Temperature-responsive Pickering high internal phase emulsions for recyclable efficient interfacial biocatalysis

Chao Wang,[a,b] Hui Chi,[a] Fan Zhang,[a,b] Xinyue Wang,[a,b], Jiarui Wang,[a,b] Hao Zhang,[a] Ying Liu,[a] Xiaona Huang,[a] Yungang Bai,*[a] Kun Xu *[a] and Pixin Wang [a,b]

Abstract: The field of biocatalysis is expanding owing to the increasing demand for efficient low-cost green chemical processes. However, a feasible strategy for achieving product separation, enzyme recovery, and high catalytic efficiency in biocatalysis remains elusive. Herein, we present thermoresponsive Pickering high internal phase emulsions (HIPEs) as controllable scaffolds for efficient biocatalysis; these HIPEs demonstrate a transition between emulsification and demulsification depending on temperature. Ultra-high-surface-area Pickering HIPEs were stabilised by Candida antarctica lipase B immobilised on starch particles modified with butyl glycidyl ether and glycidyl trimethyl ammonium chloride, thus simplifying the separation and reuse processes and significantly improving the catalytic efficiency. In addition, the switching temperature can be precisely tuned by adjusting the degree of substitution of the modified starches to meet the temperature demands of various enzymes. We believe that this system provides a green platform for various interfacial biocatalytic processes of industrial interest.

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Experimental Procedures

Materials

Acidified wax corn starch (M_v = 200000) was obtained using a previously reported method. Butyl glycidyl ether (BGE) and glycidyl trimethylammonium chloride (GTAC) were purchased from Tokyo Chemical Industries (Tokyo, Japan). Candida antarctica lipase B, a recombinant from Aspergillus oryzae, and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Shanghai, China). Tris(hydroxymethyl)methyl aminomethane, sodium hydroxide, hydrochloric acid, hexane, and hexyl hexanoate were supplied by Xilong Scientific Co., Ltd. (Shantou, China). Deionized water was used in all experiments.

Synthesis of Starches-Butyl glycidyl ether-Glycidyl trimethyl ammonium chloride (StBG)

StBGs with different degrees of substitution were obtained using the following steps. First, 8.10 g (with approximately 0.05 mol of anhydroglucose units) of acidified wax corn starch was dispersed in 20 mL deionized water, mixed with 20 mL of a sodium hydroxide solution (including 1 g NaOH), and stirred for 1 h at 75 °C. When the hydroxyl activation of starch was complete, 13 g of BGE (0.1 mol) and a certain quantity of GTAC were added and reacted at 75 °C for 5 h. Thereafter, the resulting suspensions were cooled in an ice bath and neutralized with hydrochloric acid. The supernatant was then removed, an appropriate quantity of tetrahydrofuran was added for ultrasonic dissolution, and the obtained solution was placed in a dialysis bag and purified with deionised water. Finally, the products were dried in a freeze-dryer and sealed for storage. In reference to our previous work,[1, 2] the degree of substitution (DS) of BGE was determined using ^{1}H NMR spectroscopy according to Equation 1:

$$DS_{BGE} = \frac{B/3}{A/4} = 1.33\frac{B}{A} \qquad (1)$$

where *A* is the integral of the peaks representing the hydroxyl and H-1 protons at 4.5–6.6 ppm, and *B* is the integral of the area of methyl protons represented at 0.8 ppm.

The DS of GTAC was calculated from the nitrogen content according to Equation 2:[3]

$$DS_{GTAC} = \frac{(162 + 130.19 \times DS_{BGE}) \times N}{1400 - 151.63 \times N} \qquad (2)$$

where *N* is the nitrogen content measured by Vario EL Cube (Elementar, Germany), and 162, 130.19, and 151.63 were the molecular weights of the anhydroglucoside unit, BGE, and GTAC, respectively.

Preparation of lipase-immobilized StBG

By changing the DS of modified starch, its response temperature can be flexibly adjusted to meet the needs of different application environments. In this study, StBG-3 was used as an example to investigate its performance at room temperature (25 °C). Typically, 0.01 g of Candida antarctica lipase B (CALB) and 0.1 g of StBG-3 were each separately dispersed in 10 mL of Tris-HCl buffer (50 mM, pH 7.4). The two dispersions were then mixed to form colloidal particles dispersed in water to obtain lipase-immobilised StBG.

