Supplementary Information

HA-incorporated nanostructure of peptide-drug amphiphile for targeted anticancer drug delivery

Huyeon Choi[a], M. T. Jeena[a], L. Palanikumar[a], Yoojeong Jeong[b], Sooham Park[a], Eunji Lee[b]*, Ja-Hyoung Ryu[a]*

[a] Department of Chemistry, School of Natural Science, Ulsan National Institute of Science and Technology, Ulsan, 44919, [b]Graduate School of Analytical Science and Technology, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Korea.

Contents

S1. Synthesis and characterization

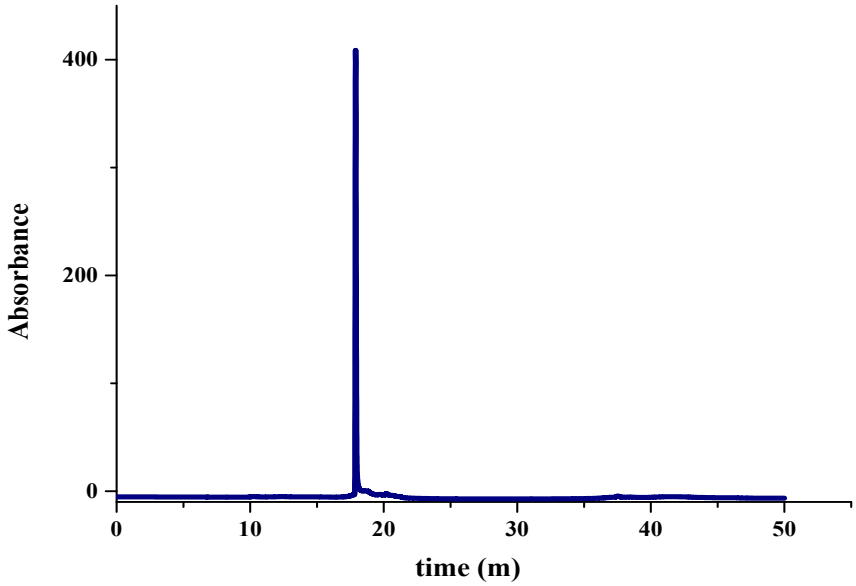
S1.1. Materials and characterization

Sodium hyaluronate was purchased from Acros Organic (Belgium), Amino acids (Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH) and rink amide MBHA resin were obtained from BeadTech (Korea), Coupling reagent (*O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, HBTU) was obtained from ApexBio (USA). 4-nitrophenyl chloroformate, *N,N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were purchased from TCI (Japan). Sodium bicarbonate ($NaHCO_3$), Triisopropylsilane and piperidine were obtained from Sigma-Aldrich (USA). *N,N*-dimethylformamide (DMF), Dichloromethane (DCM) HPLC grade was purchased from DAEJUNG (South Korea) . Dimethyl sulfoxide (DMSO) HPLC grade was from Fischer chemical (USA). Molecular sieves 4A, 4-8mesh and Magnesium sulfate ($MgSO_4$) obtained from SAMCHUN CHEMICALS (South Korea). 4-Dimethylaminopyridine (DMAP) were purchased from GL Biochem Ltd (Shanghai, China), 12N Hydrochloric acid (HCl) was obtained from OCI Company Ltd (Korea). Camptothecin (CPT) is obtained from Ontario Chemicals Inc. (Canada). Ethyl acetate (EtOAc) and DCM were obtained from SK Chemical (Korea). KCK peptide and KCK-CPT prodrug were characterized using ESI-TOF. Camptothecin derivatives were characterized using 400MHz FT-NMR (Agilent Technologies). TEM (JEM-1400) and Fluorometer (Varian, CARY Eclipse Fluorometer) Also, size was measured by Zetasizer (Malvern ZS).

