucleation centers and smaller crystals.

The methods described above differ in their technical complexity, in the level of control and in the rate of the precipitation process. The mixing method requires a special set-up[6] for both the double jet and the three-cell system. In the mixing method, the presence of other soluble counter ions (e.g., Na^+, Cl^-)[6] is inevitable, whereas in the Kitano method, calcium and (bi) carbonate are the only ions in solution and it does not involve the presence of additional counter ions (e.g., Na^+, Cl^-). Furthermore, the mixing method requires relatively large volumes and therefore it is not suitable for working with expensive biopolymers. The advantage of the double jet is that it is possible to control the rate of solution injection and that it is a rapid process in comparison to other methods.

The advantage of the Kitano method and the vapor diffusion method is that the formation of calcium carbonate is controlled by diffusion of CO_2 into/out of a $CaCl_2$ solution, thus allowing to probe slower nucleation and precipitation processes[11,12]. Furthermore, calcium carbonate formation by diffusion of CO_2 may resemble calcification processes in vivo[13-15]. In this method, well-defined and separated crystals are formed[16]. Last, the effect of single or multiple biopolymers on calcium carbonate formation can be tested. This enables a systematic study of the effect of a series of additive concentrations on calcium carbonate formation as well as a study of mixtures of biopolymers – all performed in a controlled manner. This method is suitable for use with a large range of concentrations and volumes of additives. The minimal volume used is approximately 50 µL and therefore this method is advantageous when there is a limited amount of the available biopolymers. The maximal volume depends on the accessibility of a larger well-plate, or the desiccator into which the plate or beaker containing $CaCl_2$ are to be inserted. The method described below has been optimized for working in a 96-well plate with a biopolymer chosen to be the protein TapA[17].

PROTOCOL:

1. Calcium carbonate crystallization

1.1. Control preparation and optimization

1.1.1. Prepare clean glass pieces. Use the same cleaning procedure to clean the glassware.

1.1.1.1. Use a diamond pen to cut pieces of a glass microscope slide so that they fit in a well of a 96-well plate.

NOTE: 5 mm x 5 mm pieces should largely fit.

1.1.1.2. Place the glass pieces in a beaker with triple distilled water (TDW) so that water covers the glass slides and sonicate in a bath sonicator for 10 min.

1.1.1.3. Decant the water, add ethanol to cover the glass slides, and sonicate in a bath sonicator for 10 min.

1.1.1.4. Dry the slides and the glassware with a stream of nitrogen gas and place them in an air plasma cleaner for 10 min at 130 W.

1.1.2. Optimize the concentration of the $CaCl_2$ used in the calcification experiments performed under the desired experimental conditions to achieve a sample rich with smooth-faceted calcite crystals (without or at least with a scarce number of vaterite crystals).

1.1.2.1. Fill the wells at the corners of a 96-well plate with ammonium carbonate powder and seal the plate using aluminum foil; cover the foil with paraffin film. Clean any residual ammonium carbonate using nitrogen gas.

CAUTION: Ammonium carbonate irritates nose and lungs; use only inside the fume hood.

1.1.2.2. Prepare a stock solution of 0.5 M $CaCl_2$. This stock solution will be used to prepare a gradient of concentrations of $CaCl_2$ solutions in the multi-well plate.

NOTE: A 10 mL stock solution is sufficient for the whole experiment.

1.1.2.3. Place the previously cut and cleaned glass pieces into five different wells. Use the closest wells to the center.

1.1.2.4. Fill each well bearing a glass piece with 100 µL of $CaCl_2$[16]. Mix TDW and 0.5 M $CaCl_2$ (stock) to achieve an increasing concentration gradient of $CaCl_2$ across the different wells. If a different sized well-plate is used, adjust the concentration of $CaCl_2$ to achieve separate calcite crystals (step 1.1.2.10, and see Discussion section).

NOTE: An increasing $CaCl_2$ gradient of 10, 20, 30, 40 ,50 mM concentrations in separate wells is used in this protocol. To increase the concentration range or the number of concentrations tested, use additional wells.

1.1.2.5. Puncture the cover of each of the wells containing ammonium carbonate 3x with a needle.

1.1.2.6. Put back the lid, seal the borders with paraffin film and keep it at 18 °C in an incubator for 20 h.

1.1.2.7. After the incubation, open the lid carefully inside a fume hood and remove the crystals formed at the water/air interface with a loop.

