Silica Nanostructure Formation From Synthetic R5 Peptide

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ABSTRACT

Diatoms have the ability to generate highly ornamented nanostructured silicified cell walls under ambient conditions and without harsh chemicals, yet the molecular mechanisms underlying biosilification are still not well understood. The idea of this study is to mimic silica biomineralization of diatom cell walls that may provide the key to the development of new routes towards novel tailor-made silicas. Here the ability of R5 peptide, a peptide from the silaffin-1 protein derived from diatom species of *Cylindrotheca fusiformis*, to generate silica nanostructure in vitro was investigated. The R5 peptide was synthesized using Fmoc Solid-Phase Peptide Synthesis and purified using reverse phase high performance liquid chromatography. MALDI analysis showed that the peptide was successfully synthesis. With the application of silicic acid as a silica precursor and the peptide as catalyse, the formation of silica nanostructure was achieved. AFM analysis of the precipitated silica from the mixture of silicic acid and the peptide revealed the nanostructure of silica spheres ranging between 50 – 300 nm in diameter. Silica precipitate was not obtained in the absence of R5 (negative control) and when the silicic acid was mixed with poly-L-lysine (positive control), a network of large aggregates of uniform size of silica spheres of about 100 nm in diameter was observed.

Keywords: diatoms, biosilification, R5 peptide, AFM and SEM

1. INTRODUCTION

Silicon based-materials have become increasingly important in many industrial applications resulting in increased in demand for well defined material formulations.[1] However, the fabrication of chemically synthesized silicon based materials generally requires harsh reaction conditions, high pressure and high temperature and is not environmentally friendly since the industrial processes generate extensive caustic chemical waste.[2]

Recently, there has been a great deal of interest focusing on the design and manufacture of devices at nanoscale in which current commercial micro and meso-fabrication methods have largely been based on two-dimensional processing principles which are not well suited to the low-cost mass production of three-dimensional micro devices.[3] An alternative approach to the manufacture of nanostructure materials is to learn from nature: a natural source of varying silica nanostructure can for example be found in the unicellular microscopic algae known as diatoms.[4]

The ability of diatoms to generate highly ornamented three-dimensional nanostructured silicified cell walls at ambient conditions and without harsh chemical has inspired scientists in the design and manufacture of nanostructure materials for many industrial applications. However, the exact mechanisms of biosilification are still not well understood.[5] The understanding of biomolecular and physico-chemical processes that steer biosilica formation is important for the design of process technological innovations and for the production of industrial silica under mild reaction conditions, environmentally friendly and more economically.[6] Hence, mimicking silica biomineralization of diatoms may provide the key to the development of new routes towards novel tailor-made silicas.

Smart Materials IV, edited by Nicolas H. Voelcker, Proc. of SPIE Vol. 6413, 64130Q, (2006)
0277-786X/06/$15 · doi: 10.1117/12.695946

Silaffin-1 (Sil1) is a protein from *Cylindrotheca fusiformis* that consists of seven highly homologous repeating units, R1 to R7[7]and the R5, a short 19 amino acid sequence, H2N-SSKKSGSYSGSKGSKRRIL-COOH has been shown to catalyse the formation of silica nanospheres within minutes when added to silicic acid at neutral pH and under ambient temperature in vitro.[8] Therefore, the aim of this study is to investigate the formation of silica nanostructures from a silica precursor catalyzed by R5 peptide.

