Supporting Information

Complete Surface Control of Peptide Nanospheres with Detachable and Attachable Polymer Brush Layers

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1. Experimental details

Materials

L-Phenylalanine (Phe), tetrahydrofuran (THF), *n*-hexane, dimethyl sulfoxide (DMSO), dichloromethane, pyridine, 4-dimethylaminopyridine (DMAP), 2-(ethylthio)ethylamine (EA), and potassium iodide were purchased from Wako Pure Chemical Industries (Japan), and were used without further purification unless otherwise noted. THF and *n*-hexane were distilled over sodium before use. DMSO was distilled over calcium hydride before use. Triphosgene, DL-dithiothreitol, and cystamine dihydrochloride were purchased from Tokyo Chemical Industry Corporation (Japan), and were used as received. *n*-butylamine was purchased from Kishida Chemical Corporation (Japan), and was used without purification. 4-Nitrophenyl chloroformate was purchased from Sigma-Aldrich (St. Louis MO) and was used without purification. Iodine was purchased from Nacalai Tesque, Inc (Japan), and was used as received. α-Aminopropyl-ω-methoxy, polyoxyethylene (H_2N-PEG-OCH_3; M_w = 2,000), methoxypolyethylene glycol (HO-PEG-OCH_3; M_w = 2,000), and α-Mercaptoethyl-ω-methoxy, polyoxyethylene (HS-PEG-OCH_3; M_w = 2,000 and 10,000) were purchased from NOF corporation (Japan), and were used as received.

Synthesis of Phe-NCA

Phe-NCA was synthesized by the reaction of L-phenylalanine (60.6 mmol) with triphosgene (20.2 mmol) in THF (200 mL) for 4 hours at 45°C, and was purified by subsequent re-precipitation

in *n*-hexane to give Phe-NCA as a white solid. The yield was 77%. Anal. Calcd (%) for $C_{10}N_1O_3H_9$: C, 63.82; H, 4.74; N, 7.33. Found: C, 63.02; H, 4.85; N, 7.45.

*Synthesis of carbonate- activated PEG (Compound **2**)*

Methoxypolyethylene glycol (**1**, M_w = 2,000; 2 g, 1 mmol) and 4-nitrophenyl chloroformate (0.8 g, 4 mmol) were dissolved in dichloromethane (10 mL). After the addition of pyridine (0.3 mL), the reaction mixture was stirred overnight at room temperature. Then the solvent was removed under reduced pressure. After the addition of dichloromethane, the solution was poured into excess diethyl ether under stirring at 0°C, and the resultant precipitate was collected by centrifugation and washed with diethyl ether; the yield was 92%.

*Synthesis of PEG-SS-NH_2 (Compound **3**)*

Cystamine dihydrochloride (1350 mg, 6 mmol) and 4-dimethylaminopyridine (1470 mg, 12 mmol) were dissolved in dry DMSO (5 mL). Into this solution compound **2** (1500 mg, 0.75 mmol) was gently dropped in 10 mL of dry DMSO, and then the mixture was stirred at room temperature for 24 hours. The mixture was extracted with chloroform, and dried over magnesium sulfate. The combined chloroform solution was dried under reduced pressure. After the addition of water and subsequent acidification to pH = 3 with 1N HCl aq., the aqueous mixture was extracted with chloroform. The combined chloroform solution was then concentrated under reduced pressure. The solution was poured into excess diethyl ether while stirring at 0°C, and the resultant precipitate was

collected by centrifugation and washed with diethyl ether; the yield was 76%.

Preparation of PEG-SS- Peptide Nanospheres

15.6 μmol of PEG-SS-NH_2 (M_w = 2,000) and 36.4 μmol of 2-(ethylthio)ethylamine as initiators were dissolved in water (4.5 mL). The pH of the initiator solution was adjusted to 10.8 with 1N NaOH aq.. Phe-NCA (520 μmol) in 500 μL of DMSO was gently dropped into the initiator solution, and then the mixture was stirred at 0°C for 24 hours. The nanospheres were purified by dialysis (Spectra/Pore membrane, 50,000 molecular weight cut off) against distilled water for 3 days, and the obtained nanospheres were centrifuged and re-dispersed in water.

Control of PEG Graft Density Based on Reduction

The peptide nanospheres were dispersed in water at 10 mg/mL, and incubated with an equivalent volume of dithiothreitol (DTT) solution (50 mM tris buffer solution, pH 8.0, 0–42 mM) at room temperature for 24 hours. The nanospheres were isolated by centrifugation, and washed with water to remove PEG detached from the nanospheres. The composition of the peptide nanospheres after reduction was estimated by ^{1}H-NMR measurements in a chloroform-*d*/trifluoroacetic acid (TFA)-*d* mixed solvent (chloroform-*d*/TFA-*d* = 4/1 v/v).

