Aragonite-Associated Mollusk Shell Protein Aggregates To Form Mesoscale “Smart” Hydrogels

Iva Perovic, Anastasia Davidyants, and John Spencer Evans*†

Center for Skeletal Biology and Craniofacial Medicine, Laboratory for Chemical Physics, New York University College of Dentistry, New York, New York 10010, United States

ABSTRACT: In the mollusk shell there exists a framework silk fibroin–polysaccharide hydrogel coating around nacre aragonite tablets, and this coating facilitates the synthesis and organization of mineral nanoparticles into mesocrystals. In this report, we identify that a protein component of this coating, n16.3, is a hydrogelator. Due to the presence of intrinsic disorder, aggregation-prone regions, and nearly equal balance of anionic and cationic side chains, this protein assembles to form porous mesoscale hydrogel particles in solution and on mica surfaces. These hydrogel particles change their dimensionality, organization, and internal structure in response to pH and ions, particularly Ca(II), which indicates that these behave as ion-responsive or “smart” hydrogels. Thus, in addition to silk fibroins, the gel phase of the mollusk shell nacre framework layer may actually consist of several framework hydrogelator proteins, such as n16.3, which can promote mineral nanoparticle organization and assembly during the nacre biomineralization process and also serve as a model system for designing ion-responsive, composite, and smart hydrogels.

INTRODUCTION

There have been rapid advances in the development of hydrogels for technological purposes, spanning applications such as drug delivery, adhesion, nanoparticle organization, cell culture, and so on.1−6 Hydrogels can be conceptualized as highly hydrated polymer networks possessing bonding or nonbonding interchain interactions, and hydrogel properties, such as diffusion, internal transport, and mechanical strength, are directly linked to the degree of swelling and the chemistries offered by the polymer network itself.6−9 Hydrogels can respond to environmental triggers, with temperature and pH responsiveness being the two most common ones.1−6 At present, there are two areas of hydrogel research that have garnered attention. The first is smart hydrogel technology involving the creation of polymer networks capable of responding to multiple environmental triggers.1,2 The second area is “composite” hydrogels, where small inorganic particles become incorporated into the gel network and enhance the mechanical properties of the gel phase.1,2,7 If we can gain further insights into engineering improvements in these areas, then hydrogel technology and the corresponding applications will advance more rapidly.

In some instances, our ability to jumpstart existing technologies can be enhanced by the study of organisms in nature. Although synthetic polymer networks are commonly used for hydrogel generation,1−7 there are natural polymeric systems that also lend themselves to hydrogel formation, such as collagen, chitosan, fibrin, agarose, hyaluronic acid, and cellulose.2 Interestingly, there are some naturally occurring hydrogel systems in nature that offer bioinspired insights into hydrogel technologies. One of these is the mollusk shell, where silk fibroin proteins and β-chitin polysaccharides combine to form a framework hydrogel environment for inorganic nanoparticle nucleation (calcium carbonates), assembly, and the creation of a fracture-resistant composite known as nacre.8−11 This silk–polysaccharide nacre hydrogel system may play an important role with regard to the formation of the mollusk shell itself, for example, creating a meshwork within the extracellular matrix that limits ion diffusion and creates nanovolume compartmentalization that controls the mineral nucleation process.8−11

Interestingly, there are many nonsilk nacre proteins12−18 that coexist within the silk–polysaccharide nacre hydrogel system as well, yet their role in the nucleation process and their relationship to the hydrogel environment are poorly understood. Several of these proteins are responsible for inhibiting calcite, controlling the assembly of mineral nanoparticles into mesoscale aragonite tablets, or controlling the nucleation process in nacre.12−18 Many of these proteins have very interesting properties, such as the presence of intrinsic disorder or an unfolded structure and amyloid-like cross-β strand aggregation-prone sequences, both of which promote protein–protein aggregation and assembly.19,20 This assembly process creates supramolecular complexes or protein phases,21−27 which have a particle and film-like character on surfaces. These protein phases have been demonstrated to control early stages of mineralization22,24,25 and modify the surfaces and interiors of existing crystals.25 However, the physical nature of...
these non-silk protein phases has not been fully explored: given their association with the silk−polysaccharide gel phase, are these nacre protein phases also hydrogels, and if so, do they possess any interesting properties that might advance our understanding and development of synthetic hydrogels?

