Supplementary information

Dual-Sensitive Nanomicelles Enhancing Systemic Delivery of Therapeutically Active Antibodies Specifically into the Brain

Jinbing Xie, [†,@,*] Daniel Gonzalez-Carter,[†,*] Theofilus A. Tockary,[†] Noriko Nakamura,[†,‡] Yonger Xue,[&] Makoto Nakakido,[‡] Hiroki Akiba,[‡] Anjaneyulu Dirisala,[†] Xueying Liu,[†] Kazuko Toh,[†] Tao Yang,[†] Zengtao Wang,[&] Shigeto Fukushima,[†] Junjie Li,[†] Sabina Quader,[†] Kouhei Tsumoto,[‡] Takanori Yokota,[^] Yasutaka Anraku,[†,‡,**] and Kazunori Kataoka[†,#,**]

[†] *Innovation Center of NanoMedicine, Kawasaki Institute of Industrial Promotion, 3-25-14 Tonomachi, Kawasaki-ku, Kawasaki 210-0821, Japan.*

[@] *Jiangsu Key Laboratory of Molecular Imaging and Functional Imaging, Department of Radiology, Zhongda Hospital, Medical School of Southeast University, 87 Dingjiaqiao, Nanjing 210009, China.*

[‡] *Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.*

[#] *Institute for Future Initiatives, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.*

[&] *Department of Pharmacy, Shanghai Jiaotong University, 800 Dongchun Rd, Shanghai 200240, China.*

[^] *Department of Neurology and Neurological Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan*

* These authors contributed equally

** Corresponding author, e-mail: anraku@bmw.t.u-tokyo.ac.jp or kataoka@ifi.u-tokyo.ac.jp

Materials

α-Methoxy-*ω*-amino poly(ethylene glycol) (MeO-PEG-NH_2) (M_W of PEG is 5,000, M_W/M_n = 1.05) was purchased from NOF Co., Ltd. (Tokyo, Japan). *β*-Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was purchased from Chuo Kaseihin Co., Ltd. (Tokyo, Japan). 1,2:3,4-di-*O*-isopropylidene-*α*-D-glucofuranoside (DIG), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ethylene oxide (EO) was obtained from Nippon Ekitan Corporation (Tokyo, Japan), and purified with CaH_2 by trap-to-trap method. Solvents used for the polymerization (THF, CH_2Cl_2 and DMF) were purified by passing through two packed columns of neutral alumina purchased from Nikko Hansen & Co., Ltd. (Osaka, Japan). Benzaldehyde, *N*-methyl-2-pyrrolidone (NMP), Dulbecco's phosphate-buffered saline (D-PBS(-)), D-(+)-glucose and paraformaldehyde in phosphate buffer were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cell lysis buffer was purchased from Promega Corporation (Madison, WI). Dithiothreitol (DTT) and Glutathione (GSH) was purchased from Thermo Fisher Scientific Inc. Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Synthesis of MeO-PEG-PLL

MeO-PEG−PLL block catiomer with PEG molecular weight (M_W) 5.5 K Da and poly-L-lysine degree of polymerization (DP) 71 was synthesized as previously reported[1]. In brief, *N*-ε-trifluoroacetyl-L-lysine-*N*-carboxy anhydride (Lys(TFA)-NCA) was polymerized from the ω-NH_2 terminal group of MeO-PEG-NH_2 *via* ring-opening polymerization. The mixture of Lys(TFA)–NCA and MeO-PEG-NH_2 in distilled DMF with containing of 1 M thiourea was stirred for 3 days at 25 °C. The solution was then precipitated into diethyl ether. The precipitated product was collected and was followed by vacuum drying to remove the reminiscent of diethyl ether to obtain a white powder of MeO-PEG-PLL(TFA). Next, the obtained MeO-PEG-PLL(TFA) was dissolved in methanol containing 1 N NaOH and stirred at 35°C for 12 h to remove protective TFA groups. The DP of PLys was determined to be 71 by comparing ^{1}H NMR integration ratios between methylene protons of the PEG chain (CH_2CH_2O) and those of the lysine unit ($(CH_2)_3CH_2NH_3$) measured by a JEOL AL 300 spectrometer (JEOL Ltd., Tokyo, Japan).

Ring opening polymerization of ethylene oxide (EO) from 1, 2:3, 4-di-*O*-isopropylidene-*α*-D-glucofuranoside (DIG)

EO polymerization was performed according to our previous work [2]. Sublimed in a reaction tube in a vacuum at 70 °C, DIG (260 mg, 1.0 mmol) was dissolved in THF. Then, EO (2.3 mL, 46 mmol) was charged with stirring in an Ar atmosphere after the drop-manner addition of 0.3 M potassium naphthalene solution in THF (3.3 mL, 1.0 mmol) in the DIG solution in THF. After stirring at room temperature for 48h, 1 mL of MeOH was added, and the mixture was dropped into ice-cold diethyl ether with stirring to obtain a white precipitate to afford DIG(6)-PEG-OH.

Terminus amination of DIG(6)-PEG-OH

The *ω*-hydroxyl group of DIG(6)-PEG-OH was converted to *ω*-amino group by the following conventional procedures: A THF solution of DIG(6)-PEG-OH (2.0 g, 1 mmol) and TEA (834 μL, 6.0 mmol) was added to a THF solution of methanesulfonyl chloride (387 μL, 5.0 mmol) in ice. Stirring at room temperature for 6 h, the mixture was dropped into ice-cold diethyl ether with continuous stirring to obtain a white precipitate, DIG(6)-PEG-OMs. After drying in a vacuum, the white solid was dissolved in 25% aqueous ammonia solution (200 mL) and stirred at room temperature for 2 days. The resulting solution was evaporated, dialyzed (molecular weight cut-off size (MWCO): 1,000) against dilute ammonia solution, then against de-ionized water, and finally it was lyophilized. Ion-exchange chromatography was performed using Sephadex C-25 (GE healthcare, Little Chalfont, UK) to remove non-aminated PEG fraction. After collecting aminated PEG fraction, the solution was evaporated and lyophilized to obtain pure DIG(6)-PEG-NH_2 as a white powder.