Re-modification of Hairless Nanospheres Based on Oxidation

The “hairless” nanospheres, whose PEG chains detached with excess DTT almost completely, were dispersed in water (0.8 mL) at 5.0 mg/mL, and were mixed with SH-monoterminated PEG

(MeO-PEG-SH; M_w = 2,000; 0.75 mg, 0.375 μmol). After the addition of iodine and potassium iodide solution ([KI] = [I_2] = 0 - 50 mM; 0.2 mL) to the dispersion, the mixture was stirred at room temperature for 24 hours. The nanospheres were isolated by centrifugation, and washed with water to remove any free PEG. The composition of the peptide nanospheres after oxidation was estimated by ^{1}H-NMR measurements in a chloroform-*d*/TFA-*d* mixed solvent (chloroform-*d*/TFA-*d* = 4/1 v/v).

Hemolysis Activity Test

Sheep red blood cells (RBCs) were stored at 4°C in Alsever's solution. Prior to use, the RBCs were washed three times with saline and re-suspended in saline at 2 × 10^8 cells/mL. The nanospheres were dispersed in a 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) containing 0.15 M NaCl, at a concentration of 2 mg/mL. The dispersion was incubated with an equivalent volume of 2 × 10^8 cells/mL RBCs in saline at 37°C for 1 hour. (final concentration: nanospheres 1 mg/mL, RBC 1 × 10^8 cells/mL in 25 mM MES, 0.15M NaCl). The dispersion was then centrifuged at 5,000 × *g* for 5 min. To determine the hemolytic activity of the nanospheres, the hemoglobin absorbance in the supernatant was measured with a microplate reader at 570 nm. To obtain 100% hemolysis, the RBCs (1 × 10^8 cells/mL) were lysed with water. In the controls, the RBCs were incubated in buffer alone.

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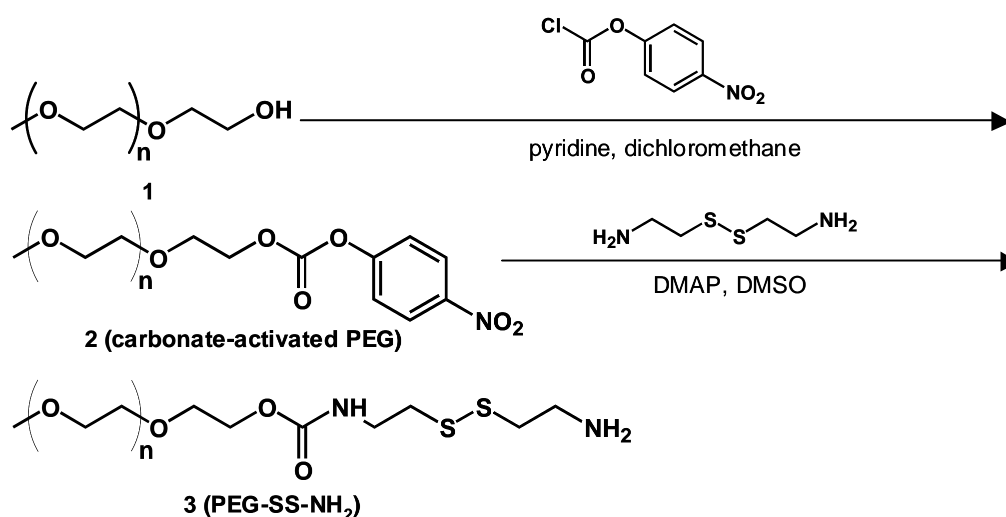
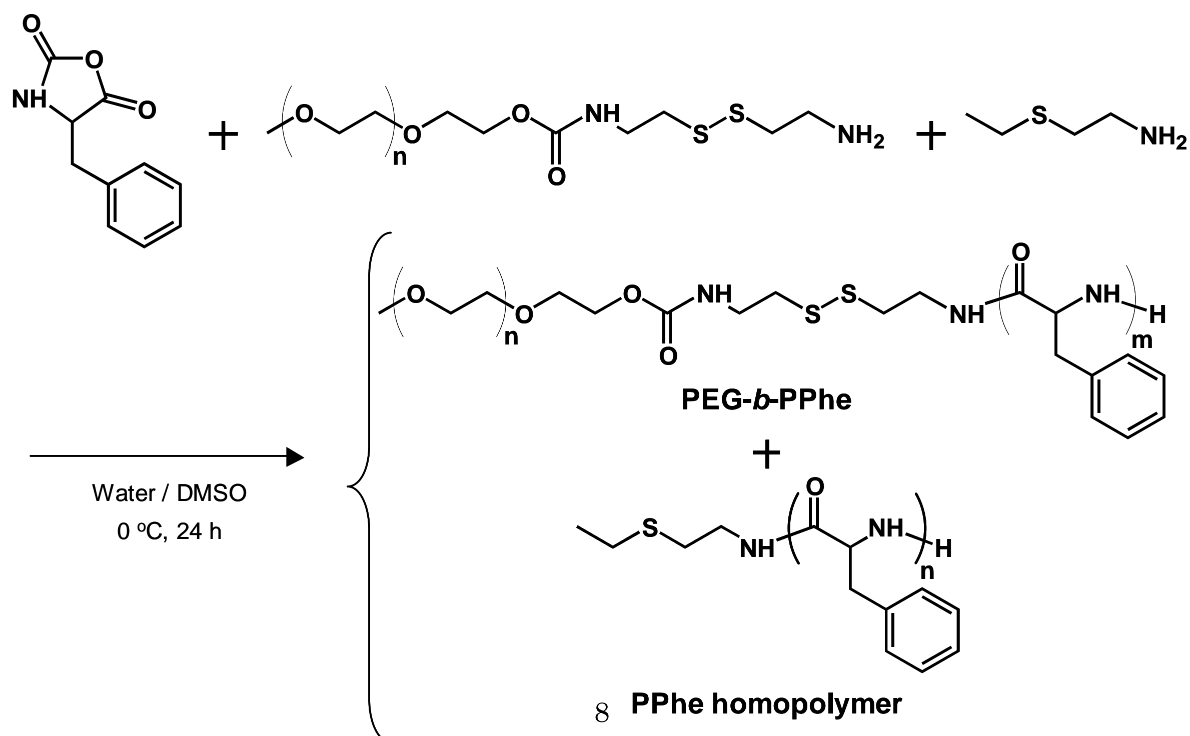
Measurements

