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Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Immobilized snailase in hollow silica nanospheres to highly efficient biotransformation of icariin to icaritin in deep eutectic solvents

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ABSTRACT: Icaritin (ICT), a biologically active isoprenylated flavonoid, which has been shown to delay liver fibrosis, inhibit cancer cell proliferation and other therapeutic effects in the treatment of hepatocellular carcinoma. However, traditional methods for icaritin preparation suffer from significant drawbacks such as high environmental pollution, energy consumption, and low yield. In this study, snailase (SNA) was immobilized for the first time in hollow silica nanospheres (HSN) through facile and novel in situ immobilization and soft template removal method to construct highly active biocatalysts (SNA@HSN) where enzymes in the cavity exhibit “immobilized but not rigid state”. The resultant SNA@HSN showed excellent activity (78% activity recovery) and stability suffered from extreme pH, temperature, and organic solvents. Meanwhile, the reusability and storage stability of SNA@HSN were satisfactory. After 5 cycles, SNA@HSN still maintained 79% original activity. Especially, SNA@HSN exhibited highly efficient biotransformation of icariin (ICA) to ICT in deep eutectic solvents (DESs) system. ICT yield in choline chloride–propylene glycol (ChCl:P, 25% v/v) system was 111% higher than that of aqueous system, reached 374 mg/L. Thus, this study developed an efficient methodology for icaritin preparation through the integration of enzymatic catalysis and environmentally friendly catalytic medium.

Keywords: ICT; immobilization of SNA; Immobilized but not rigid state; DES

1. Introduction

Significant anticancer, osteoprotective, neuroprotective, and anti-inflammatory qualities are displayed by icariin (ICA) flavonoid glycosides' bioactive aglycone [1-4], icaritin (ICT). Its anticancer actions include blocking the CDK2/Cyclin E complex, suppressing PD-L1 expression, and activating the TRAIL/Fas-caspase apoptotic pathway [5-8]. Wnt/ β -catenin and STAT-3 pathway activation drive osteogenic effects, while NLRP3 inflammasome inhibition and A β generation modulation provide neuroprotection [9-14]. ICT has a

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Received 3 September 2025

Received in revised form 21 October 2025

Accepted 18 March 2026

17% greater bioavailability than its precursor ICA due to improved lipophilicity and blood-brain barrier permeability [15]. Its soft capsule formulation has been authorized for advanced hepatocellular carcinoma treatment [16,17]. However, the low natural extraction yield prevents ICT from being produced on a large scale. Meanwhile, chemical synthesis faces environmental and toxicity challenges in harsh environments [18–20]. By contrast, enzymatic catalysis offers benefits in mild reaction conditions and product purity. For example, the α -L-rhamnosidase/ β -glucosidase cascade was developed to convert icariin C to ICA [21]. A dual enzyme system was constructed using *Thermus thermophilus*-derived β -glucosidase (TthBg3) and *Aspergillus*-derived α -L-rhamnosidase (AtRha), which sequentially hydrolysed Icaritin C to Icaritin hyposide I ($98.3 \pm 2.1\%$ conversion) and ultimately produced ICT, with a total molar conversion of 91.2%, which was significantly better than that of the chemical method in terms of efficiency [22]. Similarly, Wang et al [23]. identified a novel *Epimedium* flavonoid-glycosidase that hydrolyzes the 7-O-glucosidic linkage of ICA to produce icariside II, followed by the hydrolysis of the 3-O-rhamnosidic moiety of icariside II to yield ICT, with a molar yield of 92.5%. However, the inhibition of glycosidase by reaction-generated sugars, instability, and non-reusability of free enzymes still restricts their application efficacy.

Snailase (SNA) is a multi-component enzymatic complex consisting of more than 20 enzyme types, encompassing cellulase, pectinase, amylase, protease, hemicellulase, mannanase, sucrase, galactanase, aminotransferases, and numerous other auxiliary enzymes, which is extracted from the digestive systems or ingluvies of mollusks classified under the genus *Limax* [24,25]. Studies have shown that SNA is capable of catalyzing the transformation of ICA into ICT, achieving a product yield of 92% under the optimal reaction conditions. In addition, SNA can not only hydrolyze the β -glucosidic bond in ICA but also cleave the α -L-rhamnosidic bond, exhibiting multifunctionality [26]. Nevertheless, Free-form SNA still faced challenges of instability during continuous reactions and difficulties in repeated recovery. Liu et al [27]. immobilized SNA onto glutaraldehyde-activated silica to enhance enzyme reusability. Results showed that after six reuse cycles, the immobilized enzymes retained 60% of their initial activity. Li et al [28]. utilized chitosan-coated magnetic nanoparticles to adsorb and immobilize SNA. After incubating free enzymes and immobilized enzymes at 60°C for 60 min, they observed that the residual activity of immobilized enzymes increased by 30% over that of free enzymes. After being reused for 9 cycles, immobilized enzymes retained 56% of their initial activity.

Generally, enzyme immobilization improves stability, reusability, and reaction selectivity to overcome these constraints [29–33]. Metal-organic frameworks (MOFs), such as Zeolitic Imidazolate Framework-67 (ZIF-67), are perfect carriers due to their biocompatible synthesis and adjustable porosity [34–36]. In our previous research, a highly active biocatalyst was constructed by using in situ immobilization and ZIF-67 as the soft template method, where enzymes in the hollow silica nanosphere cavity exhibit “immobilized but not rigid state” [37]. During this process, glutathione (GSH) was specifically chosen to assist ZIF-67 in rapidly encapsulating SNA due to its higher cysteinyl content, which facilitated metal ion enrichment. Subsequently, the ligand was able to rapidly coordinate with the metal ion and thus enhanced the enzyme encapsulation

efficiency, ultimately resulting in the formation of the SNA@GZIF-67. However, ICA and its intermediates (like icarisiide I) have poor aqueous solubility, which hinders the interaction between the enzyme and the substrate. Deep eutectic solvents (DES) are good substitutes for traditional organic solvents in enzymatic catalysis because of their many benefits, which include excellent thermal stability, non-flammability, recyclability, and extensive solvation capacity [16,38,39]. The previous reports showed that DES was an excellent solvent for improving aqueous solubility of hydrophobic intermediates and enzyme stability [40–42]. Therefore, using DES as a green reaction medium makes it possible to produce ICT more cheaply and efficiently.

In this study, we proposed an efficient and green synthesis method of ICT. SNA was immobilized in hollow silica nanospheres through a facile and novel in situ immobilization and soft template removal method to construct SNA@HSN, and used to synthesize ICT in the DES system. Compared with free SNA and traditional SNA@GZIF-67, the synthesized SNA@HSN exhibited significant improvements in the catalytic properties of enzymes along with high stability, and showed higher ICT yield in the DES system than that of the aqueous system.

2. Experimental section

2.1 Materials

SNA was a brown lyophilized powder with a solubility of 200 mg/mL in water, purchased from Solarbio Life Sciences. Tetraethyl orthosilicate (TEOS), 3-aminopropyltriethoxysilane (APTES), Pluronic F-127 (F-127), 2-Methylimidazole (2-MeIM), cobalt nitrate hexahydrate, and glutathione were obtained from Aladdin (Shanghai, China). Baohuoside I (98%, HPLC), Icariin I (98%, HPLC), Icaritin (98%, HPLC) were purchased from Yuanye Biotechnology Co (Shanghai, China). Choline chloride, D-sorbitol, 4-nitrophenyl β -D-glucopyranoside and urea were obtained from Macklin (Shanghai, China). Glycerol, 1,2-propanediol, and ethylene glycol were purchased from Fuchen Chemical Reagent Co (Tianjin, China). All the other reagents used in this study were of analytic or biological grade.

