Bioinspired Silicification Reveals Structural Detail in Self-assembled Peptide Cages

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Abstract

Understanding how molecules in self-assembled soft-matter nanostructures are organized is essential for improving the design of next-generation nanomaterials. Imaging these assemblies can be challenging and usually requires processing, *e.g.* staining or embedding, which can damage or obscure features. An alternative is to use bioinspired mineralization, mimicking how certain organisms use biomolecules to template mineral formation. Previously, we have reported the design and characterization of Self-Assembled peptide caGEs (SAGEs) formed from *de novo* peptide building blocks. In SAGEs, two complementary, three-fold symmetric, peptide hubs combine to form a hexagonal lattice,

which curves and closes to form SAGE nanoparticles. As hexagons alone cannot tile onto spheres, the network must also incorporate non-hexagonal shapes. While the hexagonal ultrastructure of the SAGEs has been imaged, these defects have not been observed. Here, we show that positively charged SAGEs biotemplate a thin, protective silica coating. Electron microscopy shows that these SiO_2-SAGEs do not collapse, but maintain their 3D shape when dried. Atomic force microscopy reveals a network of hexagonal and irregular features on the SiO_2-SAGE surface. The dimensions of these (7.2 nm ± 1.4 nm across, internal angles 119.8° ± 26.1°) accord with the designed SAGE network and with coarse-grained modelling of SAGE assembly. The SiO_2-SAGEs are permeable to small molecules (<2 nm), but not to larger biomolecules (>6 nm). Thus, bioinspired silicification offers a mild technique that preserves soft-matter nanoparticles for imaging, revealing structural details <10 nm in size, whilst also maintaining desirable properties, such as permeability to small molecules.

In nature, organisms construct soft-matter nanostructures to define and stabilize the form and function of the cell. Natural self-assembled functional soft-matter architectures include: coat proteins that protect viral genetic cargo and facilitate cell penetration for replication; protein and lipid structures that are used to compartmentalize, manufacture and transport materials in bacterial and mammalian cells; and combinations of carbohydrate-based materials and lipid-protein membranes that define bacterial and plant cell walls. Biomolecular engineers take inspiration from these natural systems to design nanostructured materials that assemble from the bottom up. The self-assembling building blocks used can be based on natural, engineered or completely *de novo* biomolecules, including: peptides,[1,2] proteins,[3,4] lipids,[5] polymers,[6] and

DNA.[7] In turn, these materials have been adapted for use in a wide range of medical and biotechnological applications.[3,5,7]

Imaging the organization of bioinspired self-assembled materials at the molecular or nanoscale is essential for both confirming the targeted designs, and for advancing understanding of how to assemble and apply these materials in the future. However, imaging nanoscale biomolecular structures is challenging, as they are small, flexible, typically not very electron dense, and assembled *via* weak non-covalent interactions. Staining, embedding, coating, or crosslinking can all help to tackle these challenges and aid the visualization of soft structures.[8] Nonetheless, such processing can also introduce unwelcome artifacts, or disrupt these delicate architectures.[8] Thus simple, non-destructive processing methods that provide contrast are still required. Here we take inspiration from nature, and demonstrate the use of bioinspired mineralization to preserve the 3D structure of soft, peptide-based, self-assembled nanoparticles.

In biomineralization biomolecules direct the precipitation of minerals to produce hierarchical composite tissues.[9,10] Over 60 biominerals occur in natural biological systems,[11] including: calcium carbonate,[12] calcium phosphate,[13,14] magnetite,[15] and silica.[16–19] For example, diatoms are unicellular eukaryotic algae that construct intricate silicified cell walls, called frustules, using biomolecules such as long-chain polyamines,[20] and proteins such as arginine- and lysine-rich silaffins,[21,22] cingulins,[23] and phosphorylated silicidins.[24] These biomolecules are thought to direct silica to precipitate from soluble silicic acid *in vivo*.[21,22] Silicification has been studied *in vitro* using positively charged bio-inspired molecules, such as lysine-rich polypeptides[22,25] and other polyamines.[26] The size of the biotemplated silica structures usually correlates with their pre-silicified size in solution.[27] As such, silica coating has been used to preserve soft-matter nanostructures faithfully.[27–31] In pioneering work from the Brinker Lab, this has been extended to

coat cells[32,33] and even whole organisms.[34] This allowed features to be clearly imaged in 3D down to ≈10 nm.[31] Permeable biomineral coatings have also been used to protect soft-matter structures for use in medicine[35,36] and catalysis.[37,38] In these ways, bioinspired silicification is able to provide contrast for nanoscale imaging and stabilize the function of soft-matter structures.

Protein cages are specifically addressable soft-matter nanostructures with potential biomedical applications in drug targeting and delivery,[39–42] vaccination,[4,43] and in biotechnology as nanoreactors.[44–46] An example of these is the Self-Assembled peptide caGEs (SAGEs, Figure 1).[1] The SAGEs are rationally designed[1] from fully characterized *de novo* peptide components.[47] Specifically, a parallel α-helical coiled-coil peptide homotrimer, CC-Tri3, is linked *via* a disulfide bond and back-to-back to one of two obligate heterodimers, CC-Di-A or CC-Di-B,[1] making two complementary hubs (hub A and hub B) each with 3-fold symmetry. When these hubs are mixed, the heterodimers are formed, and these drive the self-assembly of what is intended to be a hexagonal network. Surprisingly, when imaged by electron microscopy (EM) and using in-liquid atomic force microscopy (AFM) closed, spherical particles are observed. This is rationalized by *in silico* molecular-dynamics simulations of small patches of the SAGE network, which consistently indicate that these multi-peptide structures curve in one direction.[1] However, hexagons alone cannot tile on a sphere and are unable to form closed, convex, regular Platonic solids.[48] C_{60} (Buckminsterfullerene)[49] combines 20 hexagons with 12 pentagons to form a closed Archimedean solid structure.[48] Thus, the SAGE network must incorporate irregularities to form spherical nanoparticles. It is not known if these consist of holes in the lattice, or if non-hexagonal or irregular polygons close the structures. To date, such features on the SAGE surface have not been observed. Therefore, we sought a method that could reveal how the peptides are organized in SAGE particles.

Here we describe the application of bioinspired silicification to form a stabilizing permeable coating on the SAGEs. We show that this allows detailed imaging of their surfaces. AFM of the silica-coated SAGEs (SiO_2-SAGEs) reveals a hexagonal network as designed. Moreover, with ≈7 nm vertex-to-vertex lengths, the size of these polygons is fully consistent with the designed underlying peptide network. The network predominantly comprises regular hexagons, but other shapes, which are required to form a closed object from a hexagonal lattice, are also apparent. This concurs with coarse-grained computational modelling that we have recently performed.[50] As the silicified SAGEs can be imaged clearly using standard EM and AFM techniques, bioinspired silicification offers a facile technique for preserving soft-matter nanostructures in 3D so that their self-organization can be studied. In addition, silicification offers a route to stabilizing soft, self-assembled materials for use in bionanotechnology and synthetic biology.[35,36,51]

Results and Discussion

The *N* and *C* termini of the SAGE Homotrimer can be Decorated with Positive Charge

Molecular dynamics (MD) simulations of a hexagonal peptide network designed for the 'parent' SAGE indicate that the arrays curve.[1] In the MD, the long axes of the trimeric coiled-coil units remain perpendicular to the plane of the array, whereas the dimeric components rotate freely about the two disulfide bonds. Moreover, the direction of curvature is consistent across the simulations, with the *N* termini of the homotrimers presented on the convex (*i.e.* external) face of the SAGEs, while the *C* termini are on the concave interior. This is our working model for SAGE structure and it provides a basis for further design.

