Supporting Information for

Multiscale and Multifunctional Emulsions by Host-Guest Interaction-Mediated Self-Assembly

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Experimental section

Chemicals. L-Aspartic acid β-benzyl ester was purchased from Chem-Impex International, Inc (USA). Triphosgene, propargyl amine (PPA), and β-cyclodextrin (β-CD) were obtained from TCI Chemicals (Japan). α-Methoxy-ω-amino-polyethylene glycol (PEG-NH$_2$) with average molecular weight ($M_w$) of 5 kDa was purchased from Laysan Bio, Inc (Alabama, USA). Indomethacin, mefenamic acid, flufenamic acid, sulindac, naproxen, ketoprofen, ibuprofen, tolfenamic acid, nadifloxacin, gemfibrozil, bezafibrate, ciprofibrate, dexamethasone, camptothecin, N-tert-butylacrylamide, N-phenylacrylamide, and azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich (USA). Docetaxel and paclitaxel were obtained from Xi’an Haoxuan Biological Technology Co., Ltd. (China). Benzyl alcohol (BA) was obtained from Amethyst Chemical Co., Ltd. Nile red and pyrene were obtained from J&K Scientific Ltd. Poly(D,L-lactide) (PLA, $M_w = 15$ kDa) was purchased from PolySciences, Inc (USA). Poly(lactide-co-glycolide) (PLGA, 50:50, with intrinsic viscosity of 0.6 dl g$^{-1}$), PLGA (75:25, with intrinsic viscosity of 0.65 dl g$^{-1}$), and poly(ε-caprolactone) (PCL, $M_w = 25$ kDa) were purchased from PolySciTech (USA). All the other reagents are commercially available and were used as received.

Synthesis of polyethylene glycol-block-poly(β-benzyl L-aspartate) (PEG-PBLA). β-Benzyl L-aspartate N-carboxyanhydride (BLA-NCA) was synthesized according to literature.$^1$ Specifically, 10 g L-aspartic acid β-benzyl ester was suspended in 100 ml anhydrous THF, and then 6.3 g triphosgene in 80 ml THF was added. This reaction mixture was stirred magnetically for 2 h at 40°C under nitrogen atmosphere. Thus obtained transparent solution was concentrated under a reduced pressure and the obtained white oil was recrystallized three times from a mixture of THF/petroleum ether, and finally dried at room temperature under vacuum. Subsequently, PEG-PBLA was synthesized by PEG-NH$_2$ initiated polymerization of BLA-NCA in DMF at 40°C.$^1$

Synthesis of polyethylene glycol-block-polyaspartamide containing PPA units (PEG-PPPA). PEG-PPPA was synthesized by a previously developed method with minor revisions.$^2$ Briefly, 1.0 g PEG-PBLA was dissolved in dry DMSO at 40°C, into which a 2-fold molar excess of PPA was added. After 48 h of reaction, the solution was dialyzed in deionized water. The final dialysate solution was lyophilized to obtain a light yellow powder of PEG-PPPA.

Synthesis of mono-6-azido-β-CD. Mono-6-azido-β-CD was synthesized by a nucleophilic substitution reaction of 6-monotosyl β-CD with a 1.2-fold molar excess of sodium azide in anhydrous DMF at 70°C for 6 days.$^2$ After the reaction solution was filtered through a 0.22 µm syringe filter, the product was precipitated from acetone, and purified by repeated precipitation from deionized water.
Synthesis of polyethylene glycol-block-polyaspartamide containing β-CD units (PEG-PCD). The Cu(I)-catalyzed azide-alkyne cycloaddition reaction was adopted to conjugate β-CD onto the PPPA block of PEG-PPPA. To this end, 100 mg PEG-PPPA and 2 g mono-6-azido-β-CD was dissolved into 15 ml DMSO. Then, 26 mg copper sulfate was added to this solution. After dropwise addition of a freshly prepared aqueous solution of sodium ascorbate (40 mg in 10 ml water), the mixture was stirred at 70°C, which was then magnetically stirred at room temperature until the solution became turquoise-blue. Finally, the reaction mixture was dialyzed against water, and a buff powder was obtained after lyophilization.