1.1.2.8. Use a tweezer to transfer the glass pieces into a beaker containing double distillated water (DDW). Remove the samples from the beaker, dry excessive water touching the borders of the slide with tissue wipers.

1.1.2.9. Place the slides in a covered Petri dish. Use a double-sided tape to fix the glass pieces onto the bottom of the Petri dish. Place the Petri dish in a desiccator for 24 h.

1.1.2.10. Observe the crystals formed on the glass pieces with a stereoscope (3.5x magnification) and/or an upright optical microscope (10x–40x magnification). If the control solutions are clean, rhombohedral crystals (most likely calcite) will be observed with an optical microscope (**Figure 2A**).

1.1.2.11. If in addition to the rhombohedral crystals, the control contains spherical crystals (most likely vaterite, **Figure 2B**), or if scan electron microscope (SEM) images show rhombohedral crystals with rough rather than smooth faces (**Figure 3A,B**), repeat the crystallization protocol making sure that the cleaning step (1.1.1) is performed correctly. Furthermore, make better care that there is no ammonium carbonate in areas on the plate other than the dedicated wells. Otherwise, continue to the next step.

1.2. Crystallization in the presence of the additives

1.2.1. To study the effect of the additives on the crystallization of $CaCO_3$, set up a multi-well plate that contains (in different wells), a control $CaCl_2$ solution without the additives, and $CaCl_2$ solutions with the additives. Use the optimal concentration of $CaCl_2$ found in section 1.1.2 for the experiment.

NOTE: The protocol below uses optimal conditions as those reported in a previous study[16].

1.2.2. Repeat step 1.1.2.2.

1.2.3. Place ammonium carbonate powder in the corners of the plate as described in step 1.1.2.1.

1.2.4. In each well where precipitation will occur, place a glass piece that was cut and cleaned as described in section 1.1.1.

1.2.5. To prepare control wells, pipette 90 µL of TDW into the control wells. Prepare at least one replicate of each well including the control. If the additive used is in a buffer solution, then pipette 90 µL of buffer instead of TDW water.

1.2.6. Prepare the additive-containing wells. Repeat step 1.2.5 by adding 90 µL of the additive

solution in water. If the additive is in buffer (instead of TDW), pre-adjust the concentration of the additive with buffer to meet the desired final concentration. Keep a total volume of 90 µL; pipette first the additive, then the buffer.

NOTE: A final concentration of 10 µM of the protein TapA in 100 mM NaCl, 25 mM Tris pH 8.0 buffer[16] is used in this protocol.

1.2.7. Add 10 µL of the 0.5 M $CaCl_2$ stock solution (prepared in step 1.2.2) to both the controls and the additives-containing wells to reach a final concentration of 50 mM $CaCl_2$.

1.2.8. Repeat steps 1.1.2.5–1.1.2.9.

2. Characterization of calcium carbonate crystals

2.1. With a scanning electron microscope, observe the calcium carbonate crystals formed in the presence of the additives at a higher resolution than that obtained by optical microcopy (see step 1.1.2.10).

2.1.1. Mount the glass pieces containing the crystals on an aluminum stub with double-sided carbon tape.

2.1.2. Coat with a layer of Au/Pd for 40−50 s.

2.1.3. Acquire the images at 5 kV acceleration voltage.

NOTE: **Figure 3A** shows a representative SEM image of calcium carbonate crystals formed in a proper control experiment, while **Figure 4** shows representative images of calcium carbonate crystals formed in the presence of the protein TapA.

2.2. Perform micro Raman spectroscopy to determine the calcium carbonate polymorphs formed. Micro Raman allows the collection of a Raman spectrum from single crystals rather than from a whole powder.

2.2.1. Use a 20x objective of the microscope to choose the crystal of interest.

2.2.2. Collect the Raman spectrum in a range of 100−3200 cm^{-1} using a 514 nm argon laser.

NOTE: **Figure 5** shows representative spectra of calcite (A) and vaterite (B). For the spectrum of aragonite, refer to reference[18].

2.3. Quantification of the mass percentage of the additives in the $CaCO_3$ precipitates

2.3.1. Verify/measure the extinction coefficient *(ε)* of the additive used. The extinction coefficient of a protein can be given by online servers[19]. If the extinction coefficient is unknown, measure

the absorbance of the additive at different concentrations, plot the absorbance vs. concentration and calculate the extinction coefficient from the slope of the curve.

