Supporting Information

Structure-Based Nanocarrier Design for Protein Delivery

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Supporting Information

Experimental Section

Materials. Monomethylterminated poly(ethylene glycol) monoamine hydrochloride (MeO-PEG-NH_2·HCl, M_W: ~5 kDa) was purchased from Jenkem Technology. (Fmoc)Lys(Fmoc)-OH and Fmoc-Lys(Boc)-OH were obtained from AnaSpec Inc. Fmoc-Arg(Pbf)-OH was purchased from Novabiochem. Cholesteryl chlorofomate was purchased from Alfa Aesar. CellTiter 96® AQ_{ueous} MTS reagent powder was purchased from Promega. Heptadecanoic acid and 2-phenylacetic acid were purchased from Acros. Diisopropyl carbodimide (DIC), *N*-hydroxybenzotriazole (HOBt), D-α-tocopherol succinate, trifluoroacetic acid (TFA), acetic anhydride, fluorescein isothiocyanate isomer I (FITC), *N*-hydroxysuccinimide (HOSu), rhodamine B isothiocyanate (RBITC), cholic acid, human insulin (M_w ~6 kDa, isoelectric point ~5.3), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide (Sulfo-NHS) and other chemical reagents were purchased from Sigma-Aldrich. Coumarin-based carboxylic acid was synthesized from 7-hydroxy-4-methylcoumarin by our previous method.[1] Dialysis membrane with 3,500 M_W cut off was purchased from Spectrum Laboratories, Inc.

Telodendrimer synthesis. The telodendrimers with eight amino groups containing four 2-phenylacetic acids, four coumarins, and four cholic acids, which are named as PEG^{5k}(LysLys-L-PA)$_4$, PEG^{5k}(LysLys-L-Co)$_4$ and PEG^{5k}(LysLys-L-CA)$_4$, respectively, were synthesized using a solution-phase condensation reaction starting from MeO-PEG-NH_2·HCl (~5 kDa) via stepwise peptide chemistry. The procedure was performed as follows: (Fmoc)Lys(Fmoc)-OH (3 eq.) reacted with the N-terminus of PEG using DIC and HOBt as coupling reagents until a negative Kaiser test result was obtained, indicating completion of the coupling reaction. PEGylated molecules were precipitated through the addition of the cold ether and then washed with cold ether twice. Fmoc groups were removed by the treatment with 20% (v/v) 4-methylpiperidine in dimethylformamide (DMF), and the PEGylated molecules were precipitated and washed three times by cold ether. White powder precipitate was dried under vacuum. One coupling of (Fmoc)Lys(Fmoc)-OH and two couplings of Fmoc-Lys(Boc)-OH were carried out respectively upon the removal of Fmoc groups to generate an intermediate of dendritic poly(amino acid) terminated with eight Boc groups and four Fmoc groups on one end of PEG. Then four PEG linker molecules (M_W: 470) were coupled to the amino groups upon the removal of Fmoc groups

with 20% (v/v) 4-methylpiperidine in DMF. After the removal of Fmoc groups on the PEG linkers, the polymers were coupled with 2-phenylacetic acid, coumarin-based carboxylic acid, cholic acid. The Boc protecting groups were consecutively removed via the treatment with 50% TFA in dichloromethane (DCM) to yield $PEG^{5k}(LysLys\text{-}L\text{-}PA)_4$, $PEG^{5k}(LysLys\text{-}L\text{-}Co)_4$ and $PEG^{5k}(LysLys\text{-}L\text{-}CA)_4$ (Scheme S1). These resulting telodendrimers were dissolved in deionized water, dialyzed against deionized water for 2 days, and then dried by lyophilization.

The synthesis procedure of the telodendrimer with eight amino groups containing eight cholic acids, which is noted as $PEG^{5k}(LysLys\text{-}L\text{-}LysCA_2)_4$, is similar with that for the telodendrimers described above, the only difference is to couple (Fmoc)Lys(Fmoc)-OH to the amino groups of the PEG linker molecules before coupling the cholic acids.

The telodendrimers with eight amino groups containing four heptadecanoic acids, four cholesterols and four D-α-tocopherol succinates, which are named $PEG^{5k}(LysLys\text{-}L\text{-}C17)_4$, $PEG^{5k}(LysLys\text{-}L\text{-}CHO)_4$ and $PEG^{5k}(LysLys\text{-}L\text{-}VE)_4$, respectively, as well as the telodendrimer with eight guanidine groups containing four D-α-tocopherol succinates, which is named $PEG^{5k}(ArgArg\text{-}L\text{-}VE)_4$, were also synthesized using a solution-phase condensation reaction starting from MeO-PEG-NH_2·HCl (~5 kDa) via stepwise peptide chemistry. The synthesis and characterization of these telodendrimers have been reported separately[2] and, therefore, are not repeated here.

Telodendrimer characterization. MALDI-TOF spectrum was recorded on a Bruker REFLEX-III instrument (linear mode) using α-cyano-4-hydroxycinnamic acid as a matrix. The spectrum baseline correction was performed for the raw data, and the peak molecular weight (M_p) and polydispersity index (PDI) were obtained based on the baseline-corrected spectrum. ^{1}H NMR spectra for telodendrimers were recorded on a Bruker AVANCE 600 MHz spectrometer at 25 °C. The concentration of the telodendrimers was kept at 5×10^{-4} M for NMR measurements. The solvent residual peak was used as reference (DMSO-d_6: 2.50 ppm). The numbers of hydrophobic groups in the telodendrimer molecules were calculated based on the average integration ratio of the peaks of the characteristic protons (~7.25 ppm for PA-containing telodendrimer, ~6.23, ~6.95 and ~6.98 ppm for Co-containing telodendrimer, and ~0.58, ~0.81 and ~0.93 ppm for CA-containing telodendrimers, respectively) and the methoxy proton of PEG at ~3.24 ppm in ^{1}H NMR spectra in DMSO-d_6. The formulas of the telodendrimers were calculated based on the relative peak integration to methoxyl integration on PEG according to

our previous method[2]. The formulas for $PEG^{5k}(LysLys\text{-}L\text{-}CA)_4$ and $PEG^{5k}(LysLys\text{-}L\text{-}LysCA_2)_4$ are obtained based on their *N*-terminal acetylated compounds of $PEG^{5k}(LysAcLysAc\text{-}L\text{-}CA)_4$ and $PEG^{5k}(LysAcLysAc\text{-}L\text{-}LysCA_2)_4$. Dynamic light scattering (DLS) studies were performed using a Zetatrac (Microtrac Inc.) instrument, and the area-based mean particle sizes were presented. TEM images were taken on a JEOL 2100 TEM instrument operating at a voltage of 80 kV. The samples were stained by uranyl acetate.

Determination of critical micelle concentration (CMC). Telodendrimers were dissolved in phosphate buffered saline (PBS). The initial micelle solution was diluted with PBS to obtain the required solutions ranging from 0.39 to 200 μg/mL. A known amount of Nile red in methanol was added to a series of vials. After methanol was evaporated under vacuum, a measured amount of polymer solutions were added to each vial to obtain a final Nile red concentration of 1 μM. The mixture solutions were left to shake overnight in the dark and at room temperature. The fluorescence emission intensity was measured using a microplate reader (Synergy 2, BioTek Instruments, Inc.) at the wavelength of 620 nm with excitation at 543 nm. CMCs were determined at the intersection of the tangents to the two linear fitting of the curve of the fluorescence intensity as a function of the log concentration of the telodendrimers.

Encapsulation of proteins. The proteins were dissolved in PBS containing 1 mM EDTA, and the telodendrimers in PBS were quickly added into protein solution. The proteins were encapsulated by the telodendrimers through electrostatic interaction, hydrogen bonding, and hydrophobic-hydrophobic interaction. All protein/telodendrimer (P/T) ratios used in this study are mass ratios.

Fluorescently labeled proteins. FITC- or RBITC-labeled insulin (noted as FITC-insulin and RB-insulin, respectively) was synthesized as follows: FITC or RBITC was dissolved in acetone (0.5 mg in 200 μL) and added dropwise to a 10 mL solution containing the appropriate amount of insulin dissolved in PBS contained 200 μM EDTA. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for 2 days to remove the unreacted dye molecules and then lyophilized.

Agarose gel retention assay. Samples in loading buffer (30% glycerol aqueous solution) were loaded into agarose gel (1.5%wt) in Tris-acetate-EDTA (TAE) buffer. The gel tray was run for 2 h at a constant current of 20 mA. The gel was imaged by a Bio-Rad Universal Hood II Imager (Bio-Rad Laboratories, Inc.).

