Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Modular Design and Self-assembly of Multidomain Peptides Towards Cytocompatible Supramolecular Cell Penetrating Nanofibers

Su Yang, He Dong*

Department of Chemistry & Biochemistry, The University of Texas at Arlington, Arlington, TX, USA

E-mail: he.dong@uta.edu

Experimental section

General Methods

4-Methylbenzhydrylamine (MBHA) rink amide resin, Fmoc-protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 5(6)-carboxylfluoresceinpiperidine, diisopropylethylamine (DIPEA), α-cyano-4-hydroxycinnamic acid, methyl-β-cyclodextrin, amiloride and filipin III were purchased from Sigma-Aldrich. Trifluoroacetic acid (TFA), triisopropylsilane (TIS), acetonitrile (ACN), dimethylformamide (DMF), acetic anhydride were purchased from Fisher Scientific and used as received. Dulbecco's modified Eagle medium (DMEM), LysoTracker Red DND-99 and Hoechst 33342 were purchased from Life Technologies. Fetal Bovine Serum (FBS) was purchased from VWR. CCK8 assay kit was obtained from Dojindo Molecular Technologies (Rockville, MD). Transmission Electron Microscopy (TEM) grids and uranium acetate dihydrate were purchased from Ted Pella, Inc.

Peptides Synthesis and Purification. The synthetic procedure followed the standard Fmoc-solid phase peptide synthesis method on a *Prelude*® peptide synthesizer. In brief, the synthesis was set up for 30 μmol scale using MBHA rink amide resin. The Fmoc group was deprotected in the presence of 20% (V/V) piperidine/DMF for 5 minutes and repeated once. The coupling reaction was carried out for 30 mins by adding Fmoc-protected amino acids, HBTU and DIPEA with a molar ratio of 4:4:8 with respect to the amino groups on the MBHA rink amide resin. After the completion of the synthesis, the N-terminus of the MDPs was acetylated using DIPEA and acetic anhydride in DMF for 1 hr. Kaiser test was performed to confirm the completion of the acetylation reaction. The acetylated peptide was cleaved in a mixture of TFA/TIS/ H_2O

(95/2.5/2.5 by volume) for 3 hrs. The cleavage solution was filtered, and the filtrates were collected. The resin was washed three times with neat TFA, and all filtrate solutions were combined and evaporated under airflow. The residual peptide solution was precipitated in cold diethyl ether, followed by centrifugation and washing with cold diethyl ether for three times. The crude peptide was dried under vacuum overnight for HPLC purification. Peptides were purified using a preparative reverse phase C4 column with a linear gradient of H_2O/ACN (5% to 95% of acetonitrile in 30 mins) containing 0.05% TFA and the elution was monitored at both 230 nm and 280 nm. The HPLC fraction was collected, combined and lyophilized for 2 days. Fluorescein terminated peptides were synthesized as follows. After the final deprotection of the Fmoc group, peptide resin was treated with 4 equivalents of 5(6)-carboxyl fluorescein (FITC), 4 equivalents of HBTU and 8 equivalents of DIPEA in DMF. The reaction mixture was stirred overnight. The completion of the coupling reaction was confirmed by the Kaiser test. The cleavage and purification steps followed the same procedure as described above. The molecular weight of each peptide was characterized and confirmed by MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid as the matrix. K10: expected $[M+H]^+$: 3225.8, observed $[M+H]^+$: 3225.8; K10-E2: expected $[M+H]^+$: 3483.9, observed $[M+H]^+$: 3483.8; K12-E3: expected $[M+H]^+$: 3869.1, observed $[M+H]^+$: 3869.4; K10-E3: expected $[M+H]^+$: 3612.9, observed $[M+H]^+$: 3612.6; F-K10: expected $[M+H]^+$: 3542.1, observed $[M+H]^+$: 3541.8. For all the experiments, peptide stock solution was prepared by dissolving lyophilized peptide powder in Tris buffer (20 mM, pH=7.4) to reach a final concentration at 1 mM and left at 4°C for a day.

Circular Dichroism (CD) Spectroscopy. Peptide solutions were diluted to 20 μM in Tris buffer (pH 7.4, 20 mM) for CD measurements on a Jasco-J710 spectrometer. The CD spectra were collected from 250 nm to 190 nm at room temperature using a 2 mm cuvette, a bandwidth at 0.1 nm, scan rate at 100 nm/min and a response time of 2 sec. Each spectrum was averaged from three scans. The mDeg of rotation was converted to the molar residual ellipticity using the following formula $\theta = \frac{1000 \times mDeg}{c * n * l}$, where c is the concentration of the peptide solution in mM, n is the number of amino acids in the peptide sequence and l is the path length of the cell used in mm.

Transmission Electron Microscopy (TEM). TEM was performed on a Hitachi H-9500 High-resolution TEM instrument. Peptide solutions were diluted to 100 μM in Tris buffer (pH 7.4, 20 mM) for TEM examination. 10 μl of the peptide solution was pipetted onto a holey carbon grid (TED PELLA 01824). After 2 mins, the excess solution was carefully removed with filter paper. 10 μl of 2 wt% uranyl acetate solution was dropped onto the grid for negative staining. The excess staining solution was removed with filter paper after 2 mins. The TEM sample was dried overnight before imaging.

