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to Monitor Mineralization in Hydrogels*

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TITLE. Rheology as a mechano-scopic method to track growth of calcium carbonate in gelatin hydrogels

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ABSTRACT. Biominerals have been widely studied due to their unique mechanical properties, afforded by their inorganic-organic composite structure and well-controlled growth in macromolecular environments. However, a lack of suitable characterization techniques for inorganic minerals in organic-rich media has prevented a full understanding of biomineralization. Here, we applied rheometry to study mineral nucleation and growth dynamics by measuring viscoelastic material properties of a hydrogel system during mineralization. Our proof-of-concept system consists of a gelatin hydrogel matrix preloaded with calcium ions and a reservoir of carbonate ions, which diffuse through the gel to initiate mineralization. We found that gels with diffused carbonate show an increase in low frequency energy dissipation, which scales with carbonate concentration and gel pH. Using this signal, and recognizing that mineralization occurs simultaneously with carbonate diffusion in our system, we have mechano-scopically tracked mineral growth *in situ*, showcasing the potential of rheometry for studying mineralization kinetics in real time.

INTRODUCTION

Six hundred million years ago, organisms began incorporating inorganic elements into organized mineral components, a process known as biomineralization.¹ The major types of biominerals synthesized by organisms today are calcium carbonates (CaCO_3), phosphates, and oxalates; iron oxides and hydroxides; and silica. Many of these occur in different polymorphs and have a variety of functions, such as structural support, calcium and iron storage, and sensing.^{2,3} These biominerals are inorganic-organic composites formed in aqueous environments at physiological conditions, often with superior mechanical properties compared to their purely inorganic

counterparts.⁴ Thus, biominerals have been widely studied from biological, chemical, and materials science standpoints in order to better understand the mechanisms involved in their formation and to learn how to mimic these “green” mineralization processes in synthetic systems.^{5–8} Yet, despite intense studies on their structure and formation, questions on early thermodynamics and kinetics of biomineral nucleation and growth still remain. Accordingly, recent advances in high resolution electron microscopy such as cryo-TEM^{9–11} and liquid cell TEM^{12,13} have been employed to capture early mineralization events, providing sub-nanometer resolution images of mineral growth frozen in time¹⁴ and real-time videos of *in situ* mineral phase transformations,¹² respectively. These and other recent studies have afforded insights into non-classical nucleation and growth pathways of biominerals, such as amorphous precursors,^{12,15,16} pre-nucleation clusters,^{9,17–19} dense liquid phases,^{20,21} and oriented aggregation by particle attachment^{11,14,22} wherein organic molecules are hypothesized to function as nucleation enhancers or inhibitors or as cluster stabilizers.^{23,24}

However, high-resolution electron microscopy techniques are static measurements of structure, not of material properties, and they do not allow studies of biomineralization within hydrogel-like organic matrices. One of the most well-known and studied examples of biomineralization is nacre, which has a brick and mortar composite structure of high aspect ratio tablets of aragonite (a CaCO_3 polymorph) within a proteinaceous organic matrix.²⁵ The organic matrix functions both as a scaffold for nucleation and growth during crystallization and as a glue in the final composite structure.²⁶ During mineralization, the organic matrix creates a hydrogel-like environment within which the aragonite tablets grow.²⁷ In addition to nacre, other biominerals, such as coral,^{28,29} otoliths,^{30,31} chiton teeth,^{32,33} bone,^{34,35} and enamel,^{36,37} have been found to mineralize in hydrogel-like organic matrices. Thus, *in vitro* hydrogel networks have

become a common method to experimentally study and mimic natural biomineralization systems,^{38–43} yet it remains difficult to detect nuclei within gels,⁴¹ and experiments are limited to studying nucleation by extrapolation from visual observation of crystal growth instead of actual nuclei formation.⁴² Furthermore, because of the crowded macromolecular environment and solution confinement in pores, mineralization in gels varies greatly from that in solution, and aqueous mineralization studies cannot substitute for mineralization in macromolecular hydrogel media. Hence, pre-nucleation and early mineral growth remain uncharacterized in hydrogel systems using current experimental techniques.