2.2 Preparation of SNA@GZIF-67

Synthesis of SNA@GZIF-67 was prepared according to our previous reports [37]. 2 mL SNA solution and 5 mg GSH were firstly dissolved in 2 mL of deionised water, and 2 mL 2-MeIM aqueous solution (300 mM) and 2 mL cobalt nitrate hexahydrate (40 mM) were added, respectively. The mixture solution was left to stand at room temperature for 2 h. Subsequently, the solid product was centrifuged (10000 r/min, 10 min) and washed with PB (100 mM, pH 7.0) for 3 times.

2.3 Preparation of SNA@GZIF-67/SiO₂ and SNA@HSN

The SNA@GZIF-67 was dispersed in 2 mL of deionised water, followed by the addition of 15 μ L of APTES solution and mixed thoroughly for 1 h at room temperature. The recovered precipitate was re-dispersed in 2 mL deionised water containing 25 mg F-127 powder mixed with 40 μ L TEOS solution and stirred continuously for 2 h at room temperature. Finally, after centrifugation and being washed for 3 times

with milliQ water, the SNA@GZIF-67/SiO₂ composite was obtained. For SNA@HSN, the SNA@GZIF-67/SiO₂ was dispersed in PB buffer (pH=7, 0.1 M) at room temperature for 1 h to obtain SNA@HSN.

2.4 Characterization

The morphology and internal structure of SNA@GZIF-67 and SNA@HSN were investigated by scanning electron microscopy (SEM, HitachiSU8600, Japan) and transmission scanning electron microscopy (TEM, JEM2100 plus, Japan), and elemental distributions were observed using the STEM mapping model of TEM. The observed fluorescently labelled proteins were verified using a confocal laser scanning microscope (CLSM, Leica TCS, SP5 Leica, Germany). Fourier transform infrared (FTIR) analyses were performed in the 400-4000 cm⁻¹ band using an IS50 spectrometer

2.5 Assay of enzyme activity

Free SNA and SNA@HSN activities were determined using p-nitrophenol- β -D-glucopyranoside as substrate. 900 μ L of sodium acetate buffer (pH 5) and 100 μ L of enzyme solution (1 mg/mL) were mixed, and the well-mixed solution was preheated at 50 °C for 10 min. Then, 1 mL of p-NPG solution (5 mmol/L) was added and reacted for 10 min. Subsequently, the reaction was immediately terminated by adding 1 mL of Na₂CO₃ solution (1 mol/L). After centrifugation, the supernatant absorbance at 410 nm was measured by the UV spectrophotometer.

2.6 The catalytic performances

The effects of reaction temperature and pH on free SNA and immobilized SNA were examined. Activities of free SNA and immobilized SNA were tested at different temperatures and pH. To test the thermal and pH stability of free SNA and immobilized SNA, free SNA and SNA@HSN were incubated at 30-70°C and pH 3-8 for a certain time, followed by an evaluation of their remaining activity. The apparent kinetic parameters $V_{\max}$ and K_m of free SNA and immobilized SNA were determined by varying the concentration of different substrates, and obtained by the Michaelis Menten equation. Reusability was determined by repetitive measurements of the activity of SNA@HSN. After each reaction, the SNA@HSN were collected and washed three times with PB (0.1 M, pH 7.0) to remove residual substrate before being used in the next reaction. To test the storage stability, free SNA and SNA@HSN were stored in PBS (0.1 M, pH 7.0) at room temperature for 30 days, and their residual activities were measured.

2.7 HPLC Analysis of the Conversion Product

To confirm that snail enzyme converts ICA into ICT, the retention times of different reference standards were determined by high-performance liquid chromatography (HPLC) and compared with the product. Dissolve 1 mg of ICA reference standard (purity $\geq 95\%$) in 1 mL of phosphate buffer (0.1 M, pH 7.0), then add 1 mg of SNA to initiate the enzymatic reaction. The reaction system was placed in a magnetic stirrer water bath at 50°C with a stirring speed of 2. Samples of 100 μ L were taken at 2, 4, 6, 8, and 12 hours. To terminate the enzymatic reaction, 900 μ L of chromatographic-grade methanol was added to the samples at each time

point. After vortexing, the samples were filtered through a 0.22 μm microporous membrane and analyzed by HPLC.

2.8 Synthesis of ICT in the DES system

By determining the stability of free SNA and the solubility of ICA in eight different DESs, finally, choline chloride:1,2-propanediol was selected as the solute for the DES reaction system. The synthesis conditions of ICT in the DES system were optimized, including DES concentration, synthesis pH, synthesis temperature, reaction time, ICA concentration, and the mass ratio of ICA to SNA@HSNA. After the reaction, 100 μL of the samples were taken from each reaction system, and 900 μL of chromatographic grade methanol was added to dissolve the samples and terminate the reaction. Then, the samples were filtered through a 0.22 μm organic filter membrane and used for HPLC analysis.

3. Results and discussion

3.1 Preparation of SNA@HSN and optimization of immobilization conditions.

The simple preparation process of the SNA@HSN was depicted in Fig. 1. Firstly, the SNA@GZIF-67 were prepared through a mild in-situ induced method. Then, a layer of mesoporous biomimetic silicon shell was formed on the surface of SNA@GZIF-67 particles by biomimetic siliconization technology (SNA@GZIF-67/SiO₂). Finally, SNA@GZIF-67/SiO₂ templates were removed with PB buffer (pH 7, 0.1 M) at room temperature. To obtain highly active SNA@HSN, the various conditions for the preparation of SNA@HSN were optimized. APTES precipitated onto the surface of SNA@GZIF-67, utilizing amino groups to provide a target for the growth of TEOS [43]. TEOS was the silicon precursor for SiO₂. The results showed that APTES concentration, TEOS concentration, and TEOS coating time had great effects on the SNA@HSN activity. As shown in Fig. 2a and 2b, the activity recovery of the SNA@HSN was only 34.8% when the concentrations of APTES and TEOS were low. However, the activity recovery of the SNA@HSN was the highest at 69.2% when the concentrations of APTES and TEOS (APTES 7.5 $\mu\text{L}/\text{mL}$) were increased to 7.5 $\mu\text{L}/\text{mL}$ and 20 $\mu\text{L}/\text{mL}$, respectively. Interestingly, with further increase of the concentrations of APTES and TEOS, the activity recovery decreased slightly, which may be due to the thickening of the silica shell layer caused by too high a concentration, resulting in an increase in mass transfer resistance.