Biocatalytic performance of Pickering emulsions stabilized by lipase-immobilized StBG

Biocatalytic performance of the Pickering emulsions stabilized by lipase-immobilised StBG was evaluated by the hydrolysis of hexyl hexanoate. The lipase-immobilised StBG suspension (2 mL) and hexane solution containing 100 mM hexyl hexanoate (8 mL) were used as the water and organic phases, respectively, and a stable high-internal-phase emulsion (HIPE) was obtained by homogenization with an IKA Ultra Turrax T18 homogeniser operating at 10000 rpm for 120 s. Subsequently, the emulsion was transferred to a 25 °C incubator for static hydrolysis. To determine the conversion of the substrate, 100 μL of each emulsion was collected at different times and analysed by gas chromatography (GC). Here, conversion (%) was defined as the ratio of the quantity of substrate consumed to the quantity of substrate added. One unit of lipase activity (*U*) was defined as the quantity of lipase required to convert 1 μmol of the substrate within 1 min. The specific activity of lipase was measured within 30 min. All measurements were repeated thrice. Additionally, four other systems were examined as references. First, free lipase was dissolved in water and mixed with eight times the volume of the hexane-containing substrate by continuous stirring for biphase interface catalysis. Second, a free lipase solution with an equal volume of hexane was added to the conventional emulsions stabilized with the anionic surfactant sodium dodecyl sulfate. Third, lipase-immobilized starch particles (lipase@StBGs) were used to stabilize ordinary Pickering emulsions at a water/hexane ratio of 5:5. Fourth, free lipase was dissolved in the aqueous phase of Pickering HIPEs stabilized by StB (no immobilized enzyme). Except for the system of free lipase, which required stirring, all other systems were left at room temperature for the reaction, and the concentrations of lipase and substrate were the same.

Biocatalytic performance of recycled Pickering emulsions stabilized by lipase-immobilized StBG

The hydrolysis of hexyl hexanoate was used to investigate the biocatalytic performance of recycled Pickering emulsions stabilized by lipase-immobilised StBG. When the hydrolysis reached equilibrium (6 h) at 25 °C, the emulsion was transferred to a water bath at 50 °C. After complete demulsification, the upper organic phase containing the product and residual substrate was removed directly with a pipette. The new organic phase was then added and cooled to 25 °C before re-emulsification using a homogenizer. Subsequently, the emulsion was placed in a 25 °C incubator and the next reaction cycle was initiated. At the end of each cycle, the obtained organic phase was analyzed by GC to measure the conversion.

Morphology

The microscopic appearances of StBG and lipase-immobilised StBG were observed using field emission scanning electron microscopy using a Merlin FE-SEM (ZEISS, Oberkochen, Germany). The sample suspensions were dropped on a silica surface at a certain temperature and freeze-dried immediately after soaking in liquid nitrogen; thereafter, a thin layer of conductive platinum was deposited using an ion-sputter coater. Optical microscopy and confocal laser scanning microscopy images were captured using a Nikon K550L and A1 confocal microscope (Nikon Imaging Japan Inc., Tokyo, Japan). Labelled lipases were obtained via covalent bonding with FITC. Briefly, 5 mL of CALB solution (1 mg/mL) was mixed with 5 mg of FITC and stirred at room temperature for 6 h. Excess FITC was removed by dialysis in deionized water.

Zeta potentials and size measurements

The zeta potentials and sizes of the StBG particles were measured using a Brookhaven nanoparticle size and zeta potential analyzer (Holtsville, NY, USA). Specifically, the zeta potentials of 0.5 wt% StBG-3 and 0.5 mg/mL CALB at a certain pH were measured using electrophoretic light scattering at 25 °C. The sizes of 0.5 wt% StBG-3 and lipase immobilised StBG-3 at different temperatures were obtained by dynamic light scattering (DLS).

Lower critical solution temperature (LCST)

To evaluate the temperature sensitivity of StBG with different substitution degrees, the UV transmittance of the StBG aqueous dispersions at different temperatures was characterized using a Lambda 35 UV–vis spectrophotometre (PerkinElmer, Waltham, MA, USA). All sample concentrations were 0.5 wt%, and the transmittance of the dispersions at 500 nm was recorded at a heating rate of 1 °C/min from 0 to 80 °C. Here, LCST is defined as the temperature corresponding to 50% transmittance of the samples during the heating process.

Interfacial tension measurement

To investigate the effect of temperature on the interfacial activity of the samples, the interfacial tension of StBG was measured using a DSA100 drop-shaped analyser (Krüss GmbH, Hamburg, Germany). In particular, the dynamic interfacial tension of StBG-3 (0.5 wt%) at the water-hexane interface was measured using the pendant drop method with a constant drop volume of 10 µL at different temperatures. The evolution of interfacial tension as a function of time was determined by analyzing the shape of the pendant aqueous drop using axisymmetric drop shape analysis software developed by Krüss. A Young–Laplace fit was used for each drop to derive the interfacial tension. A pendant drop formed at the tip of the needle inside a glass cuvette filled with hexane. The evolution of the drop was recorded using a camera over the course of 10 min until adsorption equilibrium was reached.

