

Supporting Information

Pickering Emulsion-Enhanced Interfacial Biocatalysis: Tailored Alginate Microparticles Act as Particulate Emulsifier and Enzyme Carrier

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Experimental

1. Materials. Lipase from *Candida sp.* expressed in *Aspergillus niger* and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich. Hexanoic acid, 1-hexanol, hexyl hexanoate and Nile red were purchased from Aladdin Industrial Corporation (Shanghai, China). Trichlorohexylsilane (C₆H₁₃Cl₃Si), trichlorooctylsilane (C₈H₁₇Cl₃Si) and trichlorodecylsilane (C₁₀H₂₁Cl₃Si) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Titanium dioxide (TiO₂) nanoparticles with a diameter of 25 nm and a purity of 99.8% were purchased from Chengdu Huaxia Chemical Reagent Co. Ltd. (Chengdu, China). Sodium alginate, paraffin oil, anhydrous calcium chloride, hydrochloric acid, Span-80, hexane, octane, anhydrous ethanol and hydrogen peroxide (H₂O₂, 30 wt.%) were obtained from Kelong Chemical Reagents Co. Ltd. (Chengdu, China). 2-Amino-2-(hydroxymethyl)-propane-1, 3-diol (Tris) was purchased from BioFroxx (Germany). Deionized water was utilized throughout the study. All the chemicals were of analytical grade.

2. Surface wettability modification of TiO₂ nanoparticles. To obtain the appropriate surface wettability of TiO₂ nanoparticles, the following experiments were carried out. Firstly, 0.6 g of purchased TiO₂ nanoparticles were added to 10 mL of 30 wt.% hydrogen peroxide solution, stirred at 800 rpm in the dark for 4 h and subsequently centrifuged at 15,000 rpm to obtain a precipitate. The precipitate was then dried at 45 °C to obtain hydroxylated TiO₂ nanoparticles. Secondly, 0.4 g hydroxylated TiO₂ nanoparticles were added to 20 mL of hexane solution containing 0.5 mL trichlorohexylsilane (C6) or trichlorooctylsilane (C8) or trichlorodecylsilane (C10). The dispersion was stirred under sealed conditions for 4 h at 800 rpm and subsequently centrifuged at 15,000 rpm to remove unreacted reagent. The resulting silylated TiO₂ nanoparticles (s-TiO₂) were dried at 45 °C. The surface wettability of s-TiO₂ nanoparticles was quantitatively evaluated in section 8.

3. Preparation of enzyme-immobilized alginate microparticles with tailored wettability. The enzyme-immobilized alginate microparticles coated by s-TiO₂ nanoparticles of tailored wettability were prepared by a mild and simple modified emulsion gelation method.¹ The general procedure is as follows: Firstly, 5 mL aqueous stock solution of lipase from *Candida sp.* was diluted in 95 mL of Tris-HCl buffer (10 mM, pH 7.4). Secondly, sodium alginate lipase solution was prepared by dissolving 0.45 g sodium alginate in 30 mL of lipase solution obtained in the first step and was left quiescent overnight at 4 °C to eliminate air bubbles. Calcium chloride solution was prepared by dissolving 2.1 g anhydrous calcium chloride in 30 mL Tris-HCl buffer. The prepared sodium alginate lipase solution and calcium chloride solution were respectively added into 45 mL of paraffin oil containing 2.7 g Span 80 and 0.3 g s-TiO₂ nanoparticles with stirring at 800 rpm for 30 min yielding water-in-oil emulsion A (sodium alginate and lipase solution as inner phase) and B (calcium chloride solution as inner phase), see Scheme S1. The average diameter of both emulsions is 1-2 μm. Span 80 surfactant is there primarily to lower the oil-water interfacial tension enabling

the formation of small droplets and the TiO₂ particles co-adsorb at the interface (as in a Pickering emulsion).

