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RESEARCH ARTICLE

Immobilization of β-glucuronidase in lysozyme-induced biosilica particles to improve its stability

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Abstract Mesoporous silica particles were prepared for efficient immobilization of the β-glucuronidase (GUS) through a biomimetic mineralization process, in which the solution containing lysozyme and GUS were added into the prehydrolyzed tetraethoxysilane (TEOS) solution. The silica particles were formed in a way of biomineralization under the catalysis of lysozyme and GUS was immobilized into the silica particles simultaneously during the precipitation process. The average diameter of the silica particles is about 200 nm with a pore size of about 4 nm. All the enzyme molecules are tightly entrapped inside the biosilica nanoparticles without any leaching even under a high ionic strength condition. The immobilized GUS exhibits significantly higher thermal and pH stability as well as the storage and recycling stability compared with GUS in free form. No loss in the enzyme activity of the immobilized GUS was found after 30-day's storage, and the initial activity could be well retained after 12 repeated cycles.

Keywords silica nanoparticles, biocatalysis, biomimetic synthesis, β-glucuronidase encapsulation, storage and recycling stability

1 Introduction

Enzymes have been widely used as biocatalysts in chemical and pharmaceutical industries due to their extraordinary catalytic efficiency and selectivity under mild and environmentally friendly conditions [1]. Nonetheless, the native enzyme itself is not an ideal catalyst for commercial applications owing to its vulnerability under harsh industrial conditions and the difficulty in separation and recovery [2]. Enzyme immobilization offers an effective solution to overcome these drawbacks by rendering the immobilized enzymes with enhanced stability, reusability, and facile separation from reaction mixture to prevent enzyme contamination in products [3]. A broad range of materials has been utilized for enzyme immobilization, such as silica gel, porous glass, chitosan, polystyrene colloidal particles, cellulose, and hydrogel. In comparison, silica materials offer a favorable microenvironment for the immobilized enzymes and convey an increased stability under harsh conditions [4–10]. Immobilization of enzyme inside the pores of sol–gel silica particles has been one of the most widely used methods [11–14]. However, the conventional sol–gel method requiring a strong acid or base as the catalyst hinders its wide application in enzyme immobilization. Biomineralization, a ubiquitous process in nature, has offered a delicate prototype for the fabrication of inorganic minerals in organisms under mild conditions [15–18]. In recent years, nanostructured silica materials inspired by the biomineralization process have attracted much attention [15,19–21]. This kind of silica, the so-called biosilica, is generated from aqueous solutions under the mediation of some specific organic substances secreted by certain polypeptide-based biomacromolecules, and can form the biomineralized materials with hierarchical structure and specific assembly [19,20]. The reaction, known as biosilicification, can be mimicked *in vitro* by utilizing synthetic peptides based on the native protein sequences, or identified from combinatorial peptide libraries [22,23].

Lysozyme is a natural cationic protein which has been successfully utilized in the biomineralization of silica [24–28]. Lysozyme as a template for biomineralization is advantageous because it is a ubiquitous protein and commercially available relative to the specific silicification

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proteins that are expensive and labor intensive to purify. The resultant bio-nanocomposites of lysozyme with amorphous silica have been found to be able to retain the native anti-bacterial activity of the proteins [24,29]. The idea of extending the biomineralization process to enzyme immobilization originated from the observation that silica-precipitating species could be entrapped during silica formation [29]. For example, lysozyme-mediated silica formation was successfully utilized to encapsulate organophosphate hydrolase (OPH) in a silica matrix immobilized directly on a waveguide surface [27,30]. Similarly, a silica-precipitating peptide was used to mediate the biosilica formation and the immobilization of horseradish peroxidase [31]. In both cases, the enzymes immobilized in the biosilica via the biomimetic mineralization approach exhibited dramatically enhanced stability.

Baicalin, a major flavonoid derived from the root of Scutellaria baicalensis Georgi, has been proved to be an important herb used in herbal medicines. Baicalein, the aglycone of baicalin, has much higher activity in pharmacological functions than baicalin, but its content in nature is very low [32–34]. β-Glucuronidase (GUS) can catalyze the conversion of baicalin into baicalein. In this study, GUS was immobilized in biosilica nanoparticles via one step precipitation reaction. Lysozyme catalyzed the formation of silica when added to a solution of prehydrolyzed tetraethoxysilane (TEOS) and GUS was entrapped in biosilica particles during silica precipitation. The immobilized GUS was used in converting baicalin into baicalein, its catalytic performance as well as the relevant stability were both investigated.

