Electronic Supplementary Material (ESI) for Green Chemistry.

Supporting Information

Hollow colloidosomes with an enzyme confined in a porous shell as Pickering interfacial biocatalysts for efficient bioconversions

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Experimental Procedures

1. Materials. Lipase (CALB) from *Candida sp.* expressed in *Aspergillus niger*, SiO_2 dispersion solution (40 wt.%) with a diameter of 22 nm and SiO_2 dispersion solution (30 wt.%) with a diameter of 12 nm were purchased from Sigma-Aldrich. Butanol, Coomassie brilliant blue G-250 and phosphoric acid were obtained from Kelong Chemical Reagents Co. Ltd. (Chengdu, China). Tributyrin was bought form Aladdin Industrial Corporation. (Shanghai, China). Sodium dihydrogen phosphate (NaH_2PO_4) was purchased from Guangdong Guanghua Technology Co., Ltd. Disodium hydrogen phosphate (Na_2HPO_4) was gotten from Tianjin Zhiyuan Chemical Reagents Co., Ltd. Fluorescein isothiocyanate (FITC) was acquired from Sigma-Aldrich. Anhydrous ethanol, sodium hydroxide, phenolphthalein and acetone were obtained from Chengdu Shiyang Chemical Reagents Co., Ltd. All the chemicals were of analytical grade.

2. Synthesis of SiO_2 colloidosomes. SiO_2 colloidosomes were synthesized according to the method of constructing Au plasmonic colloidosomes reported previously.[1] 1 mL of SiO_2 dispersion (40 wt.%), mixed with PBS solution (pH = 7.0, 2 mL) in a 50 mL polyethylene (PE) tube thoroughly, then, added butanol (30 mL), were emulsified by centrifugal disperser at 14,000 rpm for 1 min. Stand for 24 h. After that, the colloidosome could be obtained and freeze-drying.

3. Synthesis of lipase-loaded hollow colloidosomes (LHCs) and lipase-loaded solid colloidosomes (LSCs). For immobilized CALB in colloidosomes, 2 mL of lipase solution (commercially available CALB solution diluted 10 times with PBS), mixed with 1 mL of SiO_2 dispersion with nanoparticles diameter of 22 nm (40 wt.%) in a 50 mL PE tube thoroughly, and then added 30 mL of butanol, were emulsified by centrifugal disperser at 14,000 rpm for 1 min. Stand for 24 h. After that, LHCs could be obtained and drying. The synthesis method of LSCs is same as LHCs, but SiO_2 dispersion with nanoparticles diameter of 12 nm (30 wt.%).

4. Encapsulation rate of lipases in colloidosomes. The encapsulation rate of lipase in colloidosomes was measured through the Bradford method.[2] More specifically, the system for preparing LHCs use the above step, centrifuge (5000 rpm, 5 min) and remove the butanol phase, and then 10 mL distilled water added to wash LHCs, centrifuge, and keep the supernatant. 1 mL of supernatant was added into 5 mL of Coomassie Blue solution (0.2% w/v) and mixed homogenously. Allowing the mixture to react for 3 min, the absorbance values were measured at 595 nm and compared to a CALB standard curve to determine the protein concentration. The encapsulation rate of lipases in colloidosomes was calculated by the equation as shown below, where m_i (mg) is the initial amount of enzyme used for immobilization, m_s (mg) is the amount of enzyme in supernatant.

$$\text{Encapsulation rate} = (m_i - m_s)/m_i \times 100\% \quad (1)$$

5. Evaluation of the surface wettability of SiO_2 colloidosomes and LHCs. The three-phase contact angle of SiO_2 colloidosomes and LHCs at the oil-water interface were measured by using the dip-coating method. To obtain compact SiO_2 colloidosomes films on a glass slide (5 × 5 mm), the glass slide was dip-coated into a 1% (w/v) SiO_2 colloidosomes dispersion in water using a withdrawal speed of 14 cm min^{-1}. The treated glass slide was dried at 25 °C for 30 min. To ensure the glass slide was fully covered by colloidosomes, the above deposition process was repeated. Subsequently the glass slide was placed at the bottom of a transparent quartz vessel. Water was then poured into the vessel. A 3 μL tributyrin droplet was carefully placed on the disk surface. The appearance of the tributyrin droplet on the substrate was photographed when the droplet was stationary. All of the contact angles measured through water were the arithmetic average of at least five measurements on the same sample. The evaluation of the surface wettability of LHCs was the same as that described above.