2.3.2. Weigh the glass pieces where the crystals formed, preferably use a microbalance.

2.3.3. Scrap the crystals off the glass into 1.2 mL of 0.1 M acetic acid solution, vortex and sonicate the sample. Store the sample at room temperature for 24 h.

CAUTION: Acetic acid is very hazardous in case of skin or eye contact; handle with caution and dispose following the regulations.

2.3.4. Weigh the glass slide after scraping off the crystals.

2.3.5. Measure the UV/vis absorbance *(A)* spectrum of the solution. If the additive is a protein, measure the absorbance at 280 nm and calculate its concentration *(C)*, using the Beer-Lambert equation:

$$C = \frac{A}{\varepsilon \cdot l}$$

where *l* is the optical path inside the cuvette.

2.3.6. Use the concentration *(C)* found in 2.3.5 and the volume used (*V* = 1.2 mL) to calculate the mass *(m)* of the additives in/on the crystals. If the concentration is in mg/mL, use the equation $C \cdot V = m$.

2.3.6.1. If the concentration is in mol/L, then calculate the moles *(n)* applying $C \cdot V = n$. Then use the molecular weight *(Mw)* to calculate the mass *(m)* of the additives ($m = n \cdot Mw$).

2.3.7. Calculate the weight percentage of the additives in/on the crystals using the equation: $\left(Wt\% = \frac{m}{\Delta m_s} \cdot 100\right)$, where *m* is the mass of the additives, and Δm_s is the mass of the calcium carbonate crystals that were scrapped off the glass piece.

REPRESENTATIVE RESULTS:

A schematic of the experimental set up is shown in **Figure 1**. Briefly, the diffusion method is used in order to form calcium carbonate crystals in 96-well plates and test the effect of biopolymers on the morphology and structure of the calcium carbonate crystals. In these experiments, ammonium carbonate is decomposed into ammonia and CO_2, which diffuse into calcium carbonate solutions, resulting in the formation of calcium carbonate crystals (**Figure 1** and **Figure 2**).

The effect of the biopolymers is evaluated by comparison of the calcium carbonate crystals formed with and without (control) the additives. Prior to the addition of the additives, the optimized calcium carbonate concentration is chosen and the cleanliness of the solutions and glassware is tested. **Figure 2A** shows a representative image of a control experiment, where

distinct rhombohedral calcium carbonate crystals are formed. These crystals are most likely calcite (see **Figure 5**). If the solutions or plastic or glassware have not been properly cleaned then spherical crystals will form (**Figure 2B**, marked with red circles), in addition to the rhombohedral calcite crystals. The spherical crystals are most likely vaterite (see **Figure 5**). An additional indication for the use of proper conditions, is the smoothness of the calcite faces in the control experiment. This can be observed with SEM, as shown in **Figure 3**. **Figure 3A** shows a proper control with smooth calcite faces, whereas **Figure 3B** shows calcite crystals with faces composed of steps. The spherical crystals here are vaterite. The control crystals need to be separated and smooth-faceted so that the effect of the additives on the crystal morphology is clear.

To demonstrate the effect of a biopolymer on the morphology of calcium carbonate, we have used here the protein TapA. **Figure 4** shows the crystals of calcium carbonate formed in the presence of TapA in the solution. The crystals are distinct from the control crystals. They form a complex spherical calcium carbonate assembly, composed of multiple calcite microcrystals (see Raman spectrum in **Figure 5**). One method to characterize the structure of the crystals is Raman spectroscopy. **Figure 5** shows the typical spectra of calcite (**Figure 5A**) and vaterite (**Figure 5B**), taken from successful (**A**) and unsuccessful (**B**) control experiments. Typical absorbance peaks[20] are in the range of 100−400 cm^{-1} (lattice modes), a peak at ~710 cm^{-1} (symmetric bending of the CO_3^{2-}) and at ~1090 cm^{-1} (symmetric stretching of the CO_3^{2-}). Note the split of the Raman shift at ~1080 cm^{-1} that is the most evident characteristic of vaterite[21]. Refer to reference[22] for a full spectrum of aragonite. The Raman spectrum of the crystals formed in the presence of TapA is similar to the spectrum of calcite (**Figure 5A**). In cases where additional peaks appear which do not correspond to a single spectrum of a single calcium carbonate polymorph, or a combination of those, they may be attributed to an excess of calcium chloride that has not been washed thoroughly in step 1.1.2.8.

