Supporting information for

A general method for synthesizing enzyme-polymer conjugates in reverse emulsions by using Pluronic as the reactive surfactant

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Materials

Albumin from bovine serum (BSA), lipase from *Candida rugosa* (CRL), lipase from *Candida sp.*, Novozymes® CALB L (CALB), horseraddish peroxidase were purchased from Sigma-Aldrich. 4-Nitrophenyl acetate, 4-nitrophenyl butyrate, 4-nitrophenyl octanoate, 4-nitrophenylpalmitate, Pluronic®F-127, Dess-Martin periodinane, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich. Sodium metaperiodate (98%), Purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole, 99+%), formaldehyde (37% w/w in aqueous solution), sodium cyanoborohydride (95%), hexanoic acid, palmitic acid, n-butyl alcohol and n-octanol were purchased from Alfa Aesar. Mono-Sulfo-N-hydroxy-succinimido Au-nanoparticles were purchased from NanoProbe. Tert-butyl hydroperoxide (70% in solution) was purchased from J & K Chemical Technology Company and extracted by toluene before use. Lipase was dialyzed against phosphate buffer for at least 48 h prior to use. Other chemicals are of analytical grade and used as received.

Protein conjugation

The hydroxyl end-groups of Pluronic F-127 were first oxidized to aldehyde groups with reference to a previous report[1]. Then 0.5 mL of aqueous solution (phosphate buffer, 50 mM, pH 7.0) containing protein at the concentration of 5-10 mg/mL was added to 4.5 mL of chloroform solution containing 50 mg of aldehyde-functionalized Pluronic. The emulsion was formed by sonication with an ultrasonic probe (scientz-IID Ultrasonic Cell Crusher, Ningbo Xinzhi corporation, 950 W) set to power intensity of 30% for 3 min (1s on, 1s off). After 2 hours reaction at room temperature, sodium cyanoborohydride ($NaCNBH_3$) was added to the reaction mixture followed by stirring for 12 hours at room temperature. Then, 2 mL of water was added to the mixture and chloroform was removed via a rotary evaporator. The remaining solution was dialyzed against phosphate buffer (10 mM, pH 7.0) to remove unreacted reagents.

Using the same procedure, replacing the enzyme with a mixture of enzyme and BSA (1:1, w/w) and increasing the enzyme concentration to ~20 mg/mL, the enzyme-Pluronic microgels were synthesized.

For the aqueous synthesis of enzyme-Pluronic conjugates, enzymes and the aldehyde-functionalized Pluronic F-127 were dissolved in phosphate buffer (10 mM, pH 7.0), with the final enzyme concentration of 2 mg/mL and the ratio of aldehyde groups of Pluronic to amino groups of enzyme being 1.1:1. The mixture was the stirred for 2 h at room temperature, followed by adding sodium cyanoborohydride (10 wt% of Pluronic F-127) and allowing the reaction proceeded for another 12 h at room temperature. Finally, the solution was dialyzed against phosphate buffer (10 mM, pH 7.0) to remove the unreacted reagents and lyophilized for future use.

Determination of amino groups of enzymes

Free enzyme or enzyme-Pluronic conjugate was first dissolved in borate buffer (100 mM, pH 9.3), followed by the addition of 3 microliter of TNBS (3%) to 1 mL of

the above solution and incubation at 30 °C for 2 hours at dark. The absorbance of the solution was then recorded on a UV-Vis spectrometer at 420 nm. Glycine solution with concentrations from 0.01 mM to 0.1 mM was subjected to the same procedure to produce the calibration curve. The amino groups of free enzyme and enzyme-Pluronic conjugates were calculated with refer to the glycine calibration curve, and thus the modification degree of the amino groups on enzyme was calculated.

Transmission electron microscopy (TEM)

The dry powder of the protein-Pluronic conjugate obtained by lyophilization was dissolved in toluene (10 μg/mL of protein concentration). 10 μL of the solution was first placed on the carbon-coated grid. After toluene was evaporated, 10 μL of sodium phosphotungstate aqueous solution (1%, pH 7.0) was applied to stain the sample. The excess of the liquid was removed after 2 minutes and the sample was dried for 24 hours at room temperature before TEM measurements.