The synthesis method for DIG-PEG-PLL was similar to MeO-PEG-PLL synthesis. In brief, *N*-ε-trifluoroacetyl-L-lysine-*N*-carboxy anhydride (Lys(TFA)-NCA) was polymerized from the ω-NH_2 terminal group of DIG(6)-PEG-NH_2 *via* ring-opening polymerization. The mixture of Lys(TFA)–NCA and MeO-PEG-NH_2 in distilled DMF with containing of 1 M thiourea was stirred for 3 days at 25 °C and then the solution was precipitated into diethyl ether. The precipitated product was collected and was followed by vacuum drying to remove the reminiscent of diethyl ether to obtain a white powder of DIG-PEG-PLL(TFA). Obtained DIG-PEG-PLL(TFA) was dissolved in methanol containing 1 N NaOH with stirring for 12 h at 35°C to remove protective TFA groups. The DP of PLys in the obtained DIG-PEG-PLL was determined to be 72 by comparing ^{1}H NMR integration ratios between methylene protons of the PEG chain (CH_2CH_2O)

and those of the lysine unit ((CH_2)$_3$CH_2NH_3).

Removal of protective groups from glucose moiety

DIG-PEG-PLL was dissolved in TFA/water (9:1, v/v) and stirred for 30 min at room temperature. The mixture was sequentially dialyzed against aqueous 0.01 N NaOH and against de-ionized water, followed by lyophilization to obtain Gluc-PEG-PLL. The complete removal of protective groups was confirmed from ^{1}H-NMR spectra.

Synthesis of thiolated MeO-PEG-PLL and thiolated Gluc-PEG-PLL

Introduction of thiol groups to the side chain of MeO-PEG-PLL and Gluc-PEG-PLL were performed using a heterobifunctional reagent succinimidyl 3-(2-pyridyldithio)propionate (SPDP) [3]. The typical synthetic procedure is described as following for the MeO-PEG-PLL-PDP: 90 mg MeO-PEG-PLL was average splitted into three groups (30 mg, 1.38 μmol). The polymer and SPDP was separately dissolved into NMP containing 5wt% LiCl. Then NMP solution containing 18.9, 28.4 and 37.9 μmol SPDP (2.5 mL) was separately added into various MeO-PEG-PLys solution (2 mL) (molar ratio of feeding SPDP: modified Lys = 4:3) and stirred at room temperature for 4 hours after the addition of 10% volume of *N*, *N*-diisopropylethylamine aiming to obtain 20, 30, and 40mol% substitution, respectively. Then, the mixture was precipitated into an approximately 20-times-excess volume of diethyl ether. The crude precipitate was washed twice with diethyl ether to obtain white powder. The polymer was dissolved in 0.01 N HCl solution, dialyzed against distilled water overnight, and lyophilized to obtain the final product. The degree of the thiol substitution for each thiolated MeO-PEG-PLL was determined as 12.78, 21.3, and 29.11 corresponding to 18%, 30%, and 41%, respectively, from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2, δ) 3.5 ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl groups (C$_5H_4$N, δ) 7.2-8.3 ppm) or the newly introduced methylene protons of 1-imino-4-mercaptobutyl groups (HS- (CH_2)$_3$-C(NH_2^+)$^-$, δ) 2.1-3.4 ppm) in ^{1}H NMR spectra taken in D_2O at 25°C. We obtained the polymer of Gluc-PEG-PLL-PDP with thiol substitution degree of 14.4, 24.48, and 30.24 corresponding to 20%, 34%, and 42%, respectively, with the same preparation method as MeO-PEG- PLL-PDP.

Evaluation of the number of encapsulated Fab in each of the nanomicelle

The encapsulated Fab number in the micelle was calculated according to the molar weight of the

