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Design and Fabrication of Reduction-Sensitive Cell Penetrating Nanofibers For Enhanced Drug Efficacy

Su Yang[1], Dawei Xu[2], He Dong[1,2,]*

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

Department of Chemistry & Biomolecular Science, Clarkson University, Potsdam, NY, USA

E-mail: he.dong@uta.edu

General Methods

MBHA rink amide resin, Fmoc-protected amino acids, O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Novabiochem. Piperidine and diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich. All other reagents and solvents for peptide synthesis and purification were purchased from Fisher Scientific and used as received. Dulbecco's modified Eagle medium (DMEM) culture medium, Hoechst 33342 and LysoTracker Red DND-99 were purchased from Life Technologies. Fetal Bovine Serum (FBS) was ordered from VWR. CCK8 assay kit was obtained from Dojindo Molecular Technologies (Rockville, MD). ^{1}H NMR (JEOL ECX 500MHz) was used to characterize the synthesized compounds. Fluorescence measurements were performed on Varian Cary Eclipse fluorescence spectrophotometer. Circular dichroism (CD) spectra were acquired on a Jasco-J715 spectrometer using a quartz cell with 1 mm path length. Transmission electron microscopy (TEM) was conducted on a JEOL 2010 high-resolution transmission electron microscope. Reversed-phase HPLC was carried out on a HITACHI L-7100 pump using a Higgins semi-prep column (proto 300 C4 10 μm, 250*10 mm). UV absorbance was measured on a micro-plate reader (Vitor2 1420 Multilabel Counter, PerkinElmer) for toxicity quantification.

Synthesis of the reductive responsive linker Fmoc-Cystamine succinate (Fmoc-CS)

Cystamine dihydrochloride (2.25g, 10 mmol) and $NaHCO_3$ (2.52g, 30 mmol) erewere dissolved in deionized water. Then 35 mL of Dioxane was added by drop with stirring. Succinic anhydride (1.00g 10 mmol) was added to the above solution. The resulting reaction mixture was stirred at room temperature for overnight. Then $NaHCO_3$(0.84g 10 mmol) was added to the mixture. Afterwards 50 mL of acetone solution containing Fmoc-OSu (3.373g 10 mmol) was added. Then the reaction was last for 10 hours. The mixture was filtered remove the insoluble materials. The

solution was concentrated by the rotary evaporator. The pH of the solution was adjusted to 1~3 by HCl. The final solution was cold down to 4C° for 2 hours, and white powder was precipitated. The precipitate was collected by filtration and dried in vacuum. 1H-NMR (500 MHz, DMSO-D6) δ 7.86 (d, J = 8.0 Hz, 2H), 7.65 (d, J = 7.4 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.30 (t, J = 7.4 Hz, 2H), 4.28 (d, J = 6.9 Hz, 2H), 4.18 (t, J = 6.9 Hz, 1H), 2.72 (q, J = 6.5 Hz, 4H), 2.38 (t, J = 6.9 Hz, 2H), 2.28 (t, J = 6.9 Hz, 2H)

Peptides Synthesis and Purification

The synthesis of MDPs followed the standard Fmoc-solid phase peptide synthesis method. Briefly, Fmoc group was deprotected in the presence 20% (V/V) piperidine/DMF for 5 minutes, and was repeated once. HBTU was used as the coupling reagent and the coupling reaction was carried out for 45 mins in the presence of diisopropyl ethyl amine (DIPEA) and five equivalents of Fmoc protected amino acids. After the completion of the synthesis, the N-terminus of the MDPs was acetylated using DIPEA and acetic anhydride in DMF for 1 hr. The Kaiser test was used to verify the completion of the reaction. The acetylated peptide was cleaved in a mixture of TFA / triisopropanolsilane (TIS) / H_2O (95/2.5/2.5 by volume). After 3 hrs, the cleavage solution was collected and the resin was washed twice with neat TFA. TFA was evaporated under air flow and the residual solution was precipitated with cold diethyl ether. The resulting precipitate was collected by centrifugation at 6500 rpm for 5 min and washed three times with cold diethyl ether. Upon completion of the final amino acid coupling, 4 equivalents of 5(6)-carboxyfluorescein were added to the resin in the presence of 4 equivalents of HBTU and 8 equivalents of DIPEA in DMF. After 24 hrs, the reaction mixture was filtered and the resin was tested by Kaiser test. If needed, coupling reaction was repeated until a negative Kaiser test result is achieved. The resin was cleaved using the same procedure as for the non-labeled peptides. The