6. Effect of substrate concentration on reaction rate and conversion rate of PIB with LHCs. LHCs with amphiphilic lipase in shell were used to stable oil-in-water Pickering emulsion, which used tributyrin as the oil phase and PBS (0.05 $mol \cdot L^{-1}$, pH = 7.0) as the aqueous phase.

In order to investigate the effect of substrate concentration on the enzymatic reaction rate and conversion rate of LHCs, the specific method was to take PE tubes, added 30 mL of PBS buffer (pH = 7.0) respectively, and then added tributyrin to make substrate concentration in each PE tube were 0, 12, 24, 48, 72, 96, and 120 $mg \cdot mL^{-1}$. After that, quickly stirred for 10 min at room temperature, then added LHCs (2 wt.%) (the blank group was added with an equal amount of SiO_2 colloidosomes). After reaction for 15 minutes on a rotary mixer（80 rpm）, 10 mL of inactivator (water:ethanol:acetone = 1:1:1) was added to terminate the hydrolysis reaction, and the butyric acid generated during the reaction was titrated with 0.05 $mol \cdot L^{-1}$ NaOH solution. The calculation of reaction rate and conversion rate are as follows, where v ($\mu mol \cdot mL^{-1} \cdot min^{-1}$) is the reaction rate, C is the conversion rate, V_{NaOH} (mL) is the net consumption of the titrated NaOH solution, c_{NaOH} ($mol \cdot L^{-1}$) is the concentration of NaOH solution, V (mL) is the total volume of the reaction solution participating in the titration, t (min) is the reaction time, and c_S ($mol \cdot L^{-1}$) is tributyrin concentration, V_S (mL) is the amount of tributyrin:

$$\text{Reaction rate: } v = (V_{NaOH} \times c_{NaOH})/(V \times t) \quad (2)$$

$$\text{Conversion rate: } C = (V_{NaOH} \times c_{NaOH})/(c_S \times V_S) \times 100\% \quad (3)$$

7. Assessment of catalytic performance of Pickering emulsions. Lipase activity was determined by fatty acids produced in hydrolysis of tributyrin. Typically, the oil-in-water Pickering emulsion was prepared by using 2 mL of tributyrin and 30 mL of PBS buffer (pH = 7.0). The control group were conventional biphasic system consisting of lipases and substrate solution, and the Pickeirng emulsion stabilized by LSCs. The experimental group was the Pickering stabilized by LHCs. All reaction time was 15 min, then added 10 mL of inactivating agent to terminate the reaction. Then titrate the fatty acid generated during the reaction using 0.05 $mol \cdot L^{-1}$ NaOH solution and calculated the enzyme activity. The enzyme activity is defined as: the amount of enzyme that is required to catalyze the hydrolysis of tributyrin to produce1 μmol of fatty acid per minute at 37 °C at pH 7.0, which is an enzyme activity unit, symbol by *U* ($U \cdot mg^{-1}$). The enzyme activity formula is as follows, where *c* ($mol \cdot L^{-1}$) is the concentration of NaOH solution, Δ*V* (mL) is the net consumption of NaOH solution generated during the titration, *m* (mg) is the amount of enzyme added, *t* (min) is the reaction time.

Enzyme activity: $U = (c \times \Delta V)/(m \times t)$ (4)

The enzyme activity retention rate (%) of LHCs is the percentage of the catalytic activity of LHCs and the activity of the free lipases in the water phase of the emulsion stabilized by devitalized LHCs. The formula is as follows:

Enzyme activity retention rate (%) = $U_{\mathrm{LHCs}}/U_{\mathrm{free\ lipases}} \times 100\ \%$ (5)

8. Assessment of the re-usability of Pickering emulsions stabilized by LHCs. To investigate the hydrolysis reaction of LHCs in oil-in-water Pickering emulsion, the reusability was studied. After a hydrolysis reaction was completed, the LHCs were precipitated by centrifugation (5000 rpm, 5 min) without adding inactivating agent, and were washed by PBS buffer, then added fresh substrate solution and buffer to start next reaction. The new reaction batch was carried out under same conditions as the previous (15 min per cycle). After each cycle, collect the supernatant and use titration to determine relative enzyme activity. The relative enzyme activity (%) is used to investigate the reusability of the Pickering emulsion stabilized by LHCs, which is defined as the percentage of residual enzyme activity in the *N*th reaction cycle and the enzyme activity in the first reaction cycle. The formula is as follows:

Relative enzyme activity (%) = $U_{N\mathrm{th}}/U_{\mathrm{first}} \times 100\ \%$ (6)

