Supporting Information

Polymer-Protein Conjugate Particles with Biocatalytic Activity for Stabilization of Water-in-Water Emulsions

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Experimental

Materials

For the preparation of ATPS, dextran (500 kDa) was obtained from Sigma and PEG (8 kDa) was from Chengdu Kelong Chemical Reagents Co., Ltd. BSA (66 kDa) was purchased from Shanghai Boao Biotech Co., Ltd. mPEG-ALD (Xi'an Ruixi Biological Technology Co., Ltd., 5 kDa) was used for the modification of the protein. BSA labelled with the fluorophore fluorescein isothiocyanate (FITC-BSA) was purchased from Sigma-Aldrich. Sodium cyanoborohydride was bought from Kelong Chemical Reagents Co., Ltd. (Chengdu, China) and was used as a reducing agent. Urease (U1500-20KU) was purchased from Sigma-Aldrich. It was used without further purification and the major protein form has a molecular mass of between 440 and 480 kDa. Urea was supplied from Hengxing Chemical Reagents Co., Ltd. (Tianjin, China). The pH was set by adding aliquots of 0.1 M NaOH or HCl. Deionized water (18.2 MΩ, Milli-Q, Millipore) was utilized in the experiments. All of the chemicals were of analytical grade and used without further purification.

Methods and Techniques

Synthesis of mPEG-protein conjugate particles

mPEG-BSA conjugate particles were prepared using a Schiff base reaction. A solution of BSA (50 mg, 0.76 μmol) at 10 mg/mL in deionized water, pH 5.0 (near the PI of BSA, pH 4.7)[1] at 25 ^{o}C was added to a beaker containing mPEG-ALD (50 mg, 10 μmol). Stirring was required until the solution became transparent. The reaction solution was sealed and maintained at 25 ^{o}C without stirring or shaking. After reacting for 48 h, the products were

dialyzed in a dialysis tube (molecular cut-off 8 kDa) for three days to remove the remaining mPEG-ALD. The products were subsequently dried at 37 ^{o}C to obtain mPEG-BSA conjugate particles. The preparation of mPEG-BSA-FITC conjugate particles and mPEG-urease conjugate particles was almost the same as above except the former particles are prepared and stored in the dark at 25 ^{o}C. Afterwards, the mPEG-urease conjugate particles are heated to 100 ^{o}C for two hours in order to get heated-denatured mPEG-BSA conjugate particles.

Preparation of water-in-water emulsions

A stock ATPS was prepared by mixing the required amounts of PEG, dextran and deionized water in a glass container and stirring until dissolved. The mixture was allowed to stand overnight. When fully settled, both aqueous phases appear clear with the upper phase being PEG-rich and the lower phase being Dex-rich. FITC-BSA (10 ppm) was used for labelling the dextran solution. mPEG-BSA-FITC conjugate particles (20 ppm) as the label of mPEG-BSA conjugate particles were added to the ATPS before emulsification. The mPEG-protein conjugate particles (0.3-0.6 wt.%) were added to the ATPS, followed by homogeneous emulsification at 21,000 rpm for 1 min using a homogenizer (S10, NingBo Scientz Biotechnology Co., Ltd., Ningbo, China) to obtain w/w emulsions.

Evaluation of wettability of mPEG-protein conjugate particles

In order to obtain compact mPEG-protein conjugate particle films on a glass slide (5 × 5 mm), the glass slide was dip-coated into a 2% (w/v) mPEG-protein conjugate particle dispersion using a withdrawal speed of 14 cm/min. The treated glass slide was dried at 38 ^{o}C for 30 min. As a result, mPEG-protein conjugate particles were deposited on the slide. In

order to ensure the glass slide was fully covered by particles, the layer with uniform and compact mPEG-protein conjugate particles deposited on surfaces is obtained by repeating the deposition process (see Figure S1). The dried glass substrate was placed at the bottom of a transparent quartz cuvette. Pre-equilibrated ATPS samples from mixing 8.5 wt.% PEG and 8 wt.% dextran were prepared. A volume of the PEG-rich phase was poured into the vessel and then a 0.7 μL drop of the dextran-rich phase (with 0.05 wt.% FITC-BSA) was formed on the mPEG-protein conjugate particle film. The appearance of the dextran droplet was photographed immediately. The three-phase advancing contact angles (θ) are the arithmetic mean of at least five repeat experiments on the same glass sample. The mesurement procedure for BSA was the same as that described above.