Synthesis of nanogold-attached protein-Pluronic conjugates

Excess amount of Au-nanoparticles (mono-sulfo-N-hydroxy-succinimido Au-nanoparticles) was reacted with bovine serum albumin (5:1, molar ratio, Au/BSA) in phosphate buffer saline solution for 1 hour at room temperature. The excess gold nanoparticles were then removed using a Millipore Amicon ultra-centrifugal filter (MWCO=10 kDa). Concentrations of the Au-nanoparticles and proteins were determined by UV/Vis spectrophotometer based on their extinction coefficients (nanogold, 155,000 M^{-1} cm^{-1} at 420 nm; enzyme, 120,000 M^{-1} cm^{-1} at 280 nm). The nanogold-attached protein was then used to prepared the protein-Pluronic conjugates.

Circular dichroism (CD)

The CD measurement was carried out on the Chirascan circular dichroism spectrophotometer (Applied Photophysics Ltd) with a path length of 0.5 mm. The

spectra was measured from 200 to 280 nm in phosphate buffer solution (10 mM, pH 7.0), with an enzyme concentration of 0.5 mg/mL.

Exclusion chromatography (SEC)

The enzyme sample was analyzed by using the TSK gel 2000 column (7.5 mm ID × 30 cm × 10 μm) (TosoH Corp., Tokyo, Japan) with a SHIMADZU liquid chromatograph. The sample was eluted by using 100 mM phosphate buffer, pH 6.7, at a flow rate of 1.0 mL/min, and monitored with UV-absorption at 403 nm for HRP and 280 nm for other proteins.

Enzymatic activity assay

The hydrolytic activity of CRL, CALB and their conjugates was determined using 4-nitrophenyl butyrate (p-NPB) as the substrate. Briefly, p-NPB was first dissolved in acetone and then diluted with phosphate buffer (50 mM, pH 7.0) containing 1.25% (w/v) Triton X-100, giving the final concentration of 0.5 mM. The reaction was started by adding 50 μL of enzyme solution (50 μg/mL in 10 mM phosphate buffer, pH 7.0) to 950 μL of substrate solution and the increase of absorbance was detected at 348 nm by using a UV/Vis spectrophotometer.

The activity of lipase in organic solvents was determined by transesterification. For CRL and CRL conjugate, palmitic acid and n-octanol were used as substrates, while for CALB and CALB conjugate, hexanoic acid and n-butyl alcohol were used. During a run, dry powder of native enzyme or enzyme conjugates with the same protein content of 0.5 mg was added into 5 mL of toluene solution containing 0.1 mol/L of fatty acid and 0.1 mol/L of alcohol. Then the reaction mixture was shaken at 40 ^{o}C and 200 rpm in a horizontal shaker. At specific times, the reaction was terminated by diluting with 10 mL of ethanol/acetone (1:1, v/v). The remaining free fatty acid in the reaction mixture was determined by titration with 0.05 mol/L of NaOH using phenolphthalein as the indicator. Conversion of 1 μmol of fatty acid per

minute in the assay conditions was defined as one enzyme unit activity (U/mg).

The peroxidase activity of horseradish peroxidase in aqueous solution was measured by using 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS) and hydrogen peroxide (H_2O_2) as the substrates under saturated condition in phosphate buffer (50 mM, pH 7.0). In a typical measurement, the assay mixture consists of 900 μL of ABTS methonal solution (0.28 mg/mL) and 50 μL of HRP or HRP conjugate in phosphate buffer (protein concentration 0.1 mg/mL). The enzymatic reaction was initiated by adding 50 μL of H_2O_2 water solution (0.03%). The increase of absorbance at 415 nm was measured by using a UV/Vis spectrophotometer.