Critical Aggregation Concentration (CAC) Measurement. CACs were determined using a previous protocol based on the fluorescence intensity change of tryptophan.[1, 2] Fluorescence measurements were taken on a Varian Cary Eclipse fluorescence spectrophotometer Increments of 2 μL of a 400 μM peptide solution was added to 500 μL Tris buffer (pH 7.4, 20 mM). Fluorescence emission spectra were acquired after each peptide addition from 295 nm to 440 nm using an excitation wavelength at 280 nm. Fluorescence intensity at 350 nm was plotted as a function of peptide concentrations for CAC determination.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed on a Beckman-Coulter Optima XL-I analytical ultracentrifuge equipped with an An-50 Ti 8-hole rotor. Double-sector centerpieces sandwiched between sapphire windows in a standard cell housing were loaded with 400 μL of sample and an equal volume of reference buffer (20 mM Tris buffer, pH 7.4). After 2 hrs of equilibration under vacuum at 20 °C, samples were centrifuged at 50,000 rpm. Data was acquired using UV absorbance optics tuned to 280 nm for each sample. Sedimentation velocity data was fitted to a continuous *c(s)* distribution model using SEDFIT software. The buffer density and viscosity at room temperature were determined to be 0.99880 g/mL and 0.01007 cP, respectively using SEDNTERP. The partial-specific volume was estimated at 0.75330 mL/g for K10, 0.74610 mL/g for K10-E2, 0.74790 mL/g for K12-E3 and 0.74290 mL/g for K10-E3. A resolution of 50 was utilized with a regularization level of 0.68. Time-invariant noise elements were removed from the data. All figures featuring *c(s)* distributions were generated in GUSSI software.[3]

Cell uptake. DMEM containing 10% FBS was used as the culture medium for all *in vitro* experiments**.** For the cell uptake study, peptide samples were prepared as follows. Each peptide, namely K10, K10-E2, K12-E3 and K10-E3 was mixed with FITC-labeled K10 with a molar ratio of 95:5 in a mixed solvent of water and acetonitrile (1:1 by volume). The mixture was lyophilized and rehydrated in Tris buffer (pH 7.4, 20 mM) to form FITC-labeled co-assembly at a final concentration of 1 mM and left at 4°C for a day. HeLa cells were seeded onto a confocal dish at a density of 10^5 cells/well and incubated overnight at 37 °C in an incubator with 5% of CO_2. After 24 hrs, DMEM culture medium was refreshed. Co-assembled peptides were diluted in Tris buffer to reach a final concentration at 200 μM. 20 μL of the diluted peptide

solution was added to the culture medium to reach a final concentration at 20 μM. After 2 hrs and 24 hrs of incubation, cells were stained with nucleus staining dye, Hoechst 33342 at 37 °C for 15 min and washed with PBS buffer for three times. For lysotracker staining, peptide containing culture medium was removed and stained with LysoTracker Red DND-99 at 37 °C for 1 hr, followed by staining with the nucleus staining dye, Hoechst 33342 at 37 °C for 15 min. Images were captured using an Olympus IX71 Inverted fluorescence microscope and processed with ImageJ software.

For endocytosis-inhibition examination, HeLa cells were seeded onto a confocal dish at a density of 10^5 cells/well and incubated overnight at 37 °C in an incubator with 5% of CO_2. Co-assembled K10-E3 was prepared using the same procedure. After 24 hrs, the culture medium was replaced with fresh medium containing 5 μM of methyl-β-cyclodextrin, 5 μM of amiloride or 5 μg/ml filipin III. After 1 hr, the culture media containing different endocytosis inhibitors were replaced with refresh medium. FITC-labelled co-assembled K10-E3 was added into the culture to reach a final concentration of 20 μM. After 24 hrs of incubation, cells were stained with nucleus staining dye, Hoechst 33342 at 37 °C for 15 min and washed three times with PBS buffer. Images were captured using an Olympus IX71 Inverted fluorescence microscope and processed with ImageJ software.

Flow cytometry. HeLa cells were seeded onto a 24-well plate at a density of 10^5 cells/well and incubated for 24 hrs at 37 °C with 5% of CO_2. Co-assembled peptides were prepared using the same procedure as cell uptake. The culture medium was replaced with 450 μL fresh DMEM medium and 50 μL of co-assembled peptides solutions (200 μM) were added. After 24 hrs, the culture medium was removed, and cells were washed with PBS buffer three times. Cells were

digested with trypsin and washed twice with PBS buffer. 2% paraformaldehyde was used for cell fixation. The mean fluorescence intensity of cells upon different peptide treatment was quantified using a The BD LSR II flow cytometer. A minimum of 10,000 events per sample was analyzed and data were processed using FlowJo software.

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Cytotoxicity measurement. HeLa cells were seeded onto a 96-well plate at a density of 10^4 cells/well and incubated at 37 °C in an incubator with 5% of CO_2. After 24 hrs, DMEM culture medium (10% FBS) was removed. 10 μL of diluted peptide solution in Tris buffer (pH 7.4, 20mM) at various concentrations (800, 400, 200, 100, 50, 25, 12.5 μM) were mixed with 90 μL DMEM culture medium in the 96-well plate. Cells incubated with 10 μL of Tris buffer were used as a control group. After 24 hrs of incubation, culture medium was removed, the mixture

of 90 μL fresh medium and 10 μL of CCK8 assay solution[4, 5] was added to each of the wells. After 1 hr of incubation at 37 °C, UV absorbance was measured at 450 nm and the cell viability was calculated using the following equation. All experiments were performed in four replicates.

$$\% \text{ cell viability} = (A_{peptide} - A_{Tris\ control}) / (A_{Tris\ control}) \times 100$$

Statistical analysis. All data were expressed as means ± standard deviation (SD). The statistical analysis was performed using Student's T-test and one-way analysis of variance (ANOVA) at confidence levels of 95%.

