ABSTRACT: The formation of the sea urchin spicule involves the stabilization and transformation of amorphous calcium carbonate (ACC) and assembly of ACC nanoparticle precursors into a mesoscale single crystal of fracture-resistant calcite. This process of particle assembly or attachment is under the control of a family of proteins known as the spicule matrix \( [\text{Strongylocentrotus purpuratus (SpSM)}] \) proteome. Recently, two members of this proteome, SpSM50 and the glycoprotein SpSM30B/C-G (in recombinant forms), were found to interact together via SpSM30B/C-G oligosaccharide–SpSM50 protein interactions to form hybrid protein hydrogels with unique physical properties. In this study, we investigate the mineralization properties of this hybrid hydrogel alongside the hydrogels formed by SpSM50 and SpSM30B/C-G individually. We find that the SpSM50 + SpSM30B/C-G hybrid hydrogel is synergistic with regard to surface modifications and intracrystalline inclusions of existing calcite crystals, the inhibition of ACC formation, and the kinetic destabilization of ACC to form a crystalline phase. Most importantly, the hybrid hydrogel phase assembles and organizes mineral particles into discrete clusters or domains within in vitro mineralization environments. Thus, the interactions of SpSM50 and SpSM30B/C-G, mediated by carbohydrate–protein binding, reflect the need for protein cooperativity for the ACC-to-crystalline transformation, intracrystalline void formation, and guided mineral particle assembly processes that are instrumental in spicule formation.

INTRODUCTION

The formation of mineralized skeletal elements (spines, tests, and spicules) in the sea urchin is an example of how mesoscale composite materials can be formed from nanoparticle assembly. This mineralization process begins with the formation of an amorphous precursor phase, amorphous calcium carbonate (ACC), and these amorphous nanoparticles aggregate together and eventually transform into a crystalline phase, magnesium-substituted calcite (MgC). This process of nanoparticle assembly is not known in detail, but it is suspected that the matrix proteins which create the organic framework for mineralization play a key role in this process. Because the deletion of some of these proteins is detrimental to spicule mineralization. Another phenomenon that occurs during skeletal element formation is the incorporation of these matrix proteins into the mineral phase itself, resulting in the formation of nanoinclusions that modulate the force resistance of the skeletal structure. Thus, matrix proteins are a major driving force for the mineralization process in the sea urchin and are responsible for the material properties of skeletal structures for organism survival.

The formation of the mineralized embryonic spicule in the sea urchin \( \text{Strongylocentrotus purpuratus} \) requires the expression of several proteins that are designated as SpSM. Many expressed SpSM proteins are intrinsically disordered and feature a globular C-type lectin-like domain at the N-terminal region, which participates in matrix assembly and mineralization. Furthermore, in 10 SpSM proteins, there is an intrinsically disordered Met/Asn/Gln/Pro/Gly-rich redundant sequence that is homologous to known elastomeric sequences. These common sequence elements may participate in matrix formation and elasticity, mineral formation, or matrix–mineral organization processes.

Of the known SM proteins, the best characterized members are SpSM50 and the SpSM30 acidic glycoprotein A–F isoforms.
expression does not interfere with spicule formation, whereas knockdown of SpSM50 expression does. 15 Reportedly, SpSM50 stabilizes hydrated ACC from transformation in vitro. 5,6 Recombinant model forms of both proteins25,26,28,30 (the insect cell expressed glycosylated rSpSM30B/C-G, a hybrid of the “B” and “C” isoform sequences; the bacteria expressed rSpSM50) have been found to individually form protein hydrogel particles that act as “smart” hydrogels25,29,28 that rapidly exchange water, 28 modify calcite crystals, 25,28,29 and regulate the timeline and solubilities of ACC formation. 25,29,30

Thus, the spicule matrix is a hydrogel environment formed by multiple spicule matrix proteins, wherein ACC formation, stabilization, transformation, and intracrystalline incorporation processes take place during spiculogenesis.

