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A Comparative Study of Secondary Structure and Interactions of the R5 Peptide in Silicon Oxide and Titanium Oxide Co-precipitates using Solid-state NMR Spectroscopy

Erika L. Buckle, Adrienne Roehrich, Branden Vandermoon, Gary P. Drobny

Department of Chemistry, University of Washington, Box 351700, Seattle, Washington 98195, United States

Abstract

A biomimetic, peptide-mediated approach to inorganic nanostructure formation is of great interest as an alternative to industrial production methods. To investigate the role of peptide structure on silica (SiO_2) and titania (TiO_2) morphologies, we use the R5 peptide domain derived from the silaffin protein to produce uniform SiO_2 and TiO_2 nanostructures from the precursor silicic acid and titanium bis(ammonium lactato)dihydroxide, respectively. The resulting biosilica and biotitania nanostructures are characterized using scanning electron microscopy. To investigate the process of R5-mediated SiO_2 and TiO_2 formation, we carry out 1D and 2D solid-state NMR (ssNMR) studies on R5 samples with uniformly ^{13}C - and ^{15}N -labeled residues to determine the backbone and side-chain chemical shifts. ^{13}C chemical shift data are in turn used to determine peptide backbone torsion angles and secondary structure for the R5 peptide neat, *in silica*, and *in titania*. We are thus able to assess the impact of the different mineral environments on peptide structure, and we can further elucidate from ^{13}C chemical shifts changes the degree to which various side-chains are in proximity to the mineral phases. These comparisons add to the understanding of the role of R5 and its structure in both SiO_2 and TiO_2 formation.

Graphic Abstract

1. Introduction

Nanostructured silica (SiO_2) and titania (TiO_2) are versatile materials with widespread applications including use as pigments, insulators, textile coatings, catalysts, medical devices, and solar cell components.^{1–10} Each specific application requires a uniquely tuned set of physical properties for the material, such as particle size, crystallinity, and morphology; thus synthetic routes that allow for fine-tuned control are necessary.¹¹ However, despite the pervasive use of both SiO_2 and TiO_2 in the industrial and commercial sectors, production is not optimal; the anthropogenic synthesis often requires extreme temperature, pressure, and pH, while maintaining limited control over crystallinity and morphology.^{12–16}

In contrast, biological organisms produce inorganic materials through a process known as biomineralization. Diatoms, a unicellular microalgae, produce SiO_2 under mild conditions from the precursor silicic acid, resulting in material of tailored size and structure.^{17–23} One of the most widely studied silicifying organisms is the marine diatom *Cylindrotheca fusiformis*. The protein implicated in regulating the process of SiO_2 biomineralization is silaffin sil1p. The primary structure of sil1p has a repetitive peptide sequence between residues 108 and 271, composed of seven units.²² The fifth of these repeating units, a 19 amino acid peptide known as R5 (SSKKS₁SGSYSGSKGSKRRIL), has the ability to precipitate SiO_2 nanospheres in a manner similar to its parent silaffin without the need for post-translational modifications.²² Mutation studies by Knecht and Wright²² have shown that R5 self-assembles to produce SiO_2 , and that the C-terminal RRIL motif is integral to this process. The authors suggest that the RRIL motif encourages a micellar self-assembly due to the arginine's guanidinium groups in close proximity to hydrophobic leucine and isoleucine residues.²² Although this claim has been questioned recently by Senior et. al.,²³ whose DLS study found no evidence of aggregate formation in solutions of R5²³, mutation studies by Lechner and Becker²⁴ support the need for the RRIL motif, however not

necessarily at the C-terminal position or in the native order. Lechner and Becker also demonstrated the importance of the amino groups of the lysine residues in SiO₂ formation. Their study found the structure of the resulting SiO₂ is dependent on the number as well as position of lysine residues within the R5 sequence.²⁴ An alternative mechanism of R5-SiO₂ precipitation involves R5 self-assembly driven by salt bridges that form between the guanidine groups of arginine residues and phosphate anions. Cationic amino groups then bring silicic acid and SiO₂ particles into close proximity, which promotes the condensation reaction.^{22,24} Additionally, peptides rich in lysine and various cationic polymers have been used to facilitate SiO₂ production with various morphologies.^{25,28}

Biomimetic approaches are also being applied to the synthesis of non-biological materials, specifically TiO₂, using the precursor titanium bis(ammonium lactato)dihydroxide (TiBALDH).^{29–36} An example of such an approach is that of Sewell and Wright,³⁶ who have shown that R5 can produce TiO₂ nanospheres under mild conditions when introduced to solutions containing TiBALDH.

