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Secondary structure and dynamics study of the intrinsically disordered silica-mineralizing peptide P₅S₃ during silicic acid condensation and silica decondensation

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Abstract

The silica forming repeat R5 of *sil1* from *Cylindrotheca fusiformis* was the blueprint for the design of P₅S₃, a 50-residue peptide which can be produced in large amounts by recombinant bacterial expression. It contains five protein kinase A target sites and is highly cationic due to 10 lysine and 10 arginine residues. In the presence of supersaturated orthosilicic acid P₅S₃ enhances silica-formation whereas it retards the dissolution of amorphous silica (SiO₂) at globally undersaturated concentrations. The secondary structure of P₅S₃ during these two processes was studied by circular dichroism (CD) spectroscopy, complemented by nuclear magnetic resonance (NMR) spectroscopy of the peptide in the absence of silicate. The NMR studies of dual-labeled (¹³C, ¹⁵N) P₅S₃ revealed a disordered structure at pH 2.8 and 4.5. Within the pH range of 4.5 to 9.5 in the absence of silicic acid, the CD data showed a disordered structure with the suggestion of some polyproline II character. Upon silicic acid polymerization and during dissolution of preformed silica, the CD spectrum of P₅S₃ indicated partial transition into an α -helical conformation which was transient during silica-dissolution. The secondary structural changes observed for P₅S₃ correlate with the presence of oligomeric/polymeric silicic acid, presumably due to P₅S₃-silica interactions. These P₅S₃-silica interactions appear, at least in part, ionic in nature since negatively charged dodecylsulfate caused similar perturbations to the P₅S₃ CD spectrum as observed with silica, while uncharged β -D-dodecyl maltoside did not affect the CD spectrum of P₅S₃. Thus, with an associated increase in α -helical character, P₅S₃ influences both the condensation of silicic acid into silica and its decondensation back to silicic acid.

Keywords

Biomineralization; Silica-Formation; Silica-Dissolution; Circular Dichroism (CD) Spectroscopy; Nuclear Magnetic Resonance (NMR) Spectroscopy

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Work performed at¹, except NMR- and SEC-measurements²

INTRODUCTION

Diatoms build their silicified cell walls by using silaffin peptides and poly(propylamine)s to accelerate and control the formation of silica from soluble precursors.^{1–6} Other peptides⁷ are also associated with the silica-matrix, such as frustulins,^{8,9} pleuralins,^{10,11} and the recently identified cingulins,¹² which are thought to be structure-guiding agents. Silaffins from *Cylindrotheca fusiformis* initiate silica-formation via phosphate mediated self-assembly,¹³ undergoing a phase separation¹⁴ to form an environment that facilitates silicic acid polycondensation. Covalently attached polyamines are then thought to catalyse siloxane bond formation by acid-base catalysis, orchestrated through an interplay between protonated and unprotonated amines.¹⁵

Without the covalently attached polyamine moieties, silaffins generally retain their ability to accelerate silica formation, albeit only at pH 7, whereas native silaffins with covalently bound polyamines are active in a slightly acidic environment.² This was shown with the peptide R5 (Figure 1), a segment of the *C. fusiformis* silaffin. It is thought that R5 first interacts with phosphate to form a micelle-like assembly¹⁶ that is a prerequisite for silica biomineralizing activity.¹³ In this micellar structure, a limited number of amino acid side chains are exposed to the bulk solution, and NMR-analysis revealed that only amino acids near the N-terminus of R5 interact with the silica-matrix upon precipitation.¹⁷ This process is similar to the behavior of poly-L-lysine (PLL) that can also phase-separate in the presence of phosphate to produce hollow silica spheres¹⁸ or hexagonal platelets¹⁹ via a surface deposition on the assembled matrix.

In the absence of silicic acid, silaffins and other mineralization promoting peptides adopt an intrinsically disordered or random coil structure.^{20–22} This was shown for the R5-peptide by CD and NMR spectroscopy²³ and for PL12 (a partial sequence of *Thalassiosira pseudonana* silaffin-34,²⁴) by NMR spectroscopy²⁵ (see Figure 1 for the respective peptide sequences). The “intrinsically disordered” structural classification has many “flavors”²⁶ and often comprises disordered ensembles that include local transient canonical structures (α -helices and β -sheets). Typically, an increase in the net charge per residue in intrinsically disordered proteins leads to an increase in the population of extended structures, as shown with arginine-rich protamine, which has an increased content of polyproline II (PPII) conformation when the cationic charge is elevated.²⁷ The presence of PPII-dominated conformations in calcium phosphate-precipitating proteins has recently been shown to play a prominent role in hydroxyl apatite mineralization.²⁸

In solution at neutral pH, poly-L-lysine also adopts an intrinsically disordered structure^{29–32} containing a high fraction of extended conformations including PPII. During co-precipitation with silica, the unordered structure of PLL is retained in polymers of less than 100 residues^{33–35} and spherical silica is deposited. On the other hand, α -helical structures appear in PLL-chains greater than 100 residues,³⁶ but exclusively if PLL is mixed with phosphate prior to silicic acid addition³⁷ (at least 2.5 phosphate ions per lysine). Notably, this structural change in PLL is not caused by silicic acid directly, but requires the presence of phosphate which mediates the assembly of PLL (in α -helical conformation) into single crystalline sheets,³⁶ and the assemblies are templating the deposition of hexagonal silica

platelets.^{19,37,38} In a control experiment without phosphate, no significant structural changes were reported.³⁷ The latter is in line with experiments where PLL was first forced into different secondary structures (by altering pH and temperature) and subsequently silica-coated via a sol-gel route (PLL-chains as polycationic templates for silica-deposition), and the secondary structure of PLL was retained.³⁵

The structure of the silaffin peptides within the peptide-silica composite precipitates is poorly understood. In a solid state NMR approach, significant perturbations in the chemical shifts of both the peptide backbone and side chain C and N atoms were reported. These were attributed to differences in the chemical environments due to interaction with silica¹⁷ (i.e. peptide-silica interaction, peptide-chain assembly), and no information about peptide folding was inferred.^{17,39} In another report,²³ R5 was studied by solution state NMR in the presence of 50 µM (undersaturated) silicic acid. No changes in the spectra were noticed, from which the authors concluded that silicic acid at this concentration did not affect the structure of R5. A solid state NMR study of the peptide PL12 (Figure 1) in silica²⁵ showed that all amino acid residues were in an unstructured conformation described as (slightly) extended for four residues and random coil for the rest. The few chemical shift perturbations observed in the presence of silica were attributed to interactions between silica and amino acid side chains. With R5, a recent NMR investigation revealed that chemical shifts were also caused by an interaction of the peptide-bond amide with silica via the carbonyl function.⁴⁰

To study structure-function relationships in silica-precipitating peptides in a more systematic manner, we designed a bacterially expressed silaffin-related peptide containing several lysine, arginine, and serine residues called P₅S₃⁴¹ (Figure 1) that was derived in terms of its primary sequence from *C. fusiformis* Sil1.² Its recombinant production provides easy access to primary structure changes by random and site specific mutagenesis. The impact of protein phosphorylation on the interaction with silica or silicic acid was studied by making use of multiple kinase target sites introduced into the P₅S₃ sequence. In solutions of highly supersaturated silicic acid (i.e. 30 mM, 15 – 30 fold supersaturated^{42,43}), P₅S₃, both in its phosphorylated and non-phosphorylated states, accelerated the formation of silica between pH 6.5 and 9.5.⁴¹ At less supersaturated concentrations of silicic acid (i.e. 8.3 mM, 4 – 8 fold supersaturated) the peptide impeded the silicic acid polymerization to silica. At globally undersaturated (i.e. 1 mM) silicic acid concentrations, P₅S₃ slowed down the dissolution of silica.⁴⁴ Here, we report on the conformation of the peptide in the presence or absence of silica as derived from NMR^{39,45,46} and CD^{19,22,47,48} spectroscopy. We show that the P₅S₃ polypeptide backbone is disordered in solution but shifts to a higher α -helical content upon binding silica. This interaction is modulated by peptide phosphorylation and leads to both facilitated silica-formation and impeded silica-dissolution.