Förster resonance energy transfer (FRET) studies. FITC-insulin and RB-insulin were used as a pair for FRET study. Equal volumes of 1 mg/mL of FITC-insulin solution and 1 mg/mL of RB-insulin solution were mixed, and telodnedrimer solutions were added into the above mixture. The fluorescence spectra with a range from 480 to 640 nm at different time points excited by 439 nm were recorded using a microplate reader (BioTek Synergy 2). The FRET ratio was calculated by the formula of $[100\% \times I_{584}/(I_{584} + I_{528})]$, where I_{584} and I_{528} were the fluorescence intensities of at 584 and 528 nm, respectively.

Bio-layer interferometry (BLI). The kinetics of protein-telodendrimer interactions were measured at 37 ºC by BLI on an Octet-RED (ForteBio). Amine Reactive Second Generation biosensors (ForteBio #18-0026) were prewetted in water for 900 s, and insulin was immobilized on the biosensors by pre-activation with EDC and Sulfo-NHS for 300 s and immersion in insulin solutions (20 μg/mL, pH 6.0) for 900 s according the protocol provided by ForteBio. The insulin-immobilized biosensors were then quenched in ethanolamine solutions (pH 8.5), immersed in insulin solution (1 mg/mL, pH 7.4) to block the nonspecific binding sites on the sensors, washed in PBS, and transferred to wells containing telodendrimers at concentrations ranging from 33 to 600 nM in PBS for 1800 s (association). Dissociation at each studied concentration was carried out in PBS for 1800 s. In each assay, a reference sensor was run by association in PBS without telodendrimers in parallel to allow background subtraction. The association constant (k_{on}) and the dissociation constant (k_{off}) were obtained by globally fitting the BLI data to a 1:1 model algorithm using Octet analysis software (v6.4). The apparent equilibrium dissociation constant (K_D) derived from kinetic fitting was calculated as k_{off}/k_{on}.

Docking calculation using Molecular Operating Environment (MOE)[3]. The protein crystal structure of human insulin was downloaded from Protein Data Bank (PDB ID: 3I40). It was visualized in MOE viewer and all ligands and waters were deleted. Hydrogen atoms were added and the whole structure was minimized by MMFF94x force field. Partial MMFF94x charges were assigned to all atoms. Induced Fit protocol was selected to set the side chains of insulin free during the docking process. All surface atoms of insulin were chosen as the active sites manually. The ligand molecules were placed in the sites with the Triangle Matcher method and ranked with the London dG scoring function. The 100 best poses were retained and further refined by energy minimization in the pocket, followed by rescoring with the GBVI/WSA dG

scoring function. All negative docking binding energies of each ligand were averaged, and the average binding energy was used as the final binding energy for comparison and analysis.

Hemocompatibility assay. One milliliter of fresh blood from healthy human volunteers was collected into 5 mL of PBS solution in the presence of 20 mM EDTA. Red blood cells (RBCs) were then separated by centrifugation at 1000 rpm for 10 min. The RBCs were washed three times with 10 mL of PBS and resuspended in 20 mL of PBS. Diluted RBC suspension (200 μL) was mixed with NP PBS solutions at serial concentrations (10, 100, and 500 μg/mL) by gentle vortex and incubated at 37 °C for 24 h. The mixtures were centrifuged at 1,000 rpm for 5 min, and then the free of hemoglobin in the supernatant was determined by measuring the absorbance at 540 nm using a UV-vis spectrometer. Incubations of RBCs with Triton-100 (2%) and PBS were used as the positive and negative controls, respectively. The percent hemolysis of RBCs was calculated using the following formula:

$$\text{RBC hemolysis} = \frac{\left(\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}\right)}{\left(\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}\right)} \times 100\% \quad (1)$$

Cell culture and MTS assays. The Chinese hamster ovary cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37 °C using a humidified 5% CO_2 incubator. Cells were seeded in 96-well plates at a density of 3000 cells/well 24 h prior to the treatment. Various telodendrimer solutions with different dilutions were added to the plate and then incubated in a humidified 37 °C, 5% CO_2 incubator. After 72 h incubation, a mixture solution composed of CellTiter 96 AQueous MTS, and an electron coupling reagent, PMS, was added to each well according to the manufacturer's instructions. The cell viability was determined by measuring absorbance at 490 nm using a microplate reader (BioTek Synergy 2). Untreated cells served as the control. Results were shown as the average cell viability [$100\% \times (OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank})$] of triplicate wells.

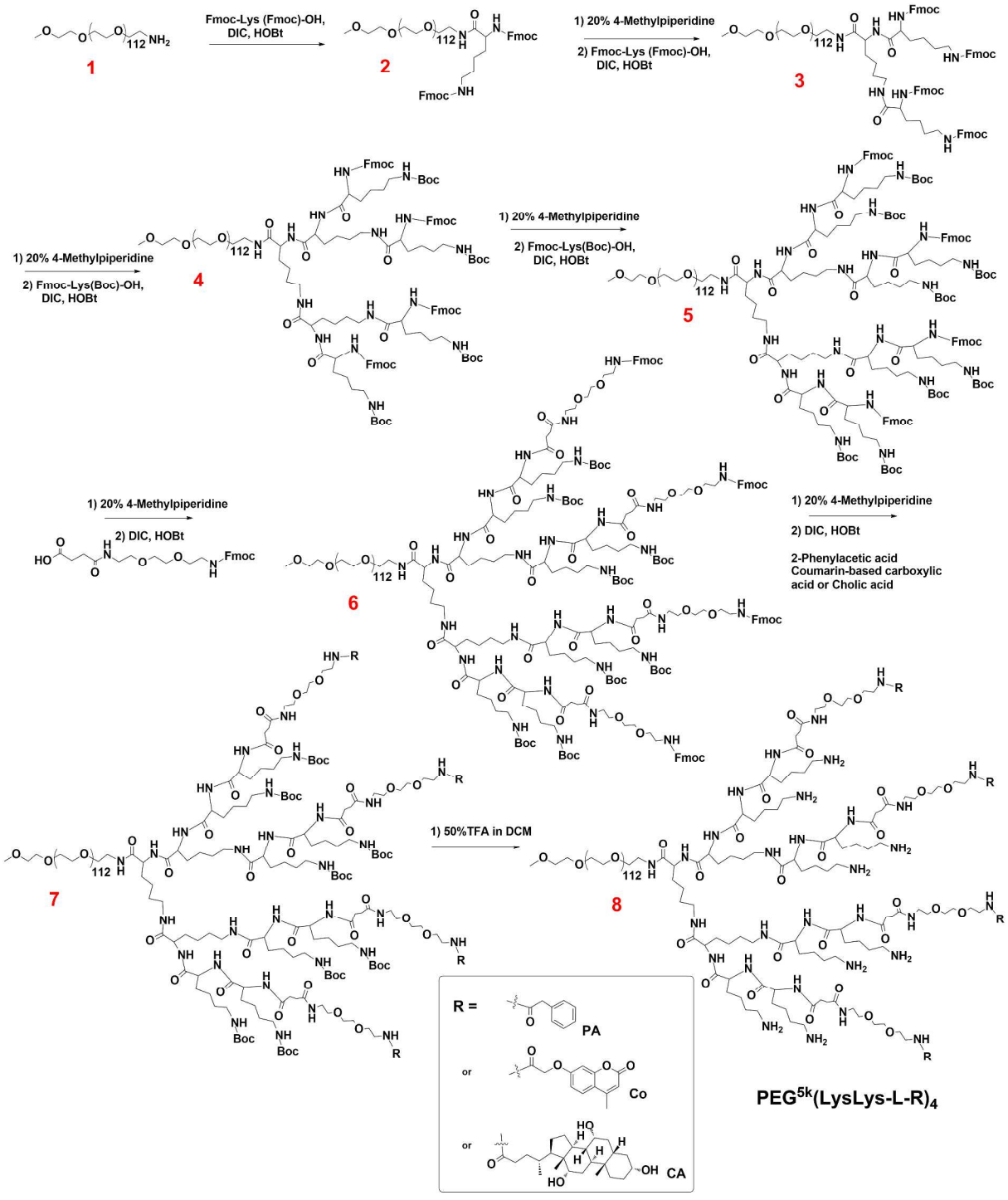
Animals. BALB/c mice, at age 8 weeks, were purchased from Charles River (Kingston, NY). All animals were kept under pathogen-free conditions according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and were allowed to acclimatize for at least 6 days prior to any experiments. All animal experiments were performed in compliance with institutional guidelines and according to protocol approved by the

Committee for the Humane Use of Animals of State University of New York Upstate Medical University.

