Supporting Information for

Filamentous Supramolecular Polymer-Drug Conjugates as Highly Efficient Drug Delivery Vehicles

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S1 Synthesis and characterization

S1.1 Chemicals

Fmoc-protected amino acids, 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluo-rophosphate (HBTU) were purchased from NovaBiochem. 5(6)-Carboxyfluorescein (FAM) and piperidine were purchased from Sigma-Aldrich. 10-hydroxycamptothecin (HCPT) was purchased from Avachem Scientific. PEG750-COOH was purchased from Rapp Polymere. All other reagents and solvents were purchased from Fisher Scientific and used as is.

S1.2 Synthesis of HCPT-GA

HCPT-GA was synthesized according the previously literature.¹ 10-hydroxycamptothecin (364 mg, 1 mmol), glutaric anhydride (342 mg, 3 mmol) and pyridine (35 mL) were placed in a 50 mL round-bottom flask. After the mixture was stirred for 48 h at room temperature, the solvent was removed by vacuum evaporation. 20 mL of 0.01M HCl was added into the residue followed by centrifugation. The resulting precipitate was washed with 0.01M HCl (20 mL) again to afford the yellow powder (430 mg, 90% yield). 1H NMR (400 MHz, DMSO-d₆), δ (ppm): 8.64 (s,1H), 8.24 (d,1H), 7.86(s,1H), 7.69-7.7(m,1H), 7.44(s,1H), 6.55(s,1H), 5.50(s,2H), 5.25(s,2H), 2.69-2.76(t,2H), 2.35-2.42(t,2H), 1.86-1.94(m,4H), 0.88-0.92(m,3H).



S1.3 Peptide Synthesis and Purification

S1.3.1 Synthesis of MDP26

The peptide WK₂(QL)₆K₂ (MDP26) (W, tryptophan was incorporated for quantitative concentration determination) was synthesized on a PS3 peptide synthesizer using the standard FMOC-solid phase peptide synthesis. Fmoc groups were deprotected with 20% piperidine in DMF for 5 min (2 times). Amino acids were coupled in the presence of HBTU and DIPEA in a molar ratio of 1:1:2.5. Fmoc amino acids were added in four equivalents of the resin. The N-terminus was acetylated in the presence of 50 equiv of acetic anhydride and 6 equiv of DIPEA in DMF. Then the peptide was cleaved from the resin with a mixture of TFA / triisopropanolsilane (TIS) / H₂O (95/2.5/2.5 by volume) for 3 hours. The TFA solution was collected and then the resin was rinsed twice with neat TFA. After evaporation of the combined TFA solutions, the residual peptide solution was triturated with cold diethyl ether. The resulting precipitate was centrifuged and washed for three times with cold diethyl ether. The crude peptide was then dried under vacuum overnight for further HPLC purification. The peptide was purified using a preparative reverse phase C18 column with a linear gradient water/acetonitrile containing 0.05% TFA. Elution was monitored at 230nm and 280 nm. ESI MS: calc. [M+H]⁺=2204.3, obsvd. [M+H]⁺=2204.9.



S1.3.2 Synthesis of MDP26-PEG

 $WK_2(QL)_6K_2$ was assembled on solid phase as described in **S1.3.1**. The N-terminus was reacted with the carboxyl terminated PEG750 using HCTU/DIPEA chemistry. The coupling reaction was performed at RT for overnight and repeated once. Cleavage and purification followed the previous procedure. ESI MS: calc.[M+H]⁺=2954, obsvd.[M+H]⁺=2936. (the mass difference between the observed and calculated mass is due to PEG polydispersity)



S1.3.3 Synthesis of HCPT-MDP26

 $K_2(QL)_6K_2$ was assembled on solid phase as described in **S1.3.1**. The N-terminus was reacted with HCPT-GA using a combination of HBTU/HOBT/DIPEA(3/3/5) as coupling and activating reagents. The mixture was stirred overnight at RT. Kaiser test was used to ensure the completion of the coupling reaction. If necessary, coupling of HCPT-GA was repeated once. Cleavage and purification followed the previous procedure. ESI MS: calc. $[M+H]^+=2437$, obsvd. $[M+H]^+=2437.2$.



HCPT-MDP26: C115H185N29O29

S1.3.4 Synthesis of FAM-MDP26

 $K_2(QL)_6K_2$ was assembled on solid phase as described in **S1.3.1**. The N-terminus was reacted with 5(6)-Carboxyfluorescein (FAM) using a combination of HBTU/DIPEA (3/5) as coupling and activating reagents. The mixture was stirred overnight at RT. Kaiser test was used to ensure the completion of the coupling reaction. If necessary, coupling of 5(6)-Carboxyfluorescein (FAM) was repeated once. Cleavage and purification followed the previous procedure. ESI MS: calc. $[M+H]^+=2335$, obsvd. $[M+H]^+=2335.6$.



