Supporting Information

Small-Sized and Robust Chimaeric Lipopepsomes: A Simple and Functional Platform with High Protein Loading for Targeted Intracellular Delivery of Protein Toxin In Vivo

Min Qiu, Zhenqi Zhang, Yaohua Wei, Huanli Sun, Fenghua Meng, Chao Deng*, and Zhiyuan Zhong*

Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, 215123, China.

*Corresponding authors. Tel./Fax: +86-512-65880098. E-mail: cdeng@suda.edu.cn (C. Deng), zyzhong@suda.edu.cn (Z. Zhong).

Materials. α-Methoxy-ω-amine-poly(ethylene glycol) (mPEG-NH_2, M_n = 5.0 kg/mol, Creative PEG Works), acrylate-PEG-NH_2 (AA-PEG-NH_2, M_n = 6.0 kg/mol, Suzhou Nord Derivatives Pharm-tech co. Ltd), saporin (SAP, Sigma), cytochrome C (CC, Sigma), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma), trypsin (Jinuo Biomedical Technology) and Phalloidin-Tetramethly-rhodamine B isothiocyanate (Sigma) were used as received. All the other reagents and solvents were purchased from Sinopharm Chemical

Reagent Co. Ltd. and used as received. α-Aminopalmitic acid *N*-carboxyanhydride (APA-NCA) was synthesized as our previous report.[1]

Characterization. ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 600 MHz using $CDCl_3$ as a solvent. The chemical shifts were calibrated against the solvent signal. The molecular weight and polydispersity index of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PL gel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using $CHCl_3$ as the eluent at a flow rate of 0.8 mL/min at 40 °C and a series of narrow polystyrenes standards for the calibration of the columns. The size of lipopepsomes was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C by a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He−Ne laser using backscattering detection. The zeta potential of lipopepsomes was determined with a Zetasizer Nano-ZS from Malvern Instruments equipped with electrophoresis. The radius of gyration (R_g) of cRGD-LPP was measured by multi-angle laser light scattering performed using a Wyatt Technology DAWN HELEOS 18 angle (from 40^o to 150^o) light scattering detector using Ga laser (658 nm, 50 mW). The concentration of cRGD-CLP varied from 0.04 to 0.1 mg/mL and concentrations were analyzed by the graphical method reported by Zimm. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 20 μL of 1 mg/mL lipopepsome suspension on the copper grid followed by staining with 1% uranyl acetate.

Critical aggregation concentration (CAC). The CAC was determined using pyrene as a fluorescence probe as previously reported.[2] The concentration of lipopepsomes varied from

1.2×10^{-5} to 0.1 mg/mL and the concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra were recorded using a FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CAC was calculated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

***In vitro* cytotoxicity assays.** The cytotoxicity of blank cRGD-CLP and CLP was determined using A549 cells. Cells were plated in a 96-well plate (1×10^4 cells/well) and cultured in RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 24 h, prescribed amounts of cRGD-CLP and CLP in 20 μL of PBS were added (final lipopepsome concentrations were fixed at 0.1, 0.2, 0.5 and 1.0 mg/mL) and incubated in an atmosphere containing 5% CO_2 for 48 h at 37 °C. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) solution in PBS (10 μL, 5 mg/mL) was added. After incubation for 4 h, the supernatant was carefully aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO for 10 min. The absorbance at a wavelength of 490 nm was measured using a microplate reader (Bio-Tek, ELX808IU). The cell viability was determined by comparing the absorbance of MTT-formazan at 490 nm with control wells containing only cell culture medium. The experiments were performed in quartets.

The antitumor activity of SAP-cRGD-CLP, SAP-CLP and free saporin was evaluated in $\alpha_v\beta_3$ integrin-overexpressing A549 human lung cancer cells. Briefly, the cells were plated in a 96-well plate (2×10^3 cells/well) using RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 24 hours, prescribed amounts of SAP-cRGD-CLP, SAP-CLP and free saporin at different saporin concentrations in 20 μL of PBS were added. The cells were incubated in an

atmosphere containing 5% CO_2 for 4 h at 37 °C. The medium was aspirated and replaced by fresh medium. The cells were further incubated in an atmosphere containing 5% CO_2 for another 92 h at 37 °C. MTT solution in PBS (10 μL, 5 mg/mL) was added. After 4 h, the supernatant was carefully aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO for 10 min. The cell viability was determined as described above.