***In vivo* efficacy.** The hypoglycemic effect, following the subcutaneous administration of insulin nanoformulations, was tested in normal mice. BALB/c mice (n = 5 per group) were fasted overnight before experiments, but allowed free access to water. The animals were administered with free insulin and insulin-loaded nanoparticle solutions (2 IU/kg) by subcutaneous injection. The mass ratio of insulin to telodendrimers in the nanoparticles is 1/10. The blood glucose levels were measured with the Easy Touch Glucose Monitoring System (MHC Medical Products, LLC).

Statistical analysis. Data are presented as means ± standard deviation (SD) unless otherwise stated. Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test. Linear regression model was fit via ordinary least square in the correlation studies. The Pearson correlation coefficient (r) and the associated P values were calculated to examine the correlation between molecular bindings obtained from molecular docking and experimental results. The level of significance in all statistical analyses was set at a probability of $P < 0.05$.

Supporting Scheme



Scheme S1. Synthesis route for $PEG^{5k}(LysLys\text{-}L\text{-}PA)_4$, $PEG^{5k}(LysLys\text{-}L\text{-}Co)_4$ and $PEG^{5k}(LysLys\text{-}L\text{-}CA)_4$ telodendrimers.

Supporting Table

Table S1. Characterization and molecular properties of the telodendrimers.

Telodendrimer	Theo. M_w[a]	M_p by MS[b]	PDI by MS[b]	Formula by NMR[c]	CMC (μM)[d]	D_h (nm)[e]	D_h with insulin (nm)[f]
$PEG^{5k}NH_2$	N/A	5,100	1.01	N/A	N/A	N/A	N/A
PEG^{5k}(LysLys-L-PA)$_4$	7,903	7,724	1.01	PEG^{5k}(LysLys-L-PA)$_{3.9}$	N/D	4±1	25±7
PEG^{5k}(LysLys-L-Co)$_4$	8,295	8,117	1.01	PEG^{5k}(LysLys-L-Co)$_{4.0}$	N/D	5±1	22±10
PEG^{5k}(LysLys-L-CHO)$_4$[g]	9,098	8,473	1.01	PEG^{5k}(LysLys-L-CHO)$_{3.0}$	1.38	18±5	22±10
PEG^{5k}(LysLys-L-CA)$_4$	8,993	8,037	1.01	PEG^{5k}(LysLys-L-CA)$_{3.7}$	N/D	3±1	12±4
PEG^{5k}(LysLys-L-C17)$_4$[g]	8,457	8,184	1.01	PEG^{5k}(LysLys-L-C17)$_{3.7}$	1.47	13±3	16±3
PEG^{5k}(LysLys-L-VE)$_4$[g]	9,498	8,892	1.01	PEG^{5k}(LysLys-L-VE)$_{3.5}$	1.62	18±6	22±5
PEG^{5k}(LysLys-L-LysCA$_2$)$_4$	11,068	9,002	1.02	PEG^{5k}(LysLys-L-LysCA$_2$)$_{3.8}$	1.65	18±5	14±4

[a]Theoretical molecular weight. [b]Peak molecular weight (M_p) and polydispersity index (PDI) acquired by MALDI-TOF analysis. [c]The values of the subscripts in the molecular formulae are calculated by based on the average integration ratio of the peaks of the characteristic protons (~7.25 ppm for PA-containing telodendrimer, ~6.23, ~6.95 and ~6.98 ppm for Co-containing telodendrimer, and ~0.58, ~0.81 and ~0.93 ppm for CA-containing telodendrimers, respectively) and the methoxy proton of PEG at ~3.24 ppm in ^{1}H NMR spectra in DMSO-d_6; the formulas for PEG^{5k}(LysLys-L-CA)$_4$ and PEG^{5k}(LysLys-L-LysCA$_2$)$_4$ are obtained based on their *N*-terminal acetylated compounds of PEG^{5k}(LysAcLysAc-L-CA)$_4$ and PEG^{5k}(LysAcLysAc-L-LysCA$_2$)$_4$. [d]Measured by fluorescent method using Nile Red dye as a probe. [e]Obtained in PBS by DLS. [f]Obtained in PBS at an insulin/telodendrimer mass ratio of 1/3 by DLS. For the DLS data, the value behind '±' describes the half-peak width of the size distribution. [g]The synthesis and characterization for C17-, CHO- and VE-containing telodendrimers have been reported in our previous publication.[2] N/A: not applicable. N/D: not detectable.

Supporting Figures

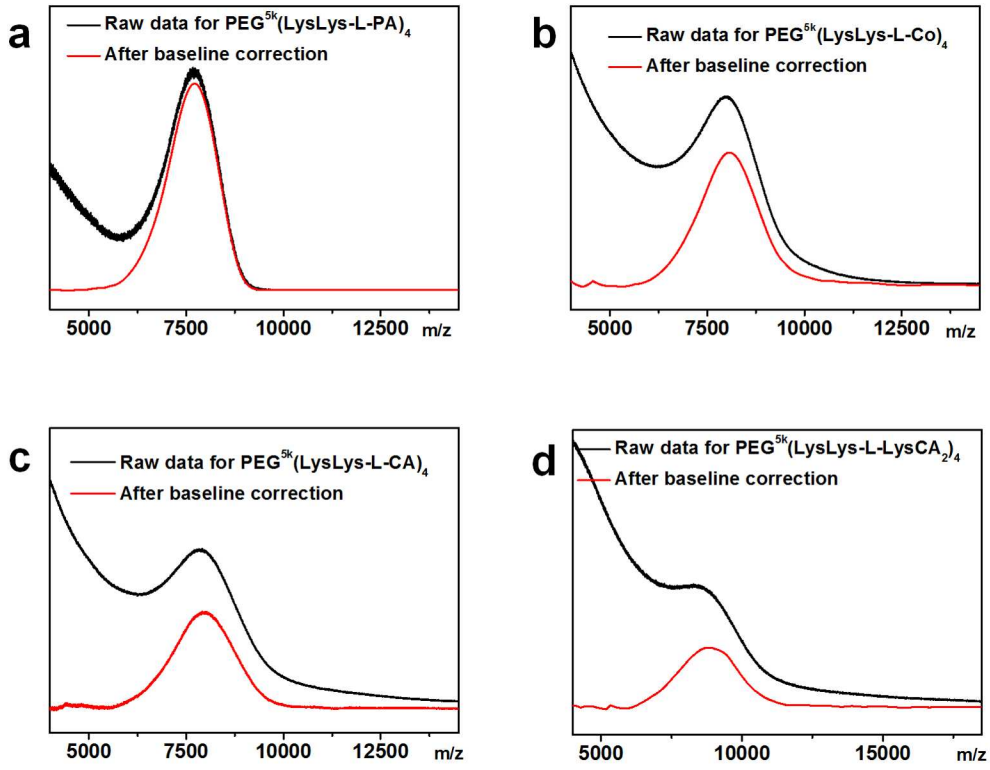


Figure S1. MALDI-TOF spectra of the telodendrimers. The elevated baselines of the raw mass spectra are due to the high laser power required in MALDI analysis to enhance the signals of the highly entangled and self-assembled telodendrimers, which has been studied in our previous publications[4]. The baseline correction was performed for the raw data to obtain polydispersity index (PDI) and more accurate peak molecular weight (M_p).