In this study, we propose rheometry as a mechanical characterization tool for observing the growth of CaCO_3 minerals in gelatin hydrogels. In particular, we use small amplitude oscillatory shear (SAOS) rheology to monitor the changes in linear viscoelastic mechanical properties of the hydrogel system with mineral deposits. While rheology has been used previously to characterize general viscoelastic properties of mineralized hydrogels,^{44,45} we specifically seek to deconstruct the unique mechanical contributions from the minerals, the hydrogel matrix, and the mineral-hydrogel interface to observe and quantify mineral growth *in situ*.

We investigated the mechanical differences between gels which have CaCO_3 particles grown directly within the gel, CaCO_3 particles physically mixed into the gel, and gels with no minerals, shown in Figure 1 as mineralized, mixed, and control gels, respectively. In all gel systems we observed increased viscous dissipation in direct correlation with Na_2CO_3 solution concentration. Since mineralization is controlled directly through carbonate diffusion in the supersaturated gel conditions used here, we used this specific carbonate mechanical signal to track the growth of minerals *in situ* and in real time. We also used scanning electron microscopy

and thermogravimetric analysis to characterize the mineral particles grown in the hydrogel composite systems.

Figure 1. Diagram of sample preparation procedures for “mineralized” and “mixed” gels, where CaCO_3 particles are either grown in the gel or manually mixed in, respectively, and for control gels without minerals. Gels are then characterized mechanically using small amplitude oscillatory shear rheology.

EXPERIMENTAL

Materials

Gelatin type A from porcine skin, calcium chloride (CaCl_2), and sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich. CaCO_3 (99.5%, 5 μm powder) was purchased from Alfa Aesar.

All reagents were used as received. All water was deionised and filtered through a Millipore Milli-Q purification system to a resistivity of 18 $\text{M}\Omega\cdot\text{cm}$.

Hydrogel Preparation

As shown in Figure 1, gelatin for mineralized and Ca control samples was dissolved in H₂O with 0.05M CaCl₂ at 50°C for 1h, while stirring, to make 10wt% gelatin solutions. For CO₃ control samples, gelatin was dissolved in pure H₂O, and for mixed samples, gelatin was dissolved in H₂O with 0.1-10wt% CaCO₃, using the same procedure. H₂O-CaCO₃ mixtures were sonicated and vortexed prior to addition of gelatin to ensure proper dispersion of CaCO₃. For all samples, 3 mL of gelatin solutions were then transferred to 60mm petri dishes to gel at 4°C for 1h. Petri dishes were sealed with parafilm to prevent evaporation, and gels were left at room temperature overnight to equilibrate before beginning mineralization or control experiments.

Mineralization Procedures

To induce mineralization, 3 mL of H₂O with 0.05M-0.75M Na₂CO₃ were pipetted on top of the calcium-loaded gelatin. Mineralization was stopped after 1h by pouring off the excess Na₂CO₃ solution. Mixed and control gels were subjected to similar conditions using NaNO₃ or pure H₂O for comparison.

Characterization

Rheology. Frequency sweeps were conducted on all gels using an MCR 302 Anton Paar rheometer with a 25mm parallel plate geometry at 25°C. To allow gels to equilibrate, they were left on the rheometer for 30 minutes at 25°C prior to starting measurements. Frequency sweeps were performed within the linear viscoelastic region at 1% strain amplitude with angular frequency ranging from 0.1-100 rad/s.

In situ experiments were done on the MCR 302 Anton Paar rheometer with a 25mm parallel plate geometry and separately on a TA Instruments DHR-3 rheometer with a custom-fabricated single cylinder Couette geometry. Gelatin solutions in 0.05M CaCl_2 were placed into the rheometers at 50°C; the temperature was then ramped down to 4°C and held at 4°C for 1h to form gels. The gels were then brought back to 25°C and left to equilibrate before adding Na_2CO_3 mineralizing solution to initiate mineralization while remaining in the rheometer. During mineralization, the storage (G') and loss (G'') moduli were monitored at multiple frequencies to capture the evolution of viscoelastic mechanical properties with the mineralization process.