Recent combinatorial studies of rSpSM50 and rSpSM30B/C-G revealed that rSpSM50 recognizes and binds to the anionic O-linked glycan moieties located within the C-terminal region of rSpSM30B/C-G, yet does not recognize or bind to the rSpSM30B/C-G protein sequence itself. 25,29,30 These protein–glycan interactions lead to the formation of a hybrid rSpSMB/C-G−rSpSM50 protein hydrogel that is dimensionally and structurally different from the hydrogels formed by the individual proteins. 30 It was postulated that rSpSM30B/C-G, which is not required for spicule matrix formation, 15 acts as a “helper” protein which boosts or modulates the activity of the required rSpSM50 protein. However, these studies did not address the mineralization activities of the hybrid rSpSM30B/C-G−rSpSM50 hydrogels and thus were unable to shed light on what changes, if any, occur to the mineralization process in the presence of this hybrid system.

To address this issue, we performed in vitro calcite mineralization experiments involving four scenarios: protein-free, rSpSM30B/C-G, rSpSM50, and 1:1 molar mixture of both proteins as utilized in our earlier combinatorial protein hydrogel studies. 30 Here, we examined the effect of each scenario on three phenomena: (a) crystal growth; (b) nonclassical nucleation [i.e., the formation of prenucleation clusters (PNCs) and ACC clusters during Ca2+ potentiometric titration]; 31−33 and (c) the organization and distribution of mineral particles. Analogous to our findings with combinatorial nacre protein studies, 31−33,35 we discovered that the rSpSM30B/C-G + rSpSM50 hybrid protein hydrogel creates synergistic mineralization effects on nucleation and crystal growth. Specifically, existing crystals become highly modified by surface nanotexturing and subsurface nanoporosities, and the formation of crystalline CaCO3 from ACC is accelerated, that is, the ACC phase is kinetically destabilized by the protein mixture. More importantly, the hybrid hydrogel guides the assembly of mineral particles into unique three-dimensional arrangements or domains not seen for either protein. Thus, protein cooperativity is a requirement for spicule mineral formation.

## EXPERIMENTAL SECTION

### Recombinant Protein Expression and Purification.

The expression, preparation, and purification of the recombinant tag-free insect cell-expressed SpSM30B/C glycoprotein (rSpSM30B/C-G) 28,30 and bacteria-expressed SpSM50 (rSpSM50) 25,30 were performed as described previously. The SF9 insect-expressed purified rSpSM30B/C-G protein sample contains three glycosylated protein variants [molecular weight (MW) = 30 848.5, 33 287.4, and 37 157.2 Da] and a small percentage of unglycosylated protein (29 678.7 Da). 28,30 For subsequent experimentation, protein samples were created by exchanging and concentrating appropriate volumes of stock solution into unbuffered deionized distilled water or other appropriate buffers using Amicon Ultra 0.5 kDa MW cutoff concentration filters. 25,30

### In Vitro Micromineralization Assays.

Calcite-specific mineralization microassays were conducted by mixing equal volumes of 20 mM CaCl2·2H2O (pH 5.5) and 20 mM NaHCO3/Na2CO3 buffer (pH 9.75) in 30 nm filtered ultrapure Molecular Biology-grade water (Fisher Scientific, USA) to a final volume of 500 µL in sealed polypropylene tubes and incubating at room temperature for 1 h. 25,28,36,39 Individual aliquots of rSpSM30B/C-G and rSpSM50 stock solutions were added to the calcium solution prior to the beginning of the reaction, with final assay concentrations of each protein of 10 µM. For scanning electron microscopy (SEM) and micro-Raman analyses, mineral and protein deposits formed during all assays were captured on 5 × 5 mm Si wafer chips (Ted Pella, Inc.) that were placed at the bottoms of the vials. Upon completion of the mineralization assay period, the Si wafers were rinsed thoroughly with calcium carbonate-saturated methanol and dried overnight at room temperature prior to analyses. 26−30

### Micro-Raman Analyses.

Micro-Raman analysis of protein-deficient, rSpSM30B/C-G, rSpSM50, and 1:1 rSpSM30B/C-G−rSpSM50 mineralization assay samples (described in Experimental Section) on Si wafers were performed as described previously with a Thermoscientific DXR Raman microscope. 26−30