Cellular uptake and intracellular protein release behaviors. The cellular uptake and intracellular protein release behaviors of FITC-CC-cRGD-CLP and FITC-CC-CLP were studied in $\alpha_v\beta_3$ integrin-overexpressing A549 human lung cancer cells through flow cytometry and confocal laser scanning microscopy (CLSM). For flow cytometry, the cells were seeded in a 6-well plate (5×10^6 cells/well) and cultured in RPMI-1640 media containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 24 h. 100 μL of FITC-CC-cRGD-CLP or FITC-CLP solution in PBS (FITC-CC dosage: 40 μg/ mL) was added to each well. After incubation at 37 ^oC for 4 h, the cells were digested by trypsin (0.25%, w/v) containing EDTA (0.03%, w/v). The cell suspensions were centrifuged at 1000 ×g for 3 min at 4 °C, washed twice with PBS, and then re-suspended in 500 μL of PBS. Fluorescence histograms were recorded with a BD FACS Calibur flow cytometer (Becton Dickinson, USA) and analyzed using FCS Express 5 Plus Research Edition.

For confocal microscopy observation, A549 cells were cultured on microscope slides in a 24-well plate (2×10^4 cells/well) using RPMI-1640 media containing 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, 100 μL of FITC-CC-cRGD-CLP or FITC-CC-CLP solution in PBS (FITC-CC dosage: 40 μg/ mL) was added to each well. After 4 h incubation at 37 ^oC, the culture medium was refreshed,

and the cells were further cultured for 8 h. After removing the culture medium, the cells were fixed with 4% paraformaldehyde solution for 15 min, cytoskeleton were stained by phalloidin-tetramethyl-rhodamine B isothiocyanate (red) for 40 min, and the cell nuclei were stained with DAPI (blue) for 5 min. The fluorescence images were obtained using a confocal microscope (Leica TCS SP2).

***In vivo* blood circulation and biodistribution.** To study the blood circulation of CLP, Cy5-labeled CLP (cRGD-CLP-Cy5 or CLP-Cy5) (Cy5 dosage = 6 nmol) was intravenously injected to Balb/c mice. Blood samples were collected at 0.05, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post-administration, and the Cy5 amount at each time point was determined through fluorescence spectrum measurement. The elimination half-life ($t_{1/2,\beta}$) was calculated by fitting the experimental data using software Origin 9 exponential decay 2 model: $y = A_1 \times \exp(-x/t_1) + A_2 \times \exp(-x/t_2) + y_0$, and then taking $t_{1/2,\beta} = 0.693 \times t_2$.

For *in vivo* biodistribution studies, orthotopic A549 tumor xenografts were acquired by injecting 5×10^6 bioluminescent A549-Luc cells suspended in 100 μL of PBS/matrigel (4/1, v/v) into the left lung parenchyma of mice, as our previous report.[2] The tumor-bearing mice were intravenously (*i.v.*) injected with Cy5-CC-cRGD-CLP, Cy5-CC-CLP, or free Cy5-CC in 200 μL PBS via tail vein (0.4 μmol Cy5-CC equiv/kg). At predetermined time points (6 h) post *i.v.* injection, the tumor and major organs were collected, washed, weighed, and then homogenized in 0.5 mL of 1% Triton X-100 with a homogenizer (IKA T25). Cy5-CC was extracted by DMSO solution and quantified by fluorescence measurement.

Histological analysis. One mouse of each group was sacrificed at day 16 for histological analysis. The heart, liver, spleen, lung, and kidney were excised, fixed with 4% paraformaldehyde solution, embedded in paraffin, and cut into slices (4 μm thick). The slices

mounted on the glass slides were stained with hematoxylin and eosin (H&E) and observed by a digital microscope (Leica QWin).

Statistical analysis. Data were expressed as mean ± SD. Difference between groups was assessed using the one-way analysis of variance (ANOVA). Survival results were analyzed by the Kaplan-Meier technique using Graphpad Prism software. A log-rank test for comparisons was used. *p<0.05 was considered significant, and **p<0.01, ***p<0.001 were considered highly significant.

References

(1) Qiu, M.; Ouyang, J.; Sun, H.; Meng, F.; Cheng, R.; Zhang, J.; Cheng, L.; Lan, Q.; Deng, C.; Zhong, Z. Biodegradable Micelles Based on Poly(Ethylene Glycol)-*b*-Polylipopeptide Copolymer: A Robust and Versatile Nanoplatform for Anticancer Drug Delivery. *ACS Appl. Mater. Interfaces* **2017,** *9*, 27587-27595.