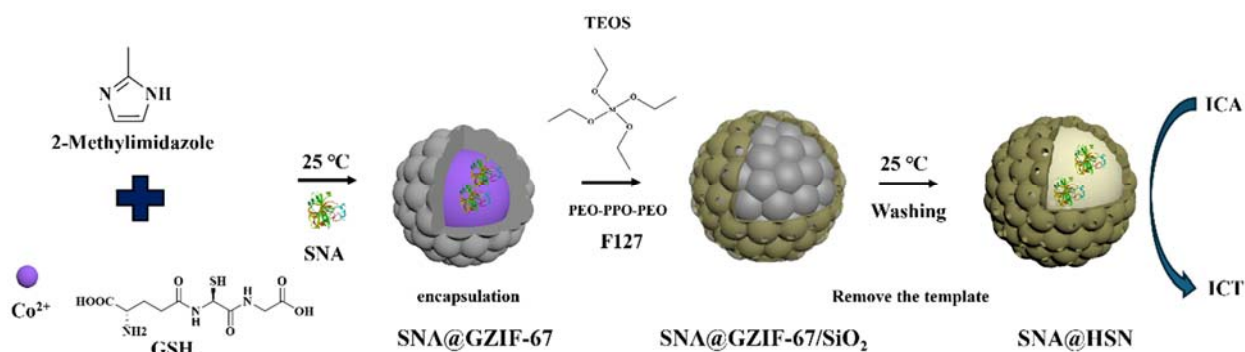


Fig. 1 Schematic for synthesis process of SNA@HSN

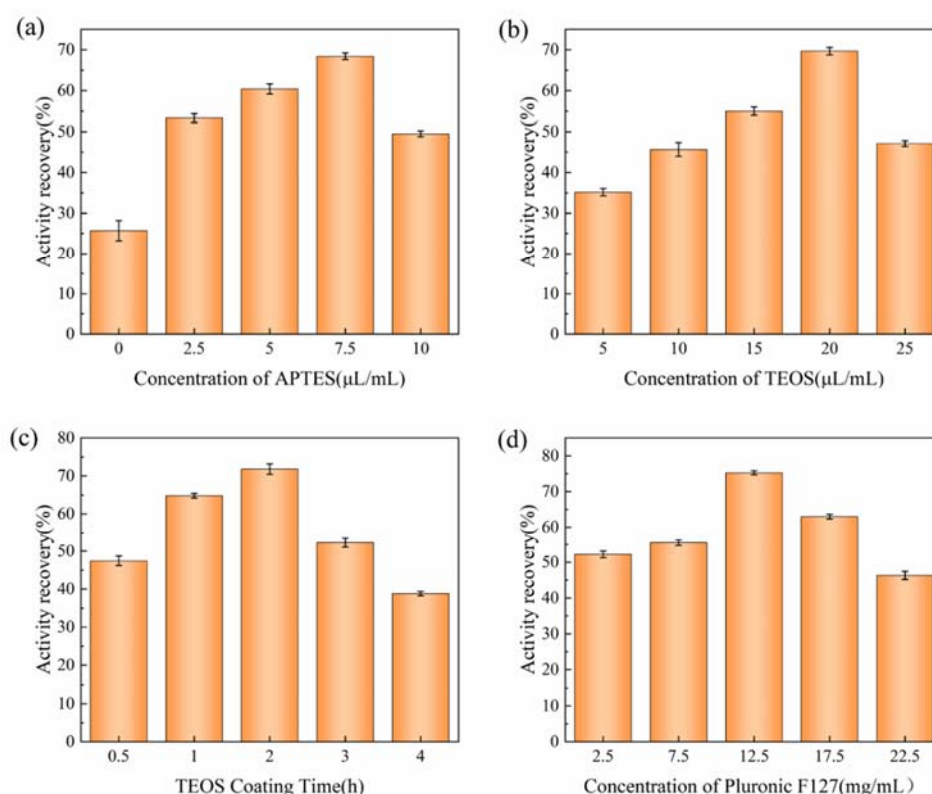


Fig. 2 Optimization preparation conditions of SNA@HSN: APTES concentration (a); TEOS concentration (b); TEOS coating time (c); Pluronic F127 concentration (d)

Besides, the effect of different TEOS coating times (APTES 7.5 $\mu\text{L/mL}$, TEOS 20 $\mu\text{L/mL}$) on activity recovery of the SNA@HSN was shown in 错误!未找到引用源。 c. The activity recovery of the SNA@HSN was low when the coating time was short, which was due to the incomplete synthesis of the silica shells, resulting in the collapse of the shells after the removal of the inner templates and enzyme leakage. The highest activity recovery of the SNA@HSN was obtained (73.6%) when the coating time was 2 h. Furthermore, as a nonionic surfactant, a certain amount of Pluronic F-127 with a hydrophilic PEO chain and a hydrophobic PPO chain enabled it to form micelles on the surface of SNA@GZIF-67 through self-assembly, guiding TEOS to form a homogeneous silica shell and forming a mesoporous silica shell [44]. However, when Pluronic F-127 concentration (APTES 7.5 $\mu\text{L/mL}$, TEOS 20 $\mu\text{L/mL}$, coating time 2 h) was higher than 12.5 mg/mL, the micelles it formed were too thick to be completely removed during washing resulting in the covering of enzyme activity sites, elevated mass transfer resistance, and reduced enzyme activity recovery (Fig. 2d). Thus, the optimized preparation conditions of SNA@HSN were 7.5 $\mu\text{L/mL}$ APTES, 20 $\mu\text{L/mL}$ TEOS, 2h coating time, and 12.5 mg/mL Pluronic F-127.

3.2 Characterization of SNA@HSN

The SEM images of SNA@ZIF-67, SNA@GZIF-67, and SNA@HSN were shown in Fig. 3. Unlike the traditional rhombic dodecahedron shape of ZIF-67, it could be seen that SNA@ZIF-67 exhibited urchin-like nanoparticles (Fig. S1, Fig. 3a). However, SNA@GZIF-67 and SNA@HSN displayed relatively homogeneous spherical particles with a size of about 200 nm (错误!未找到引用源。 b and 3c). It was

indicated that GSH could be involved in the construction process of the hybrid crystal as a candidate ligand rather than the ordinary guest molecule being embedded in the ZIF-67 crystal. X-ray photoelectron spectroscopy (XPS) pattern of obtained GZIF-67 and GSH+ZIF-67 (physical mixture of GSH and ZIF-67) indicated that sulfur peak at 161.5–162.5 eV in GZIF-67 result (Fig. 4b) profile should correspond to Co-S [37], whereas there is no according peak in the profile of GSH+ZIF-67 (Fig. 4d). As illustrated in Figure 4a, GSH displayed a characteristic S-2p peak that is also detected in GZIF-7, confirming the involvement of GSH in the synthesis of GZIF-67. Meanwhile, the formation of the Co-S bond between the cobalt ion and sulfur in GZIF-67 further demonstrated that GSH molecules are adopted as a candidate ligand for GZIF-67.