2 Materials and methods

2.1 Materials

Lysozyme was purchased from Blood Institute of Tianjin Academy of Medical Sciences. β-Glucuronidase (GUS) (EC 3.2.1.31) from *Escherichia coli* (type IX-A, lyophilized power, 1000000–5000000 units·g^{-1} protein) were purchased from Sigma Chemical Company. Tetraethoxysilane (TEOS) (19.3~22.8% SiO_2) from Tianjin Kewei Chemical Co. Ltd. was used as the silica precursor. Baicalin and baicalein standards for analysis were obtained from the National Institute for the Control of Pharmaceutical and Biologic Products of China. Baicalin (purity $\geqslant$ 98%) used as the substrate was purchased from Sichuan Xieli Pharmaceutical Co. Ltd. All the other chemicals are of analytical reagent grade.

2.2 Synthesis of lysozyme-induced silica nanoparticles and GUS encapsulation

Lysozyme (10 mg·mL^{-1}) was dissolved in Tris-HCl buffer solution (pH 7.0, 30 mmol·L^{-1}). The protein solution (1 mL) was added to 7 mL of the prehydrolyzed TEOS solution which was diluted 40 times. After the reaction mixture was standing for 5 min at room temperature, the resultant precipitate was collected by centrifugation for 5 min at 3000 rpm, and then washed three times with deionized water to remove the unreacted silicon precursor.

Similarly, a protein mixture of lysozyme (10 mg·mL^{-1}) and GUS (0.8 mg·mL^{-1}) was prepared by dissolving them in Tris–HCl buffer solution (pH 7.0, 30 mmol·L^{-1}). The protein mixture (1 mL) was added to the diluted prehydrolyzed TEOS solution (7 mL). The silica nanoparticles containing GUS were obtained after centrifugation, washed, and freeze-dried.

2.3 Transmission electron microscopy (TEM) and selected-area electron diffraction (SAED)

TEM observation was performed on a JEM-100CXμ instrument, and the crystallinity of the nanocomposites was measured by SAED attached to the TEM.

2.4 ^{29}Si MAS NMR

Solid-state ^{29}Si MAS NMR spectra of the nanocomposites were recorded on a solid-state NMR spectrometer. The sample was placed in ZrO sample tube and was spun at 3 kHz. The ^{29}Si resonance frequency was 59.6 MHz and pulse delay time was 5 s.

2.5 Pore-size distribution

The pore-size distribution of silica nanoparticles was determined by nitrogen adsorption-desorption isotherm measurements performed at 77 K on a Tristar 3000 gas adsorption analyzer. The samples were degassed at 313 K for 24 h prior to measurement. Pore-size-distribution curves were calculated based on the adsorption branch of nitrogen isotherms using the Barrett-Joyner-Halenda (BJH) method.

2.6 Immobilization efficiency

The enzyme encapsulation capacity was evaluated in terms of immobilization efficiency. The supernatant after centrifugation of the precipitates was collected and its residual GUS activity was determined by conducting enzymatic conversion reaction. Since both the two proteins involved in the co-precipitation, lysozyme and GUS, might exist in the supernatant, it was difficult to determine the GUS content inside exactly, so the enzyme immobilization efficiency was determined by comparing the difference between the residual activity of the supernatant and that of the original protein mixture, which was expressed in the following equation (Eq. (1)):

$$\text{Immobilization efficiency}/\% = \frac{[\text{GUS}]_{\text{supernatant}}}{[\text{GUS}]_{\text{dissolved}}} \times 100\%. \quad (1)$$

where $[GUS]_{supernatant}$ and $[GUS]_{dissolved}$ were the activity of GUS in the supernatant solution and in the original lysozyme-GUS solution, respectively.

2.7 Enzyme leakage

The dried biosilica particles with enzymes inside were immersed in Tris-HCl buffer solution and removed at specified time intervals by centrifugation. The activity of the supernatant was measured and the enzyme leakage as a function of immersion time was studied in terms of the change of encapsulation efficiency with time. To further test the binding strength between the enzyme and the biosilica, the GUS-containing biosilica was immersed in saturated KCl solution at 37°C for 24 h. The supernatant solution after centrifugation was detected by the micro-Bradford method. The absorbance of the above solution was measured at 595 nm by a UV spectrophotometer (Hitachi U-2800).