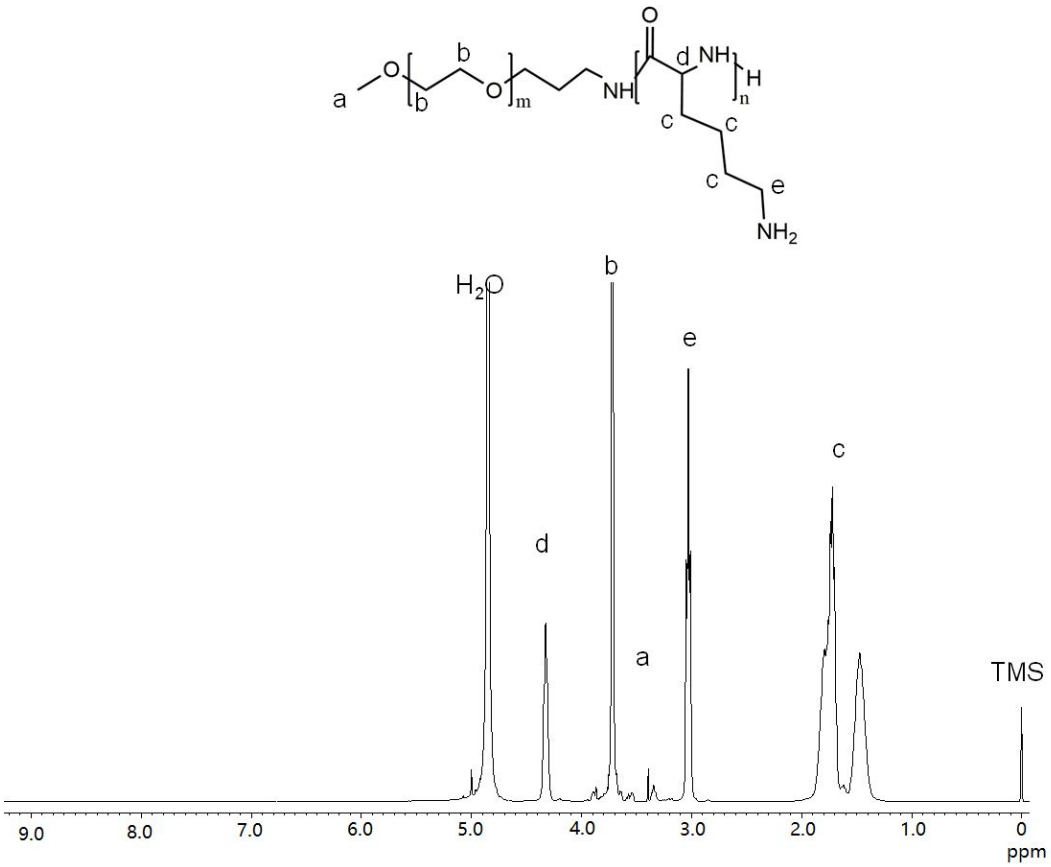
nanomicelle. To enhance the stability of nanomicelles, we added 200 μL of 10 mg/mL N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) solution into 400 μL of Cy3-labelled Fab encapsulated nanomicelle solution and incubated for 15 hours to form the covalent crosslinking in the core of the nanomicelle. The molecular weight of the nanomicelles was then evaluated with an analytical ultracentrifuge system (ProteomeLab^{TM} XL-1 protein characterization system) (Beckman Coulter Inc., U.S.A.) by monitoring the absorbance of the Fab-conjugated Alexa647. G25-PM-Fab was used as a representative sample to evaluate the encapsulated Fab number in each of the nanomicelle by the method described as follows: Firstly, the average molar weight of the nanomicelle (W_M) is obtained by analytical ultracentrifugation (AUC). AUC was conducted based on the sedimentation velocity method according to a previous report using an analytical ultracentrifuge [Optima XL-1 (Beckman Coulter, Inc., CA); rotor: AnTi60 rotor; cell: 1.2 mm two-channel Epon-filled centerpiece; detected signal: UV absorbance at 650 nm; temperature: 20°C; 18,000 rpm (26,081 G)] [4]. Radial absorbance data were collected in continuous scanning mode at 0.002 cm increments and 3 min intervals for a total of 120 scans. Acquired sedimentation boundaries were fitted based on the Lamm equation using the continuous molecular weight distribution model in the SEDFIT software to calculate the distribution of sedimentation coefficient with the following parameters: resolution 100 S, s_{min} 0 S, s_{max} 150 S, friction 1.2 μ, buffer density 1.02 g/cm^3, viscosity 0.01002 poise, and partial specific volume 0.73 cm^3/g. Hence, W_M was calculated as 1,910 kDa (Fig. S6). Following ultracentrifugation of the nanomicelles, the Fab and polymers remaining in the supernatant which did not participate in the formation of nanomicelles were measured (measurement was based on the fluorescence intensity of Alexa647-labelled Fab and polymers, each labelled in separate experiments) after being collected with a Vivaspin 6 (3 times, the cut off MW is 100K Da, 10 mM pH 7.4 phosphate-buffer solution). Subtracting the amount of free Fab/polymer from the original feeding ratio (*i.e.* charge ratio of positive/negative is 2:1) allowed for the actual association molar ratio of polymer/Fab in G25-PM-Fab to be determined as 1.6. The known average molecular weight of Fab (W_F, 50,000 Da) and of the polymers MeO-PEG-PLL-PDP$_{-30}$ (W_P) and Gluc-PEG-PLL-PDP$_{-34}$ (W_G) (19927 Da and 21109 Da, respectively), and the measured W_M were then employed to calculate the encapsulated Fab number (N_f) and polymer number (N_p) per nanomicelle respectively, based on the following equations.

$$N_f = \frac{\mathrm{Wm}}{\mathrm{Wf} + 1.6 \times (75\%\mathrm{Wp} + 25\%\mathrm{Wg})}$$