(2) Chen, P.; Qiu, M.; Deng, C.; Meng, F.; Zhang, J.; Cheng, R.; Zhong, Z. pH-Responsive Chimaeric Pepsomes Based on Asymmetric Poly(Ethylene Glycol)-*b*-Poly(L-Leucine)-*b*-Poly(L-Glutamic Acid) Triblock Copolymer for Efficient Loading and Active Intracellular Delivery of Doxorubicin Hydrochloride. *Biomacromolecules* **2015,** *16*, 1322-1330.

(3) Wu J., Zhang J., Deng C., Meng F., Cheng R., Zhong Z., Robust, Responsive, and Targeted PLGA Anticancer Nanomedicines by Combination of Reductively Cleavable Surfactant and Covalent Hyaluronic Acid Coating. *ACS Appl. Mater. Interfaces* **2017**, 9, 3985-3994.

Table S1. Characterization of PEG-*b*-PAPA-*b*-PBLA triblock copolypeptide.

Entry	Copolypeptide	M_n (kg/mol)			M_w/M_n[b]	Yield (%)
		Design	^{1}H NMR[a]	GPC[b]		
1	PEG-*b*-PAPA-*b*-PBLA	5.0-11.0-3.5	5.0-10.1-2.7	24.7	1.31	83
2	AA-PEG-*b*-PAPA	6.0-11.0	6.0-11.3	19.0	1.27	80

[a]Calculated from ^{1}H NMR. [b]Determined by GPC (eluent, $CHCl_3$, flow rate, 0.8 mL/min; temp., 40 ^oC; standard, polystyrene).

Table S2. Characterization of blank lipopepsomes.

Entry	Lipopepsomes	Size[a] (nm)	PDI[a]	Zeta[b] (mV)	CAC[c] (mg/L)
1	cRGD-CLP	80±2.8	0.14	-9.5	1.46
2	CLP	83±2.1	0.17	-7.2	1.03

[a]Size and PDI of lipopepsomes were determined by DLS. [b]Measured by electrophoresis at 25 ^oC in PB. [c]Determined by fluorescence measurement.

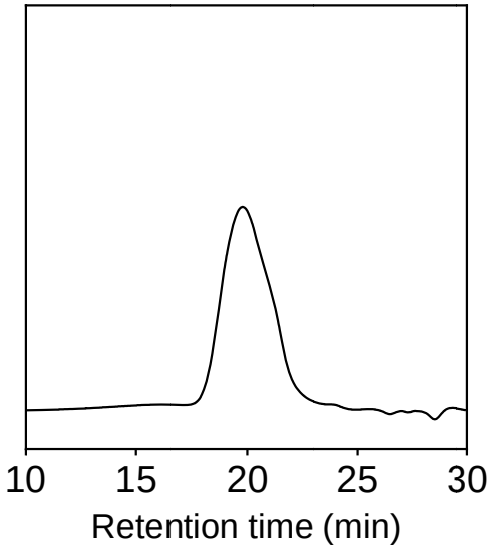

Figure S1. GPC chromatogram of PEG-*b*-PAPA-*b*-PBLA.