crude peptide was then dried under vacuum overnight for further HPLC purification. Peptides were purified using a preparative reverse phase C18 column with a linear gradient of binary water/acetonitrile solvent containing 0.05% TFA. Elution was monitored at 280 nm. The HPLC fraction was collected, combined and dried in the lyophilizer for 3 days. The molecular weight of each peptide was confirmed by by MALDI-TOF mass spectrometry using α-cyano-4-hydroxycinnamic acid as the matrix. K(SS)E: expected $[M+H]^+$: 4105, observed $[M+H]^+$: 4105; FITC-K(SS)E: expected $[M+H]^+$: 4421, observed $[M+H]^+$: 4421; K(C6)E: expected $[M+H]^+$: 3983, observed $[M+H]^+$: 3984; FITC-K(C6)E: expected $[M+H]^+$: 4299, observed $[M+H]^+$: 4299. 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 800 μM and left at 4°C for a day.

Circular Dichroism (CD) Spectroscopy

MDPs were diluted to 100 μM in Tris buffer (pH 7.4, 20 mM) for CD measurements. The data were collected from 250 nm to 190 nm at room temperature with a scan rate of 100 nm/min, a bandwidth of 1 nm and a response time of 2 sec. The final spectra were an average of five scans. The mDeg of rotation was converted to the molar residual ellipticity via the formula $\theta=(mDeg*1000)/(c*n*l)$, 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)

MDPs were diluted to 100 μM in Tris buffer (pH 7.4, 20 mM). 10 μl of the peptide solution was dropped onto a holey carbon grid (TED PELLA 01824). After 1 min, 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 and the TEM sample was dried for overnight before imaging.

Zeta Potential Measurement

Zeta Potential was performed on a Malvern Zetasizer. MDPs were diluted to 40 μM in Tris buffer (pH 7.4, 20 mM). The zeta potential was averaged on three measurements with a total number of 50 scans in each.

Critical Aggregation Concentration (CAC) Measurement

CACs were determined using a previous protocol based on the fluorescence intensity change of tryptophan. Peptide stock solution (160 μM) was added into 200 μL Tris buffer (20 mM, pH=7.4) with an increment of 2 μL each time. Fluorescence emission was acquired from 295 nm to 440 nm with the excitation wavelength at 280 nm. Fluorescence intensity at 350 nm was plotted as a function of the peptide concentration. The CAC was determined at the crossing point in which the linearity deviates from the initial trend.

Cytotoxicity measurement

HeLa cells were seeded onto a 96-well plate at a density of 10^4 cells/well and incubated for 24 hrs at 37 °C in an incubator with 5% CO_2. The culture medium was replaced and 10 μL of peptide solution (160 μM) was added to reach a final concentration of 16 μM. DOX was added to each well plate to reach final concentrations ranging from 0.05 μM to 4 μM. After 24 hrs of incubation, the CCK-8 assay was used to quantify cell viability by monitoring the UV absorbance at 450 nm. All the experiments were performed in four replicates.

Cell uptake

HeLa cells were seeded onto a confocal dish at a density of 1 x 10^5 cells/well. FITC-labeled peptides were diluted in Tris buffer to a final concentration of 160 μM. 20 μL of the peptide solution was added to the cell culture medium to reach a concentration at 16 μM. After 2 hrs and 24 hrs of incubation, cells were washed with PBS buffer for three times. Images were captured using a laser scanning confocal microscope (Leica DMi8, Germany) 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 37 °C. Culture medium was replaced with 200 μL fresh DMEM medium and 20 μL of FITC-labeled peptide solutions (160 μM) were added. After incubation with FITC-labeled MDPs for 2 hrs and 24 hrs, cells were washed with PBS buffer for three times. Cells were digested with trypsin and washed twice with PBS buffer. 2% paraformaldehyde was used for cell fixation. Cell uptake of the FITC-labeled peptide was quantified using a BD FACS Calibur flow cytometer. A minimum of 10,000 events per sample was analyzed and data were processed using FlowJo software.