Emulsion A was added into emulsion B, and the mixed emulsion was stirred at 800 rpm for 4 h to enable crosslinking yielding enzyme-immobilized Alg (E@Alg@s-TiO₂) microparticles. Subsequently, 30 mL anhydrous ethanol was added to the mixed emulsion to demulsify it with stirring at 800 rpm for 5 min. The precipitates were collected by centrifugation at 8,000 rpm for 5 min and washed with hexane three times, and then dried at 37 °C for 4 h. Finally, E@Alg@s-TiO₂ microparticles with diameters of 2-5 μm were obtained. The surface wettability of E@Alg@s-TiO₂ microparticles was quantitatively evaluated in section 8. The loading rate refers to the ratio of the immobilized lipase to the weight of Alg@C6-TiO₂. The immobilization efficiency refers to the ratio of the immobilized lipase to the initial introduced lipase in Tris-HCl solution. The concentration of lipase was determined by Bradford's method using Coomassie Brilliant Blue reagent.² The calculations of enzyme loading rate and immobilization efficiency were based on eqs. (1) and (2):

$$\text{Loading rate (mg g}^{-1}\text{)} = (m - C_1 V_1) / W \quad (1)$$

$$\text{Immobilization efficiency (\%)} = (m - C_1 V_1) / m \times 100\% \quad (2)$$

where m (mg) is the amount of lipase introduced into Tris-HCl solution, C_1 (mg mL⁻¹) and V_1 (mL) are the lipase concentration and the volume of supernatant, respectively and W (g) is the weight of Alg@C6-TiO₂.

For comparison, alginate microparticles coated with s-TiO₂ nanoparticles (Alg@s-TiO₂) and pure alginate microparticles were also prepared by the above experimental steps with some alterations. For Alg@s-TiO₂ microparticles, the sodium alginate solution did not contain lipase. For Alg microparticles, no s-TiO₂ nanoparticles were present in the oil phase and no lipase was present in the water phase.

4. Preparation of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles. In this experiment, a certain amount of E@Alg@s-TiO₂ microparticles of concentration between 0.5 and 2 wt.% (based on the water weight) as emulsifier was dispersed in hexane as the continuous phase with a vortex mixer; Tris-HCl buffer was used as the dispersed phase. The water phase was added to the oil phase (volume ratio 1:1), followed by shaking for 100 times per min for 2 min (see Movie S2). The emulsion type is evaluated in section 9.

5. Calculation of the increased interfacial area in the form of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles compared with the biphasic system. According to eqs. (3) - (9), we calculated the area of one droplet and multiplied it by the number of droplets. The number of droplets equals the total volume of water phase in all droplets divided by the volume of one droplet.

$$V_{\text{one droplet}} = \pi D^3 / 6 \quad (3)$$

$$V_{\text{water phase in all droplets}} = V_{\text{initial water}} \times (1 - \phi_0) \quad (4)$$

$$n = V_{\text{water phase in all droplets}} / V_{\text{one droplet}} \quad (5)$$

$$S_{\text{one droplet}} = \pi \times D^2 \quad (6)$$

$$S_{\text{all droplets}} = n \times S_{\text{one droplet}} \quad (7)$$

$$S_{\text{biphasic}} = l \times w \quad (8)$$

$$F = S_{\text{all droplets}} / S_{\text{biphasic}} \quad (9)$$

where $V_{\text{one droplet}}$ is the volume of one droplet, $V_{\text{water phase in all droplets}}$ is the total volume of water phase in all droplets, $V_{\text{initial water}}$ is the volume of the initial water, $S_{\text{one droplet}}$ is the surface area of one droplet, $S_{\text{all droplets}}$ is the total surface area of all droplets, S_{biphasic} is the interfacial area of the biphasic system, ϕ_0 is the volume of dispersed water relative to the volume of the initial water (see section 13), n and D are the number and average diameter of the droplets respectively, l and w are the length and width of the vessel with the oil-water biphasic system respectively, F is the factor representing the increased interfacial area in the form of droplets compared with the biphasic system.