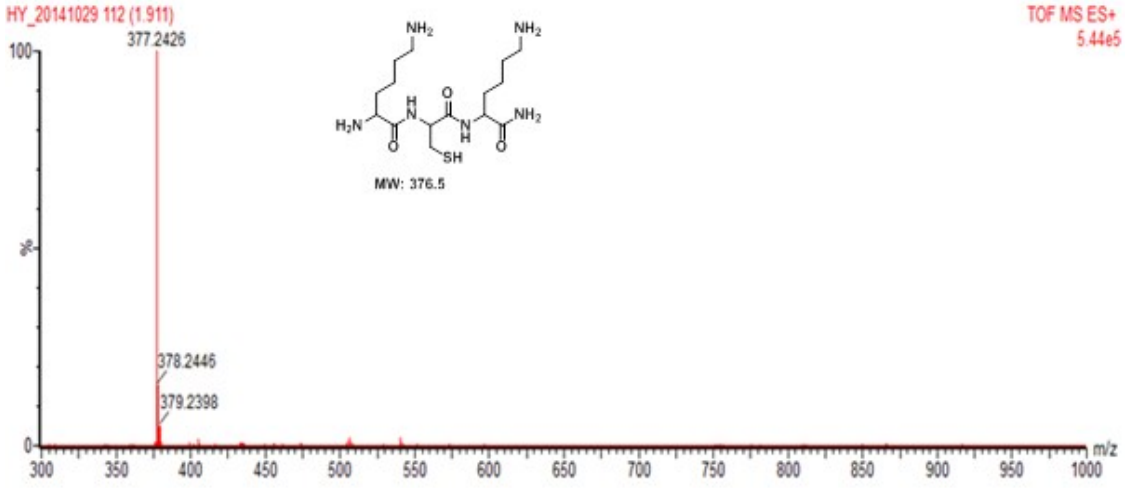
S1.2. Peptide (KCK) synthesis

Solid phase peptide synthesis (SPPS) was performed by microwave-assisted peptide synthesizer (CEM). 200 mg of resin was swollen in DMF solution. After 30 min, operate machine to synthesize peptide. Peptide scale is 0.1 mM. Amino acid solution was prepared by dissolving Fmoc-Cys(Trt)-OH 0.176 g in 3 mL DMF and Fmoc-Lys(Boc)-OH 0.281 g in 6 mL DMF. Fmoc deprotection of the Fmoc-amino acids was performed by adding 20% piperidine solution in DMF. HBTU and DIEA act as coupling reagent and base. After finishing final deprotection of Fmoc protecting group, make cleavage cocktail (TFA: Triisopropylsilane: Water = 4.75 mL: 0.25 mL: 0.25 mL). Add cleavage cocktail into resin with stirring for 4 hours at room temperature. Then, precipitate with cold diethyl ether. The

peptide is purified with High Performance Liquid Chromatography (HPLC) using Preparative HPLC with water/Acetonitrile. The obtained amount is 45.8mg. (Fig.S1) ESI-MS (Fig.S2): 376[M+H]

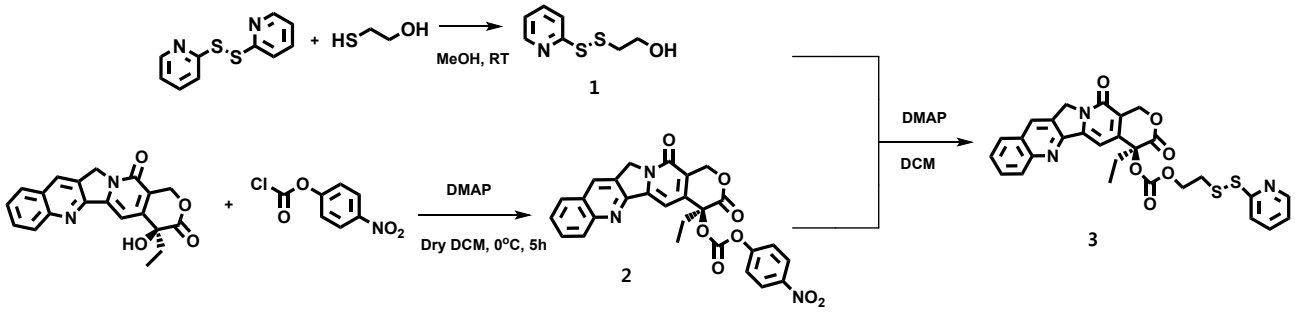


FigS1. HPLC analysis of KCK peptide.



FigS2. ESI-MS analysis of KCK peptide.

S1.3. CPT derivative synthesis



Scheme1. Synthesis of CPT derivatives

1) Synthesis of **2-(pyridin-2-yldisulfanyl) ethanol (1).**

This reaction was previously reported for the formation of activated disulfides [1]. 2-2'-dipyridyl disulfide (DPSP) (20 g, 0.0908 mol) was dissolved in dry MeOH (20 mL). 2-mercaptoethanol (7.09 g, 0.0908 mol) and acetic acid (several drops) as catalyst was added dropwise to the mixture. After 12h, solvent (methanol) was removed in vacuo. The product was extracted by DCM. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. Purification was done by silica chromatography with (Hexane: EtOAc = 2:1). After purification, byproduct was shown as yellow color and our product as transparent. The yield is 64.4%. δ_H (400 MHz, $CDCl_3$) 2.91-2.99 (2H, m), 3.80 (2H, br s), 7.15 (1H, m) , 7.41 (1H, dt), 7.54-7.63 (1H, m), 8.47-8.53 (1H, m).

2) Synthesis of **Camptothecin-4-nitrophenyl carbonate (2).**

Camptothecin (1 g, 2.87 mmol) and 4-nitrophenyl chloroformate (2.03 g, 10.04 mmol) were dissolved in dry DCM at $0^{\circ}C$. DMAP (2.109 g, 17.2 mmol) was added to the mixture. After stirring for 5 hours at $0^{\circ}C$, the mixture was washed by 1N HCl (200 mL). The product was dried over $MgSO_4$ and solvent was removed in vacuo. Purification was done by silica chromatography with (DCM: EtOAc = 1:1). The yield is 71.1%. δ_H (400 MHz, $CDCl_3$) 1.01-1.12 (3H, m), 2.16-2.45 (2H, m), 5.24-5.38 (2H, m), 5.42 (1H, d), 5.72 (1H, d), 7.36-7.44 (3H, m) , 7.70 (1H, ddd), 7.86 (1H, ddd), 8.17-8.27 (3H, m), 8.43 (1H, s).