Additional characterisation

^{1}H NMR spectra of the samples (dissolved in DMSO-d6) were measured using an AV400 spectrometer (600 MHz, Bruker, Ettlingen, Germany) at 600 M and 25 °C. Fourier-transform infrared (FT-IR) spectra were obtained using the KBr pellet method with a PerkinElmer Spectrum 100 spectrophotometre. Rheological analysis of the HIPEs was performed at room temperature using an AR 2000ex rheometer (TA Instruments, New Castle, DE, USA) in the plate-plate mode at various frequencies (0.1 to 10 Hz).

Results and Discussion

Table S1. Characterisation of StBG.

Sample	AGU:BGE:GTAC[a]	Area of protons (S)[b]		Nitrogen Content (%)[c]	DS_{BGE}[d]	DS_{GTAC}[e]	LCST (°C)[f]
		5.8-4.4 ppm (OH-2,3,6,8,15; H-1)	0.87 ppm (H-13)				
StBG-1	1:2:0.025	1	1.41	0.09	1.88	0.022	23.03
StBG-2	1:2:0.050	1	1.38	0.09	1.84	0.022	27.33
StBG-3	1:2:0.075	1	1.14	0.15	1.52	0.034	31.25
StBG-4	1:2:0.100	1	1.12	0.19	1.49	0.043	37.01
StBG-5	1:2:0.125	1	0.98	0.24	1.31	0.051	40.34
StBG-6	1:2:0.150	1	0.96	0.32	1.27	0.068	43.57

[a]Molar material ratio for starch etherification based on the quantity of anhydroglucose units contained in starch.

[b]Obtained by ^{1}HMR spectroscopy; for convenience of calculation, the area of the OH-2,3,6,8,15 and H-1 protons was set to 1.

[c]Measured using a Vario EL Cube.

[d]Determined by ^{1}H NMR spectroscopy according to Equation 1.

[e]Calculated from nitrogen content according to Equation 2.

[f]Defined as the temperature corresponding to 50% transmittance of the samples during the heating process and characterised by UV–vis spectrophotometry at 500 nm.

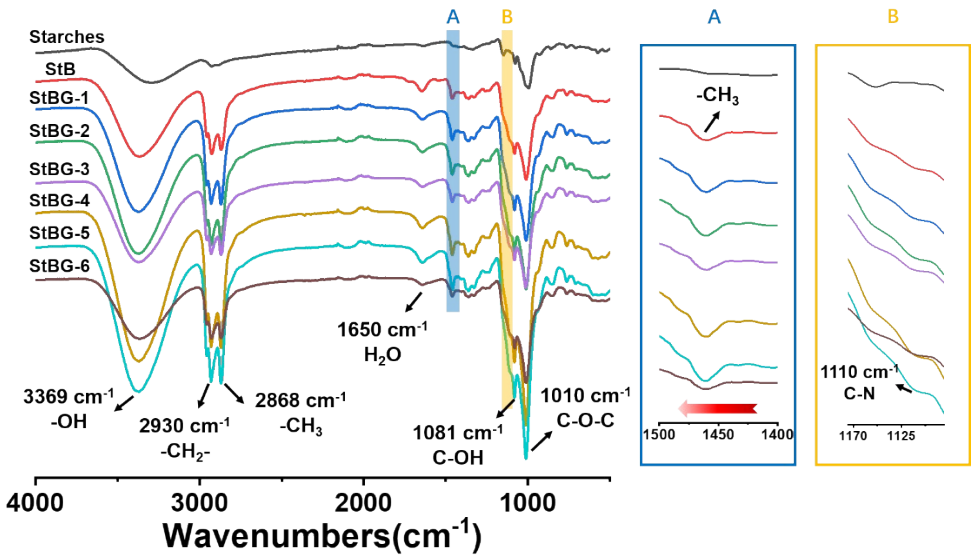

Figure S1. FT-IR spectroscopy of starches St-BGE and StBG-n modified with the same amount of BGE. In addition, StBG-n were also modified with GTAC and named StBG-1 to StBG-6 according to the quantity of bound GTAC. (A) and (B) magnified versions of the blue and yellow areas, respectively, of the main spectra.

FT-IR spectra of the samples revealed that BGE and GTAC were successfully bonded to starch molecules. Specifically, compared to the unmodified starch, new peaks appeared at 2868 and 1460 cm^{-1}, which could be attributed to the stretching vibration of the $-CH_3$ group on the substituted BGE and GTAC (Figure S1). Notably, the peak at 1460 cm^{-1} shifted towards higher frequencies with increasing DS of GTAC, confirming the presence of quaternary ammonium groups. The C-N stretching vibration peak at 1110 cm^{-1} and the shift of the methyl stretching vibration peak revealed that quaternary amine groups were introduced into the starch molecules (Figure S1B). In addition, the absorption bands at 3369, 2930, 1081, and 1010 cm^{-1} were assigned to the stretching vibrations of the -OH, $-CH_2-$, C-OH, and C-O-C groups, respectively.