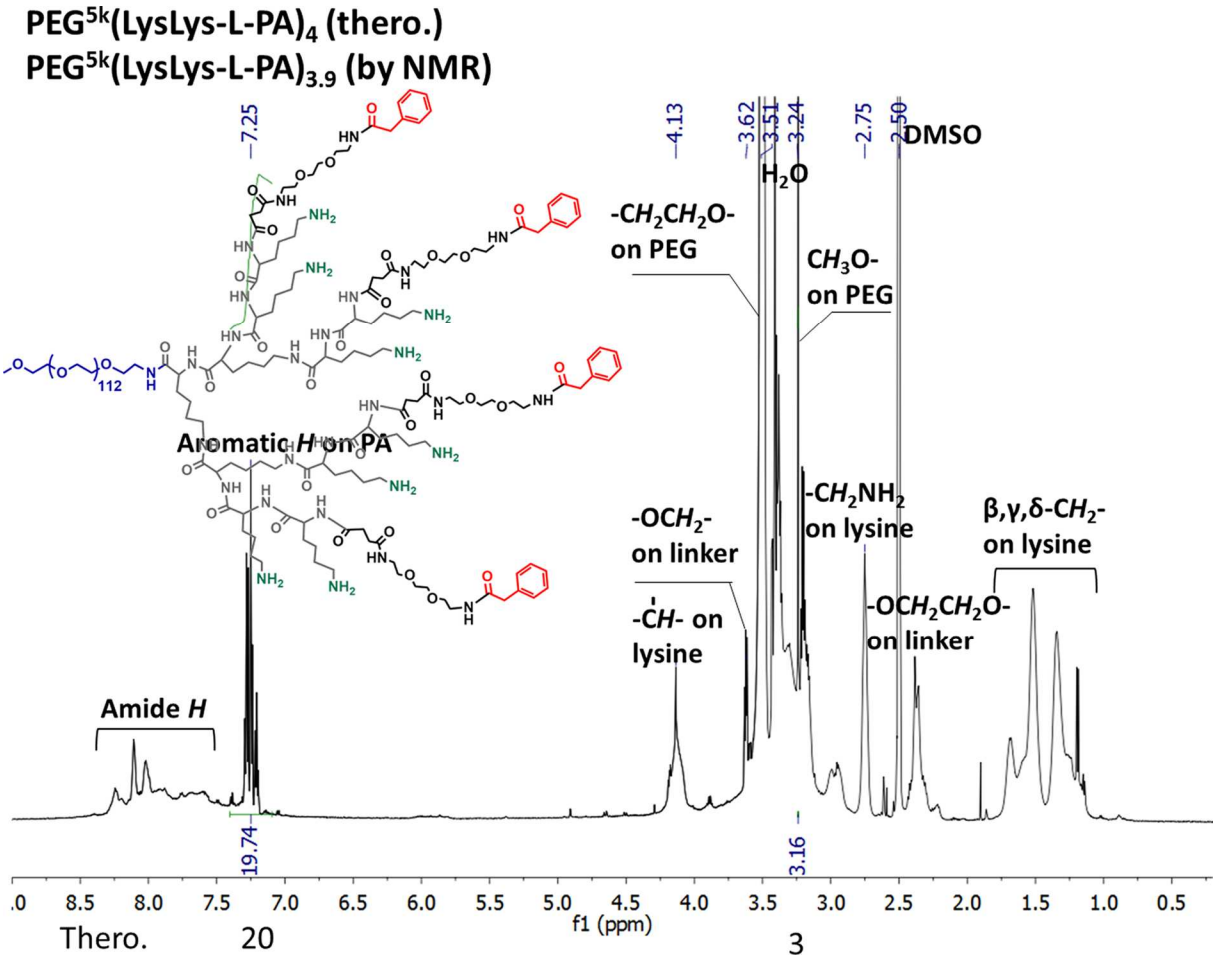


Figure S2. ^{1}H NMR spectrum of PEG^{5k}(LysLys-L-PA)$_4$ in DMSO-d_6.

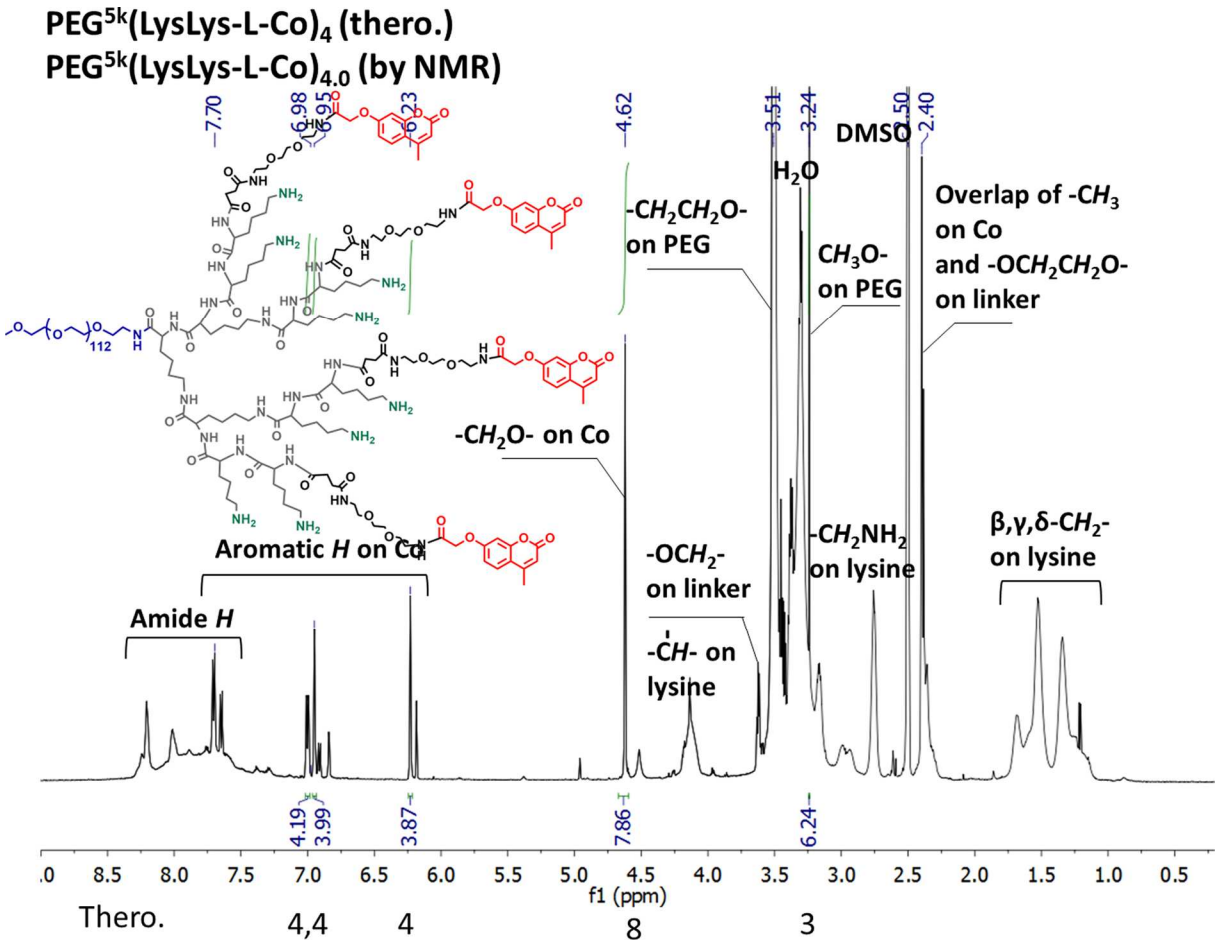


Figure S3. ^{1}H NMR spectrum of PEG^{5k}(LysLys-L-Co)$_4$ in DMSO-d_6.