Although much effort has been put forth to characterize the mechanism of R5's role in SiO₂ precipitation, significantly less work has been done to elucidate the role it plays in TiO₂ precipitation. Elucidating the structure of R5 within both SiO₂ and TiO₂ co-precipitates of similar morphology is an important first step in understanding how R5 directs the formation of TiO₂. By identifying the interactions of the amino acid residues of R5 with the surrounding SiO₂ or TiO₂, and the degree to which the structural principles that underlie the TiO₂-precipitating activity of R5 resemble or do not resemble the SiO₂-precipitating activity of R5, we can form comparisons between the two systems. By comparing the lesser-studied R5-TiO₂ to R5-SiO₂, we augment our understanding of the R5-TiO₂ system.

Here, we use solid state ¹³C NMR to characterize the structures of neat R5, R5 co-precipitated with TiO₂ (i.e. R5-TiO₂) and R5 co-precipitated with SiO₂ (i.e. R5-SiO₂), to obtain information on the structural changes that R5 undergoes within each mineral environment. We use chemical shift data and TALOS-N³⁸-predicted torsion angles to determine the structures of the backbone for R5 neat, R5-SiO₂, and R5-TiO₂. We compare the structure of R5 within the TiO₂ co-precipitate to that within the SiO₂ co-precipitate, providing important insights into the behavior of R5 during mineral precipitation. The perturbation of side-chain ¹³C chemical shifts are used to obtain information on the proximity of various amino acid side-chains in R5 to the surrounding mineral as well as the role that various side-chains might play in mineral precipitation. Finally, the conclusions drawn from our structural study of R5 in SiO₂ and TiO₂ co-precipitates are compared to several recent solid-state NMR studies of peptides and proteins in biosilica composites.

2. Experimental

2.1. Materials.

All natural and uniformly labeled ¹³C and ¹⁵N amino acids were purchased from Sigma Aldrich (St. Louis, MO). Preloaded Fmoc-protected Wang resin was purchased from EMD Millipore (Billerica, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) and used without purification.

2.2. Peptide Synthesis.

Peptides were synthesized on a CEM Liberty Blue peptide synthesizer using a standard 9-fluorenylmethoxycarbonyl (Fmoc) and *tert*-butyl protection scheme. Preloaded Fmoc-protected Wang resin was used for solid phase synthesis. Peptides were cleaved from the resin in a 10 mL solution of 95:2.5:2.5 trifluoroacetic acid: triisopropylsilane (TIS): water mixture per 1.0 gram of peptide/resin. The resulting filtrate was added dropwise into cold *tert*-butyl methyl ether, followed by centrifugation and three rinses of the resulting solids with 40 mL of cold *tert*-butyl methyl ether. Peptides were purified using RP-HPLC (Varian ProStar HPLC, Alltima WP C4 column, 5 mL/min, eluent A: water with 0.2% TFA, eluent B: acetonitrile with 0.2% TFA), using a gradient of 15–35% B over 40 minutes. Chromatograms were generated by observing the UV absorbance at 274 nm, and the analyte was verified by mass spectrometry. The fractions were then lyophilized, resulting in the pure peptide.

2.3. SiO₂ Precipitation.

Orthosilicic acid (Si(OH)₄) was freshly made before each precipitation by dissolving 0.15 mL tetramethyl orthosilicate in 0.85 mL 1 mM HCl to form 1 M Si(OH)₄. Orthosilicic acid (100 µL per 5 mg R5) was added to a solution of R5 dissolved in phosphate-citrate buffer (1.00 mL, 100 mM, pH=7.0) and vortexed. The solution was incubated for 5 minutes at room temperature. The precipitated R5-SiO₂ was separated from the mixture via centrifugation at 15000 x g for 10 minutes. The resulting precipitate was rinsed with Millipore water 3 times and dried *in vacuo*.

