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Chitosan-mediated formation of biomimetic silica nanoparticles: An effective method for manganese peroxidase immobilization and stabilization

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Our work here, for the first time, reported the use of chitosan-mediated biomimetic silica nanoparticles in enzyme immobilization. In order to make clear the relationship among silicification process, silica nanoparticle structure and immobilized enzyme activity, a mechanism of chitosan-mediated silicification using sodium silicate as the silica source was primarily evaluated. Chitosan was demonstrated effectively to promote the silicification not only in accelerating the aggregation rate of sodium silicate, but also in templating the formation of silica nanoparticles. Although the whole biomimetic silicification process contained polycondensation–aggregation–precipitation three stages, the elemental unit in precipitated silica was confirmed to be nanoparticles with 100 nm diameter regardless of the chitosan and silicate concentration used. Furthermore, the effect of enzyme on silicification process was also investigated. The introducing of manganese peroxidase (MnP) to silica precursor solution had no obvious effect on the silicification rate and nanoparticle morphology. The residual activity and embedding rate of immobilized MnP were 64.2% and 36.4% respectively under the optimum conditions. In addition, compared to native MnP, the MnP embedded in chitosan/silica nanoparticles exhibited improved stability against organic solvent and ultrasonic wave. After ultrasonic treatment for 20 min, 77% of the initial activity was remained due to the protective effect of chitosan/silica nanoparticles, while native MnP lost almost all of its original activity.

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[**Key words:** Chitosan; Biomimetic silicification; Manganese peroxidase; Silica nanoparticle; Immobilization]

Silica has received extensive attention across a broad range of research fields as the second most abundant oxide component in the Earth's crust and mantle. Especially in recent 10 years, significant advancements in understanding of molecular mechanism of biosilicification in numerous marine organisms have facilitated the rapid progress in biomimetic synthesis of silica materials in vitro. As has been clarified in the previous studies, the formation of biosilica was regulated by polypeptide (1) and polysaccharide (2) through the repeated phase-separation mediated templating mechanism (3) and the aggregation-based mechanism (4). The polypeptide separated from biosilica-forming organisms has been primarily demonstrated to be effective in promoting biomimetic silicification in vitro. Furthermore, silaffins (5), papain and even synthetic polyamines (6,7) were used as templates to regulate silica morphogenesis in vitro under mild conditions. By contrast, polysaccharide (8,9) mediated biomimetic silicification process has long been neglected. Considering the totally different conformation of polysaccharide and polypeptide in solvent system, the function of polysaccharide in silicification was also worth of deep investigation, and the confirmation of the roles of polysaccharide played in biomimetic silicification would inspire better understanding of the polysaccharide function in biosilica formation for various organisms.

Chitosan, a typical cationic polysaccharide having multiple free amino groups, was one of the few number of polysaccharides has been proved effective in bioinspired silica synthesis at ambient temperature, but the role of chitosan in silicification process has not yet been thoroughly clarified at present. In most literatures (10,11), the proposed mechanisms suggested that terminal amine groups of chitosan played a bi-functional role in silicification including catalyzing the hydrolysis/condensation of silica source and the subsequent aggregation of silica. Varying the incubation time of chitosan solution with phosphate ions before silicification, the morphology of the resulting silica would evolve from spherical to tabulate to starfruit-like structures (12). While Chang et al. (13) considered that chitosan only facilitated the aggregation of nanoparticles, but did not significantly change the rate of silica synthesis and the size of the nanoparticles. Demadis et al. (14) reported that phosphonated chitosan was able to inhibit silicic acid condensation at neutral pH via a combination of electrostatic and hydrogen-bonding interactions. Since the role of chitosan in silica nanoparticle formation was not consistent at present, it was still worth pursuing the actual mechanisms underlying chitosan promoted silicification.