Proton nuclear magnetic resonance (^{1}H-NMR) spectra of the PEG-SS-peptide nanospheres were measured with a VARIAN UNITY INOVA 600 spectrometer (600 MHz; Varian Technologies Japan Limited, Japan) using a chloroform-*d*/ TFA-*d* mixed solvent (chloroform-*d*/TFA-*d* = 4/1 v/v) for compositional analysis of the peptide nanospheres before and after reduction or oxidation and ethanol-d_6 for surface composition analysis. Scanning electron microscopic (SEM) images were obtained with a JSM-6701F (JEOL, Japan). The particle size distribution of the PEG-SS-peptide nanospheres was measured by the dynamic light scattering (DLS) method using a Zetasizer Nano ZS (Malvern Instruments, UK).

2. Synthesis of PEG-SS-peptide nanospheres



Scheme S1. Synthesis of PEG-SS-NH_2.



Scheme S2. Synthesis of PEG-SS-peptide nanospheres with disulfide linkage between the hydrophobic core and high-density PEG brush layer.

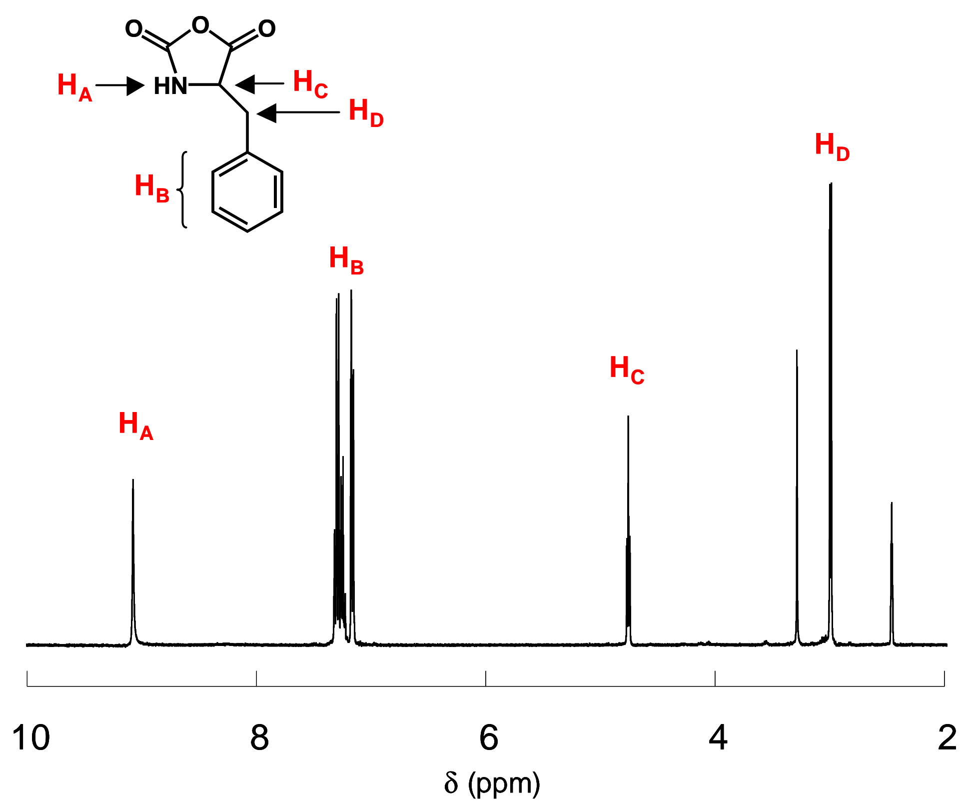


Figure S1. ^{1}H NMR spectra of Phe-NCA in DMSO-d_6.