2.4. TiO₂ Precipitation.

TiBALDH (100 µL per 5 mg R5, 1M) was added to a solution of R5 dissolved in phosphate-citrate buffer (1.00 mL, 100 mM, pH=7.0) and vortexed. The solution was incubated for 30 minutes at room temperature. The precipitated R5-TiO₂ was separated from the mixture via centrifugation at 15000 x g for 10 minutes. The resulting precipitate was rinsed with Millipore water 3 times and dried *in vacuo*.

2.5. SiO₂ and TiO₂ Morphology Characterization.

SEM images were taken on a FEI Sirion XL30 scanning electron microscope operating at variable voltages. Precipitates were dispersed onto a carbon tap, mounted on aluminum studs, and sputter-coated for 60–90 seconds with Au/Pd.

2.6. Solid-State NMR.

All solid-state NMR experiments were conducted using a 16.4 T magnetic field (proton resonant field of 700.18 MHz) on a Bruker Avance III spectrometer fitted with a ¹H {¹³C, ¹⁵N} 3.2 mm MAS probe. The ¹³C NMR signal was enhanced using cross-polarization (CP) with a ¹H-¹³C contact time of 1.1 ms, and a magic angle spinning (MAS) rate of 10–15 kHz ± 5Hz was maintained with a Bruker MAS controller unit. One-dimensional ¹³C CP-MAS experiments were performed with a proton 90 degree pulse time of 2.75 µs and a recycle delay of 2s. The number of scans for the neat R5 and the R5-TiO₂ complex ranged from 2k–32k. To obtain the resolution needed to confidently assign all the chemical shifts in the

R5 samples, 2D ^{13}C - ^{13}C dipolar assisted rotational resonance (DARR)³⁹ experiments were performed. The 2D spectra were collected with 30 or 60 ms mixing times and a recycle delay of 1.5s, with 128 points in the indirectly detected dimension and 512 points in the directly detected dimension. All chemical shifts reported were indirectly referenced to tetramethylsilane (TMS) in the solid-state using adamantane ($\delta=38.48$).⁴⁰

3. Results

3.1. Peptide-Mineral Precipitation.

Figure 1 displays SEM images of the R5-TiO₂ (Fig. 1a) and R5-SiO₂ (Fig. 1d) co-precipitates. Both peptide-mineral co-precipitates are approximately spherical, but with varying average diameters; an average diameter of 734 ± 180 nm for the R5-TiO₂ co-precipitates and 594 ± 93 nm for the R5-SiO₂ co-precipitates. The size distribution histograms for both the R5-TiO₂ co-precipitates (Figure 1b) and R5-SiO₂ co-precipitates (Figure 1e) are shown. The spherical morphologies of the R5-TiO₂ particles are consistent with those observed by Sewell and Wright³⁶, although the average size of the R5-TiO₂ particles we observe is larger than they report. The average size of the R5-SiO₂ particles we observe is consistent with those observed in the literature.^{18,24,36}

Figures 1c and Figure 1f illustrate the differences in R5 activity between the TiO₂ and SiO₂ systems. TiO₂ is only precipitated above 1 mM R5, while SiO₂ is precipitated above 0.5 mM R5. While both systems appear cooperative in nature based on the shape of the curves, it is more pronounced for the R5-TiO₂ system, suggesting that in order to induce TiO₂ formation, more R5 molecules must aggregate. This is consistent with the larger particle sizes seen for R5-TiO₂ co-precipitates, see Figure 1b and 1e. SiO₂ precipitation levels out at a much lower concentration of R5 than TiO₂ precipitation, which also produces more product at the highest concentration tested.

Both R5-TiO₂ and R5-SiO₂ consist of R5 peptide that is embedded in a mineral nanostructure. Inductively coupled plasma optical emission spectrometry (SI Table 1) reveals that phosphorus is present in both mineral co-precipitates after extensive washing of the precipitated samples. The only source of phosphorous is phosphate anions present in the buffer, so logically it must be attributed to phosphate embedded within the co-precipitate, indicating an incorporation of the phosphate-citrate buffer within the co-precipitates. Examining the secondary structures of R5-TiO₂ and R5-SiO₂ can provide insight into how R5 interacts with the phosphates present in each mineral environment.