2.8 Enzymatic conversion reaction and productivity

The activity of free or immobilized GUS was evaluated by calculating the conversion of baicalin to baicalein. Equal amount of free GUS or immobilized GUS (*ca.* 0.15 mg) was introduced into a beaker containing 8 mL of 0.09 $mmol \cdot L^{-1}$ baicalin and 0.1% w/v Na_2SO_3, both of which were dissolved in Tris-HCl buffer (30 $mmol \cdot L^{-1}$, pH 7.0). Na_2SO_3 was used here as an antioxidant. The beaker was tightly sealed and the reaction was performed at 37°C under stirring. The amount of baicalein produced in the reacting solution was measured by HPLC (HP1100, Agilent) equipped with Agilent ZORBAXSB-C18 column and recorded with reaction time. A mixture of methanol: H_2O: H_3PO_4 (60: 40: 0.2) was employed as the mobile phase at a flow rate of 1 mL/min. The detection wavelength was set at 274 nm. The enzyme activity unit was defined as the amount of GUS needed to produce 1.0 μmol of baicalein per hour at 37°C, pH 7.0. The productivity was defined as the mole ratio of the amount of baicalein produced to the original amount of baicalin in the feed. The Michaelis constant (K_m) and the maximum reaction rate (V_{max}) were determined by varying the substrate concentration from 0.06 to 0.90 $mmol \cdot L^{-1}$.

2.9 Optimal temperature and pH

The optimal reaction conditions including temperature and pH were determined by changing the conversion temperature from 30°C to 70°C and the pH from 4.0 to 9.0, respectively. The highest activities of free and immobilized enzymes under the optimal conditions were assigned to be 100%; the relative activities of free and immobilized enzymes were defined as the ratio of their activities to the highest activities, respectively.

2.10 Thermal stability and pH stability

The thermal stability for free or immobilized GUS was studied by introducing them into Tris–HCl and incubating for 1 h at temperatures ranging from 30°C to 70°C. Then, the residual activity was measured at optimal temperature. Similarly, the pH stability was investigated by incubating free or immobilized GUS in Tris-HCl buffer solution with a pH value of 4.0-8.0 for 1 h followed by measuring the activity at optimal pH.

2.11 Storage stability

Free or immobilized GUS was stored at 4°C for certain periods of time. The storage stability was denoted by storage efficiency, which was defined as the ratio of free or immobilized enzyme activity after storage to their initial activity (Eq. (2)).

$$\text{Storage efficiency}/\% = \frac{\text{enzyme activity after storage}}{\text{initial enzyme activity}} \times 100\%. \quad (2)$$

2.12 Recycling stability

The silica precipitates containing GUS were collected by filtration after each reaction batch (37°C, pH 7.0, reaction time 60 min) and the activity of the first batch was taken as 100%. After one cycle, the silica precipitates containing enzyme were removed from the reaction medium and then washed with distilled water and buffer solution (Eq. (3)).

$$\text{Recycling efficiency}/\% = \frac{\text{enzyme activity in the } n^{\text{th}} \text{ cycle}}{\text{enzyme activity in the } 1^{\text{st}} \text{ cycle}} \times 100\%. \quad (3)$$