For this study, tetra-arginine (R4) or tetra-lysine (K4) peptides were appended to either the *N* or *C* terminus of the homotrimer (CC-Tri3, Figure 1, Table 1 & S1 – S3). The aim being that, when assembled into SAGEs, these constructs would present positively charged residues on the external or internal surfaces of SAGEs for *N*-terminal or *C*-terminal appendages, respectively. In turn, these should act as templates to localize and precipitate silica from aqueous silicic acid onto the peptide cages. In this way, we aimed to create SiO_2-SAGE particles. As controls, negatively charged tetra-glutamate (E4) variants were also generated, which should be poor at biotemplating silica.[25]

Table 1. Summary of the main peptide sequences used to assemble decorated SAGEs for silicification. Systematic names of the trimer components used to make the hubs, their amino-acid sequences, and their overall charge as calculated using pepcalc.[52] See supplementary information for further details of the naming system (Table S1), mixing peptide hubs to form SAGEs (Table S2), and the full amino-acid sequences for all peptides used in this study (Table S3).

trimer name	sequence	net charge at pH 7
CC-Tri3	Ac-GEIAAIKKEIAAIKCEIAAIKQGYG-Am	0.9
E4-CC-Tri3	Ac-EEEEGGGEIAAIKKEIAAIKCEIAAIKQGYG-Am	-3.1
CC-Tri3-E4	Ac-GEIAAIKKEIAAIKCEIAAIKQGYGGGEEEE-Am	-3.1
K4-CC-Tri3	Ac-KKKKGGGEIAAIKKEIAAIKCEIAAIKQGYG-Am	4.9
CC-Tri3-K4	Ac-GEIAAIKKEIAAIKCEIAAIKQGYGGGKKKK-Am	4.9
R4-CC-Tri3	Ac-RRRRGGGEIAAIKKEIAAIKCEIAAIKQGYG-Am	4.9
CC-Tri3-R4	Ac-GEIAAIKKEIAAIKCEIAAIKQGYGGGRRRR-Am	4.9

Peptides were synthesized, purified and assembled into SAGE peptide hubs (Figure S1, Table 1 & S1 – S4 for sequences, naming and characterization). For the *C*-terminally decorated trimers,

CC-Tri3-K4 and CC-Tri3-R4, circular dichroism (CD) spectra gave α helicities similar to the undecorated parent homotrimer (CC-Tri3), and as predicted based on the peptide sequences (Figure S2 & S3 and Table S5). Moreover, these three constructs had very similar thermal unfolding transitions, with midpoints (T_Ms) of 54 °C, 57 °C, and 56 °C, respectively (Figure S2 and Table S5). By contrast, appending positive charge to the *N* termini reduced the α helicities and also the T_M values to 33 °C and 34 °C for the K4 and R4 variants, respectively (Figure S2 & S3, and Table S5). CC-Tri3 variants with negatively charged E4 blocks at the *N* and *C* termini showed the opposite behavior: the former was as folded and stable as the parent (T_M = 65.5 °C), whereas the latter was as destabilized (T_M = 31 °C) as the *N*-terminal K4 and R4 variants, Figure S2 & S3, and Table S5. These changes in stability of CC-Tri3 when charge is appended mirror a recent study of free-standing α-helical peptides.[53] They can be explained in terms of repulsive interactions between the blocks and the partial charges associated with the helical backbone, which are positive and negative for the *N* and *C* termini, respectively.[53] Analytical ultracentrifugation (AUC) sedimentation velocity (SV) and sedimentation equilibrium (SE) experiments conducted at room temperature with the parent and decorated homotrimers returned molecular weights consistent with trimeric coiled-coil assemblies for each construct in solution, Figure S4, Table S6.

Together, these data for the modified CC-Tri3 peptides show that they are stably folded and trimeric at room temperature, albeit with some of the thermal stabilities compromised. Therefore, we continued with these variants, using them to construct decorated hubs for the assembly of modified SAGE particles.

Positive Charge Localizes Silica Biomineralization onto SAGEs

Herein, we refer to the unmodified parent assemblies as SAGE, Figure 2a, and the SAGE variants as follows: E4-SAGE, K4-SAGE and R4-SAGE for the *N*-terminally decorated particles, Figure 2e; and SAGE-E4, SAGE-K4 and SAGE–R4 for the *C*-terminally decorated structures, Figure 2i. Acidification of sodium silicate forms a mixture of metastable ortho- and oligosilicic acids.[54] For simplicity, we refer to this as 'silicic acid'. Polymerization of these mixtures were monitored by fluorescence of 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)methoxy)phenyl)oxazole (PDMPO) without and with the various SAGE particles (Figure S5). The PDMPO indicated that the different SAGEs did not appear to alter the amount or rate of bulk silica precipitation. The silicification of SAGEs to form discrete biomineralized particles was optimized to form electron dense particles (Figure 2 & S6).

As controls, silicification of the peptide hubs alone—*i.e.*, negatively charged hub A, or positively charged hub B—formed mesh networks with poor contrast, Figure S7; and similar precipitates were observed with short positively charged peptides, GYGKKKK and GYGRRRR, Figure S8. By comparison, with both hub A and hub B present—*i.e.*, with SAGEs formed—silica particles were observed, and these were particularly clear with excellent contrast for the K4- and R4-decorated SAGEs, Figures 2, S6 & S7.

In more detail, the *C*-terminal tetra-arginine decoration, SAGE-R4, gave large, slightly interconnected, and polydispersed spheres (305 nm ± 120 nm). The tetra-lysine variants, K4-SAGE and SAGE-K4, assembled into an interconnected mesh and smaller, slightly interconnected spheres (Figure 2g & k and S6e & h). The most monodisperse and unconnected nanospheres were formed by *N*-terminal tetra-arginine decorated R4-SAGEs, Figure 2h & S6f. These were 213 nm ± 84 nm in diameter, Table S7, Figure 2h, S6h & S9. This is a significant increase in size and decrease of the distribution compared with unmineralized R4-SAGEs

(109 nm ± 84 nm), which were difficult to measure as they gave poor contrast (Figure S10). Similarly, the silicified R4-SAGEs gave better quality scanning electron microscope (SEM) images, revealing clearer particles when compared to unmineralized R4-SAGEs (Figure S11). Energy dispersive X-ray (EDX) spectra confirmed the presence of silicon (Si Kα = 1.74 keV) and oxygen (O Kα = 0.53 keV) in the SiO_2-R4-SAGEs (Figure S12). The absence of any coherent electron diffraction patterns, however, indicated that the silica coatings are likely amorphous rather than a crystalline polymorph of silica.

In summary to this section, a combination of TEM, SEM and EDX demonstrates that SAGEs can be modified with cationic tetra-peptides to generate discrete, spherical, silicified nanoparticles, which we refer to as SiO_2-SAGEs. The best of these preparations were made from R4-SAGEs, which we used next to optimize conditions for preparing SiO_2-SAGEs.

The Formation of Discrete SiO_2-SAGEs can be Optimized

R4-SAGEs were mineralized with a range of silicic acid concentrations (0 mM – 36 mM), imaged (Figure S13), and grainsized (Table S7). Without silicic acid, or at low concentrations (6 mM), it was difficult to discern structures by TEM, and the grains were polydisperse. At 36 mM silicic acid, large interconnected SiO_2-R4-SAGE particles were clear (217 nm ± 58 nm). We propose that high silicic acid concentrations deposit a thicker layer of SiO_2, leading to particle aggregation. Optimal mineralization occurred at 12 mM – 24 mM silicic acid, leading to discrete SiO_2-R4-SAGE spheres with sizes distributed around ≈200 nm in diameter (Figure 2h).

Next, we varied the peptide concentration between 2 μM – 25 μM peptide for K4 and R4 SAGE variants, and mineralized these with 24 mM silicic acid at 20 °C (Figure S14). At low peptide concentrations (2 μM – 5 μM), the K4- and R4-SAGE constructs formed discrete mineralized spheres similar to those noted above. At the higher peptide concentrations (10 μM –

25 μM), much larger micron-sized mesh and network structures were observed, which is consistent with our observations that unmineralized SAGEs aggregate at high μM concentrations.

