Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information

Ordered silica mineralization by regulating local reaction conditions

Takaaki Hatanaka*, Masataka Ohashi, Nobuhiro Ishida*

Toyota Central R&D labs Inc., 41-1, Nagakute 480-1192, Japan

* To whom correspondence should be addressed.
E mail : takaaki-h@mosk.tytlabs.co.jp, n-ishida@mosk.tytlabs.co.jp

Materials

A siloxene nanosheet (SiN) was prepared according to a previous report. Briefly, Zintl Phase $CaSi_2$ was immersed in 100 mL of 37% HCl at 0°C. The mixture was stirred continuously for 2 days under an Ar atmosphere. After filtration and rinsing with EtOH, the Weiss siloxane $Si_6H_3(OH)_3$ solid product was obtained. Si nanoparticles and SiO_2 nanoparticles were purchased from Sigma-Aldrich (St. Louis, MO). Tetramethyl orthosilicate (TMOS) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan)

Each nanomaterial was washed with a 1:1 mixture of methanol and acetone, and then ultrasonicated for 10 min using a Bioruptor UCD-250 ultrasonicator (Cosmo Bio, Tokyo, Japan). After washing twice with isopropanol, the samples were dispersed in Tris-buffered saline (TBS; 50 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween 20, and then washed twice more with isopropanol. After each washing step, centrifugation was carried out at 6,000 rpm for 10 min.

Phage display system and peptide screening

T7 phage libraries displaying $SCX_{9\text{-}12}CS$ random peptides, where X represents the randomized amino acids generated by mixed oligonucleotides on a DNA template, were constructed using the T7Select 10-3b system (Merck Millipore, Billerica, MA). The T7 phage displays an average of 5–15 copies of the peptide on the phage particle surface. Treated SiN (500 μg) were added to the constructed $SCX_{9\text{-}12}CS$ libraries (5×10^{10} plaque forming units; pfu), and then incubated for 1 h at room temperature. Subsequently, SiN were washed 5–20 times with TBS buffer containing 0.1–0.3% Tween 20. For proliferation of the T7 phage bound to the surface of SiN, 10 mL of *Escherichia coli* BLT5403 (Merck Millipore) proliferated to the log phase was mixed with the nanoparticles and incubated at 37°C by shaking until bacteriolysis. After bacteriolysis, phages were recovered from the culture supernatant according to the manufacturer's instructions, and the recovered phage solution was used for the next round of screening.

Identification of peptide sequences

DNA fragments inserted in the vector of the monoclonal T7 phage were amplified by PCR using PrimeSTAR Max DNA polymerase (Takarabio, Shiga, Japan). The PCR reaction was initiated at 98°C for 3 min, followed by 30 cycles of 98°C for 10 s, 55°C for 10 s, and 72°C for 5 s using a Veriti 96-well thermal cycler (Applied Biosystems, Waltham, MA). The oligonucleotide primers used in this reaction had the following synthetic sequences (Eurofins Genomics, Tokyo, Japan).

T7 forward sequencing primer: 5′-GGA GCT GTC GTA TTC CAG TC-3′ (20 mer)

T7 reverse sequencing primer: 5′-AAC CCC TCA AGA CCC GTT TA-3′ (20 mer)

The peptide sequence was determined through analysis of the DNA sequence using a Genetic Analyzer 3130 and a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

Peptide synthesis

All peptides were synthesized by a custom peptide synthesis service (Scrum, Tokyo, Japan). The synthetic peptides were prepared by solid phase synthesis using the 9-fluorenylmethyloxycarbonyl (Fmoc) group. The synthetic peptides were *N*-terminally biotinylated using a Gly-Gly-Gly spacer from the g10 protein of the T7 phage. After removal of the protecting groups from the 4-hydroxymethyl phenoxymethyl polystyrene (HMP) resin, the peptides were mildly oxidized to form intramolecular disulphide bonds. The generated disulphide-constrained peptides were purified by reverse phase high performance liquid chromatography (HPLC). After lyophilization, the peptides were dissolved in the appropriate buffers and used for the assays after centrifugation. The purity of these peptides and the formation of disulphide bonds was confirmed by HPLC-mass spectrometry.

Enzyme linked immunosorbent assay (ELISA)

To detect the binding abilities of isolated phages or synthetic peptides to the silicon composites, we performed ELISA. Selected phage mixtures were mixed with 200 μg of silicon composite and incubated for 1 h at room temperature. After washing five times with HEPES-T buffer (50 mM HEPES, 150 mM NaCl, 0.1% Tween 20, pH 7.0), the number of remaining phages on the silicon composite was determined according to the manufacturer's instructions.

The synthetic peptides were mixed with 200 μg of silicon composite using the same condition as above. After washing five times with HEPES-T buffer, horseradish peroxidase (HRP)-conjugated streptavidin (Novagen) diluted (1:5,000) in HEPES-T buffer containing 0.5% bovine serum albumin was added to the sample and incubated for 1 h. Each sample was washed five times with HEPES-T, followed by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Wako Pure Chemical Industries, Osaka, Japan). In each washing step, centrifugation was performed at 6,000 rpm for 2 min. After stopping the reaction with 1N HCl, the absorbance of each sample was measured at 450 nm using a microplate reader (Molecular Devices Spectra Max Plus 384, Sunnyvale, CA).

Silica mineralization

Peptide solutions (1–200 μM) were mixed with TMOS solution (1–100 mM) and incubated at room temperature. All experiments were carried out in buffered conditions (50 mM Tris, pH 7.5).

Dynamic light scattering (DLS) measurement

A zeta sizer nano ZSP (Malvern Instruments Ltd, Worcestershire, UK.) was employed to quantify the size of particles generated by the mineralization experiment. All measurements were performed with a 658.0 nm monochromatic laser and recorded at a scattering angle of 90° in order to minimize the reflection effect.