Synthesis of poly(N-tert-butylacrylamide) and poly(N-phenylacrylamide). Poly(N-tert-butylacrylamide) (PNtBAm) and poly(N-phenylacrylamide) (PNPAm) were synthesized by free radical polymerization of 5 mmol monomer in 40 ml THF at 70°C for 48 h using AIBN (0.1 mmol) as an initiator. PNtBAm was precipitated from n-hexane, while PNPAm was precipitated from diethyl ether.

Measurements. $^1$H NMR and $^1$H-$^1$H COSY spectra were recorded on an Agilent 600 MHz spectrometer. Fourier transform infrared (FT-IR) spectra were acquired on a Perkin-Elmer FT-IR spectrometer (100S). Confocal laser scanning microscopy (CLSM) was performed on a Carl ZEISSLSM780NLO fluorescence microscope. Scanning electron microscopy (SEM) images were taken on a S-3400N II electron microscope (Hitachi, Japan).

Fabrication and characterization of emulsions based on PEG-PCD and BA. As a general procedure, emulsions were formed by adding various volumes of BA in 1 ml of aqueous solutions containing PEG-PCD at different concentrations, which was followed by vortexing for 30 s. For catastrophic phase inversion studies, after the oil phase was added into the aqueous phase, the mixture was gently shaken to give rise to emulsions with relatively large sizes. Drug-loaded emulsions were fabricated through the similar procedures, by dissolving different drugs into the related oil phase. The drug concentration in final emulsions was 3 mg ml$^{-1}$, while the oil/water volume ratio was 0.06:1. The concentration of PEG-PCD was 10 mg ml$^{-1}$.

The particle size of emulsions was measured by dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS instrument. Unless stated otherwise, measurements were implemented at 25°C. Transmission electron microscopy (TEM) observation was carried out on a JEM-1400 microscope (JEOL, Japan) operating at an acceleration voltage of 100 kV. To stabilize the assembled different structures, 50 wt.% glutaraldehyde was used for crosslinking.

In some cases, samples were observed by super-resolution fluorescence microscopy (SRFM) on a DeltaVision OMX Blaze microscope (GE Healthcare, USA). SRFM used herein was provided with a TIRF (Total Internal Reflection Fluorescence) module that can enhance the signal-to-noise ratio and increase imaging contrast with detection limitation of about 100 nm.
**Preparation of complex emulsions.** To prepare water-in-oil-in-water (w/o/w) complex emulsions, primary w/o emulsion was first obtained by emulsifying inner aqueous solution containing PEG-PCD into BA by vortexing for 30 s. Thus obtained w/o emulsion was emulsified with the outer water phase containing PEG-PCD by gently shaking. The similar procedures were followed to fabricate oil-in-water-in-oil (o/w/o) complex emulsions. In both cases, the oil phase was doped with Nile red for observation by CLSM.

**Isothermal titration calorimetry.** Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal iTC200 Microcalorimeter (GE Healthcare, USA) at 25°C. The reference cell was filled with distilled water. An initial 0.4 µl injection was discarded from each data set in order to remove the effect of titrant diffusion across the syringe tip during the equilibration process. The experiment consisted of injecting 2 µl (19 injections, 0.4 µl for the first injection only) of BA at a concentration of 0.1 mM into the reaction cell initially containing 200 µl of PEG-PCD solution at 1 mM. A background titration was performed using an identical titrant with distilled water placed in the sample cell. The result was subtracted from each experimental titration to account for the heat of dilution. The titrant was injected at 2 min intervals to ensure that the titration peak returned to the baseline prior to the next injection. Each injection lasted 4 s. To ensure a homogeneous mixing in the cell, the stirring speed was kept constant at 1000 rpm. Results of the titration curves were analyzed using Origin software supplied by MicroCal under a single binding site model. Similar procedures were adopted for measuring ITC curves of BA/β-CD in aqueous solution at 25°C and 50°C.