Statistical analysis

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

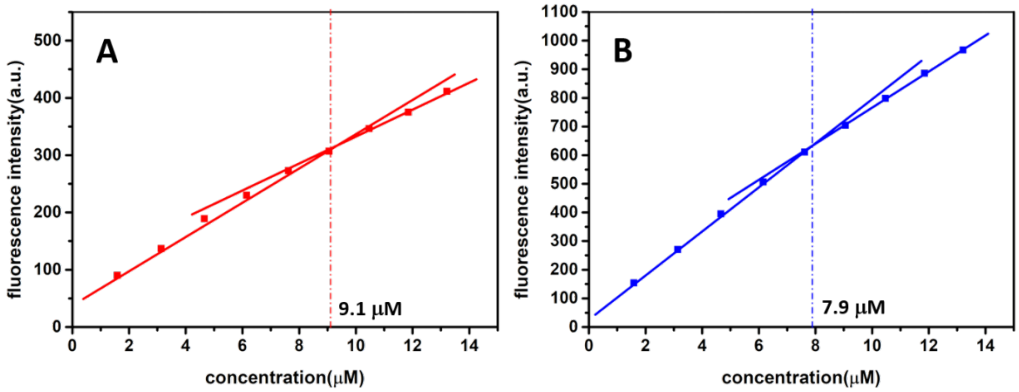

Figure S1. CAC determination through fluorescence measurements of peptides as a function of concentration in Tris buffer (20 mM, pH 7.4) (A) K(SS)E and (B) K(C6)E

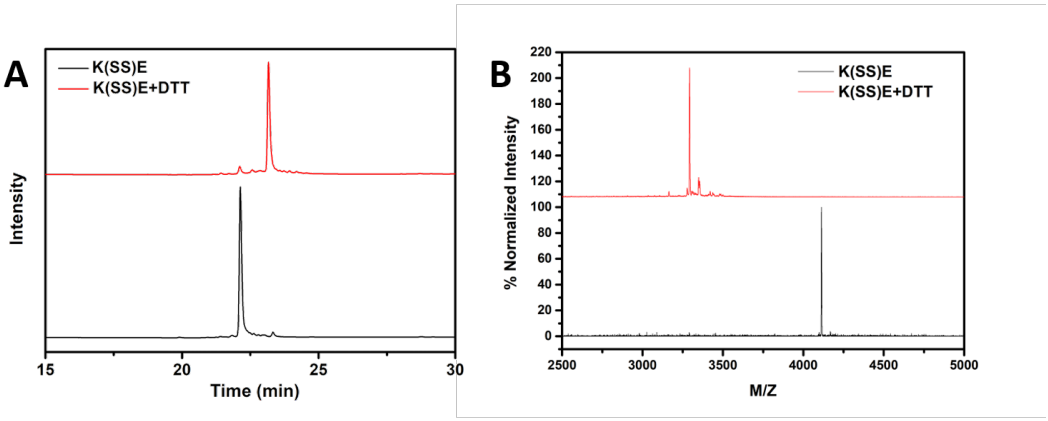

Figure S2. (A) HPLC of K(SS)E incubated with and without DTT. (B) MALDI spectra of K(SS)E incubated with and without DTT