3.2. ^{13}C Chemical Shift Assignments for R5 neat, R5-TiO₂, and R5-SiO₂ Peptide.

^{13}C chemical shift assignments for R5 neat, R5-TiO₂, and R5-SiO₂ are provided as Supplementary Information (SI Tables 2–7). To obtain site specific chemical shift assignments, both one-dimensional ^{13}C CPMAS and two-dimensional (2D) dipolar assisted rotational resonance (DARR) ^{13}C - ^{13}C experiments were performed on R5-TiO₂. We were able to use DARR to resolve and assign most of the ^{13}C spectrum of R5 in TiO₂ containing up to three uniformly ^{13}C - and ^{15}N -enriched amino acids. To assign the chemical shifts for the entire peptide, seven isotopically enriched samples were analyzed, as shown in Table 1.

The only amino acids not assigned by this study were S7, Y8 and the C-terminal L19. Roehrich and Drobny¹⁸ conducted the same set of experiments on the same seven enriched samples for both neat R5 peptide and on the R5-SiO₂ co-precipitate, to which comparisons will be made.¹⁸

Figure 2 shows a typical DARR spectrum obtained from the selectively ¹³C labeled peptide R5-GSKc TiO₂, which demonstrates the assignment of the ¹³C spins in G 13, S14, and K15. The horizontal and vertical lines delineate cross-peaks which indicate networks of dipolar-coupled ¹³C spins. To ensure the observation of only intra-residue cross-peaks, DARR spectra were taken with mixing times of 30 ms and 60 ms. In a few cases, however, unique chemical shifts could not be assigned; for example, it was not possible to assign the two $\delta^{13}\text{C}$ shifts for I18.

Changes in chemical shift (ΔCS) for backbone and side-chain ¹³C nuclei in the neat versus in co-precipitated samples occur to varying degrees at sites along the peptide backbone and side-chains. The ¹³C chemical shift perturbations of the backbone ¹³CO, ¹³C α , and ¹³C β chemical shifts are associated with a change in secondary structure as a result of precipitation with SiO₂ or TiO₂. To study systematically the degree to which R5 peptide ¹³C chemical shifts have been perturbed upon co-precipitation with SiO₂ and TiO₂, ΔCS values are obtained by subtracting the chemical shift of the ¹³C spin in neat R5 from the corresponding ¹³C spin in the R5-TiO₂ or R5-SiO₂ co-precipitate. A positive ΔCS indicates a downfield perturbation of the chemical shift (higher ppm, less shielded) in the co-precipitate versus the neat solid peptide, while a negative ΔCS indicates an upfield perturbation of the chemical shift (lower ppm, more shielded). Bar charts of backbone ΔCS values are shown in Figure 3. In both R5-SiO₂ and R5-TiO₂, significant perturbations are observed in the S1, S2, and I18 ¹³CO shifts, the K3, K4, and S5 ¹³C α shifts, and the S5, S14, and R17 ¹³C β shifts. The chemical shift perturbations are similar for the S1 ¹³CO, S2 ¹³CO, K4 ¹³C α shifts in R5-TiO₂ and R5-SiO₂, indicating similar structural changes in these regions. The perturbations in the I18 ¹³CO, the S5 ¹³C β , and the R17 ¹³C β are larger in magnitude for R5-SiO₂ than for R5-TiO₂, while the perturbations in the I18 ¹³CO, K3 ¹³C β , and the S14 ¹³C β are larger in magnitude in R5-TiO₂ than in R5-SiO₂.