### Focused Ion Beam (FIB) Sectioning of Crystals.

Imaging of internal crystal morphology was performed on crystals retrieved from 1:1 rSpSM30B/C-G−rSpSM50 assays using a Zeiss AURIGA small dual-beam FIB—scanning electron microscope. Prior to imaging, all samples were initially coated with a 4 nm iridium layer and then coated with 50 nm of Au prior to performing FIB. 25,29,36,39 A 30 kV 120 pA gallium ion beam was oriented perpendicular to the sample by tilting the sample stage to 54° and subsequently utilized to mill 15 nm serial cross sections. SEM images of cross-sectioned surfaces were then obtained. 25,29,36,37 Images were taken shortly after cross-sectioning to limit the exposure of the uncoated surfaces to the electron beam. 25,29,36,37 Images of the surfaces containing electron-beam damage were created for comparison with images of undamaged surfaces.

### Calcium Potentiometric Titrations.

The quantitative potentiometric titration experiments were conducted utilizing a computer-controlled system (Metrohm), which is composed of two Titrandos devices (Titrando 809) operating three dosing devices (800 Dosinos) for CaCl2, NaOH, and HCl addition, respectively. 25,29,35−39 During the course of titration, carbonate buffer (10 mM, 20 mL) was titrated with slow addition of CaCl2 solution (10 mM) at a constant rate of 20 µL/min. Meanwhile, the pH value was kept constant at pH 8.5 by automatic counter-titration of NaOH (10 mM) and HCl (10 mM, diluted from 0.1 N), which were used to balance the ion
associations between Ca$^{2+}$ ions and carbonate species as well as the outgassing of CO$_2$, respectively. Simultaneously, a Ca(II) ion-selective electrode (Metrohm, no. 6.0508.110) and a pH electrode (Metrohm, no. 6.0256.100) were used to monitor the calcium potential and pH values, respectively, which were recorded in the system and further used for extracting quantitative information as discussed in detail elsewhere.\textsuperscript{38} The quantitative potentiometric titrations were performed in the absence and presence of rSpSM30B/C-G, rSpSM50 (50 and 500 nM), and the 1:1 molar mixture of both proteins in which 50 and 500 nM of each protein were included; hence, for the 1:1 mixture, the protein concentrations were 100 and 1000 nM, respectively.

**Micro-Computerized X-ray Tomography (CT) Studies.** For micro-CT ($\mu$CT) imaging and quantitation of mineral deposits, we performed the same in vitro mineralization assays described in the preceding section but utilized a final assay volume of 200 $\mu$L and hinged-lid sealable plastic polypropylene lab microvials.\textsuperscript{26} Subsequently, four sample scenarios were created: a control scenario (no protein was added), two protein scenarios using individual aliquots of rSpSM50 and rSpSM30B/C-G stock solutions (final protein concentrations = 1.5 $\mu$M), and a combinatorial scenario using a 1:1 molar mixture of rSpSM50 + rSpSM30B/C-G (1.5 $\mu$M each). At the end of 60 min, each mineralization assay vial was mounted to the stage and scanned using Bruker micro-CT SkyScan high resolution model 1172G (Bruker Scientific, Kontich, Belgium). The scanning process involved a round scanning trajectory and step-and-shoot protocol, with each image requiring 0.3\textdegree/step and 780 ms exposure time.\textsuperscript{26} Approximately 1300 images with a final resolution of 4.99 $\mu$m per pixel were acquired for each sample over an 80 min period; thus, the total mineralization assay elapsed time was 140 min. For each sample, the sequence of images was processed as described in our earlier $\mu$CT studies.\textsuperscript{26}