In this report, we examine an intrinsically disordered, aggregation-prone framework oyster shell nacre protein, n16.3 (pI = 4.82; 108 AA, MW = 12947 Da Da Pinctada fucata),\textsuperscript{16,18} which is one member of the framework proteome that resides within the β chitin/silk fibroin gel coating around nacre aragonite mesocrystal tablets.\textsuperscript{12,13} This protein possesses amyloid-like cross-β strand aggregation sequences and a nearly equivalent number of anionic (18) and cationic (14) side chains (Figure 1).\textsuperscript{22,23} n16.3 is known to aggregate in aqueous solutions, forming mesoscale protein phases that modify crystal growth directions and introduce nanotexturing to the surface of growing calcium carbonate crystals.\textsuperscript{21} Furthermore, n16.3 protein phases are also known to capture and organize mineral nanoparticles (Figure 2) and stabilize an amorphous calcium carbonate precursor, ACC, during the early stages of nucleation.\textsuperscript{22} Using a recombinant version of n16.3 (r-n16.3)\textsuperscript{21–23} and biophysical techniques, we confirm that these nacre protein phases are in fact mesoscale porous hydrogel particles. These hydrogel particles change their dimensionality, organization, and internal structure in response to pH and ions, particularly Ca(II), which indicates that these behave as ion-responsive or smart hydrogels. Thus, in addition to silk fibroins, the gel phase of the mollusk shell nacre framework layer may actually consist of several framework hydrogelator proteins, such as n16.3. As discussed below, we believe that the nacre protein-based hydrogels can serve as a model system for understanding the role that disorder, aggregation propensity, and electrostatics play in hydrogel formation, structure, and the ability to capture and organize inorganic nanoparticles in solution.

### MATERIALS AND METHODS

**Recombinant Synthesis, Purification, and Preparation of r-n16.3.** The gene synthesis, cloning, bacterial expression, and purification of r-n16.3 were performed by GenScript USA (Piscataway, NJ: http://www.genscript.com/) using their proprietary OptimumGene system and recombinant expression systems, as described elsewhere.\textsuperscript{21–23} For subsequent experimentation, r-n16.3 samples were created by exchanging and concentrating appropriate volumes of stock solution into unbuffered deionized distilled water (UDDW) or other appropriate buffers using Amicon Ultra 0.5, 3 kDa MWCO (Millipore Corporation). For the studies listed in this report, the following buffer conditions were utilized: 10 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES), pH 8.0 (denoted as low ionic strength conditions), 8.9 mM NaH₂PO₄, pH 4.0, 10 mM HEPES/30 mM NaCl, pH 8.0, 10 mM HEPES/1 mM CaCl₂, pH 8.0, and 10 mM HEPES/10 mM CaCl₂, pH 8.0. All buffers were prepared in 30 nM filtered Fisher atomic force microscopy (AFM) water (Fisher Scientific) and filtered at 0.2 μm after pH adjustment to purge particulates.

**Light Microscopy Imaging of r-n16.3 Protein Hydrogel Particles.** For detection of mesoscale protein hydrogel particles, 5 μL of 30 μM r-n16.3 solution in the appropriate buffer (see above) was placed on a clean glass slide with a glass coverslip and imaged using bright field microscopy (100× objective, Nikon DS-U3 Light Microscope). Note that higher protein concentrations were required to generate sufficiently large hydrogels for visualization purposes.