Bar charts of side-chain ΔCS values are shown in Figure 4 for both the R5-SiO₂ and R5-TiO₂ systems. In R5-TiO₂, $\Delta\text{CS} > 6$ ppm for the ¹³C ϵ , while in R5-SiO₂ ΔCS values for ¹³C γ , ¹³C δ , and ¹³C ϵ range from -3 ppm to -5 ppm. In previous ssNMR studies of poly-lysine adsorbed onto SiO₂,³⁷ an upfield perturbation of lysine ¹³C ϵ spin chemical shift was attributed to proximity to the negatively-charged SiO₂ surface. Downfield perturbation of the side-chain ¹³C spin chemical shifts has been similarly interpreted as indicating proximity to positive charge centers in mineral surfaces.³⁷ Side-chain chemical shift trends in R5-SiO₂ and R5-TiO₂ suggest similar close associations between amino acid side-chains and the inorganic oxide components. Based on these data, K3 is likely interacting with the mineral in both the SiO₂ and TiO₂ systems, but the side-chain of K3 in R5-SiO₂ shows a greater degree of mineral exposure all along the side-chain. In contrast, the ¹³C γ /¹³C δ /¹³C ϵ spins for K12 and K15 in R5-SiO₂ show much smaller or negligible ΔCS . However we observe a significant upfield shift for ¹³C δ of K15 in R5-TiO₂, which is not present in R5-SiO₂.

As mentioned earlier, previous work has shown the arginine residues in R5 are necessary for peptide self-assembly, either by the arginine side-chain's involvement with adjacent hydrophobic residues to effect micelle-like self-assembly²² or by formation of phosphate salt bridges between the arginine guanidinium groups.²⁴ The occurrence of guanidinium-phosphate interactions would be expected to perturb the electronic environment of the arginine side-chains. Accordingly, significant chemical shift perturbations are observed in R5-SiO₂ and R5-TiO₂ for ¹³C γ , ¹³C δ , and ¹³C ζ in R16 and R17.

3.3. Comparison of Local Peptide Structure in R5 neat, R5-TiO₂, and R5-SiO₂.

The chemical shifts obtained from the ¹³CO, ¹³Ca, and ¹³C β spins in neat R5, R5-TiO₂, and R5-SiO₂ DARR spectra were used to produce TALOS-N³⁸ input files. TALOS-N³⁸ output files consist of predicted torsion angles, which were used to visualize the changes in secondary structures for both R5 neat, R5-TiO₂, and R5-SiO₂ peptides using Chimera⁴¹ (shown in Figure 5). Φ/Ψ torsion angle values for R5-SiO₂ and R5-TiO₂ generated by TALOS-N³⁸ are shown in the Supplementary Information (SI Tables 8–10). Based on the TALOS-N³⁸ analysis of ¹³CO, ¹³Ca, and ¹³C β chemical shifts, the majority of Φ torsion angles occur between –80 degrees and –150 degrees in the neat R5 peptide. Furthermore, the majority of Ψ torsion angles occur between 40 and 180 degrees. Outliers occur at K4-S5, and near the glycine residues G6-S7 and G10-S11. Aside from these amino acids, the majority of the peptide chain of the neat R5 peptide from S7 to I18 is in an extended conformation. The observed secondary structures for R5 in TiO₂ and SiO₂ are relatively similar and deviate from the neat structure in similar ways. In particular, R5 in SiO₂ and in TiO₂ both have significant changes in Φ values at the K3-K4 and K4-S5 positions. There is also a significant change in both Φ/Ψ at G13-S14. In general, most of the aforementioned large torsion angle changes in R5-TiO₂ are matched by R5-SiO₂, with the exception of K3-K4 where the change in the Φ torsion angle in SiO₂ is not as large as in TiO₂. As a result of these torsion angle changes, both forms of mineral-associated R5 adopt extended structures of the N-terminal S2-K3-K4-S5 segment, but deviate from an extended conformation in the G6-I18 region.

In both R5-SiO₂ and R5-TiO₂, perturbations in the backbone torsion angles near S2, K3, K4, and S5 are accompanied by a large δ CS for the side-chain of K3 and to a somewhat lesser extent in the side-chain of K4 (See Figure 4a–c). It is also interesting to note that the δ CS for the ¹³C ζ spins in the side-chains of R16 and R17 in R5-SiO₂ and R5-TiO₂ (see Figure 4d) are also accompanied by structural changes of the backbone in the immediate vicinity of these residues, but appear to be more modest than the structural changes that occur at and around K3 and K4. Although chemical shifts of side-chain ¹³C spins beyond ¹³C β are not correlated with protein secondary structure, a large δ CS for these more distal sites do reflect changes in electronic environment associated with peptide precipitation with SiO₂ and TiO₂. While we cannot quantify the origins of the δ CS for ¹³C γ /¹³C δ /¹³C ϵ /¹³C ζ spins to the degree that we can quantify the δ CS for ¹³CO/¹³Ca/¹³C β , we can combine our δ CS results with data from other studies of peptide interactions with SiO₂ and TiO₂ to obtain insight into how R5 interacts with SiO₂ and TiO₂ precursors. This will be discussed further in the next section