Scanning Electron Microscopy (SEM). Grown CaCO_3 crystals were separated from the gelatin by heating the gel above 50°C, washing with water and freeze-drying. Uncoated crystals were examined using a Zeiss Merlin High-resolution SEM at 1-3kV accelerating voltage and 300 pA beam current, using depth of field mode, to determine their size and morphology.

Thermogravimetric Analysis (TGA). A TA Instruments Q500 TGA was used to measure the mass fraction of the CaCO_3 particles. Pieces of the gelatin- CaCO_3 samples were freeze-dried prior to TGA experiments. The samples were ramped from 20-800°C at a rate of 5°C/min. All water was assumed to have evaporated by 150°C, at which point the starting sample mass was taken. The CaCO_3 mass was taken to be the remaining mass after all gelatin had decomposed.

RESULTS AND DISCUSSION

Mineralized CaCO_3 characterization

To create mineralized gels with different mineral contents, we varied the concentration of Na_2CO_3 mineralizing solution from 0.05M to 0.75M. We calculated the volume fraction of grown particles from mass fractions measured using TGA, assuming CaCO_3 density of 2.711 g/cm^3 and gelatin solution density similar to that of water (1 g/cm^3).⁴⁶ As shown in Figure 2a, we achieved a range of CaCO_3 volume fractions from 0-1.8%, with higher Na_2CO_3 concentration resulting in higher mineral volume fractions. We also characterized the particle sizes using SEM to understand how the average particle sizes and size distributions changed with varying Na_2CO_3 concentration. Particle sizes generally decreased with increasing Na_2CO_3 concentration to an average of $\sim 10\mu\text{m}$, as depicted in Figure 2a. Figure 2b-f illustrates representative SEM images of particles grown using each Na_2CO_3 concentration with insets of the particle size distributions. The particle size distribution obtained from the 0.05M Na_2CO_3 solution may indicate a bimodal distribution, which would account for the large standard deviation in sizes. For a detailed methodology of our particle size analysis, see the Supplementary Information.

Figure 2. Properties of mineralized CaCO_3 particles grown in gels. (a) CaCO_3 volume fraction (closed circles) and average particle size (open circles) as a function of the concentration of Na_2CO_3 solution used for mineralization. (b-f) Representative SEM images of particles grown with increasing Na_2CO_3 solution concentration (top left of each image). Overlays show particle size distributions in μm . The density of the particles in the images are a result of the sample

preparation procedure for SEM and are not a reflection of the particle density within the gel. All scale bars are 100 μm .

In addition to enabling particle size measurements, SEM images depicted rhombohedral particle morphologies for all samples, indicative of the calcite polymorph. X-ray powder diffraction (XRPD) of mineralized particles confirmed calcite as the dominant polymorph. Additional details and data from XRPD can be found in the Supplementary Information.

Rheological characterization

We conducted frequency sweeps to measure the storage moduli (G') and loss moduli (G'') of all gel samples. All gels showed solid-like behavior, with $G' > G''$ across all frequencies measured, as shown in Figure 3a. These data are taken from one sample from each of the four types of gels and are representative of the mechanical behavior of the whole group (please see the Supplemental Information for the complete set of data). In correlation with the presence of carbonate, we observed two marked differences in mechanical behavior of mineralized and CO_3 control gels compared to mixed and Ca control gels: higher values of G' across the frequency spectrum and an increase in G'' at low frequencies. Because of the high reactant concentrations—and thus, high levels of supersaturation—used in our experiments, mineralization correlates closely with the diffusion of carbonate ions into the calcium-loaded gel. Therefore, the mechanical differences associated with the presence of carbonate can be used as indirect signals of the growth of minerals. In particular, we chose the increase in G'' at low frequency as a signature of the presence of particles in mineralized systems.

To simplify the presentation of rheological data based on this low frequency dissipation signature, we use the loss tangent ($\tan(\delta) = G''/G'$). Figure 3b depicts the same data as in Figure

3a converted to $\tan(\delta)$. Here, the increase in $\tan(\delta)$, or energy dissipation, at low frequencies is even more clear. Because this signature is evident only at low frequencies, we are able to further simplify the differences between samples by using a single value of $\tan(\delta)$ at $\omega = 0.1$ rad/s, normalized by the minimum value of $\tan(\delta)$, which we call $\Delta\tan(\delta) = (\tan(\delta)_{\omega=0.1\text{rad/s}} - \tan(\delta)_{\min})$.