**AFM Imaging of r-n16.3 Assemblies.** We investigated the dimensional and morphological characteristics of r-n16.3 assemblies (1.3 μM and 380 nM final protein concentrations) deposited onto mica substrates under different buffer conditions (see above and the Figure legends). AFM experiments were executed at 25 °C using an Asylum MFP-3D standalone AFM operating in tapping mode in buffer solution. V-shaped Si₃N₄ cantilevers (reported spring constant 0.09 N/m) were used for imaging. A precise drive frequency in fluid (~9 kHz) was calculated for each cantilever before imaging by overlaying the thermal spectrum over the frequency sweep. All samples (100 μL) were aliquoted onto a freshly stripped surface of mica (Ted Pella, Inc., 0.9 mm thick) and incubated for a period of 15 min at ambient temperature before measurement. Igor Pro 6.01 software (http://www.wavemetrics.com) was used for image acquisition at a scan rate of 2 Hz. The Wydgydon Software package (www...
The aggregation of r-n16.3 (30 μM final concentration) was studied in the above-mentioned buffers. The samples were prepared and allowed to sit for 5 min before analysis. Aggregation measurements were performed using a multi-parameter cell analyzer CytoFLEX (Beckman Coulter, CA). Each sample solution (100 μL) was analyzed at a continuous flow rate of 10 μL/min using four laser excitation lines of 405, 488, 561, and 640 nm to register two light-scattering parameters (forward-scattered light (FSC)-A) and side-scattered light (SSC)-A) and the number of events for each sample. Data were collected using the CyToExpert 1.2.11.0 software designed for the instrument and processed using the FlowJo software (TreeStar, OR).

**Mineralization Assays.** Stock concentrations of r-n16.3 were prepared using 0.2 μM filtered UDDW. Mineralization assays were adapted from published protocols and were conducted by mixing equal volumes of 20 mM CaCl2·2H2O (pH 5.5) and 20 mM NaHCO3/Na2CO3 buffer (pH 8.9) to a final volume of 500 μL in sealed polypropylene tubes and incubating at room temperature for 5 min. Aliquots of r-n16.3 stock solution were added to the calcium solution before the beginning of the reaction, with final protein assay concentration of 30 μM. The pH of the reaction mixture was measured and found to be approximately 8.0–8.2. For TEM studies, a 10 μL aliquot of the mineralization assay supernatant was withdrawn at the completion of the assay period, spotted onto Formvar-coated Au TEM grids (200 square mesh; Ted Pella, Inc.), and washed with 0.2 μm filtered calcium carbonate saturated methanol. TEM and electron diffraction were performed using a Philips CM12 transmission electron microscope equipped with a tungsten filament electron beam source. All imaging and diffraction analyses were performed at 120 keV. A diffraction pattern of a polycrystalline gold standard was used as a calibration scale for all subsequently recorded diffraction patterns. The selected-area diffraction patterns were analyzed and indexed using the CrysTBox software package (www.fzu.cz/crybbox).

**Bioinformatics.** To determine the location of disordered sequence regions within the n16.3 sequence, we employed the DISOPRED331 and IUP_PRED32 prediction algorithms using default parameters. Subsequently, we used TANGO, AGGRESCAN,34 and ZIPPER DB35 with default parameters to globally identify putative cross-β strand sequence regions that exhibit association propensities (Figure 1).

### RESULTS AND DISCUSSION

**r-n16.3 Forms Hydrogel-Like Particles That Respond to pH and Ionic Strength Conditions.** As shown in Figure 3, top panel, light microscopy studies reveal that r-n16.3 at pH 8.0 (similar to the pH of nucleation studies) forms translucent particles that appear gel-like. These particles have irregular morphologies and appear to be porous as evidenced by the presence of void-like regions within the particles. On freshly cleaved mica surfaces, tapping-mode AFM imaging (middle and bottom panels) shows that these particles have an elongated morphology not unlike that seen in the light microscope under identical conditions (Figure 3). Note that relative to plain mica surfaces, mica surfaces that contain the r-n16.3 protein have a higher surface roughness value, Rq, indicating the presence of a protein coating or film on the mica surface (Figure 3, histogram plot).

We next studied these protein particles under two different conditions: 8.9 mM NaH2PO4, pH 4.0, and 10 mM HEPES, 30 mM NaCl, pH 8.0, with both buffer conditions possessing the same ionic strength (0.15 M). Table 1 summarizes the protein phases in each of the four buffers used (Figure 3). Under acidic conditions, the protein particle morphology changed from a translucent gel-like particulate to an irregular particulate that displays highly porous features. The porous features give the particles an irregular particulate morphology. Under alkaline conditions, the protein particles display an irregular elongated particulate morphology that is consistent with the light microscope analysis. The AFM analysis of the mineralization supernatants show that the protein particle morphology under alkaline conditions is highly porous and irregular. The AFM analysis of the mineralization supernatants show that the protein particle morphology under alkaline conditions is highly porous and irregular.