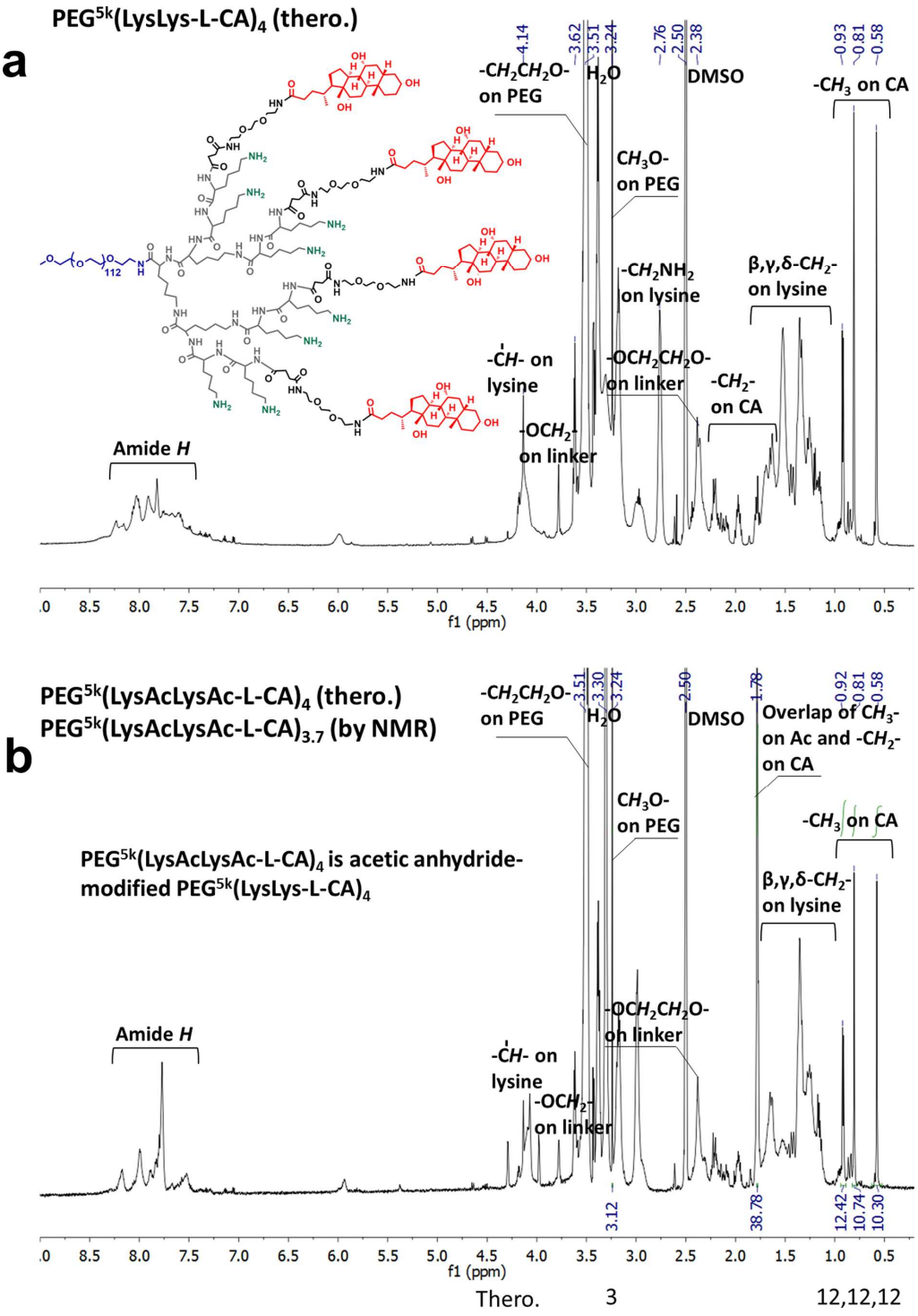


Figure S4. ^{1}H NMR spectra of PEG^{5k}(LysLys-L-CA)$_4$ (a) and PEG^{5k}(LysAcLysAc-L-CA)$_4$ (b) in DMSO-d_6. The interactions between amino groups and hydroxyl groups significantly affect the integration of the methoxy protons on PEG. Therefore, the formula by NMR was obtained based on the *N*-terminal acetylated compound of PEG^{5k}(LysAcLysAc-L-CA)$_4$.

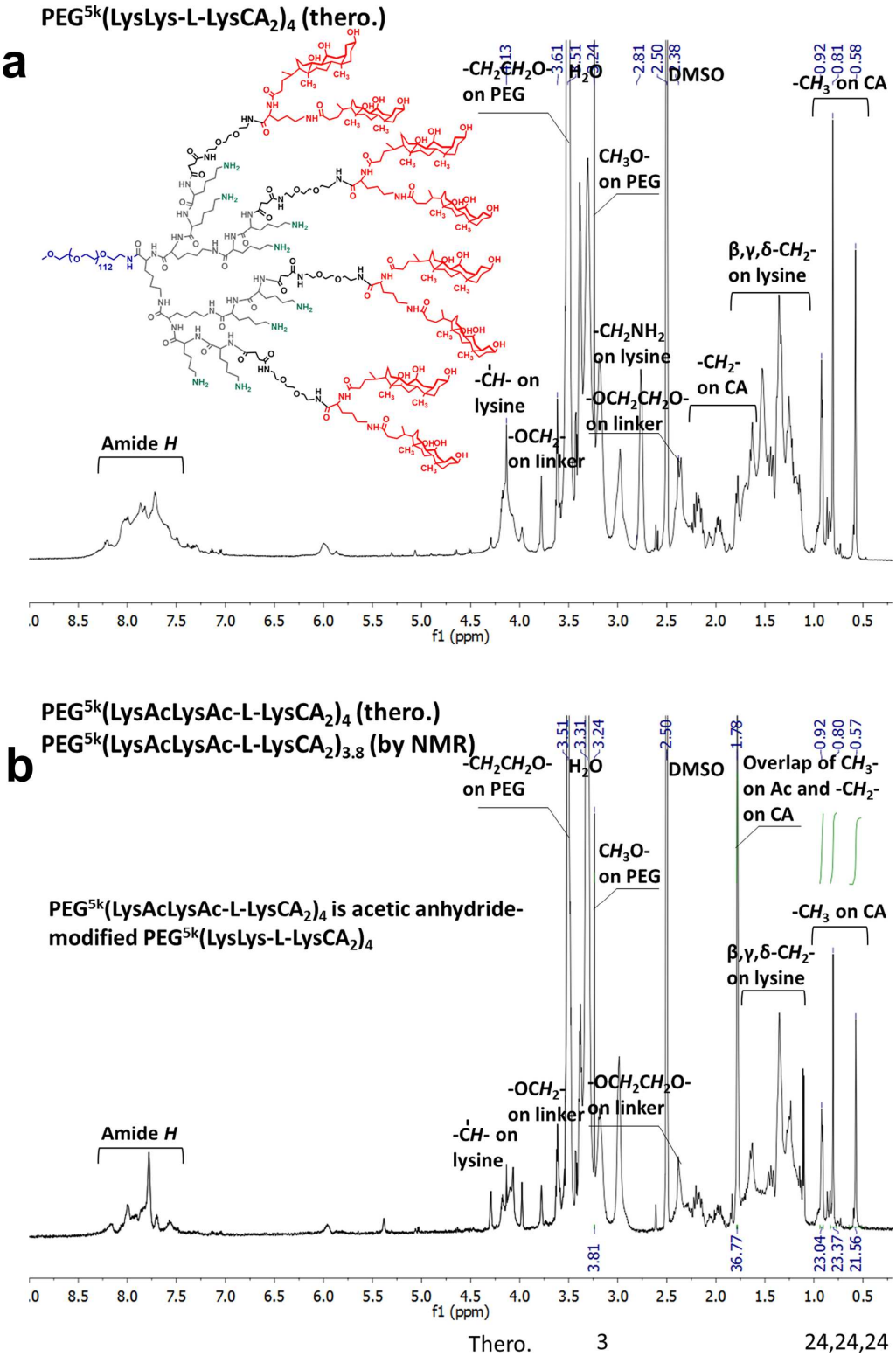


Figure S5. ^{1}H NMR spectra of PEG^{5k}(LysLys-L-LysCA$_2$)$_4$ (a) and PEG^{5k}(LysAcLysAc-L-LysCA$_2$)$_4$ (b) in DMSO-d_6. The formula by NMR was obtained based on the *N*-terminal acetylated compound of PEG^{5k}(LysAcLysAc-L-LysCA$_2$)$_4$.