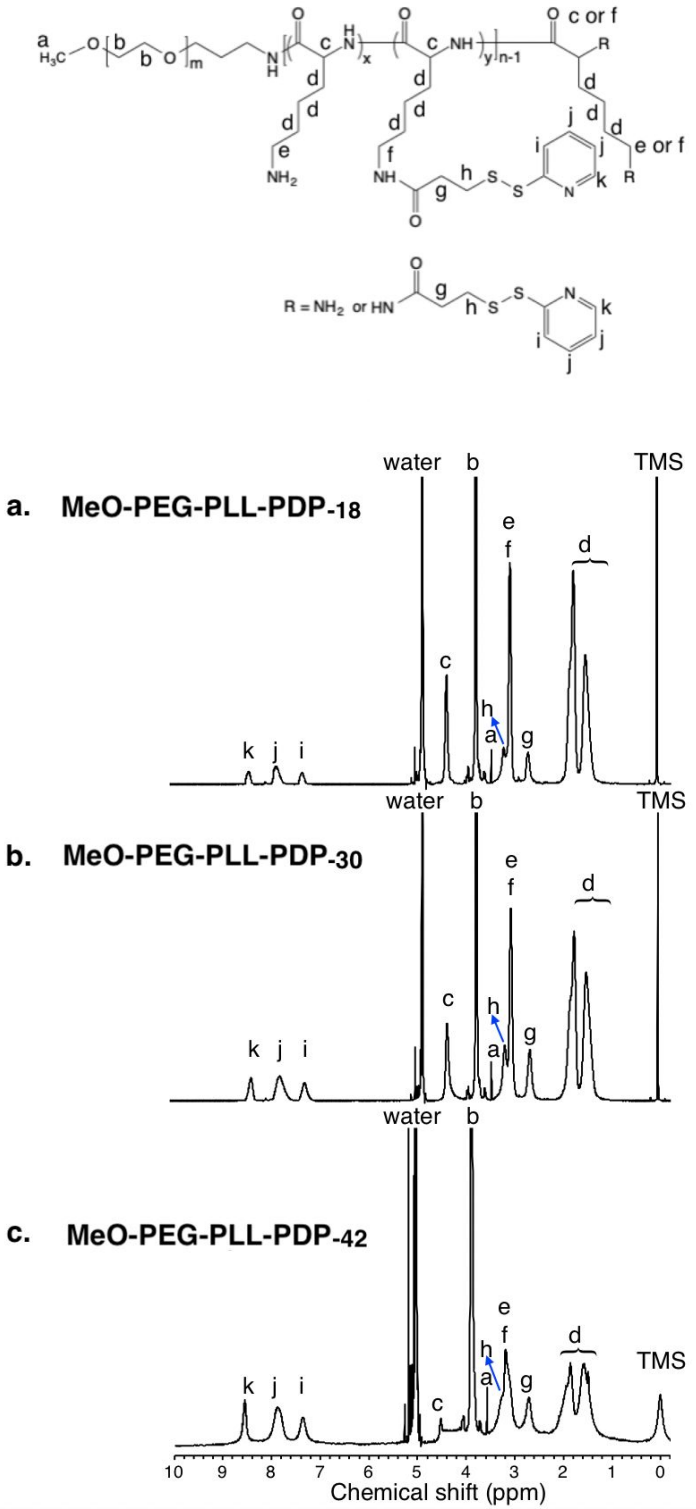
$$N_p = 1.6\ N_f$$

***In vitro* Fab release from the nanomicelles**

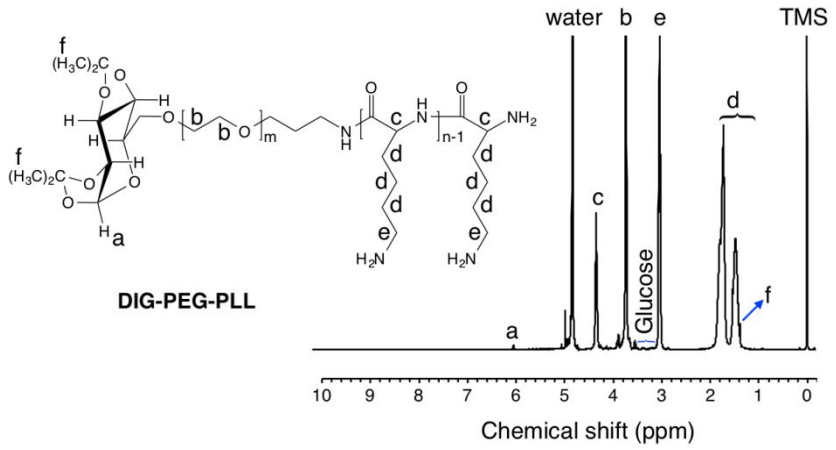
The prepared G25-PM-Fab was divided into 4 groups with incubation under pH 7.4, pH 5.0, pH 7.4 and 2 mM GSH, pH 5.0 and 2 mM GSH conditions (100 mM phosphate-buffer saline, 150 mM NaCl), respectively, at 37°C. The released Fab was collected with a Vivaspin centrifugal filter (Sartorius, Germany) with a 300,000 Da cut off size at a speed of 800 rpm/min at various incubation times of 0.25, 0.5, 1, 2, 4, 6, and 12 hours, respectively. The fluorescence intensity of the Fab labelled Alexa647 was measured for the quantitation of released Fab with an Infinite M1000 Pro microplate reader (Tecan, Switzerland).



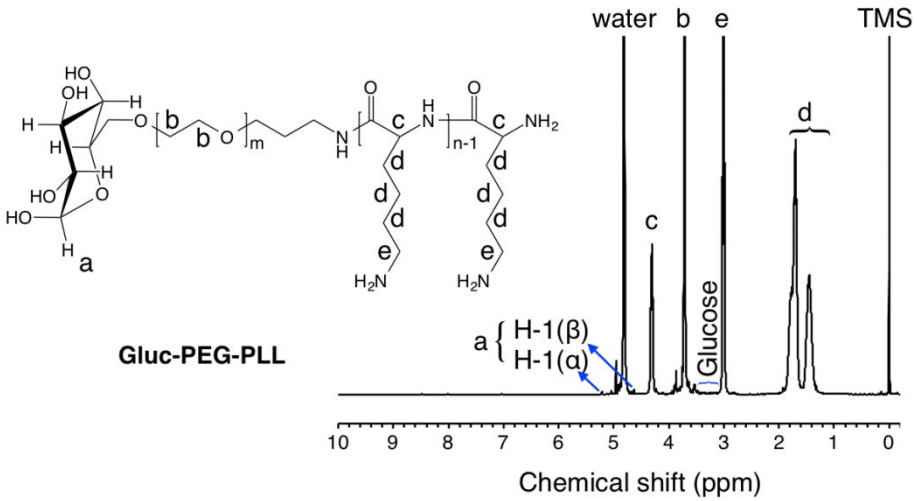
Supplementary Figure S1. ^{1}H-NMR spectrum of MeO-PEG-PLL in D_2O at room temperature.



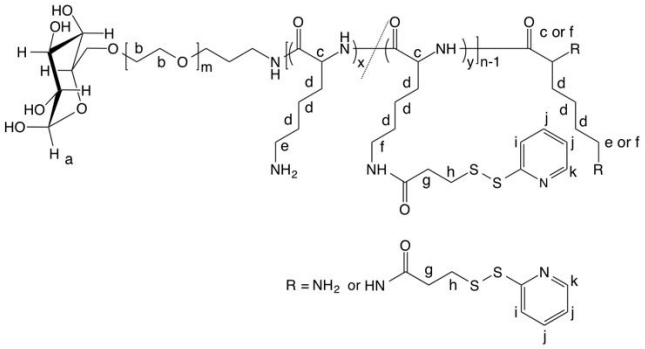
Supplementary Figure S2. ^{1}H-NMR spectra of MeO-PEG-PLL-PDP-x with introduction of 3-(2-pyridyldithio)propionate (PDP) groups into various percentages of lysine residues in the side chain of the poly(L-lysine) segment (Solvent: D_2O; Room temperature).