---

**Figure 3.** Imaging of 1.3 μM r-n16.3 protein phases in 8.9 mM NaH2PO4 (pH 4.0), 10 mM HEPES (pH 8.0), and 30 mM NaCl/10 mM HEPES (pH 8/NaCl). Light microscope images taken at 100× magnification of 30 μM r-n16.3 samples. AFM tapping-mode amplitude images are plotted at 1 μm x 1 μm. "AFM data set" refers to statistical measurements of mean particle heights, diameters, and surface roughness or Rq factor, ±S.D., taken for 30 particles under each buffer condition. The Rq values for plain mica under each buffer condition were subtracted from protein values. Scale bars in light microscope images = 10 μm and in AFM = 200 nm.
same ionic strength value as 10 mM HEPES/10 mM CaCl₂. As we shall see, similarities in ionic strength values allow cross-comparisons between these conditions and later Ca(II) studies. We will first consider low pH conditions (pH 4.0), which are below the pI (4.85) of this protein. Note in Figure 3 that there are dimensional changes in mesoscale hydrogel particles in response to protein side chain protonation. At pH 4.0, the particles become smaller in diameter but retain the same particle heights relative to pH 8.0, as confirmed by both light microscopy (top panel) and tapping-mode AFM (middle, bottom panels), respectively. However, at pH 4.0, the Rq value is \( \sim 3 \times \) that measured at pH 8.0, indicating that the protein film thickness has increased at pH 4.0 (histogram plot). Since r-n16.3 has 18 carboxylate residues (Asp, Glu) (Figure 1), it is clear that carboxylate protonation at low pH values induces charge shielding and alters the electrostatics of the protein molecules.22,23 Furthermore, mica surfaces are anionic, and charge shielding on the anionic and cationic residues,23 respectively, on protein molecules and induce morphological, interfacial, and dimensional changes to the protein hydrogel particles. Moreover, we would also expect charge shielding to induce particle rearrangements on anionic mica surfaces.22 In the presence of NaCl this is exactly what we observe: Light microscopy reveals large hydrogel particles that appear to have smaller particles clustered or associated at the particle interfaces (Figure 3, top panel). Similar clustering phenomena were also observed in AFM imaging on mica surfaces, where rounded morphologies and the presence of large and small particle diameters were noted along with evidence of particle chain formation (Figure 3, middle and bottom panels). Furthermore, although the protein particle diameters are similar to those observed at low ionic strength, the Rq value increases by a factor \( \sim 6 \times \) relative to low ionic strength conditions, which indicates that protein film formation on mica has significantly increased in the presence of NaCl (Figure 3, histogram plot). From these results, we conclude that r-n16.3 protein molecules assemble to form mesoscale hydrogel particles both on surfaces and in solution that are dimensionally and interactivity responsive to pH and salt conditions.

**Introduction of Ca(II) Ions Leads to r-n16.3 Hydrogel Particle Organization.** We now consider what happens to r-n16.3 hydrogel particles when they are exposed to Ca(II) ions relative to low ionic strength conditions at pH 8.0. Here, the most relevant scenario is 10 mM CaCl₂, pH 8.0, which mimics the conditions found within in vitro mineralization assays (Figure 2)22 minus the carbonate species. For AFM studies in the presence of Ca(II), we used protein concentrations of 380 nM, which minimize the degree of aggregation buildup that interferes with AFM tip movement and placement,22 thereby allowing greater imaging detail to be observed rather than obscured by the Ca(II)-induced aggregation process. We also examined a lower Ca(II) ion concentration, 1 mM, at pH 8.0, to determine any trends that Ca(II) ions introduce into r-n16.3 aggregation and hydrogel formation.

