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COMMUNICATION

Lysozyme–silica hybrid materials: from nanoparticles to capsules and double emulsion mineral capsules†

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Alterations in the synthesis methodology concerning emulsion templated biocatalyzed silicification provide control over the morphology of SiO₂-hybrid structures.

Controlling the morphology and monodispersity of SiO₂-structures has been a subject of intense investigation due to their wide range of applications in coating technology, catalysis, controlled release studies, micro-reactors and composite materials.^{1–5} Methods to prepare monodisperse silica capsules *via* a two-step sol–gel process using various templating methodologies *e.g.* by emulsion or polymer aggregation have been reported.^{2,6,7,10} Recently, the formation of SiO₂ structures *via* biocatalysis has drawn much attention *via* the use of enzymes^{8–10} but also with small peptide fragments *e.g.* for SiO₂ surface coating.¹¹ SiO₂ hybrid granular and also hollow spheres were prepared using lysozyme as a biocatalyst for biomimetic SiO₂ synthesis from alkoxy-silane emulsions under mild aqueous conditions.^{12–14} Although this method is simple, controlling the morphology remains a challenge. So far hollow spheres in the range of 500 nm to 15 μ m *via* sonication or solid nano-hybrid particles in the range of 250 to 1000 nm have been produced. Broadening this scope would expand the possible applications for biomineralized SiO₂ structures tremendously as seen for other systems *e.g.* non-denaturing conditions for proteins,^{15,16} double emulsion capsules¹⁶ or inverted emulsion capsules for encapsulating water-soluble components,¹⁷ especially, without changing the specific chemical composition which can affect catalytic activity or stability of other components.

Here we present a straightforward approach to obtain a wide variety of stable biocatalyzed SiO₂-hybrid structures without changing the specific chemical components. Lysozyme is able to catalyze SiO₂ formation from SiO₂-precursors like (tetraethoxy-)silane (TEOS). By altering the sequence of addition, the agitation method (mechanical or sonochemical mixing)

Agitation^{time}: vortex (V) / stirring (S) / ultrasound (US). I.M.: intermediate product.
 *Lysozyme (900 μ L) 5.0 mg mL⁻¹, ** Lysozyme 2.5 mg mL⁻¹, ***Lysozyme (100 μ L) 300 mg mL⁻¹

Scheme 1 Overview of the method variation and the corresponding lysozyme–silica-hybrid structures which were obtained.

and relative amount of the individual components, nanoparticles, capsules with controlled size exhibiting various surface morphologies, inverted capsules and double emulsion mineralized capsules were obtained. The results demonstrate the possibility of tuning the system without any additives. The standard composition of the reaction mixture used to produce the biocatalyzed SiO₂ structures was lysozyme (5 and 2.5 mg mL⁻¹) in 0.05 M glycine buffer pH 9.0 (900 mL) and 100 mL TEOS. A schematic overview of the synthetic procedures and resulting hybrid structures (HS) is shown in Scheme 1.

Proteins, including lysozyme, are able to self-assemble at polar–apolar interfaces and form a “Pickering”-type emulsion.^{16,18} Interfacial tension measurements confirm that the buffer–TEOS interface is stabilized by lysozyme (Fig. S1, ESI†). After 24 h of reaction time as well as at intermediate stages the morphology of the resulting structures was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The (chemical) composition was determined by thermogravimetric analysis (TGA) and energy-dispersive X-ray spectroscopy (EDX).

With the conventional approach, namely 900 μ L lysozyme solution and 100 μ L TEOS mixed *via* sonication (15 min, 35 kHz), small capsules are formed due to efficient emulsification (Fig. 1a).^{12–14} These capsules have a broad size distribution (300 nm to 15 μ m) and are stable up to 10 days but not for longer periods (Fig. S2, ESI†). Varying the agitation method provides very different structures *e.g.* 15 min of vortexing (2500 rpm) resulted in large capsules in the range of 50–100 μ m with a much thinner shell (Fig. 1b) while slow stirring (15 min, 5 oscill. min⁻¹) provides smaller irregularly

