Supporting Information

Protein-loaded comb-shape copolymer-based pH-responsive nanoparticles to improve the stability of proteins

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S1. Synthesis of lactobionic acid-poly (ethylene oxide)-ε-ploylysine-g-PLGA (LPEP)

Primary amines of EPL were protected by p-tolualdehyde (PT) groups according to the method of Hak Soo Choi et al. [Chemphyschem. 6 (2005) 1081-1086], with minor modifications. Briefly, 2 mmol of EPL were suspended in methanol (15 ml) and 3 mmol of PT were added to the solution while stirring at room temperature under a nitrogen atmosphere. After 6 h, chloroform (15 ml) and toluene (50 ml) were added to the mixture to extract the reactant. After removing the solvents, the mixture was precipitated in ethyl acetate and retrieved by filtration using a 0.2 μm membrane filter. The product was washed 3 times with n-hexane and water, respectively, and dried in vacuo for 48 h to give the PT-protected EPL (PTEPL). ^{1}H NMR (DMSO-d6): δ 7.85-7.21 (CH=CH of PT, 4H), 2.46 (CH_3 of PT, 3H), 10.05 (HC=O of PT, 1H); 1.40-1.89 ($C\delta H_2$-$C\gamma H_2$-$C\beta H_2$ of EPL, 6H), 3.25 ($C\varepsilon H_2$ of EPL, 2H), 3.94 (CαH of EPL, 1H).

The reaction of the primary amine of H_2N-PEG-LAC with the terminal carboxyl group of PTEPL was catalysed by EDC·HCl and HOBt as coupling agents. EDC·HCl (2 mmol) in DMF (5 ml) was poured into a solution of PTEPL (1 mmol) and HOBt (2 mmol) in DMF (15 ml) with one drop of triethylamine (TEA). The solution was stirred at room temperature for 0.5 h and allowed to be added dropwise to H_2N-PEG-LAC (1.5 mmol) in 30 ml DMF at 45 ℃. After 3 days under N_2, the product was poured into 200 ml of cold diethyl ether under vigorous stirring. The precipitate was filtered out and washed three times with ethanol and water, respectively, and dried in vacuo for 48 h to give the PTEPL-PEG-LAC. ^{1}H NMR (DMSO-d6): δ 3.12 (C_aH_2-N of PEG, 2H), 3.59 (CH_2-CH_2-O of PEG, 4H), 3.81 (C_bH_2-C_a of PEG, 2H); the other ppm values of the product were described above.

Deprotection of PT groups from PTEPL-PEG-LAC was achieved according to the method described previously [Chemphyschem. 6 (2005) 1081-1086]. Briefly, the PTEPL-PEG-LAC was dissolved in DMF, and then an excess amount of HCl aqueous solution (0.1 mol/L) was added to the organic solution to remove the protecting PT groups. The phase was shaken using a separatory funnel to separate the aqueous sample from the organic extractant. The water phase was concentrated and dried in vacuo at 50 ℃ to give EPL-PEG-LAC. The ppm values in ^{1}H NMR spectrum of the product (EPL-PEG-LAC) were described above.

A typical coupling reaction procedure of PLGA with EPL-PEG-LAC was as follows: EDC·HCl in DMF (20 ml) was poured into a solution of PLGA and HOBt in DMF (50 ml) with one drop of triethylamine (TEA). The solution was stirred at room temperature for 0.5 h and allowed to be added dropwise to EPL-PEG-LAC (1 mmol) in DMF at 45 ℃. After 3 days under N_2, the product was poured into 200 mL of cold diethyl ether under vigorous stirring. The precipitate was filtered out and washed with water and methylene chloride three times to remove small impurities and PLGA, respectively (The product was able to dissolve in DMF, but could not dissolve in methylene chloride). The filtrate, a yellow powder, was then acquired by vacuum drying as the final production (LPEPL).

It is noteworthy that the coupling reaction (step 2 and step 4) should be performed at a lower temperature (below 45 ℃) and under nitrogen atmosphere because oxidation of amine groups took place on exposure to air at high temperature.

The number of PLGA chains grafted onto LAC-PEG-EPL increased as the feeding ratio of PLGA to LAC-PEG-EPL increased in step 4 as shown in Table 1. In addition, with a

similar LAC-PEG-EPL/PLGA ratio, the graft degree of PLGA decreased as its molecular mass increased. The obtained results indicated that the steric hindrance of the association of PLGA with LAC-PEG-EPL backbone might increase the approaching resistance between LAC-PEG-EPL and the other free PLGA in step 4. During the same reaction time, the grafting degrees of PLGA could be controlled by the feeding ratio of PLGA to LAC-PEG-EPL, which is a very important factor to stabilize protein and adjust release rate of the carriers.