As noted in previous AFM studies of r-n16.3 at lower concentrations (i.e., 77 nM or 1/5 of 380 nM), the introduction of 10 mM Ca(II) leads to an increase in protein particle diameters, heights, and film thicknesses on mica surfaces, in response to Ca(II) interactions with Asp, Glu residues, and the anionic mica surface.22 As shown in Figure 4, we note these same effects at 380 nM r-n16.3 and discover new Ca(II)-induced effects at this higher protein concentration. Relative to the pH 8.0 low ionic strength scenario, the introduction of 1 mM Ca(II) leads to a clustering effect that can be visualized both at the light microscope and AFM tapping-mode levels. In the light microscope (top panel), we observe mesoscale protein hydrogel particles that consist of clusters of smaller particles that are less translucent and create a
coarsening effect to the overall hydrogel appearance. On mica surfaces (middle and bottom panels), we also observe a similar clustering effect, and in some cases, we can actually observe the formation of linear chains. This clustering effect intensifies at 10 mM Ca(II) concentrations, where in the light microscope, we observe further loss in particle translucency, increased coarsening, and the appearance of hydrogel particle chains forming and organizing in solution. Similarly, on mica surfaces, 10 mM Ca(II) solutions induce protein particle organization into fibers with irregular morphologies. At this time, we do not fully understand why Ca(II) ions induce a more linear or fibrous configuration to r-n16.3 hydrogels in solution and on surfaces. We speculate that side chain—Ca(II) affinities or Ca(II)-mediated side chain—side chain salt bridging interactions between protein molecules may be responsible for the induction of linear or fiber-like clusters. Obviously, other conditions that induce simple charge shielding (i.e., pH 4.0, NaCl) are incapable of producing this effect (Figure 3). In conclusion, our data demonstrates that, in comparison to protonation and monovalent cation shielding (Figure 3), the introduction of Ca(II) triggers changes in r-n16.3 hydrogel structure, dimension, and organization (cluster-to-fibrous transition) in solution and on surfaces (Figure 4).

r-n16.3 Hydrogel Particles in Flow Exhibit Particle Size and Internal Granularity Changes in Response to Ionic Solutions. One of the more intriguing aspects of our data set is that light microscope images of r-n16.3 reveal changes not only in particle morphology but also in the internal porosities as a function of pH and ionic species (Figures 3 and 4). To confirm the external and internal changes that occur with mesoscale r-n16.3 hydrogel particles as a function of buffer conditions, we applied a technique typically used for analyzing transparent micron-sized cell populations, flow cytometry (Figure 5), to map physical changes in translucent mesoscale protein particles under buffer conditions that parallel our light microscopy and AFM imaging studies (Figures 3 and 4). There are two light-scattering parameters that one can monitor for particles under constant flow: (1) FSC (x-axis) to determine the particle size distribution and (2) SSC (y-axis) to measure refracted and reflected light that occurs at any interface within the particles where there is a change in refractive index (RI) that results from variations in particle granularity or internal structure. (Bottom panel) One-D particle number plots, the number in the upper left corner of each plot denotes the particle count number obtained for that plot. Note that the pH 4.0, pH 8.0/NaCl, and pH 8.0/10 mM Ca(II) scenarios are equivalent in ionic strength.

As we observe in Figure 5, top panel, low ionic strength conditions at pH 8.0 generate a very limited distribution of particle sizes and internal structure or granularity. When we transition to pH 4.0, the particle number count more than doubles (bottom panel), and we note that particle size distributions and internal structure increase proportionally (top panel), indicating that Asp and Glu protonation induces changes not only in the hydrogel protein molecules but within the hydrogels themselves. In the presence of 30 mM NaCl at pH 8.0, the particle number counts (bottom panel), particle size distributions, and internal structure (top panel) are found to be very similar to low ionic strength conditions, i.e., there are limited distributions. Although this would appear to contradict the data obtained from AFM imaging studies (Figure 3, top panel), one must remember that the electrostatics of the anionic mica surface play an important role in particle behavior and physical state, and this factor is absent from the flow cytometry experiments.