MATERIALS AND METHODS

All solutions were prepared with ultra pure water (electrical resistance 18.6 MΩ). Tetramethoxysilane (TMOS) was purchased from Sigma–Aldrich GmbH, Fluka, Taufkirchen (Germany). Buffer systems (at different concentrations as outlined in the appropriate sections) for measurements at different pHs: sodium acetate/acetic acid, pH 4.5; bis-Tris-HCl (bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane hydrochloride), pH

6.5; Tris-HCl (tris(hydroxymethyl)aminomethane hydrochloride), pH 7.5 and 9.5. A Tris/Tricine-system using 16.5% acrylamide gels was used for SDS-PAGE.^{49–51}

Protein expression and purification

The peptide P₅S₃ consists of five protein kinase A target sites⁵² (RRASL) interspersed with three silaffin²-derived lysine-rich partial sequences (GKSKKL) and an amino-terminal tyrosine-containing extension (GSYS) for UV/vis-spectroscopic quantification via absorption at 280 nm (using the absorption coefficient of tyrosine⁵³). In some experiments, a P₅S₃ construct with a carboxy-terminal hexahistidyl-tag (-EHHHHHH) was used. These experiments are not specified because in instances where experiments were performed on both peptides (with and without the hexahistidyl-tag) no significant differences in the results were observed.

Full details of the expression and purification of recombinant P₅S₃ in *Escherichia coli* have been reported earlier.⁴¹ In brief, the peptide was expressed as a fusion protein with ketosteroid isomerase (KSI), which mediates the deposition of the expressed protein in inclusion bodies.⁵⁴ Cells were grown in LB-medium^{55,56} to an OD₆₀₀ of 2 – 2.5 before induction of expression by IPTG (isopropyl β-D-1-thiogalactopyranoside). After overnight incubation the cells were harvested by centrifugation, lysed enzymatically with lysozyme/benzonase, and the insoluble residue containing the KSI-P₅S₃ fusion protein washed with detergent buffer. The P₅S₃-target peptide was liberated by cyanogen bromide cleavage⁵⁷ and purified by cation exchange chromatography. Using volatile ammonium carbonate for elution allowed subsequent drying and desalting by repeated lyophilization.

For the NMR experiments, P₅S₃ was uniformly ¹³C and ¹⁵N labeled using a modified expression procedure. Since the yield of KSI-P₅S₃ was largest following induction at high cell densities,⁴¹ a pre-growth step in rich medium was applied prior to growth in minimal medium.⁵⁸ This was possible because there was no detectable expression of the recombinant product prior to IPTG-induction⁴¹ enabling efficient isotope labeling in minimal medium. Cells were initially grown in LB-medium containing 5.6 mM glucose to an OD₆₀₀ of 2 – 2.5, harvested by centrifugation (5 min, 5000 g) and transferred into an equal culture volume of M9 minimal medium^{58,59} containing 99% isotope-enriched D-(¹³C₆)-glucose (11.1 mM) and ¹⁵NH₄Cl (18.7 mM) (Euriso-top GmbH, Saarbrücken, Germany). The remaining components of the minimal medium were 42.2 mM disodium hydrogenphosphate, 22.0 mM potassium dihydrogenphosphate, 8.6 mM sodium chloride, 1 mM magnesium sulphate, 100 μM calcium chloride, 10 μM iron chloride, 105 μM chloramphenicol and 270 μM ampicillin. After a 2-hour adaptation to the medium, expression was induced with IPTG (1 mM) and the cells incubated overnight. Purification was performed following the standard protocol⁴¹ briefly described above.

Nuclear magnetic resonance (NMR) spectroscopy

The NMR data were all collected at 20 °C on a double-labeled (¹³C, ¹⁵N) sample (~1 mM) of P₅S₃ using four-channel Varian Inova-500, -600 and -750 spectrometers equipped with triple resonance probes and pulse field gradients. Two-dimensional ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra were obtained under a variety of buffer

conditions similar to those used for the CD studies, however, 2% acetic acid (2% CD₃CO₂D, 7% D₂O, 91% H₂O) was chosen for the collection of backbone assignment NMR data because the quality of the data was best under this condition. This data consisted of standard two-dimensional ¹H-¹⁵N and ¹H-¹³C HSQC experiments and three-dimensional HNCACB, HNCA, CC-TOCSY-NNH, ¹⁵N-edited NOESY (90 ms mixing time), and HNN experiments using the Varian Biopack suite of pulse programs. The average backbone amide ¹⁵N T₁/T_{1ρ} ratios, measured with a modified ¹H-¹⁵N HSQC experiment to record one-dimensional spectra,^{60,61} was used to estimate an overall rotational correlation time (τ_c) for P₅S₃ at 20 °C. Felix2007 (MSI, San Diego, CA) and Sparky (v3.115) were used to process and analyze, respectively, all the NMR data. Indirect methods (DSS = 0 ppm) were used to reference the ¹H, ¹³C, and ¹⁵N chemical shifts.⁶²

Size exclusion chromatography (SEC)

The oligomeric state of P₅S₃ at high peptide concentration was estimated from its retention time on a Superdex30 HiLoad 16/60 size exclusion column (GE Healthcare, Piscataway, NJ) pre-equilibrated at room temperature (~23°C) with elution buffer (2% acetic acid, 150 mM NaCl, pH 2.8) using a flow rate of 1.0 mL/min and a calibration curve previously obtained under similar conditions.⁶³

Circular dichroism (CD) spectroscopy

Circular dichroism data was collected on a Jasco J810 spectropolarimeter (Jasco GmbH, Gross-Umstadt, Germany) equipped with a Jasco CDF-426S Peltier temperature controlling element set at 23 °C. Quartz cuvettes with either a 10 or 0.1 mm (mountable cuvette) path length were used with P₅S₃ concentrations of 1 or 100 μM, respectively. Steady-state wavelength spectra (average of at least 5 repetitive scans) were recorded at pH 4.5, 7.5, and 9.5, at different NaCl concentrations and in the presence or absence of the detergents sodium dodecylsulfate (SDS, C. Roth GmbH, Karlsruhe, Germany) and n-dodecyl-β-D-maltoside (dodecyl maltoside (DM), Merck Chemicals GmbH, Schwalbach, Germany). All spectra are presented in mean residue ellipticity [θ]_{mrw} with unit × 10⁻³, deg*cm²*dmol⁻¹.^{64,65} Secondary structure populations were estimated by deconvoluting the CD spectra using the ContinLL algorithm in the CDPro software package.^{66,67}

It was often necessary to wait a considerable length of time before P₅S₃ reached a steady-state (e.g. the CD spectrum stopped changing with time). Therefore, time-resolved CD data were collected at a single wavelength to monitor the structure of P₅S₃ during silica formation and dissolution. The silica formation experiments started with either ortho- or precondensed silicic acid to infer the structural effects of the respective silica-species.