The ^{1}H NMR spectrum of the copolymer LPEP is demonstrated in Figure 1. The peak at δ 2.5 ppm was from the deuterated solvents DMSO (DMSO-d6). The peaks at δ 3.6-4.2 should be assigned to the protons on CH, CH_2 units of LAC. The peak at δ 3.5 arose from the methylene protons of the PEG segments. The peaks at δ 1.4-1.9, 3.3 and 4.0 came from $C\delta H_2$-$C\gamma H_2$-$C\beta H_2$, $C\varepsilon H_2$ and $C\alpha H$ of EPL, respectively. The peaks at δ 5.2, 4.9 and 1.5 arose respectively from the methane, methylene and methyl protons of the PLGA segments.

The molecular weights and structures of the copolymers were further confirmed by GPC, which were shown in Table 1. The products were much pure because only a single peak was appeared and the polydispersity index of molecular weights was small in the GPC measurement (the spectra were not shown). With the feeding ratio of LAC-PEG-EPL to PLGA at 1:4.4 (LPEP355) as well as careful fractionation, we obtained the product as three PLGA chains grafting onto one LAC-PEG-EPL molecule. Because the molecular weight of LAC-PEG-EPL is 9.44 kDa (0.36 kDa+5.00 kDa+4.08 kDa), after PLGA (5.00 kDa) chains reacted with LAC-PEG-EPL, the corresponding coupled product had about 15.1 kDa (≈5.00 kDa×3) larger Mn than the original LAC-PEG-EPL. Similarly, with the feeding ratio of

LAC-PEG-EPL to PLGA at 1:8.1 (LPEP655) and 1:16.7 (LPEP855), the products had six and eight PLGA chains grafting onto one LAC-PEG-EPL molecule, respectively. The results confirmed that the reaction occurred as expected and the graft degrees of PLGA could be controlled by the feeding ratio of PLGA to LAC-PEG-EPL.

The thermal behavior of the copolymer was investigated by DSC [36] and the data were also shown in Table 1. Compared with the value 32.3 ℃ for PLGA, the Tg of LPEP355 was found to have increased to 36.2 ℃. This might be due to the introduction of the more PLGA segments onto Lac-PEG-EPL, which would need more space and thermal motion energy to move during the glass transition. DSC thermograms of LPEP355, LPEP655 and LPEP855 were also carried out and shown in Table 1. Tg of the samples were 36.2 ℃, 40.7 ℃ and 43.5 ℃, respectively. Tg increased slightly with increasing the PLGA graft degree in the copolymer, which demonstrated that the increase of PLGA graft degrees might result in a more dense structure of the copolymer and need more thermal motion energy to move during the glass transition.

S2. Determination of surface composition of NPs with ^{1}H-NMR

LPEP was dissolved in 0.2 ml of DMSO-d6 at the concentration of 10 mg/ml and 2 ml D_2O was set in a 10 ml centrifugal tube as aqueous phase. Finally, Lac-PEG-EPL-g-PLGA solution was added dropwise to the aqueous phase under stirring over 1 h. The mixture solution was then transferred into an ultrafiltration tube (100 kDa cutoff), centrifuged at 4 ℃ (8000 rpm, 30 min) and washed by D_2O 3 times for purification. NPs were subsequently re-suspended in 1.0 ml D_2O for ^{1}H NMR analysis.