The most dramatic results are observed when Ca(II) is introduced (Figure 5). Here, we again observe proportional

Figure 5. Flow cytometry experiments conducted with 30 μM r-n16.3. (Top panel) Two-dimensional density plots of FSC as a function of SSC. Here, FSC (x-axis) determines particle size distributions and SSC (y-axis) measures refracted and reflected light that occurs at any interface within the particles where there is a change in RI that results from variations in particle granularity or internal structure. (Bottom panel) One-D particle number plots, the number in the upper left corner of each plot denotes the particle count number obtained for that plot.
increases in particle size distributions and internal granularities (top panel), as we go from 1 to 10 mM CaCl₂ concentrations. These phenomena correlate with the imaging coarseness and loss of translucency observed in our light microscope studies of these hydrogel particles under identical conditions (Figure 4). Furthermore, the particle number counts increase by a factor of \( \sim 2 \times 10 \text{mM Ca(II)} \) and \( \sim 5 \times 10 \text{mM Ca(II)} \) relative to low ionic strength conditions at the same pH value. We note that at 10 mM CaCl₂, the proportionality in particle size distributions and internal granularities mirror those seen at pH 4, and this may reflect the similarities in the charge shielding effects that protons and Ca(II) ions exert on r-n16.3 hydrogels. However, the range of FSC and SSC parameters is not identical [i.e., Ca(II) conditions feature a larger range of values], and we note that the particle numbers obtained for Ca(II) are higher than those obtained for pH 4 (Figure 5, lower panel). Thus, we believe that the effect of pH 4.0 and 10 mM CaCl₂ conditions on protein hydrogel particles is not truly identical. In summary, low pH and Ca(II) ions introduce the most significant changes in r-n16.3 hydrogel particle number, size distributions, and internal structure (Figure 5). Overall (Figures 3–5), we conclude that the framework r-n16.3 protein is a hydrogelator that forms ion-responsive mesoscale gels both on surfaces and in solution.

In conclusion, in addition to the silk fibroin–chitin polysaccharide gel layer, which coats mesoscale nacre aragonite tablets in the mollusk shell, \(^{6–11}\) we have now identified another framework aragonite protein of the mollusk shell, n16.3, as a hydrogelator. The fact that this hydrogel can capture and organize mineral nanoparticles (Figure 2) \(^{21,22}\) makes this a composite hydrogel system. \(^{1,2}\) Interestingly, this protein is associated with the silk fibroin–chitin polysaccharide gel layer, and thus, it is plausible that n16.3 and perhaps other framework protein hydrogelators may be subcomponents of this silk–polysaccharide gel nacre layer phase. We note that other invertebrate skeletal systems, specifically the sea urchin calicite spicule \(^{36}\) and the nacre layer of the red abalone, \(^{37}\) also possess nonsilk fibroin proteins that are hydrogelators, and this suggests that protein hydrogels play an important role in the biomineralization process within a wide variety of calcium carbonate-based organisms.

The intriguing response of the r-n16.3 hydrogel particles to different ionic conditions (Figures 3 and 4) indicates that the protein molecules (Figure 1) and the hydrogel particles that they form are ion-responsive or smart, with the most dramatic results obtained with Ca(II) ions. At this time, there are several unanswered questions regarding why Ca(II) induces such dramatic changes in hydrogel organization (i.e., particulate to fibrous, Figure 4) and hopefully these will be addressed by future studies. In general, the discovery of porous nacre protein hydrogels represents a tremendous step forward in our understanding of the biomineralization process in mollusk shell nacre. Potentially, hydrogel porosities provide several important features for the nucleation process and crystal building: volume confinement, compartmentalization, assembly, and organization. \(^{36,39}\) As shown in Figure 2, first, r-n16.3 hydrogels are capable of hosting and organizing calcium carbonate mineral nanoparticles within their porous matrices, and we believe that the size of these mineral nanoparticles is most likely limited by the pore sizes that exist within the hydrogel particles. Second, the porous matrices introduce a diffusion or kinetic barrier to nucleating ion complexes, as witnessed by r-n16.3 increasing the time interval for prenucleation cluster (PNC) formation \(^{40–43}\) compared to protein-deficient controls. \(^{22}\) Third, the ion responsiveness may act as a trigger for hydrogel participation in CaCO₃ nucleation events, that is, hydrogels change size, morphology, association, and organization (Figure 4) and internal structure in response to Ca(II) ions (Figure 5), which in turn can affect the assembly and organization of mineral nanoparticles (Figure 2), \(^{21,22}\) which has been proposed as an important event in the crystallization by the particle attachment \(^{38}\) mechanism that leads to aragonite tablet formation.