The activity of HRP or HRP conjugate in organic solvent was determined using 1,2-diaminobenzene and tert-butylhydroperoxide as the substrates[2]. 10 mL of 50 mM 1,2-diaminobenzene in anhydrous toluene and 1 mL of 1.1 M tert-butylhydroperoxide in anhydrous toluene were mixed. Then the reaction mixture was shaken at 40 °C, 200 rpm in a horizontal shaker. The reaction was initiated by adding dry power of native enzyme or enzyme conjugates with the same protein content of 0.5 mg. After 10 min reaction, the absorbance at 470 nm of the supernatant was recoded.

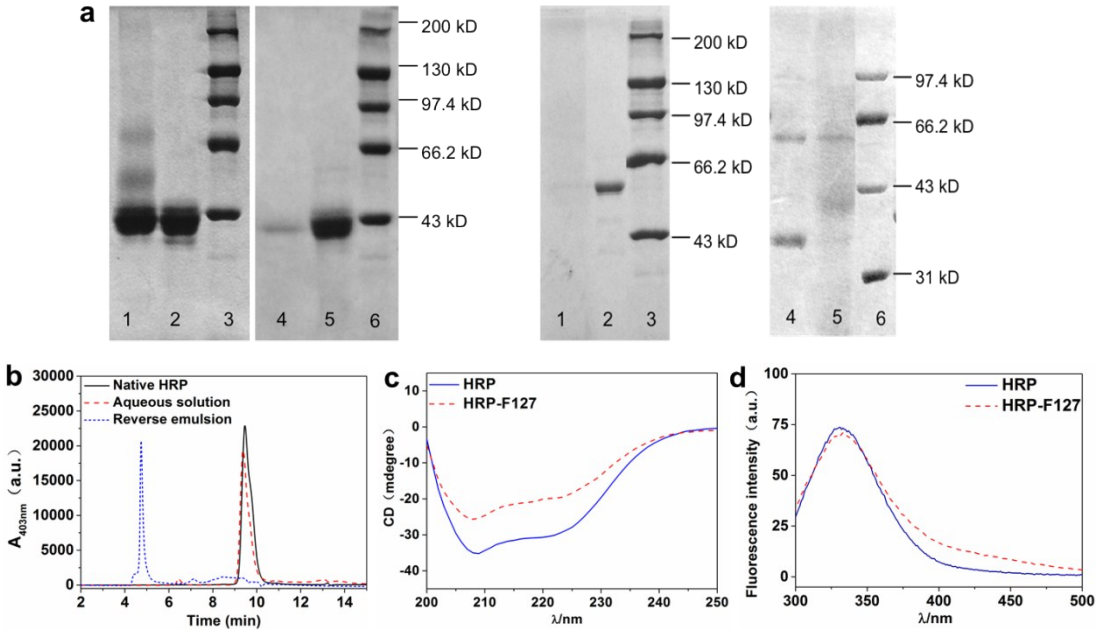

Figure S1. (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein-Pluronic conjugates. Left from 1-6: 1) HRP conjugate

synthesized in aqueous solution, 2) native HRP, 3) protein molecular weight marker, 4) HRP conjugate synthesized in reverse emulsion, 5) native HRP, 6) marker. Right from 1-6: 1) CRL conjugate synthesized in reverse emulsion, 2) native CRL, 3) marker, 4) native CALB, 5) CALB-Pluronic conjugate synthesized in reverse emulsion, 6) marker; (b) SEC of native HRP, HRP-Pluronic conjugate synthesized in aqueous solution and in reverse emulsion; (c) Circular dichroism spectra of HRP and HRP-Pluronic conjugate; (d) Fluorescence spectra of HRP and HRP-Pluronic conjugate.

