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Functional analysis of the biomimetic silica precipitating activity of the R5 peptide from *Cylindrotheca fusiformis*†

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A synthetic site-directed mutagenesis study of the non post-translationally modified silica precipitating R5 peptide reveals that the RRIL motif is critical in the formation of active silica precipitating assemblies.

Diatoms are unicellular, eukaryotic algae that form a diverse array of nanopatterned silica structures.[1–2] In contrast to many current materials engineering approaches to the synthesis of patterned silica,[3] biogenic silica is formed rapidly under mild conditions. Silaffins are highly post-translationally modified peptides derived from the Sil1 protein of *Cylindrotheca fusiformis* that have been implicated in the biosilicification process.[4] Within the native peptide, many of the lysines have been modified to long-chain polyamine moieties,[5,6] and the serines have been post-translationally phosphorylated (NatSil-1A).[7] Silaffins from a variety of diatoms, as well as other long-chain polyamines have been shown to promote silica condensation from a solution of monosilicic acid.[5,6,8] Recent work has shown that the post-translationally modified silaffins self-assemble into supramolecular structures providing a template for silicic acid polycondensation.[7] While silaffins effectively precipitate silica nanospheres under mildly acidic conditions, the non-modified R5 peptide (H_2N-SSKKSGSYSGSKGSKR-RIL-CO_2H) is capable of precipitating silica at neutral pH.[4] Recently, it has been demonstrated that sheer stresses can affect the morphology of R5 precipitated biomimetic silica.[9] Here, we report a synthetic site-directed mutagenesis study of the full length non-post-translationally modified R5 peptide. The results suggest that the C-terminus RRIL motif serves as an organizing element that enables the formation of a supramolecular assembly of peptides creating a locally high concentration of amine containing residues that promote *in vitro* silica precipitation.

A series of R5 truncates (Table 1) was designed to dissect the peptide in order to explore its functionality. Select synthetic mutants were then synthesized‡ to examine the role of individual amino acid moieties. Using automated FMOC peptide synthesis protocols, R5 (**1**), the truncates (**2–8**) and the mutant peptides (**9–11**) were synthesized and purified by HPLC (Supporting Information†). The peptides were then assayed for their ability to rapidly precipitate silica nanospheres from a solution of metastable silicic acid.

Table 1 Silica precipitating peptides and their activity

Peptide	Sequence	Specific Activity[a]	Particle Size (nm)
1	SSKKSGSYSGSKGSKRRIL	3.59 ± 0.16	250–450
2	SSKKSGSY	0.08 ± 0.05	n/a
3	SGSKGSKRRIL	3.35 ± 0.25	180–400
4	KSGSYSGSKGSKRRIL	3.29 ± 0.21	125–200
5	SGSKGSKRR	2.70 ± 0.19	150–300
6	SSKKSGSYSGSKGSK	1.09 ± 0.23	85–130
7	LIRRSSKKSGSY	3.17 ± 0.22	60–300
8	SSKKSGSYRRIL	2.88 ± 0.32	60–300
9	SGSKGSKAAIL	0.24 ± 0.16	n/a
10	SGSKGSKEEIL	0.17 ± 0.08	n/a
11	SGSKGSKNNIL	0.16 ± 0.07	n/a

[a] nmoles of silica per min·nmole peptide

Silica precipitation assays[4] demonstrated that the R5 peptide (**1**) had an activity profile consistent with previously published results. The truncate (**2**), representing the first 8 amino acids of R5 had negligible activity. In contrast, truncate (**3**), containing the final 11 amino acids of R5, demonstrated activity similar to that of the R5 peptide itself. A control solution of silicic acid without peptide did not form any precipitate. For those peptides with demonstrated silica precipitation activity, the amount of precipitated silica is proportional to the concentration of the active peptide. The significant difference in activity between truncate **2** and **3** was unexpected given previously published results that had showed simple primary amines could promote silica precipitation. Although both truncates contain lysine and serine residues, only (**3**) has the RRIL motif. When RRIL was appended to either the N- or C-terminus of the truncate (**7** and **8**, respectively), silica precipitation activity levels were restored. A truncate of the first three amino acids (SSK) of the N-terminus (**4**) also demonstrated near normal levels of activity. Longer truncates (**5** and **6**) without RRIL had some, albeit reduced, levels of silica precipitating activity, undoubtedly due to the presence of multiple primary amines. To further probe the role of the RRIL motif, a series of synthetic mutants of truncate **3** were synthesized and assayed for silicification activity. Mutant **9** in which the arginines are replaced with alanines demonstrated minimal activity. When the arginines were replaced with aspartic acid (**10**) or asparagines (**11**) in order to probe possible involvement of charge, hydrogen bonding or repeating amines, minimal levels of silica precipitation were detected (Fig. 1).