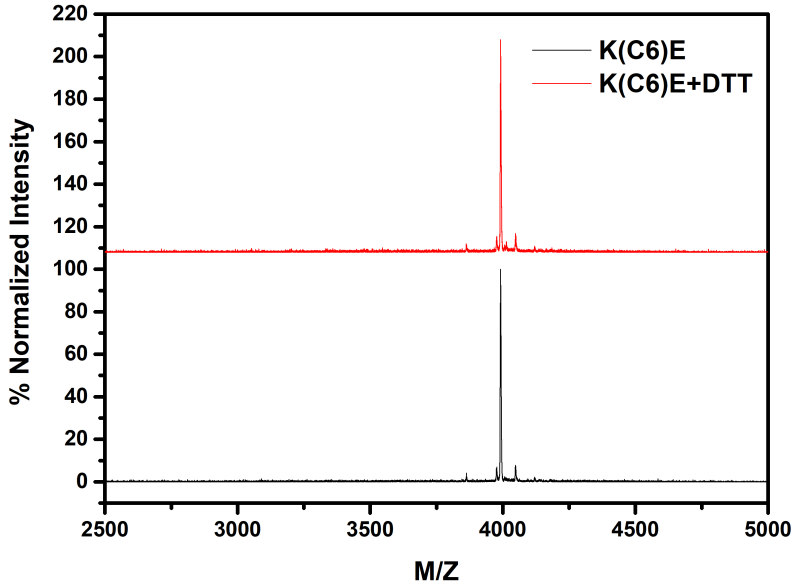

Figure S3. MALDI spectra of K(C6)E incubated with and without DTT

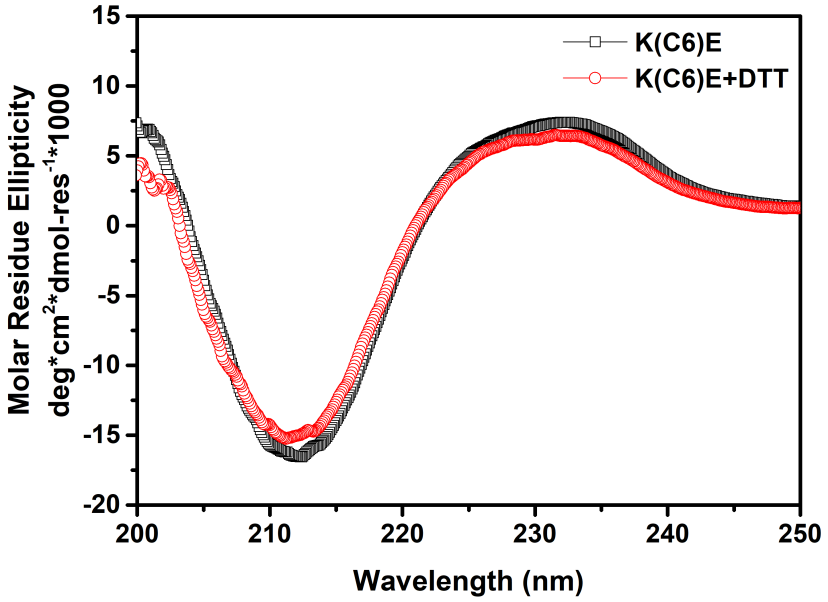

Figure S4. CD spectra of K(C6)E treat with and without DTT. Peptide concentration: 100 μM in Tris buffer (20 mM, pH =7.4), DTT concentration: 1 mM in Tris buffer (20 mM, pH =7.4).

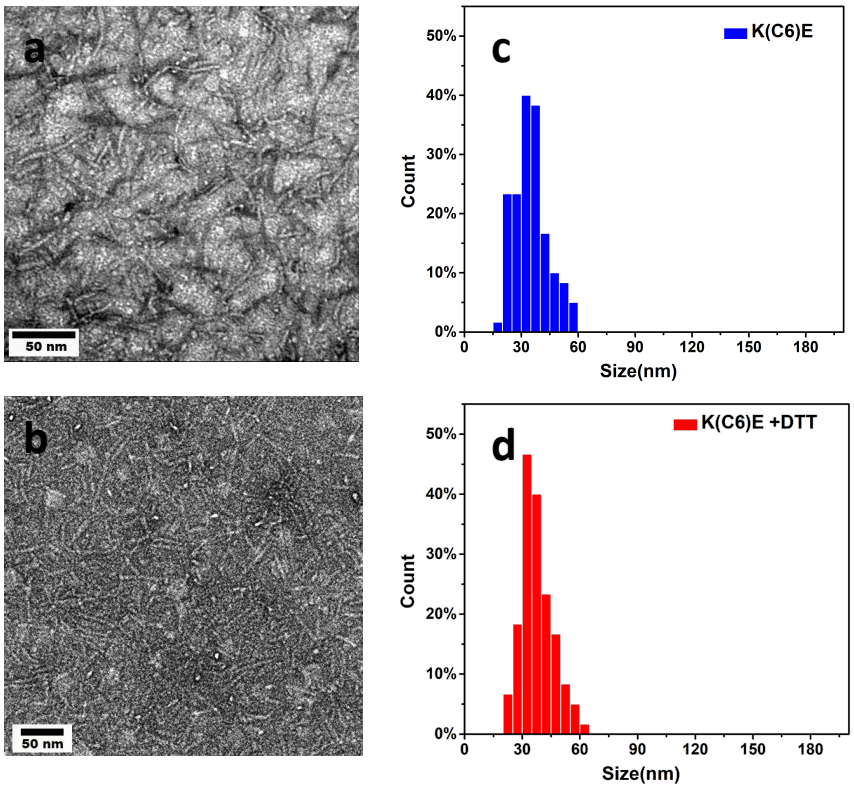

Figure S5. TEM images of the nanofibers formed by K(C6)E incubated without (**a**) and with (**b**) DTT. Statistical measurements of length and length distribution of K(SS)E nanofibers without (**c**) and with (**d**) DTT based on a total number of 100 fibers.