SEM-EDX analysis

Scanning electron microscopy (SEM)-energy dispersive X-ray spectroscopy (EDX) analysis was performed using a TM3000 microscope (Hitachi High-Technologies, Tokyo, Japan) operated at 5 keV or 15 keV. The particles generated during mineralization experiments were separated by centrifugation at 15,000 rpm for 10 min. The supernatant was removed and the precipitate was washed with pure water. The samples dispersed in ethanol were then dropped onto a nano-percolator (JEOL, Tokyo, Japan), a carbon sheet with 1 μm pores. The nano-film generated at the air-water interface during mineralization was harvested by pipetting. The harvested samples were dropped onto a nano-percolator and then washed with pure water. All samples were measured after drying under vacuum conditions.

TEM analysis

Transmission electron microscopy (TEM) analysis was conducted using a JEM-2100F microscope (JEOL, Tokyo, Japan) operated at 200 kV. The samples were prepared using the same procedure as SEM analysis. Samples dispersed in ethanol or water were dropped onto carbon-coated copper grids and dried under atmospheric conditions before obtaining the images. X-ray diffraction analyses were conducted with Cu Kα radiation using an X-Pert Pro Alpha 1 diffractometer equipped with an incident beam Johannsen monochromator and an Xcelerator linear detector (PANalytical, Almelo, The Netherlands).

Measurement of Si concentration in the precipitate

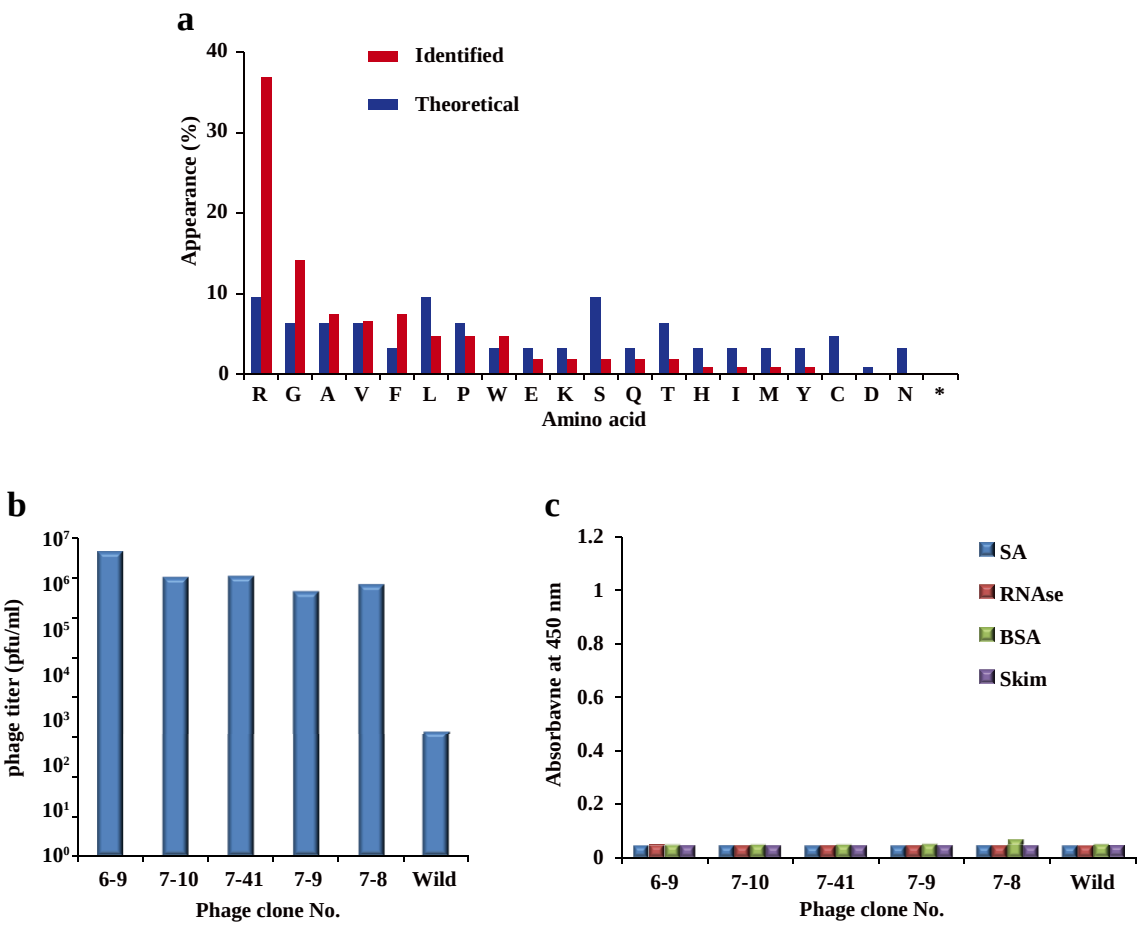
The amount of Si obtained during mineralization was determined using the molybdenum blue method with a water quality measurement kit (LR-SiO2D, Kyoritsu Chemical-Chek Lab. Corp. Japan). Absorbance of the blue silicomolybdate complex at 812.8 nm was determined using a V-530 Spectrophotometer (Jasco). Calibration of this method using a TMOS solution showed a linear relationship between concentration and absorbance over the entire concentration range used.

Samples were prepared by the following method. Typically, a 50-mM solution of TMOS and the desired amount of peptide (100-300 μM) was mixed thoroughly and left to react for 10 min. The solutions were centrifuged at 6000 rpm and the supernatant was discarded. Then 0.1 M NaOH

(1 mL) was added and incubated at 80°C for at least 1 h. After diluting the treated samples, the concentration of silicic acid was determined according to the manufacturer's instructions.