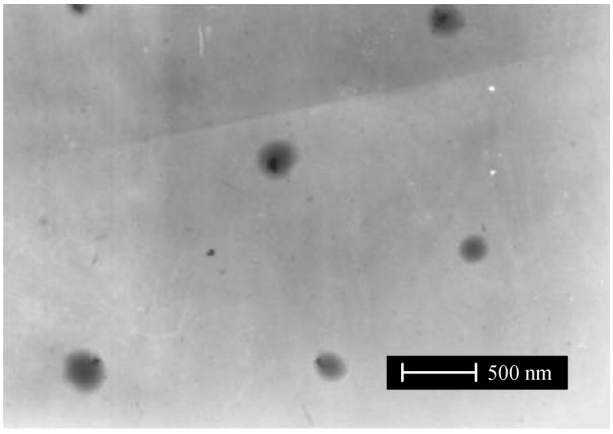
3 Results and discussion

3.1 Preparation and characterization of biosilica

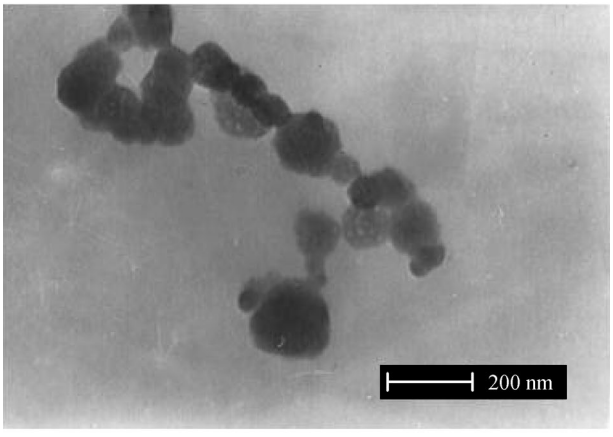
3.1.1 Morphology and structure

The biosilica nanoparticles were prepared via the biomimetic silicification method. Precipitation of silica particles occurred immediately upon the addition of lysozyme (10 $mg \cdot mL^{-1}$) into the dilute TEOS hydrolyzed solution. The morphology and the particle size of the resultant lysozyme-

containing silica precipitates were observed by TEM (Fig. 1). The lysozyme/silica composite particles are spherical with a size of about 200 nm (Fig. 1(a)). The biosilica nanoparticles with immobilized GUS are somewhat more likely to aggregate rather than a completely separate nanoparticle as shown in Fig. 1(b).



(a)



(b)

Fig. 1 TEM images of (a) the pristine lysozyme-induced biosilica particles and (b) those with GUS inside

A typical nitrogen absorption-desorption isotherm at 77 K for the lysozyme/silica composites and the corresponding pore size distribution curve are shown in Fig. 2. The nitrogen sorption isotherm for the lysozyme/silica composites shows a type IV isotherm characteristic of mesoporous materials according to the IUPAC nomenclature. The corresponding pore size distribution data of silica particles was calculated from the adsorption branch of the nitrogen adsorption isotherm by the BJH method. The pore diameter of the silica particles is ~4 nm.

As shown in Fig. 3, the chemical structure of the silica at the atomic scale was studied by ^{29}Si NMR to analyze the degree of silicate condensation of the formed biosilica catalyzed by lysozyme. The three peaks at −91.5, −100.6 and −110.3 ppm are attributed to the $Q^2[Si(OSi)_2(OH)_2]$, $Q^3[Si(OSi)_3(OH)]$ and Q^4 $[Si(OSi)_4]$ with relative percentages of 1.45%, 34.12% and 64.43%, respectively. The high total content of Q^3 and Q^4 up to 98.55% indicates a fairly high proportion of condensed silanols, suggesting that lysozyme may be helpful in forming a well-condensed silica network.

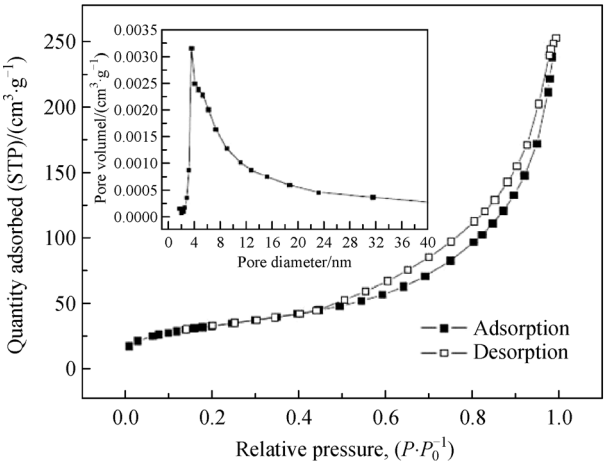


Fig. 2 N_2 adsorption-desorption isotherm and pore size distribution of the biosilica particles