In the final section of the protocol, we have measured the percentage (weight/weight) of the organic content inside or on the calcium carbonate crystals. The crystals were dissolved in acetic acid and the biopolymer was released into the solution. In cases where the biopolymer has a characteristic absorbance spectrum, its concentration in the solution can be determined. In the case of proteins containing aromatic side groups, as in the case study here of TapA, the absorbance at 280 nm is used. The absorbance spectrum of TapA, measured following dissolution of the crystals in acid, is shown in **Figure 6** (green) together with the spectrum of the control (acid-dissolved calcium carbonate crystals without the additive; black). Using Beer-Lambert's law (see step 2.3.5) and using an extinction coefficient of 29,700 $M^{-1}\,cm^{-1}$, we have found that the mass percent of TapA was 1.8% ± 0.2%. Measuring the absorbance of the solution following the crystal dissolution in acid is possible when the biopolymers do not aggregate at low pH. A null signal of the absorbance of the solution containing the additive is indicative of its aggregation. In this case, different analysis methods, such as thermal gravitational analysis (TGA) may be used to estimate the mass of additives present inside/on the crystals.

FIGURE LEGENDS:

Figure 1: Schematic description of the fast vapor diffusion method for the formation of calcium

carbonate crystals. A calcium-containing soluble salt (e.g., calcium chloride) is placed near an ammonium carbonate powder. Here we show two wells in a 96-well plate. The plate is then sealed, and the ammonium carbonate decomposed into ammonia and carbon dioxide that diffuse into the calcium-containing well, resulting in the precipitation of calcium carbonate crystals (shown here by the SEM image of a calcite crystal).

Figure 2: Optical microscope images of the calcium carbonate crystals. A clean control contains mostly calcite, which is characterized by rhombohedral crystals (**A**). When the control sample includes spherical crystals (such as those marked by a red circle) (**B**), repeat the cleaning protocol as suggested in section 1.1.1.

Figure 3: Scanning electron micrographs of calcium carbonate crystals formed in two control experiments. (**A**) An image of a sample that contains mostly rhombohedral crystals (calcite). (**B**) A micrograph of a sample with broken calcite facets and spherical crystals that are most probably vaterite. In this case, control experiments need to be repeated. This figure has been modified from Azulay et al.[16].

Figure 4: SEM images of calcite crystals formed in the presence of the protein TapA. Scale bars represent 50 µm (**A**) and 10 µm (**B**), respectively. This figure has been modified from Azulay et al.[16].

Figure 5: Raman spectra of two polymorphs of calcium carbonate. (**A**) Calcite. (**B**) Vaterite.

Figure 6: UV/vis absorbance spectra of TapA (green) and a buffer solution (100 mM NaCl, 25 mM Tris pH 8.0; black). The absorbance was used to calculate the concentration of TapA in the calcium carbonate crystals, following their dissolution in acid.

DISCUSSION:

The method described here is aimed at forming calcium carbonate crystals in the presence of organic additives and evaluating the effect of organic biopolymers on the morphology and structure of calcium carbonate crystals in vitro. The method is based on the comparison of the crystals formed in the presence of the organic additives to the calcite crystals formed in the control experiment. We have shown how to use the diffusion method to form the calcium carbonate crystals, how to characterize their morphology using optical and electron microscopy, how to characterize their structure using Raman spectroscopy, and how to determine the organic content (weight/weight percentage) of the crystals.

We described the protocol that we have used to evaluate the effect of a bacterial extracellular protein, TapA, on the morphology and structure of calcium carbonate, but the protocol can be expended to any other polymer that is biologically purified or synthesized. In addition to the effect of a single biopolymer, this method can be used with mixtures of biopolymers in order to evaluate any mutuality between different polymers in their effect on calcium carbonate precipitation. We have limited the experimental set-up to a 96-well plate; however, any other set-up where calcium carbonate solutions are positioned and physically separated from the

ammonium carbonate source (i.e., the solutions and powder are placed in a sealed vessel), is possible. Typical vessels used are multi-well plates and a typical concentration range of 10−50 mM is used for an experimental set-up with 96-well plates[10,16,23]. A sealed beaker or a desiccator can also be used.