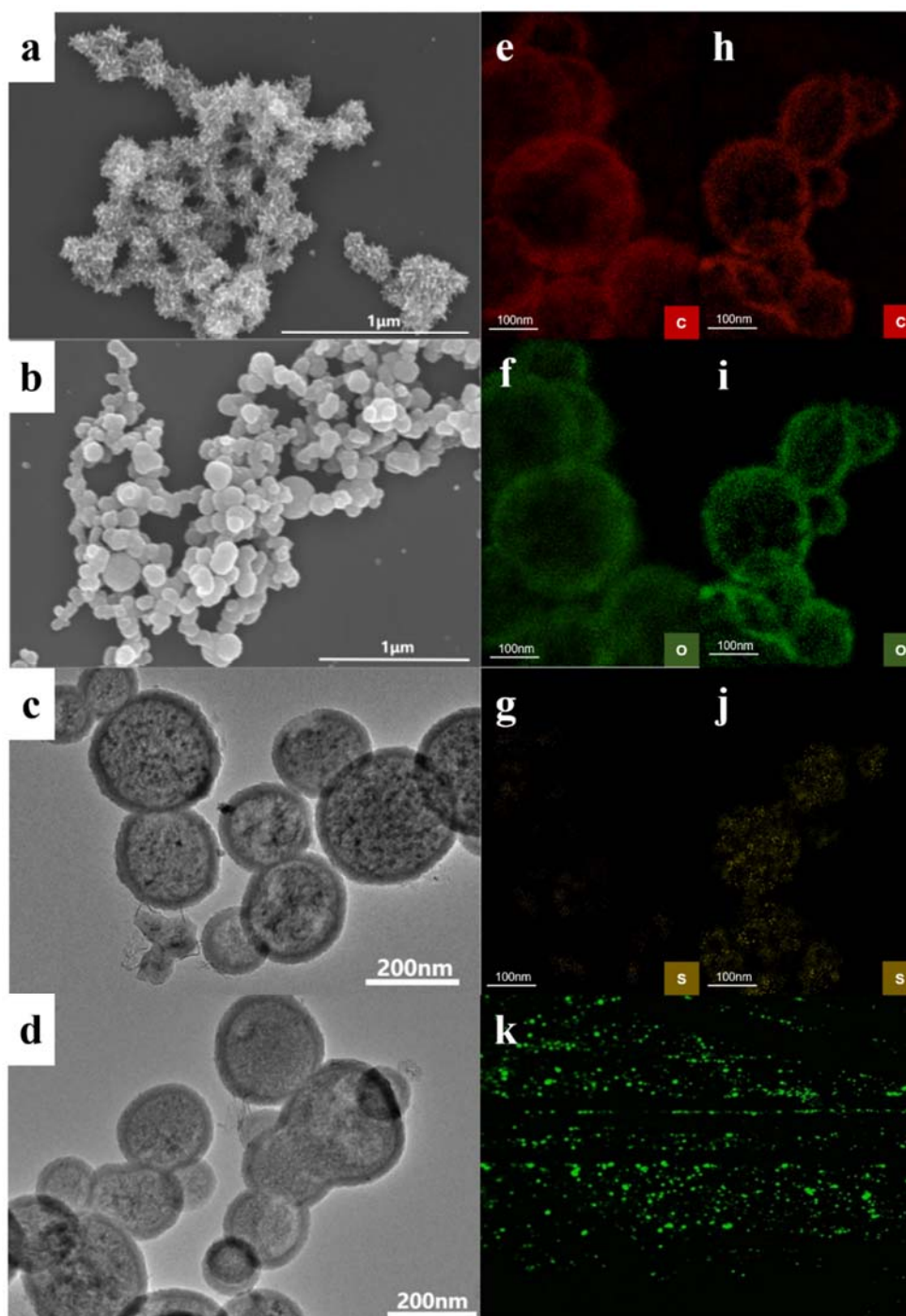


Fig. 3 SEM of SNA@ZIF-67 (a), SNA@GZIF-67 (b), and SNA@HSN (c); TEM of SNA@HSN (d); EDS mapping images of HSN (e, f, g) and SNA@HSN (h, i, j); CLSM image of SNA@HSN (k).

TEM images indicated that SNA@HSN was hollow core-shell structure spherical particle with rough surface, which proved that the GZIF-67 template inside had been removed after etching with PB buffer (Fig. 3d). The elemental distribution map showed that there were clear distribution of S element in the carrier cavity of the SNA@HSN (Fig. 3j). However, no S elements in the HSN were observed (Fig. 3g). It was indicated that SNA was immobilized inside the cavity of HSN. Fluorescein isothiocyanate (FITC) can be fluorescently labeled by binding the isothiocyanate group to the SNA. It is possible to determine whether the enzyme is immobilized from the perspective of fluorescence [45]. The bright green fluorescence was observed in the CLSM image of SNA@HSN (Fig. 3k), further demonstrating the success of the SNA immobilization.

Besides, the N₂ adsorption desorption isotherm of SNA@HSN exhibited a type IV isotherm characteristic with an obvious hysteresis loop, indicating the existence of mesopores (Fig. 4e and 4f). Moreover, the pore size of SNA@HSN was mainly concentrated in the mesoporous region with 9.325 nm average pore size (Table 2). In contrast, for the conventional ZIF-67, the pore size distribution was confined solely to the micropore region, primarily concentrated within 1–2 nm (Fig. S2a, S2c). The N₂ adsorption-desorption isotherm of GZIF-67 also displayed a hysteresis loop, and the pore size distribution showed a shift toward the mesopore region (Fig. S2b, S2d). This indicated that the incorporation of GSH and the adjustment of precursor concentration altered the pore structure of conventional ZIF-67. Meanwhile, SNA@HSN exhibited a lower specific surface area than SNA@GZIF-67/SiO₂. Obviously, after removing the GZIF-67 core template, the number of mesopores increased, causing an increases in pore volume and a decrease in the specific surface area of SNA@HSN.

Table 1 Surface area, pore volume and average pore size of SNA@GZIF-67/SiO₂ and SNA@HSN

samples	Surface area (m ² /g)	Pore volume (cm ³ /g)	Average pore size (nm)
SNA@GZIF-67/SiO ₂	1132.3	0.423	5.976
SNA@HSN	773.5	0.588	9.325

In addition, Fourier transform infrared spectroscopy (FTIR) spectrum showed that a significant absorption peak in the 1100–1000 cm⁻¹ wave number range was observed in SNA@GZIF-67@SiO₂ and SNA@HSN (Fig. 4g), which corresponded to the stretching vibration of the Si-O-Si bond, confirming the formation of the silicon layer [46]. Meanwhile, characteristic peaks were clearly observed at 1310 cm⁻¹ in both SNA@GZIF-67 and SNA@GZIF-67@SiO₂, corresponding to the imidazole group and amide III band in GSH [47]. However, the signature peak could not be observed at 1310 cm⁻¹ in the SNA@HSN sample [48]. These results suggested that GZIF-67 core template has been removed.