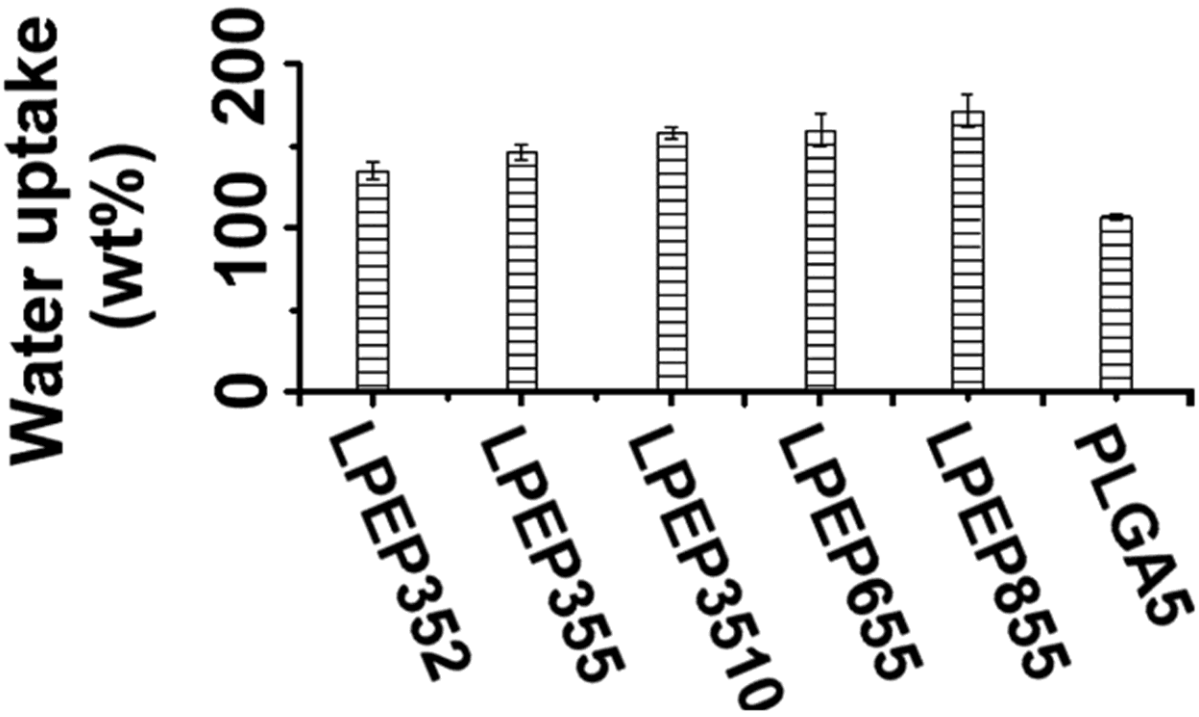
S3. Water uptake of NPs

Briefly, a sample of the NPs in weight of 50 mg was dispersed in 1 ml double distilled water, and shaken at 100 rpm at 37 ℃ using a constant-temperature shaker (SHA-B, Guohua Co., Ltd., China). A blank ultrafiltration tube (100 kDa cutoff) was weighed on an electronic microbalance (AE 240, Mettler, Switzerland) to an accuracy of ±0.01 mg (W_a). After 48 hours, the nanoparticle solution was transferred into the ultrafiltration tube (10 kDa cutoff), centrifuged at 25 ℃ (5000 rpm, 30 min), and the NPs were blotted off carefully in between sheets of tissue paper to remove the surface-adhered liquid, and then weighed together with the ultrafiltration tube on the electronic microbalance to an accuracy of ±0.01 mg (W_b). The weight of the swollen sample was acquired as the absolute value of the difference of W_a and W_b. The percentage of equilibrium water uptake was calculated as follows:

$$Water\ uptake\ (\%) = (W_w - W_d)/W_d \times 100\% \qquad (eq.\ 1)$$

where W_w is the weight of the swollen sample, and W_d is the initial weight. All samples were analyzed in triplicate and the error bars in the plot were the standard deviation.

The water uptake of NPs was shown in Figure S3.



Supplemental Figure S3. Water uptake of LPEP NPs and PLGA NPs.

S4. Degradation of NPs

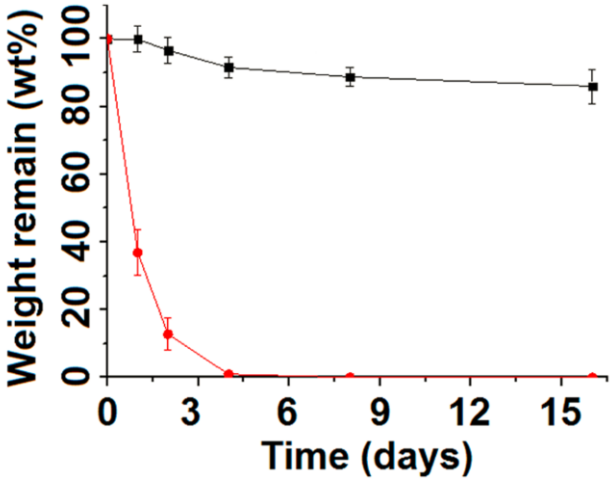
Aliquot 300 mg freeze-dried NPs into 5 centrifuge tubes, with 60 mg per tube, marked as ABCDE. Each tube then was filled with 5 ml D_2O to re-disperse the NPs with horizontal shaking at 37 ℃. At predetermined time points (day 1, 2, 4, 8, 16), ABCDE were centrifuged at 25,000 rpm for 20 min, respectively. Each supernatant fluid was measured by ^1H NMR to investigate Lac-PEG-EPL-g-PLGA NPs degradation in detail. In addition, the sedimentation was lyophilized and then weighed on an electronic microbalance to an accuracy of ±0.01 mg. All samples were analyzed in triplicate and the error bars in the plot were the standard deviation.