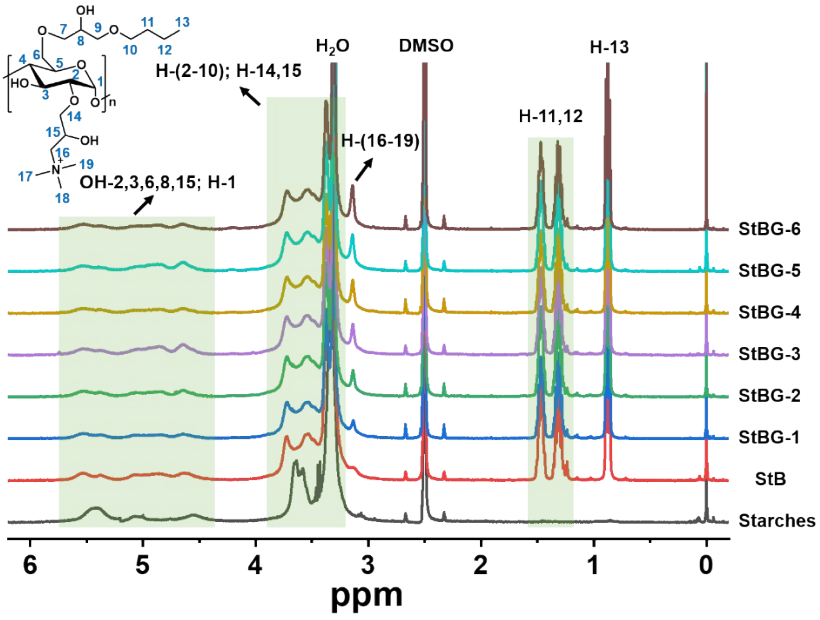

Figure S2. ^1H NMR spectroscopy of starches St-BGE and StBG-n modified with the same amount of BGE. In addition, StBG-n starches were also modified with GTAC and named StBG-1 to StBG-6 according to the quantity of bound GTAC.

The composition of the obtained products was confirmed by ^1H NMR spectroscopy. Peaks arising from anhydroglucose in the starches and modified starches were assigned according to literature.[2] Typically, the peaks in the 4.4–5.8 ppm range correspond to OH-2, 3, 6, and H-1 of the anhydroglucose units (AGUs) on the starch skeleton and OH-8 and 15 of the substituent. Chemical shifts of the six remaining protons of the AGUs (H-2, 3, 4, 5, and 6), seven protons of the BGE substituent (-O-CH2-CHOH-CH2-O-CH2-, i.e. H-7, 8, 9, and 10), and three protons of the GTAC substituent (-O-CH2-CHOH-, i.e. H-14 and H-15) were observed between 3.2 and 4.0 ppm. There was a new sharp peak at 3.1 ppm, compared to the starches and St-BGE, which could be attributed to the protons on the quaternary amine (H-16, 17, 18, and 19). The peaks at 1.3 and 1.45 ppm corresponded to the methylene groups of the butyl portion of the substituent (H-11, H-12). The characteristic triplet at 0.75 ppm was attributed to the methyl substituent. The peaks at 3.3 and 2.5 ppm correspond to H_2O and dimethyl sulfoxide (DMSO), respectively.

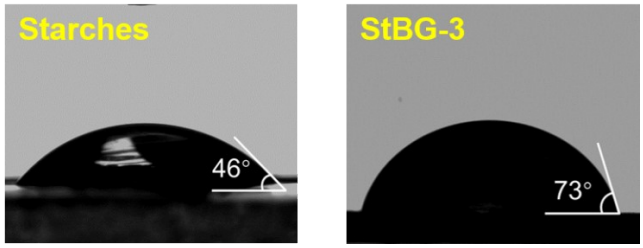

Figure S3. The three-phase contact angles of original starch and modified starch (StBG-3).

The three-phase contact angles of original starch and modified starch (StBG-3) were shown in Figure S3. Specifically, original starch was very hydrophilic with a water contact angle of 46°. After modification, the water contact angle of StBG-3 increased to 73°, indicating that the introduction of BGE and GTAC did improve the hydrophobicity of starch.