**RESULTS AND DISCUSSION**

rSpSM30B/C-G + rSpSM50 Alters Existing Crystal Growth. In earlier investigations of individual\textsuperscript{15,26,29} and combined\textsuperscript{36–39} biomineralization proteins, we used a calcite-based micromineralization assay protocol with a 60 min time interval that allows hydrogelation of the nacre proteins to take place in tandem with mineral nucleation. As shown in Figure 1, SEM imaging of mineral deposits that formed within individual rSpSM30B/C-G and rSpSM50 assays confirms the presence of calcite and reveals the typical crystal morphological features noted in past studies of the individual proteins.\textsuperscript{25,29} The rSpSM30B/C-G protein hydrogel deposits onto forming calcite crystals and promotes \{104\} faceted crystals that feature nanotextured surfaces along their lengths (Figure 1).\textsuperscript{29} Presumably, facets or flat surfaces appear because of some mineral surfaces nucleating much more slowly relative to other surfaces.\textsuperscript{29} In contrast, rSpSM50 induces the formation of highly modified rhombohedral calcite crystals that contain organized nanotexturing and evidence of significant mineralized protein hydrogel adsorption (Figure 1).\textsuperscript{25} When the two spicule matrix proteins are present as a 1:1 molar mixture, the resulting crystal morphologies are unique, with a loss of rhombohedral morphology, introduction of extensive nano-texturing, and significant mineralized protein hydrogel deposition (Figure 1, white arrows).\textsuperscript{38,39} There is also a detectable level of vaterite crystals that form in the rSpSM30B/C-G, rSpSM50, and 1:1 mineralization assays (Figure S1, Supporting Information). Because our assays do not include Mg(II), we can neither comment on the potential formation of MgC in the presence of either protein nor on the impact that Mg(II) has on protein hydrogelation, which is considerable.\textsuperscript{39} Nonetheless, the combination of the two SpSM proteins is calcite inhibitory and generates unique effects on crystal surfaces not seen with the individual proteins themselves.\textsuperscript{25,29} Given that both rSpSM30B/C-G and rSpSM50 protein hydrogels deposit onto forming calcite crystals and create numerous intracrystalline nanoporosities relative to the calcite generated in protein-free conditions,\textsuperscript{25,29} we performed FIB sectioning of calcite crystals generated in 1:1 mixture assays (Figure 2; Supporting Information, Figure S2). As expected, the hybrid hydrogels that form from both proteins are incorporated into growing crystals via repetitive hydrogel deposition,\textsuperscript{25,29,36–38} thereby creating numerous intracrystalline nanoporosities that create a “woven” appearance throughout the crystal interior, unlike the large, centrally located nanochambers created by rSpSM50\textsuperscript{25} and rSpSM30B/C-G.\textsuperscript{29} The intracrystalline incorporation of rSpSM50 + rSpSM30B/C-G correlates with the intracrystalline detection of spicule matrix proteins during spiculogenesis.\textsuperscript{7–9,16} which

![Figure 1](image1.png)  
**Figure 1.** SEM images of mineral deposits retrieved from micromineralization assays. 1:1 refers to the equimolar rSpSM30B/C-G–rSpSM50 mixture. Mineralized protein deposits are indicated by white arrows. Protein-free control image is shown as an inset to the rSpSM30B/C-G image (where scale bar = 2 $\mu$m). Note that vaterite crystals are also observed in both the rSpSM30B/C-G and rSpSM50 mineralization assays (Figure S1, Supporting Information).

![Figure 2](image2.png)  
**Figure 2.** SEM images of (A) representative Ir-coated 1:1 rSpSM30B/C-G–rSpSM50 calcite crystal and background-mineralized hydrogel deposits; (B) FIB sectioning of the same crystal, revealing the intracrystalline nanoporosities that are distributed throughout the crystal interior. Scale bar = 200 nm. Enlargement of the image in (B) can be found in the Supporting Information (Figure S2). In contrast, the calcite generated in protein (-) conditions features few, if any, intracrystalline nanoporosities.\textsuperscript{7–9,16}
induces elastic deformation to the single-crystal calcite of this skeletal element.7–9