The residual mass was calculated according to the following formula:

$$Residual\ mass\ (\%) = W_c/W_i \times 100\% \qquad (eq.\ 2)$$

where W_i and W_c represented the initial weight of the sample and that of constant weight after dried on the predetermined day, respectively.

The molecular weight of the lyophilized sedimentation was also estimated by Gel Permeation Chromatography (GPC) using a Waters system (Waters, USA).



Supplemental Figure S4. Weight loss of EPL versus time in the solution of lactic acid (red circle ●) and PBS (0.1 M, pH 7.4) (black square ■). The concentration of lactic acid was 0.2 mmol/ml; the concentration of EPL was 0.5 mg/ml.

S5. Cell toxicity assays and acute toxicity study

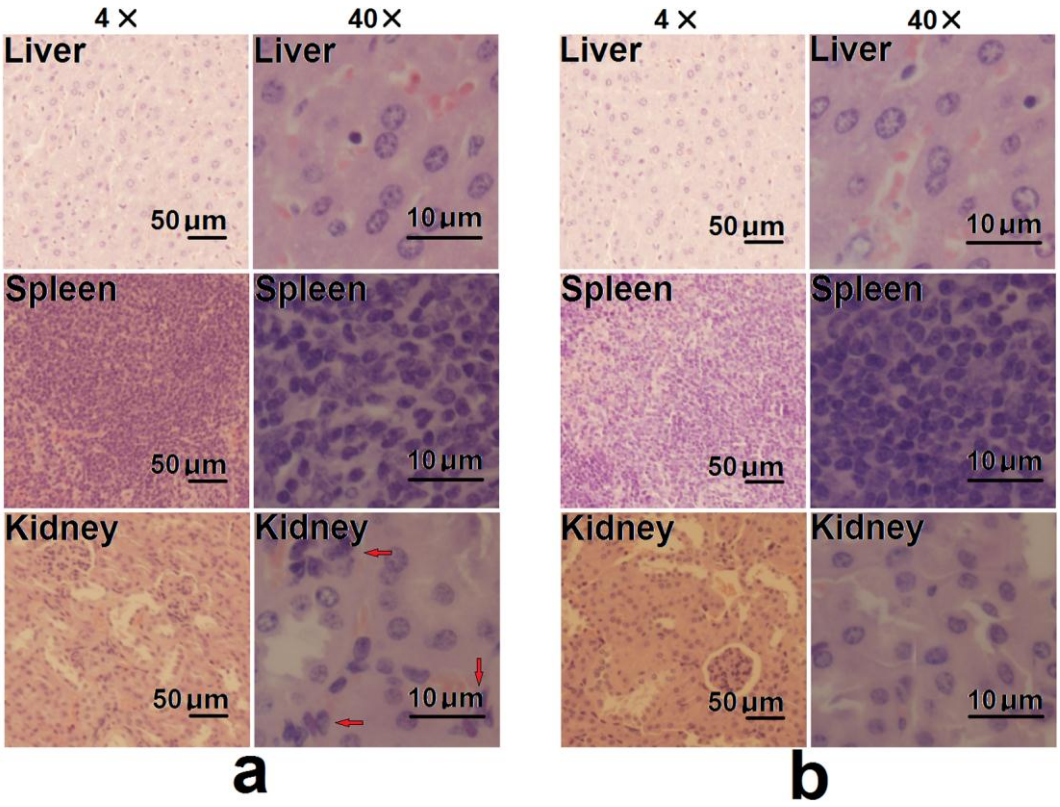
The cell line was cultured in DMEM (pH 7.4) in a humidified atmosphere (5% CO_2/95% air). The cells were seeded into 96-well plates at 5,000 cells per well. The plates were then returned to the incubator and the cells were allowed to grow to confluence for 24 h. The blank nanoparticle solutions were diluted with culture medium to give suitable concentrations. Then the medium in the wells was replaced with the pre-made culture medium-sample mixture (200 μl). The plates were returned to the incubator and maintained in 5% CO_2 at 37 ℃ for 48 and 96 h. Each sample was tested in triplicate. After incubation,

the fresh culture medium and 20 μL of MTT solution at a MTT concentration of 1 mg/ml were used to replace the mixture in each well. The plates were then returned to the incubator and incubated for another 4 h in 5% CO_2 at 37 ℃. Then, the culture medium and MTT were removed. DMSO (150 μl) was subsequently added to each well to dissolve the formazane crystals. The plate was placed in 5% CO_2 at 37 ℃ for 15 min before measurement. The optical density was read on a microplate reader at 492 nm. Cells untreated with the solution of blank NPs were used as the control and their viability was set to 100%. Cell viability was determined as a percentage of the control. Each experiment was performed in triplicate and the results were reported as the average (mean ± standard deviation) of triplicated wells. The cell viability in each well was calculated as follows:

$$Cell\ viability\ (\%) = (Abs_{test\ cells} / Abs_{control\ cells}) \times 100\% \qquad (eq.\ 3)$$

which $Abs_{test\ cells}$ and $Abs_{control\ cells}$ represent the amount of formazan determined for cells treated with the different concentration of NPs and for control cells (nontreated), respectively.