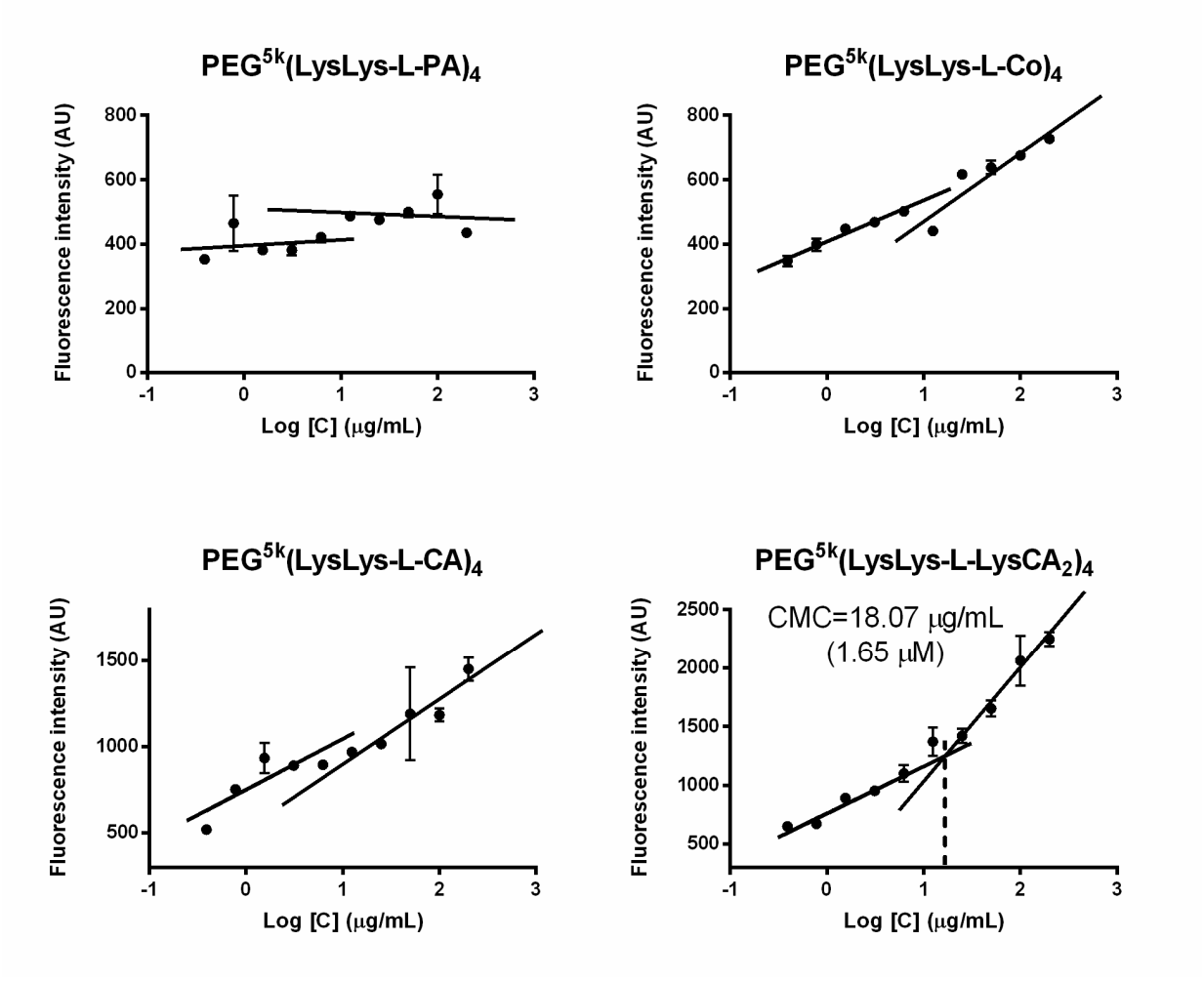


Figure S6. CMCs of the PA-, Co, CA- and $LysCA_2$-containing telodendrimers in PBS. The CMC results for the C17-, CHO-, and VE-containing telodendrimers have been shown in our previous publication.[2]

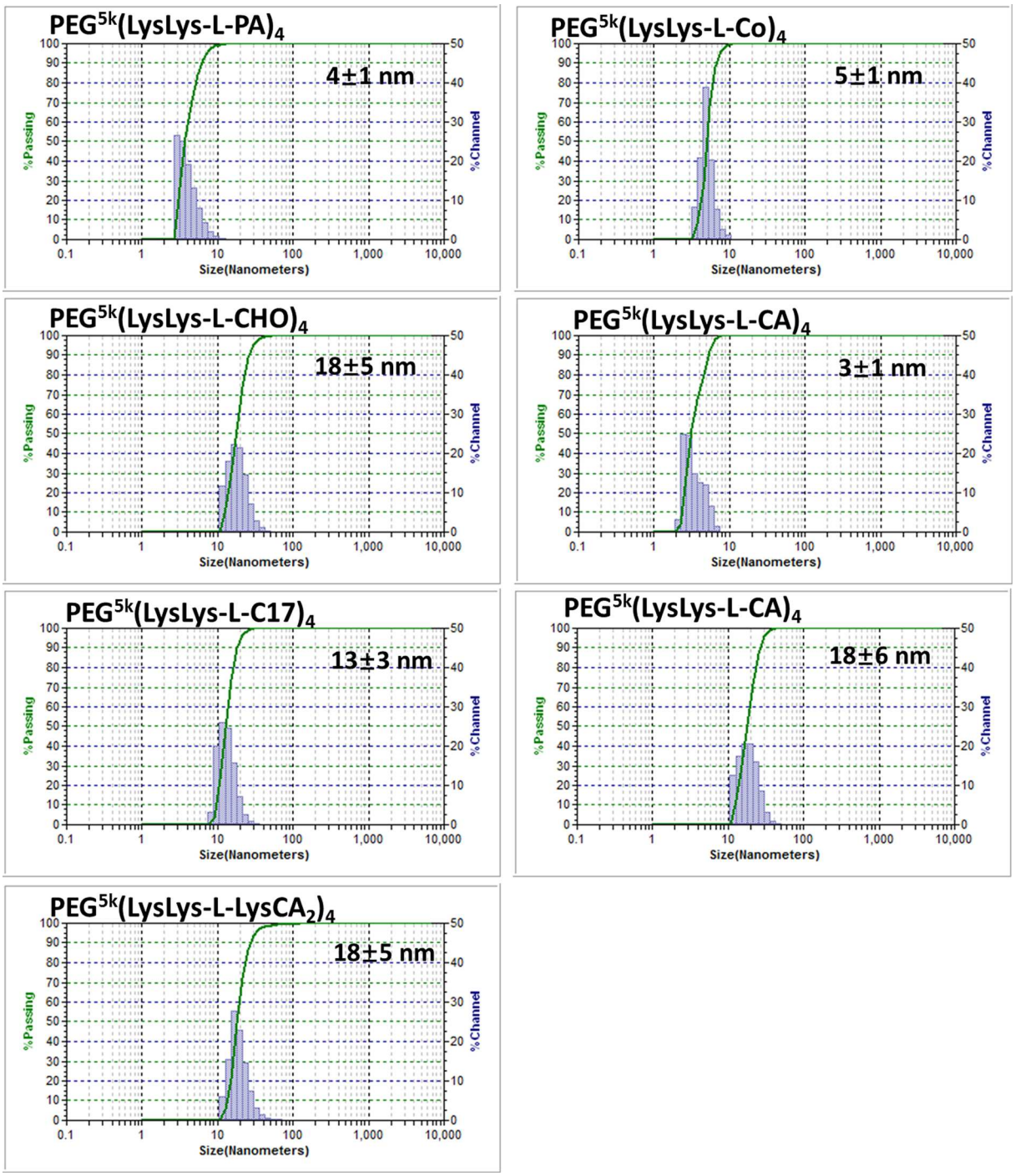


Figure S7. Hydrodynamic diameters of the telodendrimers in PBS at a telodendrimer concentration of 1 mg/mL. The value behind '±' describes the half-peak width of the size distribution.

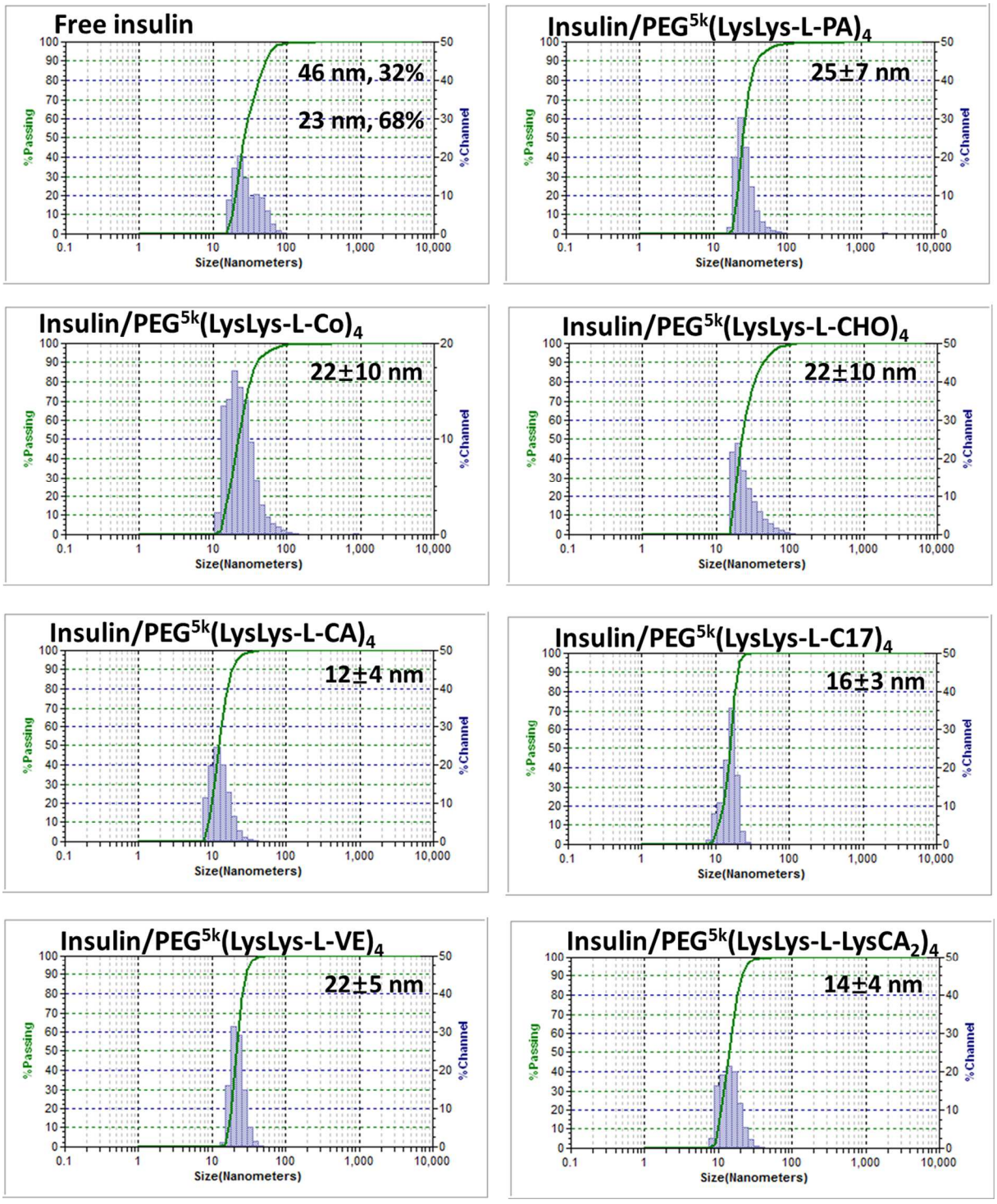


Figure S8. Hydrodynamic diameters of the telodendrimers after loading of insulin at a loading ratio of insulin to telodendrimer of 1/3 by weight in PBS at a telodendrimer concentration of 1 mg/mL. The value behind '±' describes the half-peak width of the size distribution.