Selective Synergistic Effects of 1:1 rSpSM30B/C-G–rSpSM50 on Nonclassical Nucleation. Having assessed the impact of the 1:1 hybrid spicule matrix protein hydrogel on existing calcium carbonate crystal growth, we next examined the influence of rSpSM50, rSpSM30B/C-G, and 1:1 rSpSM30B/C-G–rSpSM50 on the nonclassical CaCO₃ nucleation of ACC at pH 8.5, which was explored by means of quantitative potentiometric titrations (Figure 3; Table 1; Figure S3, Supporting Information).31–39 These experiments examine the formation of PNCs, the direct molecular precursors to phase-separated nanodroplets that assemble and dehydrate into ACC particles in solution upon nucleation.35 Previously, we have studied rSpSM30B/C-G and rSpSM50 separately using potentiometric methods at different pH regimes;25,29 in this current study, we revisit these two proteins again, this time at pH 8.5, using larger volumes (20 mL) to further minimize dilution effects.35,29

In the prenucleation regime, the slope of free Ca²⁺ development represents the stability of the PNCs.31,32 For rSpSM30B/C-G, rSpSM50, and 1:1 mixture titrations, there is insignificant change in the prenucleation gradient in comparison with the reference titration curve, indicating that the individual and combined proteins do not interfere with the formation equilibria of solute ion clusters at all tested concentrations (Table 1; Figures 3A and S3A, Supporting Information).31,32 This implies that rSpSM30B/C-G, rSpSM50, and the 1:1 mixture have no effect on the thermodynamic stability of PNCs.

However, a different story emerges for the nucleation timeline. The nucleation of solid calcium carbonate is signified by the steep decrease in free Ca²⁺ (Figure 3A) and ion product (Figure 3B). In the presence of 50 nM rSpSM30B/C-G, rSpSM50, and the 1:1 mixture, the nucleation event is slightly delayed, corresponding to nearly equivalent scaling factors33 of 1.15, 1.07, and 1.23, respectively (Table 1). At 500 nM, the inhibition of the nucleation event increases slightly, corresponding to scaling factors of 1.48, 1.50, and 1.61, respectively (Table 1). Thus, the combination of the two proteins is slightly inhibitory for the formation of solid mineral precursors.

We also witness a synergistic effect on ACC solubility and stability, which is highly relevant for spiculogenesis.7–9 After the nucleation of solid calcium carbonate, the amount of free calcium ions drops to a threshold, representing the solubility product of initially formed phases (Figures 3B and S3B, Supporting Information), which is typically proto-calcite ACC (pc-ACC)34 at pH 8.5. In the presence of 50 nM rSpSM50, there is no obvious change in the solubility of initially formed phases relative to the reference experiments within experimental accuracy (Figure S3B, Supporting Information). At 500 nM, the solubility of initially precipitated phases is slightly lower than the reference scenario (Figure 3B and Table 1), which is consistent with previous results.35 In contrast, the influence of rSpSM30B/C-G in promoting the formation of less soluble phases is significantly more pronounced, and based

![Figure 3](image)

**Figure 3.** (A) Amount of free Ca(II) ions and (B) ion product of calcium carbonate in the absence and presence of 500 nM rSpSM30B/C-G, rSpSM50, and the 1:1 protein mixture at pH 8.5 as a function of time. The dashed black line signifies the amount of added Ca(II) ions during titration. The curves represent the average amounts of three individual reference experiments and two protein experiments. The error bars signify ±1-σ standard deviation (see Table 1).

| sample                  | slope        | scaling factor | solubility (M)² |
|-------------------------|--------------|---------------|-----------------|
| reference               | 0.722 ± 0.006| 1.00 ± 0.01   | 3.08 × 10⁻⁸ ± 1.59 × 10⁻¹⁰ |
| rSpSM30B/C-G (50 nM)    | 0.724 ± 0.038| 1.15 ± 0.02   | 2.39 × 10⁻⁸ ± 6.49 × 10⁻¹⁰ |
| rSpSM30B/C-G (500 nM)   | 0.711 ± 0.004| 1.48 ± 0.19   | 2.31 × 10⁻⁴ ± 4.43 × 10⁻¹⁰ |
| rSpSM50 (50 nM)         | 0.728 ± 0.001| 1.07 ± 0.02   | 3.17 × 10⁻⁴ ± 3.50 × 10⁻¹⁰ |
| rSpSM50 (500 nM)        | 0.702 ± 0.013| 1.50 ± 0.01   | 2.86 × 10⁻⁴ ± 3.57 × 10⁻¹⁰ |
| 1:1 mixture (50 nM)     | 0.748 ± 0.002| 1.23 ± 0.09   | 2.20 × 10⁻⁴ ± 2.45 × 10⁻¹⁰ |
| 1:1 mixture (500 nM)    | 0.722 ± 0.007| 1.61 ± 0.05   | 2.01 × 10⁻⁴ ± 1.14 × 10⁻⁸ |