9. Characterization techniques. Scanning electron microscope (SEM, JEOL-JSM 7001F, Japan) was used to characterize the morphology of SiO_2 colloidosome, LSCs and LHCs. Samples was prepared that was 1% (w/v) colloidosome dispersion, dropped on a piece of monocrystalline silicon, and dried at 25 °C for 30 min, sprayed with a gold coating to remove supernumerary electrons. Confocal laser scanning microscope (CLSM, Leica TCP SP5, Germany) was used to record microscopic images with the excitation wavelength of 488 nm. Before tested, lipases were labeled with fluorescein isothiocyanate (FITC) and then immobilized. Fourier transform infrared spectroscopy (FTIR) spectra were measured with a Bruker VECTOR22 spectrometer using KBr in the range from 4000 cm^{-1} to 400 cm^{-1}. Fluorescence inverted microscope (IX71-A12FL/PH, Olympus, Japan) was used to excite the green fluorescence of FITC-labelled CALB at 488 nm to obtain a fluorescence image. Optical microscope (Phoenix Co. Ltd., XSP-24, China) and Moticam 2000 camera were used to observe the surface morphological characteristics of the emulsion droplets. The pictures were captured using Motic Images Plus 2.0 software, then processed and analyzed by Image Pro Plus software.

Results and Discussion

10. Formation mechanism diagram of LHCs.

Figure S1 shows the self-assembling mechanism for the fabrication of LHCs. The lipases and SiO_2 nanoparticles are evenly dispersed into the water phase, and the outer phase is butanol. After emulsification, the water emulsion droplets containing the SiO_2 nanoparticles and lipases are suspended in butanol. These SiO_2 nanoparticles spontaneously absorb at the emulsion interface, driven by the minimization of the total interfacial free energy[3] (Figure S1a). Because water has the appropriate dissolubility in butanol, after a while, a small amount of water diffuses into surrounding butanol, making the emulsion dispersed phase volume smaller, the surface area reduced, and the original nanoparticle layer is compressed, which leaded to the nanoparticles are closely packed and wrapped on the surface of droplets (Figure S1b). As water continues to diffuse into butanol, SiO_2 nanoparticles and lipases in water accumulate into shell (Figure S1c), forming hollow and multilayer colloidosomes where the enzyme is confined in porous shell (Figure S1d).[1]

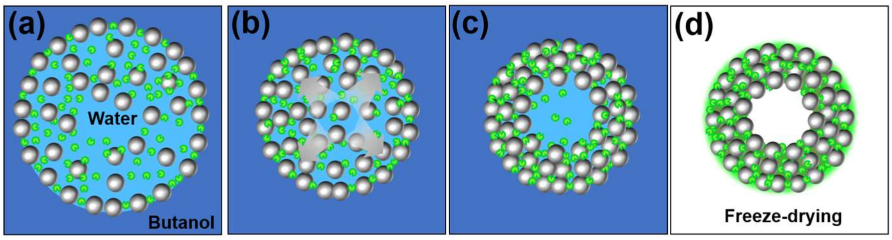

Figure S1. Formation mechanism diagram of LHCs.

11. Effect of SiO_2 nanoparticle concentration (C_{SiO2}) on the morphology of colloidosomes.

In the process of preparing colloidosomes, the moderate concentration of nanoparticles is essential for the formation of regular colloidosomes.[1] As shown in Figure S2a, when C_{SiO2} is low, colloidosomes with sphericity can be formed. As the concentration increases, the colloidosomes exhibit better spherical structure and dispersion. Further increasing the C_{SiO2} leads to larger size of colloidosomes, which is not conducive to stabilize Pickering emulsion. Moreover, when the concentration is very high, a large amount of SiO_2 nanoparticles dispersed in the water phase will aggregate together, causing disordered precipitation of SiO_2 nanoparticles. And the effect of C_{SiO2} on the mean diameters of the colloidosomes (d) shown in Figure S2b. Therefore, the C_{SiO2} is 2 wt.% that formed a regular shape of colloidosomes, which is more conducive to subsequent experiments. That is, the system of preparing colloidosomes is: 1 mL of SiO_2 dispersion (40 wt.%) and 2 mL of PBS solution fully mixed, then add 30 mL of butanol.