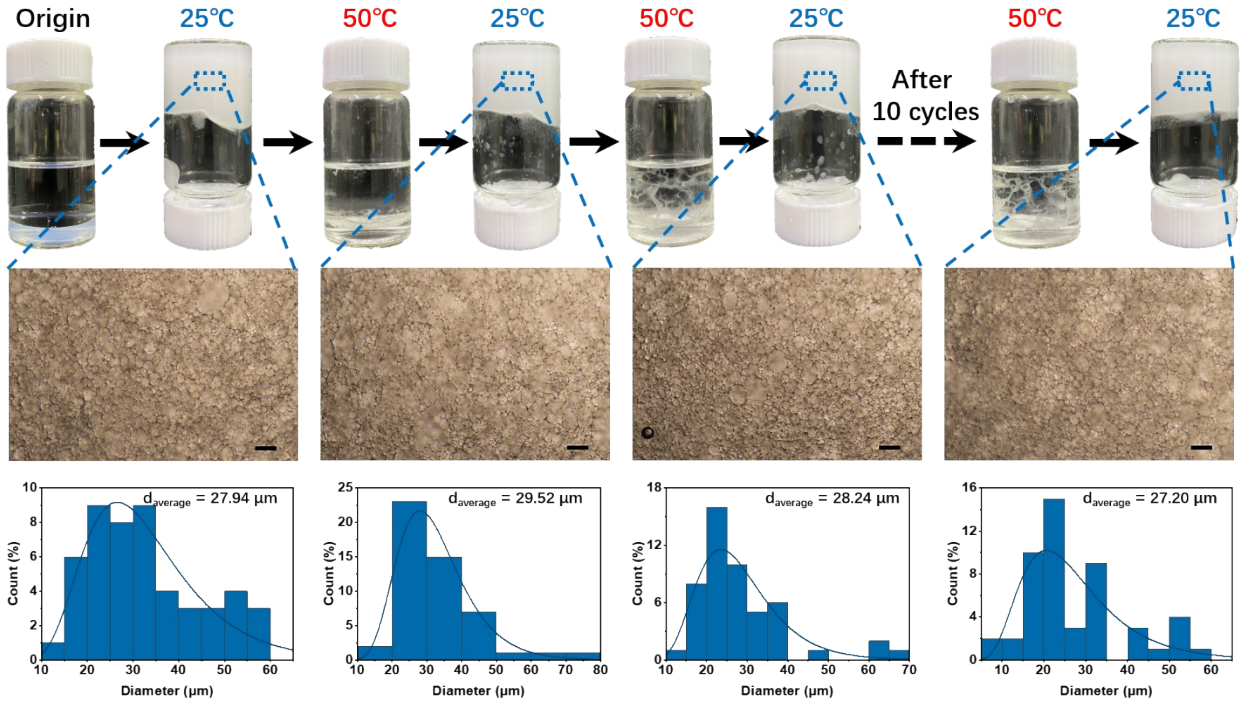

Figure S4. Photographs of the reversible emulsification/demulsification process of HIPEs stabilised by StBG-3 (upper). Microscopic photographs of the emulsion obtained after each cycle; the black scale bar corresponds to 100 μm (middle). The particle size distribution and average diameter of emulsion droplets obtained after each cycle (lower). The emulsions in this experiment were obtained at a water/hexane ratio of 2:8 with an emulsifier concentration of 0.5 wt% and re-homogenisation at 1000 rpm for 120 s after each cycle.

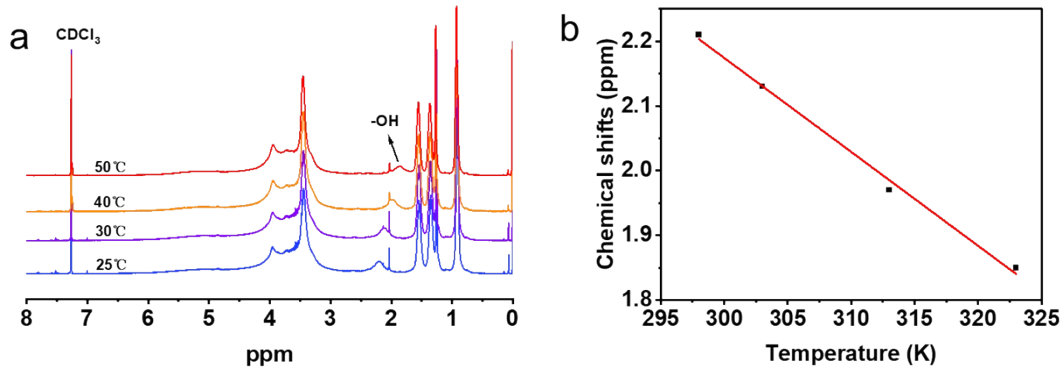

Figure S5. (a) ^{1}H NMR spectra of modified starch (StBG-3) at different temperatures. And the ^{1}H NMR spectra of the samples (dissolved in $CDCl_3$) were measured using an AV400 spectrometer (600 MHz, Bruker, Ettlingen, Germany) at 600 M. (b) The chemical shift of hydroxyl proton peak on the introduced side chain group (BGE) as a function of temperature.

