Biomineralization

Biomimetic Control of Size in the Polyamine-Directed Formation of Silica Nanospheres**

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The intricate silicified cell walls of diatoms are probably the most outstanding examples of nanoscale-structured materials in nature.[1] The degree of complexity in these hierarchically organized biominerals has never been matched in artificial materials.[2] Nanofabrication of silica in diatoms occurs under ambient conditions at slightly acidic pH values and results from specific interactions between biomolecules and silicic acid derivatives. Such biomolecules are the silaffin peptides and long-chain polyamines (both of which were isolated from diatom biosilica[3–5]) and the silicateins (isolated from sponges).[6] Silaffins isolated from *Cylindrotheca fusiformis* mainly consist of modified lysine and serine residues. The serine groups are phosphorylated and the lysine residues are

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converted into three different derivatives: ε-*N*-dimethyllysine, phosphorylated ε-*N*-trimethyl-δ-hydroxylysine, and lysines covalently linked to long-chain polyamines, *N*-methylated derivatives of a polypropyleneimine.[7,8] Silaffins as well as long-chain polyamines have been shown to rapidly direct the formation of silica nanospheres from silicic acid in vitro[3,4] which has been confirmed[9] and adapted to technical applications.[10]

In the past, a huge amount of knowledge has been accumulated with respect to the inorganic chemistry of silicic acid and its polymeric derivatives.[11] Nonporous silica nanospheres could be synthesized by controlled hydrolysis of silicon alkoxides in methanol/NH_3 mixtures[12] and the underlying mechanism of size control has been investigated.[13,14] The past decade has seen important advances in the ability to fabricate porous solids and several methods have been developed for the synthesis of different silica morphologies summarized in recent reviews.[2,15,16] By applying this knowledge to the unique structure of silaffins, a fascinating aspect emerges: Nature has designed a molecule that is perfectly adapted to the chemistry of silica formation, as briefly outlined. Silicic acid polymerization involves three distinct stages. First, monomeric silicic acid polymerizes by condensation of silanol groups to form dimers, trimers, and cyclic oligomers. Oligosilicic acid species have a strong tendency to polymerize further in such a way that siloxane-bond (Si–O–Si) formation is maximized. These early processes create highly branched polysilicic acids as nuclei for silica formation. Second, the nuclei grow to form spherical particles either by continuous polymerization with monomeric and oligomeric silicic acids or by fusion of particles. Finally, the silica nanospheres can precipitate by flocculation, a process that intimately connects silica spheres and produces hard silica, as is found in the cell wall of diatoms. The structural elements found in silaffins are likely to be involved in each of these three stages, thereby accelerating the formation of hard silica by orders of magnitude. More than 40 years ago, quaternary ammonium ions were recognized as structure-directing agents in the synthesis of zeolite molecular sieves.[17] The tetramethylammonium cation favors the formation of symmetric oligosilicate anions such as the cubic octamer $Si_8O_{16}^{8-}$, and this control of silicate speciation influences the nucleation phase of silica formation.[18] Owing to their unique ε-*N*-trimethyl-δ-hydroxylysine residue, silaffins may exert a similar control. Polyamines were shown to catalyze the polycondensation of silanol groups[19] and are known to act as efficient flocculation agents.[11] Recently, an additional type of modification, a high degree of phosphorylation of silaffins, was shown to be essential for the formation of silica nanospheres in vitro.[7]

Herein, we define the function of phosphate anions in the polyamine-directed formation of silica nanospheres with the polyamines extracted from the cell wall of the diatom *Stephanopyxis turris* as a model. These polyamines consist of 15–21 *N*-methylpropyleneimine repeating units attached to putrescine. The polyamines were able to precipitate silica nanospheres from a silicic acid solution after a few minutes, even under acidic conditions that presumably represent the physiological situation[20] (approximately pH 5). After a short lag phase, silica started to precipitate (Figure 1a, blue line). However, this precipitation was strictly dependent on the presence of phosphate. In the presence of acetate anions only (the buffer system), no precipitate was formed at all (Figure 1a, green line). Silica precipitation in the presence of increasing phosphate concentrations produced nanospheres with increasing diameters (Figure 1b, red line). It is the particle size of the silica nanospheres that is strictly controlled by the concentration of phosphate anions. Defined particle diameters between 30 and 700 nm were obtained and the resulting size distributions were close to monodisperse. Replacement of orthophosphate by pyrophosphate, an anion