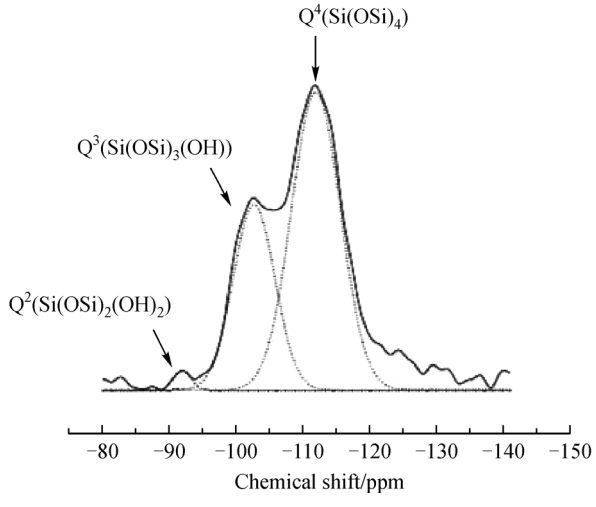
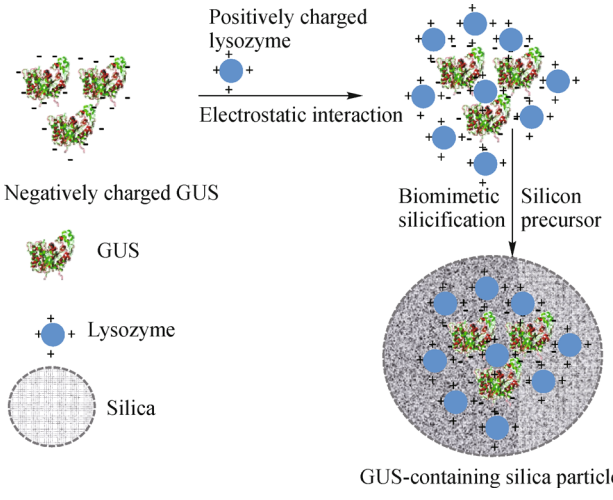


Fig. 3 ^{29}Si NMR of the lysozyme-induced biosilica

3.2 Immobilization procedure, immobilization efficiency and enzyme leakage

The mixture (1 mL) of lysozyme (10 mg·mL^{-1}) and GUS (0.8 mg·mL^{-1}) was added to the diluted prehydrolyzed TEOS solution (7 mL) and the precipitate appeared immediately. The mechanism for the GUS immobilization process was tentatively illustrated in Scheme 1. At pH 7.0, GUS and lysozyme are negatively and positively charged, respectively, because GUS has a typical isoelectric point (pI) of 3.0-5.0 and lysozyme has a pI of 11.3. When the negatively charged GUS is mixed with the positively

charged lysozyme solution at pH 7.0, the amino groups of lysozyme are prone to be attracted to GUS through electrostatic interaction, leading to enzyme aggregates. When these aggregates are kept in contact with the prehydrolyzed TEOS solution, the free cationic amino groups of lysozyme attract the negatively charged silicon precursor via electrostatic and hydrogen bonding interaction. Furthermore, an increase in precursor concentration around the aggregates induces and templates the silicification process and ultimately results in GUS-containing silica particles with a diameter of about 200 nm. The immobilization efficiency of enzyme is about 84.3% by measuring the residual activity of the supernatant during the process of preparation. To investigate the possible leakage of enzymes out of the silica carrier, the biosilica with immobilized enzymes was immersed in saturated KCl solution for 24 h and no protein was detected in the buffer solution, indicating that the GUS molecules are tightly immobilized inside the biosilica without any leakage.



Scheme 1 Schematic view of the immobilization process of GUS in biosilica particles

3.3 The activity and stability of GUS immobilized in biosilica

3.3.1 Optimal temperature and pH of immobilized GUS

The effect of temperature on the activity of free or immobilized GUS was assayed in the range of 30–70°C. As depicted in Fig. 4(a), the optimal reaction temperatures for free and immobilized GUSs are 60°C and 50°C, respectively. After immobilization in the biosilica, the optimal reaction temperature of GUS was lowered by 10°C, which may be attributed to the conformational changes of the GUS molecules due to the strong electrostatic interactions between GUS and lysozyme. Two similar examples (reduction by 20°C and 5°C in the optimum temperature after immobilization, respectively)