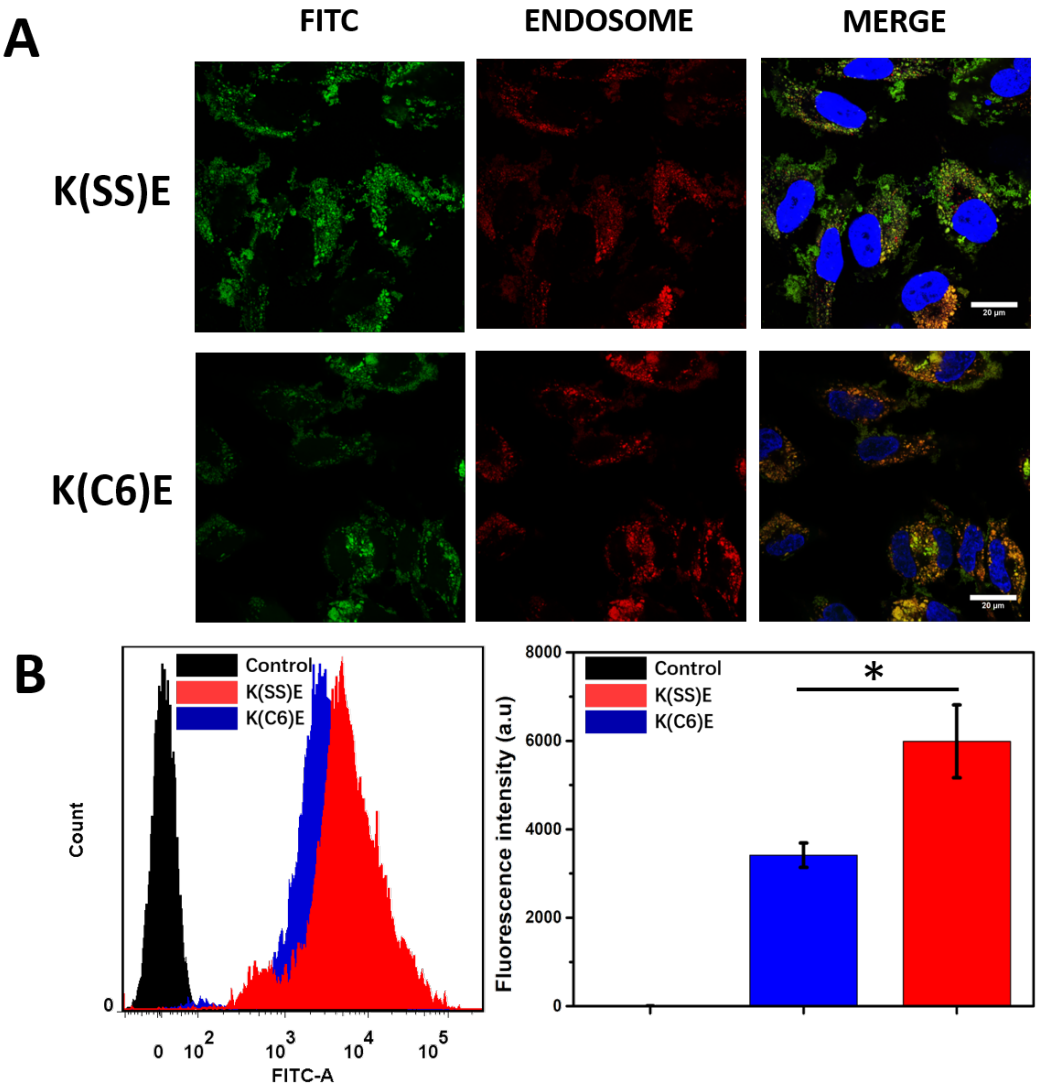

Figure S6. (A)Cell uptake of FITC-labeled K(SS)E and K(C6)E upon incubation with HeLa cells for 24 hrs. (B) Cellular uptake of K(SS)E and K(C6)E evaluated by flow cytometry. Scale bar: 20 μm. Statistically significant differences are indicated by *p $\leqslant$ 0.05.