**Fluorescence spectroscopy measurement of different systems.** Sample solutions for fluorescence measurements were prepared as described previously. Briefly, pyrene in acetone was added to each of a series of 2 ml vials, and acetone was evaporated. A total of 1 ml of various concentrations of BA/PEG-PCD at various oil/water ratios was added to each vial and then heated at 50°C for 10 h, and then left to cool for 10 h at room temperature. The final concentration of pyrene was $6.0 \times 10^{-7}$ M. Steady-state fluorescent spectra were acquired using a F-7000 fluorescence spectrophotometer (Hitachi, Japan) with a slit width of 2.5 nm for both excitation and emission. All spectra were run on air-equilibrated solutions. For fluorescence excitation spectra, the emission wavelength was 390 nm, with scan speed of 240 nm min$^{-1}$. All the experiments were carried out at 25°C.

**Interfacial tension measurements.** The maximum bubble pressure method was used to measure interfacial tension of different oil-water systems, using a surface tension measuring instrument (Model DP-AW-II, Sangli, China). Specifically, the tip of a capillary tube was adjusted to ensure it is immersed in the oil/water interface. The gas flow into the device creates bubbles at a capillary orifice at a constant and controlled frequency. These bubbles grow with increasing in the internal pressure, due to the energy cost upon generation of the bubble surface. The pressure inside the bubble was monitored and the maximum value was
identified, corresponding to the point when the bubble reached a hemispherical shape. The resulting maximum pressure drop $\Delta P_{max}$ across the bubble surface was then substituted into the Young-Laplace equation to determine the surface tension $\sigma$ based on the equation

$$\sigma = \frac{r}{2} \Delta P_{max} = K \Delta P_{max}$$

where $r$ is the radius of the surface curvature. For a bubble with a hemispherical shape, $r$ is equal to that of the capillary orifice. $K$ is a constant calculated using distilled water under the same experimental conditions.

**Calculation of hydrophilic-lipophilic balance (HLB) values.** HLB values of different compounds were calculated according to the group contribution method established by Davies. Specifically, the HLB value is given by the following equation.

$$HLB = 7 + \sum(\text{hydrophilic group numbers}) + \sum(\text{lipophilic group numbers})$$

**Fabrication of polymeric nanoparticles based on assembled emulsions.** To prepare polymer nanoparticles using BA/PEG-PCD nanoemulsions, different polymers were directly dissolved in BA, which were then emulsified into pre-formed BA/PEG-PCD nanoemulsions. The obtained nanoemulsions were stirred at 50°C for 4 h. The solidified polymeric nanoparticles were collected by centrifugation and washed with deionized water for additional characterization.

**Preparation of porous structures based on different polymers.** Emulsions were obtained by vortexing of 1 ml of aqueous solution containing PEG-PCD at 5 mg ml$^{-1}$ and 80 µl BA with or without polymer materials, which were then coated on freshly cleaved mica for observation via SEM. As control experiments, the BA solutions containing different polymers at 10 mg ml$^{-1}$ were coated on freshly cleaved mica, SEM observation was conducted after the solvent was evaporated.

**In vivo pharmacokinetic study after oral delivery of drug-loaded nanoemulsions.** All the animal care and experimental protocols were performed with review and approval from the Animal Ethical and Experimental Committee of the Third Military Medical University. Twelve male Sprague-Dawley rats (200-250 g) were randomly assigned into two groups. Indomethacin-loaded nanoemulsions and free indomethacin in a formulation of suspension were orally administered via gastric gavage at a dose of 3.0 mg kg$^{-1}$. All the rats fasted overnight before administration. At defined time points, blood samples were collected. Indomethacin concentrations in plasma were quantified by high performance liquid chromatography. According to the similar procedures, the plasma drug concentration-time curves of free paclitaxel and paclitaxel-containing nanoemulsions were examined after oral administration at 10 mg kg$^{-1}$. In this case, the plasma paclitaxel concentration was quantified by liquid chromatography-mass spectrometry.
**Acute toxicity evaluation on BA/PEG-PCD nanoemulsions.** Male Kunming mice (18-23 g) were randomly divided into 2 groups ($n = 5$). In each mouse, BA/PEG-PCD nanoemulsions was administered via oral gavage at 33 ml kg$^{-1}$, while the same volume of saline was administered to mice in the control group. For the used nanoemulsions, the oil/water volume ratio was 0.06:1, while the PEG-PCD concentration was 10 mg ml$^{-1}$. After 2 weeks, animals were euthanized. Blood samples were collected for hematological analysis (Sysmex KX-21, Sysmex Co., Japan) as well as quantification of biomarkers relevant to hepatic and kidney functions. Lipid levels were also measured. Major organs including heart, liver, spleen, lung, kidney, and thymus were isolated and weighed. In addition, histopathological sections of major organs were prepared and stained with hematoxylin-eosin (H&E). The organ index was calculated according to the following equation.