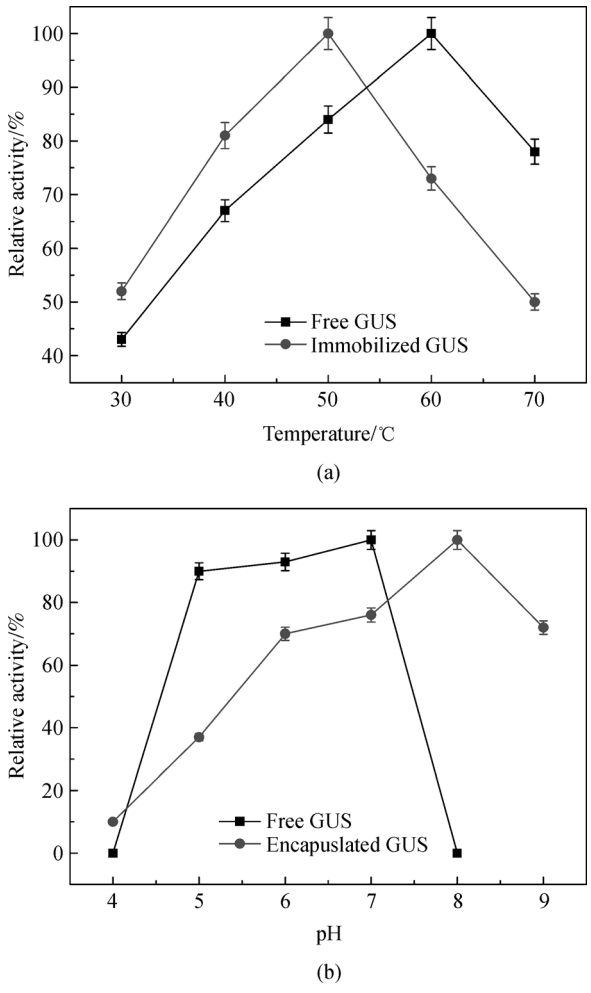


Fig. 4 Effect of (a) temperature and (b) pH on the activity of free and immobilized GUSs

have been reported in literatures [35,36]. As for the optimal reaction pH, a notable shift was also observed (Fig. 4(b)). The highest activity of the immobilized GUS was achieved at pH 8, while that of free GUS was achieved at pH 7. The increase in the optimal reaction pH after immobilization was tentatively explained as follows. Lysozyme is a weak polyelectrolyte and positively charged at the experimental pH, so it could tune the local pH to some extent due to its abundant $=NH/=NH_2^+$, $-NH_2/-NH_3^+$ pairs (so called buffering effect). Under this condition the local pH within the particle is lower than the bulk pH. And the pH change in the microenvironment is envisaged to be smaller than that happened in the bulk solution. In addition, the surface of the lysozyme/GUS/silica composite particles is covered by abundant silanol (Si–OH) groups. The residual silanol (Si–OH) groups could dissociate into Si–O- species at high pH. Thus the local pH within the carrier is supposed to be below the pH of the bulk solution and somewhat more acidic. Therefore, for the immobilized GUS, the pH of the bulk solution would shift to alkaline to ensure that the enzyme molecules are in a neutral environment. The

thermal and pH stability of the immobilized GUS was further studied in the following section.

3.3.2 Thermal and pH stability of free and immobilized GUSs

The thermal and pH stability of free and immobilized GUSs was determined by incubation under different temperature or pH for an hour. The immobilized GUS shows a similar trend in thermal stability with the free counterpart, but the remaining activity of the immobilized one is about 6%-14% higher than that of the free one under the same thermal treatment conditions (Fig. 5(a)). The activity of the immobilized and free GUS decreases quickly when the incubation temperature reaches up to 60°C, and after incubation at 70°C, both the immobilized GUS and the free GUS lose enzymatic activity completely. It is well established that thermal inactivation starts with the unfolding of the protein molecule which is followed by irreversible changes due to aggregation. The biosilica matrix could protect the immobilized GUS inhibiting the unfolding motions of the immobilized GUS, thus preserving its activity at higher temperatures.

The pH stability of immobilized GUS is higher than the free GUS when the pH is lower than 5 or higher than 7.5 (Fig. 5(b)). The free GUS lose all its activity at acidic pH 4.0, whereas the activity of immobilized GUS still retain ~20% at the same pH. The immobilized GUS achieves the highest activity at basic pH 8.0 while the activity of the free GUS decreases to 70% at the same pH. The enhanced tolerance of immobilized GUS against the acidic and alkaline changes in medium is tentatively explained by the confinement effect of the biosilica cages. The physical cage confinement inhibits to some extent the unfolding–refolding motions of the enzyme inside and thus has a favorable effect on the retaining of enzyme activity. In addition, the "water blanket" effect may remarkably reduce the negative effect of pH variation on the protein. More specifically, within the pores of the biosilica, GUS molecules are only surrounded by one or two layers of water molecules because of space limitation. It can be supposed that each GUS molecule retains about 100 water molecules as a blanket against the silica cage wall. Even at pH = 0, only two water molecules would be protonated to give H_3O^+. This would not be enough to give rise to the denaturation of GUS. A similar mechanism holds for the protecting ability under the alkaline condition.