Finally, what insights do nacre protein hydrogels offer material science and nanotechnology? We believe that there are several important lessons that one can glean from this study. The first is that disordered, aggregation-prone polymers containing a nearly equivalent number of anionic and cationic side chains, such as r-n16.3 (Figure 1), \(^{22,23}\) can form smart hydrogels in solution under a wide variety of conditions (Figures 2–5). The presence of both anionic and cationic charged groups allows for molecular electrostatic responsiveness in different ionic media and, as discussed earlier, may play a role in the formation of the hydrogels themselves via complementary ion pairing. Furthermore, charged side chains may participate in the attraction of nascent PNCs that form during the early phases of the nucleation process \(^{38–43}\) and may explain why r-n16.3 hydrogel particles nucleate intragel mineral nanoparticle deposits over time, forming a composite hydrogel (Figure 2). \(^{22}\) The second important feature is the change in exterior dimensionality and interior granularities or structure in response to environmental conditions (Figure 5). On the basis of our light microscope imaging (Figures 3 and 4), we speculate that the changes in granularities reflect changes in hydrogel porosities, either in terms of the number of porosities, their size, and/or their location within the protein hydrogel particles. In turn, these adjustments to ionic media may eventually influence the formation, size, and distribution of mineral nanoparticles \(^{35,39}\) within the hydrogels (Figure 2). Collectively, these nacre protein hydrogel features are worthy of further exploration in future studies, such that we can apply these concepts to new and useful composite, responsive smart hydrogels for materials and nanotechnology applications.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jse1@nyu.edu.

ORCID

John Spencer Evans: 0000-0002-9565-7296

Present Address

Cornell Medical Center, New York, New York 10065-4896, United States (I.P.).

Author Contributions

All authors have given approval to the final version of the manuscript.

Funding

This research was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Materials Sciences and Engineering, under Award DE-FG02-03ER46099.

Notes

The authors declare no competing financial interest.
ACKNOWLEDGMENTS

We thank Dr. Eric Chang for help with running the AFM and TEM imaging studies and Dr. Martin Pendola for his help with processing the flow cytometry data sets. This report represents contribution number 85 from the Laboratory for Chemical Physics, New York University.

ABBREVIATIONS

n16.3, nacre protein 16, isoform 3, Pinctada fucata; r-n16.3, E. coli produced recombinant form of n16.3; ACC, amorphous calcium carbonate; PNC, prenucleation cluster; FSC, forward-scattered component; SSC, side-scattered component