6. Assessment of catalytic performance of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles. The catalytic performance of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles was investigated *via* the esterification of 1-hexanol and hexanoic acid in a water-hexane mixture. Typically, 8 mL of Pickering emulsion stabilized by 2 wt.% E@Alg@s-TiO₂ microparticles was prepared as described above, with 4 mL of substrate solution containing 400 mM 1-hexanol and 400 mM hexanoic acid in hexane being used as the continuous phase. As controls, free lipase in the biphasic water-hexane system and free lipase positioned in the inner phase of the Pickering emulsion stabilized by Alg@s-TiO₂ microparticles were also used to catalyze this esterification reaction. The reaction conditions of the controls were the same as those given for E@Alg@s-TiO₂, including substrate concentration, amount of lipase and oil:water ratio. All the esterification reactions were carried out on a rotating shaker (80 rpm) at 37 °C. To determine the concentration of product (hexyl hexanoate), 100 µL samples of oil phase were extracted at different reaction times (t), centrifuged (12,000 rpm, 5 min) and analysed by gas chromatography (GC).

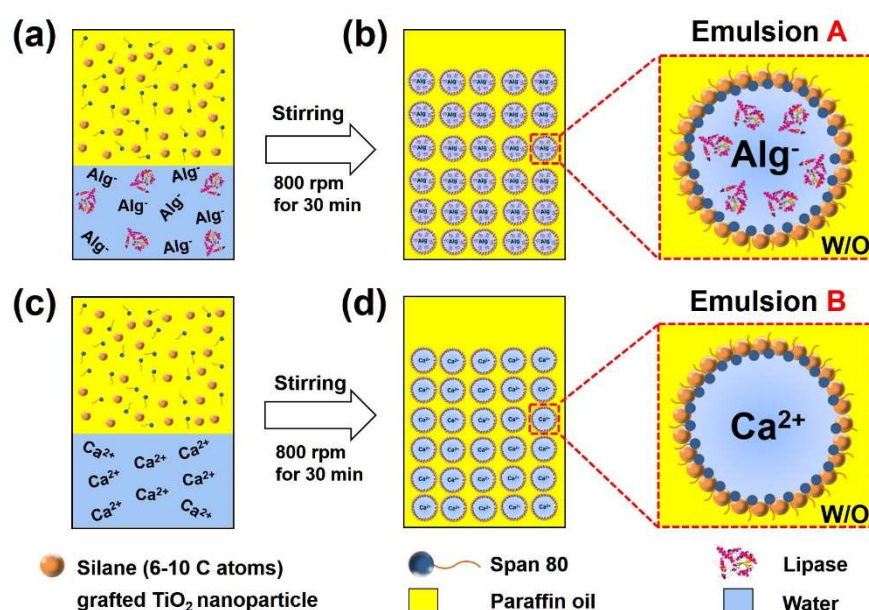
The concentrations of hexanoic acid, 1-hexanol and hexyl hexanoate were determined by using a GC system (9790 II, Zhejiang FULI, China) equipped with a flame ionization detector (FID) on a Kromat KB-5 column (30 m × 0.32 mm × 0.5 µm). Conversion (%) was defined as the ratio of the amount of product to the theoretical amount. One unit of lipase activity (U) was defined as 1 µmol product within 1 min. The relative activity (%) represents the ratio of residual enzymatic activity to enzymatic activity in the first reaction cycle. The specific activities (U mg⁻¹) of free lipase of the biphasic system, free lipase in the inner phase of an emulsion and lipase immobilized at the droplet interfaces of an emulsion were measured under the same conditions within 30 min. All the measurements were repeated three times.

7. Assessment of the re-usability of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles. The re-usability of Pickering emulsions stabilized by E@Alg@s-TiO₂ microparticles was investigated by using the esterification of 1-hexanol and hexanoic acid in a water-hexane mixture (24 h per cycle). After each reaction cycle, the oil phase containing the substrate/product was separated from the emulsion layer through gravitational sedimentation. Samples of oil phase were extracted and analysed by GC and the excess oil phase was then removed. Then the Pickering emulsions were carefully rinsed several times with fresh hexane to remove any residual substrate and product. A new volume of hexane and substrate was then introduced for the next reaction cycle.