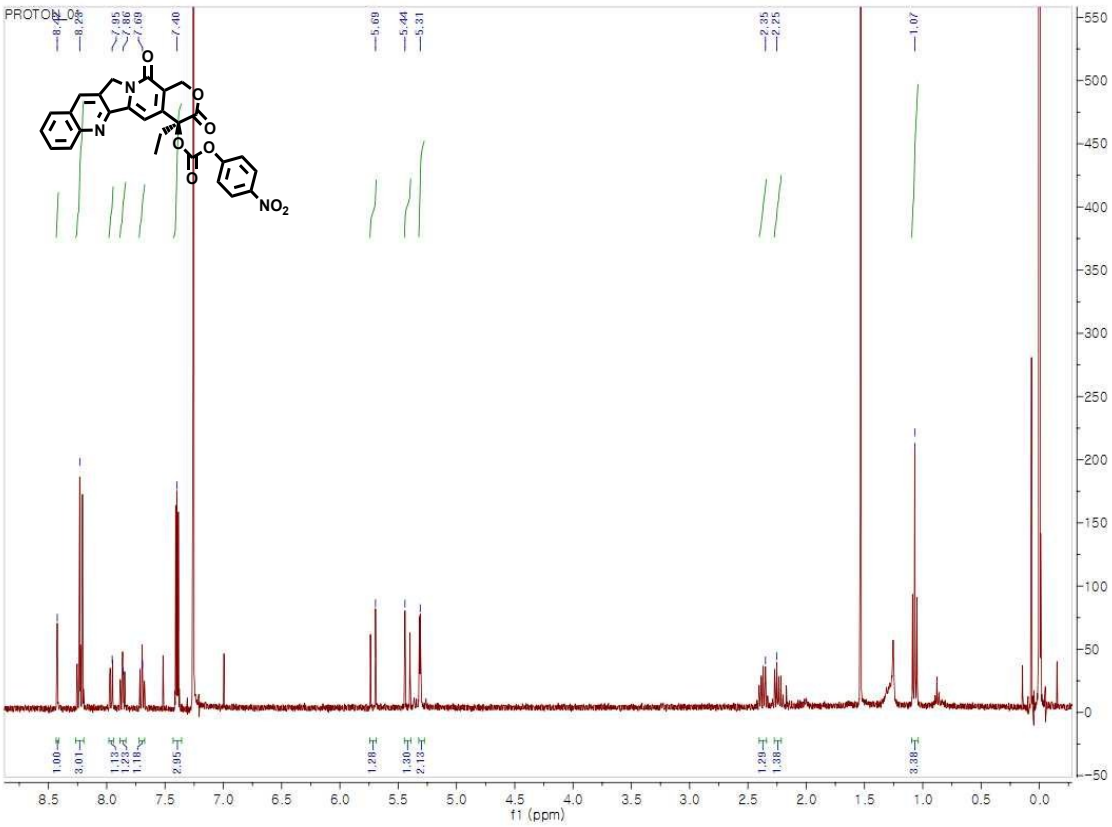


Fig. S3. NMR spectra of **Camptothecin-4-nitrophenyl carbonate (2).**

3) Synthesis of **Camptothecin-(4-pyridyldisulfanyl)ethyl carbonate (3).**

After synthesizing Camptothecin-4-nitrophenyl carbonate (2) (1g, 0.583mol)**,** 2-(pyridin-2-yldisulfanyl) ethanol (1)(0.18g, 0.96mol) and DMAP (0.133g, 1.089mol) dissolved to dry DCM, reflux at 55℃ for 12h. After cooling, wash with 1M $NaHCO_3$(215 ml) and the product was dried over $MgSO_4$ and solvent was removed in vacuo. Purification was done by silica chromatography with (DCM: EtOAc = 1:1). The yield is 79.1%. δ_H (400 MHz, $CDCl_3$) 1.01 (3H, m), 2.10-2.21 (1H, m), 2.24-2.34 (1H, m), 3.06 (2H, t), 4.30-4.42 (2H, m), 5.27-5.30 (2H, m), 5.39 (1H, d), 5.69 (1H, d), 7.03 (1H, td), 7.34 (1H, s), 7.62 (1H, d), 7.63 (1H, m), 7.65-7.70 (1H, m), 7.83 (1H, m), 7.94 (1H, dd), 8.22 (1H, d), 8.39 (1H, s), 8.42 (1H, dt)