^{1}H NMR spectra is an important method to study hydrogen bonds, and $CDCl_3$ is the most commonly used solvent for studying intermolecular hydrogen bonds that are susceptible to polar solvents (such as DMSO-d6).[4] Studies have shown that the chemical shift of intermolecular hydrogen bond varies greatly with temperature.[5, 6] As predicted, the chemical shift of the hydroxyl proton peak on the introduced side chain group (BGE) shown a strong temperature dependence. As shown in Figure S4a, the chemical shift of the hydroxyl proton peak was 2.21 ppm at 25 ° C, while when the temperature raised to 50 ° C, the chemical shift moved to the high field to 1.85 ppm. And its chemical shift with temperature was 14.51×10^{-3} ppm K^{-1} (Figure S4b), which proved that hydrogen bonds did exist and that they existed in the form of intermolecular hydrogen bonds (> 4.0×10^{-3} ppm K^{-1}),[5] i.e. the hydrogen bonds between the modified starch and the water molecules.

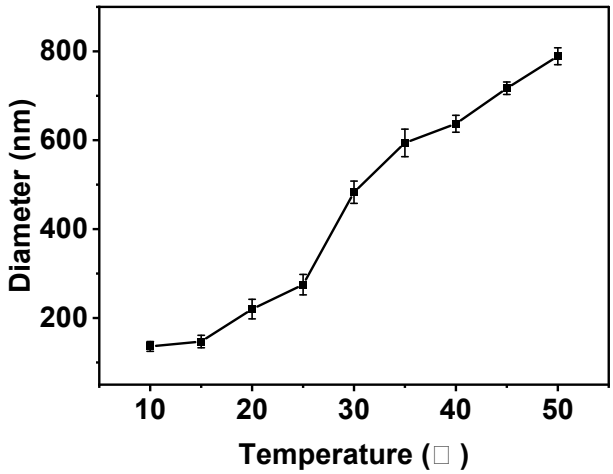

Figure S6. The DLS measurements of starch particles (StBG-3) in water dispersion at different temperature.

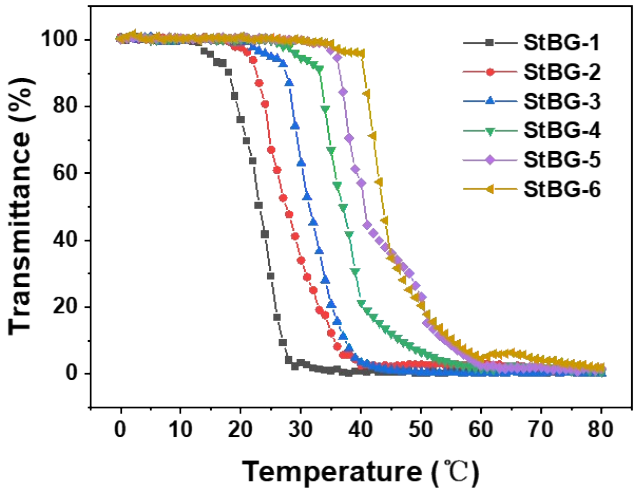

Figure S7. Transmittance changes for 0.5 wt% aqueous dispersions of StBG-n at 500 nm with a heating rate of 1 °C/min from 0 to 80 °C.