This method is easy to use and it is compatible with low concentrations and low volumes of the biopolymeric additives. Working in a multi-well plate allows the screening of multiple parameters at the same time in one multi-well plate experiment. This method may be sensitive to the relative position of the calcium carbonate wells with respect to the position of the ammonium carbonate powder. Therefore, care needs to be taken to always use wells at the same position in the multi-well plate and also to check that changing the location of the wells does not affect the results. Normally, using a large enough distance between the wells where the experiments take place and the ammonium carbonate powder, ensures that the results are reproducible. In addition, it is critical to adjust the concentration of $CaCl_2$ so that separate crystals are formed in the control experiment, as described in section 1.1.2. The concentration of the additives should also be optimized to exceed a minimal concentration below which no effect is observed. Note that the method is highly sensitive to the concentration of the additives; different additive concentrations may induce a different effect on the morphology and structure of calcium carbonate crystals[24].

One major limitation of this method is that ammonia and CO_2 both diffuse into the calcium chloride test solutions and therefore there is poor control of the pH throughout the experiment. As a result of the diffusion of ammonia, the pH in solution increases (when ammonia becomes ammonium), as shown in the equilibrium equations[5,6] ($(NH_4)_2CO_{3(s)} \rightarrow 2NH_{3(g)} + CO_{2(g)} + H_2O_{(l)}$, $NH_{3(aq)} + H_2O_{(l)} \rightarrow NH_4^+{}_{(aq)} + OH^-{}_{(aq)}$, $Ca^{2+}{}_{(aq)} + CO_{2(aq)} + 2OH^-{}_{(aq)} \rightleftharpoons CaCO_{3\,(s)} + H_2O_{\,(l)}$) and it favors the formation of calcium carbonate.

Compared to the additional methods described in the introduction, this method is technically simple. Due to the slow precipitation process, crystal growth can be followed in real time, using absorbance or scattering techniques from the transparent multi-well technique. In addition, in order to follow the kinetics of the crystal growth, one can also probe the crystal morphology and structure at different time points, rather than after 20 hours, as performed in our study. This method can be expanded to study the precipitation of other salts of carbonate bearing a small enough Ksp, such as magnesium, barium and cadmium carbonates.

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DISCLOSURES:

The authors have nothing to disclose.

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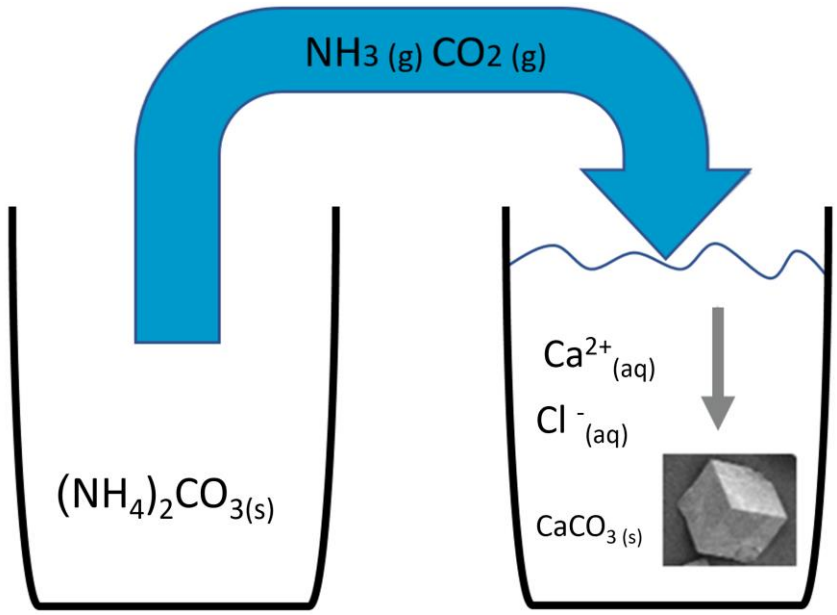