Study of biocatalytic activity of mPEG-urease conjugate particles

In order to investigate the biocatalytic activity of mPEG-urease conjugate particles, the experiments were carried out as follows. Two samples of the prepared ATPS (C_{PEG} = 8.5 wt.%, $C_{dextran}$ = 8 wt.%) were poured into two vials of 25 mL respectively. To one was added 5.2×10^{-3} μmol mL^{-1} urease and solid heated-denatured mPEG-urease conjugate particles as a control, and to the other was added mPEG-urease conjugate particles followed by homogeneous emulsification at 21,000 rpm for 1 min using the homogenizer. The mPEG-urease conjugate particles were synthesized using 5.2×10^{-3} μmol mL^{-1} urease and 0.5 μmol mL^{-1} mPEG. Finally, 20 mg mL^{-1} urea was added to the above two vials respectively and they were immediately shaken vigorously by hand. The enzymatic reaction happens at once. Urease can catalyze the hydrolysis of urea to ammonium and carbonate ions in water. The

conductivity of these ions was determined as a function of reaction time (Mettler-Toledo Instruments Co., Greifensee, Switzerland). The enzymatic reaction mostly occurs within droplets (containing urease) or at the interface of emulsion droplets (containing mPEG-urease conjugate particles). The ammonium and carbonate ions can diffuse from the dispersed phase or the interface of w/w emulsions to the outer aqueous phase.

Characterization techniques

Fourier transform infrared (FTIR, IR Prestige-21, Shimadzu, Japan) spectroscopy was used to verify the surface chemistry of mPEG-protein conjugate particles during the preparation process. The FTIR specimens were prepared using the KBr disc technique. ^{1}H-NMR experiments were performed on a Varian 400 NMR spectrometer. The surface morphology of mPEG-protein conjugate particles was examined by field emission scanning electron microscopy (SEM, JSM-7001F, JEOL, Japan) with an accelerating voltage of 15 kV. The particle diameter and zeta potential of the mPEG-protein conjugate particles were determined using a Malvern Zetasizer Nano-ZS90 instrument. The measured electrophoretic mobilities are converted into zeta potentials using the Smoluchowski equation. The zeta potential of mPEG-protein conjugate particles was measured in deionized water. The zeta potential of the particles was measured as a function of pH. The microstructure of mPEG-protein conjugate particle-stabilized water-in-water emulsions was observed with a fluorescence microscope (Th4-200, Olympus Corporation). The emulsion droplet size distributions were examined by optical microscopy after the emulsions had been equilibrated at 25 °C for 1 h. All the droplets were viewed with an XSP-24 research microscope (Phoenix

Co. Ltd, China) fitted with a Moticam 2000 camera. The images were captured using Motic Images Plus 2.0 software, then processed and analyzed by Image Pro Plus software.

Supplementary Data

Average particle size as a function of the reaction time

Table S1. Diameter of mPEG-protein conjugate particles determined by dynamic light scattering (DLS) for different Schiff base reaction times.

Time/hr	24	48	72
Average particle diameter/nm	157	306	374

SEM of the glass slide after particle deposition

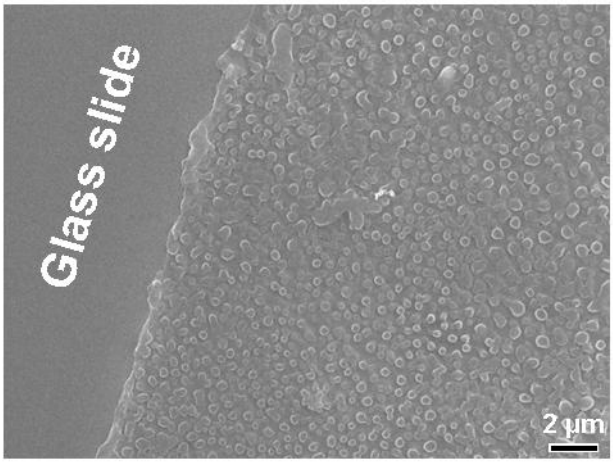

Figure S1. SEM image of the glass slide after deposition of mPEG-protein conjugate particles.