8. Evaluation of the surface wettability of s-TiO₂ nanoparticles and Alg@s-TiO₂ microparticles. The equivalent three-phase contact angle of s-TiO₂ nanoparticles and Alg@s-TiO₂ microparticles at the oil-water interface were measured by using the dip-coating method. To obtain compact s-TiO₂ nanoparticle films on a glass slide (5 × 5 mm), the glass slide was dip-coated into a 1% (w/v) s-TiO₂ nanoparticle dispersion in hexane 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 nanoparticles, the above deposition process was repeated. Subsequently the glass slide was placed at the bottom of a transparent quartz vessel. Hexane was then poured into the vessel. A 3 μL water drop was carefully placed on the disk surface. The appearance of the water droplet on the substrate was photographed when the droplet was stationary. All of the contact angles measured through water are the arithmetic average of at least five measurements on the same sample. The evaluation of the surface wettability of Alg@s-TiO₂ microparticles is the same as that described above.

9. Characterization techniques. The surface chemistry of hydroxylated TiO₂ and s-TiO₂ nanoparticles was verified by Fourier transform infrared spectroscopy (FTIR, Prestige-21, Shimadzu, Japan). The samples were prepared by mixing the nanoparticles with KBr at 1:100 mass ratio and pressing. Dynamic light scattering from s-TiO₂ nanoparticles was measured by a Malvern Zetasizer Nano-ZS90 instrument (Malvern Instruments Ltd., UK). The samples were prepared by dispersing the s-TiO₂ nanoparticles in ethanol. The morphology of s-TiO₂ nanoparticles was investigated by field emission transmission electron microscopy (FE-TEM; JEM- 2100F, JEOL, Tokyo, Japan). The samples were prepared by dispersing in ethanol and coating on a carbon-coated copper grid. The grid was then allowed to dry before being imaged. The morphologies of alginate microparticles and Alg@s-TiO₂ microparticles were investigated *via* scanning electron microscopy (SEM, JEOL-JSM 7001F, Japan) equipped with energy dispersive spectrometry (EDS). The samples were freeze-dried and sputter-coated with gold for observation. Fluorescence images were obtained using a confocal laser scanning microscope (CLSM, Leica TCP SP5, Germany), exciting the blue fluorescent channel at 405 nm for TiO₂ nanoparticles, green fluorescent channel at 488 nm for FITC and red fluorescent channel at 543 nm for Nile red. Emulsion droplets were viewed with an optical microscope (Phoenix Co. Ltd, XSP-24, China) fitted with a Moticam 2000 camera. The pictures were captured using Motic Images Plus 2

software, then processed and analyzed by Image Pro Plus software. The arithmetic mean diameter is calculated by measuring the size of about 50 emulsion droplets.



Scheme S1 Formation of water-in-oil emulsion A (sodium alginate and lipase solution as inner phase) and B (calcium chloride solution as inner phase) from a biphasic system (a, c) yielding a Pickering emulsion (b, d).

Results and Discussion

10. Surface morphology and characterization of pure alginate microparticles.

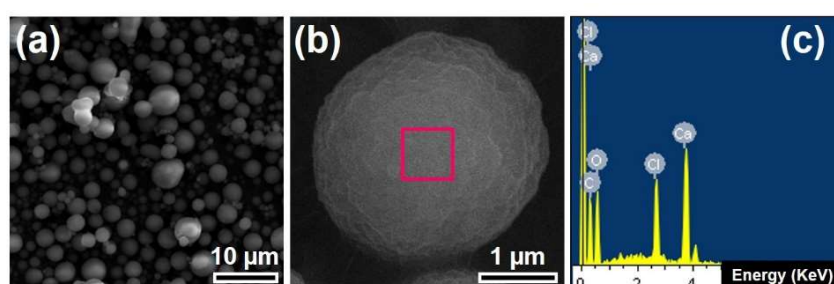


Fig. S1 SEM images (a) $\times 2000$ and (b) $\times 30000$ and (c) EDS element mapping for pure alginate microparticles.