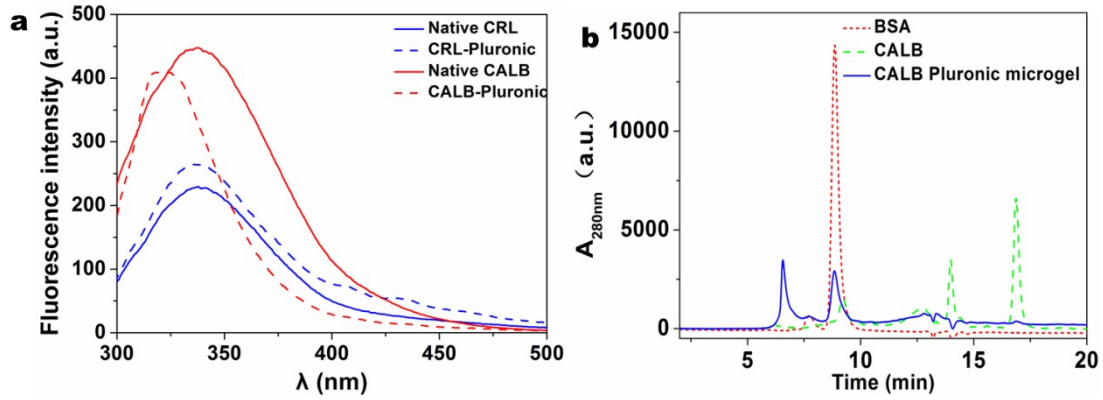

Figure S2. (a) Fluorescence spectra of native enzyme and enzyme-Pluronic conjugate for CRL and CALB; (b) SEC for determining the protein concentration in the supernatant after the synthesis of CALB-BSA microgel, BSA: native BSA as control, CALB: native CALB as control; CALB Pluronic microgel: the supernatant after the synthesis.

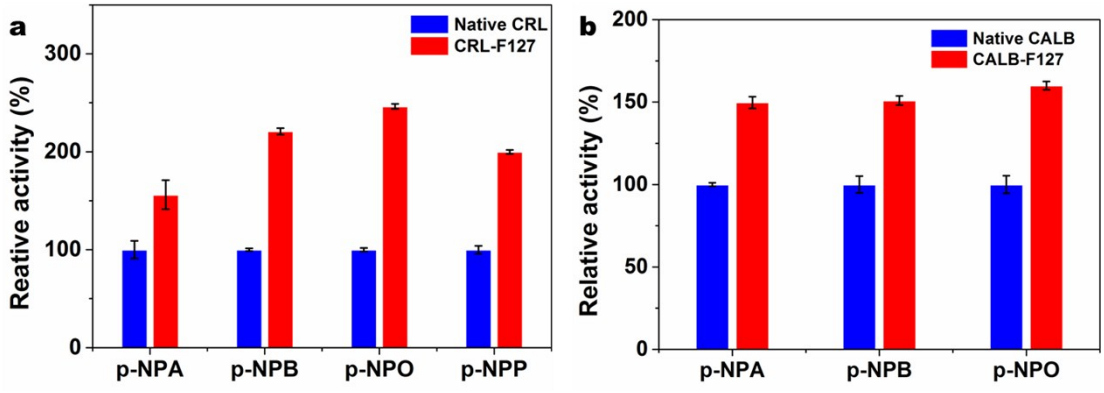

Figure S3. (a) Relative activity of native enzyme and enzyme-Pluronic conjugates by using substrates having different side chains (p-NPA: p-nitrophenyl acetate; p-NPB: p-nitrophenyl butyrate; p-NPO: p-nitrophenyl octanoate; p-NPP: p-nitrophenyl

palmitate). For native CRL, V_{max}(p-NPO)> V_{max}(p-NPB)> V_{max}(p-NPP)> V_{max}(p-NPA), for CRL-Pluronic conjugate, it has the same selectivity order. For native CALB, V_{max}(p-NPA)> V_{max}(p-NPB)> V_{max}(p-NPO), for CALB-Pluronic conjugate, it has the same selectivity order.

References

1. A. K. Muszanska, H. J. Busscher, A. Herrmann, H. C. van der Mei and W. Norde, *Biomaterials*, 2011, **32**, 6333-6341.
2. H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino and S. Inagaki, *Chemistry of Materials*, 2000, **12**, 3301-3305.