Figure 1

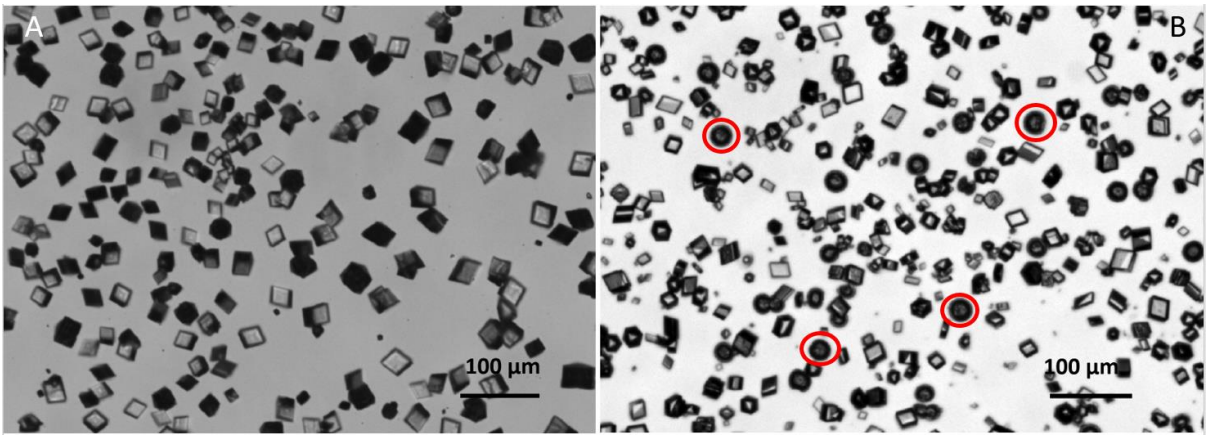

Figure 2

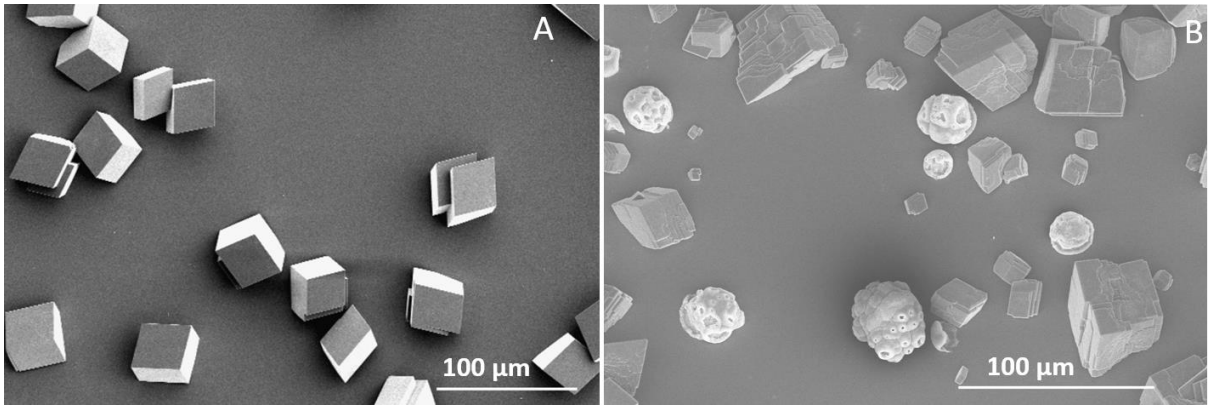

Figure 3

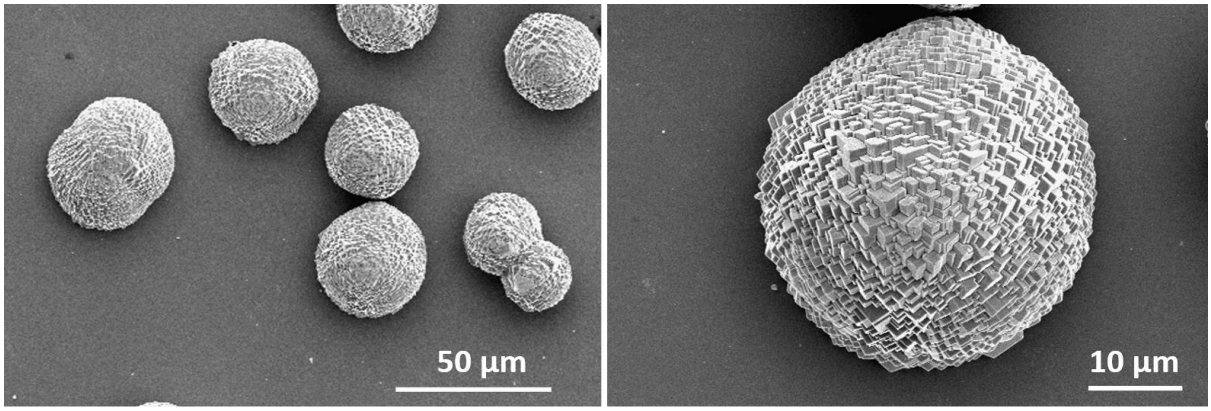

Figure 4

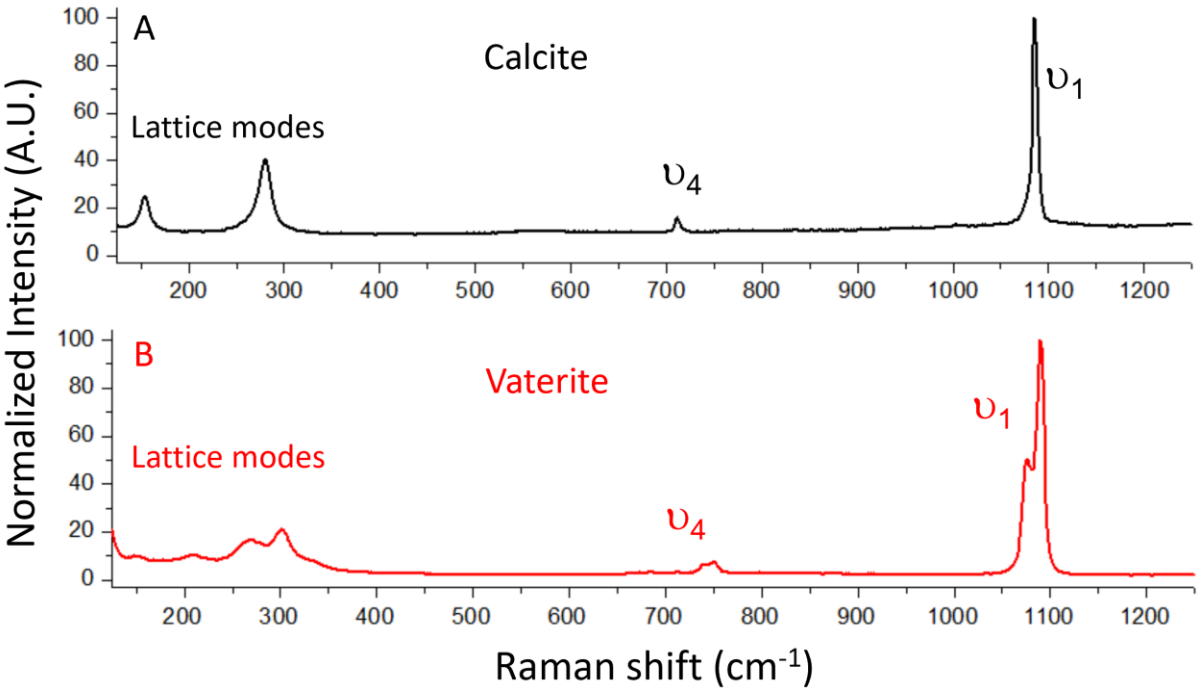

Figure 5

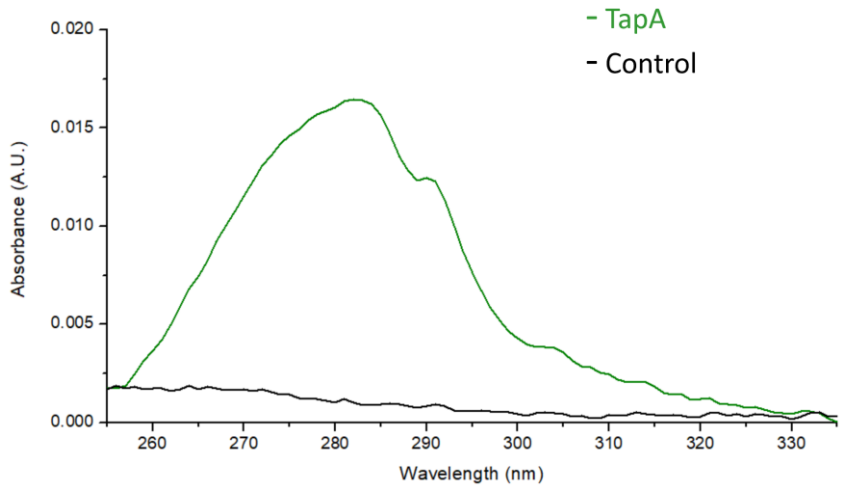

Figure 6

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetic acid	Gadot	64-19-7	
Ammonium carbonate	Sigma-Aldrich	506-87-6	
Calcium chloride dihydrate	Merck KGaA	10035-04-8	
Ethanol Absolute	Gadot	64-17-5	
Micro-Raman	Renishaw		inVia Reflex spectrometer coupled with an upright Leica optical microscope
Microscope	Nikon		Eclipse 90i model
Nis elements Br software	Nikon		For microscope imaging
Scanning Electron Microscope	ThermoFisher Scientific		FEI Sirion microscope
Spectrophotometer	JASCO		V-670 model
Sputter coater	Polaron		SC7640 model

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Signature: Liraz Chai LC Date: 01-01-2019

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04-02-19

Prof. Xiaoyan Cao
Review Editor
Jove

Dear Professor Cao,

Thank you for your letter concerning our manuscript entitled "**Calcium carbonate formation in the presence of biopolymeric additives**" (JoVE59638). We are grateful for the reviewers for their comments and we have now fully addressed all the editorial and reviewers' comments and queries and the detailed response is attached below.

On behalf of my co-author, David Azulay, I thank you for considering our manuscript for publication in *Jove*.

Sincerely,
Liraz Chai

Liraz Chai

Detailed response to the editorial comments:

1. 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   Response: Done.

2. Please revise lines 31-32 and 55-56 to avoid previously published text.