2. MATERIALS AND METHODS

2.1 Synthesis of the R5 peptide

Solid Phase Peptide Synthesis (SPPS). R5 peptide was synthesized using Solid Phase Peptide Synthesis. Prior to coupling, the amount of the reagents (HOBt, HBTU and DIEA) and excess of amino acids used in the synthesis was determined. 100 mg of Fmoc-L-Leu-wang resin was weighted and added to the glass vessel. The resin was swelled with 10 ml dichloromethane (DCM) with shaking for about an hour. The DCM was drained by attaching vacuum to the bottom of the vessel and turn water on. After draining the DCM, a 10 ml of N,N-dimethyl formamide (DMF) was then added to the resin and swelled for another 3 hours. The DMF was drained and the Fmoc group on the resin bound amino acid was removed with 5 ml of 25% piperidine in DMF by shaking for 10 minutes. Deprotection with 25% piperidine in DMF was repeated twice. It was then rinsed with 10 ml of DMF for 5 minutes (4 times). Following this, the next protected amino acid was added. 3 times excess of the mass of the next amino acid, 3 times excess of HOBt and 3 times excess of HBTU were weighted and placed in a tube. 5 ml of DMF was added to the tube and mixed using vortex. The mixture was then added to the reaction vessel. 9 times excess of the DIEA was added to the mixture and then shaken for about 2 hours. After 2 hours coupling, the resin was drained and washed with 5 ml DMF for 5 minutes repeated 3 times.

Kaiser Test. To test whether coupling occurred, the Kaiser test was performed by placing a few beads in a small vial and adding a few drops of all three Kaiser Test solutions. The vial was then placed in a beaker of heated water for about 2 minutes. The colour change of the beads was observed. If the beads turn black, the coupling had failed and recoupling or capping had to be performed.

Cleavage the peptide. After the completion of the peptide assembly, the peptide was cleaved from the resin. Prior to cleavage, the Fmoc group of the last amino acid to be coupled was removed with 5 ml of piperidine and shaken for 15 minutes (2 times). The resin was then washed three times with 5 ml DMF for 5 minutes, three times with 5 ml DCM for 5 minutes, three times with 5 ml methanol for 5 minutes and three times with 5 ml diethyether for 5 minutes. Following this, the resin was drained and the beads were dried overnight in a dessicator with dessicant.

Cleavage the peptide was accomplished using a cleavage mixture containing 9.0 ml of trifluoroacetic acid (TFA), 0.5 ml of thioanisole, 0.3 ml of 1,2-ethanedithiol (EDT) and 0.2 ml of anisole. Nitrogen was bubbled through the cleavage mixture and then added to the vessel. The peptide on the resin was allowed to react with the cleavage mixture for several hours (2-4 hours) with shaking. The samples were drained into a tube and aliquated them into six 1.5 ml eppendorf tubes. The tubes were placed in centrivap concentrator LABCONCO and spun for 15 minutes or until the volume was 1 ml or less. The spun down solutions were added drop wise into the cold diethyl ether. A precipitate formed and the sample was then incubated in the fridge overnight.

The precipitated solution was aliquoted in two 50 ml centrifuge tubes (equal volume) and centrifuged at 3000 rpm and 4^{o}C for 15 minutes. The supernatant was removed and fresh cold diethyl ether was added followed by another centrifugation. Additional cold diethyl ether and centrifugation were repeated 3 times. After the last centrifugation, the supernatant was removed until below 5 ml and placed in four Eppendorf tubes. Again, the samples were centrifuged at 13000 rpm for 30 seconds. The supernatant was discarded and 1 ml of glacial acetic acid was added to the tubes. The

samples were sonicated for 20 minutes and placed in cold vacuum to remove the solvent. The dried peptide was stored in the freezer for further analysis.

2.2 Purification of the Synthetic R5 peptide using Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The crude peptide was purified using RP-HPLC (The Hitachi D-7000). The column used for separation of the components of the synthetic peptide was C8 (VYDAC 208TP510). Two solvents were used, solvent A and B. The A and B are commonly used to refer to the aqueous and organic solvent respectively. The solvent A contain 10 ml of HPLC grade acetonitrile, milliQ water (make up to 1 litre) and before adding 1 ml Trifluoroacetic acid (TFA), the solvent was degassed for about 1 hour to remove air bubble. The solvent B contains 50 ml of milliQ water and was made up to 500 ml with HPLC grade acetonitrile. The solvent was degassed for 1 h before adding 0.35 ml TFA.