Orthosilicic acid was prepared by hydrolysis of 150 mM TMOS (tetramethoxysilane) in 1 mM HCl for 15 min (23 °C, 1,400 rpm orbital shaker).

Precondensed silica for the formation-assay was prepared by the autopolycondensation of a 60 mM silicic acid solution in 10 mM Tris-HCl pH 9.5 for 15 min (23 °C, 1,400 rpm orbital shaking), conditions where the condensation is rapid.^{42,68} The buffer was inactivated with a known amount of HCl and the sample added to an equal volume of a 200 μM P₅S₃-solution buffered to pH 6.5 (10 mM Bis-Tris). The final pH of 6.5 was chosen for this assay because

the autopolycondensation of silicic acid is slow enough to distinguish the effect of precondensed and orthosilicic acid on P_5S_3 in the respective experiments.

The silica dissolution experiments used silica prepared by allowing a 30 mM silicic acid solution buffered with 15 mM Tris-HCl at pH 7.5 to autocondense for 15 min (23 °C, 1,400 rpm orbital shaking). This silicic acid solution was then diluted to 1 mM and P_5S_3 added to a final concentration of 1 μ M. In all the experiments the final pH was verified with pH indicator strips, with an error of approximately ± 0.5 pH units.

P_5S_3 -induced silica formation in the presence of NaCl

A solution containing 100 μ M P_5S_3 was supplemented with Tris-HCl buffer (50 mM, pH 7.5), 30 mM acidically hydrolysed TMOS (15 min in 1 mM HCl), and different concentrations of NaCl. After 30 min at 23 °C and 1,400 rpm orbital shaking, silica was harvested by centrifugation (5 min at 18,400 g), washed by resuspension in water, and harvested again. Both the supernatant and pellet were stored. Molybdenum blue analysis^{41,69} was used to quantify the silicic acid in the pellet following hydrolysis in 1 M NaOH for 1 hour at room temperature. For localization of P_5S_3 , NaOH was added to the supernatant to achieve a concentration of 1 M, the peptide concentrated and desalted by trichloroacetic acid (TCA) precipitation,^{44,70} and analyzed by SDS-PAGE.

P_5S_3 -desorption from pre-formed silica with NaCl

Silica was produced by mixing 0.5 mM spermine (Sigma–Aldrich GmbH, Fluka, Taufkirchen (Germany)) with 30 mM TMOS (after HCl hydrolysis) buffered to pH 7.5 with 50 mM Tris-HCl. After 30 min the silica was harvested by centrifugation (5 min at 6,570 g). The pellet was resuspended in 1 M NaCl buffered to pH 7.5 with 50 mM Tris-HCl to remove any surface-bound spermine and re-pelleted by centrifugation (5 min, 6,570 g). This was repeated twice in Tris buffer without NaCl. After the final centrifugation step the P_5S_3 was bound to the silica by resuspending the pellet in 15 μ M P_5S_3 (dissolved in 50 mM Tris-HCl, pH 7.5). After incubation at 23 °C for 2.5 min the P_5S_3 exposed silica was collected by centrifugation, the pellet washed with 50 mM Tris-HCl, pH 7.5, and re-pelleted by centrifugation. The desorption of P_5S_3 from the silica was examined in parallel assays with solutions containing 0.3 – 1.0 M (in steps of 0.1 M) NaCl buffered in 50 mM Tris-HCl pH 7.5. The silica was suspended in the NaCl solutions, immediately centrifuged (5 min, 6,570 g), and the released P_5S_3 in the supernatant analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

NMR and SEC analysis of P_5S_3 in acidic aqueous solution

The peptide P_5S_3 has a molecular weight of 5,555 Da. Size exclusion chromatography and rotational correlation time calculations indicated that P_5S_3 behaved as a monomer under the acidic conditions used to collect the NMR data. Using a flow rate of 1.0 mL/min, P_5S_3 eluted off a 16/60 Superdex30 size exclusion column with a retention time of 53 min that corresponds to a ~ 7.5 kDa peptide on the column's calibration curve.⁶³ Such a value is closer to a monomeric (~ 5.5 kDa) than to a dimeric (~ 11 kDa) species. The shorter than expected (64 min) retention could be due to the peptide adopting a more elliptical versus

spherical structure. The estimated isotropic overall rotational correlation time, τ_c , inferred from the average ^{15}N spin relaxation ratio was 3.2 ± 0.3 ns at 20 °C, a value consistent with an ~5.5 kDa peptide⁶⁰ (http://www.nmr2.buffalo.edu/nescg.wiki/NMR_determined_Rotational_correlation_time). These observations are in contrast to the P_5S_3 aggregates reported earlier from transmission electron microscopy (TEM).⁴¹ However, the copper TEM-grid was coated with glow-discharged carbon, creating a negative surface that may mimic polyanionic additives (like phosphate) which are known to mediate self-assembly of silaffins.¹³

Figure 2 is a ^1H - ^{15}N HSQC spectrum for P_5S_3 collected at 20 °C, pH 2.8, in 2% acetic acid. Aside from the penultimate N-terminal and the C-terminal amides (S2 and L50 respectively), all the amide proton resonances fall within a narrow proton chemical shift range of 0.3 ppm that is indicative of either an all α -helical or a largely unstructured peptide.⁷¹ The analysis of other sets of NMR data conclusively eliminates the former alternative. First, the chemical shifts of side chain $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ carbons, assigned in the ^1H - ^{13}C HSQC spectrum of P_5S_3 in Figure 3 and residue-tabulated in Table 1, show little chemical shift dispersion. Aside from intensity differences due to the different number of the seven different residues in P_5S_3 , the ^1H - ^{13}C HSQC spectrum looks essentially like the spectrum for seven individual “random coil” amino acids. This can also be seen in Table 1 where the ^{13}C chemical shift range is never greater than 0.9 ppm for any identical side chain carbon and the $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts are largely consistent with random coil carbon chemical shift values (cns_solve_1.1).⁷² Second, the ^{15}N -edited NOESY data display no amide i to $i+3$ or other NOE patterns typical of α -helical peptides and proteins (data not shown).⁷³ Third, a deuterium amide exchange experiment performed by re-suspending lyophilized peptide into 2% $\text{CD}_3\text{CO}_2\text{D}$ and 98% D_2O followed by the rapid collection (less than 10 min later) of an ^1H - ^{15}N HSQC spectrum show that all the amide protons exchange rapidly with deuterons (data not shown), indicating the absence of a stable network of backbone amide-to-amide hydrogen bonds. Note that at pH 7.0 only a few amide cross peaks were present in the ^1H - ^{15}N HSQC spectrum for P_5S_3 , and under solution conditions approaching a pH of 8.0 no amide cross peaks were observed. The disappearance of amide resonances at increasing pH is likely due to conformational exchange and averaging,⁷⁴ or, extensive aggregation.^{61,71} It was because of this extensive line broadening of the backbone amide protons at higher pH that the NMR data for the backbone assignments were collected at pH 2.8 in 2% acetic acid.