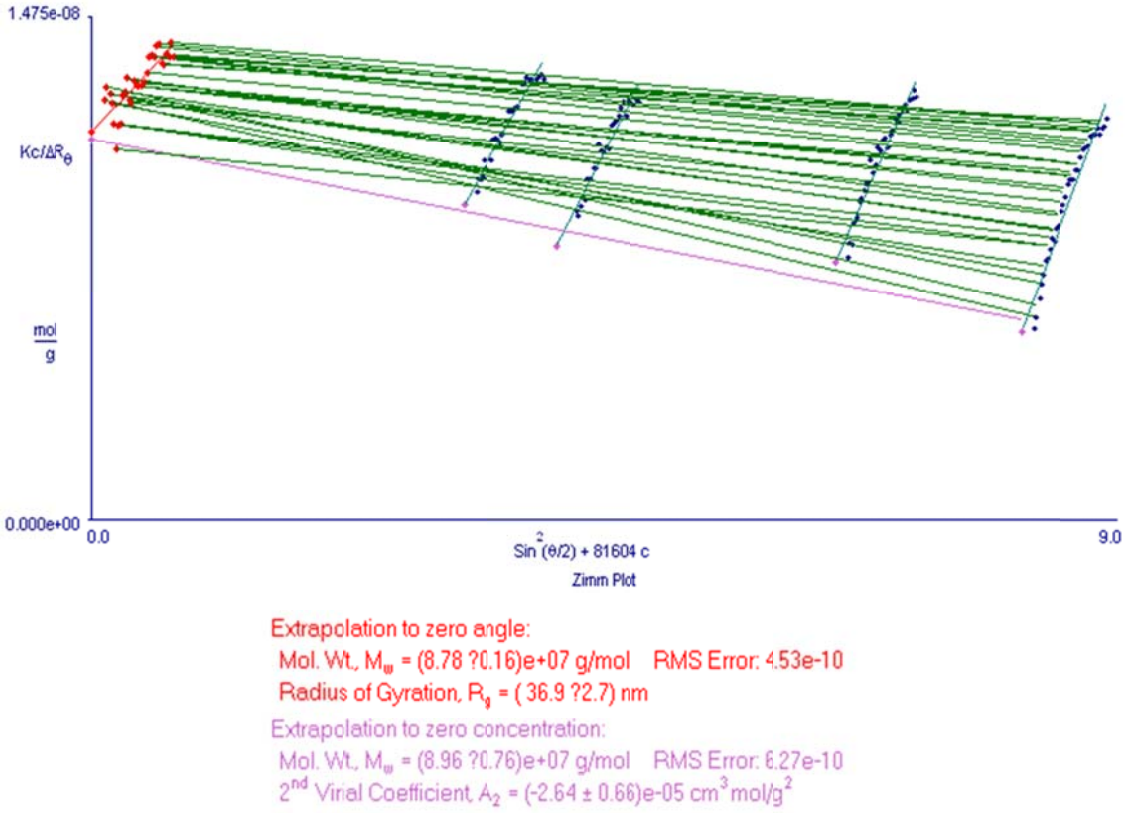

Figure S2. Static light scattering (SLS) measurements of cRGD-CLP (concentrations varying from 0.04, 0.05, 0.08, to 0.1 mg/mL) in DI water.

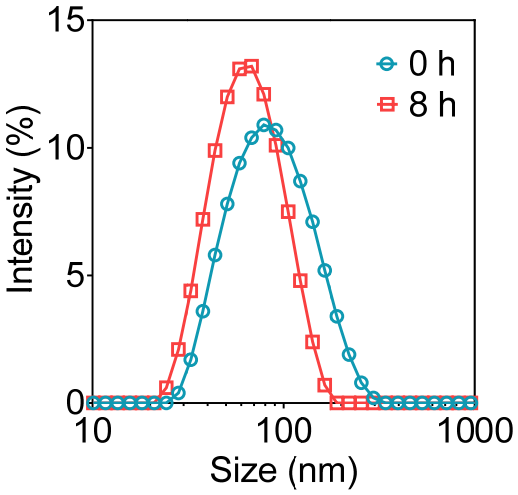

Figure S3. Stability test of blank cRGD-CLP against 10% FBS in PB.

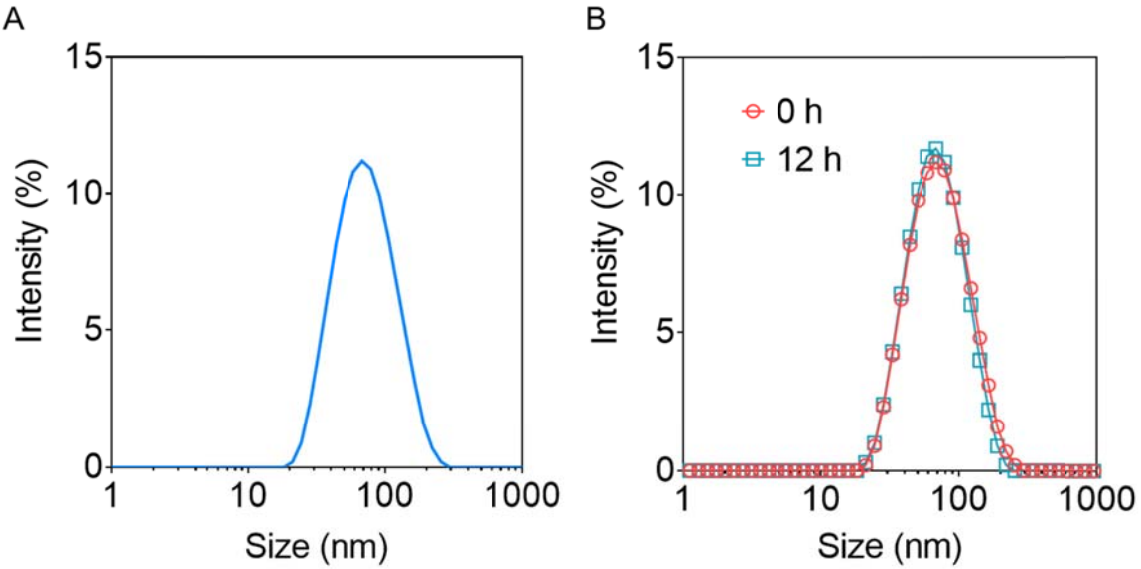

Figure S4: (A) Size and distribution of cRGD-CLP following loading with 2.0 wt.% SAP in PB. (B) Stability test of SAP-cRGD-CLP against 10% FBS in PB.