The sample was dissolved in 1 ml solvent A. The samples were separated by reverse phase HPLC on a C8 column by application of 5 – 40% of solvent B in 30 minutes. The fraction from each peak was collected. Each of the fractions from the crude peptide was analyzed with MALDI-MS and the fractions of selected peaks were then lyophilized and stored frozen at -20^{o}C for further analysis.

2.3 Maldi-MS (Matrix Assisted Laser Desorption Ionization Mass Spectrometry)

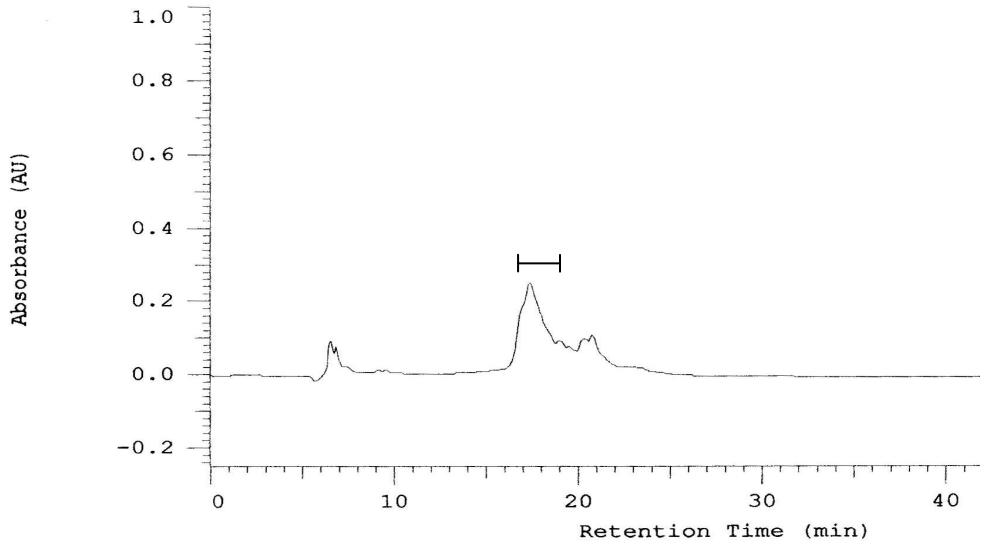
The matrix used for the synthetic R5 peptide was α-cyano-4-hydroxycinnamic acid (CNCA). The matrix solution was prepared by dissolving 10 mg of CNCA in a mixture of 495 µl of acetonitrile, 495 µl of ethanol, 10 µl of 0.1% aqueous trifluoroacetic acid (TFA). The solution was vortexed and then centrifuged for 30 seconds at 7000 rpm. Only the supernatant was used for the preparation of the samples. Following this, 20 µl of the matrix solution was mixed with 20 µl of the peptide fraction from the HPLC.