The SAGE system is modular, which means that the hub modules can be mixed and matched to tune the SAGE properties. Thus far, both hub A and hub B carried the E4, K4 or R4 decorations, *i.e.* they were 100% decorated. To vary this, first we prepared SAGEs with undecorated parent hub A plus hub B fully decorated with K4 or R4 (Figure S15). These were silicified using standard conditions of 2 μM peptide, 24 mM silicic acid, 10 mM phosphate (PI) buffer pH 7.4, 20 °C, 24 hours. Compared with 100% decoration, these 50% decorated SAGEs form smaller particles, particularly for the R4 variants. R4-SAGEs displaying between 50%-100% tetra-arginine were prepared by combining R4-hub B and mixtures of hub A with and without the R4 decoration (Figure S16). The less highly charged SAGEs, *i.e.* 50% - 80% R4-SAGE, formed smaller interconnected mineralized silica particles, with average diameters of 60 nm – 90 nm, Table S8; whereas the 90% and 100% R4-SAGEs formed the larger but nonetheless discrete structures noted above.

100% R4-SAGEs were also assembled and silicified at a range of temperatures (4 °C – 37 °C). This range should not significantly perturb the silicification process,[55] but, as the midpoint unfolding temperature (T_M) of CC-R4-Tri3 is 34 °C, this range could affect homotrimer folding, and thus SAGE assembly (Figure S17). At 4 °C, the mineralized particles were small (77 nm ± 26 nm) interconnected spheres. At 20 °C, the particles were larger (213 nm ± 84 nm) and discrete, and were larger still (330 nm ± 168 nm) and clumped together at 25 °C. At 37 °C, smaller (105 nm ± 42 nm) interconnected spheres were formed. Thus, the optimal temperature for assembling discrete mineralized R4-SAGEs is at ≈20 °C, where the CC-R4-Tri3 should be

well folded, but there is enough thermal energy to avoid forming the small, interconnected structures assembled at lower temperatures.

Finally, the components for 100% R4-SAGEs were mixed at between pH 5.5 – 8.0, and the products silicified at the assembly pH. At low pH, silica formation is known to be slowed, but the 24-hour mineralization used should be ample for silica formation.[55] TEM images (Figure S18) reveal that silicification at neutral pH (7.0 and 7.4) led to discrete spherical particles; whereas at higher pH values (7.5 – 8.0), interconnected SAGEs resembling those formed at higher (36 mM) silicic acid concentrations were formed, which we attribute to rapid and more complete precipitation of silica. At slightly lower pH values (6.5 and 6.0) spheres were discernible, but these were embedded in meshes. Finally, at pH 5.5 only thin sheets are visible, which we ascribe to the precipitation of silica by largely unfolded aggregated peptide.

Thus, we find that for R4-SAGE particles, which provide the best substrates for silicification, there are optimal conditions for mineralization to produce SiO_2-R4-SAGEs. These are summarized in Figure 3, and comprise: 2 – 5 μM peptide, 90 – 100% R4-decorated hubs, 20 °C, pH 7.0 – 7.4 PI buffer, mineralized with 12 – 24 mM silicic acid for 24 hours.

The 3D Structure of SiO_2-SAGEs can be Visualized by EM Tomography and AFM

We recorded TEM tilt series for SiO_2-R4-SAGEs and used these to construct tomograms and 3D models of the mineralized particles (Movie S1 – S4, Figure S19). The tomograms show that the particles did not collapse significantly when dried onto TEM grids; *i.e.*, dried SiO_2-R4-SAGEs retained their 3D structure. SiO_2-R4-SAGEs were also imaged by tapping-mode atomic force microscopy (TM-AFM) and PeakForce AFM (PF-AFM), Figure S20. AFM measurements of unmineralized SAGEs show significant collapse of the peptide particles upon drying to an aspect ratio (AR) between the height z and the diameter in x and y of ≈0.1.[1] By

contrast, the dried SiO_2-R4-SAGEs had an aspect ratio of ≈0.7 in AFM. Thus, the silica coating reinforces SiO_2-R4-SAGEs against collapse.

We were interested to see if silicification also preserved the structure of the peptide building blocks and pores between the hub subunits anticipated from the original design, Figure S21. PF-AFM was used to map variations in the height, adhesion and dissipation properties of the surface of a cluster of SiO_2-R4-SAGEs (Figure 4). Despite variations in the topography due to the near-spherical shape of the SiO_2-R4-SAGEs, the surfaces of the nanoparticles could be imaged. This revealed a polygonal lattice texture on the surface of all the SiO_2-R4-SAGEs imaged (Figure S22).

Interrogation of these patterns on individual SiO_2-R4-SAGEs with PF-AFM identified polygons with vertex-to-vertex spacings of 7.2 nm ± 1.4 nm (Figure 5). This is entirely consistent with the SAGE design.[1] Moreover, these features were apparent in the height, adhesion and dissipation plots, with regions of increased height and adhesion co-localized, Figure 5e, h & i. The network was also seen in phase contrast in TM-AFM (Figure S23f & i). Ridges, created by the peptide framework within the biotemplated silica, should present as raised lattice structures on the curved SAGE surface with increased height, nanoscale roughness and flexibility when compared to a silica-filled pore (Figure S21). This was indeed observed in PF-AFM, with raised ridges corresponding to higher adhesion (roughness) and dissipation (flexibility) when compared to the pore centers, and confirmed by positive phase shifts on ridges in TM-AFM.

Interestingly, the AFM data also revealed variations in the originally intended hexagonal network (Figure 5j & l). To help quantify the extent of these variations, a model for a smooth non-symmetric convex surface was fitted to the height data to flatten the dome shape of the

particle (Figure S24b). This generated a topology map of the particle, highlighting variance due to surface features (*i.e.* pores and ridges). The internal angles of the polygonal structures from unflattened images were distributed around 120° (119.4° ± 26.9°, Figure S24d). Nonetheless, other angles—*e.g.* for pentagons (108.0°), heptagons (128.6°) and irregular polygons—also fell comfortably within the distribution.

The internal angles measured from coarse-grained modelling of SAGE assembly (see Mosayebi *et al.*[50] and Figure S24c & d) showed a major peak centered on hexagonal polygons (122.0° ± 25.0°), which agrees well with the experimental data. However, there was also a small peak for squares at 89.6° ± 8.6°, which is not observed experimentally. The squares are likely to be due to the simulated network, which comprises obligate heteromeric pairings of pure hubs (*i.e.*, hub A and hub-B only), not being able to access odd-sided polygons, *e.g.* pentagons, to close the SAGEs. In the experimental system, however, non-hexagonal polygons could form by non-cognate peptide interactions (*i.e.*, CC-Di-B to CC-Di-B, or CC-Di-A to CC-Di-A), especially as hubs of same type are brought into close proximity during assembly. Alternatively, as the peptide hubs are inherently dynamic, the homotrimers may exchange to form mixed hubs, which would also increase the chances of incorporating non-hexagonal structures. The current simulations do not allow for subunit exchange in hubs, and penalize homomerization of the heterodimer components, both of which would facilitate the formation of the odd-sided polygons by SAGE hubs during assembly. This will be corrected in future coarse-grained models.

These observations indicate how the peptide lattice adapts to close, and leads us to refine our initial hypothesis for SAGE assembly and structure, which centered on the formation and propagation of hexagonal peptide arrays, Figure 1.[1] The inclusion of non-hexagonal and irregular shapes, as revealed herein using the SiO_2-SAGE system and supported by coarse-grained

computational modelling of SAGE assembly (*vide infra*),[50] presents a clear mechanism for closure. The variations that we see are not evenly spaced on the surface (Figure 5f – m, S24) as required in Archimedean solids,[48] so the SAGE peptides self-assemble to form more complex, irregular polyhedra.