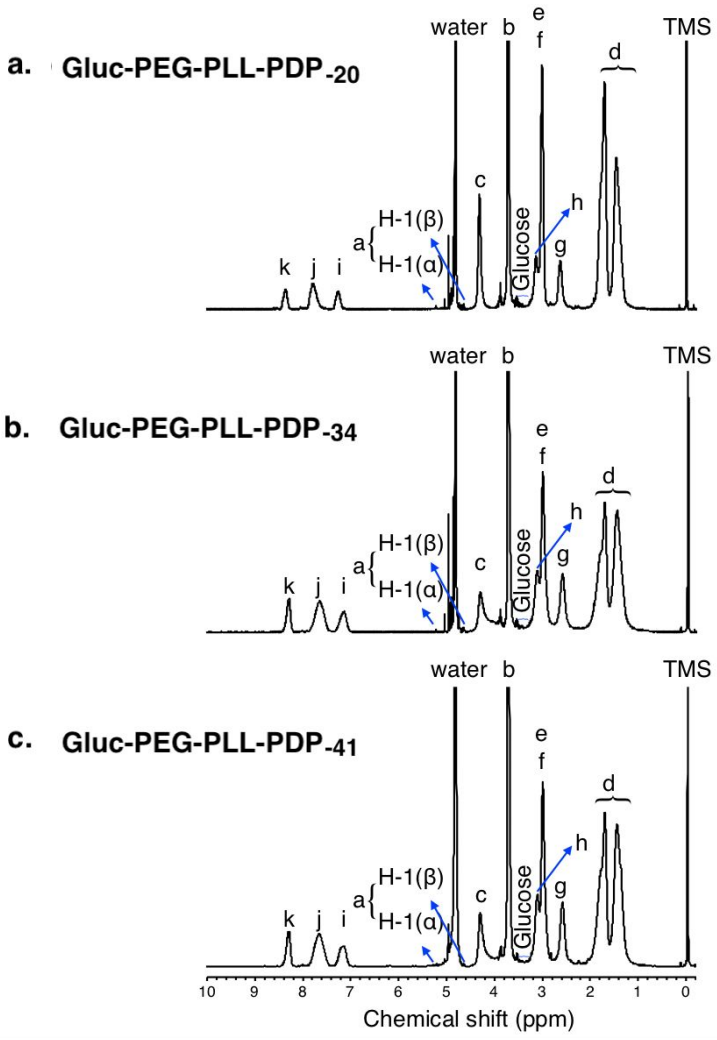


Supplementary Figure S3. ^{1}H -NMR spectrum of DIG-PEG-PLL in D_2O at room temperature.

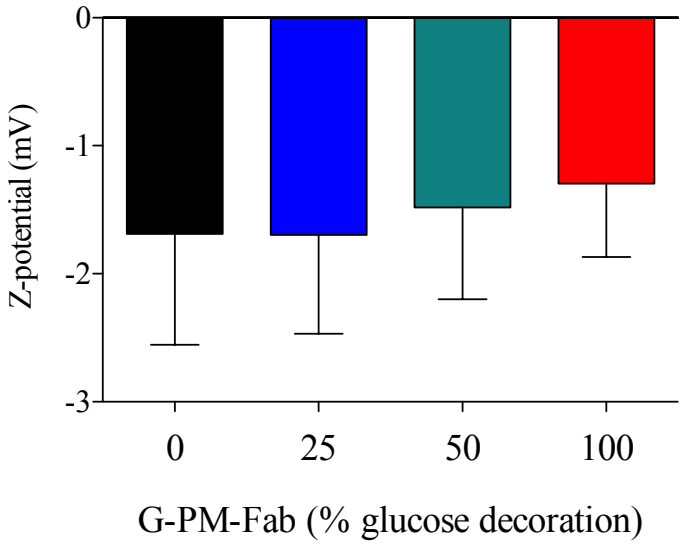


Supplementary Figure S4. ^{1}H-NMR spectrum of Gluc-PEG-PLL in D_2O at room temperature.