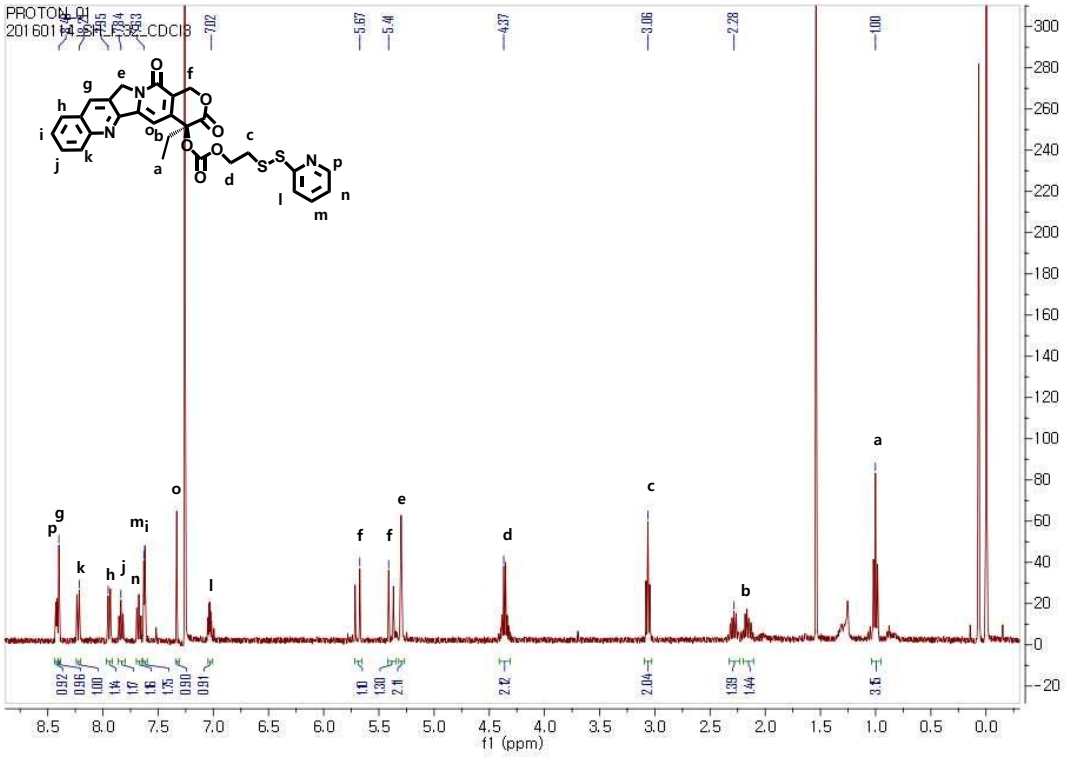


Fig. S4. NMR spectra of **Camptothecin-(4-pyridyldisulfanyl)ethyl carbonate(3).**

S1.4. Prodrug synthesis (KCK -CPT)

Purified peptide (KCK) 25 mg (0.066 mmol) and CPT-4-PDEC (37mg, 0.066mmol) were dissolved in dry DMSO and stirred for 24 h at room temperature. Then, KCK-CPT is purified with HPLC using water and acetonitrile. After purifying, the product were collected, frozen by liquid nitrogen, and lyophilized. The yield is 32%. The purity is 97%. The product is characterized by ESI-TOF (Fig.S4). ESI-MS: 827.3[M], 828.3[M+H].

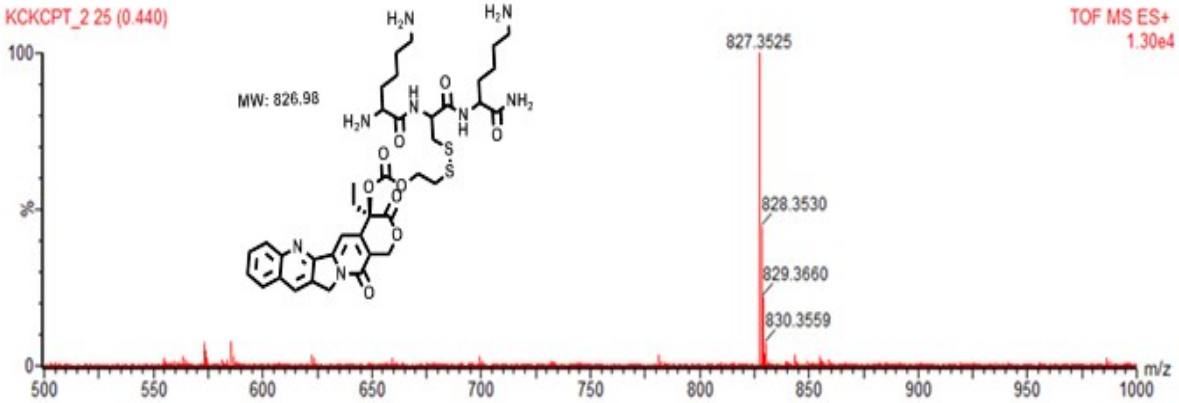
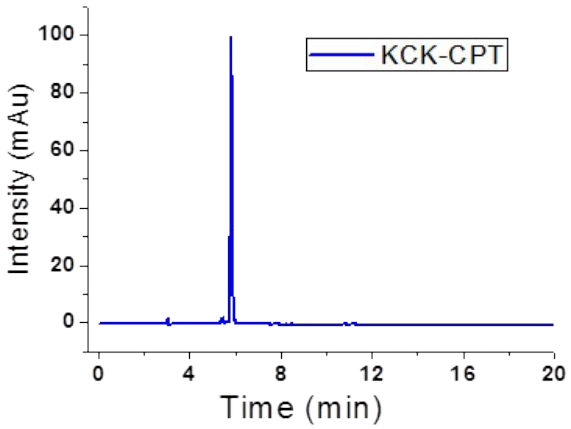


Fig. S5. ESI-MS profile of the KCK-CPT prodrug.



FigS6. HPLC analysis of KCK-CPT peptide.

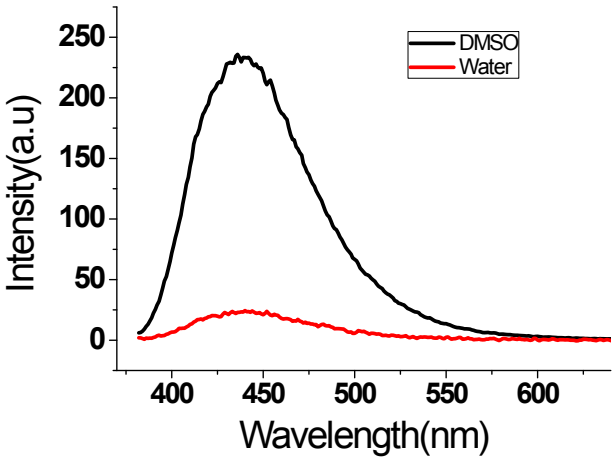


Fig. S7. Fluorescence emission spectrum change of KCK-CPT in 1 mM DMSO and aqueous solution. (λ_{ex} = 365 nm) Black solid line is 1 mM DMSO solution and Red solid line is 1 mM aqueous solution.

S1.5. Characterization by TEM

A drop of each sample in aqueous solution was placed on a formvar/carbon-coated copper grid and allowed to evaporate under ambient conditions. When sample was stained, a drop of uranyl acetate solution (2 wt%) placed onto the surface of the sample-loaded grid. The sample deposited about 1 min at least, and excess solution was wicked off by filter paper. The specimen was observed with a JEM-1400 operating at 120 kV. The data were analyzed with Gatan Digital Micrograph program.