On the fourteenth day after intravenous injection of the acute experiment, the mice were sacrificed and three internal organs (liver, kidney and spleen) were acquired, and the tissues were immediately fixed with 10% formalin, dehydrated and embedded in paraffin wax. Each embedded specimen was sectioned and the sections were stained with hematoxylin and eosin (H&E). Images were visualized under an Olympus IX81 microscope equipped with Meta Image Series [Int. J. Pharm. 420 (2011) 266-273] as shown in Supplemental Figure S5.



Supplemental Figure S5. H&E-stained section of EPL, PLGA NPs and LPEP NPs. (a) H&E-stained section of liver, spleen and kidney after injection of EPL in vivo. (b) H&E-stained section of liver, spleen and kidney after injection of LP355 NPs in vivo.

S6. pH inside PLGA and LPEP NPs

Fluorescent dye encapsulated NPs were prepared as described in "2.3.1" with some modification, in which the model protein was replaced by SNARF-1 dextran. Briefly, the polymer was dissolved in 0.1 ml of DMF at the concentration of 10 mg/mL as an organic phase. SNARF-1 dextran was dissolved in 1.0 ml double distilled water of 4.0 mg/ml as an aqueous phase. Finally, the organic phase was added dropwise to the aqueous phase under stirring over 1 hour. The mixture solution was then transferred into a dialysis membrane (0.35 kDa cutoff), and dialyzed against 5000 mL of PBS (0.1 M, pH 7.4) for 36 hours to removed

DMF. At predetermined times, Fluorescent dye loaded NPs solution was transferred into an ultrafiltration tube (100 kDa cutoff, 400 μl) and centrifuged at 4 ℃ (8,000 rpm, 30 min). The NPs were washed by 20 μl×3 double distilled water and the eluate was combined with the initial eluates. The concentration of free SNARF-1 dextran in the final eluate was measured by fluorescence spectrophotometer. The loaded SNARF-1 dextran in NPs was calculated by subtracting free SNARF-1 dextran from the total SNARF-1 dextran. SNARF-1 dextran loaded NPs were re-dispersed in PBS (0.1 M, pH 7.4) to maintain the concentration of SNARF-1 dextran at 0.8 mg/ml. 15 μl of NP solution was drawed out and put uniformly between two coverslips for obtaining confocal images immediately.

In order to map the microclimate pH inside NPs, a ratiometric method was utilized, based on imaging with a laser scanning confocal microscope (LSCM, Leica TCS SP2) as the modified procedures described previously [J. Control. Release 101 (2005) 163-173]. Briefly, SNARF-1 dextran encapsulated in the NPs was excited at 488 nm by an Ar/He laser, and two images at 580 and 640 nm were taken, the ratio image I_{640}/I_{580} was correlated with pH from a standard curve independent of dye concentration. An image at a wavelength corresponding to the isosbestic point (610 nm, pH-insensitive) of the dye was also examined to estimate the concentration of the dye. All measurements were conducted using a 63×oil immersion objective with numerical aperture of 1.4.

The standard curve of pH versus fluorescent intensity ratio (I_{640}/I_{580}) was determined using 0.1 mol/L sodium phosphate buffer solutions from pH 5.8 to 8.0. Confocal images at different wavelengths (580, 610 and 640 nm) were obtained from fixed pH solutions and the standard curve of pH versus I_{640}/I_{580} was acquired by choosing the appropriate image

processing steps. The data of standard curves could be fitted with the following equation:

$$pH = CON + lg\ ((R-R_{min})/(R_{max}-R)) \qquad (eq.\ 4)$$

where R_{min} and R_{max} represent the 640/580 ratios of the fully protonated and fully deprotonated SNARF-1 dextran, respectively. CON is equal to pKa+lg (I_{f580}/I_{b580}), in which I_{f580} and I_{b580} are the fluorescence values at 580 nm for the deprotonated and protonated forms of the dye, respectively [Biochem. Pharmacol. 57 (1999) 1037-1046]. The parameters, CON, R_{min}, R_{max}, are constant at the same concentration and can be acquired after fitting with the equation above. The criterion for selecting the most appropriate model was based on the adjusted coefficient of determination ($R^2_{adjusted}$).