FT-IR spectra

The FTIR spectra of BSA and mPEG-BSA conjugate particles in the range of 3600-400 cm^{-1} are shown in Figure S2. The spectrum of BSA and mPEG-BSA conjugate particles are pretty similar. The spectrum of native BSA (Figure S2a) shows the typical absorption bands

of C=O at 1630-1690 cm^{-1}. Comparing Figure S2a and Figure S2b, the peaks of PEG-BSA conjugate particles (Figure S2b) shows a remarkable increase in intensity at 1640-1690 cm^{-1}, which demonstrates the typical absorption bands of a double bond between carbon and nitrogen. In addition, the appearance of the peaks at 2921-2853 cm^{-1} and 1035-1125 cm^{-1} are attributed to aliphatic $-CH_3$ and C-O-C stretching vibrations respectively which are the specific groups in mPEG-ALD. However, there is no new absorption peak of the aldehyde group at 1720-1740 cm^{-1} in the spectrum of mPEG-BSA conjugate particles, suggesting that the mPEG-ALD is completely grafted onto the native BSA surface.

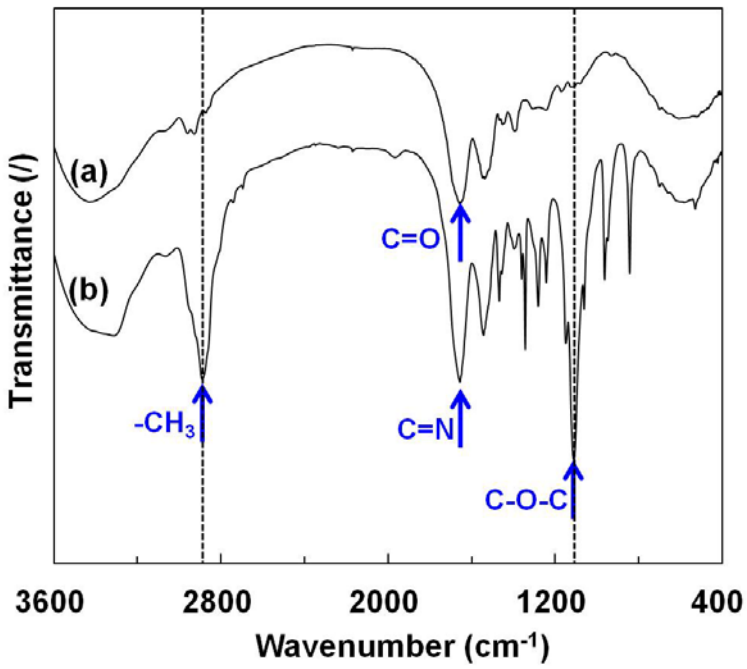

Figure S2. FT-IR spectrum of (a) BSA and (b) mPEG-BSA conjugate particles.

^{1}H NMR spectra

Further, synthesis of the mPEG-BSA conjugate particles is also confirmed by ^{1}H NMR spectra as given in Figure S3. In Figure S3a, the resonances at 1.26, 2.24, 3.34 and 3.39 ppm

are ascribed to BSA. The ^{1}H NMR spectrum of mPEG-ALD in Figure S3b shows the characteristic resonances at 3.72 and 8.46 ppm are separately assigned to the O-CH_2CH_2-O and CHO structure. In the ^{1}H NMR spectrum of mPEG-BSA conjugate particles (Figure S3c), the characteristic resonances at 1.26, 2.24, 3.34 and 3.39 ppm of BSA and 3.72 ppm of mPEG-ALD are retained, while the resonance of CHO at 8.46 ppm disappears. These all demonstrate that mPEG is successfully grafted onto BSA.