Figure 3. (a) Representative graph of the storage and loss moduli of mineralized (circle), mixed (square), and control samples (Na_2CO_3 control – star, CaCl_2 control – asterisk) from 0.1-100 rad/s angular frequency. (b) The same data from (a) replotted as $\tan(\delta)$ to simplify the data representation and highlight the enhanced low frequency dissipation signature of the mineralized and CO_3 control samples.

To better understand how the low frequency dissipation signature correlates with gel mineralization, in Figure 4 we plot $\Delta \tan(\delta)$ for gels with respect to (a) particle volume fraction and (b) Na_2CO_3 mineralizing solution concentration. Figure 4a shows that $\Delta \tan(\delta)$ increases with particle volume fraction in mineralized samples while there is no increase in $\Delta \tan(\delta)$ with mixed in particle volume fraction. However, Figure 4b shows that similar values of $\Delta \tan(\delta)$ are achieved in CO_3 control samples with no particles. These data confirm that the presence of mineral alone does not lead to increased dissipation and that the magnitude of $\Delta \tan(\delta)$ correlates with the amount of carbonate in the gel. To further test this correlation, we diffused 0.5M Na_2CO_3 into mixed gels and observed a corresponding increase in $\Delta \tan(\delta)$ independent of CaCO_3 particle content (see Figure 4a and 4b).

Figure 4. Variation in $\Delta \tan(\delta)$ ($\tan(\delta)_{\omega=0.1} - \tan(\delta)_{\min}$) of all gels with respect to (a) CaCO_3 volume fraction in the gel and (b) Na_2CO_3 mineralizing solution concentration. Samples represented include mineralized (circle), mixed (square), Na_2CO_3 control (star), CaCl_2 control (asterisk), and mixed + Na_2CO_3 (triangle). Notice the collapse of data points from (a) to (b) of mixed + Na_2CO_3 samples with varying CaCO_3 volume fraction but identical Na_2CO_3 solution concentration.

It has been widely observed that the mechanical properties of gelatin can depend strongly on the system pH.^{47–49} Specifically, studies have shown increases in storage moduli of gelatin gels at higher pH,⁵² which may explain the higher moduli observed for gels exposed to Na_2CO_3 (see Figure 3a) given that Na_2CO_3 is a base. However, the link between pH and viscoelastic dissipation in hydrogels has not been thoroughly explored. When plotting $\Delta \tan(\delta)$ against the pH of CO_3 control gels, we observe a tight linear correlation (see Figure 5), but gels exposed to basic pH using NaOH—without the presence of carbonate—do not show the same mechanically dissipative behavior. These results strongly suggest that carbonate-specific interactions with the

Figure 5. $\Delta \tan(\delta)$ with respect to gel pH for Na_2CO_3 control samples (star) and gels exposed to NaOH (plus sign) to produce a high pH environment without the presence of carbonate.

gel network, rather than gel pH, is the major source the observed dissipation. One possible explanation of this carbonate-induced dissipation is the association of divalent CO_3^{2-} ions with the gelatin matrix. Simulations by Tlatlik et al. revealed that hydrogen bonding between PO_4^{3-} and HPO_4^{2-} ions and the gelatin triple helix caused some bending of the gelatin molecule, increasing flexibility.⁵³ A similar association mechanism could be occurring between CO_3^{2-} ions and gelatin triple helices in our system, leading to increased dissipation at longer timescales (corresponding to lower frequencies) due to gelatin helical bending.

In situ mineralization

Next, we wanted to utilize the low frequency mechanical dissipation signal to monitor changes in mineralization *in situ* in real time. Figure 6 demonstrates a clear increase in $\tan(\delta)|_{\omega=0.1}$ over time during *in situ* mineralization in a gel, whereas $\tan(\delta)|_{\omega=0.1}$ remains constant for the Ca control.

While further analysis is required to fully understand what aspects of mineral growth are encapsulated in the time evolution of $\tan(\delta)$, our results confirm the ability of rheology to nondestructively probe mineral growth in gels in real time.