   Response: We have revised lines 31-32, 55-56.

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   Response: The copyright permission is uploaded. Figure legends 3,4 have been changed accordingly.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

   Response: We have revised the goal statement (lines 63-67).

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

   Response: Done.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that

cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: Done.

7. 1.1.2.1: Please specify the amount of powder ammonium carbonate added

Response: We have changed the text in line 156 (step 1.1.2.1), to account for our accurate actions. We do note weigh the ammonium carbonate, but rather fill in the well, as is now instructed.

8. 1.1.2.2: Please list an approximate volume to prepare.

Response: We have added a note to line 162 (step 1.1.2.2), advising the reader to prepare a 10 mL stock solution.

9. Please number the figures in the sequence in which you refer to them in the manuscript text.

Response: Done.

10. Discussion: Please discuss critical steps of the protocol.

Response: Critical steps of the protocol have been added in lines 379, 389-394.

11. Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.

Response: An updated list is now uploaded.

Detailed response to the reviewers' comments.

Reviewer: 1

The presented manuscript is well written and contains all the necessary information to perform the described experiment.

Response: We thank the reviewer for the positive feedback.

Reviewer: 2

In this, paper Azulay et.al present their results on biomineralization of calcium carbonate in the presence of biopolymers. The motivation of this research is quite interesting and the subject itself is very relevant to biomineralization. Overall, this work is very original and in my opinion, the results presented in this study are significant for understanding CaCO3 biomineralization. I can only come up with a few arguments that I think the authors should correct this well-written article:
1) The authors should briefly give a note on the thermodynamic stability of each crystalline form of CaCO3 this is a key point the crystallization of calcium carbonate.
2) Another comment that the authors should give kinetics details on the bio mineralization of calcium carbonate.
3) Figure 5- Raman peaks should be assigned to vibrational modes of CaCO3.
In conclusion, I recommend the publication of this paper after the authors' response to the comments raised in this report.

Response: we thank the reviewer for the positive feedback, and our answers are outlined below.

1. We have included a reference to the thermodynamic stability of the different polymorphs of calcium carbonate in the original text (lines 43-45).
2. We thank the reviewer for this comment. Although a kinetic study is out of the scope of this protocol, we have now included this suggestion in the discussion (lines 403-406).
3. We thank the reviewer for this note, and we have now assigned the peaks on the Raman spectrum in Figure 5.

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Title: Biopolymers from a Bacterial Extracellular Matrix Affect the Morphology and Structure of Calcium Carbonate Crystals

Author: David N. Azulay, Razan Abbasi, Ilanit Ben Simhon Ktorza, et al

Publication: Crystal Growth and Design

Publisher: American Chemical Society

Date: Sep 1, 2018

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