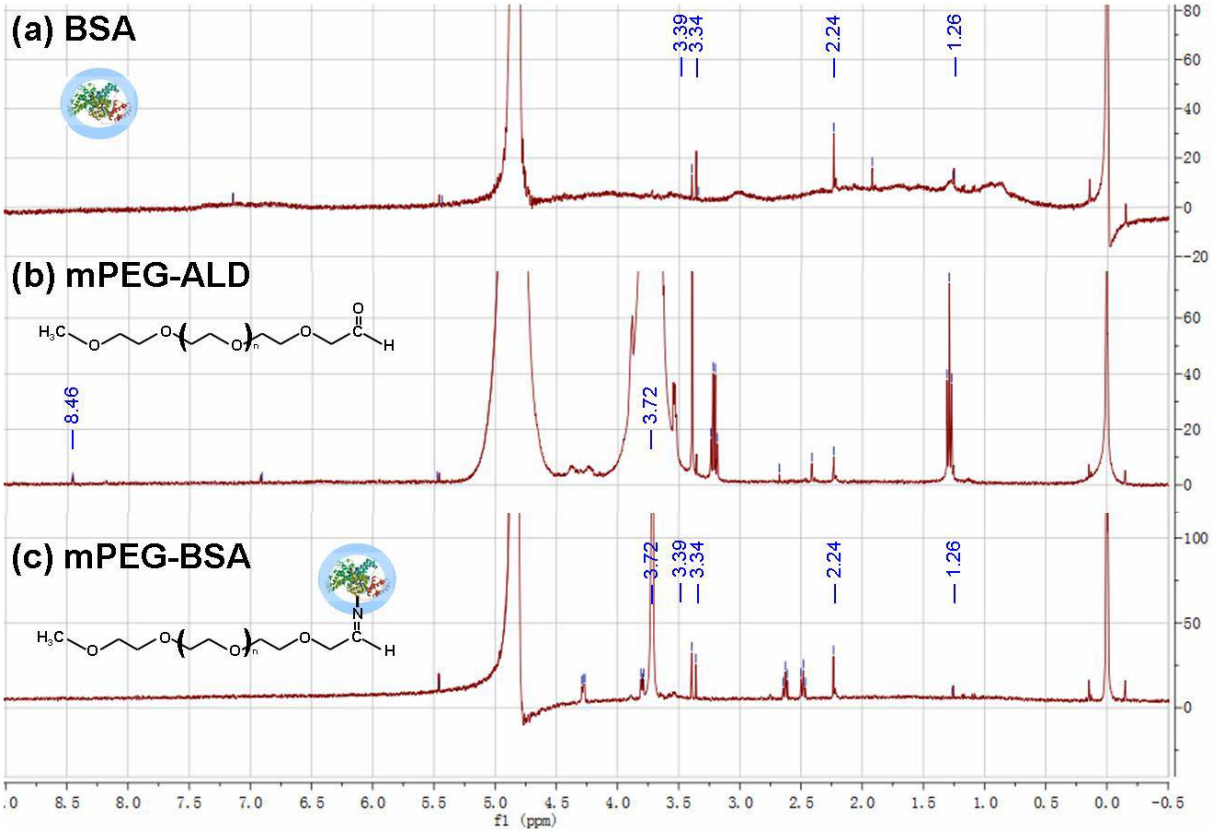

Figure S3. ^{1}H NMR spectrum of (a) BSA, (b) mPEG-ALD and (c) mPEG-BSA conjugate particles.

Partitioning of urease and urea in PEG/dextran system

It has been reported that proteins of higher molecular weight are partitioned more readily

to the dextran-rich phase (bottom phase) in a PEG/dextran system.[2] In our experiment, the concentrations of urease in the two phases were determined using the Bradford method.[3] Table S2 shows that 90% of urease is partitioned into the Dex-rich phase. Urea partitions to a similar extent in both phases (Table S3).