11. Chemical characterization and size of s- TiO_2 nanoparticles.

To confirm the surface functional groups on hydroxylated and C6 silane grafted TiO_2 nanoparticles, FTIR spectroscopic analysis is performed. The spectra of hydroxylated TiO_2 nanoparticles and C6- TiO_2 nanoparticles (Fig. S2a and S2b) show the typical absorption bands of the Ti-OH group at $1650\ \text{cm}^{-1}$ which clearly demonstrates the

presence of hydroxyl groups. In Fig. S2b, the appearance of the two peaks at 2921 cm^{-1} and 2853 cm^{-1} are attributed to aliphatic $-\text{CH}_3$ and $-\text{CH}_2-$ stretching vibrations respectively, indicating the presence of hydrophobic alkyl chains on nanoparticles surfaces. Compared with Fig. S2a, the existence of two peaks at 1125 cm^{-1} and 1035 cm^{-1} due to Si-O-Ti stretching vibration (see Fig. S2b) confirms the grafting of silane groups on the TiO_2 surfaces.

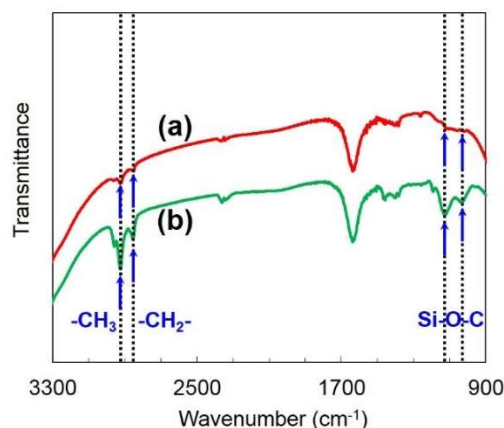


Fig. S2 FT-IR spectra of (a) hydroxylated TiO_2 and (b) C6- TiO_2 nanoparticles.

To investigate the diameter of s- TiO_2 nanoparticles, transmission electron microscopy (TEM) and dynamic light scattering (DLS) are performed. As shown in Fig. S3a, the TEM image shows C6- TiO_2 nanoparticles of diameter ranging from 20 to 30 nm, close to the diameter of the primary TiO_2 nanoparticles suggesting that the chemical surface modification has little influence on their size. A closer examination of the TEM image shows that some of the nanoparticles form randomly shaped aggregates. DLS measurements (Fig. S3b) reveal that the C6- TiO_2 nanoparticles have a uniform size distribution with an average diameter of 158 nm and a polydispersity index of 0.12. Moreover, SEM images (Fig. 2b) further reveal some aggregates of C6- TiO_2 nanoparticles with a diameter of approximately 150 nm on Alg@C6- TiO_2 microparticle surfaces.

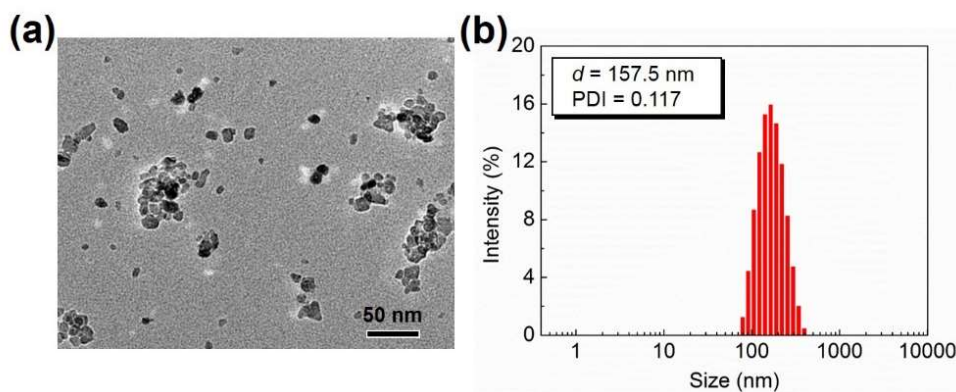


Fig. S3 (a) TEM image and (b) DLS size distributions of C6- TiO_2 nanoparticles.