SiO_2-SAGEs are Permeable to Small Molecules and Peptides, but not to Proteins

Permeable biomineral coatings have been used to protect enzymes,[37,38] drug cargos,[56] and vaccines.[35,36,51] With a view to using SiO_2-SAGEs in these contexts, we tested their permeability to small molecules and proteins. To do this, we set up a reporter system, which uses the modularity of SAGE system to introduce a fluorophore into the peptide fabric of the SAGEs, Figure 6a. A R4-SAGE preparation was doped with 2.5% of hub B harboring carboxyfluorescein (hub B-fl). When silicified, these R4-SAGE-fls formed particles indistinguishable from R4-SAGEs by TEM, Figure 6b. Moreover, the particles were fluorescent, and correlative light and electron microscopy (CLEM)[57,58] showed that this fluorescence co-localized with the particles observed by TEM, Figure 6c & d, and S25. Consistent with this, prior to silicification R4-SAGE-fls were difficult to visualize by TEM, but fluorescent particles were clear by light microscopy (LM), Figure S26. We found that higher concentrations of silicic acid (36 mM) reduced detection of the fluorescence signal, and, therefore, used intermediate silicic acid concentrations (12 – 24 mM) to form thinner silica coatings.

To monitor small-molecule ingress into SiO_2-R4-SAGE-fls, we used the fluorescence quencher Trypan blue, which must contact the fluorophore to quench it.[59,60] Trypan blue is small (≈1.5 x 0.5 x 0.5 nm), and was able to quench the fluorescence of both R4-SAGE-fls and SiO_2-R4-SAGE-fls, Figure 6f, j & n. The SAGE structures were still visible in bright-field LM

images (Figure S26), indicating that Trypan blue can traverse the silica coating to effect quenching.

For delivery applications, small-molecule cargos must be able to exit the host vehicle. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) reduces the disulfide bonds used to construct the SAGE hubs, and so disassembles SAGE particles.[1] TCEP treatment reduced the number and intensity of fluorescent SiO_2-R4-SAGE-fls particles, Figure 6g, k & o, consistent with disruption of SAGEs and the release of peptides from the biomineralized structures (CC-Tri3 is ≈3.0 x 1.5 x 1.5 nm when folded). Nonetheless, particles were still visible by bright-field LM and TEM after this treatment, Figure S26 – S27, demonstrating that the biomineral replicas remain intact after TCEP treatment to remove the peptides.

Finally, to test if larger proteins penetrated the silica barrier, R4-SAGE-fls and SiO_2-R4-SAGE-fls were treated with the protease trypsin. For the former, non-mineralized particles fluorescent puncta were lost, Figure 6h, indicating proteolysis of the SAGE peptides and release of the fluorophores. However, with the SiO_2-R4-SAGE-fls these objects remained visible indicating that the silica coat protected the peptides from proteolysis by trypsin, Figure 6l & p. The trypsin may be too large (≈7.0 x 6.0 x 5.5 nm) to penetrate the silicified SAGEs, or may adhere to the silica coat becasue it has a pI of ≈10. However, as the pI of CC-Tri3 (≈9) and trypsin are similar and the smaller CC-Tri3-fl does appear to traverse the silica coating, it is unlikely that charge alone is responsible for preventing proteolysis of the SiO_2-SAGEs by trypsin. Together, these data indicate that SiO_2-SAGEs are permeable to small molecules (<2 nm across), including fluorescence quenchers, reducing agents and the peptide building blocks of the SAGEs themselves, but not to larger (≈6 nm across) protein molecules such as trypsin.

Conclusions and Future Directions

Herein we demonstrate that SAGE particles can be decorated with positive charge, assembled and controllably silicified under mild conditions in aqueous buffer. We find that an *N*-terminal tetra-arginine tag—to give R4-SAGEs—forms discrete silica-coated particles. We have tuned the particle size between ≈50 – 200 nm by controlling the proportion of tetra-arginine in the SAGE particles; and by optimizing silicic acid concentration, and also the conditions for SAGE assembly (peptide concentration, temperature and pH). We envisage this ability to control particle size and functionalization could allow silicified SAGE particles to be tailored for use in a range of biotechnological applications. We find that SiO_2-SAGEs particles do not form below pH 6.0, and that they aggregate above pH 7.5. This suggests that SAGE particles assembled at or near neutral pH could then be disassembled under acidic conditions. Such pH-triggered disassembly could be used to release encapsulated cargos controllably within target cells, as acidification during endosomal trafficking should lead to disassembly and thus delivery.[61]

SiO_2-SAGEs maintain their 3D structure when dried, which facilitates imaging of the underlying polygonal peptide network. The network shows predominantly hexagonal shapes ≈7 nm across, consistent with our original design and *in silico* models.[1,50] However, and interestingly, it also reveals the presence of non-hexagonal and irregular polygons with internal angles distributed evenly about the hexagonal 120°. These are necessary to close the network, as hexagons alone cannot tile on a sphere. These experimental measurements largely concur with a coarse-grained computational model for SAGE assembly that we are developing,[50] which shows the dominance of a hexagonal arrangement of hubs, and highlights the importance of non-hexagonal polygons to achieve closure. However, the simulations suggest that square arrangements of hubs should be more abundant than we observe experimentally. This current

model disfavors mechanisms that can access odd-sided polygons in the lattice, *e.g.* homotypic association of the two heterodimer components or exchange within the homotrimer core to form mixed hubs. The AFM data show that peptide network within SiO_2-R4-SAGEs is able to form internal angles consistent with odd-sided polygons rather than closing using squares. In turn, this indicates that defects formation *via* one of the above two or other mechanisms must be in operation during SAGE assembly. These new concepts and the experimental data from the SiO_2-SAGEs are being incorporated into a revised coarse-grained model to explore the formation of odd- and even-sided hub assemblies.

As the SAGEs are closed by small shape defects rather than by large holes in the lattice, any larger cargos contained within SAGEs should not leak out. This bodes well for using them to support active proteins and enzymes;[45,46] for drug encapsulation, targeting and delivery;[42,62] and as antigen presentation vaccine platforms.[3,4] Biomineral coatings are currently used to protect drug cargos,[56,63] vaccines[36,51] and enzymes[37,64] by stabilizing biomolecules against thermal and other degradation. Thus, bioinspired silicification of SAGEs functionalized for use in medical or biotechnological applications could afford similar protection to these particles. SAGEs could also be designed to template other materials.[65,66] For example, SAGEs displaying antigens could be coated with adjuvantive[51] and/or protective[36] calcium phosphate to create stabilized vaccine formulations with improved efficacies and shelf-lives. SAGEs displaying ligands for cell-surface receptors and disease treatment could also be decorated with gold nanoparticles to combine targeting, imaging and treatment of diseased cells;[67] or SAGEs coated with platinum nanoparticles could evolve hydrogen more effectively than disordered Pt nanoparticles.[68] The SAGE system has clear attributes to facilitate such applications, including its modularity, our ability to redesign it, and the ease with which the SAGE components can be functionalized.

Therefore, by mixing and matching appropriately modified hub components, there is considerable potential to develop a range of tailored multifunctional organic, inorganic, and/or biologically decorated SAGEs for future applications.

Materials and Methods

For extended materials and methods, see the Supporting Information.

Peptides were synthesized using Fmoc solid-phase synthesis[69] on Rink amide resin in a Liberty™ microwave peptide synthesizer on 0.1 mM scales (see Tables S1 – S3). Each amino acid was coupled (5 eq. of Fmoc amino acid, 4.5 eq. of hydroxybenzotriazole (HOBt), 10 eq. of *N*,*N*'-diisopropylcarbodiimide (DIC) in 7 mL dimethylforamide (DMF) with 25 W microwave irradiation at 50 °C for 5 minutes), washed (5 x 7 mL DMF), deprotected (20% (v/v) morpholine in DMF, with 20 W microwave irradiation at 75 °C for 5 minutes), and then washed before the next amino acid was added. Assembled peptides were acetylated on resin (3 eq. acetic anhydride, 4.5 eq. of N,N-diisopropylethlamine (DIPEA) in 7 mL DMF for 30 minutes). Peptides were cleaved from the resin with 94% (v/v) trifluoroacetic acid (TFA), 2% (v/v) 1,2-ethanedithiol (EDT), 2% (v/v) water and 2% (v/v) triisopropylsilane (TIPS) for 3 hours. Peptides were filtered, precipitated using diethyl ether (Et_2O), pelleted (4000 xg, 10 minutes, 4 °C), and freeze dried.