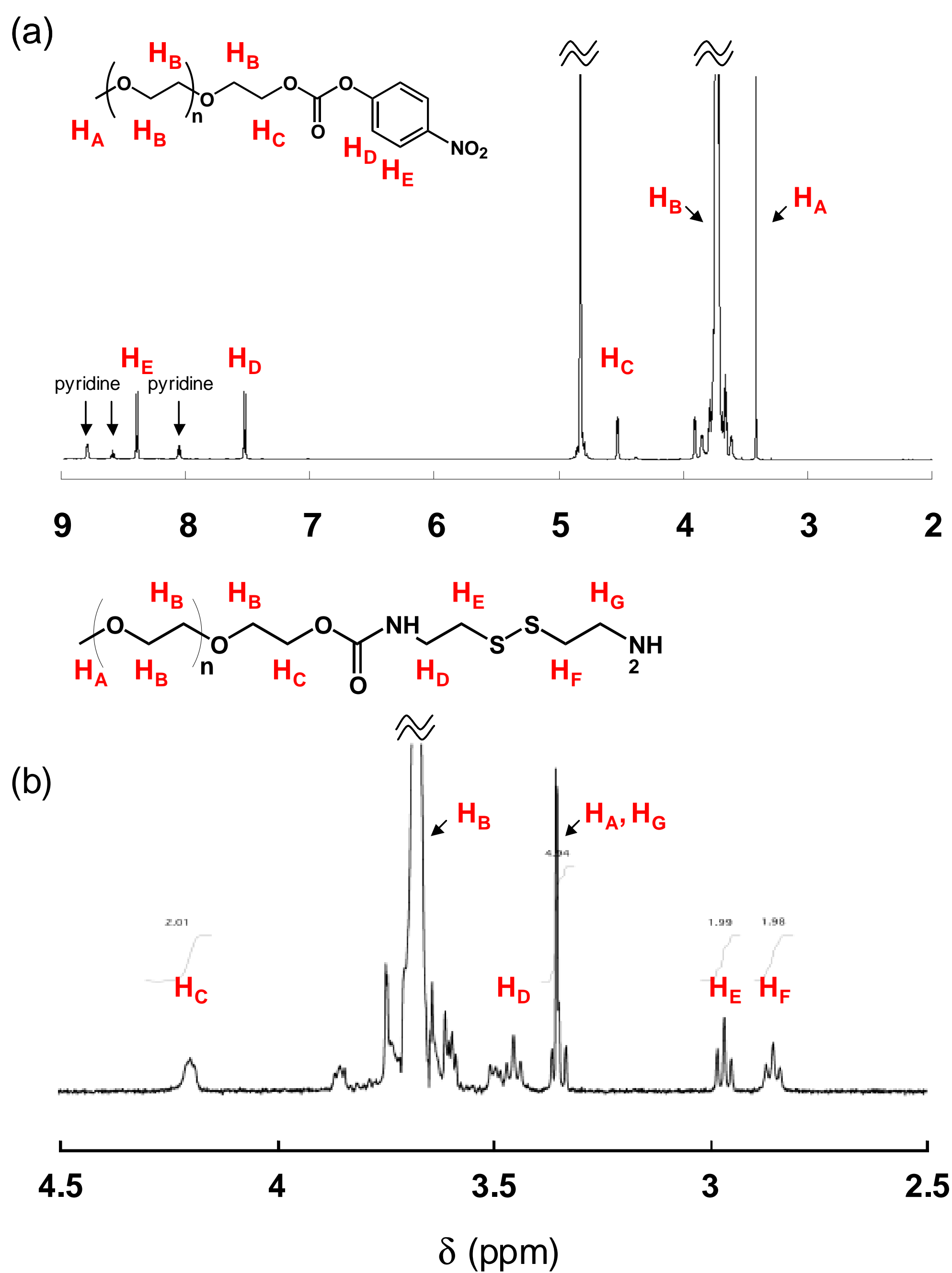


Figure S2. ^{1}H NMR spectra in D_2O of (a) carbonate-activated PEG and (b) PEG-SS-NH_2.