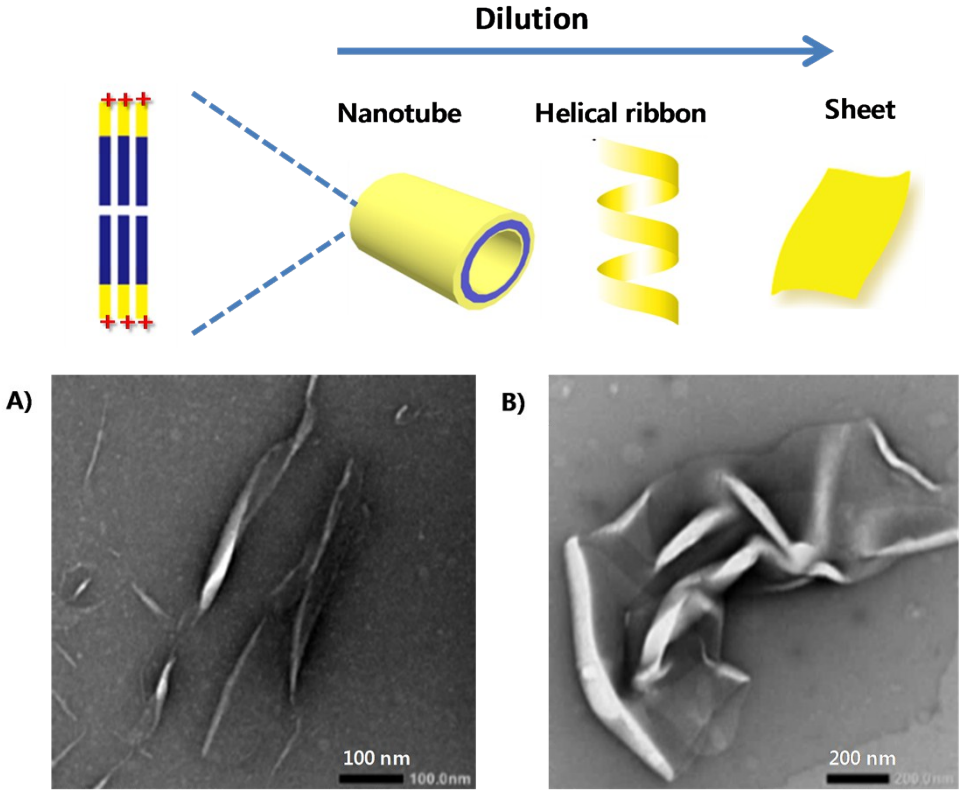


Fig. S8. Self-assembled nanostructures of KCK-CPT. A-B) curved sheet nanostructure of 400 µM and 50 µM aqueous solution of KCK-CPT.

S.1.6 Colloidal stability analysis and Critical transition concentration determination

To determine the colloidal stability of micelle formed by HA-KCK-CPT, 100 μM of HA-KCK-CPT was prepared both in water as well as in 1X phosphate buffered saline (PBS). The solutions were stirred overnight and sonicated. The size was measured using Malvern Zetasizer ZS series (United Kingdom). In order to confirm the colloidal stability, the measurements were reoeated for 3 days. The stability of the HA-KCK-CPT micelle has been checked in both water as well as PBS by using DLS. Both in water and PBS, it is stable for at least 3 days with a consistent size of about 55nm (Fig. S9). In the case of FBS, We tried to check size distribution in DLS. However, original peak from FBS solution was observed intensely due to high concentration of FBS proteins. Moreover, the peaks were overlapped with our samples' size distribution. As shown in Fig. S9 c-d, we have observed there is no difference of fluorescence intensity with in presence and absence of FBS for 24 h, which indicates that there is no significant change of colloidal stability.