The primary amino acid sequence of P_5S_3 is composed of only seven different amino acids, K, R, S, L, A, G and Y, and an 11-residue sequence, RRASLGKSKKL, is repeated three and 7/11 times. Such residue redundancy and sequence repetition in the 50-residue peptide, coupled with its random coil properties, made assignment of the ^1H - ^{15}N HSQC spectrum challenging. An NMR experiment particularly useful for making the assignments was the HNN experiment,⁷⁵ an experiment often used to assist the assignment of intrinsically disordered proteins.^{76,77} Using the HNN experiment together with a suite of classical backbone assignment NMR experiments (HNCA, HNCACB, and CC-TOCSY-NNH) it was possible to unambiguously assign backbone amides to all but two of the 49 expected cross peaks (the N-terminal $-\text{NH}_2$ group is usually not observed) in the ^1H - ^{15}N HSQC spectrum of P_5S_3 as illustrated in Figure 2. Cross peaks could not be unambiguously identified for the

amide of R6 and R38 but they are likely underneath the cross peaks assigned to other arginine amide residues. As can be seen in Figure 2, the amide resonances for each type of residue are generally clustered together within a narrow ^{15}N chemical shift range. This observation, together with the overall narrow proton chemical shift range already discussed (0.3 ppm for all residues except S2 and L50), is further evidence that P_5S_3 alone in solution is predominantly a random coil.⁷¹

CD analysis of the effect of pH on the structure of P_5S_3 in solution

Figure 4 shows CD spectra of P_5S_3 in aqueous solution at pH 4.5, 7.5, and 9.5 (solid lines) at 23 °C. All three spectra are characterized by a large negative minimum band near 200 nm and a small maximum band near 220 nm. These CD spectral features are attributed to the presence of some extended polypyrrolone II (PPII) structure⁷⁸ because such a profile is slightly different from the CD spectral characteristics of a random coil (unstructured/disordered) structure;^{64,65} a large negative band near 195 nm and an (almost) complete absence of the band near 220 nm.⁷⁹ For P_5S_3 at pH 7.5, we previously reported that the PPII features are most prominent at low temperature (4 °C) and shift towards random coil as the temperature is raised (60 °C),⁴¹ a temperature dependent behavior noted also in the CD spectra of other proteins and peptides with PPII character.^{79,80} The spectra shown here for P_5S_3 at different pH values have a small red shift in the minima of the major negative band near 200 nm and a decrease in the intensity of the maximum near 220 nm as the pH is raised from 4.5 to 9.5. As concluded for the spectra of P_5S_3 at different temperatures⁴¹ this pH dependent structural transition may reflect an increase in random coil structure as the pH is raised. However, it may also reflect a partial transition into either β -sheet or α -helical structures which both have a minimum in this spectral region.⁶⁵ Considering the high lysine content in P_5S_3 , the decrease in the maxima of the 220 nm band may be due to an increase in α -helical content, in analogy to the pH dependent structure of poly-L-lysine.^{30,31,81} This is because the pK_a of the lysine side chain N_ϵ amine is ~ 9.8 ,⁸² and therefore, increasing the pH to 9.5 reduces the positive charge on the ten lysine side chains in P_5S_3 and this may result in a partial transition from an extended to an α -helical conformation.

Comparison of the NMR and CD structural analyses of P_5S_3

A ^1H - ^{15}N HSQC spectrum for P_5S_3 was collected at pH 4.5 and 20 mM buffer (Tris), which are essentially the same solution conditions as those used to collect CD at pH 4.5, except that a higher peptide concentration was required (~ 0.5 mM (NMR) versus 0.1 mM (CD)). This HSQC spectrum (data not shown) was essentially identical to the spectrum presented in Figure 2 (collected in 2% acetic acid, pH 2.8), suggesting the peptide was predominantly in the same structural state at pH 4.5. As discussed earlier, it was not possible to use NMR spectroscopy to characterize the peptide at pH 7 due to the disappearance of amide resonances in the ^1H - ^{15}N HSQC spectra. The CD spectra for P_5S_3 reported here and earlier⁴¹ suggest the presence of some PPII structure at pH 4.5, 7.5, and 9.5 although the different CD features between random coil and PPII structures are often not easy to distinguish and their populations difficult to quantitate.⁷⁹ The NMR data presented here indicates that P_5S_3 adopts a random coil structure, but unfortunately, NMR spectroscopy is a poor method to recognize and identify PPII structures since the NMR characteristics of PPII structures are similar to random coil structures.^{83,84} Moreover, one of the methods often

used to identify PPII regions with NMR, the measurement of $^3J_{\text{HN}\alpha}$ values,⁸⁵ is not practical for P_5S_3 due to the extensive overlap of most of the amide resonances in the ^1H - ^{15}N HSQC spectrum (most of the $^3J_{\text{HN}\alpha}$ measurements to characterize PPII regions are for short peptides (< 10 residues) while P_5S_3 is composed of 50 residues). Hence both methods, CD and NMR spectroscopy, indicate P_5S_3 is largely random coil (disordered/unstructured) at pH 4.5 with CD spectroscopy suggesting the presence of a population of PPII structures.

Conformational changes in P_5S_3 upon interaction with silica at different pH values

As shown before,⁴⁴ the pleiotropic effects of P_5S_3 on the formation or hydrolysis of silica involves the interaction of the peptide with silica or oligomers of silicic acid. We used UV-CD to assess the impact of such interactions on the secondary structure of the peptide. Note that all CD measurements were done at 0.1 mm optical pathlength to minimize signal distortion by light scattering. Figure 4 shows the CD spectra for P_5S_3 at three different pH values, in the absence and presence of silicic acid at two different molar ratios. At pH 4.5 no significant changes in the CD spectra were observed up to a P_5S_3 :silicic acid molar ratio of 1:600. Since no precipitation was observed at pH 4.5 and silicic acid has a very slow condensation rate at this pH,⁶⁸ it can be assumed that primarily orthosilicic acid was present and that this silicic acid species did not influence the P_5S_3 secondary structure.