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Polymer mediated formation of silica composite materials have attracted much attention from fields of enzyme immobilization, because the polymer/silica composites ingeniously combined the desirable characteristics of both organic polymer and inorganic silica compounds. Entrapping enzyme in biomimetic silica nanoparticles exhibited advantages in both embedding rate and enzyme residual activity. For instance, Naik et al. (15) prepared R5 peptide mediated silica matrix from TMOS to co-entrap the R5 peptides and enzyme during the generation of silica matrix, the immobilized catalase and horseradish peroxidase (HRP) showed activities comparable to their native formations. Betancor et al. (16) reported the immobilization of nitrobenzene nitroreductase in PEI-mediated silica particles, the embedding rate and activity yield of the enzyme were 99.5% and 58.7%, respectively. So far, the encapsulation of biomolecules within silica nanoparticles using bioinspired silicification process has already been extensively used to enzymes including oxido-reductases (17), hydrolases (18) and isomerases (19). However, the types of silica mediating agents were found to have significant effect on enzyme activity and affinity to substrate. Roth et al. (20) revealed that luciferase embedded in cysteamine and ethanolamine mediated silica retained 87% and 17% of the original activity respectively. Therefore, enzyme embedded in polysaccharide-mediated silica might obtain distinctive results compared with polypeptide-mediated silica. Unfortunately, very few studies were available on enzyme encapsulation within the biomimetic silica nanoparticles that using polysaccharide as a template, especially for chitosan promoted silica nanoparticle. Furthermore, studies on effect of enzyme encapsulation on biomimetic silicification have also been generally neglected.

In this work, chitosan promoted silica nanoparticles was for the first time used to encapsulate MnP. As the naturally existed cationic polysaccharide, chitosan has far cheaper price and cost than artificially synthesized polypeptide. More importantly, chitosan-mediated silicification might suitable for proceeding at acidic pH value, which met well with the suitable pH value for maintaining MnP activity. The regulating function of chitosan in both kinetic behavior of silica polymerization and the growth pattern of nanoparticle were systematically analyzed. In addition, the effect of manganese peroxidase (MnP) on silicification process was investigated. The stability of immobilized MnP in organic solvent and ultrasonic wave was also evaluated.

MATERIALS AND METHODS

Reagents Manganese peroxidase (MnP) from white-rot fungus (*Phanerochaete chrysosporium*) and ABTS (≥98%) were purchased from Sigma–Aldrich Co. (USA). Chitosan with an average molecular weight of 200,000 g/mol was purchased from J&K Chemical Co. (Beijing, China). Sodium silicate ($Na_2Si_3O_7$: 27% w/w SiO_2, 4% w/w NaOH) was obtained from Xilong Chemical Co. (Shantou, Guangdong, China). Ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals and reagents were of analytical grade and used without any further purification.

Preparation of chitosan/silica composites Chitosan was dissolved in 2% w/w acetic acid solution. Sodium silicate solution with concentration of 45 to 100 mmol/L was prepared by dissolving sodium silicate in 50 mmol/L sodium acetate solution and the pH value was adjusted to 7.0 by the addition of acetic acid. After agitating 3 mL of the sodium silicate solution for 10 min at room temperature, 300 μL of chitosan solution was added to yield a final concentration of 0 to 0.2% w/v. The resulting solution with a final pH of 5.5 was mixed homogeneously and then left undisturbed for silicification.

Determination of silicic acid concentration Variation in concentration of silicic acid and small silica oligomers during silicification process were periodically determined by the molybdosilicate titration method (21), using a standard curve established with different concentrations of sodium silicate solution. At regular time intervals, 100 μL of silicification solution was taken and added to 3 mL of deionized water. Equal volume (200 μL) of 1.5 mol/L H_2SO_4 and 0.08 mol/L ammonium molybdate were then added (22). The mixture was left to stand for 10 min in order to allow monomeric silicic acid and small silica oligomers to react with the heptamolybdic acid, and ultimately form the yellow silicomolybdic acid $H_8Si(Mo_2O_7)_6$. The optical density of the final solution was measured at 400 nm using a 2800 UV/VIS spectrophotometer.

Morphology and composition characterization of chitosan/silica composites The variation of chitosan/silica composite diameter during silicification process was determined by dynamic light scattering (DLS) using the ZetaPlus apparatus (Brookhaven Instruments Corp., USA) at a wavelength output of 660 nm. The measurement was conducted at a scattering angle of 90° with 2 mL of 45 mmol/L sodium silicate in quartz tube as a control solution. Then 0.2 mL of chitosan solution (0.002 to 0.2% w/v) were added directly to the sodium silicate control solution in order to initiate the silicification reaction. At different reaction time from 0 to 200 min, quartz tube containing silicification solution was placed into DLS apparatus. After 3 min measurement, the mean diameter of chitosan/silica composites could be determined from peak point of DLS data. While the quartz tube was fetched out and stay still until next detection. The relation between composite diameter and silicification time could thus be confirmed.