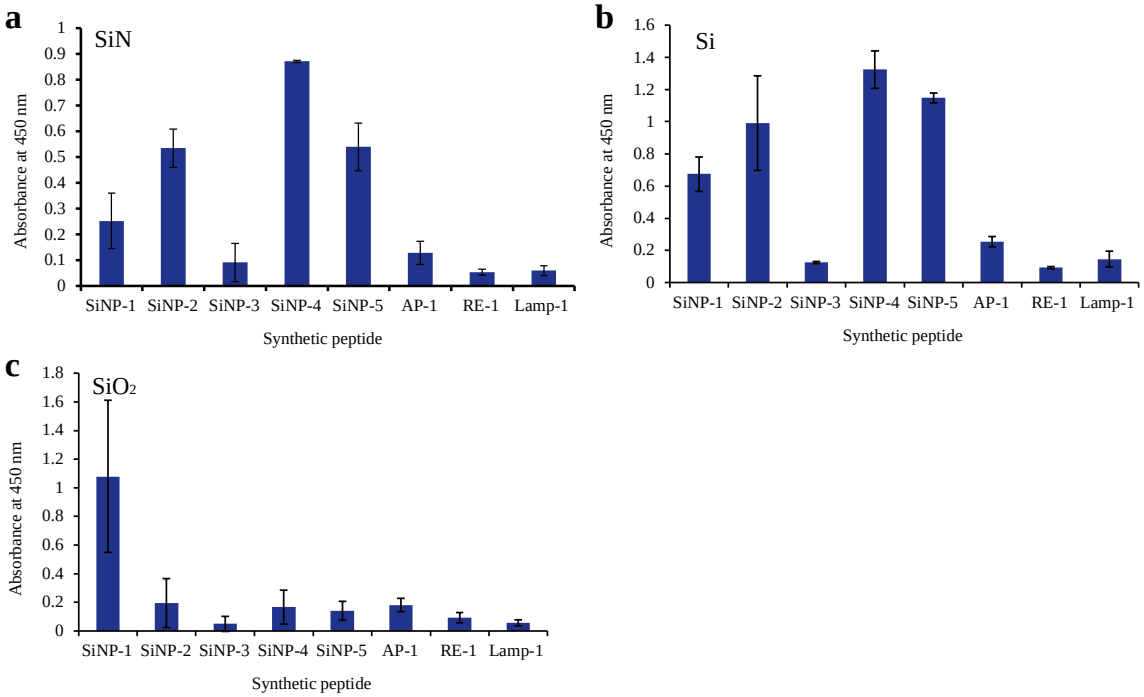
AFM analysis

Atomic force microscopy (AFM) images were taken in the dynamic force mode (DFM) with a Seiko E-sweep SPM equipped with a SII NanoNavi probe station (HITACHI High-Technologies, Tokyo, Japan). Samples were prepared by dropping a dilute solution containing the generated film in water onto mica.



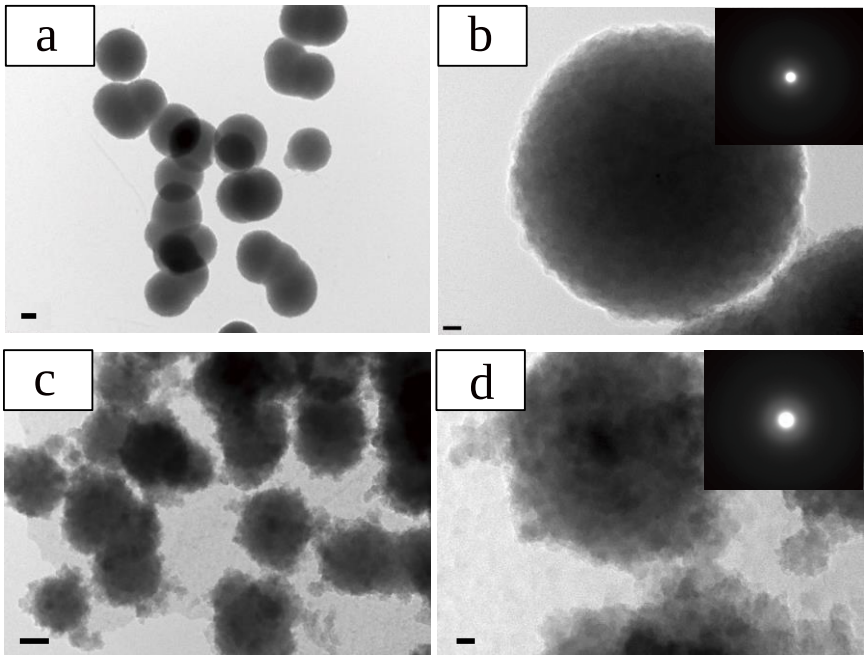
Supplemental Figure 1. Identification of SiN binding phages

(a) The appearance frequencies of amino acids observed in the candidate peptide sequences are listed in Supplementary Table 2. This theoretical value was based on the NNK codon, where N = A, T, G, or C and K = T or G. **(b)** The binding ability of isolated phage clones with SiN. **(c)** The binding ability of isolated phage clones with proteins. SA; Streptavidin. BSA; Bovine serum albumin. Skim; Skim milk. The wild phage was used as a control. These results indicate that Arg is important for SiN recognition, and that the isolated clone recognizes SiN but not typical proteins.