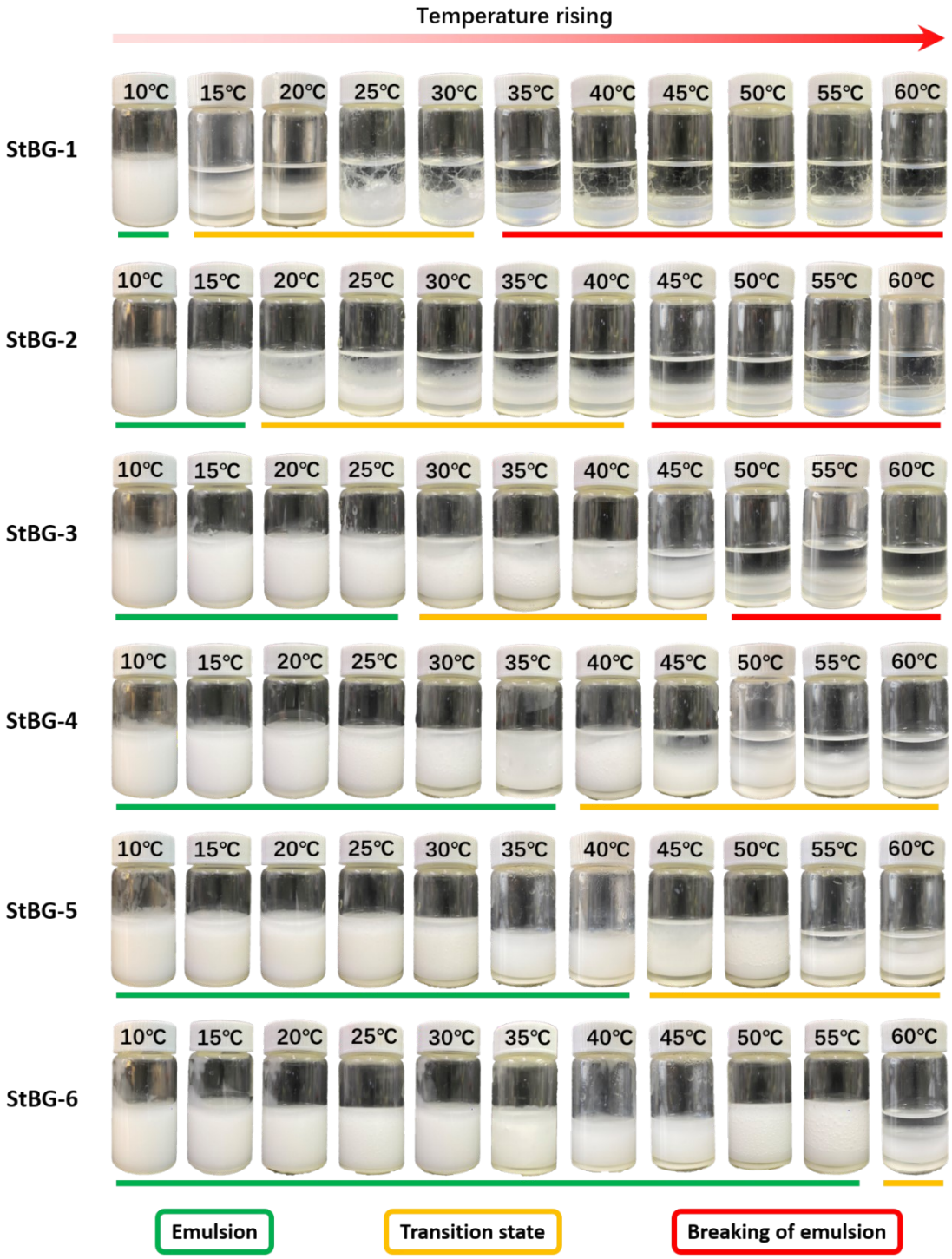

Figure S8. Photographs of emulsions stabilised by StBG-n at different temperatures. Except for the emulsifier, all other conditions are the same, including homogenisation rate (10000 rad/min, 120 s), emulsifier concentration (0.5 wt%), and water/organic (water/hexane) ratio (2:8).

Together with the results from Figure S4, it can be clearly seen that the resulting emulsion state corresponds strongly to the temperature responsiveness of the StBG. In particular, when the solution state of StBG did not change (T < LCST), the resulting emulsion remained stable. The emulsion began to partially demulsify when StBG was in the 'temperature window'. When StBG precipitated out of the solution, that is, when the transmittance was at the lowest value (T > LCST), the emulsion was completely demulsified. This indicates that the LCST can be used as a 'switching temperature' to accurately predict the emulsification and demulsification temperatures of emulsions. The temperature switch can be precisely regulated by adjusting the DS of StBG to meet the requirements of various practical environments.

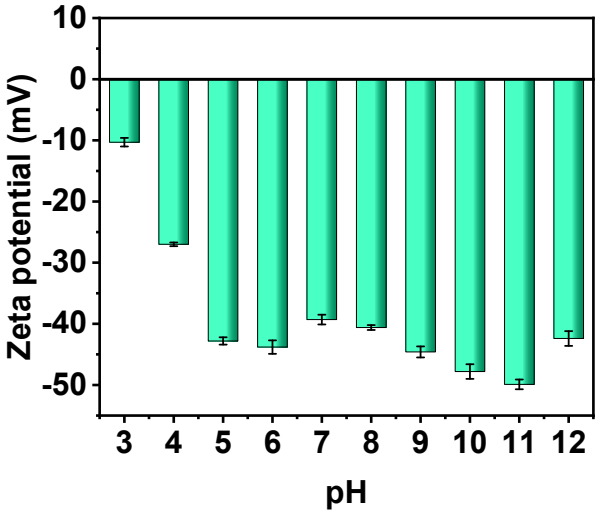

Figure S9. Zeta potential of StB with different pH values, as determined by electrophoretic light scattering. The concentration of samples was 0.5 wt%, and the measurement temperature was 25 °C.

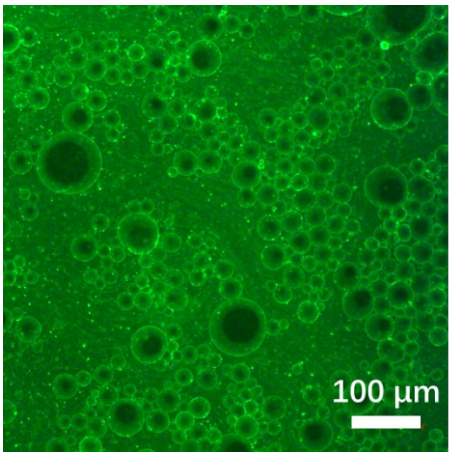

Figure S10. Confocal laser scanning microscopy image of emulsions stabilised by fluorescent-labelled lipase and StB. The water/organic ratio was 5:5. The labelled lipase was obtained by covalent bonding with FITC.