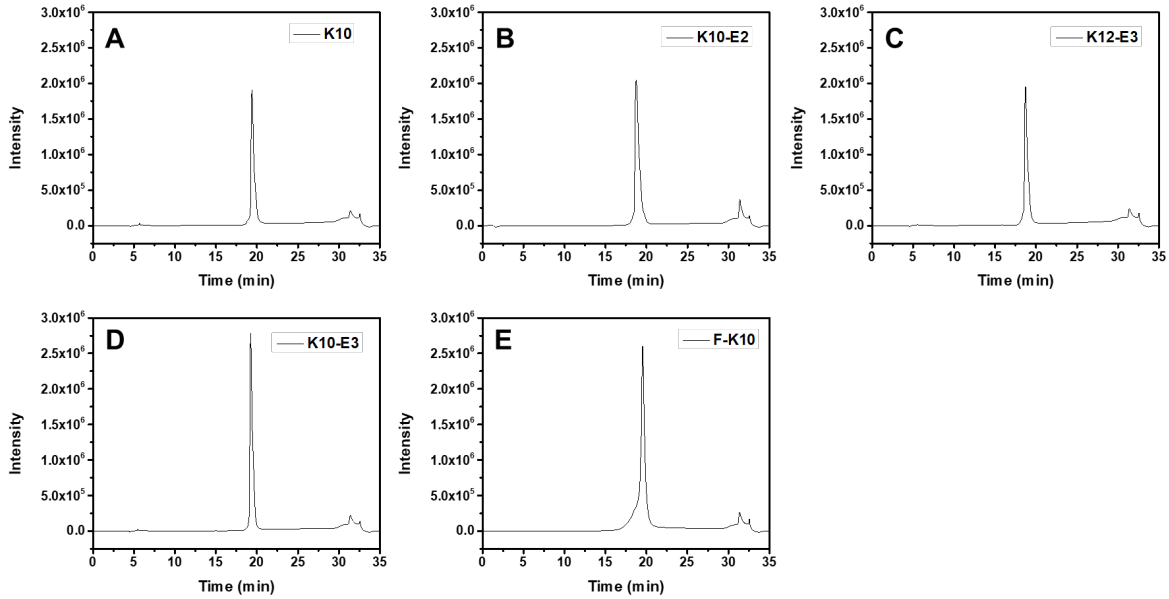

Figure S1. HPLC chromatograms of (**A**) K10 (**B**) K10-E2 (**C**) K12-E3 (**D**) K10-E3 and (**E**) F-K10.

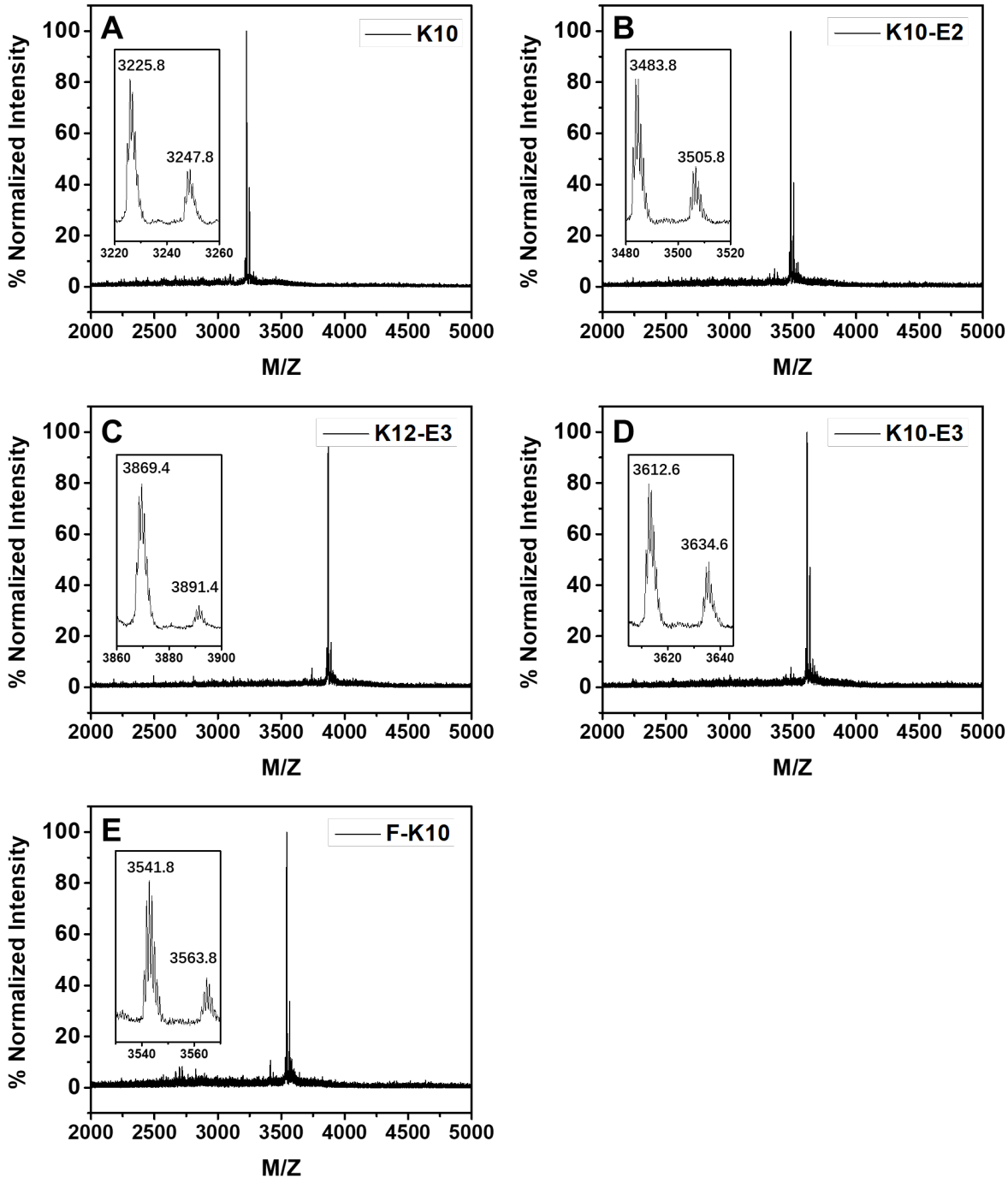

Figure S2. Mass spectra of (**A**) K10 (**B**) K10-E2 (**C**) K12-E3 (**D**) K10-E3 and (E) F-K10.