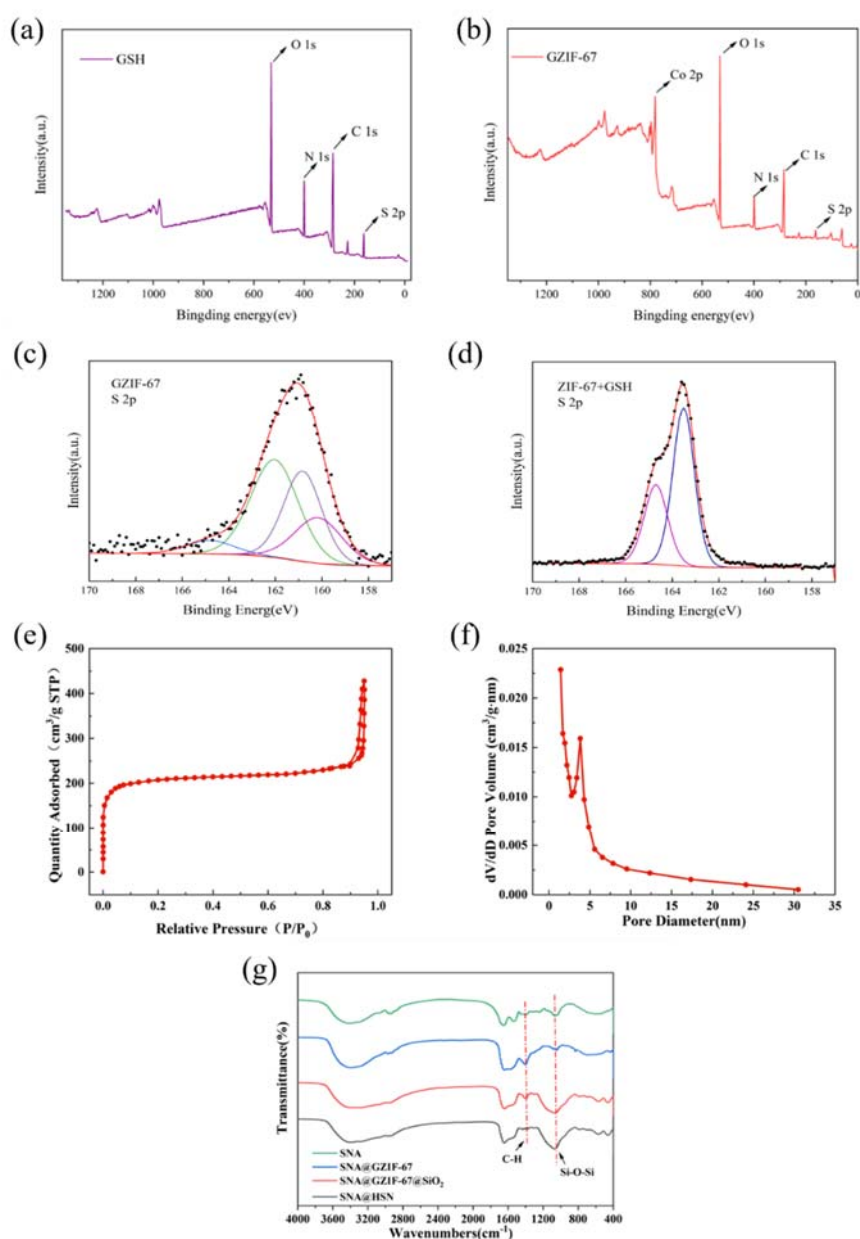


Fig. 4 XPS spectroscopy results of GSH (a), GZIF-67(b), GZIF-6(c), and ZIF-67+GSH(d); N₂ adsorption-desorption isotherm of SNA@HSN(e); Pore size distribution of SNA@HSN(f); FTIR spectra of SNA@HSN (g).

3.3 Assay of enzymatic properties

The optimal temperature and pH of the free and immobilized SNA were investigated over a wide range of temperatures (30 ~ 70 °C) and pH (4.0 ~ 8.0). the results were showed in 错误!未找到引用源。a, immobilized SNA exhibited the maximum relative activity at 50 °C, which was consistent with free SNA. However, the immobilized SNA exhibited higher activity than free SNA in the temperature range of 55 ~ 70°C, indicating that the immobilized SNA has a good thermal adaptability. Similarly, the optimal pH of immobilized SNA was the same as its free form (pH 5.0) (Fig. 5b). Meanwhile, it is noteworthy that the pH adaptation of immobilized SNA was greatly improved compared with free SNA.

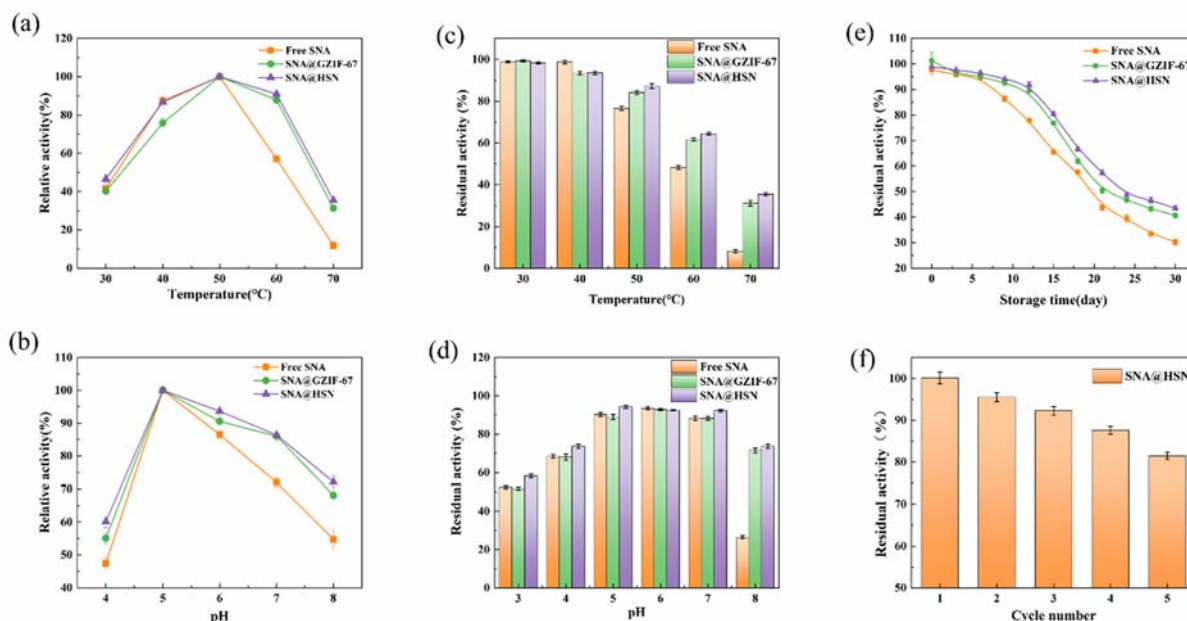


Fig. 5 Catalytic performances of free SNA and SNA@HSN. Optimum temperature (a); Optimum pH (b); Thermal stability (c); pH stability (d); Storage stability (e); Reusable stability (f).

Generally, immobilized enzymes are often expected to have excellent temperature and pH stability in order to enable the enzyme to be applied to more extreme conditions. As shown in Fig. 5c, compared with the free SNA, the thermal stability of both SNA@GZIF-67 and SNA@HSN was enhanced. SNA@HSN retained 35% of its initial activity after 1 h of incubation at 70 °C, while the free SNA was almost completely inactivated. This result indicated that the protection of the silica shell enhanced the thermal stability of SNA. Besides, the pH stability was shown in Fig. 5d. The free SNA only retained about 25% of its initial activity when incubated in an alkaline environment for 1 h (pH 8), while the SNA@GZIF-67 and SNA@HSN still retained 70% and 74% of their initial activity, respectively. This result suggested that the metal framework structure of GZIF-67 and the rigid structure of the silica shell of HSN could provide protective effects for the enzyme molecules under neutral and alkaline conditions, thus reducing the protein denaturation of the enzyme molecules due to the extreme pH environment. The industrial applicability of biocatalysts is mainly dependent on their reusability and storage stability. Fig. 5e showed that SNA@HSN still retained 45% of its initial activity after 30 days of storage (Fig. 5e). However, free SNA only maintained 30% of its initial activity. Besides, SNA@HSN could retain 80% of its initial activity after 5 cycles, which is useful for subsequent applications in patchouli synthesis.

3.4 Kinetic Characterization

Table 2 compared the kinetic characteristics of free SNA, SNA@ZIF-67, SNA@GZIF-67, and SNA@HSN. The results demonstrated that the K_m values of immobilized SNA were all higher than those of free SNA, whereas the V_{max} values decreased to varying extents. It may be due to the fact that the immobilization increases the mass transfer resistance and decreases the affinity of the enzyme to the substrate. However, SNA@GZIF-67 showed an increase in V_{max} value and a decrease in K_m value compared to

traditional SNA@ZIF-67, which suggests that the addition of glutathione caused an increase in the affinity of the enzyme for the substrate. Smaller K_m values of SNA@HSN implied that it has a stronger affinity for the substrate, probably because the SNA molecules in the hollow silica spheres have more freedom, exhibit “immobilized but not rigid state”, making it easier to bind the substrate.