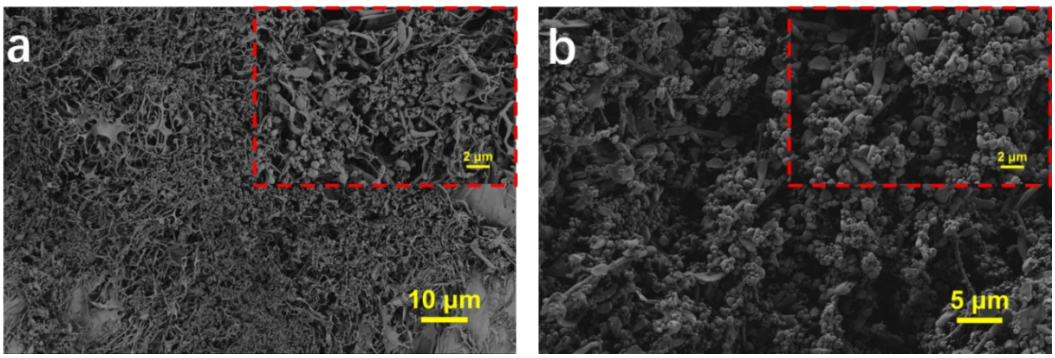

Figure S11. Scanning electron microscopy images of lipase-immobilised StBG-3 (lipase@StBG-3) at different temperatures: (a) 25 °C; (b) 50 °C. The insets are overviews at higher magnification.

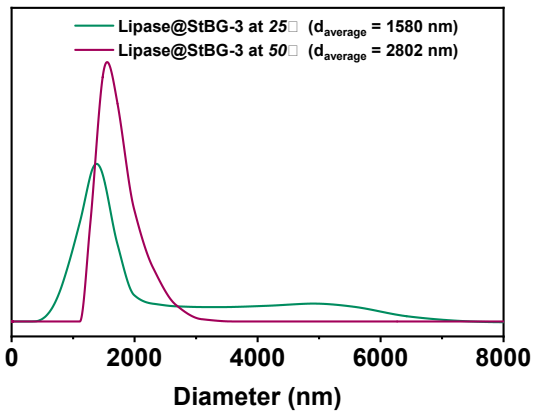

Figure S12. Size distributions of lipase-immobilised StBG-3 at temperatures below (25 °C) and above (50 °C) the LCST. The results were obtained by dynamic light scattering with 0.5 wt% sample concentrations.

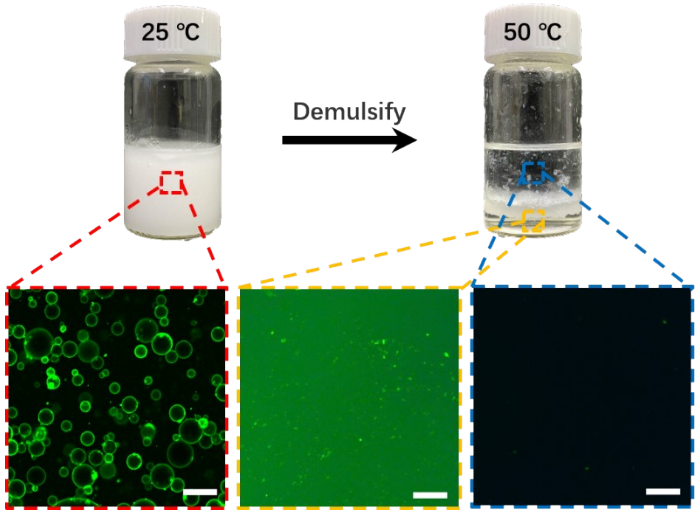

Figure S13. Confocal laser scanning microscopy images before and after demulsification: emulsion (red outline), water phase (yellow outline), and organic phase (blue outline). Scale bar: 50 μm.

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Author Contributions

Chao Wang: data curation, formal analysis, investigation and writing of the original draft—lead.
Hui Chi: formal analysis and project administration—supporting
Fan Zhang: formal analysis and investigation—supporting
Xinyue Wang: validation—supporting
Jiarui Wang: investigation—supporting
Hao Zhang: investigation—supporting
Ying Liu: validation—supporting
Xiaona Huang: formal analysis—supporting
Yungang Bai: funding acquisition, project administration, and validation—supporting
Kun Xu: funding acquisition, formal analysis, project administration, and validation—supporting
Pixin Wang: funding acquisition, formal analysis, and project administration—supporting