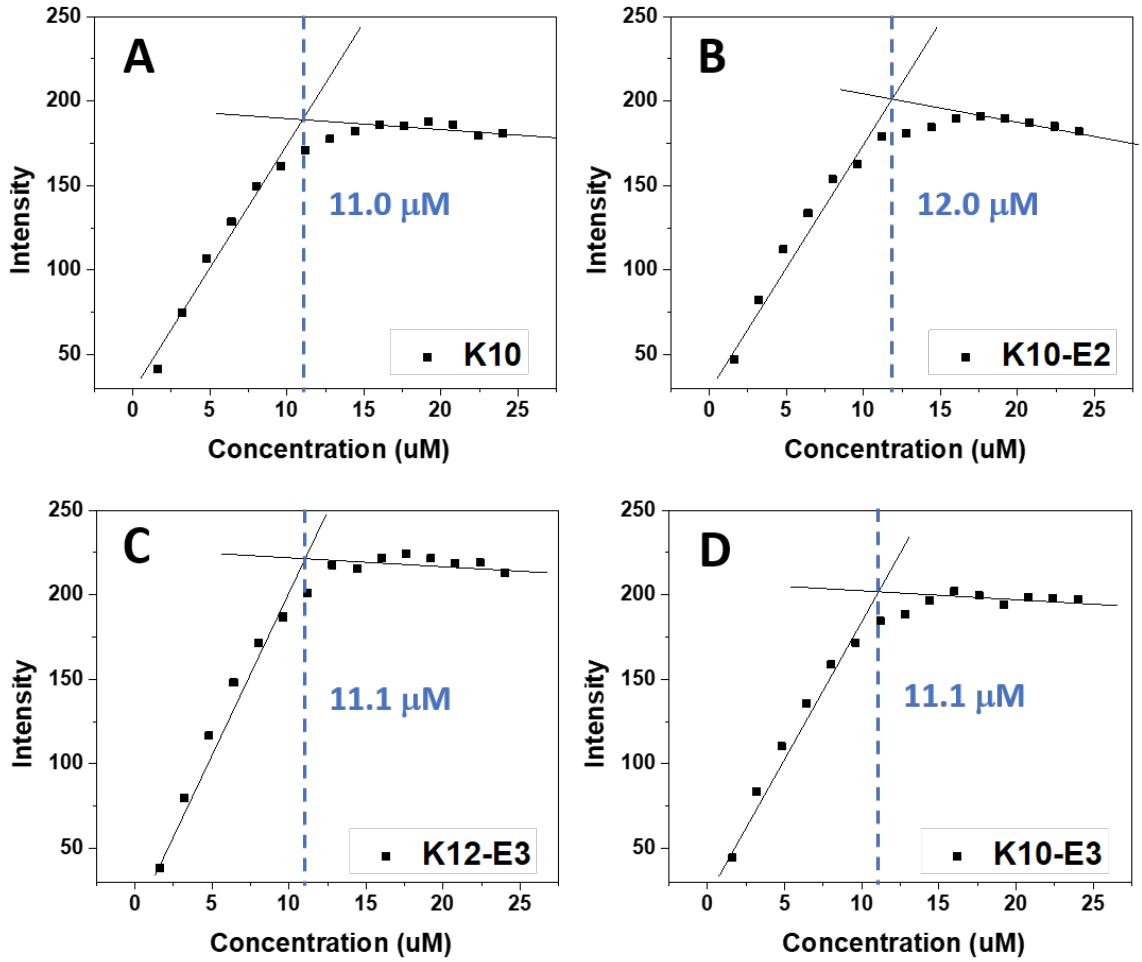

Figure S3. CAC determination of (**A**) K10, (**B**) K10-E2 (B), (**C**) K12-E3 and (**D**) K10-E3 by plotting the tryptophan fluorescence intensity as a function of peptide concentrations.