The mean values and the error, ±1 standard deviation are from N = 3 and N = 2 repetitions of the reference experiments and protein titrations, respectively. The scaling factor is the quotient of the average nucleation time in protein-containing titrations and that in the corresponding reference titration.33 The solubilities of initially formed phases are between that of monohydrocalcite30 and vaterite41 corresponding to 2.51 × 10⁻⁸ and 1.22 × 10⁻⁶, respectively.
upon the solubilities (Table 1), we are witnessing the accelerated formation of either monohydrocalcite (which may be very transient and difficult to detect experimentally) or vaterite (Figure 3B, Table 1; Figure S3B, Supporting Information), which explains why vaterite is observed in rSpSM30B/C-G assays. However, when compared to the individual proteins (50 nM or 500 nM), the solubility threshold of less soluble phases is reached even faster in the 1:1 scenario, especially at 500 nM (i.e., the gradient of the drop in the free calcium or ion product observed upon nucleation is steeper than in the other cases; Figure 3B; Figure S3B, Supporting Information). This indicates that the combination of rSpSM50 and rSpSM30B/C-G synergistically drives the formation of thermodynamically more stable crystalline phases from ACC.

To summarize, rSpSMs, either individually or in combination, do not affect the PNC equilibria at the investigated concentrations. However, rSpSM30B/C-G and the 1:1 mixture exhibit slight inhibitory effects on the nucleation event (Figure 3A), and the initially precipitated phases are thermodynamically more stable than in the reference experiments (Figure 3B and Table 1). Note also that for the 1:1 mixture titrations, the solubility threshold of more stable phases is established faster. From this, we conclude that there is synergy between rSpSM30B/C-G and rSpSM50 within the hybrid hydrogels, which accelerates the formation of crystalline CaCO₃ from ACC. We speculate that the ACC phase is kinetically destabilized by the hybrid protein hydrogels, but further experimentation would be needed to confirm this. If this mechanism is correct, then it would be highly relevant for the spicule mineralization process, given that ACC to calcite transformation is somehow triggered during spiculogenesis. This process may be facilitated by the ability of each spicule matrix protein to form hydrogels that regulate water binding, thereby controlling the hydration—dehydration events that occur with ACC prior to calcite formation.

Hybrid 1:1 rSpSM30B/C-G–rSpSM50 Hydrogel Creates a Unique Distribution of Mineral Particles. In previous studies of biomineralization protein hydrogels, we utilized µCT imaging to noninvasively detect and analyze X-ray attenuating calcium carbonate mineral particles (5 μm or greater in size) and distinguish the quantity, distribution, and organization of mineral particles within a protein hydrogel environment or protein-free scenario. The hydrogels themselves possess scarce X-ray attenuation properties, and thus because of low imaging contrast, they will not be directly detected by µCT. As shown in Figure 4, the transverse (XY-plane) and sagittal (XZ-plane) imaging planes reveal distinctive differences between both spicule matrix proteins and the protein-free control assays. First, the obvious difference is the number of mineral particles generated within the three scenarios, with rSpSM50 generating the largest number (by ∼2×) and rSpSM30B/C-G the smallest number (∼1.9×) relative to (−) protein conditions (Figure 4, top row). Second, in the sagittal views (Figure 4, bottom row), one can clearly see that both spicule matrix proteins create stratified mineralized phases at the bottom of the vials compared to the control scenario, with rSpSM50 generating the highest degree of stratification compared to either rSpSM30B/C-G or the negative control scenario. As stated in our earlier µCT studies, we believe that this stratification phenomenon is protein-specific.