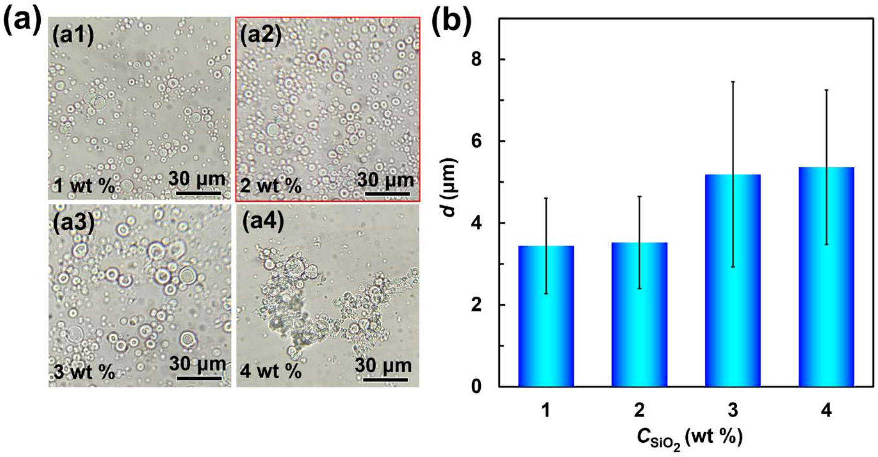

Figure S2. Optical microscopy images of colloidosomes with varied SiO_2 nanoparticles concentrations; C_{SiO2} (wt.%): (a1) = 1 wt.%, (a2) = 2 wt.%, (a3) = 3 wt.%, (a4) = 4 wt.%. (b) Effect of C_{SiO2} (wt.%) on the mean diameters of the colloidosomes.

12. Spectral characterization of SiO_2 dispersion and SiO_2 colloidosomes.

FTIR spectra is used to analyse the bonding between silica of colloidosome. As shown in Figure S3, the spectra indicate the existence of asymmetric stretching of Si-O-Si at 1113 cm^{-1}, and symmetric stretching and bending vibration of Si-O at 800 cm^{-1} and 474 cm^{-1} respectively. The peak near 1636 cm^{-1} is the H−O−H bending vibration peak of water. The broad peak near 3454 cm^{-1} is the antisymmetric stretching vibration peak of structural water-OH. Moreover, there is no chemical bond change after the SiO_2 dispersions form colloidosomes.

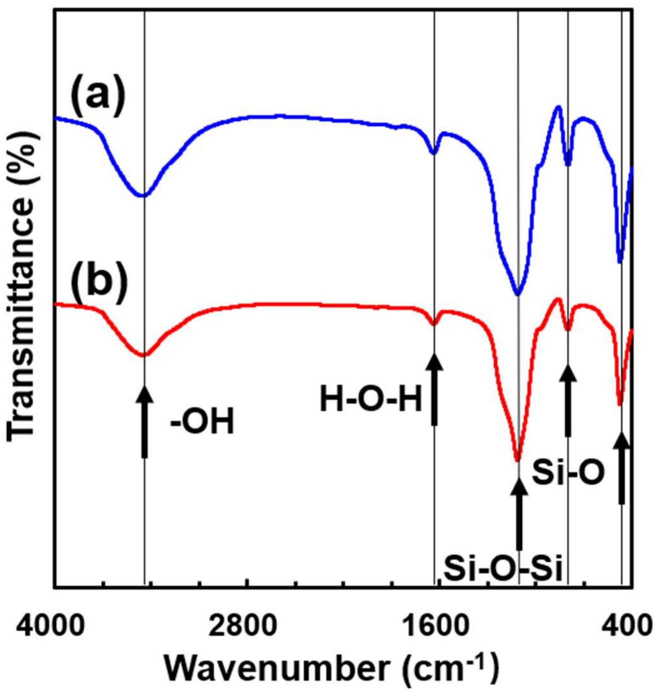

Figure S3. FTIR spectra of SiO_2 dispersions (a) and SiO_2 colloidosome (b).

13. Encapsulation of FITC-labelled CALB in colloidosomes.

In order to visually observe the lipase-loaded colloidosomes formation process, a water drop with the diameter of 1mm containing FITC-labelled CALB and SiO_2 nanoparticles is added to the butanol. As shown in Figure S4, the water drop gradually shrinks as water diffuses to butanol. The fluorescent protein cannot cross the water-butanol interface, and self-assembles to form a shell with SiO_2 nanoparticles over 24 hours. Figure S5 demonstrates the lipase-loaded colloidosomes (2−6 μm) formation process by emulsification at 0 h and 24 h.

According to the formation mechanism of colloidosome, with the diffusion of water to butanol, the water phase volume becomes smaller rapidly, and the nanoparticles at the interface of the droplet solidify to realize rigid colloidosomes. The drop (1mm) prepared by dropping method in Figure S4 is much larger than the droplet (2−6 μm) formed by emulsification in Figure S5. For Figure S4, due to the big drop diameter, the concentration of nanoparticles at the interface is higher than that in center with the fast diffusion of the water phase, which leads to the stretching and extrusion of the nanoparticles, to a certain extent, causing the top of the droplet to fold inward until the assembly of internal nanoparticles is completed,[4] resulting the collapsing structure. However, these colloidosomes are formed by emulsification in a very small size (Figure S5), during the formation of LHC, the diffusion distance is short, and the nanoparticles at the interface quickly accumulate and solidify to form a strong shell structure without serious stretching and extrusion between particles, resulting in intact sphere.