$$\text{Organ index} = \frac{\text{The organ weight (g)}}{\text{The body weight (g)}} \times 100\%$$

**Safety statement.** For all the materials used and all experiments performed, no unexpected or unusually high safety hazards were encountered.

**Statistical analysis.** Statistical analysis was performed by SPSS 19.0 using one-way ANOVA test for experiments consisting of more than two groups, and with a two-tailed, unpaired $t$-test in experiments with two groups. Statistical significance was assessed at $p < 0.05$.

**References**

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Figure S1. Synthesis of a hydrophilic diblock host copolymer containing β-CD units. (a-c) Schematic route of synthesis of a host diblock copolymer with one PEG block and another block containing β-CD units (PEG-PCD). (a) Synthesis of an alkyne containing PEG diblock copolymer PEG-PPPA. (b) Synthesis of a functional β-CD bearing an azide group at the primary site (Mono-6-azido-β-CD). (c) Synthesis of PEG-PCD by a “click chemistry” reaction between alkyne and azide. (d) FT-IR spectra of different polymers. (e) Representative $^1$H NMR spectrum of PEG-PPPA in DMSO-d$_6$. (f) $^1$H NMR spectrum of PEG-PCD in D$_2$O. $M_w$ of the PEG block was 5 kDa.
Figure S2. Characterization of host-guest interactions between PEG-PCD and BA. (a-b) ITC curves (a) as well as calculated thermodynamic parameters and the binding constant (b) of β-CD and BA in aqueous solution. (c) Chemical shift changes in phenyl protons of BA in the presence of PEG-PCD in D$_2$O. (d) $^1$H-$^1$H COSY spectrum of BA/PEG-PCD in D$_2$O. For PEG-PCD-containing samples, 10 mg copolymer and 10 or 20 µl of BA were dissolved in 1 ml D$_2$O for NMR measurement.
**Figure S3.** Characterization of the BA/PEG-PCD system in aqueous solutions. (a) Interfacial tension of BA and deionized water in the absence or presence of 10 mg ml\(^{-1}\) PEG-PCD pre-incubated with varied volumes of BA. (b) Interfacial tension between BA and different aqueous solutions. For the PEG-PCD group, it was quantified with 10 mg ml\(^{-1}\) PEG-PCD without pretreatment with BA. In other groups, 1 ml of aqueous solution containing various concentrations of PEG-PCD was treated with 10 µl BA. (c) Excitation spectra of pyrene in aqueous solutions of PEG-PCD at 10 mg ml\(^{-1}\) with increased volumes of BA. (d) The fluorescence intensity ratios at 338 and 333 nm of pyrene excitation spectra in aqueous solution of PEG-PCD at 10 mg ml\(^{-1}\) as a function of BA volume. Data in (a,b) are mean ± s.d. (n = 3).
Figure S4. TEM images of vesicles formed by self-assembly of BA and aqueous solution of PEG-PCD (10 mg ml$^{-1}$) at an oil/water volume ratio of 0.01:1.
Figure S5. The stability of assembled BA/PEG-PCD o/w nanoemulsions at enhanced temperature. (a-b) Temperature-dependent changes in size distribution profiles (a) as well as the mean diameter and PDI values (b) of BA/PEG-PCD nanoemulsions assembled at an oil/water volume ratio of 0.04:1. (c) Changes in the mean diameter and PDI values with increased and decreased temperature for BA/PEG-PCD nanoemulsions at a volume ratio of 0.06:1. (d-g) The effect of temperature on host-guest interactions between BA and PEG-PCD. (d-e) $^1$H NMR spectra of different samples in D$_2$O at 50°C. The spectra in (e) show a zoomed in image of proton signals due to phenyl of BA. The PEG-PCD concentration was 10 mg ml$^{-1}$. The volume ratio of BA to D$_2$O was 0.02:1. (f-g) ITC curves (f) as well as calculated thermodynamic parameters and the binding constant (g) of 20 mM β-CD and 1 mM BA in aqueous solution at 50°C. $^1$H NMR spectra suggested slightly impaired host-guest interactions between BA and PEG-PCD. However, ITC measurement showed comparable interactions between β-CD and BA at either 25 or 50°C. Data in (b,c) are mean ± s.d. (n = 3).