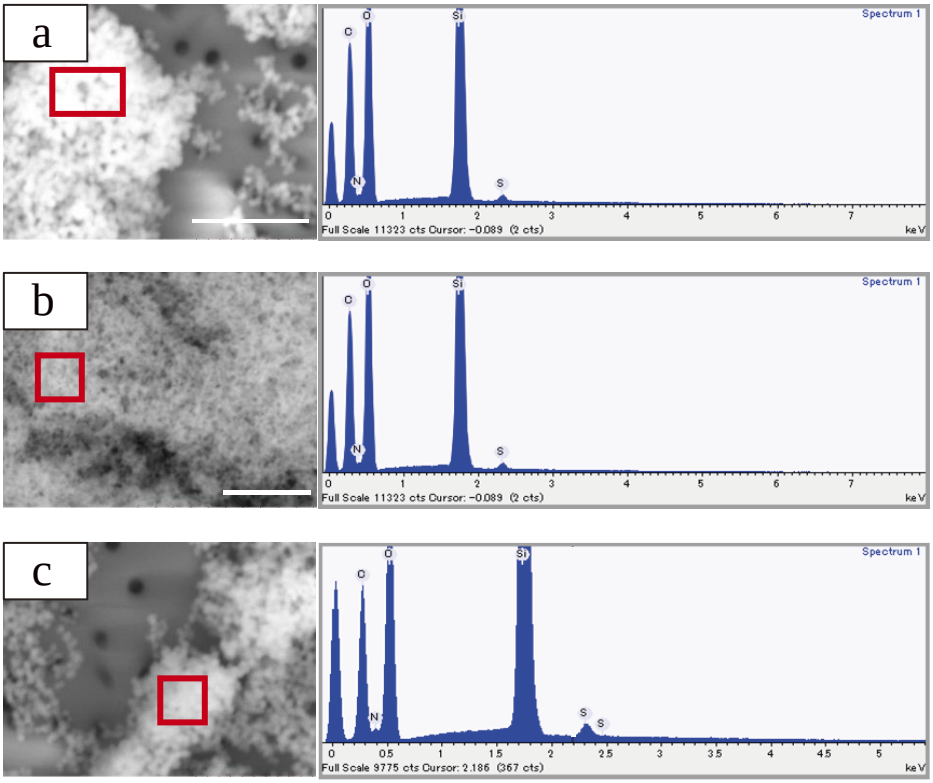
Supplemental Figure 2. The binding ability of synthetic peptides with three kinds of Si composite materials

(a) SiN, **(b)** Si nano particles, and **(c)** SiO_2 nano particles were used for the binding experiment. The diameter of Si nanoparticles and SiO_2 nanoparticles was 10-20 nm and 100 nm, respectively. Error bars represent the standard deviation of three individual experiments. These results indicate that SiNPs, except for SiNP-3, bind to the metal Si surface. Interestingly, SiNP-1 also recognizes SiO_2, indicating that its binding property differs from that of the cationic SiNPs and anionic SiNP.



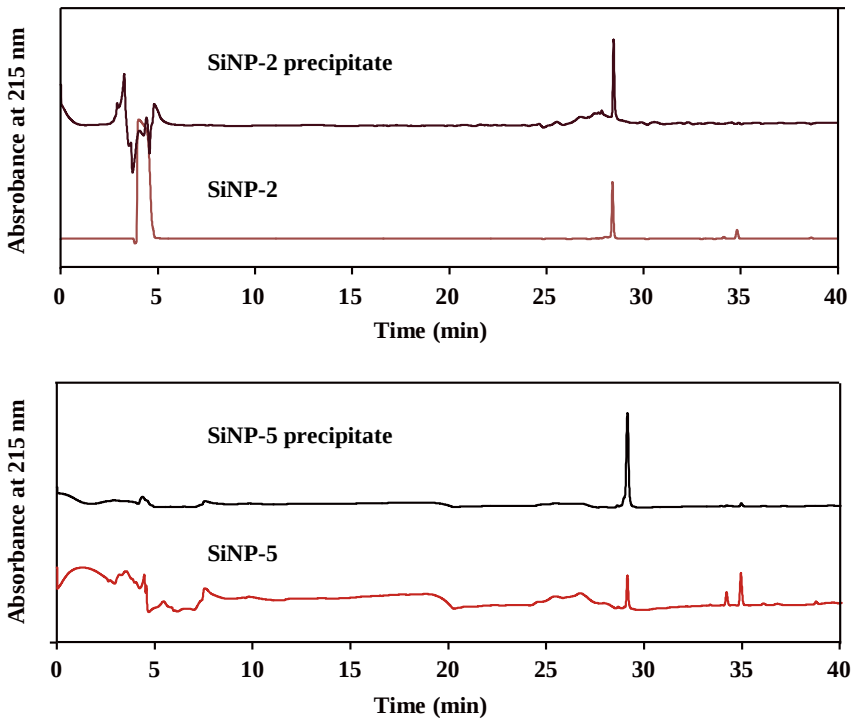
Supplemental Figure 3. Precise structural analysis of the generated particle

TEM image of generated particles induced by **(a, b)** SiNP-2 and **(c, d)** SiNP-4. The small panels in **b** and **d** indicate the diffraction pattern. Scale bars: **(a, c)**: 100 nm, **(b, d)**: 20 nm. These results indicate that the reaction of SiNP and TMOS generates spherical particles in the amorphous phase.