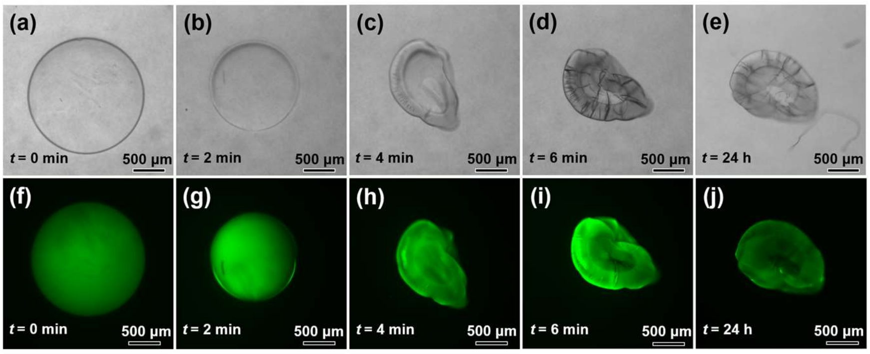

Figure S4. Formation process of lipase-loaded colloidosome (1mm) prepared by dropping method from visualization, fluorescent images taken at different times.

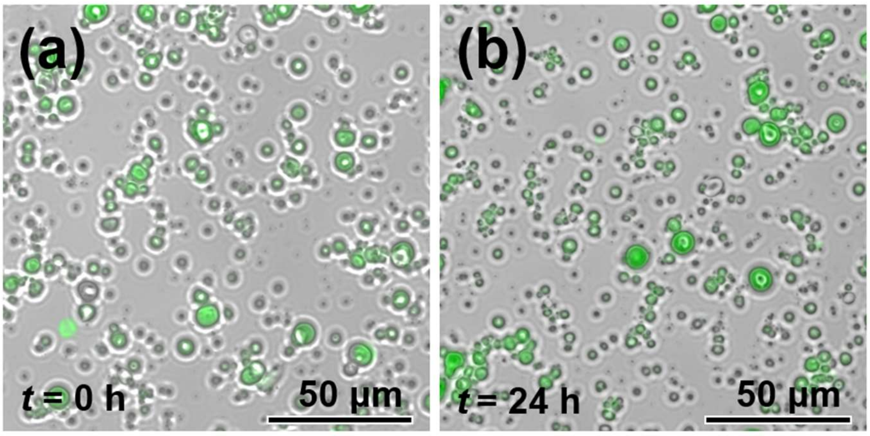

Figure S5. Formation process of lipase-loaded hollow colloidosome (LHC) by emulsification with the diameter of 2−6 μm at 0 h (a) and 24 h (b).

14. Evaluation of the leakage of lipase from LHCs in water.

In order to investigate the leakage of lipase during use of LHCs, the LHCs are subjected to a ten-hour immersion experiment in PBS solution (Figure S6). Fluorescent images show that the FITC-labelled CALB encapsulated in colloidosomes not obviously diffuse into water during ten hours and the amount of enzyme leakage are 3.9% after 24 hours, and the spherical shape of LHCs remains intact in experiment. In addition, these colloidosomes are stable for greater than 300 days when they are incubated in the water solutions or at the interfaces.