Table S2. Partitioning of urease in PEG/dextran system.

Enriched phase	PEG	dextran
Urease content (%)	10	90

Table S3. Partitioning of urea in PEG/dextran system.

Enriched phase	PEG	dextran
Urea content (%)	55	45

Average particle diameter as a function of pH

The average diameter of mPEG-BSA conjugate particles at pH 4-10 is obtained by DLS (Figure S4). It decreases from 600 nm to 300 nm between pH 4 and 6 remaining more or less constant up to pH 10. This demonstrates the greater aggregation of particles at pH 4-6 than at higher pH as a result of the isoelectric point of native BSA (pH 4.7). The insets show photos of the aqueous dispersion of 0.3 wt.% mPEG-BSA particles. The result demonstrates that transparent stable aqueous dispersions are obtained at all pH values.

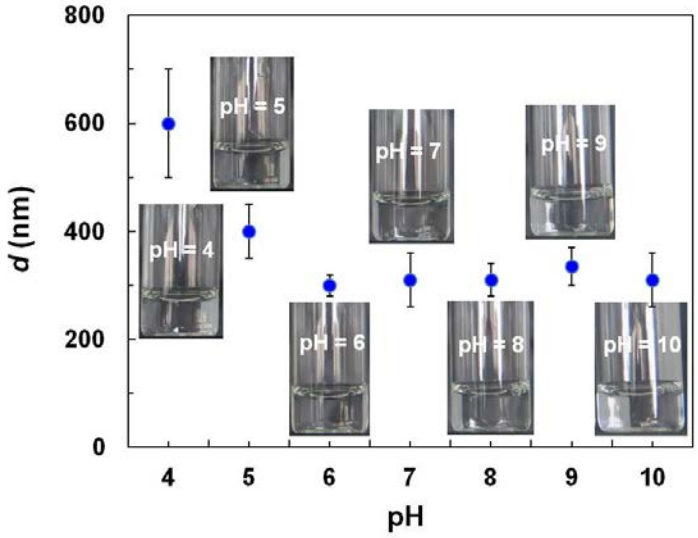

Figure S4. Effect of pH on the average diameter (*d*) of mPEG-BSA particles in water (1 mg mL^{-1}). The inset photos are of the 0.3 wt.% aqueous dispersion.

Effect of pH on the stability of water-in-water emulsions

The stability of Pickering emulsions stabilised by charged particles is sensitive to the particle charge. It is well known that the amidogen and carboxyl groups within proteins can become charged to different extents depending on pH. Protein molecules are cationic (anionic) at pH values below (above) the pI. The evolution of Dex-in-PEG emulsions with time at different pH values is shown in Figure S5. In order to observe the stability of emulsions within a short time, 0.3 wt.% mPEG-BSA conjugate particles are used to stabilize the w/w emulsions. We find that the emulsion stability is extremely sensitive to pH. At pH 4 and 5, the emulsions are stable for at least 30 min, whereas they rapidly completely destabilize within 10 min between pH 6 and 8. The emulsions exhibit good stability without any phase separation at pH 9. At pH 10, the emulsions again show signs of destabilization, completely phase separating within 10 min (Figure S5a-c). There is no emulsion phase inversion between pH 4

and 10 (Figure S6) and Figure S7 shows that the pH value has little effect on the wettability of mPEG-BSA conjugate particles. As shown in Figure S5d, the zeta potential of mPEG-BSA conjugate particles in water decreases as pH increases from 4 to 9, but increases slightly at pH 10. Since the PI of native BSA is 4.7[1] we also see that the zeta potential of mPEG-BSA conjugate particles is close to zero at pH 4-5. Accordingly the aggregation of mPEG-BSA conjugate particles near PI (Figure S4) enhances the stability of the w/w emulsions (Figure S5a-c and Figure S6a).[4] At pH 6-8, the emulsions rapidly destabilize (Figure S5a-c and Figure S6b-e) because the increase of the charge of the particles leads to a decrease in the extent of aggregation (Figure S4). The optimum stability of emulsions at pH 9 (Figure S5a-c and Figure S6f) can be explained by the fact that charged droplets are enough to repel each other due to electrostatic repulsion of the increased charges of the mPEG-BSA conjugate particles. At pH 10 we can observe that the zeta potential of mPEG-BSA conjugate particles decreases to -23.5 mV which leads to the destabilization of emulsions again (Figure S5a-c and Figure S6g). From Figure S5-S7, the results reveal that pH is an interesting variable for the stability of Pickering emulsion stabilized with polymer-protein conjugate particles.