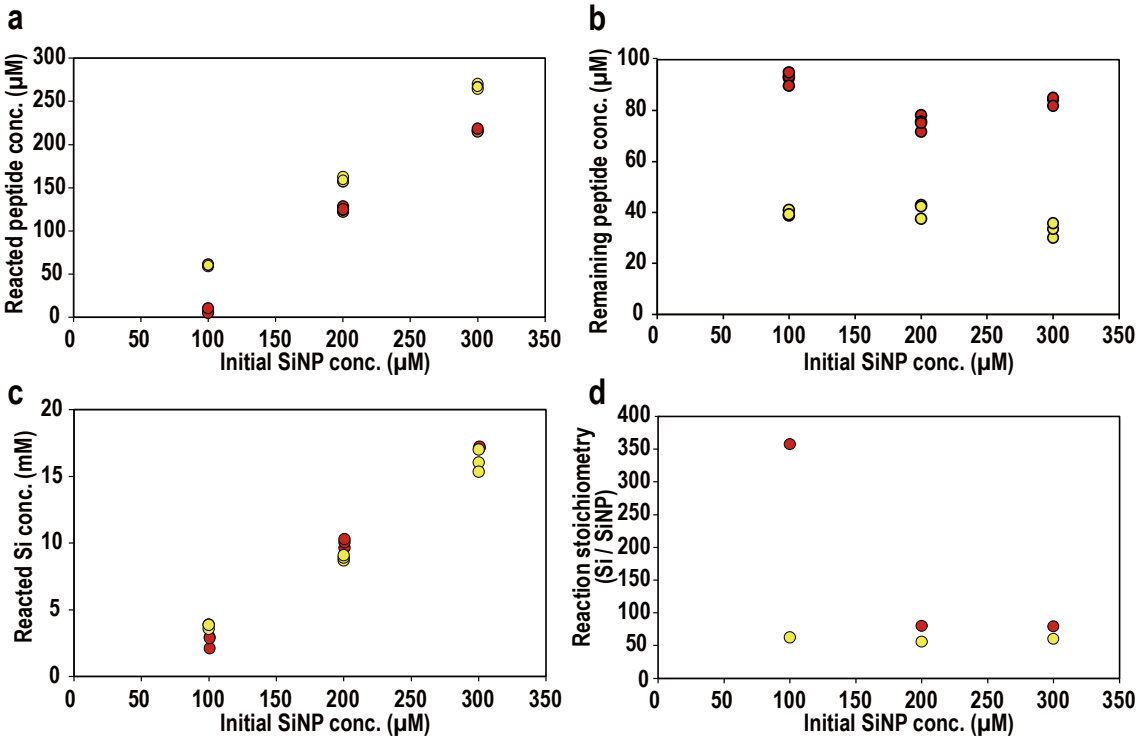
Supplemental Figure 4. The content of generated nanoparticles

SEM (left) and EDX analysis (right) of the precipitated particles induced by **(a)** SiNP-2, **(b)** SiNP-4, and **(c)** SiNP-5. Scale bars: 10 µm. The red square in the left-hand panel indicates the point used for EDX analysis. These results indicate that the mineralized particles contain Si, C, O, N, and S, which come from the silica and peptide.



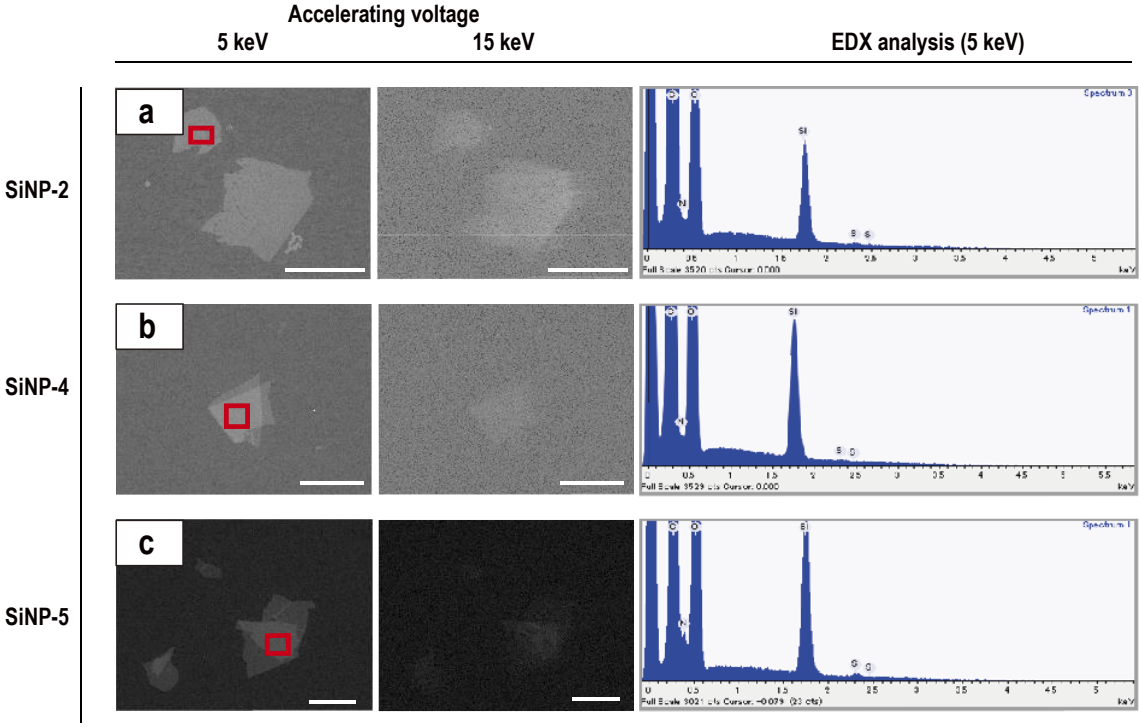
Supplementary Figure 5. The precipitated silica particles contain SiNP

The precipitated particles were dispersed in pure water and sonicated for 10 min. Centrifugation and filtration was performed before analysis by RP-HPLC. The upper line shows the precipitated sample and the lower shows SiNP.