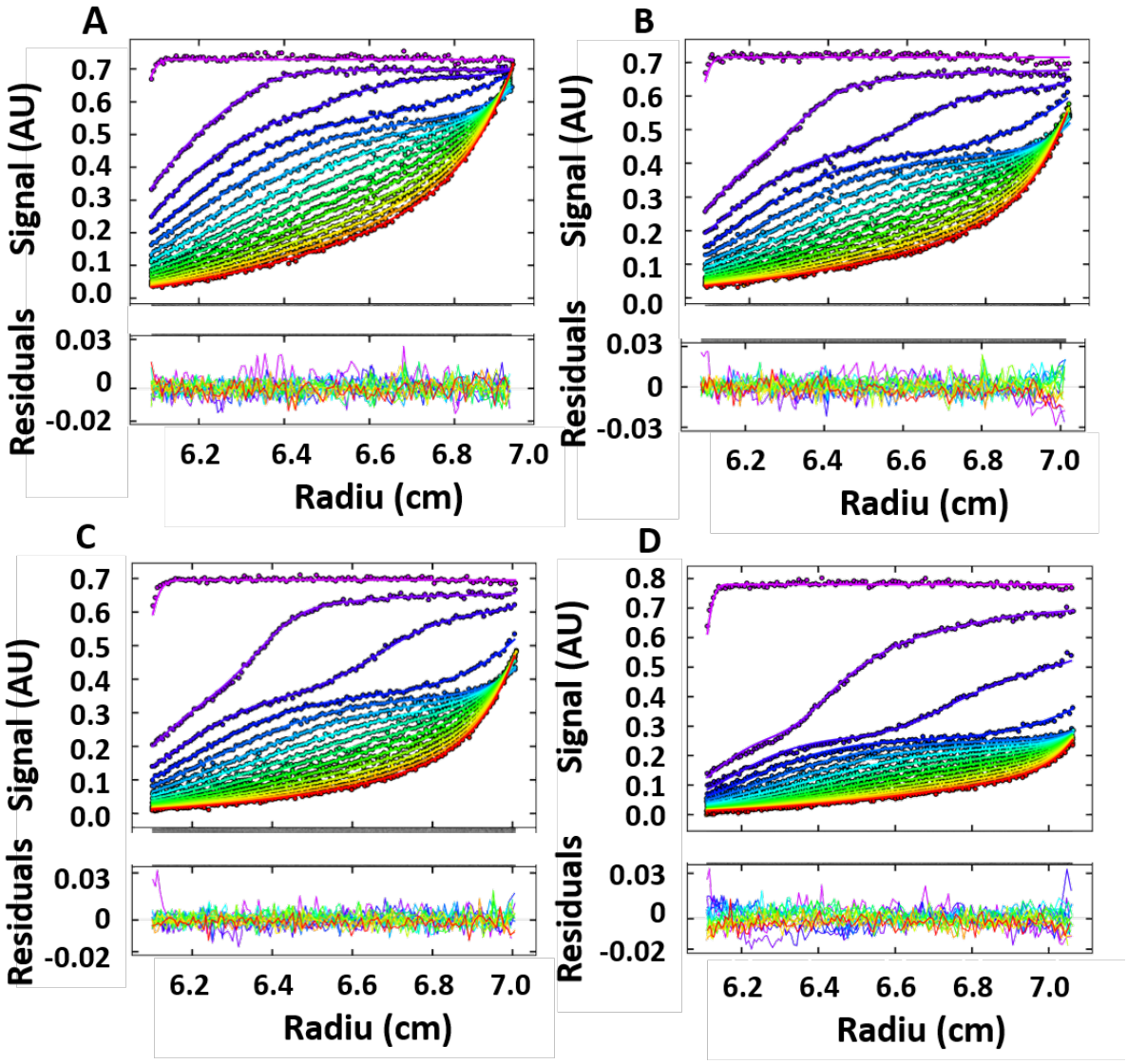

Figure S4. Raw sedimentation profiles by monitoring the absorbance at 280 nm versus cell radius and residual plots supplied by SEDFIT software showing the fitting goodness. (**A**) K10, (**B**) K10-E2, (**C**) K12-E3 and (**D**) K10-E3.

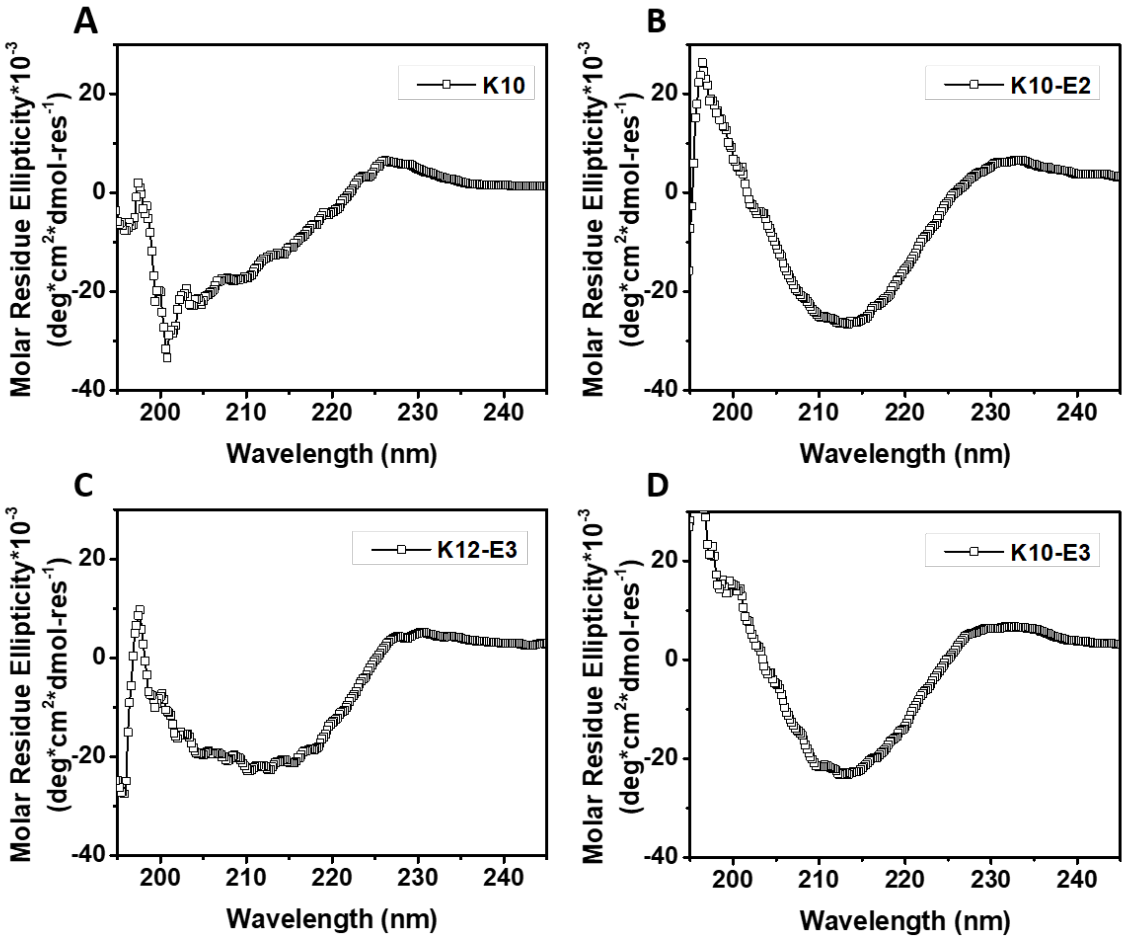

Figure S5. CD spectra of (**A**) K10, (**B**) K10-E2, (**C**) K12-E3 and (**D**) K10-E3 in Tris buffer (pH 7.4, 20 mM). Peptide concentration: 20 μM.