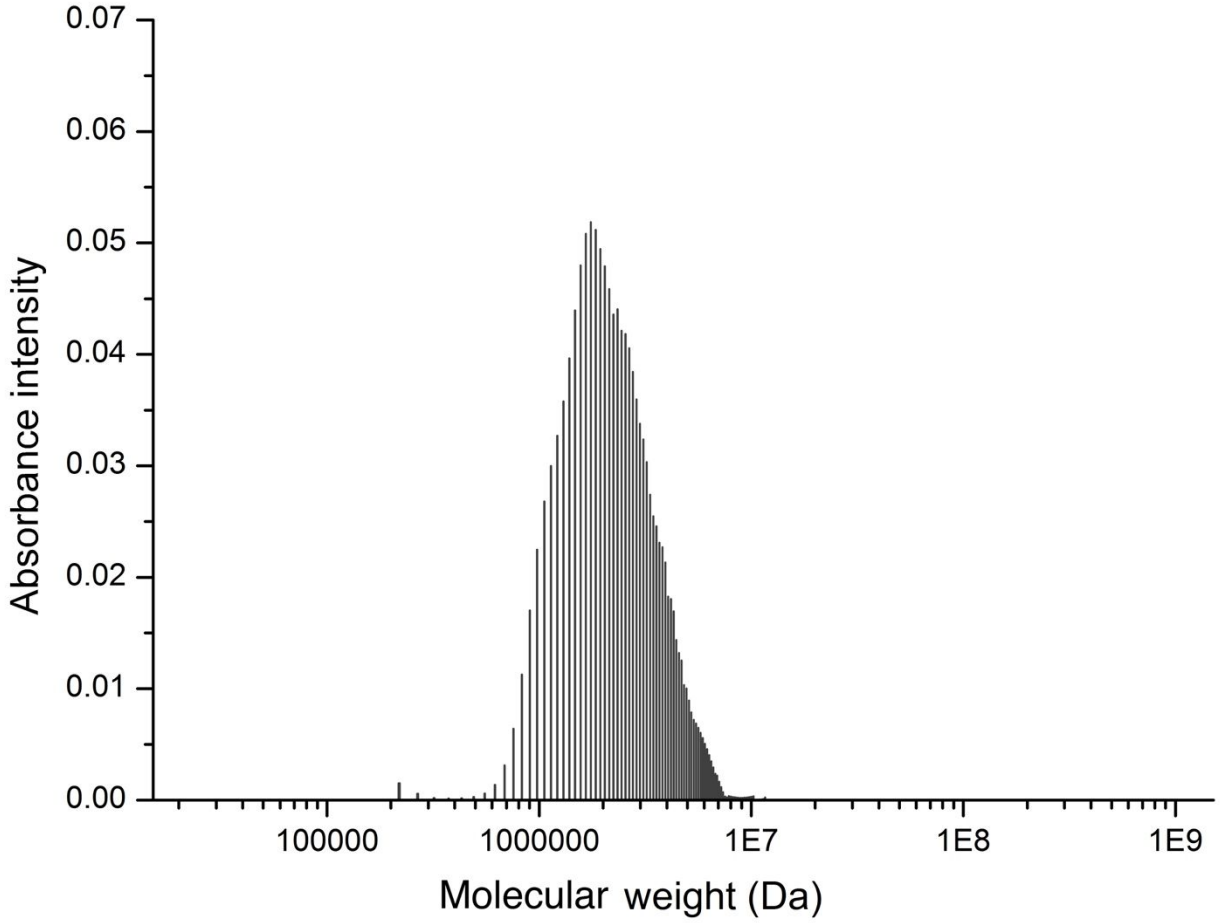




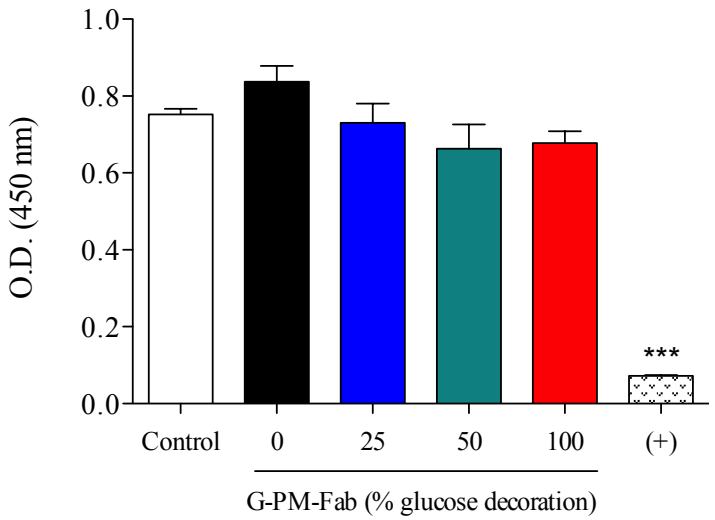
Supplementary Figure S5. ^{1}H-NMR spectra of Gluc-PEG-PLL-PDP-x with introduction of 3-(2-pyridyldithio)propionate (PDP) groups into various percentages of lysine residues in the side chain of the poly(L-lysine) segment (Solvent: D_2O, Room temperature).



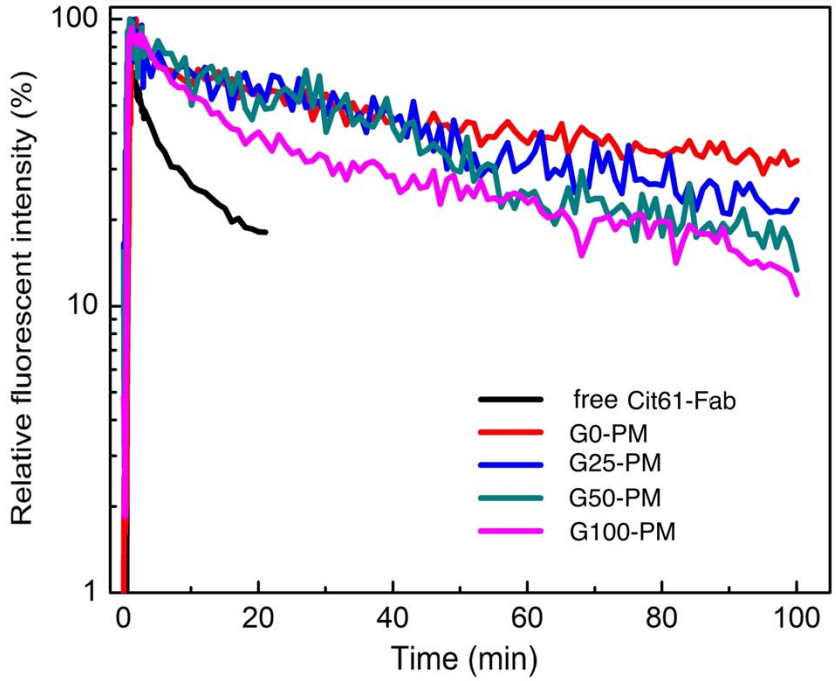
Supplementary Figure S6. Z-potential measurements of G-PM-Fab nanomicelles with varying degree of glucose decoration (Results represent mean ± SEM of 5 independent nanomicelle syntheses).



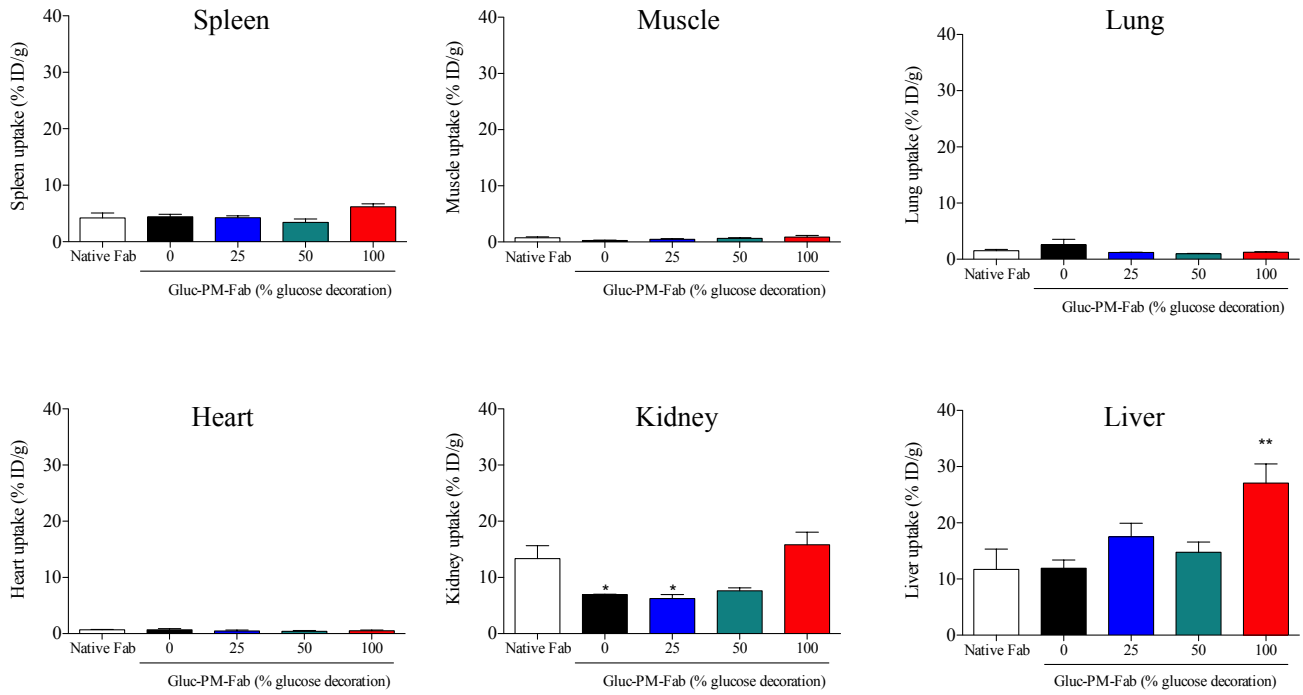
Supplementary Figure S7. The molecular weight distribution of the G25-PM-Fab as measured with an analytical ultracentrifuge system.