REFERENCES

(1) Delgkars, K.; Tadele, T. S.; Olthuis, W.; van den Berg, A. Hydrogel-based devices for biomedical applications. Sens. Actuators, B 2010, 147, 765.
(2) Buwalda, S. J.; Boere, K. W. M.; Dijkstra, P. J.; Feijen, J.; Vermondenh, T.; Hennek, W. E. Hydrogels in a historical perspective: From simple networks to smart materials. J. Controlled Release 2014, 190, 254.
(3) Cui, J.; Bjornmalm, M.; Liang, K.; Xu, C.; Best, J. P.; Zhang, X.; Caruso, F. Super-Soft hydrogel particles with tunable elasticity in a microfluidic blood capillary model. Adv. Mater. 2014, 26, 7295.
(4) Du, X.; Zhou, J.; Shi, J.; Xu, B. Supramolecular hydrogelators and hydrogels: From soft matter to molecular biomaterials. Chem. Rev. 2015, 115, 13165.
(5) Huang, H.; Herrera, A.; Prakash, O.; Sun, X. S. Structural transformation and physical properties of a hydrogel-forming peptide studied by NMR, transmission electron microscopy, and dynamic rheometer. Biophys. J. 2012, 103, 979.
(6) Patanarut, A.; Luchini, A.; Botterell, P. J.; Mohan, A.; Longo, C.; Vorster, P.; Petricoin, E. F., III; Liotta, L. A.; Bishop, B. Colloids Surf, A 2010, 362, 8.
(7) Bai, H.; Polini, A.; Delattre, B.; Tomsia, A. P. Thermoresponsive composite hydrogels with aligned macroporous structure by ice-templated assembly. Chem. Mater. 2013, 25, 4551.
(8) Levi-Kalisman, Y.; Falini, G.; Addadi, L.; Weiner, S. Structure of the nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using cryo-TEM. J. Struct. Biol. 2001, 135, 8.
(9) Nudelman, F.; Shimon, E.; Klein, E.; Rousseau, M.; Bourrat, X.; Lopez, E.; Addadi, L.; Weiner, S. Forming nacreous layer of the shells of the bivalves Atinna rigida and Pinctada margaritifera: an environmental- and cryo-scanning electron microscopy study. J. Struct. Biol. 2008, 162, 290.
(10) Ma, Y.; Feng, Q.; Bourrat, X. A novel growth process of calcium carbonate crystals in a silk fibroin hydrogel system. Mater. Sci. Eng., C 2013, 33, 2413.
(11) Keene, E. C.; Evans, J. S.; Estroff, L. A. (2010) Silk fibroin hydrogels coupled with the n16N - beta-chitin complex: An in vitro organic matrix for controlling calcium carbonate mineralization. Cryst. Growth Des. 2010, 10, 5169.
(12) Fang, D.; Xu, G.; Hu, Y.; Pan, C.; Xie, L.; Zhang, R. Identification of genes directly involved in shell formation and their functions in pearl oyster, Pinctada fucata. PLoS One 2011, 6, No. e21860.
(13) Zhang, G.; et al. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 2012, 490, 49–54.
(14) Kinoshita, S.; Wang, N.; Inoue, H.; Maeyama, K.; Okamoto, K.; Nagai, K.; Kondo, H.; Hirota, I.; Asakawa, S.; Watabe, S. Deep sequencing of ESTs from nacreous and prismatic layer producing tissues and a screen for novel shell formation-related genes in the pearl oyster. PLoS One 2011, 6, No. e21238.
(15) Marie, B.; Joubert, C.; Tayale, A.; Zanella-Cleon, I.; Belliard, C.; Piquemal, D.; Cochennec-Laureau, N.; Marin, F.; Gueguen, Y.; Montagnani, C. Different secretory repertoires control the biominer-
(36) Jain, G.; Pendola, M.; Rao, A.; Colfen, H.; Evans, J. S. A model sea urchin spicule matrix protein self-associates to form mineral-modifying protein hydrogels. *Biochemistry* 2016, 55, 4410.

(37) Pendola, M.; Jain, G.; Davidyants, A.; Huang, Y. C.; Gebauer, D.; Evans, J. S. A nacre protein forms mesoscale hydrogels that "hijack" the biomineralization process within a seawater environment. *CrystEngComm* 2016, 18, 7675.

(38) De Yoreo, J. J.; Gilbert, P. U. P. A.; Sommerdijk, N. A. J. M.; Penn, R. L.; Whitelam, S.; Joester, D.; Zhang, H.; Rimer, J. D.; Navrotsky, A.; Banfield, J. F.; Wallace, A. F.; Michel, F. M.; Meldrum, F. C.; Colfen, H.; Dove, P. M. Crystallization by particle attachment in synthetic, biogenic, and geologic environments. *Science* 2015, 349, No. aaa6760.

(39) Wallace, A. F.; Hedges, L. O.; Fernandez-Martinez, A.; Raiteri, P.; Gale, J. D.; Waychunas, G. A.; Whitelam, S.; Banfield, J. F.; De Yoreo, J. J. Liquid-liquid separation in supersaturated CaCO₃ solutions. *Science* 2013, 341, 885.

(40) Gebauer, D.; Kellermeier, M.; Gale, J. D.; Bergstrom, L.; Colfen, H. Pre-nucleation clusters as solute precursors in crystallization. *Chem. Soc. Rev.* 2014, 43, 2348.

(41) Gebauer, D.; Volkel, A.; Colfen, H. Stable prenucleation of calcium carbonate clusters. *Science* 2008, 322, 1819.

(42) Gebauer, D.; Colfen, H. Prenucleation clusters and non-classical nucleation. *Nano Today* 2011, 6, 564.

(43) Demichelis, R.; Raiteri, P.; Gale, J. D.; Quigley, D.; Gebauer, D. Stable prenucleation mineral clusters are liquid-like ionic polymers. *Nat. Commun.* 2011, 2, No. 590.