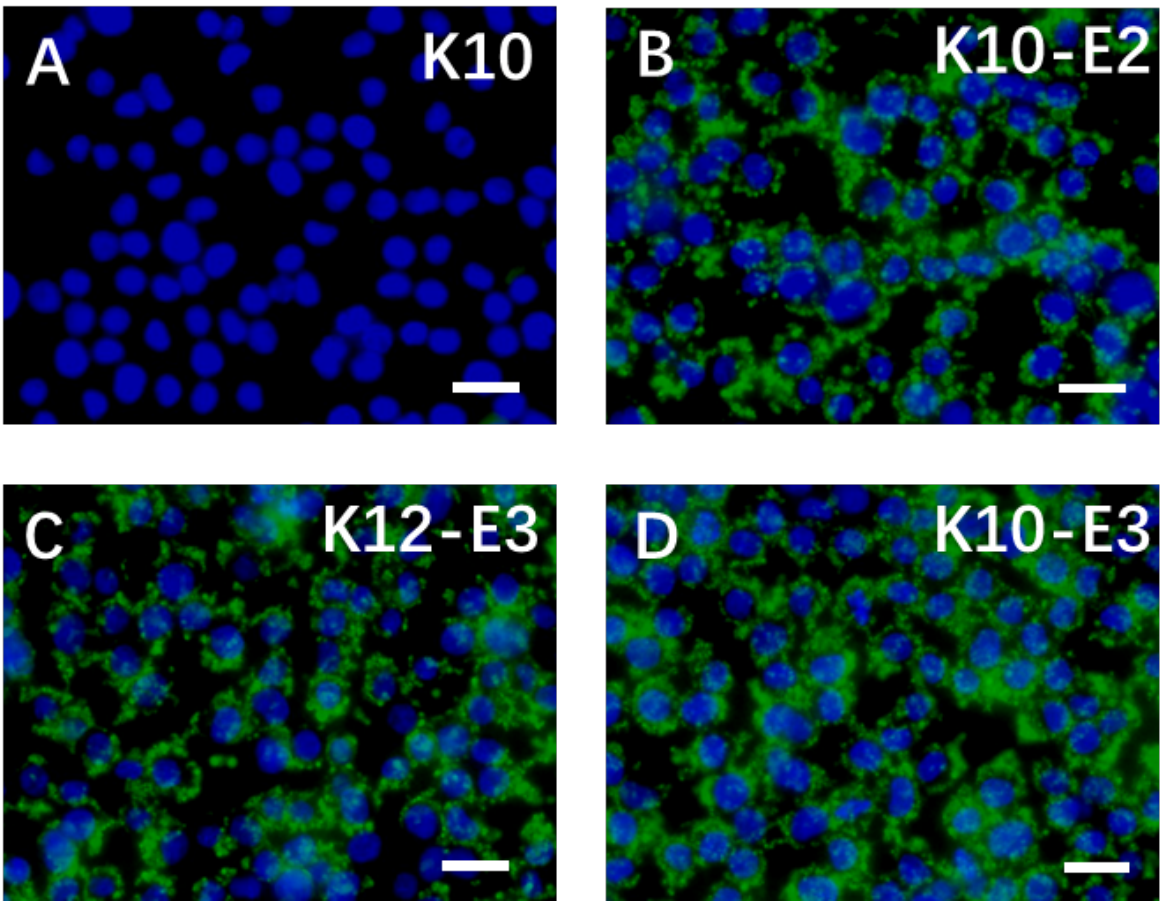

Figure S6. Fluorescence microscopic images of HeLa cells upon incubation with FITC-labeled (**A**) K10, (**B**) K10-E2, (**C**) K12-E3 and (**D**) K10-E3 for 2 hrs. Scale bar = 25 μm.

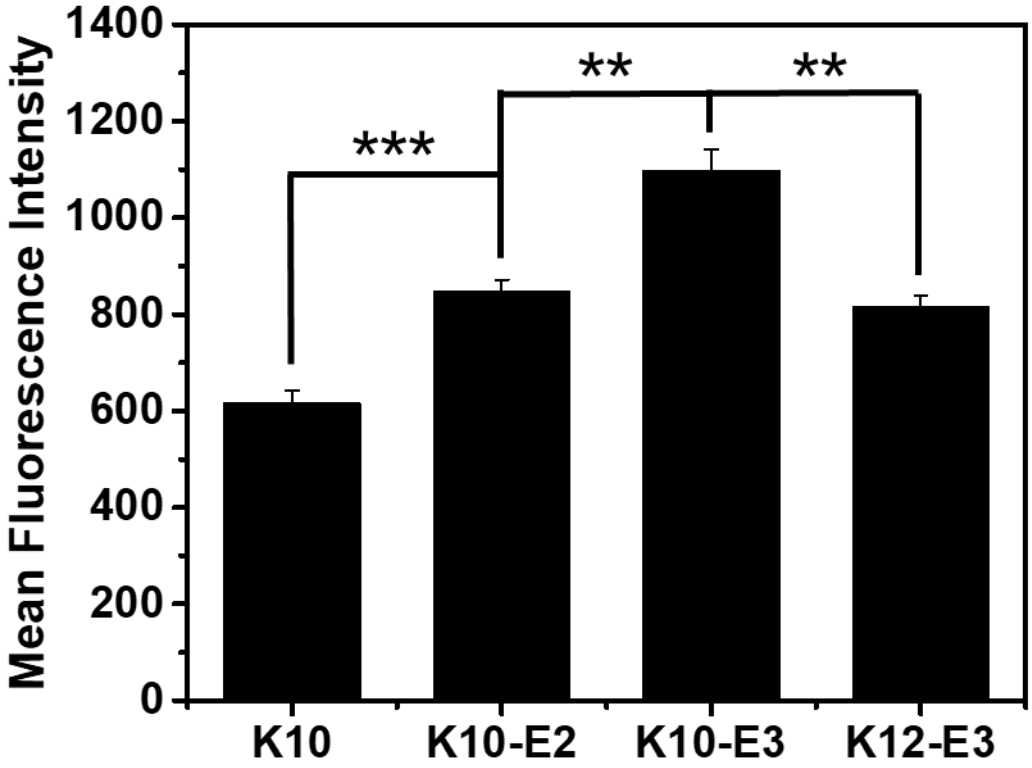

Figure S7. Fluorescence mean intensity of HeLa cells upon incubation with different MDPs as measured by flow cytometry. Peptide concentration: 20 μM. Statistically significant differences are indicated by **p<0.01, ***p<0.001.