An appropriate volume of PBS should be added into fluorescent dye loaded NPs, ensuring the SNARF-1 dextran loaded in NPs with the concentration of 0.8 mg/ml. SNARF-1 dextran encapsulated in the NPs was excited at 488 nm by an Ar/He laser, and three images at 580, 610 and 640 nm were taken, the ratio image I_{640}/I_{580} was correlated with pH from a standard curve at dye concentration of 0.8 mg/ml. In the pH mapping method, we selected the rigorous processing algorithm (averaged 27 frames, plus frame averaging, neighborhood averaging, median filter and threshold application). The overall procedure of image processing is summarized as described by Lei Li et al. [J. Control. Release 101 (2005) 163-173]. Briefly, the fluorescent images from three different wavelengths were read into three corresponding two-dimensional matrices, r_{xy} (580 nm), g_{xy} (640 nm) and i_{xy} (610 nm) (x, y represent the coordinate of the pixel). Then, image-processing algorithms (frame averaging, neighborhood averaging and median filter) was used to process three matrices and three new matrices R_{xy} (580 nm), G_{xy} (640 nm) and I_{xy} (610 nm) were acquired. Then,

$RATIO_{xy}=G_{xy}/R_{xy}$. If I_{xy} is larger than threshold, $RATIO_{xy}= G_{xy}/R_{xy}$, else $RATIO_{xy}=0$. The new signal distribution was fit with a Gaussian distribution, and the corresponding standard deviation was obtained. By using a standard curve obtained from fixed pH solutions, the pixel-by-pixel pH was then obtained.

The standard curve of pH vs. intensity ratio was fitted with eq. 4 as shown in Supplemental Table S6.

Supplemental Table S6 Kinetic fitting results of SNARF-1 dextran standard curve

CSD	$pH = CON + \lg((R-R_{min})/(R_{max}-R))$			
	CON	R_{min}	R_{max}	$R^2_{adjusted}$
0.8 mg/ml	7.63	0.72	2.48	0.9996

CSD represents concentrations of SNARF-1 dextran in PBS (0.1 M, pH 7.4).

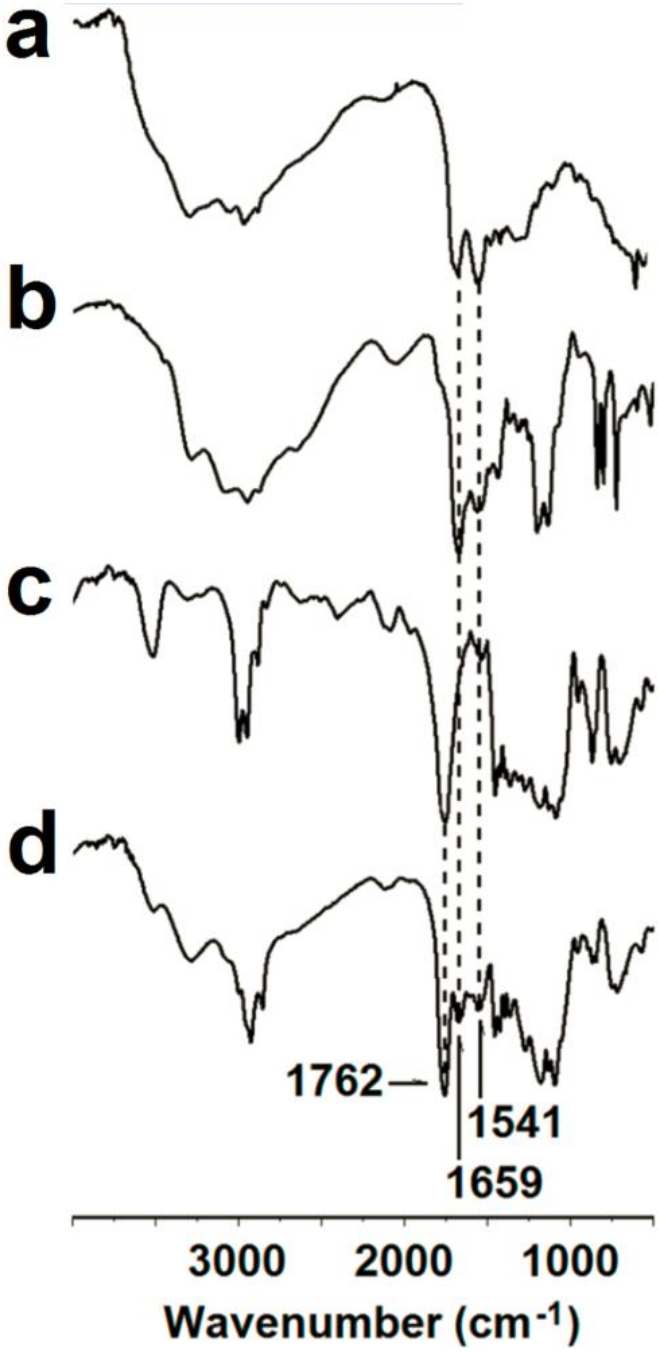
S7. Circular dichroism spectroscopy and FTIR