3.5 Application and optimization of DESs in the synthesis of ICT

In order to choose a suitable combination of DES for enzyme catalysis, eight DES were selected for testing the catalytic performance of SNA. As shown in Fig. 6a, SNA exhibited comparable enzyme activity to that in the buffer after 1 h of treatment in choline chloride:1,2-propanediol (ChCl:P) solution. However, in some DES, such as choline chloride: citric acid (ChCl:Ca), the enzyme activity showed a rapid decline to only 18% of the initial activity. This may be associated with DES destroying the structure of the SNA. A similar phenomenon occurred in Bisht's research. After incubation in ChCl:Ca, α -chymotrypsin also exhibited a significant decrease in activity. It was explained that the removal of the enzyme's essential water layer led to loosened conformation [49]. After determining the appropriate DES type, we further investigated the concentration of DES (Fig. 6b). As the concentration of ChCl:P increased from 10% to 50%, both free SNA and SNA@HSN exhibited a decreasing trend in activity. However, the activity of SNA@HSN remained above 90%, whereas the activity of free SNA had dropped to approximately 70%, which indicated that the enzyme exhibits a certain extent of stability after immobilization. Moreover, the maximum concentration of ICT by using SNA@HSN was obtained when the concentration of ChCl:P was at 25% (Fig. 7a). Thus, the optimized ChCl:P concentration was 25%. Besides, the optimal pH and temperature for the synthesis conditions of ICT using SNA@HSN in the DES system were at 7.0 and 55 °C (Fig. 7b and 7c). 错误!未找到引用源。 7d demonstrated that the concentration of ICT begins to approach equilibrium after 12 h under the catalytic promotion of SNA@HSN. However, the concentration of ICT by SNA@HSN decreased with decreasing enzyme-to-substrate ratio (E/S ratio) (错误!未找到引用源。 7e). Considering the cost-effectiveness of industrialisation, a 1:1 E/S ratio was chosen as the equilibrium point. Furthermore, effects of the substrate ICA concentration on the ICT yield were shown in Fig. 7f. The results showed that the ICT yield increased with an increase in ICA concentration. By adjusting the substrate concentration, we found that the highest concentration of ICT was achieved at an ICA concentration of 1 mg/mL. Based on this, the yield and molar conversion of ICT could reach a maximum of 37.4% and 69%, respectively. Compared to previous studies [22], this conversion rate lacked a significant advantage. Based on the results in Table 1, mass transfer issues between the enzyme and substrate appeared to be the primary limiting factor. Therefore, regulating the pores of the carrier should be a key area for future improvement. Finally, the icaritin yield by SNA@HSN in choline chloride–propylene glycol (ChCl:P, 25% v/v) system was 111% higher than that of the aqueous system. This enhancing effect was primarily derived from the solubilizing function of DES on the substrate and its ability to stabilize the conformation of enzymes [50].

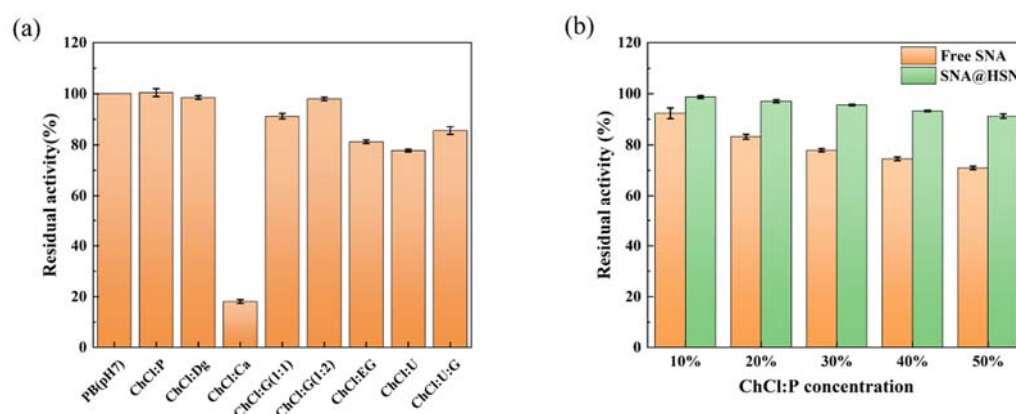


Fig. 6 Stability of free SNA in different DES systems (a); SNA@HSN in different concentrations of ChCl:P (b).

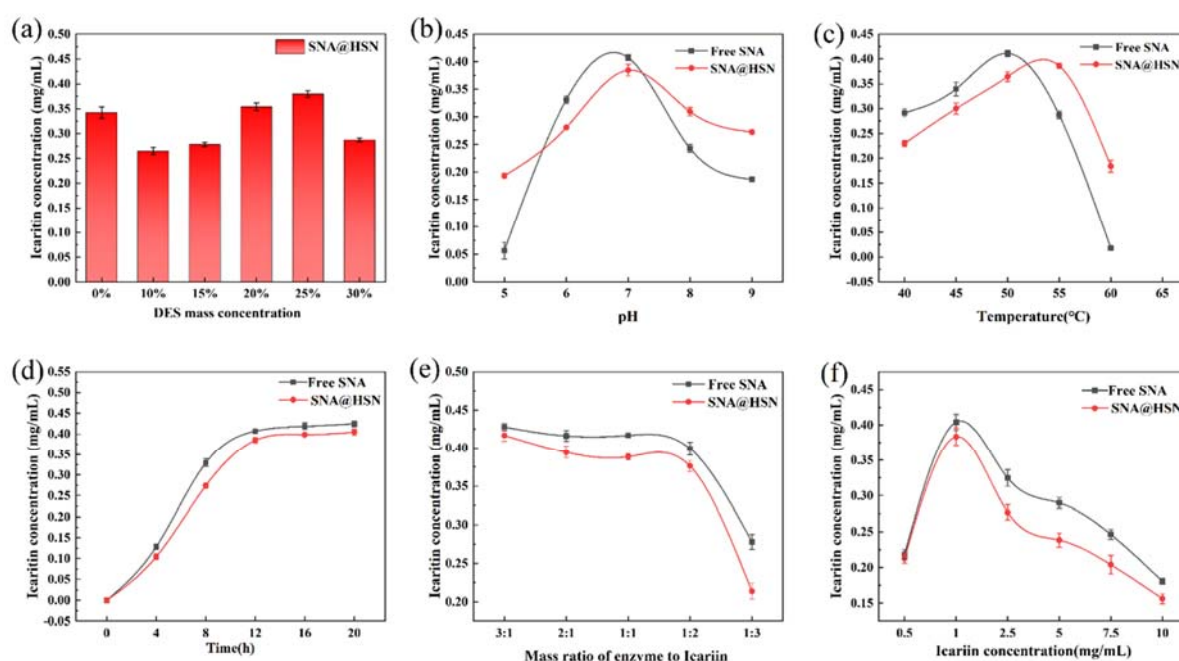


Fig. 7 Effect of synthesis conditions on ICT production in the DES system. DES concentration (a); Reaction pH (b); Reaction temperature (c); Reaction time (d); Substrate-to-enzyme mass ratio (e); Substrate concentration (f).

Table 2 Kinetic constants of free SNA; SNA@ZIF-67; SNA@GZIF-67; SNA@HSN

Samples	$K_m(\text{mmol/L})$	$V_{\max}(\text{mmol}(\text{L} \cdot \text{min})^{-1})$
Free SNA	0.1302	0.1674
SNA@ZIF-67	0.2132	0.0986
SNA@GZIF-67	0.1632	0.1316
SNA@HSN	0.1518	0.1479

4. Conclusion

In conclusion, a highly active biocatalyst (SNA@HSN) was successfully constructed by immobilizing SNA in hollow silica nanospheres using an in situ immobilization and soft template removal method. The SNA in hollow silica nanospheres exhibited “immobilized but not rigid state”. The resultant SNA@HSN displayed excellent activity (78% activity recovery) and stability against extreme pH, temperature, and organic solvents. Furthermore, the reusability and storage stability of SNA@HSN are also satisfactory. In

particular, SNA@HSN exhibited highly efficient biotransformation of ICA to ICT in the DESs. The ICT yield in the choline chloride–propylene glycol (ChCl:P, 25% v/v) system was 111% higher than that of the aqueous system, reaching 374 mg/L. This study provided an effective integration strategy of enzymatic catalysis and environmentally friendly catalytic medium for the production of ICT.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

Dr. J. D. Cui thanks supports from Open Project of National Edible Fungi Processing Technology Research and Development Center (project No. 20250022) , Key R & D Project of Henan Province (project No. 231111112100), China, the Key Projects of Tianjin Natural Science Foundation (project no. 19JCZDJC38100), China, the National Natural Science Foundation of China (project no. 32402222), the Natural Science Foundation of Guangdong Province (project no. 2023A1515110754), and the Science Project of Department of Education of Guangdong Province (project no. 2024KSYS007), China.