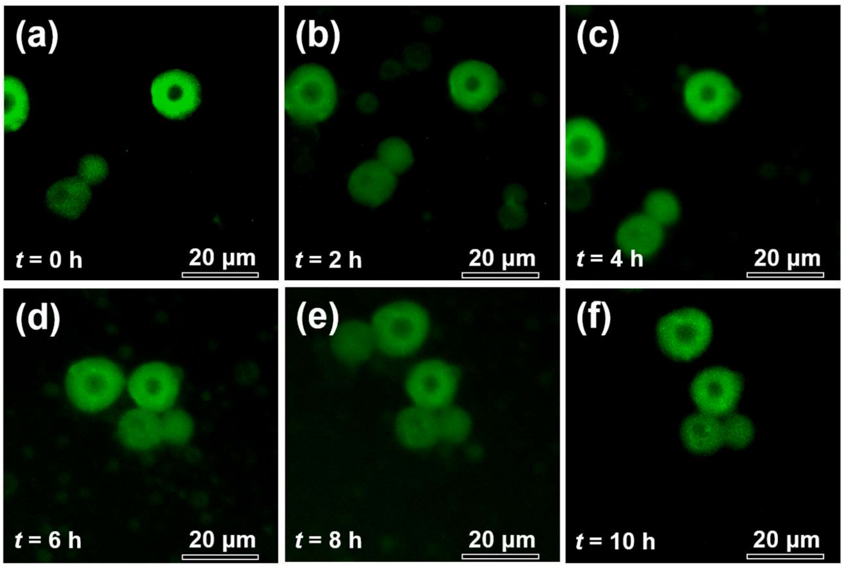

Figure S6. Immersion experiment of FITC-labelled CALB from LHCs in water, fluorescent images taken at different times.

15. Evaluation of the surface wettability of SiO_2 colloidosomes and LHCs.

Three-phase contact angle testing is a useful way to reveal the contest result of solid particles at oil-water interface by water and oil. In order to prove that the amphiphilic lipase changes the wettability of colloidosomes, we measure the three-phase contact angle of SiO_2 colloidosomes before and after loading lipase. As is seen in Figure S7, the contact angle of the LHCs is 122° (Figure S7b), which become smaller than SiO_2 colloidosomes of 170° (Figure S7a), making LHCs have a suitable wettability to stabilize oil-in-water Pickering emulsion.

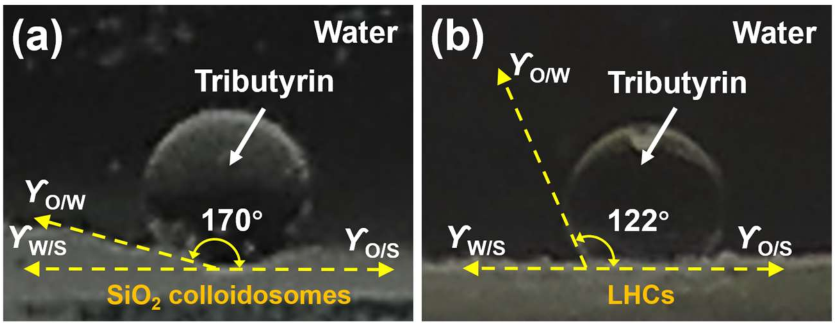

Figure S7. Three-phase contact angle of SiO_2 colloidosomes (a) and LHCs (b).

16. Effect of LHCs concentration (C_{LHCs}) on the diameters of Pickering emulsion droplets.

It is well known that reaction rate of Pickering interfacial biocatalysis is extremely limited by the oil-water interface area. In order to explore the influence of the C_{LHCs} on the average diameter of emulsion droplets (D), the C_{LHCs} of 0.5, 1, 1.5 and 2 wt.% are selected for experiments. It can be seen from Figure S8a that the size of Pickering emulsion decreases as the C_{LHCs} increases. Figure S8b shows the value of droplet size decrease as the C_{LHCs} goes up, which can be explained by the fact that small droplets incompletely covered by particles will coalesce, yielding larger droplets until the interface is completely stabilized by particles.[5,6] Moreover, there is no significant difference of Pickering emulsion diameter between the C_{LHCs} of 1.5 wt.% and 2 wt.%, while the latter lead to a waste of colloidosomes. Therefore, the C_{LHCs} of 1.5 wt.% (D = 176 μm) with the relative high surface area is chosen in subsequent experiments.

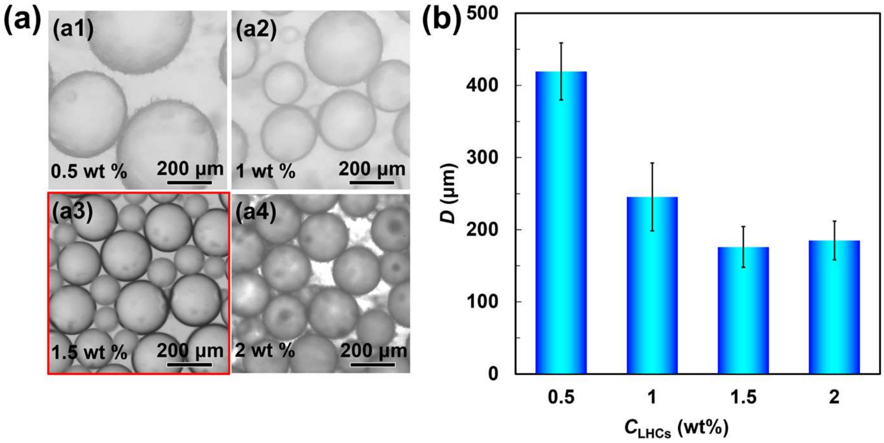

Figure S8. Optical micrograph of Pickering emulsions stabilized with varied LHCs concentrations; C_{LHCs} (wt.%): (a1) = 0.5 wt.%, (a2) = 1 wt.%, (a3) = 1.5 wt.%, (a4) = 2 wt.%. (b) Effect of *C* (wt.%) on the mean diameters of the Pickering emulsion droplets.