12. Characterization of Pickering emulsion stabilized by E@Alg@s-TiO₂ microparticles.

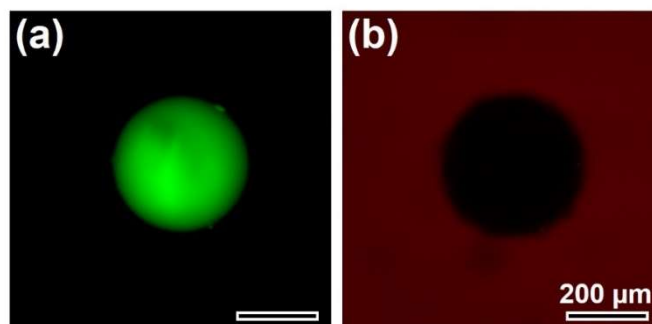


Fig. S4 CLSM images of a water-in-hexane Pickering emulsion stabilized by E@Alg@C6-TiO₂ microparticles with single channel emission of (a) FITC in water and (b) Nile red in hexane.

13. Influence of concentration of E@Alg@s-TiO₂ microparticles on Pickering emulsion stability.

To explore the influence of E@Alg@C6-TiO₂ microparticle concentration on the stabilization of water-in-hexane emulsions, the droplet diameter (D) and the fraction of dispersed water ϕ_0 (defined as the volume of dispersed water relative to the volume of the initial water) is used as indicators for coalescence. Fig. S5a shows the optical microscopy images of emulsions prepared with different concentrations of E@Alg@C6-TiO₂ microparticles (C). Increasing C from 0.5 to 2 wt.% leads to the formation of stable emulsions (Fig. S5a1-a4) of decreasing average diameter as illustrated in Fig. S5b, which can be explained by the theory of limited coalescence.³ Fig. S6 shows the size distributions of the Pickering emulsions with varied E@Alg@C6-TiO₂ microparticle concentration for Fig. S5(a). Macroscopic photos of emulsions (Fig. S5c) demonstrate the decrease of ϕ_0 with increasing particle concentration (Fig. S5d). At low particle concentration, the emulsion undergoes significant coalescence after preparation, leading to high ϕ_0 and relatively large drops. When the particle concentration reaches a certain value (2 wt.%), the emulsions become very stable against coalescence and the droplets remain constant of smallest diameter. Therefore, we choose 2 wt.% as the concentration of E@Alg@C6-TiO₂ microparticles in subsequent experiments.

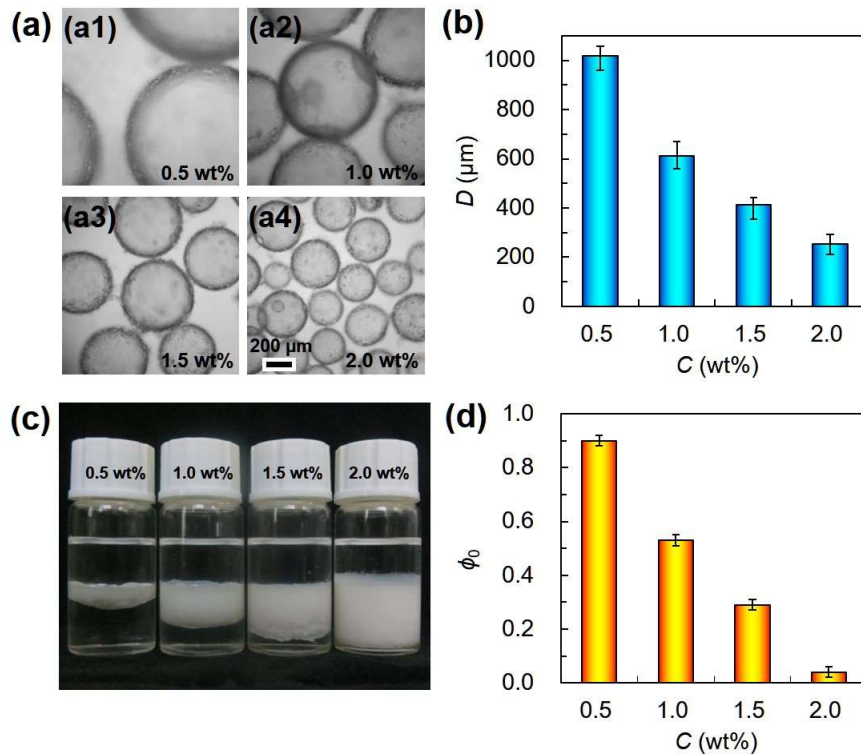


Fig. S5 (a) Optical microscopy images of water-in-hexane Pickering emulsion droplets with varied E@Alg@C6-TiO₂ microparticle concentration C (wt.%): (a1) 0.5, (a2) 1.0, (a3) 1.5 and (a4) 2. (b) Effect of C on the mean diameter of emulsion droplets. (c) Photos of vessels containing emulsions with varied E@Alg@C6-TiO₂ microparticle concentration. (d) Effect of C on ϕ_0 . Emulsions are 1 h old at room temperature.