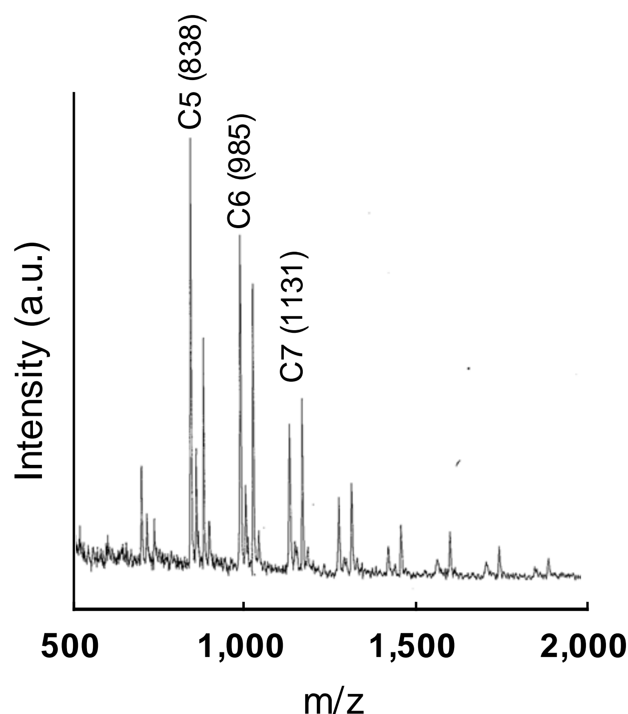


Figure S3. MALDI-TOF mass spectrum of the PEG-SS-peptide nanospheres.

Molecular characterization

In the same procedure as previous report (Ref. 5b), we evaluated each DP of the two components, PPhe and PPhe-*b*-PEG in the PEG-SS-peptide nanospheres synthesized in this study as follows. MALDI-TOF mass gave molecular weight of only PPhe within the nanospheres, because we used dithranol as matrix, which is optimum one for PPhe, not for PEG moiety. Therefore, we determined the DP of PPhe to be 6 based on the results of MALDI-TOF mass. On the other hand, ^{1}H NMR measurement of the peptide nanospheres in chloroform-*d*/TFA-*d* (4/1 v/v) showed the molar ratios of total Phe units, PEG-SS-NH_2, and 2-(ethylthio)ethylamine (EA), as 70 : 1 : 7.8, respectively. The

7.8 molar ratio of EA indicated the existence of the 7.8-fold molar ratio of PPhe homopolymer.

Therefore, the DP of Phe units in PPhe-*b*-PEG should be calculated as follows: 70 - (7.8 x 6) = 23.2.

Accordingly, we estimated the DP of PPhe and PEG-*b*-PPhe to be 6 and 23.2, respectively.

3. DLS measurements of PEG-SS-peptide nanospheres after detaching and attaching PEG brush layer

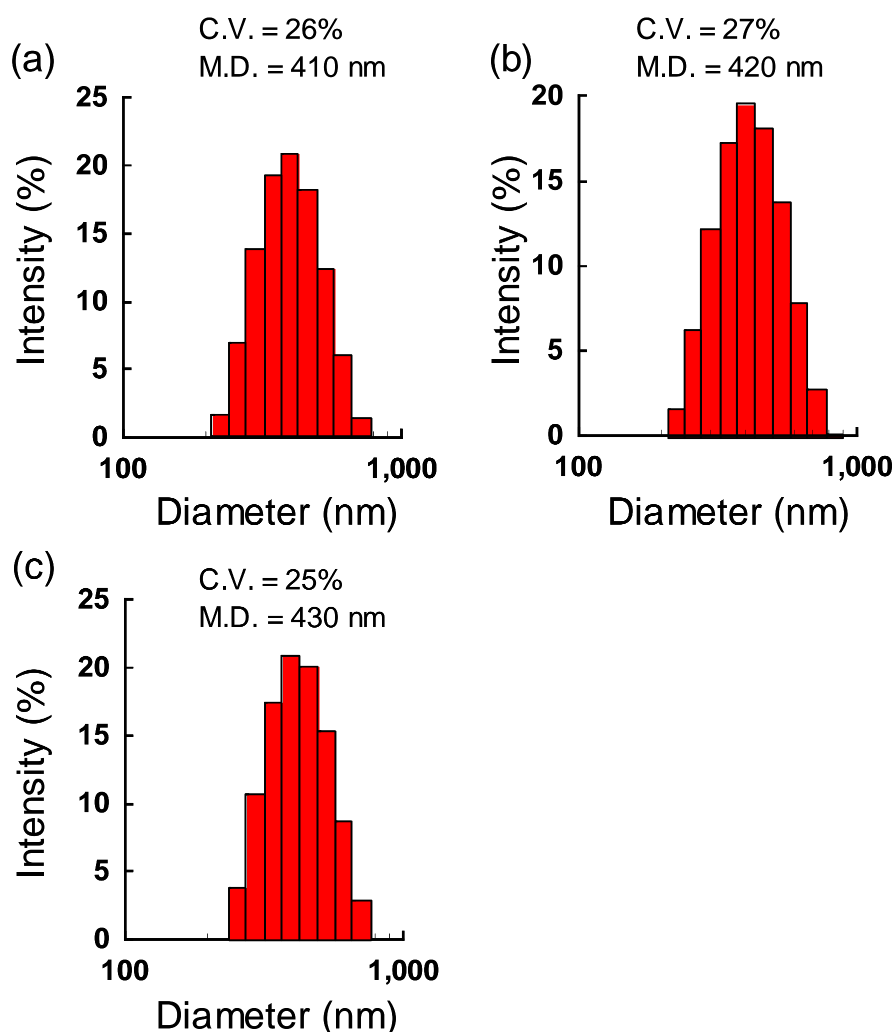


Figure S4. Size distribution of the PEG-SS-peptide nanospheres before reduction (a) and after reduction (b), and after remodification (c) measured in ethanol, which is a good dispersant for both samples before and after reduction, by dynamic light scattering. Coefficient of variation (C.V.) is standard deviation/mean diameter (M.D.).