17. The time profile of the substrate conversion of LHCs in PIB system.

To explore the time profile of the substrate conversion, we had carried out catalytic reactions of different times. As shown in Figure S9, the conversion reaches 85% within 6 hours. The hydrolysis of tributyrin usually produces glyceryl dibutyrate and butyric acid within 15 minutes. The production amount of butyric acid and the reactant consumed quantity of tributyrin are 1:1, which is convenient for testing the catalytic performance of the LHCs by acid-base titration.

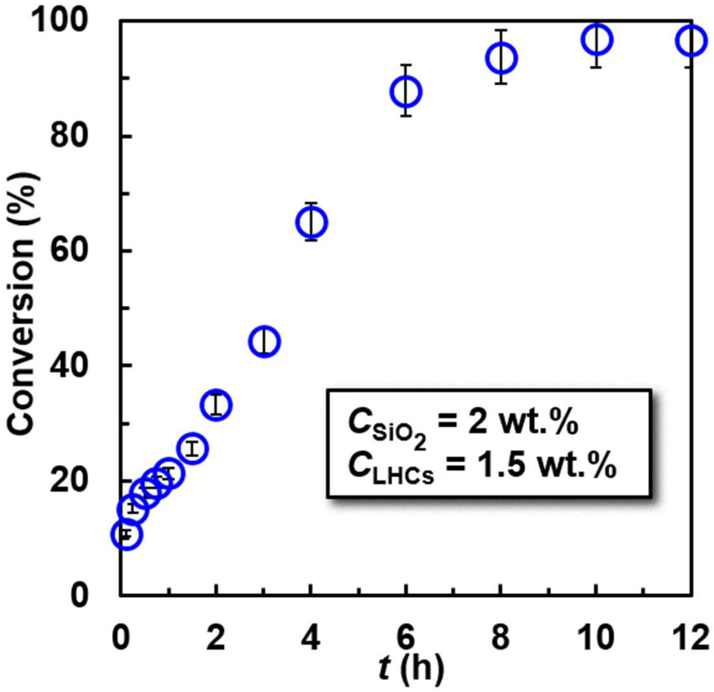

Figure S9. The time profile of the substrate conversion of LHCs in PIB system. C_{SiO2} = 2 wt.%, C_{LHCs} = 1.5 wt.%.

18. Morphology of LSCs and LHCs.

In order to compare the catalytic difference between solid and hollow structure, we prepared LSCs and LHCs. As is shown in Figure S10, the LSCs with solid structure formed by SiO_2 nanoparticles (*d* = 12 nm) and lipase (Figure S10a−b), while the LHCs with hollow structure assembled by SiO_2 nanoparticles (*d* = 22 nm) and lipase (Figure S10e−f). Moreover, the FITC-labelled CALB is entrapped in the LSCs (Figure S10c) and the shell of LHCs (Figure S10g). It is obviously that the diameter of SiO_2 nanoparticles is the important factor of colloidosomes structure. As the size of the constituent particles increases, the number of nanoparticles per unit volume decreases, thus hollow colloidosomes with thin shell are formed.[7] It can be seen from the Figure S10d that LSCs have a spherical shape with the size of 2−5 μm is slightly smaller than LHCs with the diameter of 2−6 μm (Figure S10h). The encapsulation rate of lipase for LSCs is determined by Bradford method, which is 79.92%, the loading amount of lipase is 28.1 mg/g (lipase / (lipase + SiO_2)).