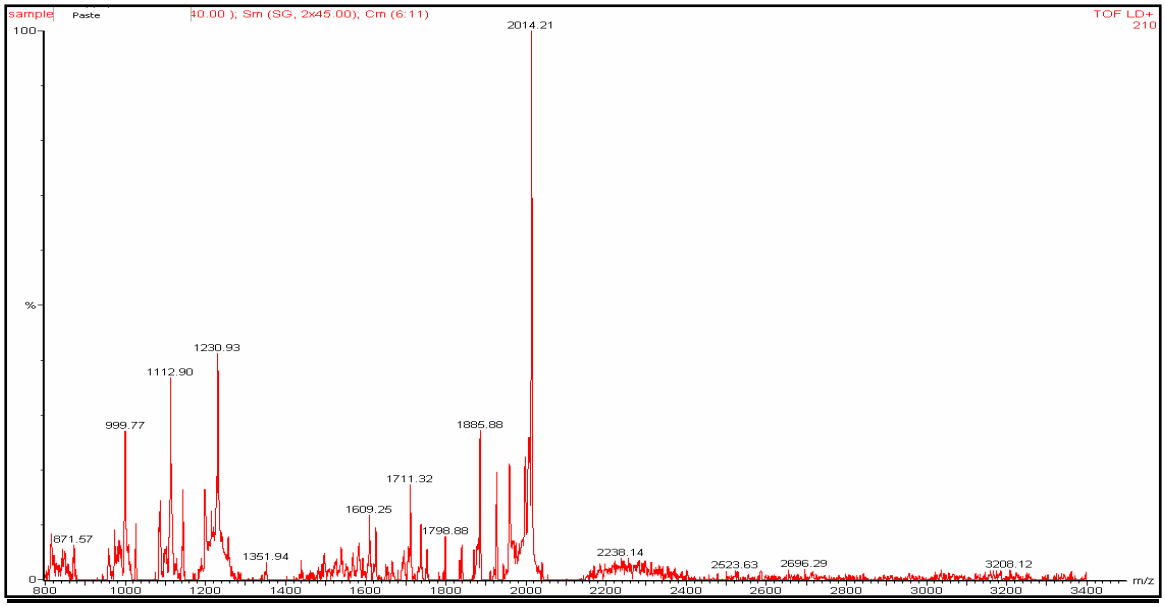
2.4 Silica precipitation assay

Silica precipitation assay was performed according to the method of Naik et al. (2003). Silicic acid solution was prepared by hydrolyzing 1 M TMOS (tetramethyl orthosilicate) with 1 mM HCl for about 30 minutes with agitation. Silicic acid solution was then mixed with the peptide stock at a concentration of 100 mg/ml milliQ water. The mixture was incubated for 15 minutes at room temperature. After incubation, the mixture was centrifuged for 3 minutes at 14000 rpm. The supernatant was carefully removed and the precipitated silica was washed three times with milliQ water. Similarly, poly-L-lysine was mixed with silicic acid solution at a concentration of 100 mg/ml and incubated for 15 minutes at room temperature followed by centrifugation. The precipitated silica was then washed 3 times with milliQ water by repetitive centrifugation and resuspension.

2.5 Atomic Force Microscopy (AFM)

The precipitated silica was mounted onto the silicon wafer and air dried. The silicon wafers were then adhered to metal pucks with double-sided adhesive. The cleaned silicon wafer without sample, the silicic acid solution incubated without the peptide and the poly-L-lysine were prepared as controls. For tapping mode AFM experiments, Olympus tapping mode etched silicon probes (Digital Instruments/Veeco Meteorology Group, Santa Barbara, California) with a resonancy frequency range of 100-500 kHz were used. All images and force measurements were acquired using a Nanoscope IV





3.2 Silica Nanostructure Formation From R5 Peptide

In the present study, a biomimetic study of silica nanostructure formation is presented. Two catalysts, the synthetic R5 peptide and a synthetic polyamine, poly-L-lysine were used in the presence of silicic acid. The silicic acid was synthesizes by dissolving 1 M TMOS in 1 mM HCl. This product was then mixed with one of the catalyst (as described in materials and methods). A control without the peptide was also treated with silicic acid solution.

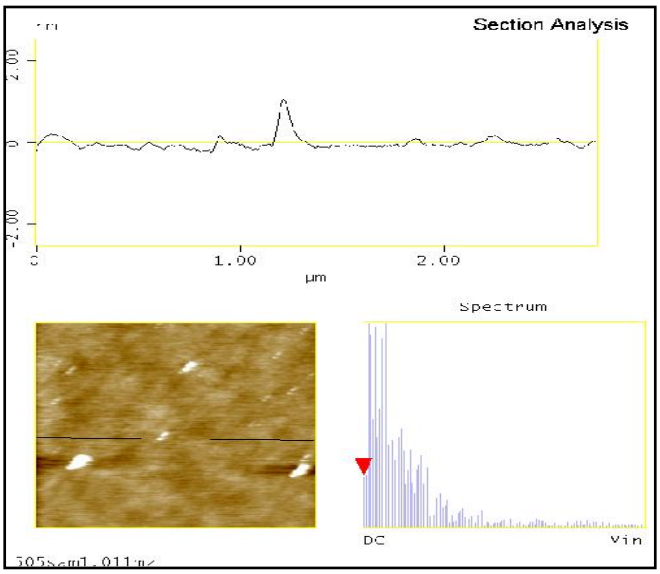
In agreemenet with previous studies, the formation of silica nanoparticles was observed in the presence of both the R5 peptide and poly-L-lysine, respectively. The morphologies of the precipitated silica were investigated by scanning electron microscopy (SEM) and atomic force microscopy (AFM) techniques.