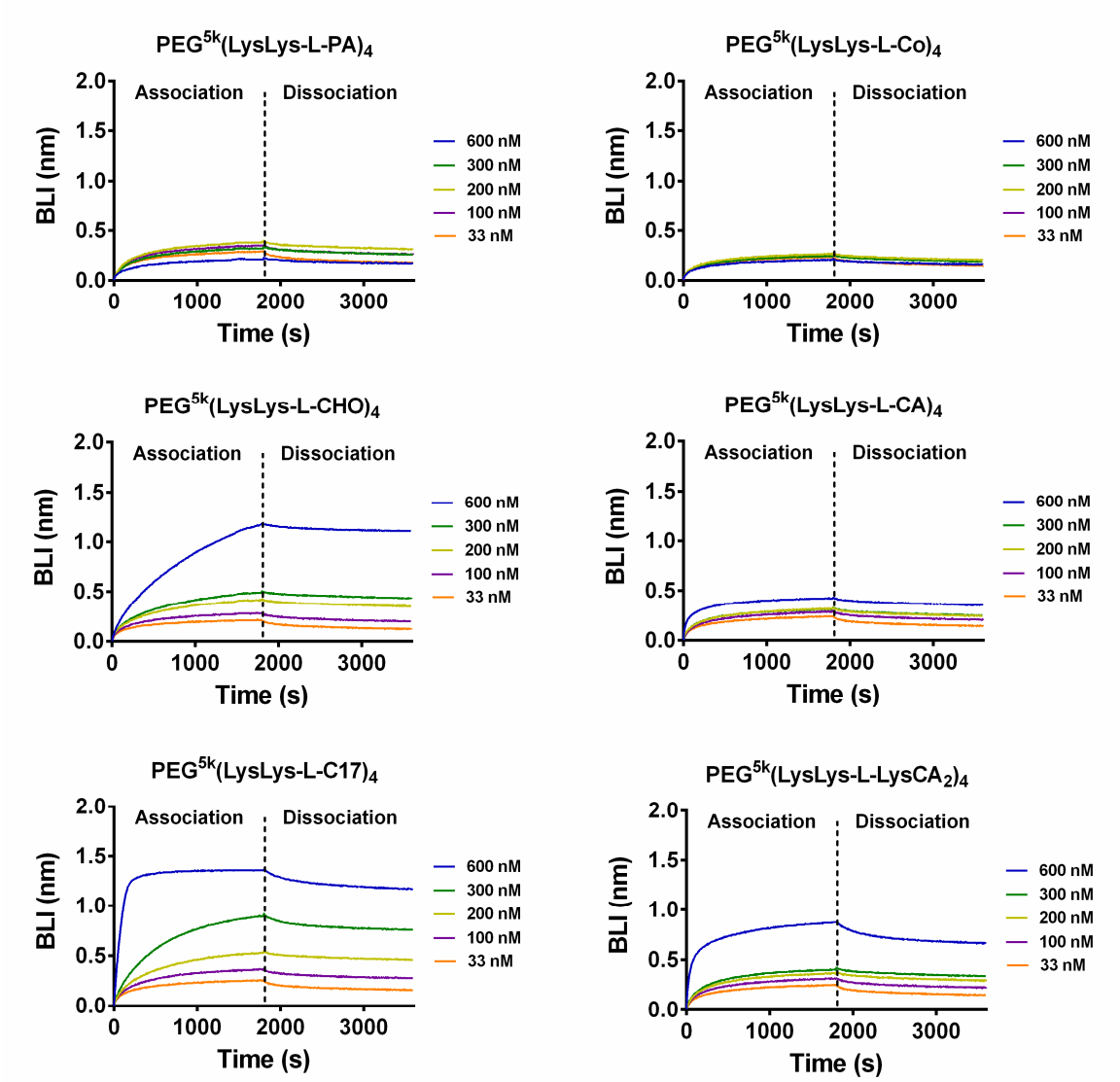


Figure S9. Kinetics for association in different telodendrimer solutions at concentrations ranging from 33 to 600 nM and dissociation in PBS.

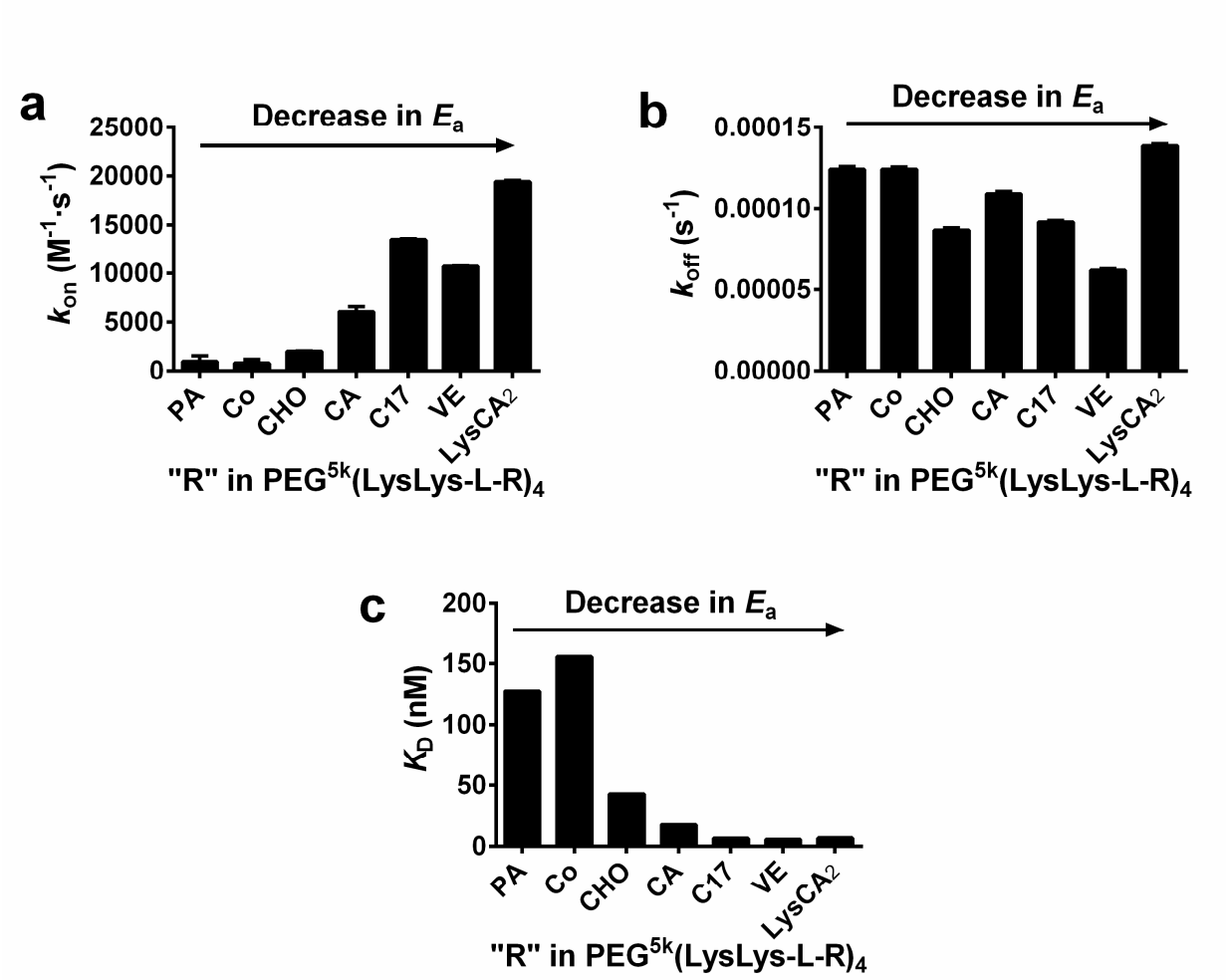


Figure S10. Association constant (k_{on}), dissociation constant (k_{off}) and equilibrium dissociation constant (K_D) obtained by measuring kinetics of the interactions between insulin and different telodendrimers.