Figure S6. Host-guest interaction-mediated emulsification under different conditions. (a) Digital photos illustrate phase separation of aqueous solution of β-CD (10 mg ml\(^{-1}\)) with Nile red-doped BA. (b) Nanoemulsification of aqueous solution of PEG-PCD at 10 mg ml\(^{-1}\) with Nile red-doped BA. (c-d) Digital photos (c) and size distribution profiles (d) showing demulsification of BA/PEG-PCD nanoemulsions in the existence of 1-adamantylamine hydrochloride (ADA).
Figure S7. Catastrophic phase inversion of assembled BA/PEG-PCD emulsions. The images show low magnification views of representative fluorescence images, indicating bicontinuous emulsions at specific oil fractions. The concentration of PEG-PCD was 10 mg ml\(^{-1}\), while the oil phase fraction was 50.0%, 66.7%, and 71.4%, respectively.
Figure S8. Catastrophic phase inversion of assembled BA/PEG-PCD emulsions at different PEG-PCD concentrations. (a-b) Fluorescence images indicating phase inversion of emulsions assembled at 5.0 mg ml⁻¹ (a) and 20.0 mg ml⁻¹ (b) of PEG-PCD. Scale bars, 10 µm.
Figure S9. SEM images of different polymers after they were dissoved in BA and coated on freshly cleaved mica. (a-d) PLGA5050 (a), PLA (b), PNPAm (c), and PNTBAm (d).
Figure S10. Assembled BA/PEG-PCD o/w nanoemulsions for solubilization of different hydrophobic drugs. (a) Chemical structures of various drugs used. (b-c) Digital photos (b) and size distribution profiles (c) of nanoemulsions containing different hydrophobic drugs. The drug concentration in these emulsions was 3 mg ml\(^{-1}\), while the oil/water ratio was 0.06:1. The PEG-PCD concentration was 10 mg ml\(^{-1}\).
Figure S11. *In vivo* safety study after oral administration in mice. (a) Body weight before and after different treatments. (b) The organ index of representative major organs at day 14 after treatment. (c-f) Typical hematological parameters of blood samples collected from mice at day 14, including WBC (c), RBC (d), PLT (e), and HGB (f). (g-h) The serum levels of biochemical markers relevant to hepatic (g) and kidney (h) functions. (i) Levels of representative lipids in blood samples collected at day 14. Mice in the nanoemulsion group were administered with BA/PEG-PCD nanoemulsions at the oil/water ratio of 0.06:1 by oral gavage at 33 ml kg$^{-1}$. The saline group was treated with the same volume of saline. At day 14 after treatments, mice were euthanized. Blood samples and major organs were collected for further analyses. WBC, white blood cell; RBC, red blood cell; PLT, platelet; HGB, hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; UREA, blood urea; CREA, creatinine; TCH, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data are mean ± s.d. ($n$ = 5).
Figure S12. Hematoxylin and eosin-stained histopathological sections of main organs from mice collected at day 14 after treatment with saline or BA/PEG-PCD nanoemulsions by oral administration at 33 ml kg\(^{-1}\). All optical images were taken at \(\times10\) magnification.