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Fig. 1 SEM images of hybrid structures prepared by single addition of 100 μL TEOS to 900 μL lysozyme solution (5 mg mL^{-1}) with different agitation methods: (a) **HS3**; (b) **HS1**; (c) **HS2** and (d) **HS5**. Insets are higher magnifications showing the surface structures in more detail (for d, a TEM image is shown).

shaped particles of 50–300 nm (Fig. 1c). The particles are mineralized together into a large cluster, the milder agitation method provides ample time for them to grow together. Particle formation was previously found for non-biocatalyzed mineralization upon applying weaker mechanical forces.⁶ The surface of the particles seems to be very rough in contrast to the particles shown in Fig. 1d (the TEM inset displays homogeneously filled structures) where the same agitation method is applied only now for the entire duration of the reaction (24 h) and provides less clustered and smoother particles. In contrast to previous works, we applied external stirring (at 5 oscill. min^{-1}) instead of internal stirring lowering the shear stress and providing a more homogenous system.^{12,13} EDX confirmed that these particles are also protein– SiO_2 hybrids due to the presence of sulfur which is indicative of the lysozyme (see Fig. S3, ESI[†]).

Precipitation of SiO_2 particles comprises four steps:¹⁹ precursor hydrolysis, nucleation, oligomer agglomeration forming primary particles and finally their further agglomeration. Based on this mechanism, upon increasing the time of stirring at constant strength, the number of monodisperse particles increases and they become more spherical due to sufficient nucleation-time. Previously, it was found that formation of primary particles and precursor hydrolysis are important for particle growth.^{20,21} It is noteworthy to mention that aggregation of oligomers leads to formation of primary particles which mainly controls further emulsion templating due to their colloidal properties.¹⁹ Based on this knowledge, the synthetic method was adjusted. First 20 μL of TEOS was added followed by mild agitation. Finally the remaining 80 μL was added leading to the same overall composition of 100 μL . The emulsion was then stirred for one day. By addition of one part TEOS followed by 30 minutes mild stirring, formation of oligomers and nucleation proceed throughout the other precipitation steps, ensuring a homogenous distribution of nuclei over the sample.²⁰ After the second TEOS addition, a new emulsion-template is formed, producing new primary particles alongside the already existing ones. As the growth rate of silica particles is a surface limited reaction, the surface-to-volume ratio is the critical factor determining the final size

of the obtained particles.¹⁹ As shown in Fig. S4 (ESI[†]), granular hybrids of polysilicate–protein particles (I.M.) are observed in the intermediate stage after the first addition. At this stage, no agglomeration occurs due to low concentration and mild agitation. This was also observed for non-biocatalyzed studies where TEOS-droplets acted both as template and precursor.^{22,23} By adding the remaining TEOS (80 μL) a new emulsion templated system is generated. Here the initially formed hybrid oligomers assemble around the emulsion droplets creating hollow SiO_2 -hybrid capsules in a regular fashion.

Using the two step procedure, different types of capsule structures emerge depending on the type of agitation without changing the overall reaction composition or reaction time with respect to the single addition procedure. While the single addition step of 100 μL of TEOS and mild stirring led to the formation of solid nanoparticles (Fig. 1d), the two step addition results in hollow structures with a shell which resembles primary particles grown together around the emulsion template (Fig. 2a). The diameter of the capsules is about 250 nm which is smaller than that of the solid particles in the one step method and significantly smaller than that reported previously,^{12,13} and more homogeneous in size. What is particularly interesting is that the shell morphology becomes smoother and more homogenous when 15 min sonication is applied (Fig. 2b). These sequences can be varied even more *e.g.* by introducing another high energy agitation step directly after the second addition (**HS8**). While all other parameters are the same as for **HS7**, the additional treatment with ultra-sound for 15 min after the second addition results in capsules with a more homogenous shell and a diameter between 500 and 2000 nm for which less agglomeration between capsules is observed (Fig. 2c). That the capsules are larger than for **HS7** can be explained by droplet-fusion due to enhanced collisions.²⁴ It is shown that one day stirring is necessary to obtain stable structures (less time results in insufficient mineralization, Fig. S5, ESI[†]). Surprisingly, lowering the lysozyme concentration to 2.5 mg mL^{-1} compared to **HS8** (5 mg mL^{-1}) resulted in nanocapsules **HS9** (Fig. 2d)

Continuous stirring for 24 h provides a homogeneous distribution within the solution and ample time for species

Fig. 2 SEM images of capsules obtained *via* the two-step preparation method: (a) **HS6** (b) **HS7** (c) **HS8** and (d) **HS9**. Inset: TEM/SEM images showing the nature of corresponding particles. All inset scale bars are 300 nm.