At pH 7.5 and 9.5, where P_5S_3 rapidly precipitates with silica,⁴¹ CD spectra changed considerably in the presence of silicic acid, i.e. upon mineralization. Most significantly, at both pH values, the large negative minimum band near 200 nm grew smaller and red-shifted towards 210 nm with increasing silicic acid concentration. Concomitantly, the small positive maximum band near 220 nm that was assigned to PPII vanished and seemed to be overlaid with a negative signal, creating a new minimum at 228 nm at pH 9.5. This transition with decreasing P_5S_3 :silicic acid ratios was more pronounced at pH 9.5 than at pH 7.5, and at the more alkaline condition the CD signal became strongly positive below 200 nm. In fact, the CD spectrum at the extreme of the conditions surveyed (pH 9.5, P_5S_3 :silicic acid ratio = 1:150), with an apparent positive maximum band around 190 nm and double minimum bands at 209 and 228 nm, is similar to the characteristic CD spectrum of an α -helix (positive maximum band below 200 nm and a double negative minimum band at 208-210 and 222 nm).^{65,86} To assure that the observed changes had their origin in a conformational change and not in artifacts from light scattering, we inspected the corresponding high-tension voltage (HT) spectra for distortions and found them to be virtually identical for all the presented CD data (SI, Figures S2-S8). Therefore, it is highly unlikely that light scattering was responsible for the observed CD signal changes. Moreover, for the highest silicic acid concentrations, we can exclude that the different extents of structural changes at pH 7.5 and 9.5 are due to some P_5S_3 staying in solution, because in similar assays (pH 7.5, with 0.1 mM P_5S_3 and 30 mM silicic acid (molar ratio 1:300), P_5S_3 quantitatively co-precipitated with silica (see Figure 8B (no NaCl)). At pH 5.5 and below, no precipitate (neither silica, nor P_5S_3) occurred in earlier experiments.⁴⁴

Overall, the CD spectra suggest a transition from a disordered/PPII structural state for “free” P_5S_3 to a state with some helical character at high pH and at silicic acid concentrations enabling condensation. This notion is supported by deconvoluting the CD spectra into

individual elements of secondary structure using the ContinLL program in the CDPro software package^{66,67} for secondary structure estimation (not shown). The disordered populations (combined “turn” and “unordered” states) decreased by ~20%. ContinLL suggested a concomitant increase in α -helical and β -strand populations, however, since the program did not perfectly reproduce the experimental spectra, and the absence of β -strand features in the CD profiles (Figure 4), this interpretation should be taken with caution.

The CD spectrum for unbound P_5S_3 was similar to that reported for the silaffin-derived peptide R5²³ except for the absence of the small maximum band near 220 nm attributed to a population of PPII structures in P_5S_3 . The NMR spectra for R5 showed that this peptide also adopts an ensemble of random coil (unstructured/disordered) conformations.²³ A silicic acid concentration of 2 mM, near the autopolycondensation threshold,^{42,43} had no effect on the CD spectrum of R5. Consistently, we noted no change in the CD spectrum of P_5S_3 at similar silicic acid concentrations at pH 7.5 (data not shown). Because little or none of the silicic acid at a concentration of 2 mM exists in a condensed state⁸⁷ (i.e. silicic acid does not condense above a degree of polymerization of three and rapidly converts back to the monomeric species), it can be concluded that monomeric silicic acid is not responsible for inducing a structural transition in P_5S_3 or R5. This notion is further supported by the observation that at pH 4.5 (very slow silicic acid condensation^{68,88}) silicic acid up to 60 mM had no effect on the CD spectrum of P_5S_3 (see Figure 4).

The CD structural transitions observed here for P_5S_3 in going from low pH and no silicic acid (disordered with some PPII extended character) to high pH and high silicic acid (increase in α -helical character) bears some similarity to the transitions reported for PLL of different sizes.^{18,19} Poly-L-lysine larger than 100 residues was disordered in solution and adopted an α -helical structure at 50 – 100 mM silicic acid concentrations upon the formation of silica.¹⁹ This conformational transition required phosphate³⁷ and was accompanied by PLL assembly into hexagonal crystals.³⁶ By contrast, as judged by CD spectroscopy, P_5S_3 did not change its secondary structure in a solution containing 50 mM phosphate at pH 7.5 in the absence of silica (see Figure S1 & S2 in SI) and the transition in the presence of silica was not dependent on phosphate since assays were Tris-buffered (i.e. no phosphate added). Poly-L-lysine less than 100 residues in size was also disordered in solution with a high fraction of extended conformations but did not exhibit the α -helical transition triggered by silica.^{29,30,32,78} This lack of secondary structure transition towards α -helix was also described for PL12 upon binding to silica surfaces.²⁵ Again, P_5S_3 with its 50 amino acids that do exhibit the secondary structure transition behaved differently compared to small PLL polymers or 12 amino acid long PL12.

What is the reason for the observed secondary structure transition with large PLL or P_5S_3 ? Neutralization of positively charged lysine side chains by negatively charged phosphate ions has been assigned to this shift for other polypeptides bearing numerous cationic charges.^{27,89,90} So the structural shift of P_5S_3 driven by silica may require an oligo- or polymeric arrangement of anionic charges. These spatially fixed charges then neutralize (part of) the 20 amino acids of P_5S_3 that contain basic side chain residues. Furthermore, at pH 9.5, where P_5S_3 adopts the most helical character in the presence of silicic acid, ~50% of the lysines are uncharged.⁸² Together, the high pH and silicate counter-ions would then

reduce the repulsion between positively charged P₅S₃ side chains, which are characteristic for extended protein structures like those found in intrinsically disordered proteins.^{27,32,78} Contributions to the CD spectral changes may also come from interactions between silica and imidates formed on the P₅S₃ backbone⁴⁴ as suggested for the silica-biomineralizing peptides KSL^{91,92}, silaffin-1,⁹³ and R5.⁴⁰

Conformational changes in phosphorylated P₅S₃ upon interaction with silica at different pHs

Self-assembly of native silaffin and its capacity to precipitate silica is dependent on the presence of phosphate ions or phosphorylation of serine residues.¹³ Previously, it had been shown that fully phosphorylated P₅S₃ still facilitated silica formation but with slightly reduced yields relative to unmodified P₅S₃.⁴¹ Since P₅S₃, as opposed to silaffin, did show structural transition and silica precipitation in the absence of phosphate (Figure 4) the question arose whether covalently attached phosphate groups will have an impact on silica dependent secondary structure dynamics. To answer this question the peptide was enzymatically phosphorylated at all of its five protein kinase A sites,⁴⁰ and CD experiments as reported in Figure 4 were repeated (Figure 5, HT traces in SI Figure S4).

The spectra of phosphorylated P₅S₃ at pH 4.5, 7.5, and 9.5 (solid lines) in the absence of silicic acid were similar to the CD spectra of non-phosphorylated P₅S₃ shown in Figure 4 (solid lines) and exhibited a large negative minimum band near 200 nm and a small maximum band near 220 nm. Although the molar ellipticity of the negative signal just below 200 nm was slightly decreased with respect to non-phosphorylated P₅S₃, these features are characteristic of a random coil structure with PPII contributions.

At pH 4.5, the CD spectrum of phosphorylated P₅S₃ did not change and no precipitation was observed upon the addition of silicic acid, again as observed with non-phosphorylated P₅S₃ at pH 4.5 (Figure 4), suggesting that phosphorylated P₅S₃ also did not interact with orthosilicic acid at this pH. At pH 7.5 and 9.5, where silica precipitation did occur, the intensity of the CD spectra for phosphorylated P₅S₃ decreased with increasing content of silicic acid and showed a slight bathochromic shift of the major signal towards 200 nm. Above 200 nm and in contrast to non-phosphorylated P₅S₃ (Figure 4) CD spectra did not change significantly upon addition of silicic acid. Deconvolution of the CD spectra using the ContinLL program reports a smaller reduction in disordered conformation (~15%) with the addition of silicic acid relative to the ~20% reported for non-phosphorylated P₅S₃ upon the addition of silicic acid. We speculate that these observed differences may be due to local electrostatic repulsion between negatively charged phosphate groups on P₅S₃ and the negatively charged acidic silica surface that alters the peptide's conformation upon binding to silica. Alternatively, phosphorylated serine side chains have been reported to form hydrogen bonds with adjacent peptide bonds;⁹⁴ this in turn would stabilize the polyproline II extended conformation,^{95,96} preventing α -helix formation.