Now, when we examine the µCT results obtained for the 1:1 rSpSM30B/C-G–rSpSM50 hybrid hydrogel mineralization assay (Figure 5), we find some surprising results: (1) There are fewer mineral particles generated in the 1:1 assay system compared to all other scenarios, that is, we observe a 2.2×, 5×, and 1.6× reduction compared to the control, rSpSM50, and rSpSM30B/C-G samples, respectively. (2) The distribution of mineral particles is completely different, with the 1:1 rSpSM30B/C-G–rSpSM50 hydrogel system arranging or guiding the mineral particles into discrete clusters, compartments, or domains (∼30 in number) as viewed in the transverse plane with very few examples of individual mineral particles noted outside these domains. This can also be seen in a three-dimensional tilt view of this sample (Figure S4, Supporting Information). In some locations, the mineral clusters appear linear or very dense (Figure 5, white arrows). We believe that the clustering effect is evidence of a protein-guided mineral assembly or compartmentalization process similar to what has been documented for mollusk shell nacre proteins. In the sagittal XZ plane, we note that a significant number of clustered mineral particles appear suspended above the vial bottom as if embedded within a supporting matrix. From these results, we conclude the following regarding the 1:1 rSpSM30B/C-G–rSpSM50 hybrid hydrogel mixture: (1) similar to our potentiometric findings (Figure 3), the 1:1 mixture limits the calcite crystal growth to a greater degree
Our present data reveal that this spicule matrix protein, SpSM50, which lead to the formation of moieties of the glycoprotein, SpSM30B/C-G, and the major independent interaction between the O-linked oligosaccharide genesis. Previously, we documented the high-affinity interactions between the two spicule matrix proteins enable mineral-containing protein hydrogel particles to coassemble, thereby creating a nanoporous, fracture-resistant mineralized skeletal element.36 Further in situ research will be required to establish these protein-directed processes within the spicule during development.

Perhaps the most compelling result from our study is the ability of the hybrid SpSM50–SpSM30B/C-G hydrogel to initiate guided assembly of mineral particles into organized clusters, compartments, or domains (Figure 5). As mentioned in the previous section, assembled ACC nanoparticles within the spicule matrix transform and constitute the mesocrystalline phase of calcite and these nanoparticles are coated with an organic matrix.7 In earlier scanning transmission electron microscopy flow-cell studies with the nacre-associated mollusk shell protein, we demonstrated the ability of a biomineralization protein to assemble mineral particles via hydrogel–hydrogel particle assembly.42 Hence, the SpSM protein family is a likely agent in mineral nanoparticle assembly and mesoscale organizational process, and our current study supports this hypothesis by demonstrating these capabilities in vitro (Figure 5). To some degree, this mineral particle assembly process could be accomplished by SpSM50 alone and to a minor extent, SpSM30B/C-G, as indicated by the presence of mineral layering (Figure 4)7,26 but this process becomes accentuated and more sophisticated when SpSM30B/C-G interacts noncovalently with SpSM50 (Figure 5; Figure S4, Supporting Information). At this juncture, we do not fully understand how the interactions of SpSM30B/C-G with SpSM50 facilitate mineral particle assembly and organization. On the basis of past nacre protein hydrogel particle assembly studies,42,43 it is possible that carbohydrate–protein interactions between the two spicule matrix proteins enable mineral-containing protein hydrogel particles to coassemble, thus bringing the mineral particles together to form a mesoscale crystal.42,43 Thus, the spicule matrix proteins of the sea urchin probably function in a similar fashion vis-a-vis mineral nanoparticle formation and assembly. It would be interesting to learn if other calcium carbonate–associated proteomes function in a similar fashion and we hope that future research will establish this.