BSA loaded NPs were prepared as described above '2.3.1'. 30 mg NPs were dispersed in 1 ml sterilized double distilled water. At specified time intervals (2, 4, 8 and 16 days), the solution was transferred into a 5 ml ultrafiltration tube (100 kDa cutoff), centrifuged at 4 ℃ (8,000 rpm, 30 min), and the concentration of protein released was measured by BCA protein colorimetric assay based on the manufacturer's instructions. The NPs were redissolved in 1 ml sterilized double distilled water for the continued release.

The stability of the released protein (with the same concentration in double distilled water) from NPs was determined by measuring circular dichroism spectra, which were recorded on a Jasco-715 Spectropolarimeter (JASCO, Tokyo, Japan) at 37 ℃ under a constant flow of nitrogen gas. Typically a cell with a 1 mm path length was used for spectra recorded between 190 and 260 nm with sampling points every 0.1 nm. The spectra

represented the average of 8-20 scans and CD intensities were expressed in mdeg.

In order to determine whether the stability of BSA was affected by contact with LPEP, the interaction between BSA and LPEP355 NPs was investigated by FTIR 29 as shown in Supplemental Figure S7.



Supplemental Figure S7. FTIR spectra of (a) BSA, (b) EPL, (c) PLGA NPs, (d) BSA loaded LPEP355 NPs.

S8. Distribution and Pharmacokinetics of BSA-FITC-loaded Carriers in vivo

The in vivo tests of carriers were carried out using eighty Kunming mice (40 male and 40 female) weighing about 20 g each. The animals were randomly distributed into eight groups (termed as ABCDEFGH), containing ten animals per group.

Testing solutions containing BSA-FITC loaded Lac-PEG-EPL-g-PLGA NPs (group A), blank Lac-PEG-EPL-g-PLGA NPs (as the control of BSA-FITC-loaded Lac-PEG-EPL-g-PLGA NPs) (group B); PEG-EPL-g-PLGA NPs (group C), blank PEG-EPL-g-PLGA NPs (as the control of BSA-FITC loaded PEG-EPL-g-PLGA NPs) (group D); EPL-g-PLGA NPs (group E), blank EPL-g-PLGA NPs (as the control of BSA-FITC-loaded EPL-g-PLGA NPs) (group F) and free BSA-FITC (group G) were used in the in vivo experiments. 400 μl solution, with a predetermined amount (the same amount of BSA-FITC for each fluorescence acquired animal), was injected though tail vein. The control group (group H) animals were injected with 400 μl sterilized sodium chloride solution (0.9%) at the same time of testing groups.

For the in vivo detection of BSA-FITC in blood, an aliquot of blood was drawn from the tail vein of each animal at predetermined time points, 1, 2, 4, 8 and 16 days, a 0.20 ml aliquot of blood from the tail vein was collected into an Eppendorf tube, which was mixed with 0.04 ml of heparin (1 wt% in sodium chloride solution (0.9%)), followed by gentle shaking. To obtain the plasma, the blood solution was centrifuged at 5,000 rpm for 10 min at 25 ℃. 100 μl plasma samples were acquired and diluted with sodium chloride solution (0.9%) to 3 ml for fluorescence spectroscopy measurement (F-4500, Hitachi, Tokyo, Japan).

The amount of BSA-FITC at different time points was determined based on a linear

standard curve established with BSA-FITC controls. We prepared BSA-FITC standard solutions of known concentrations with 100 μl animal plasma each solution from group H for the standard curve. The amount of existed BSA-FITC in vivo at predetermined time points was estimated by comparison with the standard curves. For each group of ten rats treated with a particular formulation of solution, the in vivo experiment was separately performed on each animal and the results were averaged.