SDS micelles as a model anionic surface

To examine the hypothesis that it is the array of negative charges on the silica surface that drives the silica-induced secondary structure changes in P₅S₃, CD spectra of the peptide

were recorded in the presence of sodium dodecylsulfate (SDS), a detergent with anionic headgroups.⁹⁷ Peptides that are intrinsically disordered in solution have been observed to adopt more rigid canonical conformations upon interaction with SDS at concentrations above and below the SDS critical micellar concentration. Such examples include the biomineralization protein amelogenin,⁹⁸ Parkinson's disease associated α -synuclein,⁹⁹ Alzheimer's disease associated amyloid A β peptide,¹⁰⁰ porcine peptide hormone motilin,¹⁰¹ and apolipoprotein fragments.¹⁰² Peptides with cationic side chains are believed to bind to the anionic headgroups on the surface of SDS micelles, which drives changes in the peptide's structure.^{103,104} Consequently, the negatively charged headgroups of SDS may mimic the negatively charged functional group on silica, and induce the same structural changes in P₅S₃.

The CD spectra of P₅S₃ in the presence of SDS at concentrations above (34.7 mM) and below (1 mM) the critical micelle concentration (cmc) of the detergent (~8 mM in water)^{105–107} are shown in Figures 6A and 6B (HT traces in SI Figure S5), respectively, with 100 or 1 μ M P₅S₃. To overcome turbidity at high SDS concentrations it was necessary to collect the CD data for 34.7 mM SDS in a cuvette with a path length of 0.1 mm to obtain sufficient signal intensity, while the 1 mM SDS assay was analysed with a 10 mm cuvette. This in turn necessitated the different P₅S₃ concentrations to achieve an appropriate CD signal intensity.

At both SDS concentrations the CD spectra for P₅S₃ changed significantly. At a sub-cmc concentration of SDS, the CD spectrum of P₅S₃ resembled that of a “text-book” α -helical peptide,⁶⁵ exhibiting a positive maximum band below 200 nm and a double negative minimum band at 208–210 and 222 nm (Figure 6A). At a supra-cmc concentration of SDS, the CD spectrum closely resembled the one observed upon silica precipitation at high excess of silicic acid at pH 9.5 (Figure 4): a significant positive signal below 200 nm with an indication of a maximum below 190 nm, a minimum band at 207 and a significant shoulder around 225 nm (Figure 6B), CD features characteristic for helical peptides. The increase of α -helical character of P₅S₃ in the presence of SDS is also indicated by secondary structure estimations by ContinLL (not shown). The transition towards α -helical structure was more pronounced below than above the cmc and likely reflects two different types of associations with SDS. Below the SDS cmc, association is driven by both electrostatic, and hydrophobic interactions, whereas above the cmc the association is likely dominated by electrostatic interactions of the positively charged side chains of P₅S₃ with the concentrated negative charges on the surface of the SDS micelles. No influence on the P₅S₃ secondary structure is observed upon adding dodecyl maltoside (DM), a detergent with the same hydrophobic tail as SDS, but, an uncharged disaccharide instead of a negatively charged headgroup (Figure 6A and S7). This points to the electrostatic interactions between P₅S₃ and SDS triggering the secondary-structural change in the peptide. Similar effects were reported with the synthetic peptide XAO (acetyl-X₂A₇O₂-amine; X = diaminobutyric acid, A = alanine, O = ornithine), which contains four positively charged side chain residues. The peptide XAO adopts an extended polyproline II conformation in water, acquires α -helical structure upon interaction with SDS, and displays no CD detectable structural changes in the presence of DM.¹⁰⁸

The effect of sodium chloride on P₅S₃-silica interactions

To further investigate the electrostatic contribution to the interaction between P₅S₃ and silica, the behavior of the peptide in the presence of sodium chloride and silica was examined. The binding affinity of P₅S₃ to a silica surface was assessed in a series of NaCl desorption experiments using preformed silica prepared by TMOS precipitation in the presence of spermine.¹⁵ Figure 7 shows that P₅S₃ was released from the silica at NaCl concentrations of 600 mM and above.

To examine the effect of NaCl on the ability of P₅S₃ to induce silica formation, the molybdenum blue assay⁶⁹ was used to quantify the amount of silica formed over a range of NaCl concentrations in the presence and absence of the peptide. In the absence of NaCl the peptide was required for silica formation (Figure 8). At 300 mM NaCl approximately 50% of the silicic acid was converted to silica in the absence of P₅S₃, however, more silica was formed when the peptide was present indicating that the peptide is still accelerating silica formation at this salt concentration. At NaCl concentrations of 600 mM or larger, under our assay conditions, all the available silicic acid was converted to silica regardless of whether or not P₅S₃ was present, and therefore, no conclusions can be drawn regarding the effect of P₅S₃ on silica formation at these NaCl concentrations.

To determine whether P₅S₃ was associated with the silica after its formation, the soluble and insoluble fractions from the molybdenum blue assay were analyzed by SDS-PAGE. As shown in Figure 8B, P₅S₃ was exclusively associated with the silica irrespective of the NaCl concentration. Consequently, P₅S₃ was occluded within the mineral matrix in these reactions because the peptide is desorbed from pre-precipitated silica at NaCl concentrations of 600 mM and above (Figure 7).

Circular dichroism spectroscopy was used to determine whether NaCl influenced the structure of P₅S₃ under the conditions showing the most pronounced structural changes in the absence of salt (pH 9.5 and a 1:150 molar ratio of P₅S₃:silicic acid). As shown in Figure 9A, NaCl, in the absence of silicic acid, had little effect on the CD spectrum of P₅S₃ up to a concentration of 1 M at pH 9.5. On the other hand, there were noticeable changes in the CD profile of P₅S₃ as a function of NaCl concentration at a 1:150 ratio of peptide:silicic acid as shown in Figure 9B. In the absence of NaCl, the CD spectrum of P₅S₃ has features resembling α -helical peptides, a positive signal below 200 nm and double minimum bands at 209 and 228 nm. These α -helical features disappeared with the addition of NaCl and at 1 M NaCl the CD spectrum exhibited a major minimum band at ~205 nm and a minor minimum band at 228 nm; a profile similar to the CD spectrum of P₅S₃ at a lower peptide:silicic acid ratio (1:75, Figure 4). So, although NaCl did not interfere with quantitative entrapment of the peptide within the silica precipitate, it did reduce the impact of silica on the secondary structure of P₅S₃. This again is consistent with electrostatic interactions between P₅S₃ and silica altering the secondary structure of the peptide. Even at 1 M NaCl the remaining electrostatic forces must still be strong enough or are complemented by other interaction forces^{44,109} to cause entrapment of the peptide in newly forming silica particles.