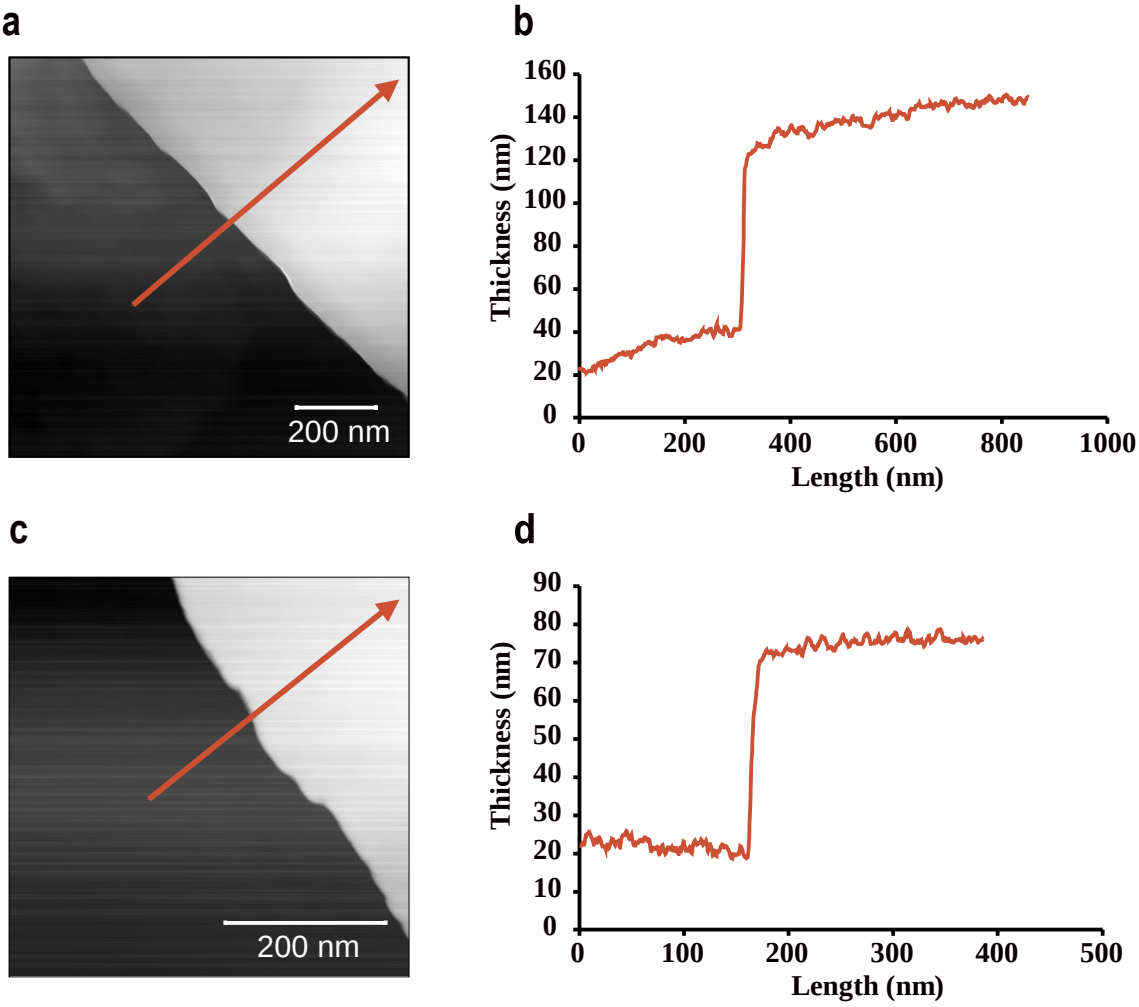
Supplementary Figure 6. Reaction stoichiometry in silica particle mineralization

(a) The reacted peptide concentration was plotted as a function of initial peptide concentration. **(b)** The remaining peptide concentration was plotted as a function of initial peptide concentration. **(c)** The reacted Si concentration was plotted as a function of initial peptide concentration. **(d)** The reaction stoichiometry ([reacted si] / [reacted SiNP]) was plotted as a function of initial peptide concentration. The concentration of the peptide in the solution was calculated from respective absorbance at 280 nm and molar extinction coefficients. Si consumption was determined using the molybdate blue method.



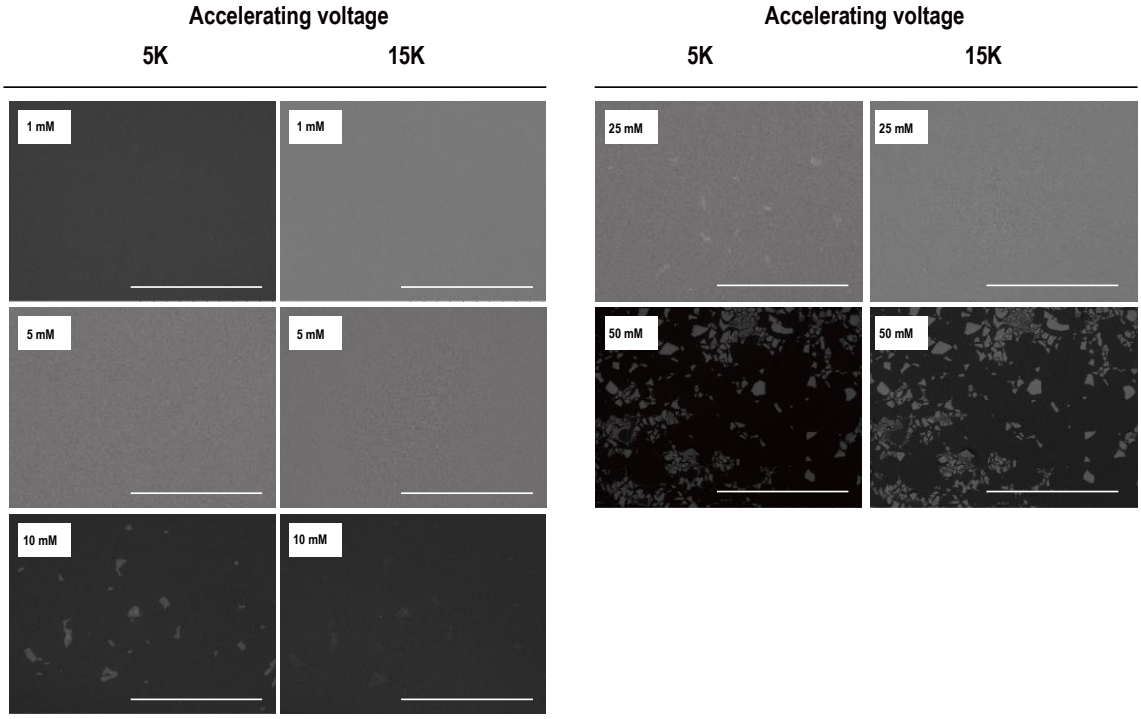
Supplementary Figure 7. SEM/EDX analysis of the generated nano-film