Scanning electron microscope (SEM, JSM-6700F, JEOL, Japan) was used to observe the morphology of nanoparticles.

In order to estimate the proportion of chitosan in chitosan/silica nanoparticles (23,24), thermogravimetric analysis (TGA) was performed on a simultaneous DSC-TGA instrument (SDT Q600, TA Instruments, USA). After the first run of heating up to 120°C and cooling to room temperature at a rate of 20°C/min, the TGA curves of each sample were obtained from the second run at the same heating rate under nitrogen atmosphere.

MnP immobilization MnP was dissolved in sodium malonate buffer solution (0.25 mol/L, pH 4.5) to give a final concentration of 1 mg/mL. Aliquots of 100 μL MnP solution and 100 μL chitosan solution were mixed and pre-incubated for 0.25 to 2 h. Then the mixture solution was added to 1 mL sodium silicate solution. The final chitosan concentrations were changed from 0 to 0.2% w/v, and the sodium silicate concentrations were ranged from 45 to 100 mmol/L. The silicification samples were left undisturbed for 15 min and then centrifuged for another 15 min at 12,600 ×*g* in order to separate the MnP-embedding silica nanoparticles. The MnP-immobilized silica nanoparticles were repeatedly washed 3 times with 1 mL buffer solution for each wash until no protein was detected in the washing solutions. MnP concentration was determined by BCA method using BSA as the protein standard. Embedding amounts of MnP in nanoparticles was calculated by initial protein amounts presented in MnP solution subtracting the total protein amounts presented in the supernatant and washing solutions. The embedding rate was the value of the embedding amount of MnP divided by the initial protein amounts.

MnP activity assay MnP activity was determined spectrophotometrically at 30°C and 468 nm as described by Field et al. (25). The method is based on the oxidation of 2,6-dimethoxyphenol (2,6-DMP) by the MnP to form aquinone dimer. The molar extinction coefficient of the product at this wavelength is 49,600 L mol^{-1} cm^{-1}. The MnP activity assay system was consisted of 200 μL sodium malonate (250 mmol/L, pH 4.5), 50 μL 2,6-DMP (20 mmol/L), 50 μL $MnSO_4$ (20 mmol/L), 550 μL water, and 50 μL of enzyme sample (containing 10 μg protein) whose MnP activity is to be determined. The reaction was initiated by the addition of 20 μL H_2O_2 (20 mmol/L), ended up by adding 1 mL H_2O_2. One unit of activity was defined as the amount of enzyme which oxidize 1 μmol of 2,6-DMP per minute. Taking the activity of native MnP as 100%, the activity recovery of embedded MnP was represented by a ratio of embedded MnP activity to its native form activity under the identical reaction conditions.

Enzyme kinetic assay The reaction kinetic assay of native and immobilized MnP (containing same amount of protein about 2 μg) were also detected by 2,6-DMP oxidation method by varying the 2,6-DMP concentration from 2 to 20 μmol/L, and the kinetic data including Michaelis constant (K_m) and the maximum reaction rate (V_{max}) were obtained from the Lineweaver–Burk plots.

Stability of native and immobilized MnP The enzyme activity assays were performed with native MnP solution containing 10 μg protein, or chitosan/silica nanoparticles embedding 10 μg MnP. Native or immobilized MnP were suspended by 1 mL buffer solution, followed by incubation in ultrasonic wave condition. The tolerance of enzyme against ultrasonic wave was investigated by continuously exposing the enzyme to 100 Hz ultrasonic wave for 3 s to 30 min. The temperature was maintained constant at 30°C during ultrasonic process by the temperature control system and circulating water system equipped in ultrasonic bath machine. The native and immobilized enzyme in the absence of ultrasonic treatment was assumed to have 100% activity. Residual activity was determined as the ratio of enzyme activity after and before ultrasonic wave treatment.

To determine the stability of native and immobilized MnP in organic solvent, MnP was dispersed in 1 mL buffer solution containing 36% v/v acetone. After incubation for 2.5 to 20 h, activity of enzymes were assayed. The activity of native and immobilized enzyme incubated in the absence of acetone was considered to be 100%. Residual activity was defined as the ratio of enzyme activity after and before organic solvent treatment.

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