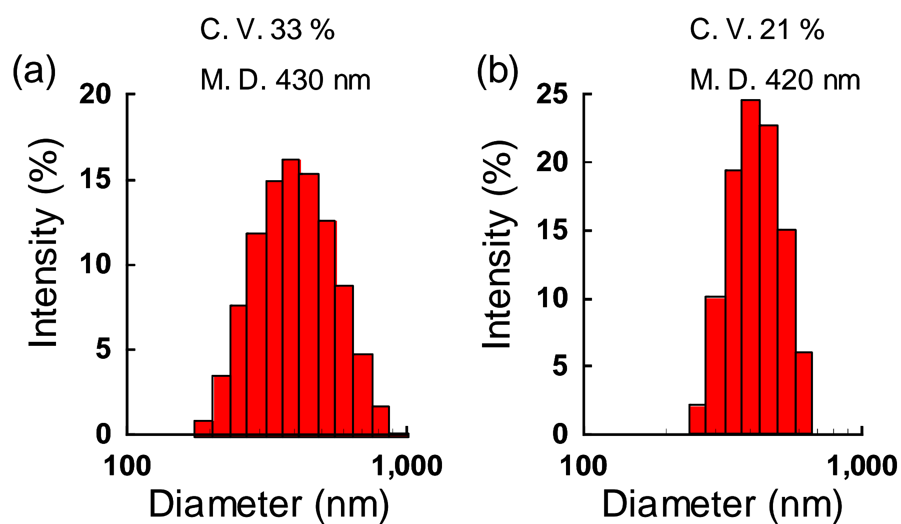


Figure S5. Size distribution of the PEG-SS-peptide nanospheres before reduction (a) and after oxidation (b) in water by dynamic light scattering. Coefficient of variation (C.V.) is standard deviation/mean diameter (M.D.).

4. Surface structural analysis of PEG-SS-peptide nanospheres after detaching and attaching PEG brush layer

We evaluated the ratio of the amount of PEG within "whole nanosphere" after reduction versus before, R_{PEG}, from the following equation based on the ^{1}H NMR measurements in chloroform-*d*/TFA-*d*:

$$R_{PEG}(\%) = \{I(PEG)_{red}/I(Phe)_{red}\}/\{I(PEG)_0/I(Phe)_0\} \times 100$$

where $I(PEG)_0/I(Phe)_0$ represents the relative intensity of the PEG methylene proton peak in the region of 4.02–3.62 ppm to the methine proton peak of Phe segments of PPhe homopolymer and PEG-*b*-PPhe in the region of 4.90–4.35 ppm before reduction, and $I(PEG)_{red}/I(Phe)_{red}$ represents the ratio after reduction. When a 5 mg/mL PEG-SS-peptide nanospheres dispersion was stirred with 42 mM DTT for 6h, the value of R_{PEG} was 28 ± 6%. This result clearly shows that about 72% of the PEG chains on the nanospheres were successfully detached, assuming that the amount of Phe segments was consistent before and after reduction. Furthermore, the ^{1}H NMR spectrum of the nanospheres after reduction in ethanol-d_6 shows no peaks at 4.3–2.7 ppm corresponding to the PEG methylene proton, in sharp contrast to the nanospheres before reduction (Fig. S6). This result indicates that there were no PEG chains on the nanosphere surface after reduction on the basis that ^{1}H NMR measurements in ethanol-d_6 provide the composition of the entire surface of the

nanospheres. Therefore, we conclude that almost all of the PEG on the surface of the nanospheres were detached upon cleavage of the disulfide bonds with a reductant to yield "hairless" nanospheres. On the basis of these findings, we defined "surface PEG percentage" as the ratio of the amount of PEG on "the nanosphere surface" after reduction versus before, and calculated from the following equation:

$$\text{surface PEG percentage} = (R_{\text{PEG}}–28)/(100–28) \times 100$$

As shown in Fig. 2a and S7, the PEG surface percentage decreased with increasing DTT concentration and reaction time, and reached to about 0%, supporting the our assumption that there were no PEG chains on the surface of the nanospheres after reduction with 42 mM DTT for 6 h as above.