Peptides were purified using reverse phase high pressure liquid chromatography (RP-HPLC) on a Kromatek (semi-micro, 5 µm, 100 Å, 10 mm ID x 150 mm L) C18 column, using a linear gradient of buffer A (0.1% (v/v) TFA in water) and 20% - 80% buffer B (0.1% (v/v) TFA in MeCN). Purified peptide peaks were analyzed by matrix-assisted laser desorption / ionization – time of flight mass spectrometry (MALDI-TOF) and analytical RP-HPLC. For 2,2'-dipyridyldisulfide (DPDS) thiol activation[70] of heterodimer A or B, ≈5 mg of heterodimer was

dissolved in 5 mL of water or phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4). 10 eq. DPDS in 1 mL methanol (MeOH) was added. After 1 hour, excess DPDS was removed using 3 x 30 mL Et_2O, and the activated peptide freeze dried, purified using RP-HPLC, characterized (MALDI-TOF and analytical RP-HPLC), and freeze dried. To form hubs, each homotrimer was mixed with an activated heterodimer in equimolar amounts in PBS (≈1 mg mL^{-1} peptide), and mixed for 3 hours at 20 ºC. Hubs were purified by RP-HPLC, and characterized (MALDI-TOF and analytical RP-HPLC, Figure S1 & Table S4).

Circular dichroism (CD) spectra were recorded at between 5 °C – 90 °C using a JASCO J-810 spectropolarimeter fitted with a Peltier temperature controller. A 1 mm path length quartz cuvette containing 50 μM peptide in phosphate buffer (PI, 10 mM potassium phosphate (8.02 mM dibasic K_2HPO_4, 1.98 mM monobasic KH_2PO_4) pH 7.4) and 250 μM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was heated at 40 °C $hour^{-1}$. The Mean Residue Ellipticity (MRE) at 222 nm was recorded every 1 °C, and full CD spectra (190 nm – 260 nm) were recorded every 5 °C. Analytical ultracentrifugation (AUC) was performed on each homotrimer (325 μM peptide in PI, 1.625 mM TCEP and 25 mM NaCl). Sedimentation velocity (SV) and sedimentation equilibrium (SE) experiments were conducted using Beckman Optima XL-A and XL-I analytical ultracentrifuges with An-60 and An-50 rotors, respectively, and at 20 °C. For SV, samples were spun at 60 krpm with 120 scans collected between 5.8 cm – 7.3 cm and data fitted using SEDFIT.[71] For SE, data were collected at 6 rotor speeds between 30 krpm – 48 krpm, and fitted with a single ideal species model using Ultrascan II.[72] The partial specific volumes ($\bar{v}$) for each peptide, buffer densities and viscosities were calculated using SEDNTERP[73] (Table S6).

Typically for silicification, SAGEs were assembled (2 μM hub A + 2 μM hub B) in PI buffer for 1 hour at 20 ºC, then 500 μL of these samples were mixed with 500 μL PI buffer containing 0 mM – 36 mM freshly prepared silicic acid,[74] and washed 3x with water after 24 hours. 0.5 μL 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)methoxy)phenyl)oxazole (PDMPO, LysoTracker®)[75] was added to 100 μL of mineralization solution to monitor the polymerization of silica in solution by fluorescence emission.

For scanning electron microscopy (SEM), samples were dried on silicon wafers fixed to an aluminum stub. Unmineralized samples were coated with ≈5 nm Au-Pd using an Emtech 575X sputter coater. Stubs were imaged in a FEI Quanta FEG-SEM. Samples were dried on carbon coated copper grids and imaged on a Tecnai 12 – FEI 120 kV BioTwin Spirit transmission electron microscope (TEM). Tilt series between angles of ≈70° and ≈-70° for tomographic reconstructions were collected using a Tecnai 20 – FEI 200 kV Twin Lens Scanning Transmission Electron Microscope (STEM). Images were collected using an FEI Eagle 4k x 4k CCD camera. Grainsizes of SiO_2-SAGEs were measured from representative TEM images using ImageJ,[76,77] and distributions fitted in Origin 2015 64 bit (Equation S3). Tilt series were aligned and reconstructed using eTomo in the IMOD[78,79] software package (University of Colorado, Boulder). 3D reconstructions of tomograms were rendered in Amira 3D (FEI Software). Movie files of the tomographic reconstruction software outputs were compiled in ImageJ[76] and compressed using HandBrake. Energy dispersive X-ray (EDX) spectra were recorded using a JEOL 2100F STEM and a Gatan Orius 11 megapixel 832 camera. X-rays were detected using an 80 mm^2 AZtec detector, and processed using AZtecTEM software. For correlative light and electron microscopy (CLEM),[57,58] fluorescent R4-SAGE-fls were imaged first using a Leica

DMI4000 B inverted epifluorescence microscope, then using a TEM (see above). Fluorescence was overlain with TEM images using the TurboReg plug-in in Fiji.[80]

Tapping Mode Atomic Force Microscopy (TM-AFM) was performed on a Multi-mode microscope with a Quadrexed Nanoscope III controller using Scout-Beta cantilevers (NuNano, Bristol, UK). PeakForce AFM (PF-AFM) was conducted on a Multi-mode VIII microscope with Nanoscope V controller and a fast scan head unit in combination with SCANASYST-AIR-HR cantilevers (Bruker, CA, USA). Data was analyzed using Nanoscope Analysis 1.5 software (Bruker, CA, USA) and scripts written in Matlab (Mathworks). Design and parameterization of the coarse-grained computational modelling is described in Mosayabi *et al.*[50]

The permeability of particles to small molecules was tested using silica-coated and unsilicified fluorescent R4-SAGE-fls. 1 mL of washed silicified SAGE was pelleted (17,000 xg, 5 minutes, 20 °C) and resuspended in 100 µL PI buffer, and 10 µL added to 30 µL PI buffer. As a control, 40 µL of unmineralized fluorescent SAGE was used. Samples were made up to a total volume of 50 µL by PI buffer (control, no additive), or PI buffer plus a fluorescence quenching molecule (10 µL 0.4% (w/v) Trypan blue, 2 hours at 20 °C), a reducing agent (10 µL 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 2 hours at 20 °C), or a protease (trypsin, 5 µL 1 mg mL^{-1}, 2 hours at 37 °C). Samples were transferred to slides and imaged on a fluorescence microscope (detailed above).

Associated Content

The authors declare no competing financial interests.

The following files in support of this manuscript are available free of charge on the ACS Publications website (http://pubs.acs.org/) at DOI XXXX:

SiO2-SAGE_SI.pdf contains: Extended methods, supplementary notes, figures and tables that include: analytical RP-HPLC, MALDI-TOF, AUC data, CD data, PDMPO fluorescence spectra, TEM images, grainsizing, SEM images, AFM plots, CLEM images, bright-field and fluorescence LM images, and diagrams.

Tomograms and reconstruction movies: tilt series 29,000x magnification - S1 29kx tomo.avi; and at 50,000x magnification - S2 50kx tomo.avi. 3D reconstructions of the 50,000 x magnification series rotating equatorially - S3 50kx 3D1.avi, and polar - S4 50kx 3D2.avi.