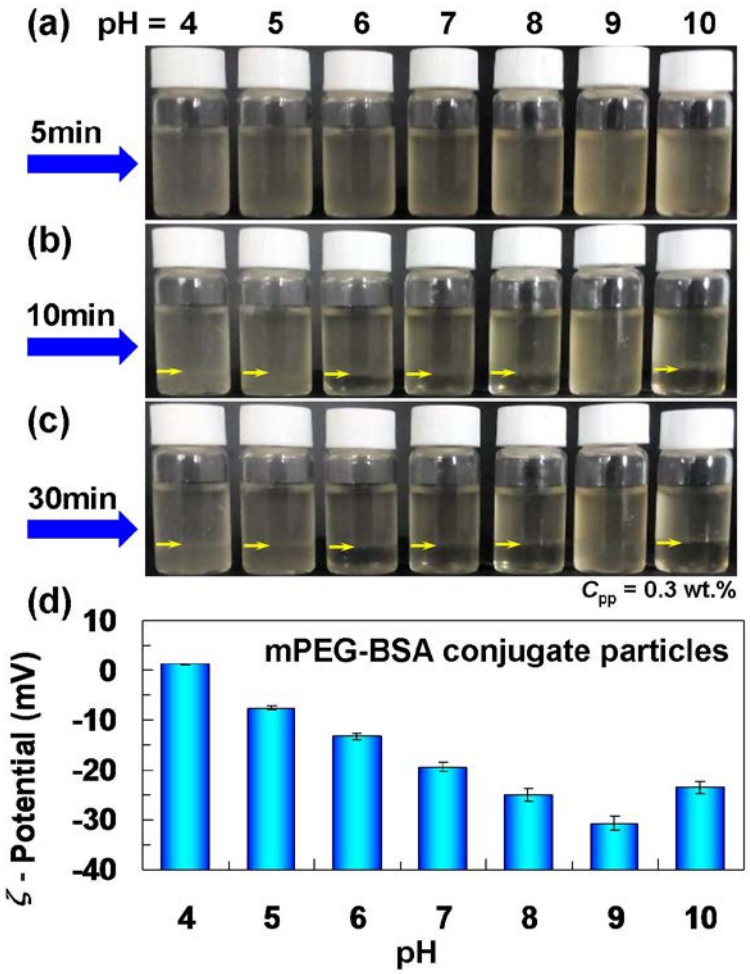

Figure S5. (a-c) Appearance of Dex-in-PEG emulsions at different times after preparation and for different pH for emulsions containing 0.3 wt.% mPEG-BSA conjugate particles, 8.5 wt.% PEG and 8 wt.% dextran. Volume fraction of Dex before mixing = 0.3, (d) Zeta potential of 1 mg mL^{-1} mPEG-BSA conjugate particles in water as a function of pH.

Fluorescence micrographs as a function of pH

Fluorescence micrographs of Dex-in-PEG emulsions with 0.3 wt.% mPEG-BSA conjugate particles at different pH values are shown in Figure S6.