4. Discussion:

Solid-state NMR has been used extensively to study the structure and interactions of peptides in biosilica composites.^{18,42–46} This is due in part to the occurrence of spin $\frac{1}{2}$ nuclei in the peptide side-chains, and the occurrence of ^{29}Si , a spin $\frac{1}{2}$ species with 4% natural abundance, in the mineral component, which together enable the study of peptide-silica interactions by solid-state NMR heteronuclear correlation methods. The interactions of peptides with the non-biological oxide TiO_2 are also of great interest, but solid-state NMR studies of such interactions is complicated by the fact that recoupling to ^{47}Ti and ^{49}Ti nuclei is impractical, making the heteronuclear correlation approach used in peptide-silica studies infeasible. In addition to a comparative study of R5 secondary structure in SiO_2 versus TiO_2 , we have also used ^{13}C chemical shifts to probe how the R5 peptide interacts with SiO_2 and TiO_2 in the respective R5-mineral composites, as well as the degree to which the R5 peptide interacts with other components of the composite, including phosphate ions and other peptides.

The presence of the spin $\frac{1}{2}$ nucleus ^{29}Si in SiO_2 makes possible direct determination of peptide-mineral contacts using heteronuclear NMR correlation methods, which in turn provides a means to interpret directly ^{13}C trends for R5 in SiO_2 and to infer environmental origins for similar ^{13}C trends for R5 in TiO_2 . Several NMR studies have shown that molecules containing amine and/or amino groups make close contact with SiO_2 surfaces via these functional groups. Schmidt and coworkers⁴² used $^{15}\text{N}\{^{29}\text{Si}\}$ REDOR to show that monomeric amino acids interact with SiO_2 surfaces via the NH_3^+ functional group. Brunner and coworkers⁴³ used ^1H - ^{13}C - ^{29}Si double CP-based HETCOR NMR techniques to show that organic polyamines in biosilica from the diatom species *Thalassiosira pseudonana* are closely associated with SiO_2 . And recently Goobes and coworkers⁴⁴ used ^1H - ^{29}Si HETCOR experiments in combination with NMR signal enhancement via Dynamic Nuclear Polarization (DNP) to show that lysine side-chain amine protons in the peptide PL12 (KA AKL FKP KASK) co-precipitated with SiO_2 are also in close contact with the SiO_2 . These NMR studies generally support the hypothesis that the ^{13}C observed in the K3 and K4 side-chains of R5 in SiO_2 originate from interactions between these side-chains and the negatively charged SiO_2 surface. The general absence of significant ^{13}C in the side-chains of K12 and K15 in R5- SiO_2 in turn indicate absence of interactions with SiO_2 at these sites. Furthermore, it is possible that the secondary structural rearrangements observed in the N-terminus of R5- SiO_2 co-precipitates, occur to optimize contacts between K3, K4, and the adjacent SiO_2 interface.

Significant ^{13}C trends are also observed for the $^{13}\text{C}\zeta$ spins of R16 and R17 in SiO_2 . The upfield shift for the $^{13}\text{C}\zeta$ spins in the arginine guanidinium groups may be due to SiO_2 contacts, but the absence of similar contacts at the adjacent K15 argues against this scenario. Given the affinity of guanidinium groups for phosphate ions,^{46,47} the fact that R5 and other unmodified silaffin peptides will not precipitate SiO_2 in the absence of phosphate,⁴⁸ and the apparent role played by arginine residues in R5 self-assembly via phosphate bridges,²⁴ it is reasonable to assume that the upfield shifts we observe for the $^{13}\text{C}\zeta$ spins in R16 and R17 in R5- SiO_2 are due to interactions with phosphate ions.