Author Contributions

JMG, JMF, PC and DNW conceived the project, designed the experiments and drafted the manuscript. JMG and LS performed the mineralization experiments, and JMG recorded TEM and SEM images and reconstructed tomograms with JMM, JC and LRH. JMG performed grainsizing. JMG and JLB synthesized the peptides, conducted the solution-phase biophysics and fluorescence microscopy. JMG, JLB and LRH recorded fluorescence and TEM images for CLEM with PV and JC. JMG and RLH recorded the AFM data and analyzed them with W-FX. JMG and GGR recorded and analyzed the AUC data. MM, NL and TBL performed the coarse-grained computational modelling. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Figure Legends

Figure 1. Schematic for Self-Assembled peptide caGE (SAGE)[1] design and silicification. A homotrimerizing coiled-coil peptide (CC-Tri3, green) is joined back-to-back *via* a disulfide bond to one of two heterodimeric sequences, namely a negatively charged CC-Di-A peptide (red) or a positively charged CC-Di-B peptide (blue). These give hub A (CC-Tri3-CC-Di-A) and hub B (CC-Tri3-CC-Di-B), respectively. When mixed, the hubs are posited to form a regular hexagonal array that closes to form SAGEs. To ensure that silica precipitation is localized onto the SAGEs

when immersed in silicic acid, the *N* termini or *C* termini of the homotrimers are decorated with positively charged peptides.

Figure 2. Representative transmission electron microscopy (TEM) images of silicic acid treated SAGE particles and controls. (a, e, i) Cartoons showing peptide modules (left) and assembled SAGEs (right). (b – d) Controls showing (b) silica precipitated in the absence of peptide, (c) with parent SAGE, and (d) parent SAGE without silicic acid added. (f – h) Silicic-acid treated SAGE with *N*-terminally appended tetrapeptides (f) E4-SAGE, (g) K4-SAGE and (h) R4-SAGE. (j – l) as in panels f – h, but for *C*-terminally decorated SAGEs. Samples were prepared at a concentration of 2 μM peptide, 24 mM silicic acid, phosphate (PI) buffer (10 mM potassium phosphate (8.02 mM dibasic K_2HPO_4, 1.98 mM monobasic KH_2PO_4), pH 7.4), 24 hours, 20 °C. Scale bars are 200 nm.

Figure 3. Summary of structures assembled during optimization of SiO_2-R4-SAGE formation. (a) A fine network or mesh of disordered mineralized peptide, (b) spheres interconnected with fine mesh, and (c) spheres interconnected with assembled mineralized peptide. Individual SAGEs that are small (d), and regularly sized (e) (highlighted in yellow in panel f) can also be produced. (f) Summary indicating how changing silicic acid and peptide concentrations, proportion of parent SAGE, pH and temperature, alter the types of assemblies observed for the SiO_2-R4-SAGEs. The "Goldilocks zone" for making individual, regularly sized, spherical SAGEs is highlighted in yellow. Arrows represent increasing values of the condition illustrated.

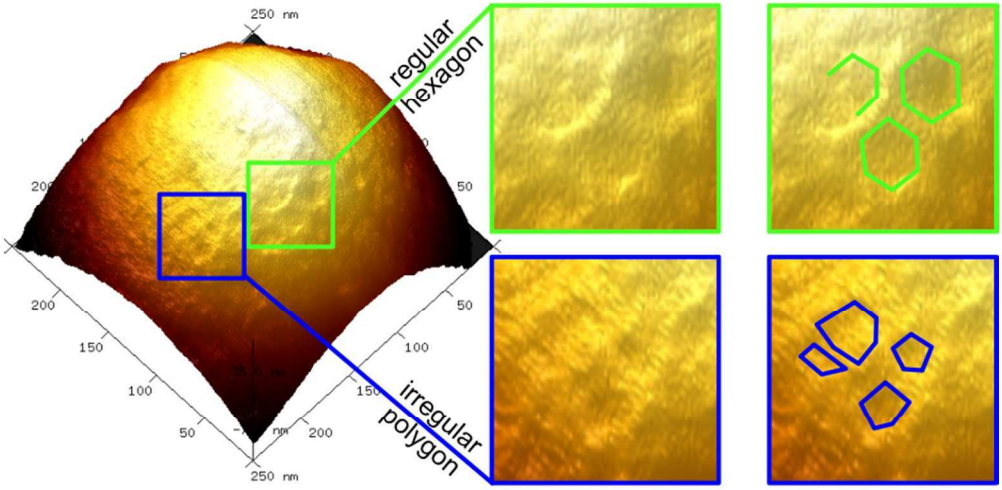
Figure 4. PeakForce atomic force microscopy (PF-AFM) of SiO_2-R4-SAGE particles on mica. 2D plots show variations recorded in (a) the height, (b) adhesion, and (c) dissipation

properties of the SiO_2-R4-SAGEs (upper parts of images) and mica (lower parts of images). (d) A 3D plot of the topography. The mica surface is smooth, whereas the SiO_2-R4-SAGE surface reveals texture, which is shown in greater detail in Figure 5. Silicification was performed with 2 μM peptide, 12 mM silicic acid, PI buffer, 24 hours, 20 °C.

Figure 5. PeakForce atomic force microscopy (PF-AFM) of an individual SiO_2-R4-SAGE particle. 2D plots zoomed in on area shown in Figure 4, which map variation in (a) height, (b) adhesion, and (c) dissipation, and a plot showing (d) height contrast. (e) The same surface as shown in (d) with the corresponding adhesion map overlaid as a blue-scale color-map, which shows that variations in topography and adhesion co-localize. (f – i) 3x magnifications for (f & g) 3D height plots and (h & i) height-adhesion overlays, revealing (f & h) irregular polygons and (g & i) regular hexagons. (j – m) Annotated version of panels f – i with shapes drawn to guide the eye. Silicification was performed with 2 μM peptide, 12 mM silicic acid, PI buffer, 24 hours, 20 °C.

Figure 6. Correlative light and electron microscopy (CLEM) and fluorescence microscope images of R4-SAGE-fl fluorescent SAGEs. (a) Schematic showing the peptide modules used to assemble R4-SAGE-fls (2 μM peptide, PI buffer, 1 hour, 20 °C). (b) High-magnification TEM image of a cluster of SiO_2-R4-SAGE-fls (mineralization conditions: 24 mM silicic acid, 24 hours, PI buffer, 20 °C). (c & d) TEM images superimposed with fluorescence microscope images of the same area to produce CLEM images. In c & d, red boxes highlight the zoomed area for the previous panel. (e – p) Fluorescence microscope images of R4-SAGE-fls (scale bars 10 μm): (e – h) unmineralized; mineralized with (i – l) 12 mM silicic acid; and (m – p) 24 mM silicic acid. Separate bright-field and overlaid images are given in Figure S26. R4-SAGE-fls

without additives (e, i, m), and after treatment with: (f, j, n) Trypan blue; (g, k, n) tris(2-carboxyethyl)phosphine hydrochloride (TCEP); and (h, l, p) trypsin.



TOC entry

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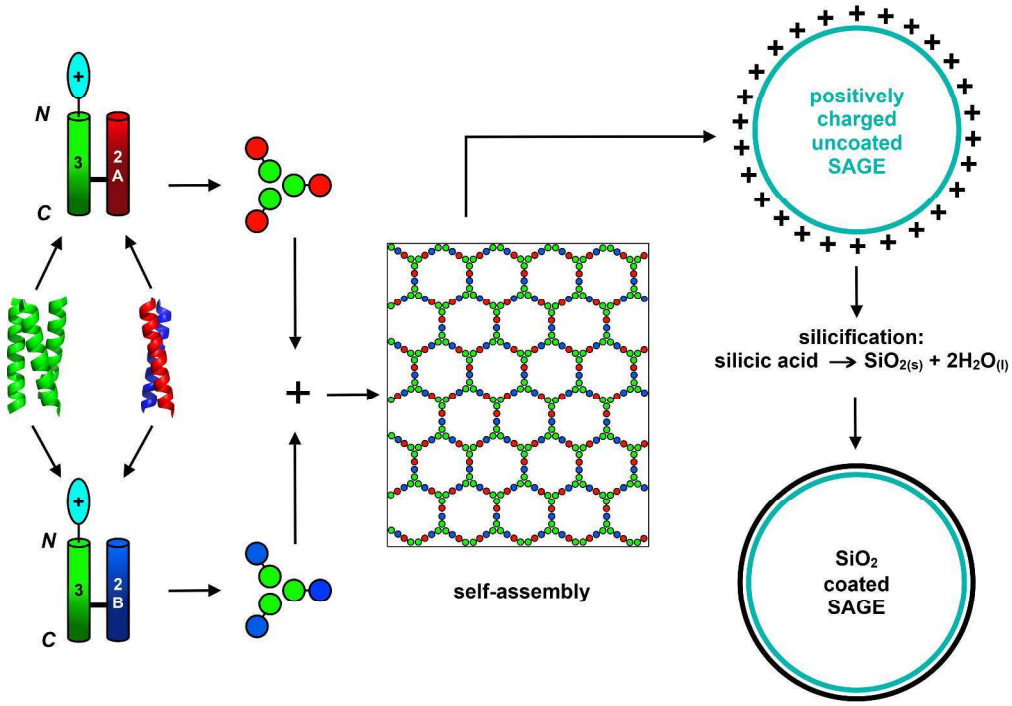