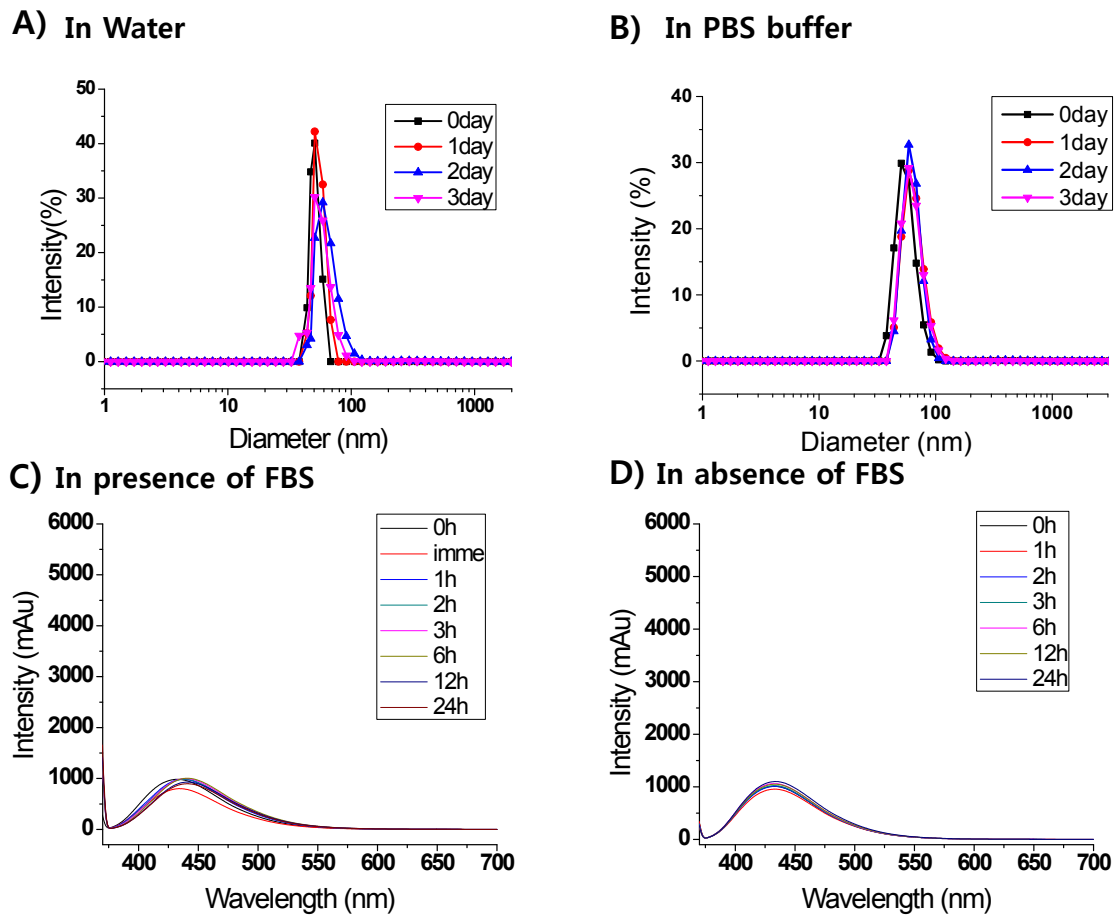


Fig. S9. Colloidal stability studies of HA-KCK-CPT by A-B) hydrodynamic diameter distribution. A) 100 μM HA-KCK-CPT in water and B) 100 μM HA-KCK-CPT in PBS buffer solution over time. C-D) Emission spectra of HA-KCK-CPT. C) Emission spectra of 100 μM HA-KCK-CPT in presence of FBS. D) Emission spectra of 100 μM HA-KCK-CPT in absence of FBS (λ_{ex} = 365 nm)

To determine the critical transition concentration of HA-KCK-CPT naofiber to micelle, 2mL of 2mL of 800 μM aqueous solution was diluted to 400 μM, 300 μM, 200 μM and 100 μM by adding MilliQ water. Each solution was stirred for at least overnight and sonicated. We determined the critical transition concentration for this transformation by dynamic light scattering (DLS) as shown in Fig. S10. The concentration dependent DLS measurement showed a difference in the distribution pattern from monomodal to bimodal from 200 μM to 300 μM. So the critical transition concentration falls in between 200 μM and 250 μM.

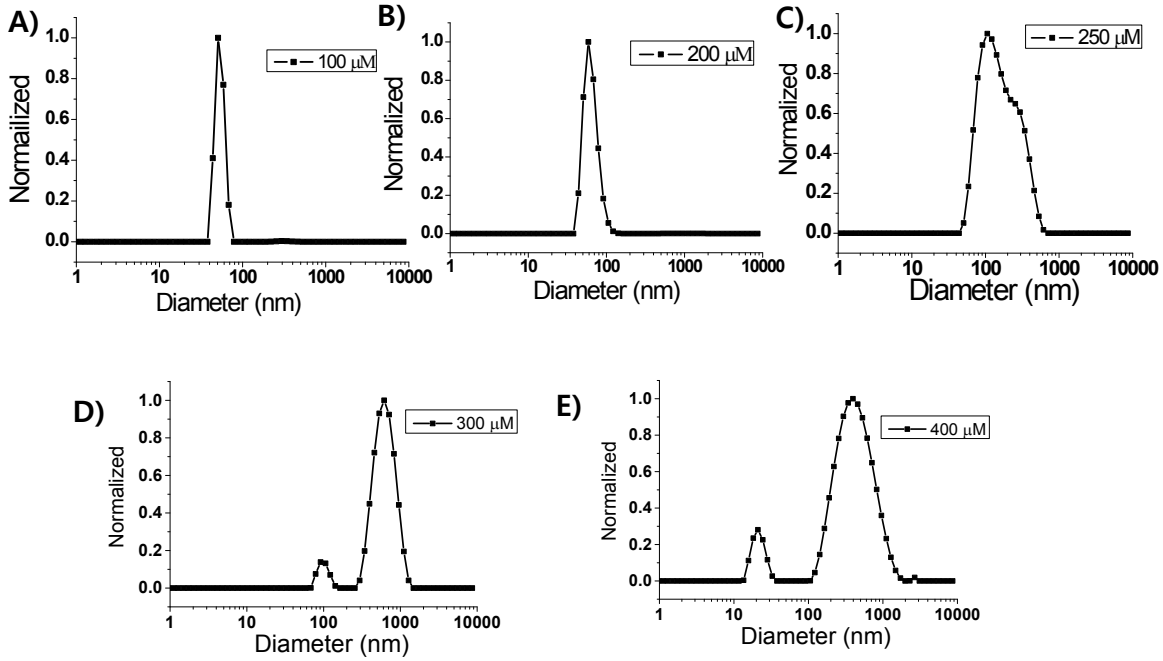


Fig. S10. Hydrodynamic diameter distribution of HA-KCK-CPT with different concentrations. A) 100 μM, B) 200 μM, C) 250 μM D) 300 μM and E) 400 μM of HA-KCK-CPT in water.