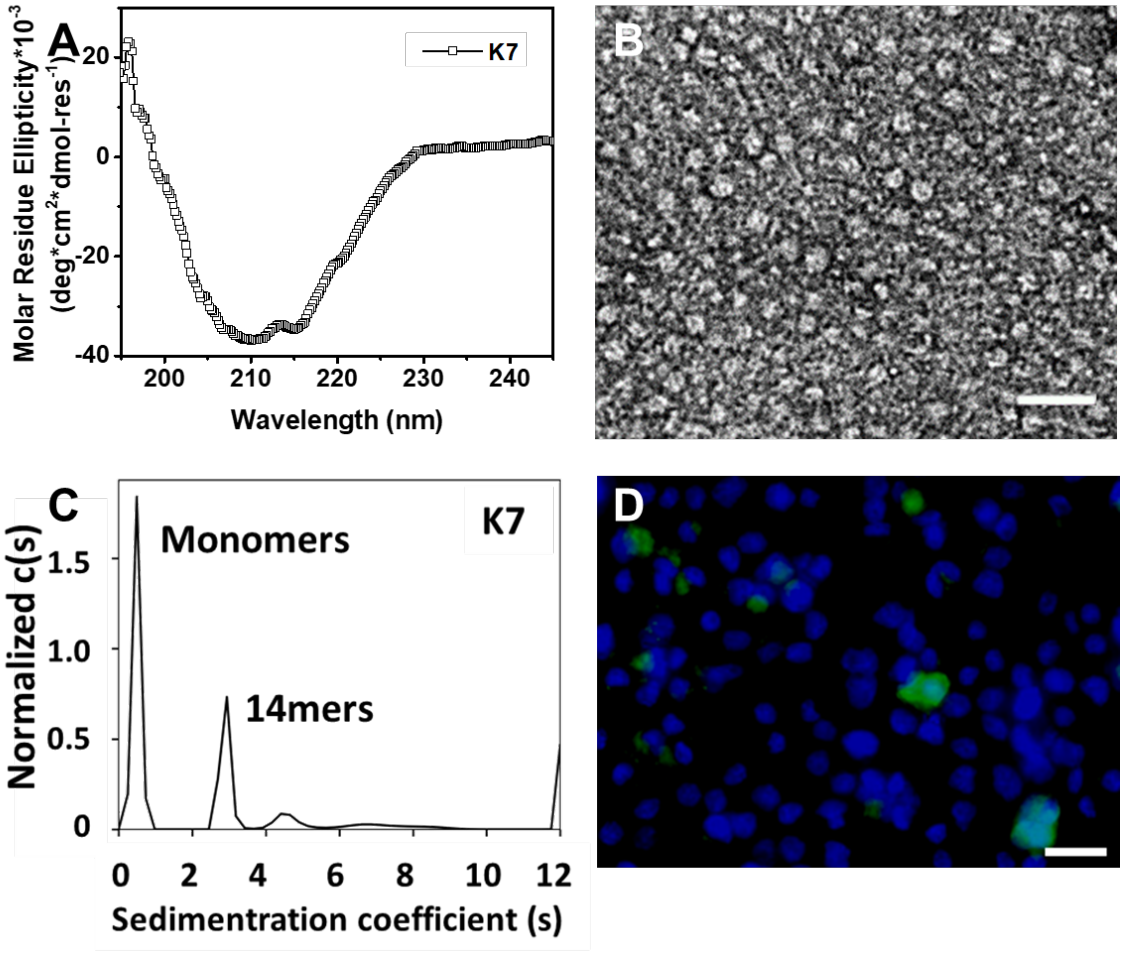

Figure S8. (**A**) CD spectrum of K7 in Tris buffer (pH 7.4, 20 mM). Peptide concentration: 20 μM. (B) Negatively stained TEM image of K7. Scale bar = 50 nm. (C) Sedimentation velocity data of K7 as a semi-quantitative measure of the assembly state. Peptide concentration: 20 mM in Tris buffer (20 mM, pH = 7.4). (D) Fluorescence microscopic images of HeLa cells incubated with co-assembled K7 (95%) and FITC-K10 (5%) for 24 hrs. Green: FITC labelled peptide, blue: nucleus staining. Scale bar = 25 μm.