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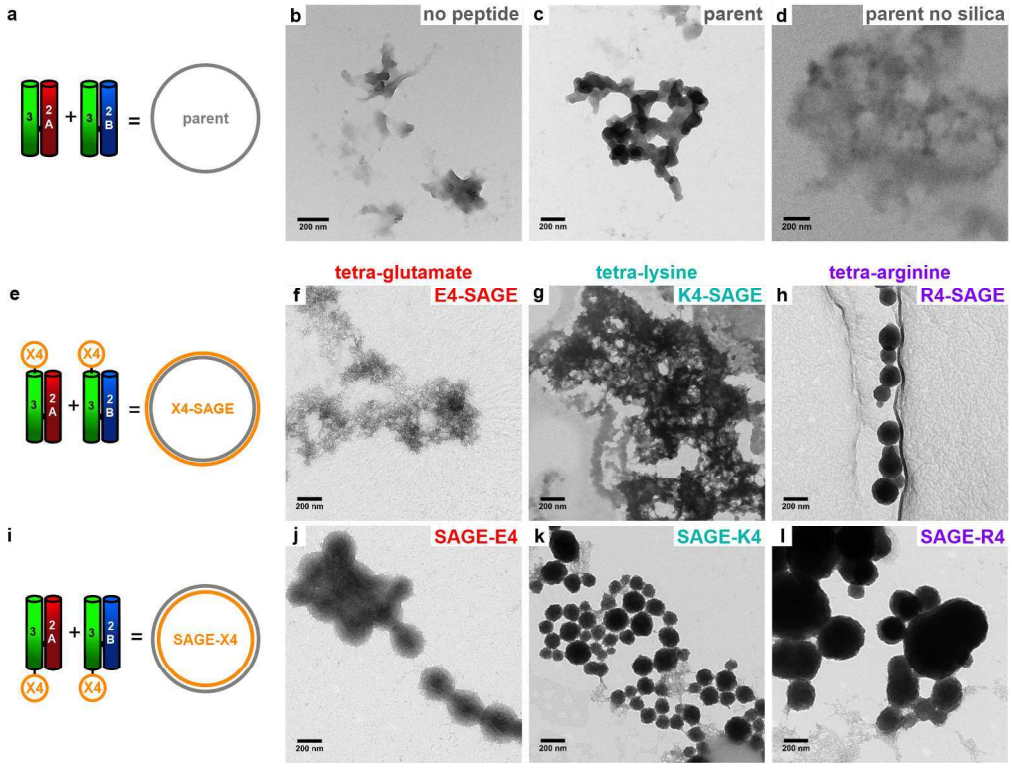

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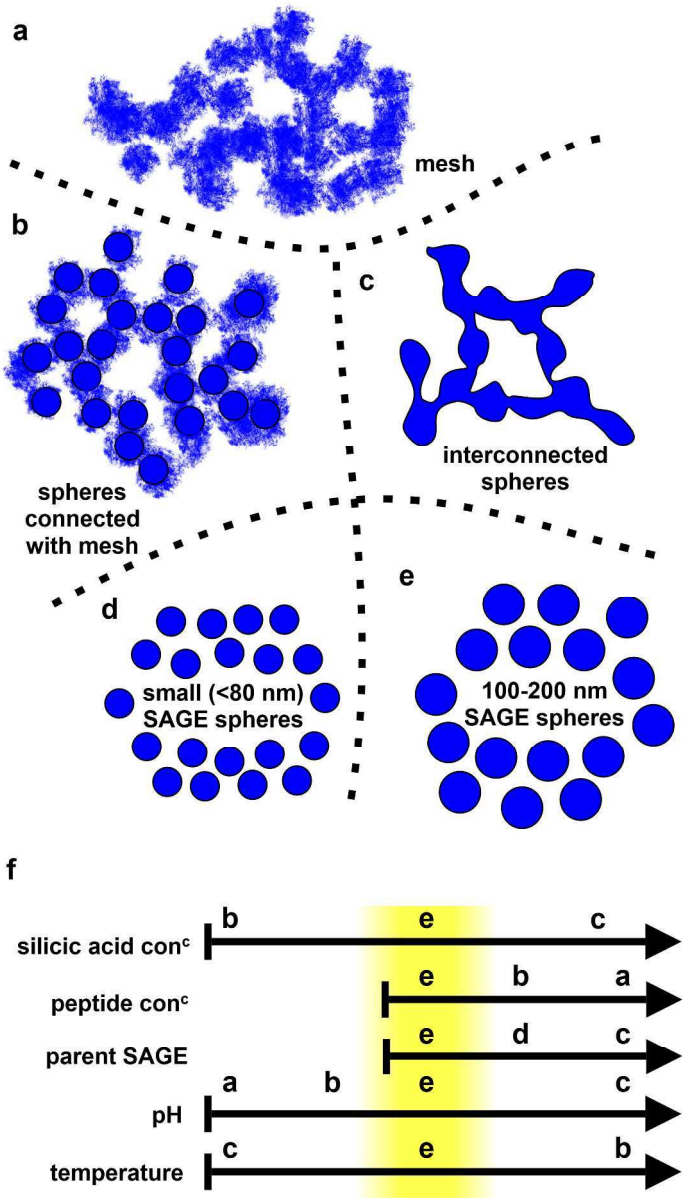

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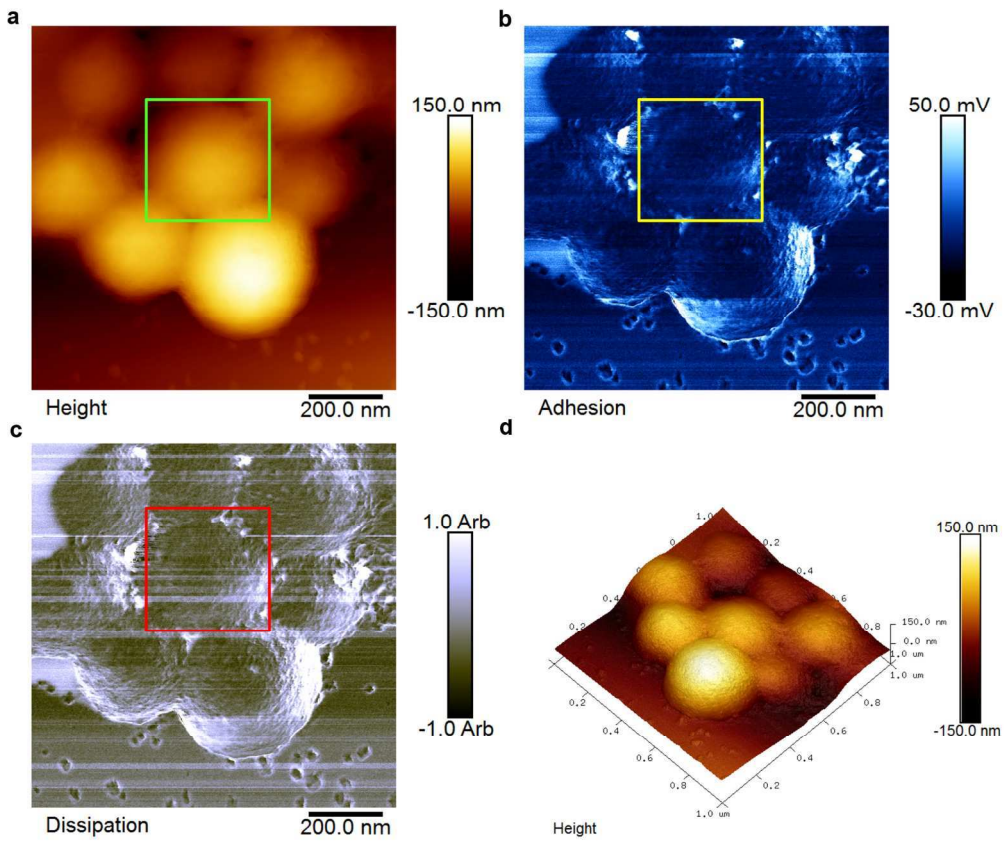