Finally, we believe that our study clarifies the importance of protein–protein interactions and cooperativity in spiculogenesis. As mentioned previously, knockdown experiments established that SpSM50, and not SpSM30, is essential for spiculogenesis.15 Yet, immunolocalization experiments clearly demonstrate that SpSM30 appears in the spicule matrix during the later stages of crystal growth and obliterates the rhombohedral morphology, increases the degree of surface nanotexturing (Figure 1), and generates woven-appearance intracrystalline nanoporosities (Figure 2). During the early stages of nucleation, rSpSM30B/C-G + rSpSM50 slightly increases the time interval for ACC formation (Figure 3A, Table 1; Figure S3A, Supporting Information), thereby limiting crystal formation (Figure 5). Furthermore, the 1:1 mixture establishes the solubility threshold of more stable phases faster, and we believe that the protein mixture kinetically destabilizes ACC to rapidly form crystalline phases (Figure 3B, Table 1; Figure S3B, Supporting Information). Given that ACC exists in hydrated and dehydrated forms during spiculogenesis and transforms into calcite4–6 and that there is an organic layer detected around ACC,7 we hypothesize that hybrid SpSM50–SpSM30B/C-G hydrogels could influence ACC hydration/dehydration via hydrogel binding and release of water over time.78 Interestingly, SpSM50 alone has been reported to stabilize ACC in vitro,3 and thus, the combination of rSpSM50 + rSpSM30B/C-G alters rSpSM50 function from the stabilizer to the transformer. Once the ACC-to-calcite transformation process takes place (Figure 3), the hybrid hydrogel phases eventually become incorporated into the calcite mineral phase (Figure 2), thereby creating a nanoporous, fracture-resistant mineralized skeletal element.36–39

Figure 5. (Top row) μCT image (along z-axis) of a representative 1:1 rSpSM30B/C-G–rSpSM50 mineralization assay vial. Scale bars = 1 mm. Numbers refer to the mineral particle number quantitated in sample volume. (Bottom row) μCT XZ-sagittal plane volumetric section (4 mm × 4 mm × 1.9 mm) of the same sample. In both images, note the unique distribution of mineral particles, which is compared to the distributions as seen in Figure 4. Arrows point to representative dense mineral clusters. Scale bar = 1 mm; height of each image = 1.9 mm. Orientation axes shown.
development and is mineral-associated.16,19,20 On the basis of our research, we believe that SpSM30 isoforms do not function independently but are interactive with SpSM50 in situ and cooperatively form hydrogel-like phases with modified functionalities that enable ACC-to-calcite transformation and guided particle assembly or compartmentalization. Thus, we believe that the minor proteins of the spicule matrix, such as SpSM30,11−16 act as “helpers” or “agents” that influence or “tune” the function of the major SpSM50 spicule matrix protein and its impact on nucleation, stabilization, assembly, and mineral organization in a temporal and/or regional fashion. Obviously, in situ protein localization studies as a function of developmental time and gene expression will be necessary to establish or refute this hypothesis.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01697.

Micro-Raman spectra and corresponding light microscopy images of representative crystals generated in (−) and (+) protein mineralization assays; enlargement of SEM image of FIB-sectioned 1:1 rSpSM30B/C-G−rSpSM50 calcite crystal; profiles of the amount of free Ca(II) ions and ion product of calcium carbonate in the absence and presence of 50 nM rSpSM30B/C-G, rSpSM50, and the 1:1 protein mixture at pH 8.5 as a function of time; and 45° tilted three-dimensional CT image of the 1:1 rSPSM30B/C-G−rSpSM50 mineralization assay (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: jse1@nyu.edu (J.S.E).

ORCID
Denis Gebauer: 0000-0003-1612-051X
John Spencer Evans: 0000-0002-9565-7296

Author Contributions
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ABBREVIATIONS

SpSM, Strongylocentrotus purpuratus spicule matrix protein; SpSM30B/C-B, C isoform hybrid spicule matrix glycoprotein; SpSM50, spicule matrix protein SpSM50; rSpSM30B/C-G, recombinant insect cell-expressed SpSM30B/C; rSpSM50, recombinant SpSM50; ACC, amorphous calcium carbonate; FIB, focused ion beam; MgC, magnesium-bearing calcite; μCT, micro-computerized X-ray tomography

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