References

- [1] Z. Bi, W. Zhang, X. Yan, Anti-inflammatory and immunoregulatory effects of icariin and icaritin, *Biomedicine & Pharmacotherapy*. 151 (2022), 113180.
- [2] S. Chen, Y. Sun, Y. Xie, Y. Liu, H. Hu, C. Xie, S. Xu, Z. Zhang, J. Zhang, Y. Shen, X. Xu, N. Qiu, Mitochondria-Targeted Icaritin Nanoparticles Induce Immunogenic Cell Death in Hepatocellular Carcinoma, *ACS applied materials & interfaces*. 17 (2025), 2899–2910.
- [3] M.-C. Jiang, X.-H. Chen, X. Zhao, X.-J. Zhang, W.-F. Chen, Involvement of IGF-1 receptor signaling pathway in the neuroprotective effects of Icaritin against MPP⁺-induced toxicity in MES23.5 cells, *European Journal of Pharmacology*. 786 (2016), 53–59.
- [4] Z. Wu, L. Ou, C. Wang, L. Yang, P. Wang, H. Liu, Y. Xiong, K. Sun, R. Zhang, X. Zhu, Icaritin induces MC3T3-E1 subclone14 cell differentiation through estrogen receptor-mediated ERK1/2 and p38 signaling activation, *Biomedicine & Pharmacotherapy*. 94 (2017), 1–9.
- [5] H. Han, B. Xu, P. Hou, C. Jiang, L. Liu, M. Tang, X. Yang, Y. Zhang, Y. Liu, Icaritin Sensitizes Human Glioblastoma Cells to TRAIL-Induced Apoptosis, *Cell Biochemistry and Biophysics*. 72 (2015), 533–542.
- [6] L. Sun, Q. Peng, L. Qu, L. Gong, J. Si, Anticancer agent icaritin induces apoptosis through caspase-dependent pathways in human hepatocellular carcinoma cells, *Molecular medicine reports*. 11 (2015), 3094–3100.
- [7] D. Mo, H. Zhu, J. Wang, H. Hao, Y. Guo, J. Wang, X. Han, L. Zou, Z. Li, H. Yao, J. Zhu, J. Zhou, Y. Peng, J. Li, K. Meng, Icaritin inhibits PD-L1 expression by Targeting Protein IκB Kinase α, *European Journal of Immunology*. 51 (2021), 978–988.
- [8] C. Zhang, X. Wang, C. Zhang, Icaritin inhibits CDK2 expression and activity to interfere with tumor progression, *iScience*. 25 (2022), 104991.
- [9] Q. Wei, B. Wang, H. Hu, C. Xie, L. Ling, J. Gao, Y. Cao, Icaritin promotes the osteogenesis of bone marrow mesenchymal stem cells via the regulation of sclerostin expression Corrigendum in /10.3892/ijmm.2023.5277, *International Journal of Molecular Medicine*. 45 (2020), 816–824.
- [10] R. Z. L. Lim, L. Li, E. L. Yong, N. Chew, STAT-3 regulation of CXCR4 is necessary for the prenylflavonoid Icaritin to enhance mesenchymal stem cell proliferation, migration and osteogenic differentiation, *Biochimica et Biophysica Acta (BBA) - General Subjects*. 1862 (2018), 1680–1692.

- [11] R. Z. L. Lim, L. Li, N. Chew, E. L. Yong, The prenylflavonoid Icaritin enhances osteoblast proliferation and function by signal transducer and activator of transcription factor 3 (STAT-3) regulation of C-X-C chemokine receptor type 4 (CXCR4) expression, *Bone*. 105 (2017), 122–133.
- [12] C.-T. Wu, T.-H. Yang, M.-C. Chen, S.-S. Guan, C.-M. Chen, S.-H. Liu, Therapeutic Effect of Icaritin on Cerebral Ischemia-Reperfusion-Induced Senescence and Apoptosis in an Acute Ischemic Stroke Mouse Model, *Molecules*. 27 (2022), 5783.
- [13] Y.-Y. Li, N.-Q. Huang, F. Feng, Y. Li, X.-M. Luo, L. Tu, J.-Q. Qu, Y.-M. Xie, Y. Luo, Icaritin Improves Memory and Learning Ability by Decreasing BACE-1 Expression and the Bax/Bcl-2 Ratio in Senescence-Accelerated Mouse Prone 8 (SAMP8) Mice, *Evidence-Based Complementary and Alternative Medicine*. 2020 (2020), 8963845.
- [14] H. Wu, X. Liu, Z.-Y. Gao, M. Lin, X. Zhao, Y. Sun, X.-P. Pu, Icaritin Provides Neuroprotection in Parkinson's Disease by Attenuating Neuroinflammation, Oxidative Stress, and Energy Deficiency, *Antioxidants*. 10 (2021), 529.
- [15] Z.-J. Li, C. Yao, S.-F. Liu, L. Chen, Y.-M. Xi, W. Zhang, G.-S. Zhang, Cytotoxic Effect of Icaritin and Its Mechanisms in Inducing Apoptosis in Human Burkitt Lymphoma Cell Line, *BioMed Research International*. 2014 (2014), 391512.
- [16] P. Xu, G.-W. Zheng, M.-H. Zong, N. Li, W.-Y. Lou, Recent progress on deep eutectic solvents in biocatalysis, *Bioresources and Bioprocessing*. 4 (2017), 34.
- [17] H.-T. ZENG, Research progress on pharmacology effects and new drug delivery system of icaritin, *Zhongcaoyao*. (2020), 5372–5380.
- [18] N. T. Huong, N. T. Son, Icaritin: A phytochemical with enormous pharmacological values, *Phytochemistry*. 213 (2023), 113772.
- [19] V.-S. Nguyen, L. Shi, Y. Li, Q.-A. Wang, Total synthesis of icaritin via microwave-assistance Claisen rearrangement, *Letters in Organic Chemistry*. 11 (2014), 677–681.
- [20] Q. Mei, C. Wang, Z. Zhao, W. Yuan, G. Zhang, Synthesis of icariin from kaempferol through regioselective methylation and para-Claisen–Cope rearrangement, *Beilstein Journal of Organic Chemistry*. 11 (2015), 1220–1225.
- [21] Y. Shen, H. Wang, Y. Lu, L. Xu, H. Yin, J. P. Tam, H. Yang, X. Jia, Construction of a novel catalysis system for clean and efficient preparation of Baohuoside I from Icariin based on biphasic enzymatic hydrolysis, *Journal of Cleaner Production*. 170 (2018), 727–734.
- [22] Q. Li, L. Ge, D. Zheng, X. Zhang, L. Zhao, Screening and characterization of a GH78 α -l-rhamnosidase from *Aspergillus terreus* and its application in the bioconversion of icariin to icaritin with recombinant β -glucosidase, *Enzyme and Microbial Technology*. 153 (2022), 109940.
- [23] Z. Wang, C. Liu, H. Yu, B. Wu, B. Huai, Z. Zhuang, C. Sun, L. Xu, F. Jin, Icaritin Preparation from Icariin by a Special Epimedium Flavonoid-Glycosidase from *Aspergillus* sp.y848 Strain, *Journal of Microbiology and Biotechnology*. 32 (2022), 437–446.
- [24] B. Zhu, J. Liu, W. Gao, Effects of Snailase Treatment on Wettability of Raw Cotton Yarns in Pre-wetting Process of Foam Sizing, *Applied Biochemistry and Biotechnology*. 182 (2017), 1065–1075.
- [25] W. Chen, G. Liu, Y. Zhang, Production of Rhamnosyl Icariside II by snailase hydrolysis of *Epimedium wushanense* extracts, *Heliyon*. 10 (2024), e23805.
- [26] D. Jia, X. Jia, J. Xue, F. Shi, E. Sun, L. Chen, Z. Zhang, Study on preparation of icariin from icariin by snail enzyme transformation, *China Journal of Chinese Materia Medica*. 35 (2010), 857–860.
- [27] C. Liu, R. Li, J. Peng, D. Qu, M. Huang, Y. Chen, Enhanced hydrolysis and antitumor efficacy of Epimedium flavonoids mediated by immobilized snailase on silica, *Process Biochemistry*. 86 (2019), 80–88.
- [28] W. Li, X. Zhang, Z. Xue, Y. Mi, P. Ma, D. Fan, Ginsenoside CK production by commercial snailase immobilized onto carboxylated chitosan-coated magnetic nanoparticles, *Biochemical Engineering Journal*. 174 (2021), 108119.
- [29] M. A. Oke, S. A. Ojo, S. A. Fasiku, E. A. Adebayo, Nanotechnology and enzyme immobilization: a review, *Nanotechnology*. 34 (2023), 385101.
- [30] Y. Jiang, L. Ma, L. Zhou, L. Ma, Y. He, X. Zhang, J. Gao, Structured interlocked-microcapsules: A novel scaffold for enzyme immobilization, *Catalysis Communications*. 88 (2017), 35–38.