SEM (left, middle) and EDX (right) analysis of the nano-film induced by **(a)** SiNP-2, **(b)** SiNP-4, and **(c)** SiNP-5. Scale bars: 100 μm. The red square in the left-hand panel indicates the point used for EDX analysis. The change of accelerating voltage (5k eV: left, 15k eV: middle) clearly altered the visibility of the nano-film. These results indicate that the mineralized nano-film has a very thin structure and contains Si, C, O, N, and S, which come from the silica and peptide.



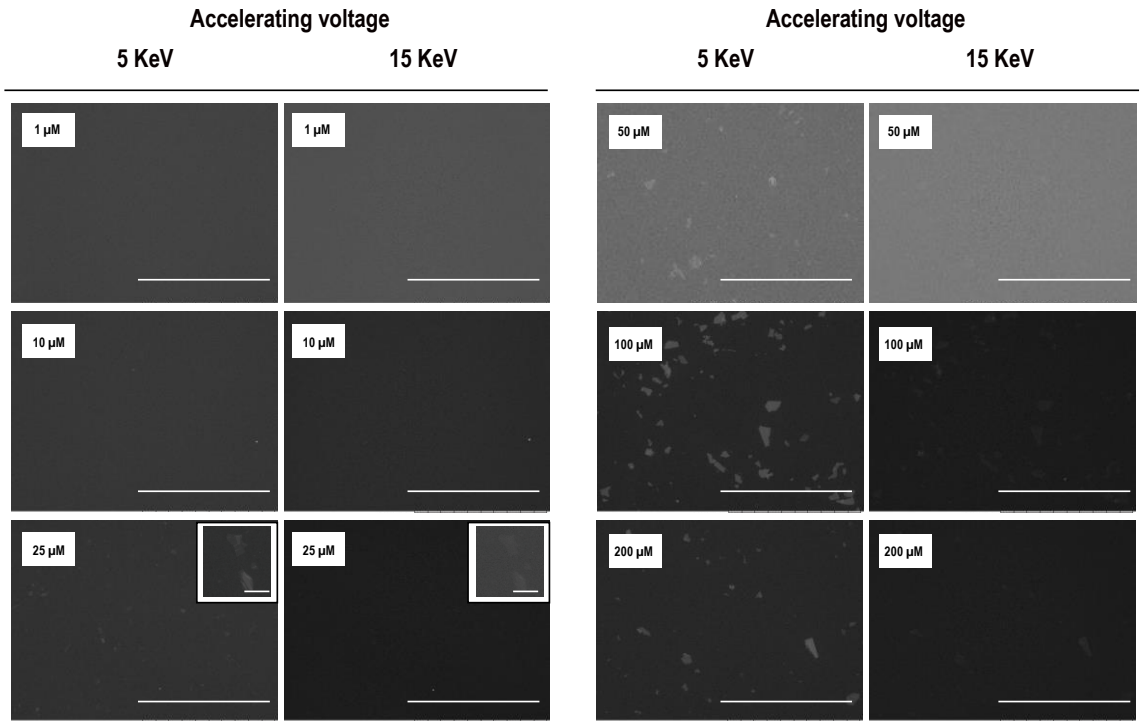
Supplementary Figure 8. Thickness and surface asperity of the silica film

The films generated by the reaction of TMOS and SiNP-2 **(a, b)**, and SiNP-4 **(c, d)** were analyzed by AFM. The colored lines in panels **a** and **c** correspond to graphs **b** and **d**, respectively. These results indicate that the thickness of the film is less than 100 nm and that its surface is almost flat.



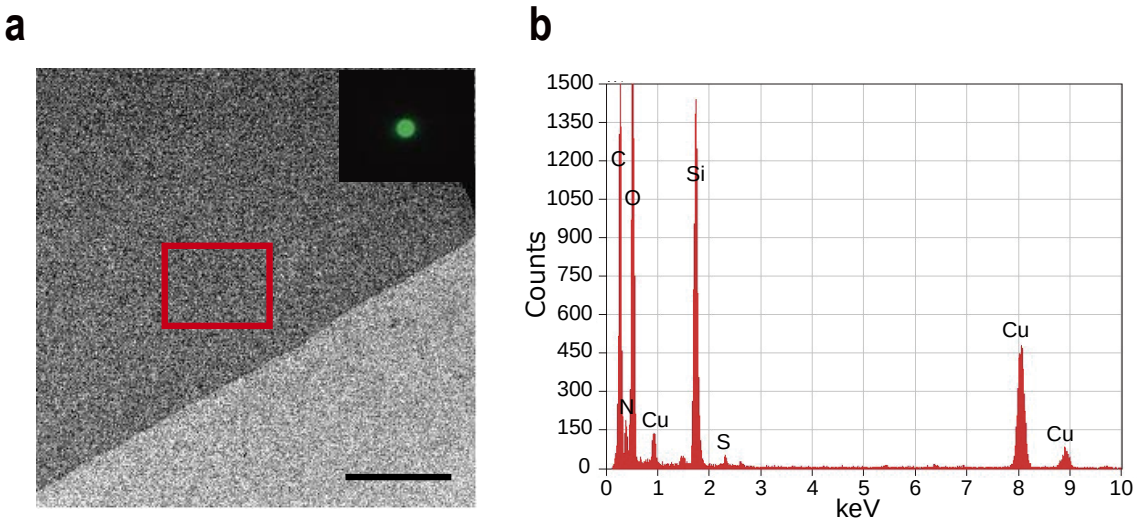
Supplementary Figure 9. The effect of TMOS concentration on nano-film formation