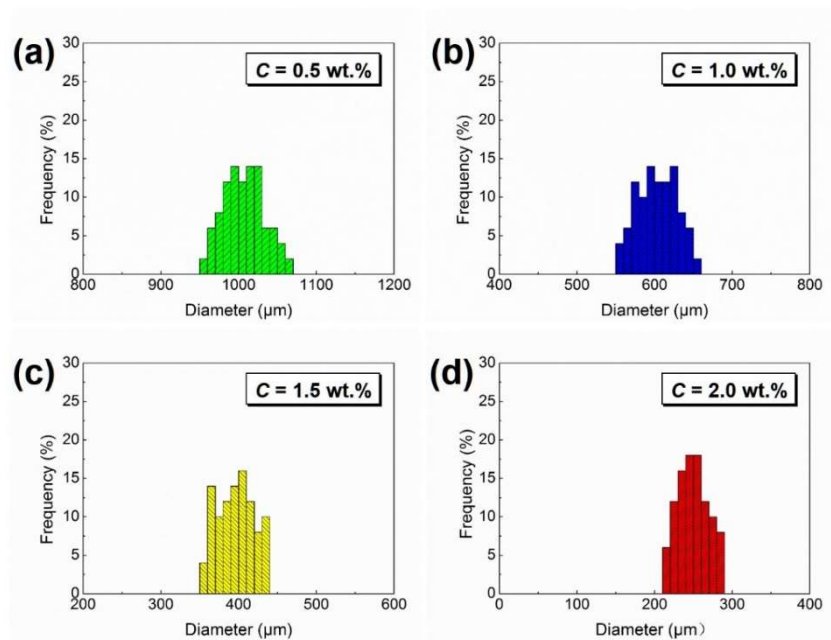


Fig. S6 Size distributions of the Pickering emulsions with varied E@Alg@C6-TiO₂ microparticle concentration of Fig. S5(a).

14. Influence of storage time on the stability of Pickering emulsion stabilized by E@Alg@s-TiO₂ microparticles.

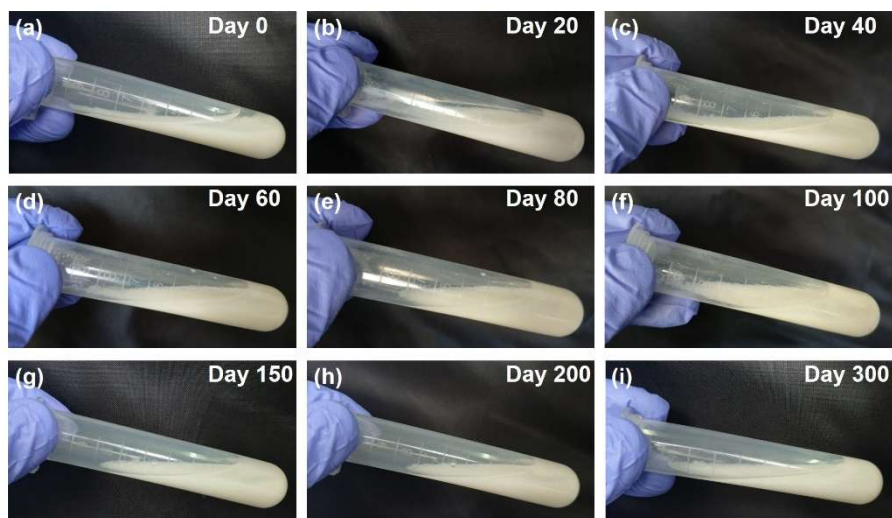


Fig. S7 Stability assay of the as-prepared water-in-hexane Pickering emulsions stabilized by E@Alg@C6-TiO₂ microparticles at different times.

References

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