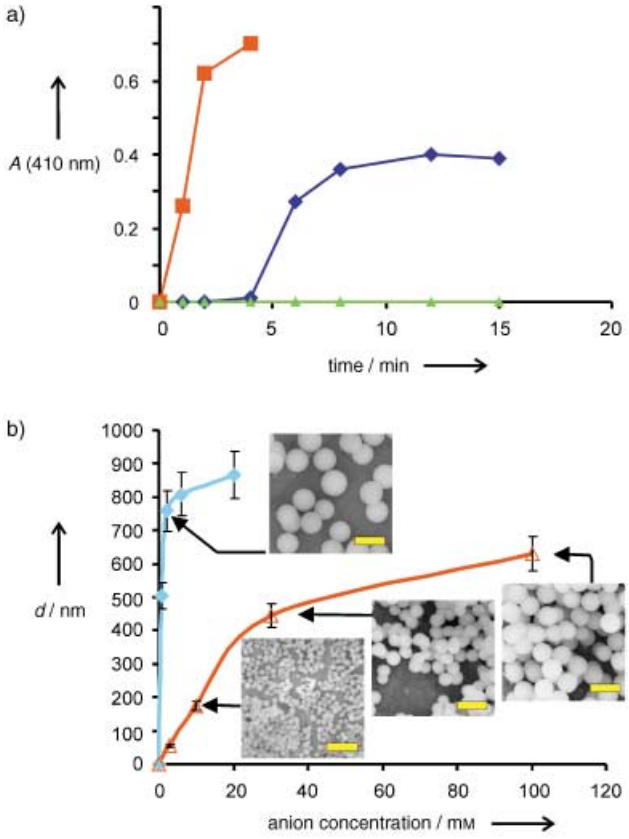

Figure 1. Characteristics of silica fabrication catalyzed by the polyamine/phosphate system. a) Kinetics of silica precipitation. The incubation mixture contained sodium phosphate (pH 5.5; 30 mM), polyamine (0.2 mM), and mono-/disilicic acid (40 mM) at 25 °C. After the times indicated, aliquots (50 μL) were removed and centrifuged. Precipitated silica was dissolved in NaOH (2 M; 5 min at 90 °C) and quantified by the molybdate method.[11] An absorbance of 1.0 corresponded to 0.5 μmol SiO_2. Blue line: All components were mixed at $t = 0$ min. Green line: Phosphate was replaced by sodium acetate (pH 5.5; 30 mM). Red line: Silicic acid was preincubated for 15 min in sodium phosphate (pH 5.5; 30 mM) and polyamine was added at $t = 0$ min. b) Silica nanosphere diameters as a function of the multivalent anion concentration. Silica fabrication was performed in a sodium acetate buffer (pH 5.5; 30 mM) as in part a) with increasing concentrations of multivalent anions. The reaction was allowed to proceed for 12 min. The resulting nanospheres were collected by centrifugation and analyzed by SEM. Response to orthophosphate and pyrophosphate is shown by the red and the blue lines, respectively. Insets: SEM micrographs of the corresponding precipitates. Scale bars: 1000 nm.

with a higher negative charge, gave rise to a drastic effect. Control of nanosphere size distribution was exerted at anion concentrations nearly two orders of magnitude lower and the maximum sphere diameter was increased to about 1000 nm (Figure 1b, blue line). Importantly, other multivalent anions can produce silicate precipitates as well, whereas monovalent anions such as chloride or acetate ions fail to do so. In particular, we have tested sulfate ions and polyvalent anions such as DNA and sodium dodecyl sulfate (see Supporting Information). The shape of the precipitates formed in the presence of the latter anions, however, is strongly different from the spherical nanoparticles obtained in the presence of phosphate ions.