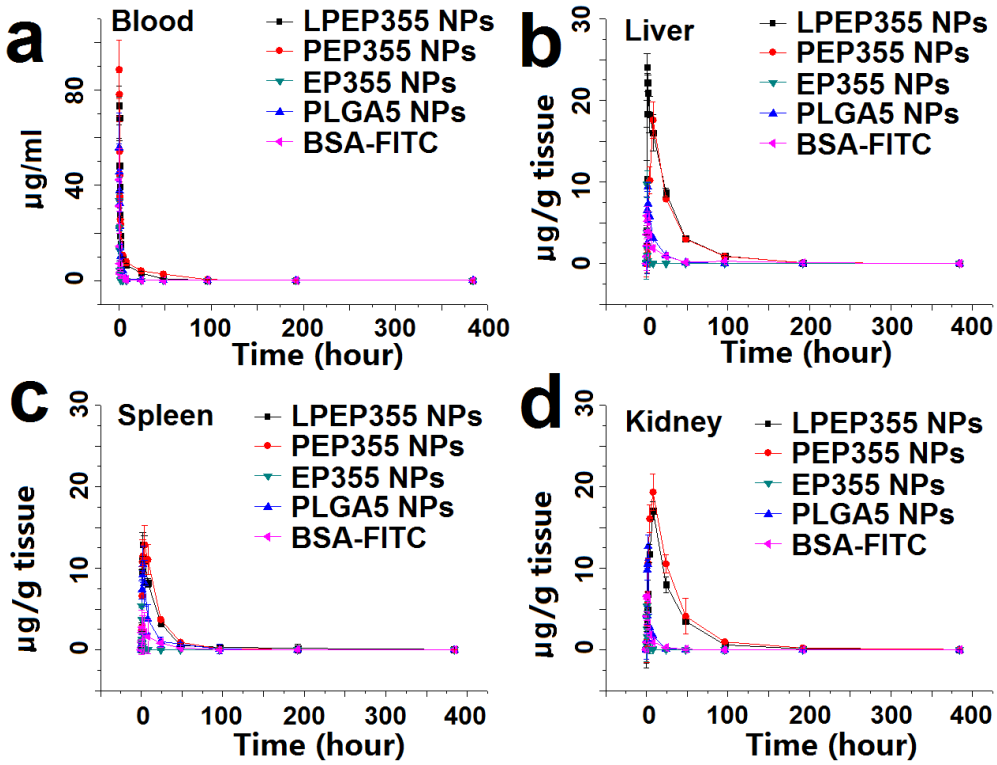
In order to detect the amount of BSA-FITC accumulated in the tissues (liver, kidney and spleen), at predetermined time points, 1, 2, 4, 8 and 16 days, the animal were euthanized and liver, kidney and spleen were removed. After washing 3 times with physiological saline to remove blood, the organ was blotted off carefully in between sheets of tissue paper to remove the surface-adhered liquid, and weighed together with the ultrafiltration tube on the electronic microbalance to an accuracy of ±0.01 mg. The organ was subsequently transferred to a homogenizer to make tissue homogenate. Subsequently, the homogenate was diluted with 1 ml physiological saline, transferred into a 15-ml centrifuge tube, centrifuged at 8,000 rpm for 30 min at 4 ℃. Then, the supernatant was collected and the sedimentation was washed with physiological saline (1 ml×2). The supernatants were combined and diluted to 5 ml. The impurities were removed by a microporous membrane with an aperture of 0.22 μl. The samples were measured by fluorescence spectroscopy (F-4500, Hitachi, Tokyo, Japan) for the concentration of BSA-FITC.

AUC_{t0-t} values were calculated by measuring the area under the BSA-FITC curves from t0 to time t using the trapezoidal rule [J. Control. Release 157 (2012) 391-397].

The distribution profiles of free BSA-FITC and carriers loaded with BSA-FITC in

Kunming mice for 384 hours after intravenous administration were shown in Supplemental Figure S8.

The plasma levels of free BSA-FITC (as the control) and other BSA-FITC loaded formations in vivo rapidly declined over time as single exponential decay curves as shown in Figure 8a. The plasma clearance rate of free BSA-FITC, PLGA5 and EP355 NPs showed a sharp decrease as shown in Figure 8a. By comparing the AUC_{t0-t} results of PEGylated carriers with that of free BSA-FITC, we found that the fluorescence levels of PEG modified carriers were markedly higher than that of the control, PLGA5 and EP355 NPs at the same time point ($p < 0.05$). This result indicated that PEGylated carriers showed favorably long circulation properties compared with free BSA-FITC and other carriers.



Supplemental Figure S8. Time course of BSA-FITC concentration in plasma (a) and tissues [liver (b), spleen (c) and kidney (d)] for 16 days after intravenous injection of free BSA-FITC solution and BSA-FITC loaded carriers.