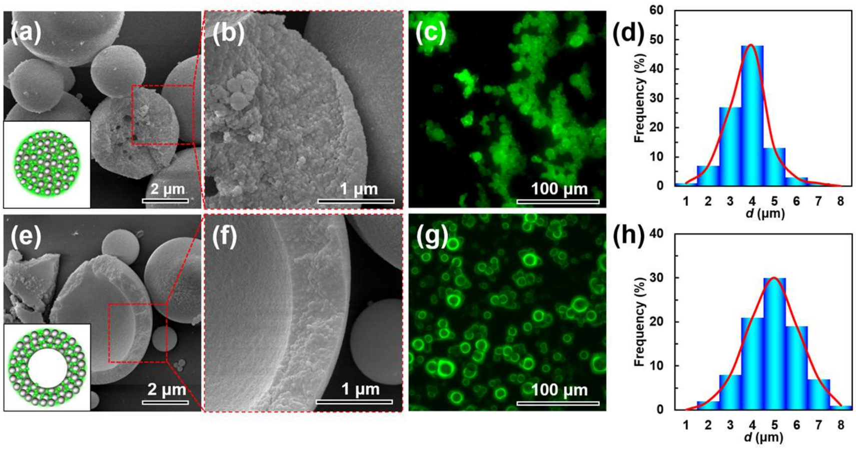

Figure S10. SiO_2 particles with different sizes to form lipase-loaded colloidosomes. (a−c) SEM image and fluorescent photograph of LSCs formed by SiO_2 nanoparticles (d = 12 nm), (d) the size distributions of LSCs. (e−g) LHCs formed by SiO_2 nanoparticles (d = 22 nm), (h) the size distributions of LHCs. Insets are schematic diagrams of LSCs and LHCs. Green indicates FITC-labelled CALB.

19. Characterization of Pickering emulsion stabilized by LSCs.

The LSCs are used for Pickering interfacial biocatalysis under the same conditions with LHCs. Figure S11 shows that the Pickering emulsion stabilized by LSCs is 170 μm, which is no significant difference of droplet diameter stabilized by LSCs and LHCs.

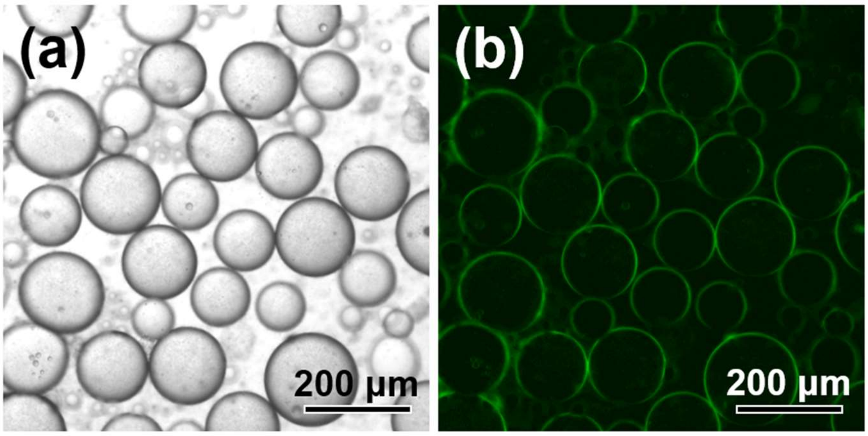

Figure S11. Micrographs of LSCs stabilized tributyrin-in-water Pickering emulsion, (a) bright-field diagram, (b) dark-field diagram.

20. Optical micrograph of the morphology of LHCs over 10 reaction cycles.

As shown in Figure S12, the appearance of LHCs in the 0 (Figure S12a) and the tenth (Figure S12b) PIB reaction cycles, indicating that LHCs has a rigid shell with an effective protection for lipases and the encapsulation rate of enzyme in LHCs after ten cycles is 82.7%.

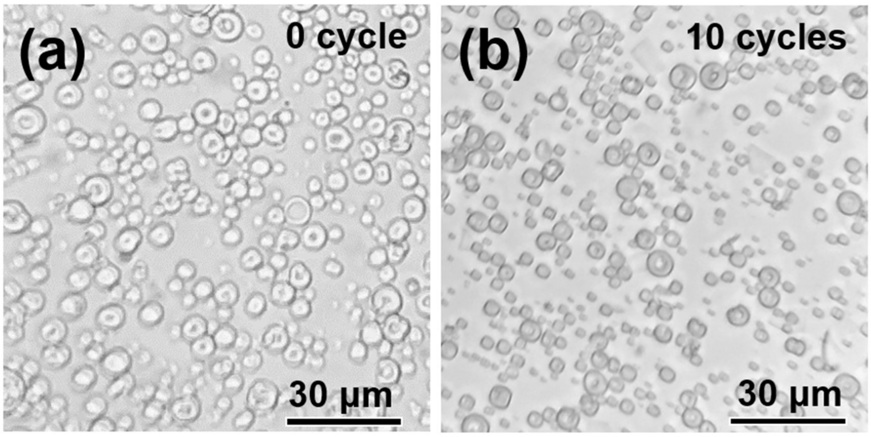

Figure S12. Optical micrograph of the morphology of LHCs in the 0 (a) and the tenth (b) PIB reaction cycles.

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