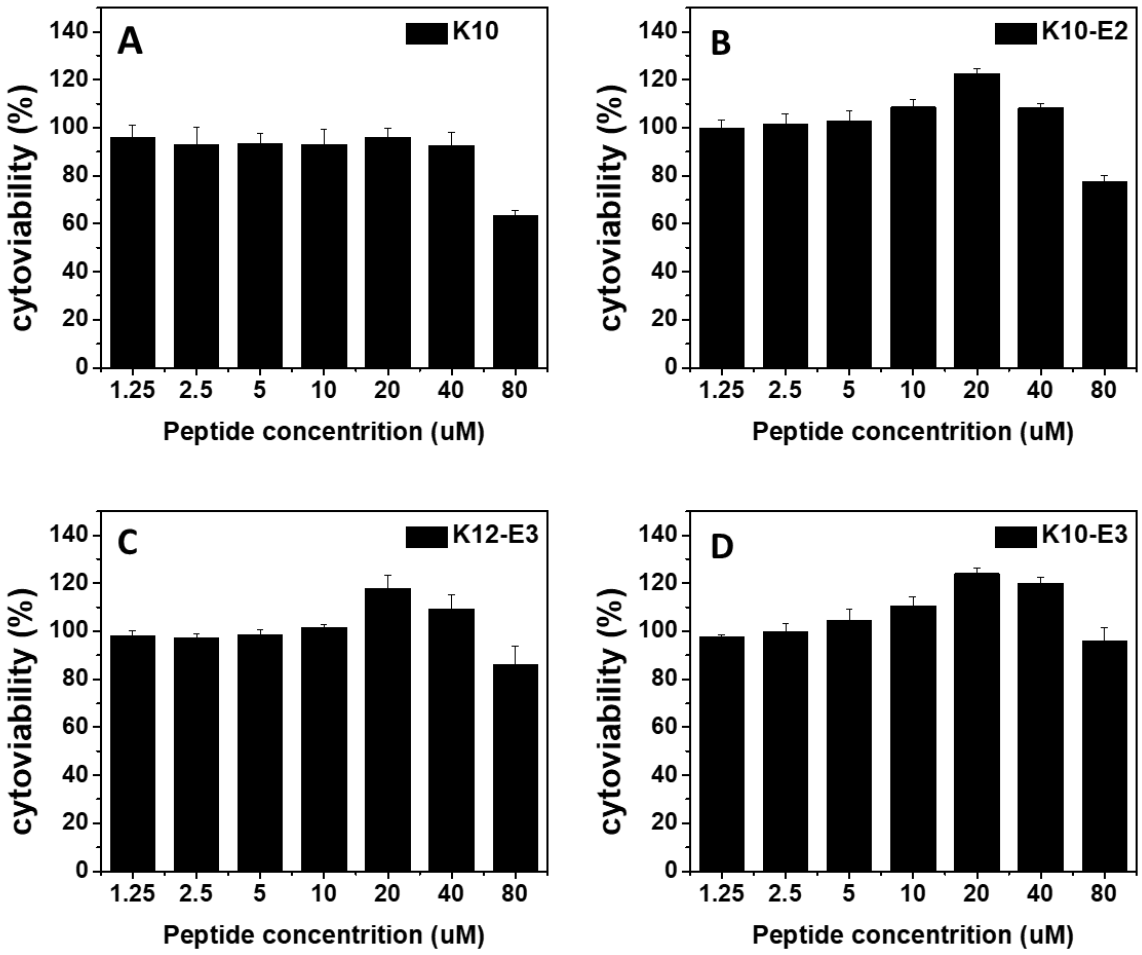

Figure S9. Viability of HeLa cells upon incubation with (**A**) K10, (**B**) K10-E2, (**C**) K12-E3 and (**D**) K10-E3. The cell viability assay was performed after 24 hrs of incubation of HeLa cells with peptides with concentrations ranging from 1.25 μM to 80 μM.

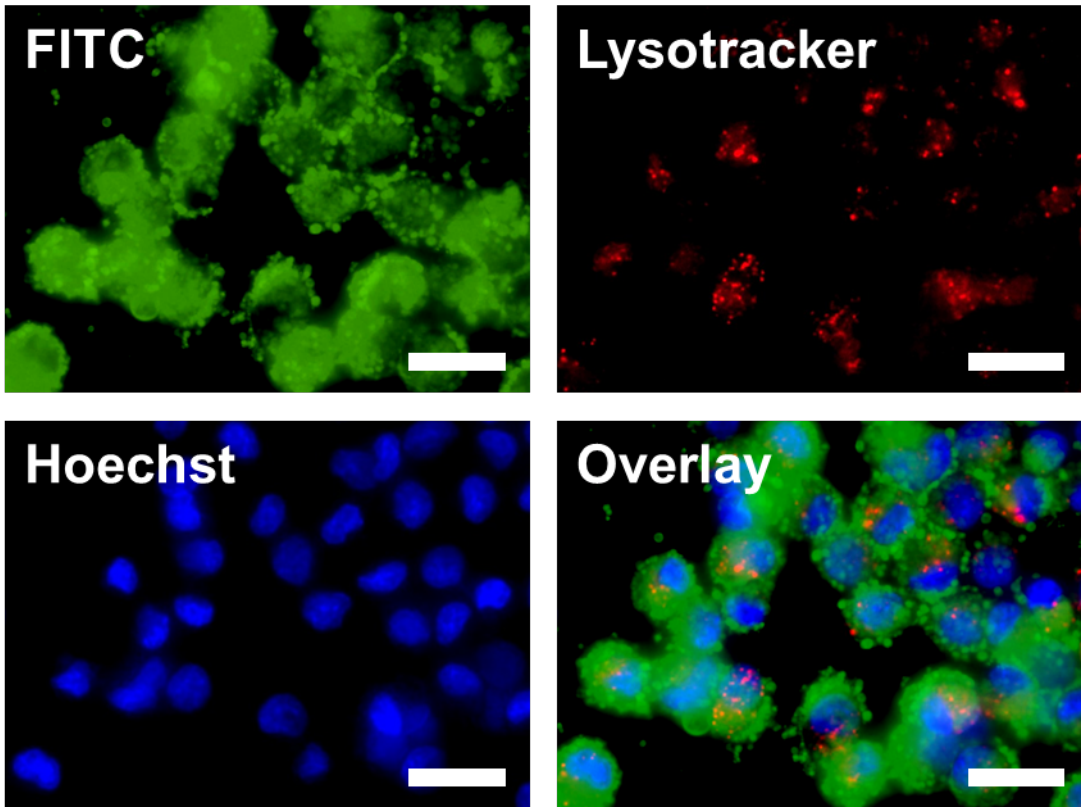

Figure S10. Fluorescence microscopic images of co-assembled K10-E3 (95%) and FITC-K10 (5%) for 24 hrs. Scale bar = 25 mm.

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3. C. A. Brautigam, *Methods Enzymol*, 2015, **562**, 109-133.
4. H. Tominaga, M. Ishiyama, F. Ohseto, K. Sasamoto, T. Hamamoto, K. Suzuki and M. Watanabe, *Anal. Commun*., 1999, **36**, 47-50.
5. M. Ishiyama, Y. Miyazono, K. Sasamoto, Y. Ohkura and K. Ueno, *Talanta*, 1997, **44**, 1299-1305.