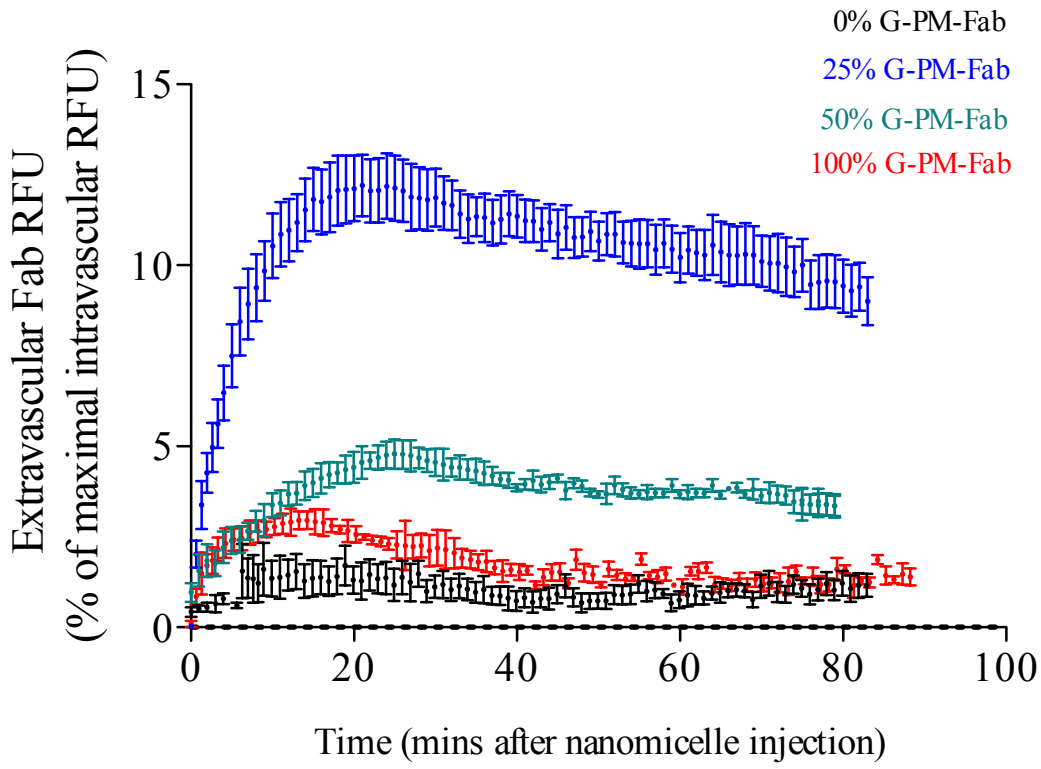
Supplementary Figure S8. The toxicity of G-PM-Fab nanomicelles towards primary rat brain endothelial cells was examined by treating brain endothelial cells with G-PM-Fab (16 μg/mL Fab concentration for all) for 20 hrs prior to measuring metabolic activity through a WST assay. As positive control ((+)) for cytotoxicity, cells were also treated with 10 μM staurosporine. Results are displayed as mean ± SEM of 4 independent wells. *** denotes $p < 0.005$ *vs.* control as determined by a one-way ANOVA with Tukey's *post-hoc* test.



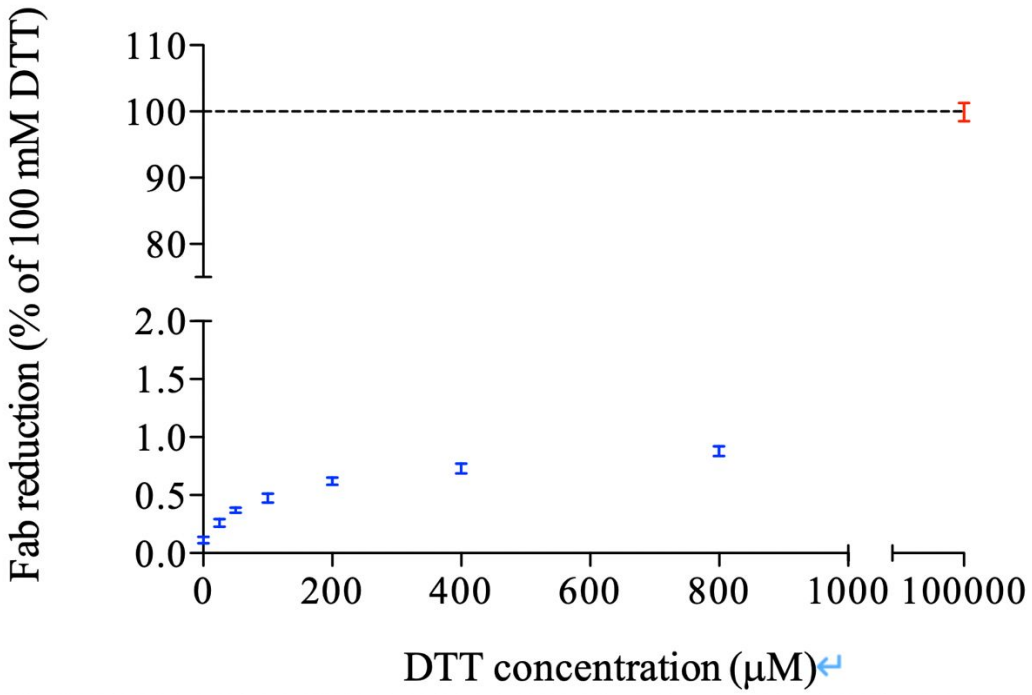
Supplementary Figure S9. Blood circulation profiles of free Cit-Fab and Gluc-PM-Fab (both with Alexa647-labelled Fab) with varying glucose decoration (0, 25, 50, 100%) as determined by quantifying Alexa647 fluorescence in earlobe vasculature of C57BL/6J mice through *in vivo* confocal laser scanning microscopy.