3.3.3 Enzymatic conversion of baicalin

The conversion reaction of baicalin catalyzed by either free or immobilized GUS was investigated by measuring the productivity of baicalein with elapse of reaction time. As shown in Fig. 6, the initial productivity using free GUS is much higher than that using immobilized GUS because the

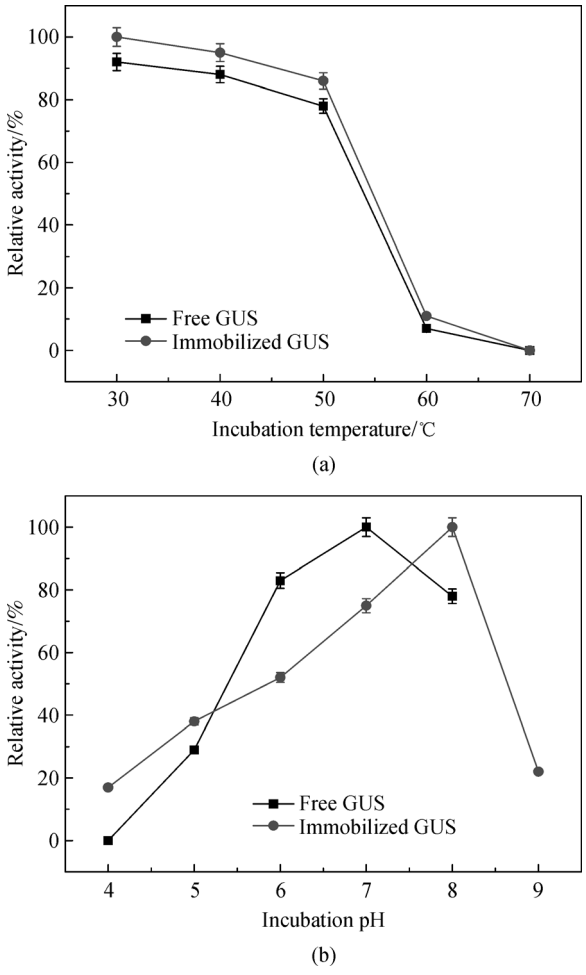


Fig. 5 (a) Thermal and (b) pH stability of free and immobilized GUSs

mass transfer resistance increases after immobilization. The productivity reaches a maximum of 80% in 2.5 h for free GUS, and a comparable level of 60% in 4 h for the immobilized GUS. The apparent specific activities of free and immobilized enzymes were 28 U·mg^{-1} and 7.6 U·mg^{-1} GUS, respectively. The reduction in activity after immobilization might be due to the harmful effect of ethanol from the hydrolysis and condensation of TEOS. The kinetic parameters including the Michaelis constant (K_m) and the maximum reaction rate (V_{max}) were calculated from the Lineweaver-Burk plots and listed in Table 1. After immobilization, V_{max} remarkably decreases, probably due to the additional diffusion resistance toward the substrates and products in the biosilica particles, while the K_m increases, indicating a weaker affinity of the substrate baicalin to the enzyme GUS. Although the apparent reaction activity of the immobilized GUS in the biosilica is much lower than that of the free GUS,the high and comparable final productivity, together with the advantage of reusability, ensure a great potential for practical application of this enzymatically active biosilica.