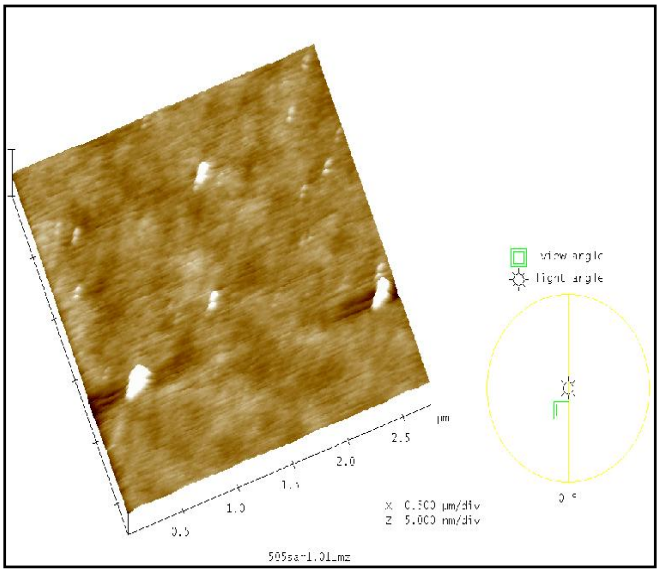
Tapping mode AFM analysis revealed the formation of nanostructure of spherical particles synthesized using the peptide. Interestingly, the nanoparticle size formed by the mixture of the synthetic R5 peptide and the silicic acid solution was not uniform in size. The cross section analysis of the sample in Figure 4A revealed the size of the particles were about 50-300 nm in diameter. This is somewhat smaller than the 400–600 nm diameter particles observed by Naik et al [9].

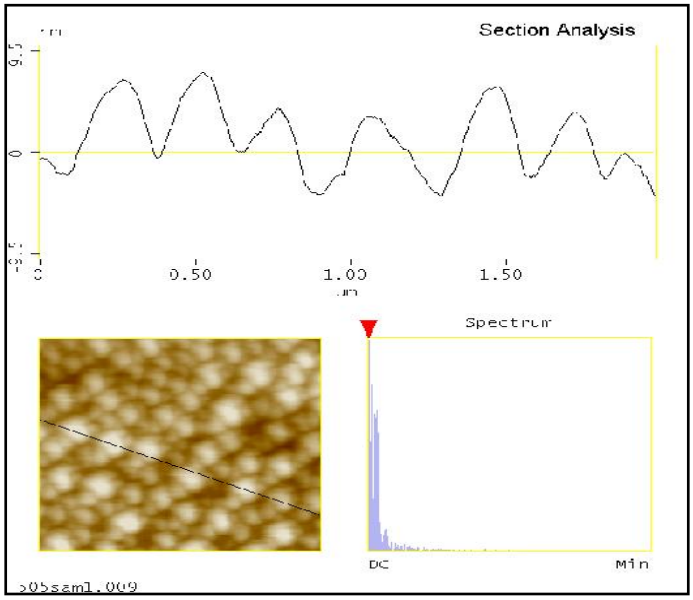
A possible explanation for the variation in size diameter was that the peptides used contain some side products including truncated peptides that might also react with the silicic acid. A study done by Knecht and Wright [10] reported a synthetic site-directed mutagenesis study of the full length non-post-translationally modified R5 peptide. The results of this study showed that the R5 peptide formed a network of spherical silica nanoparticles 250-450 nm in diameter, whereas R5 truncates resulted in the formation of smaller particles ranging from 60-400 nm.