Infrared analysis of isolated and exhaustively washed silica nanospheres clearly indicates that the peptide has co-precipitated with the silica. Vibrations for the amide I, amide II and C–O vibrations from the precipitating peptide are clearly discernable (Supplementary Material†) in the IR spectrum of the silica precipitate.[10] Within the precipitate, the molar ratio of silica to peptide ranges from 13 to 18. In comparison, the molar ratio of silica to silaffin-1A with its post-translationally modified lysines (but not serines) is reported as 12.[4] The precipitates were examined by both scanning and transmission electron microscopy. In SEM, the R5 peptide (**1**) precipitate is comprised of a network of roughly spherical silica particles 245–450 nm in diameter (Table 1). This is somewhat smaller than the 400–600 nm diameter particles observed by Naik *et al.*[11] The truncate (**3**) showed a similar size distribution of particles ranging from 180–400 nm in diameter. The remaining active truncates (**4–8**) showed a broader distribution of smaller particles with diameters from 60–300 nm. TEM analysis of the network of silica particles revealed that the particles were often connected through an overgrown neck and clearly revealed the incorporation of smaller spheres into larger ones. These observations are consistent with the morphogenic silicification

† Electronic supplementary information (ESI) available: HPLC and MALDI of peptides (11 pgs); EMs of silica particles (4 pgs); IR data (3 pgs); DLS data (1 pg) and mechanistic detail (1 pg). See http://www.rsc.org/suppdata/cc/b3/b309074d/

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process proposed by Kröger *et al*. arising from self-assembled peptide aggregates of at least 700 peptide molecules.[7]

Dynamic light scattering measurements of selected peptides revealed that the RRIL motif was crucial for the formation of active silica precipitating assemblies. The R5 peptide (**1**) showed aggregates with sizes of approximately 825 nm. The truncate of the second half (**3**) and the truncate of the first half with an appended RRIL motif (**7**) formed aggregates sized approximately 700 nm and 950 nm, respectively. These peptide assemblies are similar in size to the block copolypeptides mimicking the activity of silicatein reported by Cha *et al*.[12] The inactive peptide **2** (identical to **7** *sans* RRIL) formed no detectable aggregates. The function of the RRIL motif in the self-assembly of the silica precipitating structure is likely mediated by the pattern of arginine's positively charged guanidinium groups in proximity to the hydrophobic leucine and isoleucine residues resulting in a micelle like assembly. When either a carboxylate (**10**) or hydrophobic (**9**) residue replaces arginine in the RRIL motif of the truncate of the second half, activity is completely lost. This suggests that the binary patterning of positive and hydrophobic residues is important in the formation of an active peptide assembly. Similar interactions resulting in the formation of biomimetic silicification assemblies have been observed in arginine based surfactants.[13]

Although the details of the assemblies between NatSil-1a and the R5 peptide differ, it is likely that similar chemistry is driving the polycondensation of silica. As has been suggested by Mizutani *et al*.,[14] the polyamines within the peptide assembly could act as an acid–base catalyst in which deprotonated residues (base) accepts a proton from silicic acid forming a reactive silanolate group and protonated residues (acid) would drive the release of water by protonation of silicic acid substrates. Further, the positively charged patches along the surface of the peptide structure would interact electrostatically with growing negatively charged silica species. This interaction undoubtedly results in the encapsulation of the template molecule within the silica.

The RRIL motif is observed in the amino acid sequence of all seven Sil-1A repeats within the Sil1 gene. Amino acid and mass spectral analysis suggest that within the biological system the RRIL motif is removed during the processing of the silaffin precursor polypeptide. Nevertheless, continued biomimetic studies of R5 and its applications to the biomimetic synthesis of silica structures dictate a careful functional analysis of amino acid residues to determine their role in the biosilicification process. By examining a series of truncates and site-directed mutants of R5, it is apparent that the RRIL sequence functions as a self-assembling motif to form the active silica precipitating peptide assembly. Such assemblies have been implicated in a number of natural and biomimetic silica precipitating systems and represent an important design criteria for the continued development of novel systems for the formation of multi-scale silica structures.

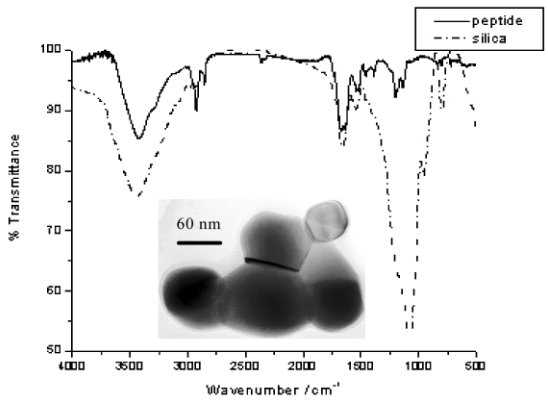

Fig. 1 IR spectra of peptide **(8)** and precipitated, washed silica showing the peptide amide band at 1662 cm^{-1}suggesting peptide encapsulation. Insert shows TEM of silica nanoparticles synthesized from peptide **(8)**

Notes and references

‡ Peptide Synthesis: Automated peptide synthesis was performed using standard FMOC procedures.[1] Peptides were cleaved with 95% trifluoroacetic acid/5% triisopropylsilane, filtered and precipitated using cold diethyl ether. Peptides were purified using a Waters Prep LC 4000 system on a Waters RCM bulk matrix C_{18} column (25 × 10 cm) using a water (0.1% TFA)/acetonitrile gradient. Peptide sequences were confirmed by MALDI-TOF mass spectrometry (Supplementary Material†). Work supported by NSF 0304124 and NSF 0196540.

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