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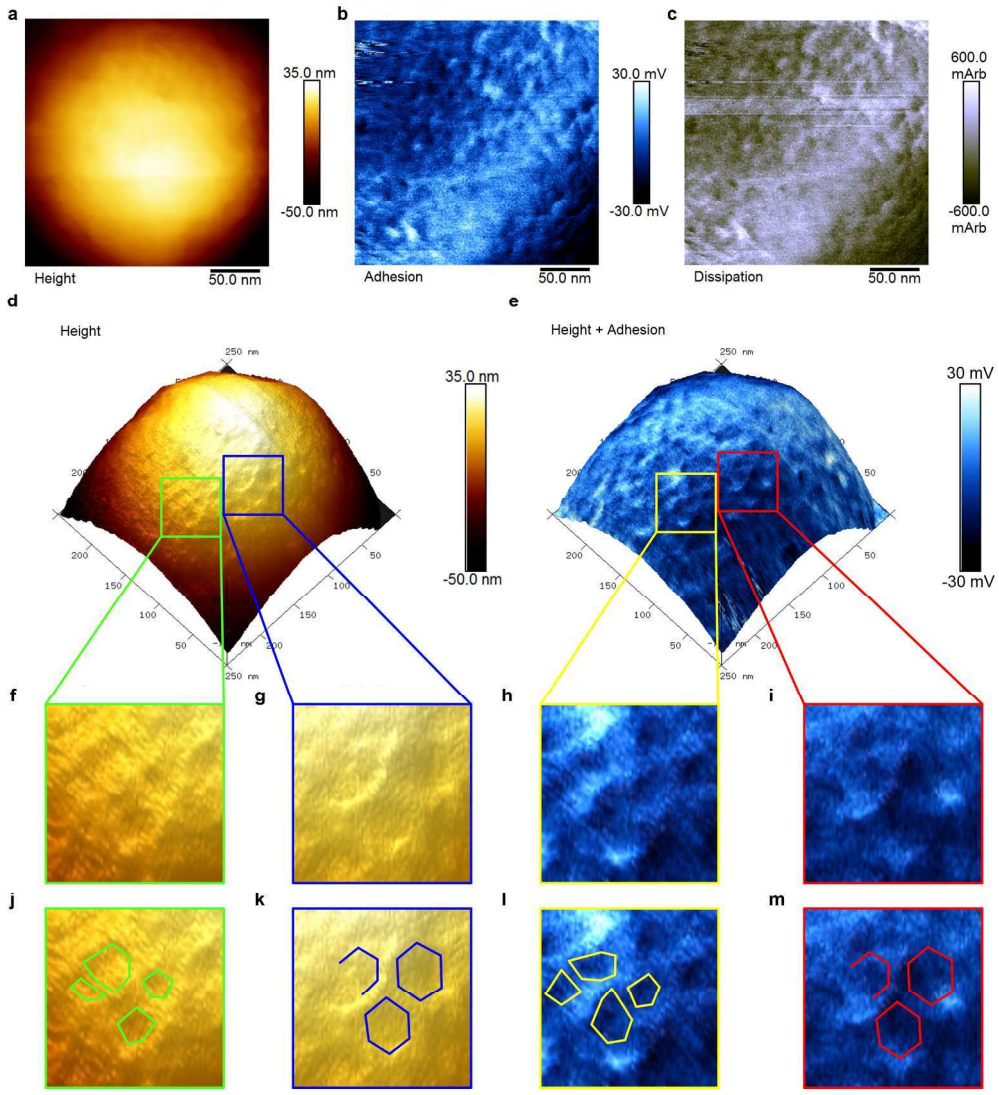

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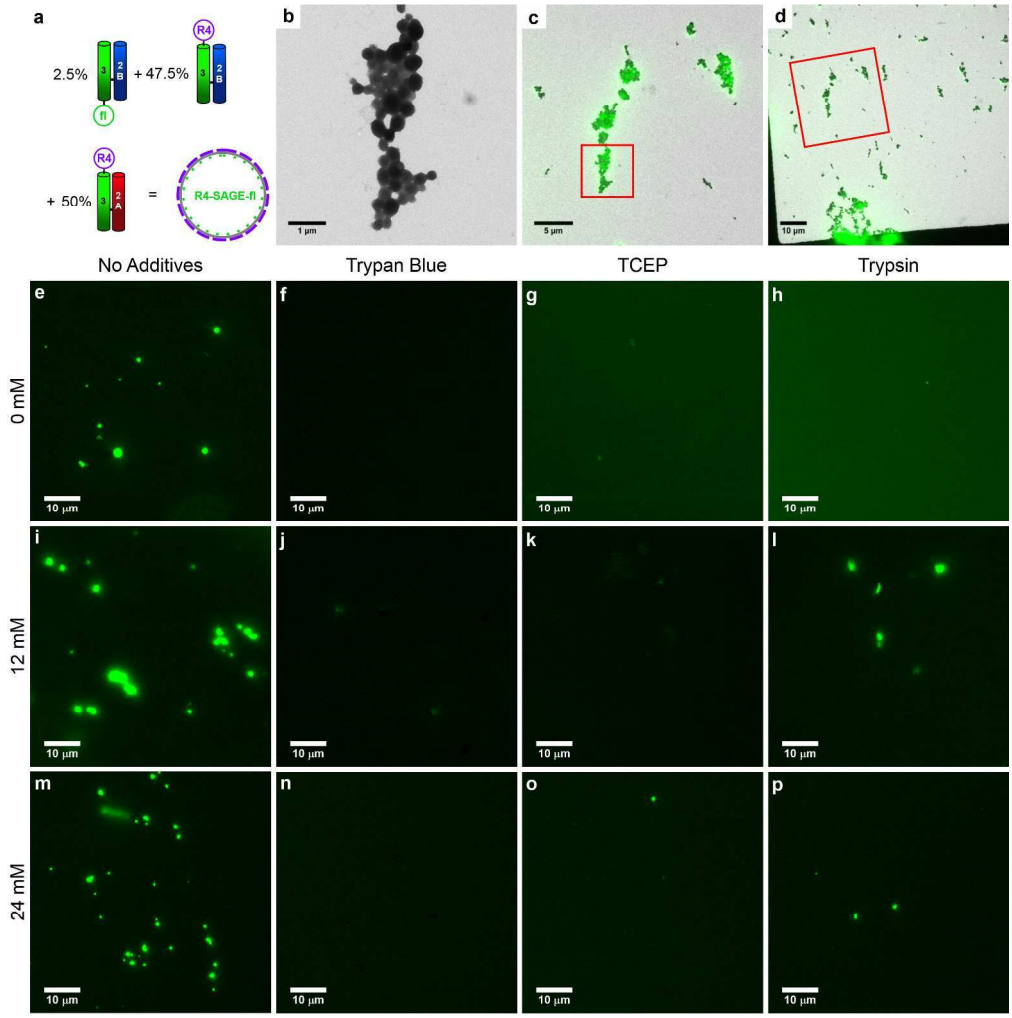

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