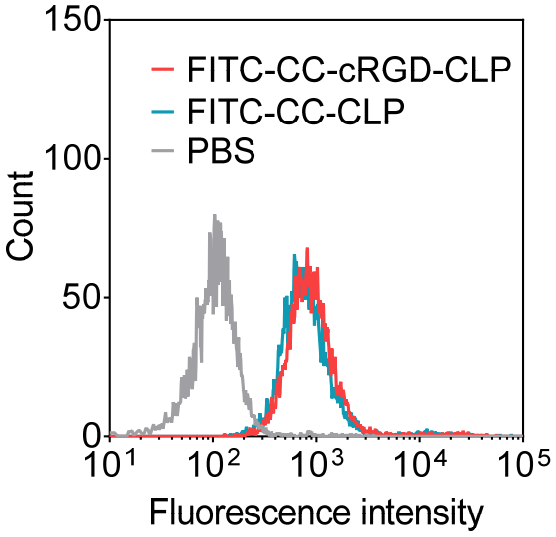

Figure S5. Flow cytometry assays of MCF-7 cells treated with FITC-CC-cRGD-CLP and FITC-CC-CLP for 4 h.

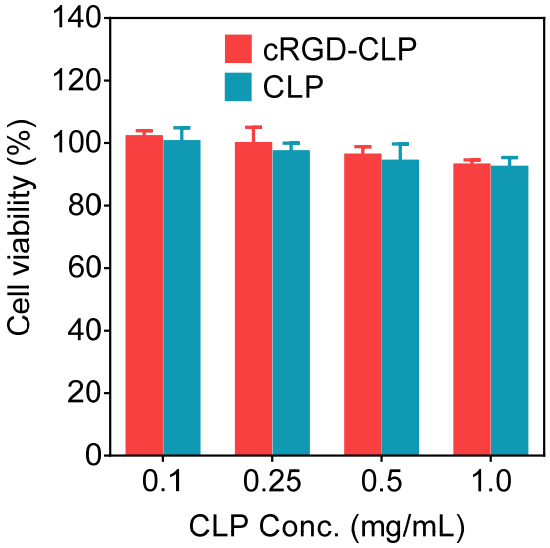

Figure S6. MTT assays of blank cRGD-CLP and CLP in A549 cells following 48 h incubation.

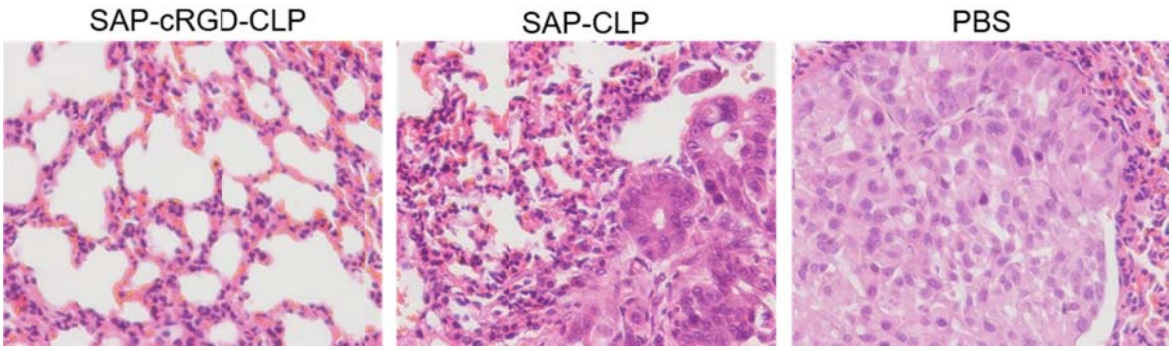

Figure S7. Histological analyses of H&E stained sections of lung excised from mice following 16 d treatment with different formulations. The images were obtained under Olympus BX41 microscope using a 40× objective.