S1.7 Drug release profile

The release profile of CPT from HA-KCK-CPT in aqueous solution is performed by High Performance Liquid Chromatography (Agilent Technologies, Santa Clara, CA). The analysis was done using ZORBAX Eclipse Plus C18 analytical column with a flow rate of 1 mL/min. The Refractive Index Detector (RID) set at 365 nm. We have performed the experiment with High Performance Liquid Chromatography (HPLC) with respect to monitor the release of CPT from HA-KCK-CPT in presence of hyaluronidase and disulfide reducing agent, GSH. The release profile for CPT has provided in the supporting information. As shown in Fig. S11A, the 20% of CPT was released from HA-KCK-CPT in presence of GSH within 20 h at 37 ℃. However, the release was increased up to 80% in presence of hyaluronidase and 10 mM GSH within 14 h at 37 ℃. Almost 100% of the CPT was released from HA-KCK-CPT in presence of 0.56 mg hyaluronidase and 10 mM GSH as shown in Fig. S11B. In addition, we observed the intermediate of cleavage product was also observed in HPLC, which is consistent with our proposed mechanism and reported literature (reference # 27). (Fig. S11C-E)

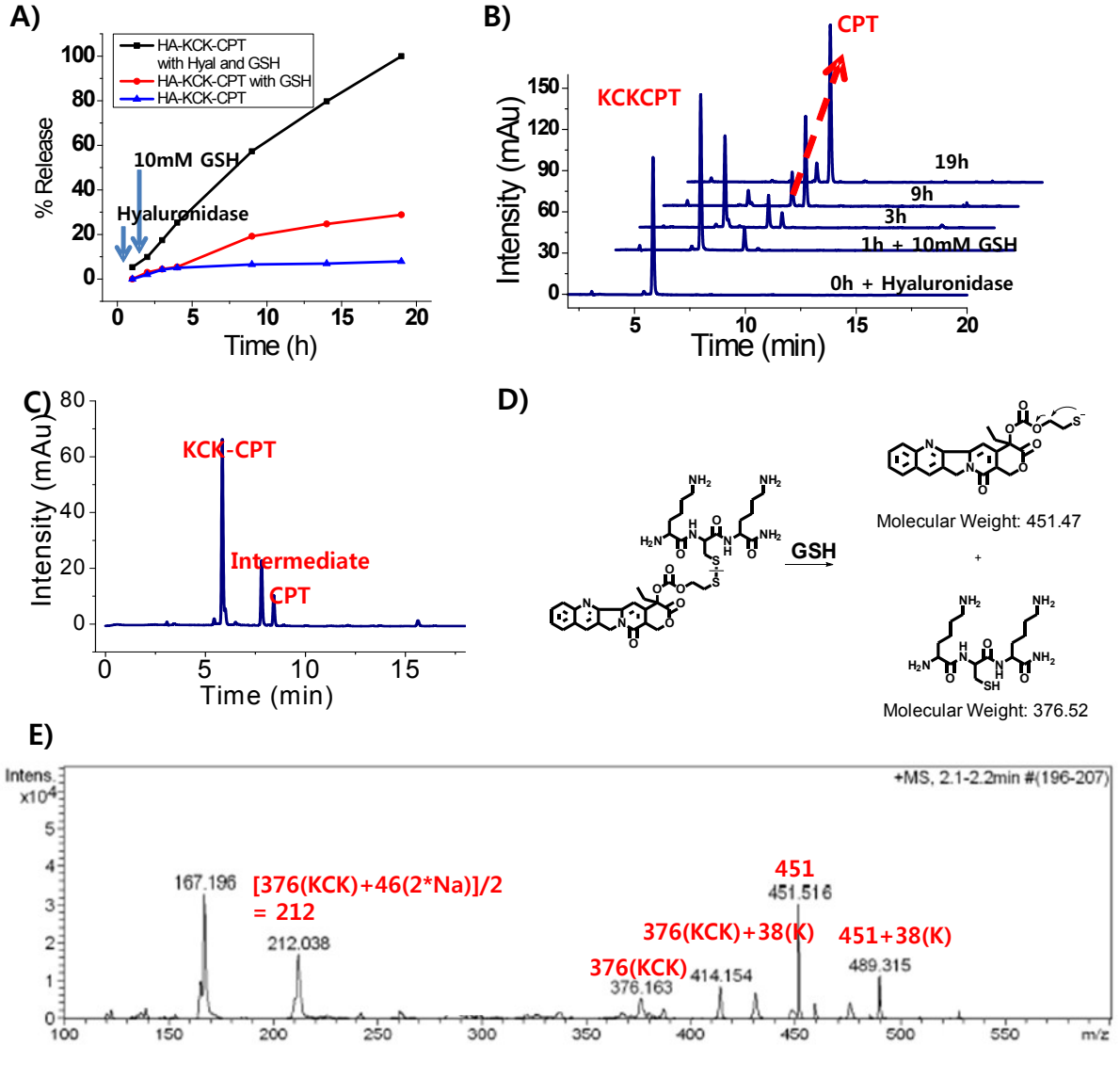


Fig. S11. GSH induced CPT release profile from HA-KCK-CPT. A) drug release percentage versus time of 100 μM HA-KCK-CPT in the presence of hyaluronidase and 10 mM GSH together (black line) and only 10 mM GSH (red line) B) HPLC analysis of GSH induced release of CPT from 100 μM HA-KCK-CPT containing 10 mM GSH at 37℃ after 1h, 3h, 9h and 19h. C) HPLC analysis of GSH induced CPT from 100 μM HA-KCK-CPT containing 10 mM GSH at 37℃ after 3h. After adding GSH, the intermediate and CPT is released from KCK-CPT D) Mechanism of HA-KCK-CPT disulfide cleavage by GSH. E) ESI-MS profile of intermediate from KCK-CPT.