The ^{13}C CS map for R5-TiO₂ is somewhat more complicated to interpret than that of R5-SiO₂. Studies of peptide binding to oxidized titanium surfaces find that the surface oxide film consists of amorphous and non-stoichiometric TiO₂.^{49,50} In contrast to surface SiO₂ where surface hydroxyl groups have pK_a's of 2.8, and thus exist predominantly as O⁻ at neutral pH, hydroxyl groups in TiO₂ have pK_a's of 2.9 and 12.7, and thus exist as O⁻ and OH₂⁺ at neutral pH.⁴⁹⁻⁵¹ The fact that amorphous surface films of TiO₂ will accumulate both positively charged lysine⁵² and negatively charged aspartate⁵³ supports the amphoteric nature of TiO₂. Thus, while ^{13}C spins in the distal positions of peptide side-chains interact predominantly with acidic hydroxyl groups in SiO₂ at neutral pH, in TiO₂ peptide side-chains are exposed to a more complicated electronic environment consisting of both acidic and basic hydroxyl groups. Accordingly, there are similarities and differences in the ^{13}C CS maps for ^{13}C spins located at the distal positions of lysine and arginine side-chains in R5-SiO₂ versus R5-TiO₂. As in R5-SiO₂, structural changes in the N-terminus of R5-TiO₂ correlate with the ^{13}C CS in the K3 and K4 side-chains, indicating that a secondary structural change may occur to optimize contacts between peptide side-chains and TiO₂ precursors. Also, as in R5-SiO₂, the $^{13}\text{C}\zeta$ spins in the guanidinium groups of R16 and R17 in R5-TiO₂ display significant ^{13}C CS but only modest structural changes.

There are some differences between the ^{13}C CS observed for the $^{13}\text{C}\epsilon$ lysine spins of K3 and K4 in R5-TiO₂ versus R5-SiO₂ that indicate differences in the two peptide-mineral interfaces. For example, while $^{13}\text{C}\epsilon$ of K3 shows a large ^{13}C CS of almost -6 ppm in SiO₂, the same spin has a ^{13}C CS of +6 ppm in TiO₂. Downfield chemical shift changes observed for ^{13}C spins in peptides adsorbed onto inorganic surfaces have been attributed to proximity to positive charge centers.⁴² A possible explanation for the downfield shift observed for the $^{13}\text{C}\epsilon$ in TiO₂ is that the distal portion of the lysine side-chain of K3 is laterally oriented relative to the TiO₂ surface, exposing the NH₃⁺ group of the lysine side-chain to an acidic hydroxyl group, while the $^{13}\text{C}\epsilon$ is oriented closer to a basic hydroxyl group or a Ti⁴⁺ ion. The same orientation of the lysine side-chain on a SiO₂ surface would result in exposure of both the NH₃⁺ and the $^{13}\text{C}\epsilon$ to acidic hydroxyl groups and an upfield shift of $^{13}\text{C}\epsilon$ would result, as observed. The $^{13}\text{C}\zeta$ spins in R16 and R17 also have a more complicated ^{13}C CS pattern in R5-TiO₂ than R5-SiO₂. Unlike the R5-SiO₂ system, R5 will precipitate TiO₂ in the absence of phosphate, albeit at lower levels.³⁶ This raises the possibility that there are alternative mechanisms for the interaction of R5 with TiBALDH, and the upfield and downfield shifts observed for the $^{13}\text{C}\zeta$ spins in R16 and R17 may result from interactions between guanidinium groups and phosphate in addition to direct interactions with TiO₂ precursors.

5. Conclusion:

This is the first solid-state NMR study of the structure and interactions of the silicifying peptide R5 in both in a biosilica composite, i.e. R5-SiO₂, and in a non-biological metal oxide composite, i.e. R5-TiO₂. This study has produced site-specific chemical shifts assignments for the majority of ^{13}CO , $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ spins in the neat peptide, and for the peptide in the two inorganic oxides, enabling a comparative study of R5 structure in all three environments. Numerous studies conclude that lysine residues in R5 are necessary for interactions with SiO₂/TiO₂ precursors. Thus the curvatures induced in the peptide structures

in the two mineral environments, which we derive from a TALOS-N³⁸ analysis of the ¹³CO, ¹³Ca, and ¹³Cβ chemical shifts, may function to maximize exposure of the K3 and K4 side-chains at the surface of the peptide aggregate to SiO₂/TiO₂ precursors.