The surface PEG percentage of the regenerated PEG brush peptide nanospheres after oxidation with thiol-monoterminated PEG (M_{w} = 2,000) was calculated in the same way as follows. We evaluated the ratio of the amount of PEG within "whole nanosphere" after oxidation versus before reduction, R'_{PEG}, from the following equation based on the ^{1}H NMR measurements in chloroform-*d*/TFA-*d*:

$$R'_{\text{PEG}}(\%) = \{I(\text{PEG})_{\text{oxi}}/I(\text{Phe})_{\text{oxi}}\}/\{I(\text{PEG})_{0}I(\text{Phe})_{0}\} \times 100$$

where $I(\text{PEG})_0/I(\text{Phe})_0$ represents the relative intensity of the PEG methylene proton peak to the methine proton peak of Phe segments of PPhe and PEG-*b*-PPhe before reduction, and $I(\text{PEG})_{\text{oxi}}/I(\text{Phe})_{\text{oxi}}$ represents the ratio after oxidation. Next, we calculated surface PEG percentage as the ratio of the amount of PEG on "the nanosphere surface" after oxidation versus

before reduction from the following equation:

$$\text{surface PEG percentage} = (R'_{\text{PEG}}-28)/(100-28) \times 100$$

The surface PEG percentage was increased with increasing oxidant concentration and reaction time, as shown in Fig. 2b and S8a, respectively, and reached up to about 100%. In contrast, it was decreased with increasing SH-PEG concentration, possibly due to the dimerization of SH-PEG (Fig. S8b).

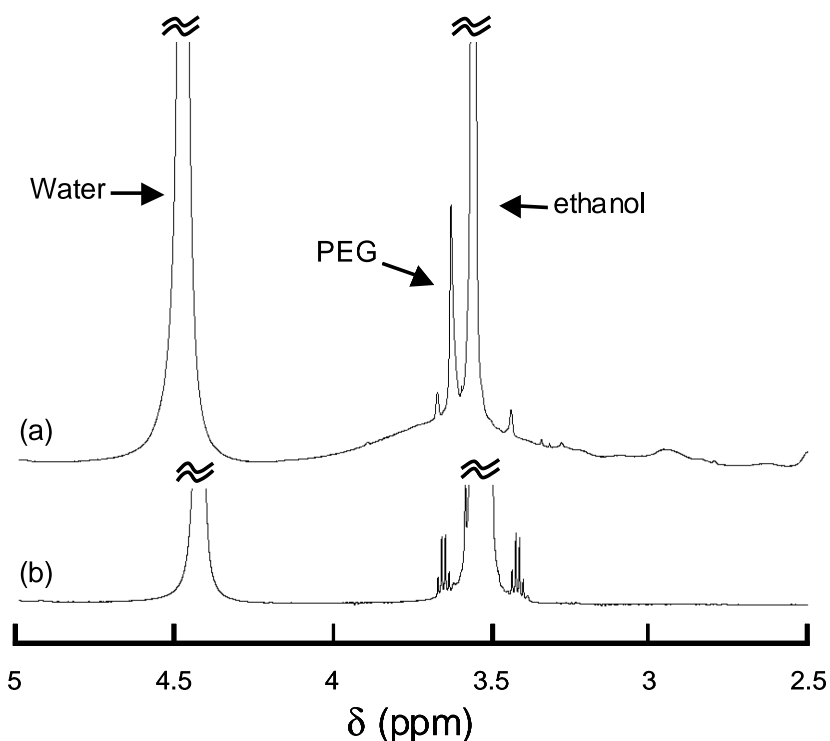


Figure S6. ^{1}H NMR spectra in ethanol-d_6 of PEG-SS-peptide nanospheres before (a) and after (b) reduction with molar excess of DTT over PEG within the nanosphere for 6 h at room temperature.

5. Time dependence of PEG detachment under reductive condition

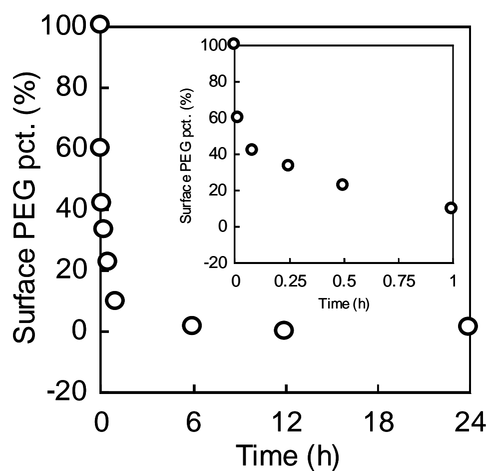


Figure S7. Time dependence of surface PEG percentage of PEG-SS-peptide nanospheres upon reduction. The PEG-SS-peptiide nanospheres (5 mg/mL) were stirred with 21 mM DTT in 25 mM tris buffer (pH 8.0) at room temperature. The inset is the expanded figure from 0 to 1 h.