S2. In vitro experiment

S2.1. Cell culture and viability analysis.

SCC-7 and KB cells were cultured (using RPM 1640 medium); HeK293T (using DMEM medium) in sterile 96-well Nunc (Thermo Fisher Scientific Inc.) microtitre plate at a seeding density of 5 x 10^3 cells/well and they were allowed to settle for 24 h under incubation at 37 °C and 5% CO_2. In-order to check cell viability, the cells were then treated with different concentrations of KCK-CPT peptide and HA-KCK-CPT peptide (0.01, 0.1, 2.0, 3.0 and 5.0 μM) in KB, SCC7 and HeK293T cells. Cell viability were measured at 48h using Alamar Blue assay with each data point measured in triplicate. Fluorescence measurements were made using the plate reader (Tecan Infinite Series, Germany) by setting the excitation wavelength at 565 nm and monitoring emission at 590 nm on the 96 well plates.

S2.2. In-situ confocal microscopy for cellular internalization

KB and SCC7 cells were seeded in one well glass cover slips at a seeding density of 2 x 10^5 cells/well. After 24 h, cells were treated with KCK-CPT peptide and HA-KCK-CPT peptide at a final concentration of 10.0 μM. The cellular uptake was monitored in the coverglass (Lab Tek II glass chamber coverglass, Thermo Fisher Scientific Inc) using Carl Zeiss LSM 700 and Olympus FV1000i microscope connected to CO_2 incubator at different time points 1h and 3h. In order to check the colocalization with lysosomes, SCC7 cells were seeded into four well chambered cover glass at a density of 2 x 10^5 cells/well. After 24h, cells were treated with 10 μM of HA-KCK-CPT peptide, after 30 min, 3.5 h and 7.5 h intervals (total time for each analysis is 1 h, 4 h and 8 h), the cells were stained with 50 nM of lysosome tracker (Lysotracker DND-26, Life Technologies) for a period of 30 min and analyzed using Carl Zeiss LSM 700 microscope connected to CO_2 incubator.

S2.3. Endocytosis of HA-KCK-CPT.

The SCC-7 cells were seeded into one well chamber cover glass (Lab Tek II, Thermo Scientific) at a seeding density of 2 x 10^5 cells/well. To inhibit clathrin- or caveolin dependent endocytosis or macropinocytosis, the cells were pre-incubated with 450 mM sucrose or 10 mM methyl-β-cyclodextrin for 30 min or 3 mM of amiloride for 15 min,

respectively. [2,3] They were then incubated with 5 μM of HA-KCK-CPT for different time points. The cellular uptake was monitored periodically using Carl Zeiss LSM 700 or Olympus FV1000 microscope connected to CO_2 incubator.

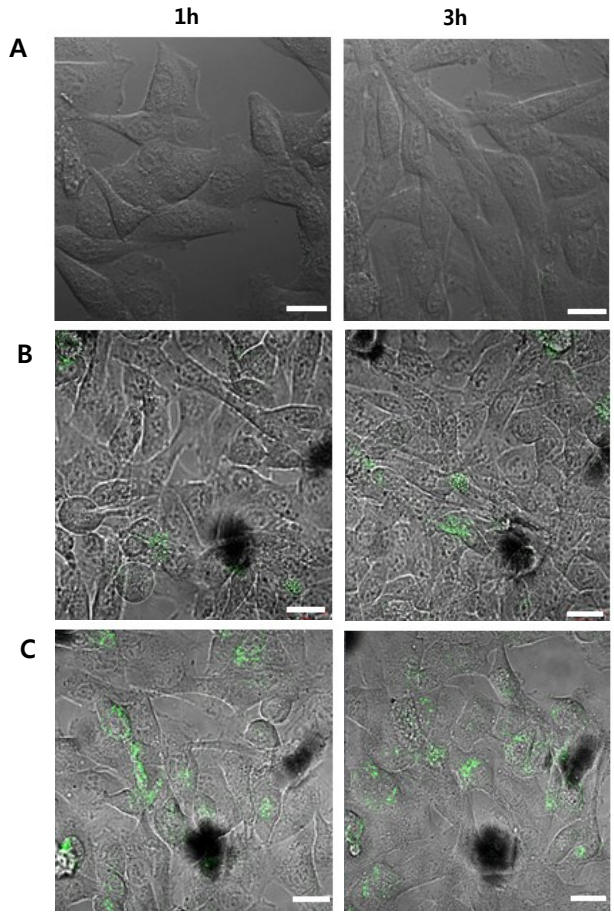


Fig. S12. CLSM images of SCC-7 cells treated by HA-KCK-CPT in the (A) presence of methyl-β-cyclodextrin for caveolae mediated endocytosis, (B) presence of Amiloride(AMI) which inhibits macripinocytosis and (C) presence of Sucrose which inhibits clathrin mediated endocytosis. (Conc: 5 μM). The scale bar is 20 μm.

S3. Reference

1. A. G. Cheetham, Y. Ou, P. Zhang, H. Cui, *Chem. Commun*., 2014, **50**, 6039-6042.
2. L. Liang, J. Li, Q. Li, Q. Huang, J. Shi, H. Yan, C. Fan, *Angew. Chem*. *Int. Ed*, 2014, **126**, 7879-7884.
3. S. Falcone, E. Cocucci, P. Podini, T. Kirchhausen, E. Clementi, J. Meldolesi, *J. Cell Sci.*, 2006, **119**. 4758-4769.