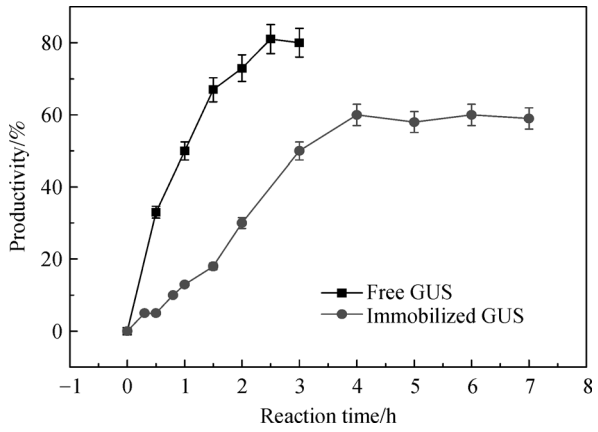


Fig. 6 Productivity of baicalein with reaction time

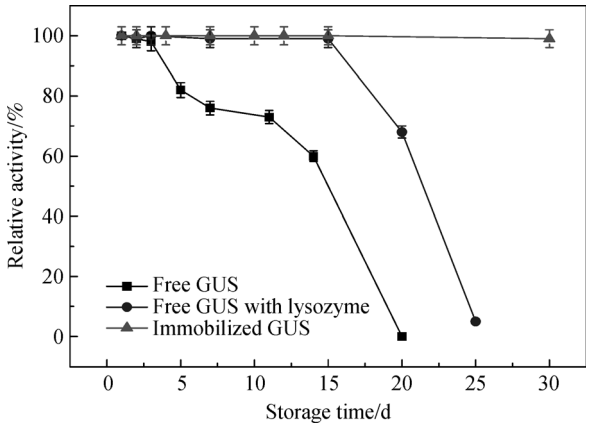


Fig. 7 Storage stability of free and immobilized GUSs

Table 1 Kinetic parameters for free and immobilized GUS

GUS	K_m /mM	V_{max} /(μmmol·min^{-1}·mg-GUS^{-1})	Apparent specific activity /U·mg^{-1}
Free	0.19	1.70	28
Immobilized	0.85	0.94	7.6

3.3.4 Storage stability

The free GUS buffer solution, GUS-lysozyme buffer solution and the immobilized GUS powder were stored at 4°C over a 30-day period and their relative enzyme activity were tested and compared. As shown in Fig. 7, the immobilized GUS retains all its initial activity over the whole storage period of 30 days while its free counterpart lose its activity sharply and shows no activity after 20 days. The stability of the free GUS is significantly enhanced in the presence of lysozyme. It has been reported that the loss of the enzyme activity was probably due to the presence of protein-degradation microbial. The inducer used for the biomimetic silicification, lysozyme, just has an antiseptic function which can protect the immobilized enzyme from the attack of microbial degradation. In addition, the small pore size of the biosilica (about 4 nm) would not allow the infiltration of the protein-degradation microbial into the biosilica matrix [15,37]. It is reasonable to conclude that the confinement effect and the stable microenvironment provided for the enzyme by the biosilica can stabilize both lysozyme and the immobilized GUS.

3.3.5 Recycling stability

A significant advantage of the immobilized GUS in the biosilica is its excellent recycling stability. As shown in Fig. 8, no appreciable loss in activity was observed for immobilized GUS after 12 repeated cycles. The high

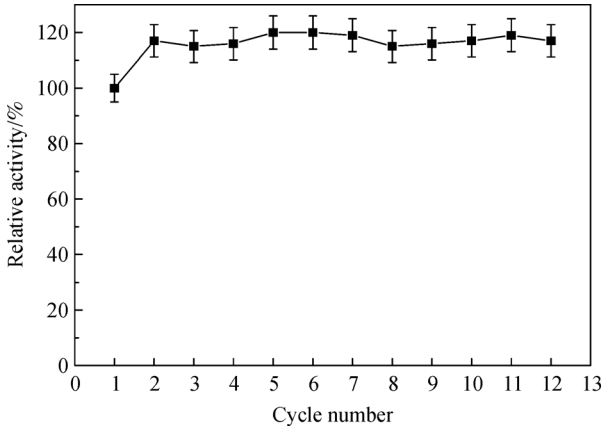


Fig. 8 Recycling stability of immobilized GUS

recycling stability could be attributed to the complete confinement of GUS in the biosilica carrier, effectively preventing the enzyme leakage during reaction and separation processes due to the small pore size of about 4 nm for the biosilica. The results are promising in potential industrial applications.

4 Conclusions

A facile procedure to prepare mesoporous biosilica nanoparticles for efficient enzyme immobilization during the biomimetic silicification process is presented. Lysozyme was used to first induce GUS to be immobilized via electrostatic attractive interactions and the resulting immobilized GUS was subsequently employed to catalyze the silicification process. The average diameter and most probable pore size of the resultant biosilica particles are around 200 nm and 4 nm, respectively. The pH stability and thermal stability of the immobilized GUS were significantly improved. Compared with free GUS, the immobilized GUS exhibits improved storage stability, and

100% of the initial activity is preserved after 30-day storage at 4°C. The immobilized GUS also exhibits excellent recycling stability. No loss in relative activity for the immobilized GUS was found during 12 repeated cycles. Baicalin can be efficiently converted into baicalein by GUS immobilized in the biosilica. The productivity of baicalein for one reaction batch catalyzed by the immobilized GUS reached 60% in 4 h. As an efficient carrier for enzyme immobilization, the biosilica particles prepared in this study may find potential application in biomedical, biosensor and other biotechnology areas.

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