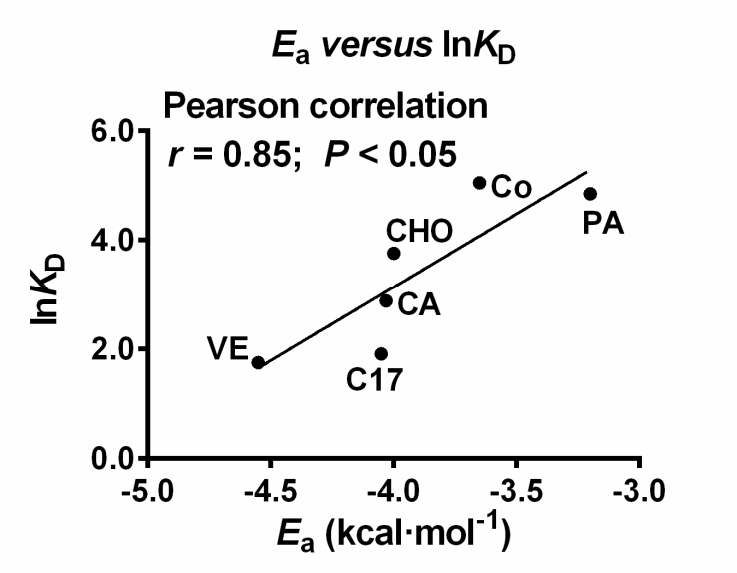


Figure S11. $\ln K_D$ for the telodendrimers containing protein binding moieties plotted against E_a for the protein binding molecules. Linear regression model was fit via Ordinary Least Square to calculate the Pearson correlation coefficient (r) and the associated P values.

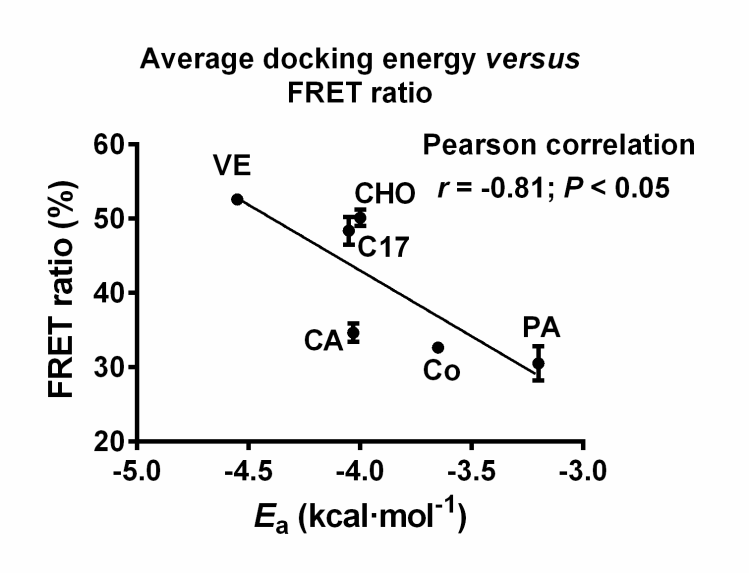


Figure S12. FRET ratios for 1/1/6 (w/w/w) mixtures of FITC-insulin/RB-insulin/telodendrimers containing different protein binding moieties plotted against the average docking energies (E_a) for the protein binding molecules. Linear regression model was fit via Ordinary Least Square to calculate the Pearson correlation coefficient (r) and the associated P values.

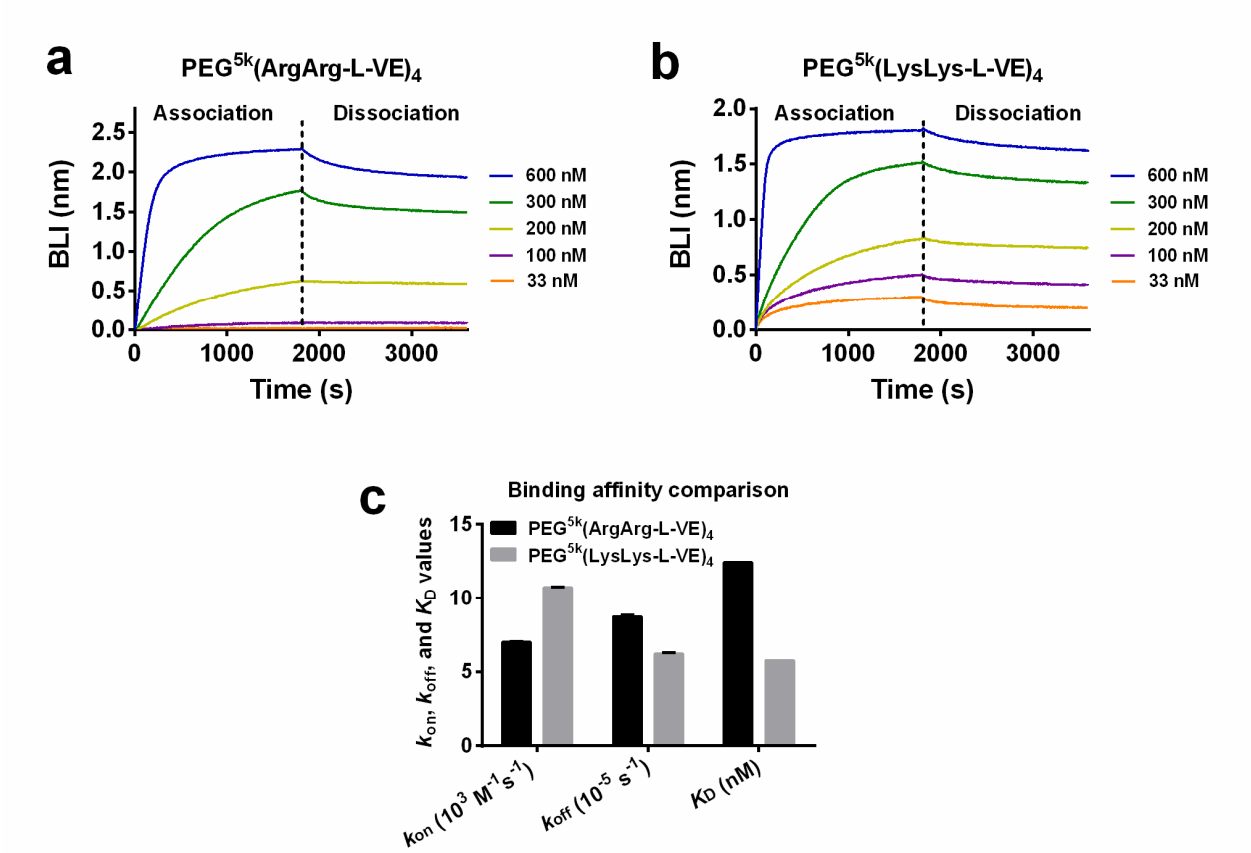


Figure S13. (a, b) Kinetics for association in arginine-containing PEG^{5k}(ArgArg-L-VE)$_4$ (a) and lysine-containing PEG^{5k}(LysLys-L-VE)$_4$ (b) telodendrimer solutions at concentrations ranging from 33 to 600 nM and dissociation in PBS. (c) Comparison of the binding affinity between arginine- and lysine-containing telodendrimers against insulin.

Supporting References

[1] Shao, Y.; Shi, C.; Xu, G.; Guo, D.; Luo, J. *ACS Appl. Mater. Interfaces* **2014**, *6*, 10381-10392.

[2] Wang, X.; Shi, C.; Zhang, L.; Bodman, A.; Guo, D.; Wang, L.; Hall, W. A.; Wilkens, S.; Luo, J. *Biomaterials* **2016**, *101*, 258-271.

[3] Molecular Operating Environment (MOE), 2014.09; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7 **2014**.

[4] Huang, W.; Wang, X.; Shi, C.; Guo, D.; Xu, G.; Wang, L.; Bodman, A.; Luo, J. *Mol. Pharm.* **2015**, *12*, 1216-1229.