Cryogenic electron tomography reveals the template effect of chitosan in biomimetic silicification†

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Chitosan (CS) can mediate the formation of spherical, tabulate, and unique starfruit-like silica in the presence of phosphate ions (Pi). CryoTEM and cryoET were used to examine the CS aggregates in the hydrated state. 3D starfruit-like CS/Pi aggregates were reconstructed, which unambiguously confirmed the templating effect of CS/Pi in biomimetic silicification.

The fascinating features of biogenic silica, such as the hierarchical multiscale structure found in the frustules of diatoms, have triggered a large range of biomimetic approaches to silica based materials.1 For the mechanism of silica formation in diatoms, two models have been proposed, i.e. the repeated phase-separation mediated templating mechanism (downscaling model)2 and the aggregation-based mechanism (upscaling model).3 The specialized biomolecules involved in these processes, e.g. silaffins and polyanimes, promote the formation of silica particles from silicic acid solutions also in vitro.4–6 Other biomimetic experiments have employed a variety of (macro)molecules,7–16 containing both nitrogen- and oxygen-abundant functional groups to effect biosilicification.17,18 Some of these experiments have revealed mechanisms in which a true copy of the template was produced,19 whereas others showed that the template changed under the mineralization conditions.20

We have recently reported the formation of unique starfruit-like silica structures through the use of a chitosan–phosphate complex as a soluble directing agent in biomimetic silicification.21 We found that the morphology of the resulting silica evolved from spherical to tabulate to starfruit-like structures (Fig. 1), depending on the incubation time of chitosan (CS) solution with phosphate ions (Pi) before silicification. To understand the mechanism involved in the templating process, we employed cryogenic transmission electron microscopy (cryoTEM) and cryogenic electron tomography (cryoET) to examine the aggregation states of CS before silicification.

CryoTEM allows the direct visualization of assembled structures in solution22,23 and at interfaces,24 by plunge-freezing the sample into liquid ethane such that the structures become embedded in a thin layer of vitreous ice and can be imaged in their native hydrated state without staining.22,23 CryoET involves the acquisition of a tilt series of cryoTEM images recorded at different tilt angles and the subsequent computer-assisted reconstruction of the volume. It has been demonstrated as a very powerful tool in studying self-assembly processes in three dimensions, both in solution and at interfaces.25–27

Upon incubation with Pi for 3.5, 22, 46, and 70 h ([Pi]/[CS repeating unit] = 1.6), the CS solutions gradually turned turbid (insets in Fig. 1). SEM studies revealed that after silicification using tetramethyl orthosilicate (TMOS) as the silica precursor,3 the resulting structures developed from spherical to tabular to starfruit-like (Fig. 1) as a function of the CS/Pi incubation time prior to the mineralization reaction.

The solutions of the un-mineralized CS/Pi complexes were vitrified and studied by cryoTEM (Fig. 2). After a short incubation period (3.5 h, Fig. 2a), spherical particles of ~15 nm in diameter were formed in the solution, as well as some thread-like structures. After 22-h incubation, aggregates with the dimensions of 50–200 nm and composed of the aforementioned particles were observed (Fig. 2b), including some denser, “beads on a string” structures (Fig. 2b, white arrows). When longer incubation times (46 and 70 h) were

Fig. 1 SEM images of silica particles, obtained after different incubation times for CS/Pi solutions: (a) 3.5 h, (b) 22 h, (c) 46 h, and (d) 70 h, followed by TMOS silicification for 4 h. Insets are the photographs of the unmineralized CS/Pi solutions after different incubation times.
applied, the projection images of the aggregates showed circular, almost electron transparent structures with superimposed electron-dense linear structures (Fig. 2c and d). These images were interpreted as starfruit-like structures with four layered leaves of which two were lying in the plane of the vitrified ice layer and the two other ones were sticking out of that plane, parallel to the electron beam. We speculate that the initial spherical particles tend to form tabular aggregates, which then stack up to form leaves of the starfruit-like structure, as exemplified by the thin black lines shown in the TEM images (Fig. 2c and d).

To verify this interpretation, cryoET was applied on a 70-h specimen. A tilt series of projection images was recorded under low-dose conditions between $-66^\circ$ and $66^\circ$. The series was reconstructed to a 3D volume (Fig. 3a and b) using a simultaneous iterative reconstruction technique (SIRT) algorithm (see Supporting Information). This confirmed that the aggregate prior to silicification indeed already had the starfruit-like morphology. This starfruit-like structure also had four leaves, which were made up of small spherical particles and one of them was only poorly developed (Fig. 3a and b).

Comparing the structure of the particles observed before (Fig. 2c and d and 3a and b) and after silicification (Fig. 1), it becomes clear that the starfruit-like structure arises from the incubation of CS with Pi and is not a consequence of a complex interaction between the organic template and the silica. Furthermore, the 2D and 3D cryo-images reveal that the multi-layered structure of fused particles found for the CS/Pi complex is accurately reproduced in the final product (Fig. 3c).

From these results we propose that in the presence of phosphate ions, CS undergoes an aggregation process, in which spherical particles are formed that in time assemble into starfruit-like structures in aqueous solutions. The fact that the layered structure is so accurately preserved in the silicified state (Fig. 3c) suggests that the silica deposition reaction may not be simply depositing on the outer surface of the organic structure. In our opinion, the detailed transcription of these structures in silica may be understood in terms of a mechanism that involves the infiltration of the small spherical aggregates with (hydrolyzed) precursors leading to a complete mineralization of the interior of the aggregates. Moreover, during the infiltration of silica precursors and silicification, the phosphate ions in the CS aggregates were not replaced by the silica (precursors). This was supported by energy-dispersive X-ray spectroscopy (EDX) data of the silica particle (Fig. 4) that showed the presence of phosphorus in the silica. Inset: TEM image of the starfruit-like silica.

Fig. 2 CryoTEM images of CS/Pi complexes before mineralization, after incubation times of (a) 3.5 h, (b) 22 h, (c) 46 h, and (d) 70 h.

Fig. 3 (a) 3D reconstructed model of the CS aggregate after 70-h incubation with vertical cross section of a leaf; (b) 3D structure without cross section; and (c) TEM image of silica obtained from 70-h incubation of CS solution followed by TMOS silicification for 4 h.

Fig. 4 EDX analysis of the silica product, indicating the presence of phosphorus in the silica. Inset: TEM image of the starfruit-like silica.
phosphorus was indeed present in the final particle, even after it was washed once with HCl (0.01 mmol dm\(^{-3}\)) and twice with water.

In conclusion, the shape of the silica structure can be unambiguously related to the templating effect of the CS/Pi complex during silicification. More specifically we demonstrated that the observed starfruit-like shape of silica particles is not due to the collapse of a spherical structure during drying (one of the possible causes we proposed before\(^{31}\)) but originates directly from the morphology of the CS/Pi aggregate. These results once again underline not only the effectiveness of cryoTEM to visualize self-assembled structures in their native hydrated state, but in particular the power of cryoET to analyze their 3D morphology.\(^{7,25-27}\)

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Notes and references