This study has also acquired chemical shift assignments for the majority of the side-chain ¹³C spins in neat R5 and for R5-SiO₂ and R5-TiO₂. The ¹³C trends for the lysine ¹³Cε spins and the arginine ¹³Cζ spins in the R5-SiO₂ precipitate are interpreted using a model in which the N-terminal lysines are exposed at the surface of the peptide aggregate while the lysine ¹³Cε spins nearer the C-terminus show smaller ¹³C, suggesting K12 and K15 are removed from SiO₂ contacts with the arginine ¹³Cζ spins, which show ¹³C that most likely indicate contacts with phosphate anions. While the secondary structure of R5 in TiO₂ resembles the structure of the peptide in SiO₂, ¹³C trends observed for lysine ¹³Cε and arginine ¹³Cζ spins in R5-TiO₂ indicate contact with a more complicated electrostatic environment, perhaps arising from proximity of some of these side-chains to acidic hydroxyl groups and others to positive charge centers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

(a) SEM image of R5-TiO₂ co-precipitates showing spherical morphologies with a mean diameter of 734 ± 180 nm. (b) Size distribution histogram for R5-TiO₂ co-precipitates. (c) TiO₂ produced as a function of R5 concentration. The concentration of TiBALDH was held constant at 0.1 M. Data are a mean from two independent repeats. (d) SEM image of R5-SiO₂ co-precipitates showing spherical morphologies with a mean diameter of 596 ± 93 nm. (e) Size distribution histogram for R5-SiO₂ co-precipitates. (f) SiO₂ produced as a function of R5 concentration. The concentration of Si(OH)₄ was held constant at 0.1 M. Data are a mean from two independent repeats.

Figure 2:
 ^{13}C - ^{13}C DARR spectrum for the R5-GSKc in the TiO_2 co-precipitate. Vertical and horizontal lines indicate assignments of ^{13}C spins in G13, S14, and K15.

Figure 3:

Backbone CS plots showing chemical shift perturbations for (a) ^{13}CO shifts, (b) $^{13}\text{C}\alpha$ shifts, and (c) $^{13}\text{C}\beta$ shifts. CS for R5 co-precipitated with TiO_2 (black) and SiO_2 (gray) are in reference to the neat peptides. Positive changes indicate a downfield shift while negative changes indicate an upfield shift relative to the neat peptide.

Figure 4:

Side-chain CS plots showing chemical shift perturbations for (a) $^{13}\text{C}\gamma$ shifts, (b) $^{13}\text{C}\delta$ shifts, (c) $^{13}\text{C}\epsilon$ shifts, and (d) $^{13}\text{C}\zeta$ CS for R5 co-precipitated with TiO_2 (black) and SiO_2 (gray) are in reference to the neat peptides. Positive changes indicate a downfield shift while negative changes indicate an upfield shift relative to the neat peptide.

Figure 5: Chimera⁴¹-generated models of (a) R5-neat, (b) R5-TiO₂, and (c) R5-SiO₂ using TALOS-N³⁸-generated torsion angles from experimentally obtained chemical shifts. The models are shown with Φ of -119 and Ψ of 113 (values consistent with the Φ/Ψ angles of surrounding residues and localized secondary structure) for the S7, Y8, and L19 positions, since the backbone chemical shifts for these residues were not determined in this study.

Table 1:

R5 Samples in Which Uniformly ^{13}C - and ^{15}N -Enriched Amino Acids Were Incorporated.

Sample Name	Label Position
R5-S	S*SKKSGSYSGSKGSKRRIL
R5-SK	SS*K*KSGSYSGSKGSKRRIL
R5-KSG	SSKK*S*G*SYSGSKGSKRRIL
R5-GSK _m	SSKKSGSYSG*S*K*GSKRRIL
R5-GSK _c	SSKKSGSYSGSKG*S*K*RRIL
R5-R	SSKKSGSYSGSKGSKR*RIL
R5-SRI	SSKKSGSYS*GSKGSKRR*I*L