Fig. 3 Fluorescence microscopy (oil-phase, red) and SEM images of double mineralization capsules **HS10** (a)/(b) and inverted emulsion capsules **HS11** (c)/(d).

to assemble at the interface and thereby forming a strong shell structure. Results show that the capsules formed *e.g.* in **HS8** are stable for at least one month (Fig. S6, ESI†) which is much longer than the conventional capsules (**HS3**) (Fig. S2, ESI†). It becomes clear that minor changes in the preparation methods have significant influences. This is further supported by the lack of structures when an additional sonication step was added between the initial stirring and the second addition (Fig. S7, ESI†). When capsules are obtained using a modified method where previously no structures were obtained it means that the yield is better or that structures become more stable. In the last case it would mean that a lower lysozyme concentration would also be sufficient. While normally no structures would be the result or at least a low yield using a 2.5 mg mL⁻¹ lysozyme solution (**HS4**, Fig. S9, ESI†), using the two-step method, hollow-nanocapsules are obtained (**HS9**, Fig. 2d).

Surprisingly, the composition of the hybrid structures with respect to the protein/SiO₂ ratio is relatively constant at 45 wt% protein and 55 wt% SiO₂ and only deviates a few percent depending on the method used (shown by TGA, S8, ESI†). Compared to pure lysozyme all the individual transitions native to lysozyme are also observed in the hybrid structures. That lysozyme is active under the synthetic conditions is recognized by the SiO₂ formation and the presence of all individual transitions in TGA depicts the presence of lysozyme. However, to obtain more details about the structure of lysozyme inside the SiO₂ structure, a comparative Raman-study was performed. From the Raman-fingerprint of pure lysozyme, it was found that the structural features can also be recognized in the hybrid material (**HS3** and **HS8**) namely the presence of the β -sheet as well as the α -helix motifs (S10, ESI†). Furthermore, the overall spectra resemble that of lysozyme very nicely with only a slight loss of structural features.

In previous work we showed that the preparation of double emulsions is possible using ultra-sound,¹⁶ using a lower TEOS content (10 μ L/1 mL lysozyme solution (5 mg mL⁻¹)) and longer sonication times in a glass vial instead of a plastic eppendorf and thus increasing the energy input to such an extent that aqueous μ -droplets are pushed into the oil droplet. Upon mineralization this should provide a double emulsion biocatalyzed

mineral capsule structure. The double emulsion was analyzed by (fluorescence-) microscopy in which Nile Red was added to visualize the oil-phase and even got incorporated into the mineral shell (Fig. 3a and Fig. S11, ESI†). The red structures inside the larger capsules are clearly visible and the double mineralization process was confirmed by SEM (Fig. 3b). In most emulsion templated systems only hydrophobic components can be encapsulated unless an inverse emulsion is used in which aqueous capsules should be formed. This was tested by using 100 μ L lysozyme solution (300 mg mL⁻¹) in 900 μ L TEOS. The higher concentration lysozyme is necessary to compensate for the lower overall amount of protein because of the lower water content. *Via* optical microscopy many capsules were observed which appeared to be highly clustered inside a much larger capsule (Fig. 3c). Analysis by SEM did not display these large capsules but many smaller ones grown together (Fig. 3d). Since the minor phase is buffer, the capsules observed should be with an aqueous interior, which was confirmed by fluorescence microscopy (inset in Fig. 3c) where red indicates TEOS (stained with Nile Red). The structures here are prone to form large clusters instead of separated capsules as shown by SEM (Fig. 3d).

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