Figure 6. Evolution of $\tan(\delta)$ at $\omega = 0.1$ rad/s over time for a gelatin gel undergoing *in situ* mineralization (circle) and a CaCl_2 control sample (asterisk).

The parallel plate geometry used above is not ideal for this type of experiment since it creates a radially inhomogeneous gradient of particle growth within the gel. Hence, further rheological data analysis is complicated because we cannot determine how much of the system has been mineralized (radially) at any given time. Therefore, we also conducted *in situ* mineralization experiments using a cylindrical Couette geometry with a transparent outer

cylindrical wall, schematically depicted in Figure 7a. This geometry has a larger surface area over which the CO_3^{2-} ions can diffuse, and the transparent outer cylinder allowed us to monitor

Figure 7. (a) Schematic of the *in situ* mineralization setup using a cylindrical Couette geometry (b) Images of the gelatin gel during *in situ* mineralization at various timepoints in the cylindrical Couette geometry. The sharp, horizontal line toward the top of each image is the interface between the top of the gel and the carbonate solution. (c) Distance of the mineralization front from the interface over time, showing growth of the mineralized fraction of the gel. Dashed lines connect data points at various times to corresponding images in (b). (d) Evolution of $\tan(\delta)$ at varying frequencies over time, indicating the greatest increase in dissipation at the lowest frequency of 0.1 rad/s.

visually the axial fraction of the gel that has mineralized. The Couette geometry also enabled us to capture live images showing crystal growth over time, as shown in Figure 7b. Figure 7c

depicts ΔL , the axial distance of the mineralization front from the gel/solution interface, over time, which represents the growth of the mineralized portion of the gel. Dashed lines connect the images in Figure 7b to corresponding time points in Figure 7c. Figure 7d portrays the time evolution of $\tan(\delta)$ of the gelatin- CaCO_3 system at nine different frequencies. Here, $\tan(\delta)$ is normalized by the initial value of $\tan(\delta)$ to more clearly show the relative changes in $\tan(\delta)$ over time at various frequencies. The largest change in $\tan(\delta)$ occurs at a frequency of 0.1 rad/s, the lowest frequency measured, confirming that probing the mineral-gel system at lower frequencies gives more sensitivity overall and especially at earlier times during mineralization. Furthermore, the essential similarity of the shapes of the mineralized $\tan(\delta)|_{\omega=0.1}$ curves in Figure 6 and Figure 7d, captured using different instruments and different measurement geometries, reveals that rheological measurements are indeed robust characterization tools for quantifying the dynamics of mineral growth in hydrogels.

CONCLUSIONS

With a proof-of-concept hydrogel mineralization system consisting of gelatin and CaCO_3 , we have successfully tracked mineral growth in real time using rheological measurements of the complex modulus, $G^*(\omega)$, as an *in situ* mechano-scopic characterization tool. We identified a unique low frequency dissipation signal originating from the presence of excess CO_3^{2-} ions within the gelatin matrix. When combined with high reactant concentrations, the start of mineralization correlates closely with the diffusion of CO_3^{2-} ions into a Ca-loaded gel. Therefore, we were able to spatio-temporally track the growth of mineral particles in the gel by monitoring the signature low frequency dissipation signal induced by carbonate diffusion. Furthermore, we

have demonstrated that we can reliably capture this mechano-scope signal *in situ* over time using two different stress-controlled rheometer geometries. In the future, we aim to study mineralization systems with stronger mineral-matrix interactions that will lead to more direct correlation between mineralization and the hydrogel viscoelastic mechanics. For example, Li et al. have shown drastic rheological differences between gels crosslinked with either Fe^{3+} ions or Fe_3O_4 nanoparticles in otherwise identical polymer matrices.⁵⁴ Utilizing a similar hydrogel system for rheological characterization of *in situ* mineralization could allow unprecedented insights into early nucleation and growth mechanisms, such as distinguishing different polymorphs in the mineralization process.

SUPPORTING INFORMATION

Supplementary information: particle size analysis, x-ray powder diffraction data, rheological data, and *in situ* mineralization details (pdf)

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