Long-chain polyamines from diatom biosilica behave like amphiphilic substances. They are extractable from an aqueous solution by chloroform/methanol (3:2 v/v). Possibly, these polyamines form aggregates in aqueous solution (microemulsions) with positively charged surfaces. If so, multivalent anions should promote higher-order assemblies of the emulsion droplets. This possibility was investigated by NMR spectroscopy and dynamic light scattering techniques. The pronounced influence of phosphate ions on the ^{1}H NMR spectrum of polyamines from *S. turris* is demonstrated in Figure 2a. In the presence of phosphate (top trace), the ^{1}H nuclei attached to the repeating unit give rise to three signals A, B, and C. In the absence of phosphate, these signals split into two or more components (bottom trace). Evidently, the polyamine molecules exist in different states in the absence of phosphate, whereas a unique state is observed for the phosphate-containing sample. Furthermore, the ^{1}H NMR chemical shifts of the signals, except for C1 and C2, strongly depend on the type and concentration of the anions added, as shown for signal A1 (Figure 2b). Pyrophosphate (PPi) ions influenced the ^{1}H NMR chemical shifts much more strongly than orthophosphate (Pi). In contrast to the polyamines from *S. turris*, short-chain amines such as spermine did not exhibit corresponding phosphate-induced chemical shifts. The linewidths of the signals A and B strongly exceed those of lines C, C1, and C2 (Figure 2a).

Carr–Purcell–Meiboom–Gill (CPMG) experiments[21,22] demonstrated a relaxation rate of only 6 s^{-1} for lines C, C1, and C2, whereas T_2^{-1} amounts to 36 s^{-1} and 47 s^{-1} for lines A and B, respectively. An estimation of the methylene ^{1}H relaxation rate[23] expected for a single and globular polyamine molecule of 1.55 kDa molecular weight dissolved in water yields a value of about 3 s^{-1} only. Although the assumption of a globular shape for the polyamine molecules may only be a rough approximation, the discrepancy between the estimated value and the values observed for methylene ^{1}H (lines A and B) is surprisingly large. Aggregation of the polyamine molecules is a reasonable explanation for this observation. However, neither T_2^{-1} nor the diffusion coefficient were found to depend on the phosphate concentration. We therefore assume that the polyamine molecules dissolved in water form a microemulsion even in the absence of phosphate ions. If so, phosphate ions could attract the preformed droplets to build larger, loosely bound assemblies. The viscosity within the polyamine droplets are expected to be different from that of water and determines T_2^{-1}, independent of the phosphate concentration. According to this model, the diffusion coefficient of the polyamine molecules is determined by the diffusion within the droplets, independent of the phosphate concentration, as was observed experimentally (see above). In summary, we conclude that the polyamines from *S. turris* form aggregates under the solution conditions described. The addition of phosphate results in the formation of larger assemblies by the attraction of such preformed droplets. This concept could be confirmed by ^{31}P NMR spectroscopy on the phosphate-containing samples (see Supporting Information) and by dynamic light scattering experiments performed on phosphate-free and phosphate-containing polyamine solutions.