Supplementary Figure S10. Biodistribution of free Fab and G-PM-Fab nanomicelles into peripheral organs as % initial dose/gram of tissue (% ID/g). Results represent mean ± SEM of 5 animals. *, ** denote p <0.05 or 0.01, respectively, *vs.* native Fab, as determined by a one-way ANOVA with Tukey's *post-hoc* test.



Supplementary Figure S11. IVRT-CLSM quantification of G-PM-Fab entry into the brain parenchyma following intravenous injection into mice. Data are average (± SEM) of fluorescence emissions detected at 5 independent parenchymal areas from a single mouse for each nanomicelle.



Supplementary Figure S12. The production of reduced thiol groups in Fab following incubation with increasing concentrations of DTT was measured through an Ellman assay. Number of reduced thiol groups in Fab following incubation with 100 mM DTT for 1 hour at 90°C (red data point) and in the native Fab sample were set as 100% and 0%, respectively. Results are displayed as mean ± SEM of n = 4.

Table S1. Effect of Fab charge conversion on nanomicelle formation. Characterization of G25-PM-Fab nanomicelles formed from complexation of PEG-PLL-PDP polymers (MeO-PEG-PLL-PDP$_{-30}$:Gluc- PEG-PLL-PDP$_{-34}$ = 3:1) with Fab modified with various percentage of Cit.

Sample name	Charge converted amines per 3D6 Fab (number)[a]	Charge converted amines per 3D6 Fab (%)[b]	z-Averaged diameter (nm±SD)	PDI[b]	Released Fab (%) at pH=5.0 /2mM GSH (at 6h)
Native Fab	0	0	_	_	_
Cit24-Fab	6.24	24	155.6±5.3	0.688	98.8
Cit43-Fab	11.18	43	77.5±3.5	0.358	97.7
Cit61-Fab	15.86	61	46.2±2.1	0.135	85.6
Cit81-Fab	21.06	81	44.5±1.4	0.093	65.4

[a] Number of amino groups per Fab occupied by citraconic anhydride.

[b] Amino groups occupied by citraconic anhydride as a percentage of total amino groups per Fab.

Table S2. Nanomicelle formation through Cit-Fab complexation with PEG-PLL polymers with different degree of PDP modification.

Sample name	Lysine residues with PDP modification (% of residues per polymer) [a, b]	z-Averaged diameter determined from DLS in PBS (nm±SD)	PDI from DLS in PBS	Diameter in PBS determined from FCS (nm±SD)	Diameter in PBS with 10% Serum determined from FCS (nm±SD)
Null crosslinking	MeO-PEG-PLL-PDP$_{-0}$ Gluc- PEG-PLL-PDP$_{-0}$	44.7±2.4	0.096	41.4±2.5	8.4±1.6
Low crosslinking	MeO- PEG-PLL-PDP$_{-18}$ Gluc- PEG-PLL-PDP$_{-20}$	45.8±2.2	0.102	41.6±1.6	23.3±2.1
Medium crosslinking	MeO- PEG-PLL-PDP$_{-30}$ Gluc- PEG-PLL-PDP$_{-34}$	46.2±2.1	0.135	40.3±1.8	39.4±2.3
High crosslinking	MeO- PEG-PLL-PDP$_{-42}$ Gluc- PEG-PLL-PDP$_{-41}$	44.9±3.2	0.278	39.6±1.7	38.1±2.1

[a] The "x" in MeO-PEG-PLL-PDP-x and Gluc-PEG-PLL-PDP-x represents % of lysine residues modified with PDP per polymer.

[b] Gluc-PEG-PLL:MeO-PEG-PLL ratio was fixed at 1:3.

Table S3. Size and polydispersity index (PDI) of Cit-Fab loaded glucosylated polymeric nanomicelles determined by DLS, TEM and FCS.

Sample	% glucose decoration[a]	z-Averaged diameter determined from DLS in PBS (nm±SD)	PDI from DLS in PBS	Diameter determined from TEM (nm±SD)	Diameter in PBS determined from FCS (nm±SD)	Diameter in PBS containing 10% of Serum determined from FCS (nm±SD)
G0-NM	0	44.3±2.6	0.106	36.6±2.2	41.8±2.0	40.2±2.1
G25-NM	25	46.2±2.4	0.135	38.4±1.4	41.7±2.3	40.6±1.6
G50-NM	50	45.8±2.0	0.124	37.8±2.3	40.7±1.9	39.3±2.4
G100-NM	100	46.3±3.2	0.148	38.1±2.1	42.2±2.4	38.3±3.1

[a] Gluc-PEG-PLL-PDP polymers as a percentage of total Gluc- and MeO-PEG-PLL-PDP polymers.

References

1. Harada, A.; Kataoka K. Formation of Polyion Complex Micelles in an Aqueous Milieu From a Pair of Oppositely-Charged Block Copolymers with Poly(Ethylene Glycol) Segments. *Macromolecules* **1996,** *28,* 5294-5299.
2. Anraku, Y.; Kuwahara, H.; Fukusato, Y.; Mizoguchi, A.; Ishii, T.; Nitta, K.; Matsumoto, Y.; Toh, K.; Miyata, K.; Uchida, S.; Nishina, K.; Osada, K.; Itaka, K.; Nishiyama, N.; Mizusawa, H.; Yamasoba, T.; Yokota, T.; Kataoka, K. Glycaemic Control Boosts Glucosylated Nanocarrier Crossing the BBB Into the Brain. *Nat. Commun.* **2017,** *8,* 1001.
3. Dirisala, A.; Uchida, S.; Tockary, T.A.; Yoshinaga, N.; Li, J.; Osawa, S.; Gorantla, L.; Fukushima, S.; Osada, K.; Kataoka K. Precise Tuning of Disulfide Crosslinking in mRNA Polyplex Micelles for Optimizing Extracellular and Intracellular Nuclease Tolerability. *J. Drug Target.* **2019,** *27,* 670-680.
4. Mochida, Y.; Cabral, H.; Miura, Y.; Albertini, F.; Fukushima, S.; Osada, K.; Nishiyama, N.; Kataoka K. Bundled Assembly of Helical Nanostructures in Polymeric Micelles Loaded with Platinum Drugs Enhancing Therapeutic Efficiency against Pancreatic Tumor. *ACS Nano* **2014,** *8,* 6724-6738.