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6. Time dependence of PEG attachment under oxidative condition
Relationship between PEG concentration and surface PEG percentage

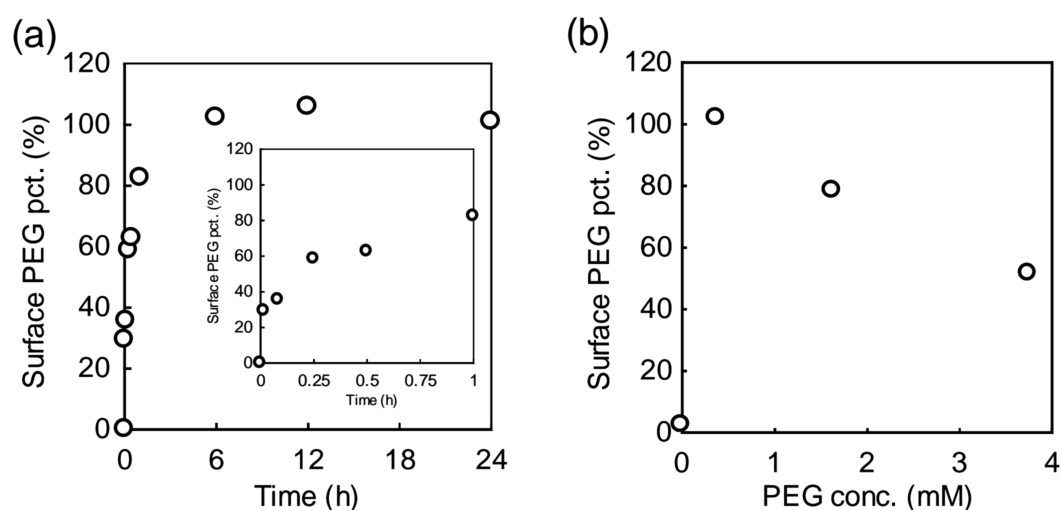

Figure S8. (a) Time dependence of surface PEG percentage of PEG-SS-peptide nanospheres after the remodification with thiol-monoterminated PEG (M_w = 2,000, SH-PEG) based on oxidation. 4 mg/mL of the "hairless" nanospheres dispersion were vigorously stirred with 0.375 mM SH-PEG (M_w = 2,000) in 10 mM KI/I_2 aqueous solution at room temperature. The inset is the expanded figure from 0 to 1 h. (b) Relationship between SH-PEG conc. and surface PEG percentage of SS-peptide nanospheres after the remodification. 4 mg/mL of the hairless nanospheres dispersion were vigorously stirred with PEG-SH (M_w = 2,000) at various concentration in 10 mM KI/I_2aqueous solution for 24 h at room temperature.

7. Immobilization of SH-monoterminated PEG with molecular weight 10,000

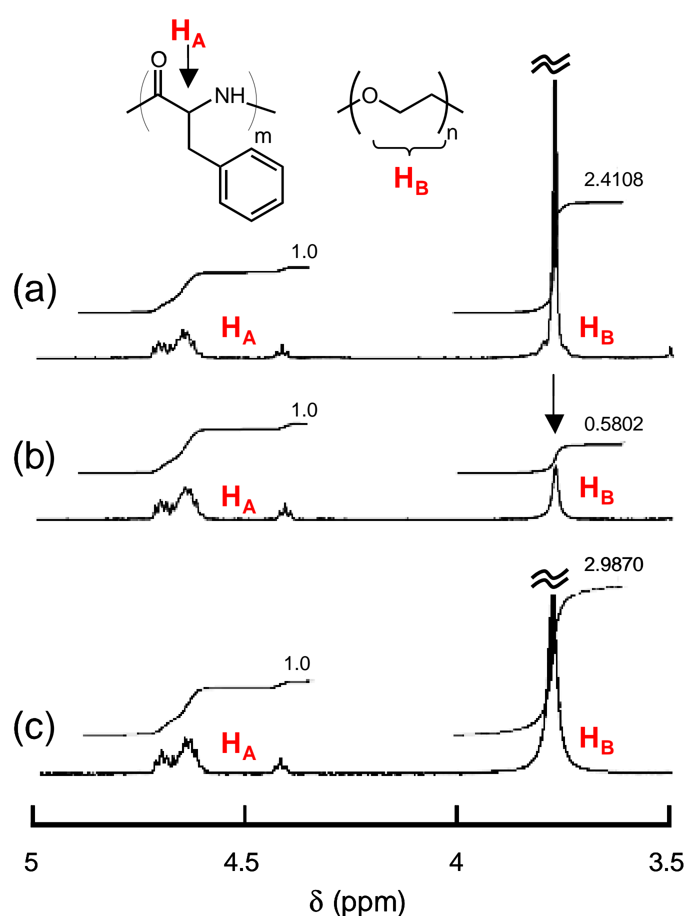

Figure S9. ^{1}H NMR spectra in TFA-*d*/chloroform-*d* of (a) PEG brush peptide nanospheres with disulfide linkage between the core and brush layer as prepared, (b) the brush layer-detached "hairless" peptide nanospheres after reduction with excess of DTT, and (c) the regenerated PEG brush peptide nanospheres after oxidation with thiol-monoterminated PEG (M_w = 10,000, SH-PEG). 4 mg/mL of the hairless nanospheres dispersion were vigorously stirred with SH-PEG (M_w = 10,000) at the concentration of 3.75 mM in 10 mM KI/I_2 aqueous solution for 24 h at room temperature.