Time-resolved CD spectroscopy of P₅S₃ during P₅S₃-induced silica formation

As shown above, secondary structure changes do not take place in P₅S₃ under conditions which do not allow for condensation of orthosilicic acid (as pH 4,5: see Figure 4) but can be observed under conditions that allow silica polymerization. We therefore tested whether the peptide was useful to monitor the dynamics of silica formation at higher pH. To accomplish this, the ellipticity at 197 nm was recorded over the course of the condensation (Figure 10A) and the full CD spectrum compared before and after silica formation (Figure 10B; corresponding HT traces in SI Figure S7). The time-resolved data were recorded at pH 6.5 because silica autopolycondensation is slower here than at higher pH,^{68,88} yet the reaction is complete in a reasonable period of time (~30 min).⁴¹ The silicic acid was introduced either in its monomeric (orthosilicic acid, acidic TMOS hydrolysate) or pre-condensed (15 min at 60 mM silicic acid, pH 9.5) form. Note that at 0 min the ellipticity at 197 nm does not always correspond to the value in the CD spectrum prior to the addition of silicic acid because of the time delay between mixing the reactants and collecting the first time-resolved data point.

The ellipticity at 197 nm was approximately -27 (all ellipticity values in the following discussion are given in [$\times 10^{-3}$, deg*cm²*dmol⁻¹]) at the start of time-resolved CD data acquisition (red, Figure 10A), an intensity similar to that observed in the CD spectrum for P₅S₃ (solid black spectrum, Figure 10B) prior to silicic acid addition. This indicates that there was no immediate interaction between silicic acid and P₅S₃ leading to a structural transition. The ellipticity at 197 nm linearly decreased over the first 10 min to ~-22 followed by a slower rate of decrease to ~-20 over the next 20 minutes. This contrasts to the time-dependent change in CD for pre-condensed silica (blue, Figure 10A) where there was a significant decrease from the initial negative ellipticity (-27) to the first data point at -23. The intensity continued to decrease, meeting the monosilicic acid trace at the 10 minute point; after that the kinetics are the same. The steady-state CD spectra recorded after the time-resolved measurements (red dotted and blue dashed lines, Figure 10B) were essentially identical, suggesting a similar reduction in the disordered conformation irrespective of whether the reaction was initiated with monosilicic acid or pre-condensed silica. Binding to the pre-formed silica (blue trace) appears to be fast, since most of the CD signal change occurs between the mixing of the reactions and measurement of the first data point (ca. 60 s). The subsequent decrease of the CD signal may be due to additional silicic acid forming oligomers after peptide addition. On the other hand, no fast CD signal change is observed upon adding the peptide to silicic acid (red trace), confirming that monomeric silicic acid does not induce a signal change; thus, the subsequent decrease in the CD signal reflects the kinetics of silica formation.

Transient P₅S₃ CD signal change during silica dissolution at globally undersaturated silicic acid concentrations

We also tested whether P₅S₃ was useful to monitor the dynamics of silica dissolution. Previously, we had shown that the rate of appearance of molybdate reactive silicic acid (i.e. silica monomers and dimers, possibly trimers and tetramers^{69,87}) from the dissolution of silica condensate at undersaturated silica concentration is retarded by P₅S₃ (Figure 11A; HT traces in SI Figure S8).⁴⁴ The peptide roughly doubles the time required for complete

dissolution. During the dissolution process, the CD signal of P₅S₃ at 197 nm was monitored (Fig. 11A, grey trace). Note that at 0 min the ellipticity at 197 nm does not correspond to the value in the CD spectrum prior to the addition of silica because of the time delay between mixing the reactants and collecting the first time-resolved data point. The CD spectra of the peptide before adding silica and after complete silica hydrolysis (Fig. 11B) were virtually superimposable. This supports the notion that the hydrolysis product, monomeric silicic acid, either does not bind to P₅S₃ or its binding does not significantly alter the secondary structure of the peptide.

Upon the addition of pre-condensed silica to P₅S₃, the CD signal at 197 nm rapidly (within ca. 60 s prior to recording) jumped from -25 to -20 ($\times 10^{-3}$, deg*cm²*dmol⁻¹) similar to the observation shown in Figure 10A (blue). Over the course of the experiment the molar ellipticity at 197 nm decreased for roughly 2 h and then reversed direction and began increasing in strength, leveling off as the concentration of molybdate-reactive silicic acid reached its steady state. This presumably reflects the hydrolysis of pre-formed silica to silicic acid. But why did the intensity of the negative CD signal at 197 nm decrease for the first 2h until 80 % of the silica was hydrolyzed to molybdate-reactive material? Three possible explanations can be envisioned.

(1) The very rapid binding of P₅S₃ to pre-formed silica, taking place between mixing and the start of the measurement and reflected by the initial signal offset, was followed by a second binding phase. The combination of these two fast processes responsible for the decrease of CD amplitude with the slower process of gradual silica hydrolysis releasing the peptide again, lead to the transient CD signal.

(2) The initial binding equilibrium between the peptide and the silica particles left part of the protein unbound. During the first period of hydrolysis, larger particles were broken down into several smaller ones, creating new silica surfaces available for P₅S₃ binding. This scenario would contradict earlier reports of decondensation experiments under similar conditions that the “number of nanoparticles remained constant throughout much of the dissolution process”.¹¹⁰ Moreover, it is hard to imagine that new surfaces are being generated by breakdown of large silica particles after some 80 % of the material has already been turned into (near-)monomeric silicic acid. Therefore, we consider this scenario to be least likely.

(3) P₅S₃ binds more strongly to, or its secondary structure is more efficiently affected by, small silica particles or even silica oligomers compared to the larger particles that are expected to result from silica precondensation.¹¹¹ It would be conceivable that the concentration of such smaller silica adducts goes through a maximum during the hydrolysis of precondensed silica. This hypothetical scenario is potentially interesting and will be tested in future experiments.

CONCLUSION

The peptide P₅S₃ is able to precipitate silica from a silicic acid solution similar to silaffins and silaffin-derived peptides R5²³ and PL12.²⁵ In solution the structure of P₅S₃ is largely

disordered with some indication of a PPII component at pH 4.5 to 9.5. As P_5S_3 polymerizes silicic acid, the CD spectrum of P_5S_3 gradually adopts features similar to α -helical peptides suggesting structural changes to P_5S_3 triggered by interactions with newly formed oligomers/polymers of silicic acid. Both the binding of P_5S_3 to pre-formed silica and change of its CD spectrum during this binding are weakened by NaCl, suggesting electrostatic interactions are involved. An array of negative charges appears sufficient to trigger the shift of P_5S_3 towards α -helical structure since this shift also occurred when the peptide interacted with SDS in aqueous solution.

The CD signal changes of P_5S_3 can be used to monitor the kinetics of peptide-induced silica condensation or the hydrolysis of pre-formed silica at non-saturated silicic acid concentrations. In the latter experiment, the molar ellipticity of the peptide exhibits a potentially interesting transient signal change. Future studies will show whether this transient signal change is due to differential binding affinities to different silica particles or to differential binding affinities to silica oligomers of different sizes, or whether these silica adducts have a different impact on the CD signal of the peptide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A) Peptide R5

SSKKSGSYSGSKGSKRRIL

B) Peptide PL12

KAAKLFKPKASK

C) Peptide P₅S₃

GSYSRRASL [GKSKKLRRASL]₃ GKLRRASL

Figure 1.