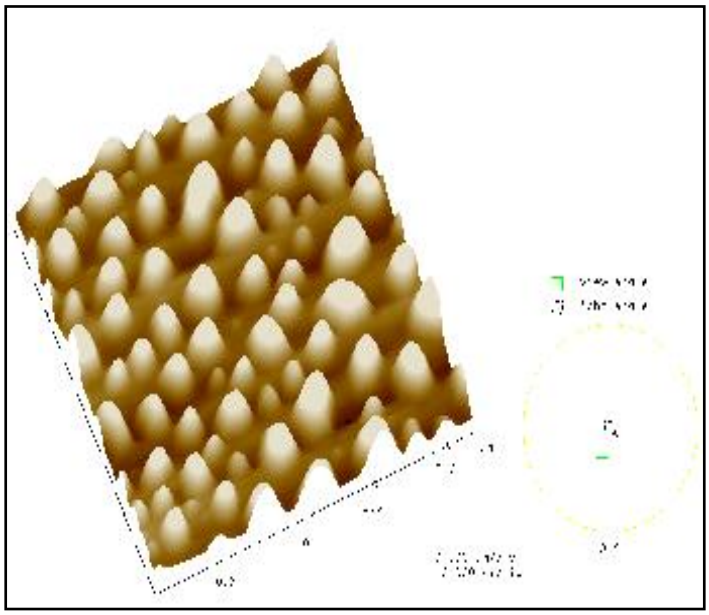
Even more interesting was that in the presence of poly-L-lysine, a heavily fused mass of uniform size of silica spheres approximately 100 nm in size was achieved, as evident in Figure 5. This result is in agreement with previous studies. The average particle sizes of silica particles formed was about 108 nm in diameter[11] and Mizutani et al[12] also have demonstrated the ability of poly-L-lysine to precipitate silica consisting of agglomerated nanoparticles of 50-100 nm in diameter.

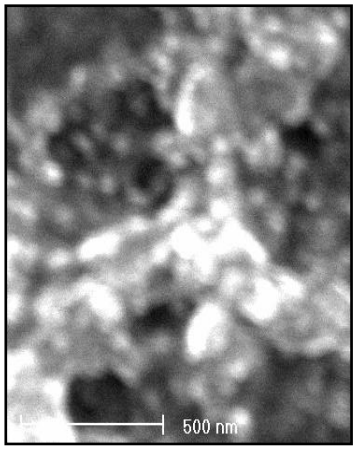
In contrast, in the absence of the peptide, no silica nanospheres formation was observed. Cross-section analysis of the control as evident in Figure 3A showed a flat surface.

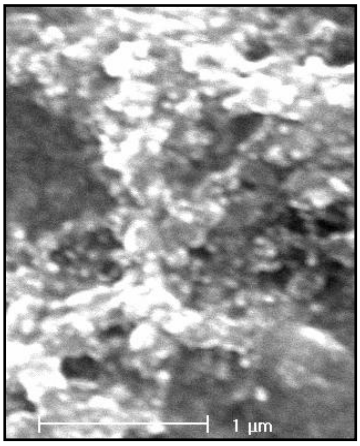


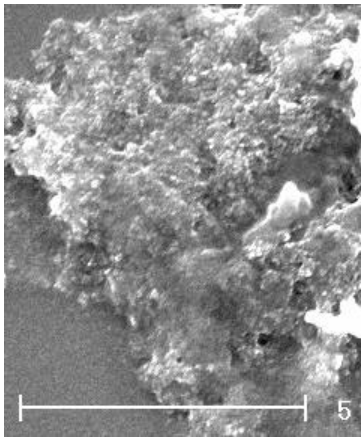












silica nanostructure was observed. The results of this project could be useful for the development of new routes towards the environmentally benign synthesis and patterning of well defined silicon based material at low cost.

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