Nano-film generation was evaluated at various TMOS concentrations (1-100 mM) with a fixed concentration of SiNP-5 (100 μM). We judged nano-film generation by the visibility change at different accelerating voltages (5 keV: left, 15 keV: right). Scale bar of the large panels: 2 mm. We could not prepare the SEM sample at 100 mM of TMOS because the mixture turned to gel during the hours of incubation. The nano-film was observed at TMOS concentrations of 10 and 25 mM and not at other concentrations. The visibility of the 50 mM sample was not changed, meaning the conditions were not favorable for nano-film preparation. These results indicate that there is an appropriate concentration of TMOS for nano-film preparation, and that this is 10 mM under our experimental conditions.



Supplementary Figure 10. The effect of SiNP concentration for nano-film formation

Nano-film generation was evaluated at various SiNP-5 concentrations (1-200 μM) with a fixed concentration of TMOS (10 mM). The nano-film was observed when the SiNP-5 concentration was over 25 μM. The small panel indicates an extended view of the generated nano-film. Scale bar of large panels: 2 mm. Scale bar of small panels: 100 μm. This result indicates that nano-film generation increases as SiNP-5 concentration increases.



Supplementary Figure 11. TEM analysis of the silica nano-film

(a) TEM image of nano-film generated by SiNP-5. The small panel indicates the diffraction pattern of the nano-film. Scale bar: 1 µm. **(b)** EDX measurement of the red square in panel **a**. The signal of copper results from the TEM grid. This result indicates that the generated nano-film has an amorphous structure like the nanoparticles.

Supplementary Table 1. Peptide libraries

Library Type	Diversity (pfu)	Concentration (pfu/mL)
SCX8CS	9.88.E+07	4.87E+11
SCX9CS	3.76.E+07	8.11E+11
SCX10CS	6.78.E+06	9.56E+11
SCX11CS	1.25.E+07	5.00E+11
SCX12CS	1.56.E+06	9.00E+11

Supplementary Table 2. Candidate peptide sequences and their characteristics

Library Type	Clone No.	Identified Sequence	Frequency	Length	Basic a.a.	Acidic a.a.	MW	PI	Synthetic peptide
SCX8CS	6-4	**SCAAMGRRVVCS**	4/96	12 a.a	2	0	1221.5	8.83	
SCX9CS	6-9	**SCAAFGFWEPACS**	2/96	13 a.a	0	1	1357.5	3.85	SiNP-1
SCX10CS	6-49	**SCRRAALGARSRCS**	3/96	14 a.a	4	0	1475.7	12.02	
SCX9CS	7-2	**SCQKGLLRRRRCS**	4/96	13 a.a	5	0	1544.9	12.1	
SCX9CS	7-18	**SCKRVGFFRTSCS**	5/96	13 a.a	3	0	1459.7	9.91	
SCX9CS	7-10	**SCGTRRFRWRRCS**	8/96	13 a.a	5	0	1652.9	12.3	SiNP-2
SCX9CS	7-57	**SCRRGRLFGRRCS**	2/96	13 a.a	5	0	1535.8	12.3	
SCX10CS	7-41	**SCPPRGVWQGEPCS**	3/96	14 a.a	1	1	1484.7	6.14	SiNP-3
SCX11CS	7-9	**SCRRRFVRLRGGRCS**	7/96	15 a.a	6	0	1791.1	12.5	SiNP-4
SCX11CS	7-8	**SCRRIRHWRPWRGCS**	3/96	15 a.a	6	0	1938.3	12.3	SiNP-5
SCX11CS	7-85	**SCRVRGYFRRGRVCS**	2/96	15 a.a	5	0	1784.21	11.9	

Basic a.a: Basic amino acids (Arg, Lys, His)

Acidic a.a: Acidic amino acids (Glu, Asp)

MW: Molecular weight

PI: Isoelectric point

Supplementary Table 3. Synthetic peptides

Synthetic peptide	Sequence	Sequence Type	Length	Basic a.a.	Acidic a.a.	MW	PI	other	Clone No.
SiNP-1	**SCAAFGFWEPACS**	SCX9CS	13 a.a	0	1	2746.8	3.85	cyclic	6-9
SiNP-2	**SCGTRRFRWRRCS**	SCX9CS	13 a.a	5	0	1652.9	12.3	cyclic	7-10
SiNP-3	**SCPPRGVWQGEPCS**	SCX10CS	14 a.a	1	1	1484.7	6.14	cyclic	7-41
SiNP-4	**SCRRRFVRLRGGRCS**	SCX11CS	15 a.a	6	0	1791.1	12.5	cyclic	7-9
SiNP-5	**SCRRIRHWRPWRGCS**	SCX11CS	15 a.a	6	0	1938.3	12.3	cyclic	7-8
AP-1	**SACDQSHPQQCG**	SACX7CG	12 aa	1	1	1242.3	5.29	cyclic	
RE-1	**SACTARSPWICG**	SACX7CG	12 aa	1	0	1233.4	8.27	cyclic	
Lamp-1	**SCLWGDVSELDFLCS**	SCX11CS	15 aa	0	3	1655.8	2.83	cyclic	

Five different sequences that showed silicon nanosheet binding ability on the phage surface were selected for peptide synthesis. These peptides were named SiNPs (Silicon Nanosheet binding Peptides)

AP-1, RE-1, and Lamp-1 were used as controls. [10]