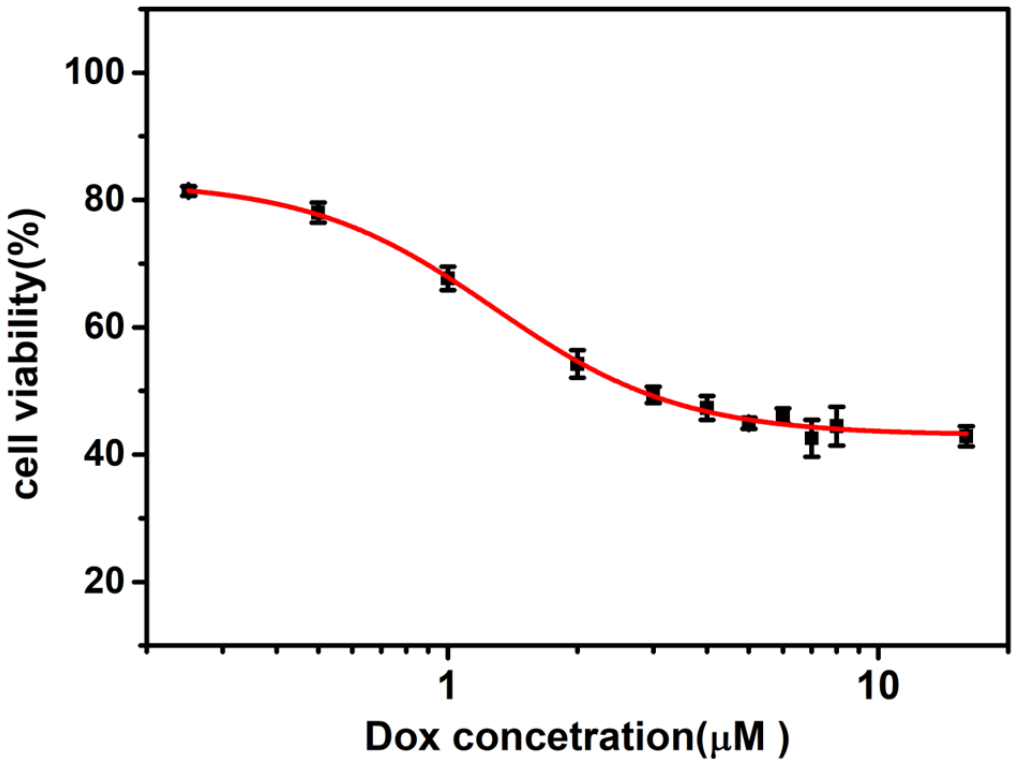

Figure S7. IC50 of Doxorubicin in Hela cell.

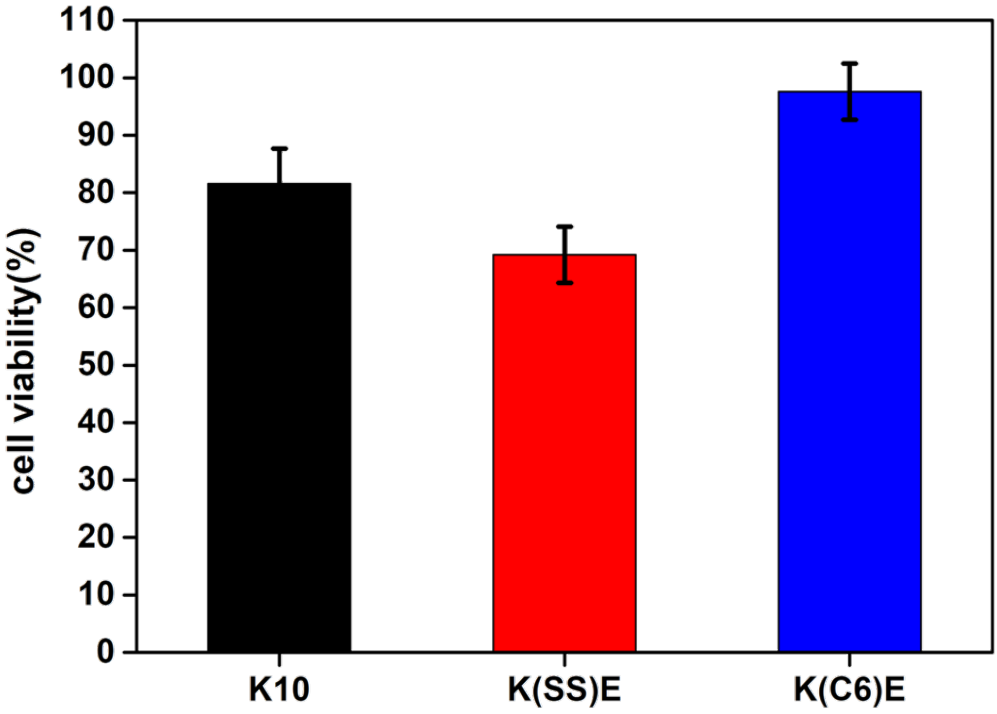

Figure S8. Cell viability of K(SS)E, K(C6)E and K10 (16mM) after 24 hours incubation with Hela cells.