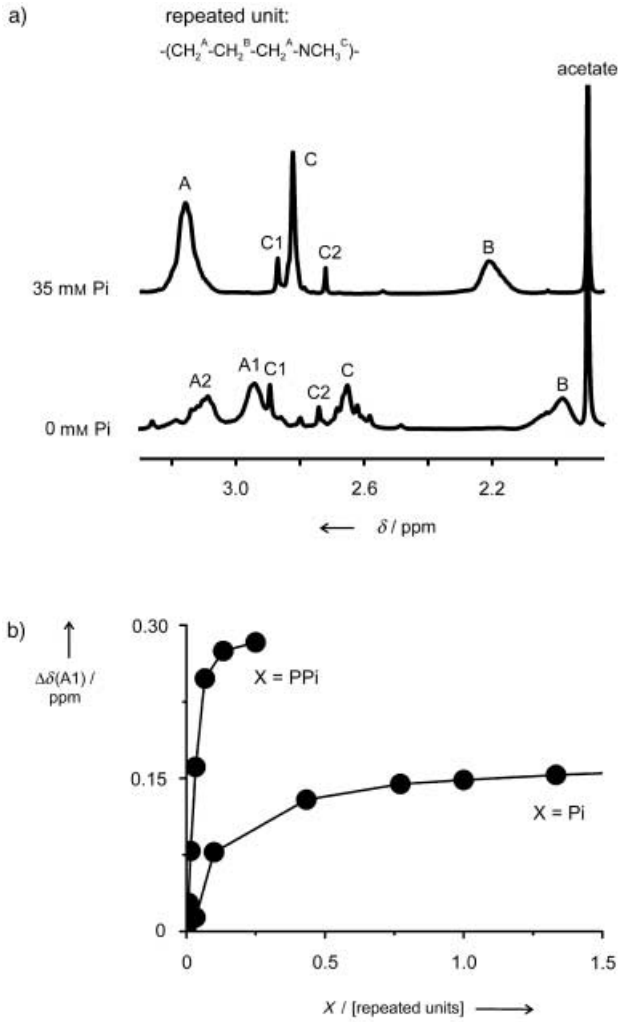

Figure 2. ^{1}H NMR spectra of polyamines from *S. turris*. a) Spectrum of a solution of polyamine (pH 6.3; 3 mM). Top: sodium phosphate (35 mM), bottom: phosphate-free sample. The assignment of the signals is indicated in the formula of the repeating unit. The small signals C1 and C2 were assigned to the methyl groups at the termini of the molecule. Note that the samples usually contained residual amounts of acetate from the purification procedure. b) Phosphate-induced chemical shift ($\Delta\delta$(A1)) of line A1 defined as the chemical shift difference between the phosphate-containing and the phosphate-free sample.

Phosphate-induced assembly of polyamines appears to be a prerequisite for silica formation, but the mechanism is unclear. Therefore, some of the partial reactions of silica formation were analyzed separately. First, the kinetics of monosilicic acid polymerization in the physiologically relevant pH range was determined in the absence or presence of *S. turris* polyamines. Monosilicic acid was easily determined by gas chromatography after conversion into the corresponding trimethylsilyl derivative.[18] Unexpectedly, the rate of monosilicic acid polymerization was not affected at all by the addition of *S. turris* polyamines, which contain tertiary amino groups exclusively (see Supporting Information). This observation is contrary to the catalytic action found for synthetic polyamines that contain secondary amino groups.[19] However, as the formation of silica nanospheres strictly depends on the presence of both *S. turris* polyamines and phosphate, we conclude that it is the condensation of oligo-/polysilicic acids that depends on the presence of polyamines and phosphate. If so, the lag phase observed in a silica precipitation experiment (Figure 1a, blue line) would represent the period required for the spontaneous polymerization of monosilicic acid. Only after the production of sufficient amounts of polymeric material, are these species further cross-linked by the action of the polyamine/phosphate system. Indeed, the lag phase was completely eliminated after the monosilicic acid solution had been preincubated for 15 min (Figure 1a, red line). Monosilicic acid ($pK_a = 9.7$) is an uncharged molecule under acidic conditions and therefore is unlikely to interact with polyamines. Only the much more acidic polysilicic acids are expected to interact with polyamine droplets through ionic interactions as well as through numerous hydrogen bonds with polyamine droplets. These interactions result in polysilicic acids becoming highly concentrated and they are therefore able to polycondensate to silica. By scanning electron microscopy (SEM) we also followed the time dependence of polyamine/phosphate-induced silica nanosphere formation in vitro (see Supporting Information).

These model studies define a cooperative function of polyamines and multivalent anions in the production of silica. In silaffins, nature has elegantly combined both structural elements in a single molecule. Self-assembly of phosphorylated silaffins has previously been documented.[7] Notably, nonphosphorylated silaffins are only capable of precipitating silica in vitro if phosphate or other polyvalent anions are added to the solutions. This behavior is in line with the observations reported herein: Silica precipitation is only triggered if phosphate or other polyvalent anions are added to solutions that contain polyamines and silica precursors. At the present time, however, we have no experimental information about the nature of the polyvalent anion acting in vivo in the polyamine-directed silica formation in *S. turris* cells.

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