- [31] S. Olshannikova, V. Koroleva, M. Holyavka, S. Pankova, A. Belenova, M. Kondratyev, A. Samchenko, A. Kabanov, V. Artyukhov, The adsorption immobilization of bromelain on chitosan matrices, *Journal of Biotechnology*. 280 (2018), S68.
- [32] N. Chen, C. Zhang, Y. Liu, X. Dong, Y. Sun, Cysteine-modified poly(glycidyl methacrylate) grafted onto silica nanoparticles: New supports for significantly enhanced performance of immobilized lipase, *Biochemical Engineering Journal*. 145 (2019), 137–144.
- [33] Z.-m. Tang, J.-w. Kang, Enzyme Inhibitor Screening by Capillary Electrophoresis with an on-Column Immobilized Enzyme Microreactor Created by an Ionic Binding Technique, *Analytical Chemistry*. 78 (2006), 2514–2520.
- [34] G. Kuang, Z. Wang, M. Bilal, Z. Wang, Y. Feng, Y. Du, J. Cui, Metal-Organic Frameworks: A Potential Platform From Enzyme Immobilization to Mimetic Enzyme, *Aggregate*. 6 (2025), e724.
- [35] Y. Hu, L. Dai, D. Liu, W. Du, Y. Wang, Progress & prospect of metal-organic frameworks (MOFs) for enzyme immobilization (enzyme/MOFs), *Renewable and Sustainable Energy Reviews*. 91 (2018), 793–801.
- [36] H. Tang, D. Fan, Y. Chen, S. Han, Exploring enzyme–MOF (metal–organic framework) catalytic systems: trade-offs between enzyme activity and MOF stability, *Green Chemistry*. 27 (2025), 2605–2628.
- [37] Y. Du, L. Zhao, Z. Geng, Z. Huo, H. Li, X. Shen, X. Peng, R. Yan, J. Cui, S. Jia, Construction of catalase@hollow silica nanosphere: Catalase with immobilized but not rigid state for improving catalytic performances, *International Journal of Biological Macromolecules*. 263 (2024), 130381.
- [38] S. Shafique, A. S. Belousov, R. Rashid, I. Shafiq, K. H. H. Aziz, N. Riaz, M. S. Khan, A. Shaheen, M. Ishaq, P. Akhter, M. Hussain, Deep eutectic solvents (DES): Structure, properties, and cutting-edge applications in green catalysis, *Journal of Molecular Liquids*. 419 (2025), 126769.
- [39] O. V. Morozova, I. S. Vasil'eva, G. P. Shumakovich, E. A. Zaitseva, A. I. Yaropolov, Deep Eutectic Solvents for Biotechnology Applications, *Biochemistry (Moscow)*. 88 (2023), S150–S175.
- [40] T. Khan, N. Das, S. Bhowmik, K. S. Negi, P. Sen, Critical Role of Water beyond the Media to Maintain Protein Stability and Activity in Hydrated Deep Eutectic Solvent, *The Journal of Physical Chemistry B*. 129 (2025), 162–175.
- [41] T. Khan, N. Das, K. S. Negi, S. Bhowmik, P. Sen, Understanding the intricacy of protein in hydrated deep eutectic solvent: Solvation dynamics, conformational fluctuation dynamics, and stability, *International Journal of Biological Macromolecules*. 253 (2023), 127100.
- [42] J. Guo, N. Zhao, Y. Zhao, H. Jin, G. Sun, J. Yu, H. Zhang, M. Yu, D. Yang, Z. Liang, Effect of Deep Eutectic Solvents on the Activity and Stability of Cellulases and Pectinases, *ACS Omega*. 8 (2023), 45678–45686.
- [43] M. Sypabekova, A. Hagemann, D. Rho, S. Kim, Review: 3-Aminopropyltriethoxysilane (APTES) Deposition Methods on Oxide Surfaces in Solution and Vapor Phases for Biosensing Applications, *Biosensors*. 13 (2023), 36.
- [44] S.-Z. Zu, B.-H. Han, Aqueous Dispersion of Graphene Sheets Stabilized by Pluronic Copolymers: Formation of Supramolecular Hydrogel, *The Journal of Physical Chemistry C*. 113 (2009), 13651–13657.
- [45] Lalith K. Chaganti, N. Venkatakrishnan, K. Bose, An efficient method for FITC labelling of proteins using tandem affinity purification, *Bioscience Reports*. 38 (2018), BSR20181764.
- [46] K. Kato, Y. Kawachi, H. Nakamura, Silica–enzyme–ionic liquid composites for improved enzymatic activity, *Journal of Asian Ceramic Societies*. 2 (2014), 33–40.
- [47] M. Picquart, L. Grajcar, M. H. Baron, Z. Abedinzadeh, Vibrational spectroscopic study of glutathione complexation in aqueous solutions, *Biospectroscopy*. 5 (1999), 328–337.
- [48] X. Zheng, Y. Li, Y. Xu, Z. Hong, M. Wei, Metal–organic frameworks: Promising materials for enhancing electrochemical properties of nanostructured Zn₂SnO₄ anode in Li-ion batteries, *CrystEngComm*. 14 (2012), 2112–2116.
- [49] M. Bisht, P. Venkatesu, Influence of cholinium-based ionic liquids on the structural stability and activity of α -chymotrypsin, *New Journal of Chemistry*. 41 (2017), 13902–13911.
- [50] M. Pätzold, S. Siebenhaller, S. Kara, A. Liese, C. Syltatk, D. Holtmann, Deep Eutectic Solvents as Efficient Solvents in Biocatalysis, *Trends in Biotechnology*. 37 (2019), 943–959.