Primary structures of silaffin-derived peptides R5 (19 amino acids), PL12 (12 AA) and P₅S₃ (50 AA), the latter being the subject of this study. The R5 and PL12 peptides are partial sequences of *Cylindrotheca fusiformis* Sil1² and *Thalassiosira pseudonana* Sil3,²⁵ respectively. On the other hand, P₅S₃ is a tailored peptide that can be produced by recombinant bacterial expression⁴¹ and is active in silicic-acid mineralization⁴¹ and – stabilization.⁴⁴ Basic amino acids are highlighted in blue (underlined).

Figure 2.

The amide assigned ^1H - ^{15}N HSQC spectrum for P_5S_3 (~1 mM in 2% acetic acid, pH 2.8, 20 °C) collected at a ^1H resonance frequency of 750 MHz. It was not possible to unambiguously assign the amides of two residues, R6 and R38.

Figure 3.

The carbon assigned ^1H - ^{13}C HSQC spectrum for P_5S_3 (~1 mM, 20 °C) collected at a ^1H resonance frequency of 750 MHz. The spectrum was collected on a sample dissolved in 2% $\text{CD}_3\text{CO}_2\text{D}$ and 98% D_2O to better resolve resonances near the chemical shift of water. The ^{13}C chemical shifts for the aromatic side chain δ and ϵ carbons are at 133.2 and 118.1 ppm, respectively, and were not observed in the shown spectrum because it was optimized for the detection of aliphatic carbons. The primary amino acid sequence of P_5S_3 contains

one tyrosine (Y), five glycine (G), five alanine (A), nine leucine (L), ten serine (S) ten arginine (R), and ten lysine (K) residues.

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Figure 4.

Room temperature (23 °C) CD spectra for P_5S_3 in the presence of silicic acid at different pH values. Spectra were recorded at pH 4.5 (no silica precipitation), 7.5, and 9.5 (both conditions with silica precipitation) with 0.1 mM P_5S_3 and 20 mM buffer (Tris-HCl or sodium acetate). Colors of the spectra indicate different peptide:silicic acid molar ratios (1:0 to 1:600) as indicated in the legends in the respective figures. Measurements were done in a 0.1 mm mountable cuvette. Spectra were recorded after silica-formation was complete (no signal change over time).

Figure 5.

Room temperature (23 °C) CD spectra for phosphorylated P_5S_3 in the presence of silicic acid at various pHs. Spectra were recorded at pH 4.5 (no precipitation of silica), 7.5, and 9.5 (both conditions with silica precipitation) with 0.1 mM P_5S_3 and 20 mM buffer (Tris-HCl or sodium acetate). Colors of the spectra indicate different peptide:silicic acid molar ratios (1:0 to 1:300) as indicated by the legends in the individual panels. Measurements were done in a 0.1 mm mountable cuvette. Spectra were recorded after silica formation was complete (no signal change over time).

Figure 6.

Room temperature (23 °C) CD spectra for P_5S_3 in the presence of various detergents at pH 7.5. A) 1 μ M P_5S_3 without detergent (*black solid line*) and in the presence of 1 mM sodium dodecylsulfate (SDS) (*red dotted*) or dodecyl maltoside (DM) (*blue dashed*). Data was collected using a cuvette with a 10 mm path length. B) 100 μ M P_5S_3 in the absence (*black solid*) or presence (*red dotted*) of 34.7 mM (1 % w/v) SDS. Data was collected using a cuvette with a 0.1 mm path length.

Figure 7.
SDS-PAGE analysis of the NaCl desorption of P₅S₃ from biomimetically prepared silica.
Desorption was performed in 20 mM Tris buffer at pH 7.5.

Figure 8.

Impact of NaCl on P₅S₃-silica co-precipitation at pH 7.5. A) Amount of silicic acid, quantitated by molybdenum blue assay, in the pellet fractions of reactions conducted without (*black bars*) or with (*white bars*) P₅S₃. The total amount of silicic acid in each reaction was 3 µmol at a concentration of 30 mM. B) Supernatant (*S*) and pellet (*P*) fractions from the P₅S₃-silica NaCl assay were hydrolyzed and then analyzed for P₅S₃ by SDS-PAGE.

Figure 9.

CD spectra for P_5S_3 (100 μ M) with various concentrations of NaCl and silicic acid at pH 9.5. Spectra were recorded with 0 mM (*black solid line*), 150 mM (*red dotted*) and 1,000 mM (*blue dashed*) NaCl. The optical density of samples containing 1,000 mM NaCl increased rapidly below 198 nm (compare HT traces in SI Figure S6), therefore, data is only shown up to this wavelength. A) No silicic acid. B) 15 mM silicic acid. Spectra were recorded after silica-formation was complete (no further signal change over time).

Figure 10.

A) Time-resolved CD signal of P_5S_3 recorded at 197 nm during silica condensation. Data were collected at pH 6.5 in the presence of 30 mM silicic acid and 100 μ M P_5S_3 . Red = addition of orthosilicic acid; Blue = addition of pre-condensed silicic acid. B) Complete CD spectra for P_5S_3 recorded prior to silicic acid addition (black solid line) and 30 minutes after the addition of either orthosilicic acid (red dotted) or pre-condensed silica (blue dashed).

Figure 11.

A) Time-resolved CD signal at 197 nm (right axis) of P_5S_3 in response to the dissolution of silica condensate at undersaturated silica concentration (grey). Data were collected at pH 7.5, 0.5 mM Tris, in the presence of pre-condensed silica with a global silicic acid concentration of 1 mM silicic acid and 1 μM P_5S_3 . Squares and triangles depict the concentration of molybdate-reactive silica (left axis) in the absence and presence of P_5S_3 ,

respectively, as previously reported in Spinthaki et al.⁴⁴ **B)** Steady-state CD spectra for P_5S_3 recorded prior to (*black solid*) and 480 min after silica addition (*red dotted*).

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Table 1

Range of carbon-13 chemical shifts for P₅S₃ collected in 2% acetic acid, pH 2.8, at 20 °C. The first column lists the amino acids with their abundance in P₅S₃ (number of residues) in round brackets. Values in squared brackets correspond to random coil carbon chemical shift values taken from CNS (cns_solve_1.1).

	C _α	C _β	C _γ	C _(other)
Y(1)	58.1 [58.1]	38.9 [38.8]	–	133.2(δ1); 118.1(δ2)
A(5)	52.8-52.9 [52.3]	19.3 [19.0]	–	–
G(5)	45.2 [45.1]	–	–	–
L(9)	55.1-56.0 [55.1]	42.2-42.7 [42.3]	27.0	24.1(δ1); 23.3-23.4(δ2)
S(10)	58.1-58.5 [58.2]	64.0-64.1 [63.2]	–	–
R(10)	56.1-56.4 [56.1]	31.0 [30.3]	26.9	43.4(δ)
K(10)	56.1-56.4 [56.5]	33.0-33.3 [32.5]	24.7	29.0(δ); 42.1(e)