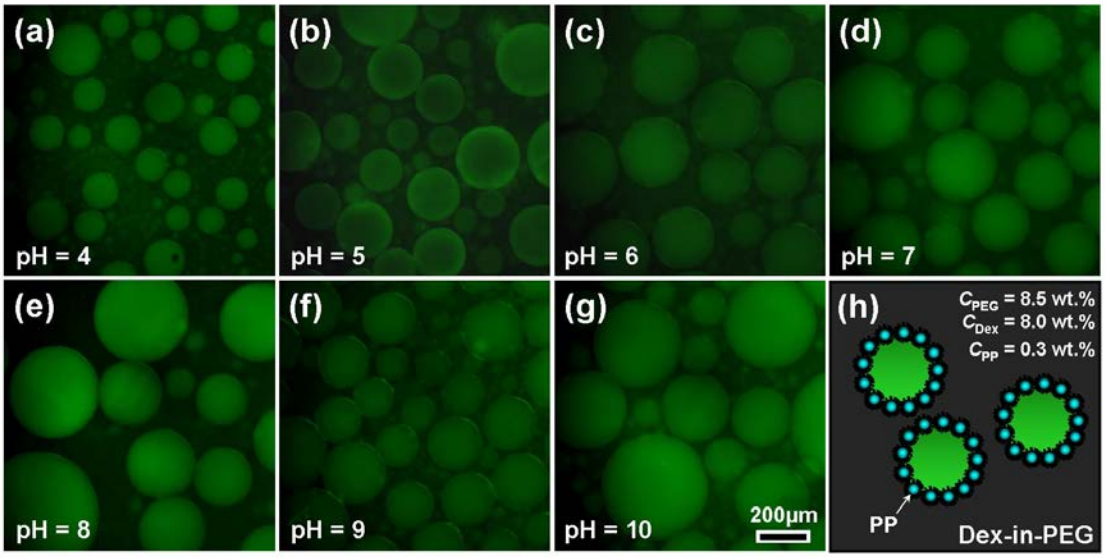

Figure S6. (a-g) Fluorescence micrographs of Dex-in-PEG emulsions showing the FITC-BSA signal for Dex phase in the presence of 0.3 wt.% mPEG-BSA conjugate particles at different pH values. (h) Sketch of Dex-in-PEG droplets.

Wetting properties as a function of pH

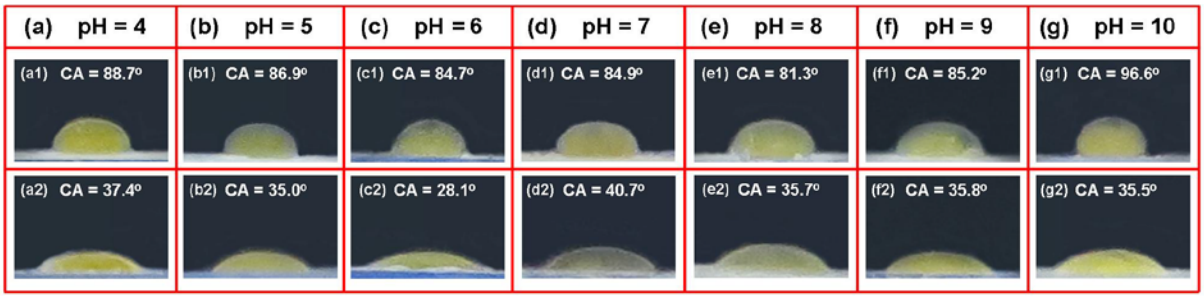

Figure S7. Side-view photos of a drop of Dex-rich phase under a PEG-rich phase on either (a1-g1) an mPEG-BSA conjugate particle film (PP) or (a2-g2) a BSA film at different pH given.

Contact angle experiments are performed to determine the influence of pH on the wettability of mPEG-BSA conjugate particles. The value of θ at different pH for mPEG-BSA conjugate particle films range slightly from 81.3° to 96.6° (Figure S7 a1-g1) and is

independent of pH for the BSA control film (Figure S7 a2-g2).

References

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(3) Bradford, M. M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

(4) Murray, B. S.; Phisarnchananan, N., Whey Protein Microgel Particles as Stabilizers of Waxy Corn Starch plus Locust Bean Gum Water-in-Water Emulsions. *Food Hydrocolloids* **2016**, *56*, 161-169.