S1.3.5 Synthesis of HCPT-MDP26-PEG

Peptide with a sequence of K(Alloc)G₃K₂(QL)₆K₂ was synthesized using standard Fmoc solid phase synthesis. The carboxyl terminated PEG750 was coupled at the N-terminus using HCTU/DIPEA chemistry. After overnight coupling reaction, the Alloc group was removed by using 0.2 equiv of Pd(PPh₃)₄ and 24 equiv of PhSiH₃ in DCM. The reaction was performed for 30 min and repeated five times. Then the peptide resin was washed with DCM (3×3 mL), DMF (3×3 mL), then twice with 0.5% DIPEA in DMF (v/v) and 0.5% (w/w) sodium diethyldithiocarbamate tritydrate (DEDTC) in DMF to remove the palladium catalyst from the resin. HCPT-GA was coupled on the side chain of the lysine residue by using HBTU/HOBT/DIPEA (3/3/5) as coupling and activating reagents. The mixture was stirred at RT for 24 h. Cleavage and purification followed the procedure described in **S1.3.1**. The synthetic route of HCPT-MDP26-PEG is shown in **Scheme SI-1**. ESI MS: calc. [M+H]⁺=3486, obsvd. [M+H]⁺=3509.7. (The mass difference between the observed and calculated mass is due to PEG polydispersity)



Chemical Structure of HCPT-MDP26-PEG



Scheme S1.Synthetic Route of HCPT-MDP26-PEG









S1.3.7 Synthesis of FAM-MDP26-PEG

The synthetic procedure follows the same described in **S1.3.6** except that 5(6)-carboxyfluorescein (FAM) was used for the coupling with the Alloc-deprotected lysine residue. ESI MS: calc. $[M+H]^+=3384$, obsvd. $[M+H]^+=3452.2$. (The mass difference between the observed and calculated mass is due to PEG polydispersity)





S1.3.8 Synthesis of FAM-MDP24-PEG

The synthesis of FAM-MDP24-PEG followed the same procedure as described in **S1.3.7**. ESI MS: calc. $[M+H]^+=2886$, obsvd. $[M+H]^+=2969.2$. (The mass difference between the observed and calculated mass is due to PEG polydispersity)



Figure S11. HPLC of FAM-MDP24-PEG

FAM-MDP24-PEG



S2 Structural Characterization

S2.1 CD Spectroscopy

CD spectra were obtained on a Jasco-J715 Spectropolarimeter using a quartz cell with 1 mm path length. All samples were prepared at a concentration of 100 μ M in 20 mM Tris buffer (pH 7.4) upon dilution from stock solution in water. Freshly prepared solution was examined by CD. CD data were collected from 250 nm to 190 nm at room temperature, with scan rate of 100 nm/min, a response time of 2 sec, and a bandwidth of 1 nm. The absorption differences were converted to mean residual ellipticity.



Figure S1. CD spectra of (a) MDP26 and MDP26-PEG; (b) HCPT-MDP26 and HCPT-MDP26-PEG; (c) FAM-MDP26 and FAM-MDP26-PEG. All PEGylated and non-PEGylated MDP and derivatives form beta sheet structures. (d) FAM-MDP24-PEG and HCPT-MDP24-PEG

S2.2 TEM

Peptide stock solution was prepared and stored in pure water at a concentration of ~ 1 mM for further characterization. TEM samples were diluted from the stock solution into Tris buffer (pH=7.4, 20mM) at a concentration of 100 μ M. After occasional vortexing for 10 mins, 10 μ l of diluted solution was pipette onto a holey carbon grid (TED PELLA 01824). After 2 min, the excess solution was carefully removed by using a piece of filter paper and samples were dried for 2 min. 10 μ l of 2 wt% uranyl acetate aqueous solution was added. After 30 sec, the excess staining solution was removed and the TEM samples were allowed to dry for about 5 h before TEM imaging. The TEM imaging was performed on a JEOL 2010 High Resolution Transmission Electron Microscope.



Figure S2. Negatively stained TEM image of MDP26-PEG.

S2.3 SAXS

MDP26-PEG and MDP26 were directly dissolved in 20 mM Tris buffer (pH=7.4) at a concentration ~1 mM. The synchrotron SAXS experiments were performed on the bioSAXS high-throughput P12 EMBL beamline located on the PETRA III storage ring at DESY, Hamburg. The instrument is equipped with 2M detector and the measurements were carried out in a Q-range of 0.0076-0.46 Å-1. The data acquisition was executed under injection of a 10 μ l amount of sample into quartz capillaries (2 mm) using 20 successive frames with 50 s exposures that were later combined in order to improve the statistics. No sign of beam radiation damage was observed under these conditions. The data were averaged after normalization to the intensity of the transmitted beam and calibrated on an absolute scale using Millipore water as a primary calibrating standard.



Figure S3. SAXS data showing the scattered intensity of PEGylated and non-PEGylated MDP26. Solid lines display a fit to a form factor for sheets with Gaussian chains attached on one side.

S3 Molecular Dynamic Simulation

All simulations in this study were performed using GROMACS.² The conjugates were described by a hybrid atomistic-coarse grained model in which MDP26 and PEG were coarse-grained using the Martini force field³ while all atoms of HCPT were explicitly represented using the CHARMM general force field.⁴ Interatomic interactions were determined by the Lorentz-Berthelot mixing rule. Time step was set to be 1 fs. NPT ensemble⁵ was employed with the temperature and pressure maintained around 300 K and 1 bar, respectively. Periodic boundary conditions were applied along all directions. The unit cell size was 31.68 nm in the length direction for the fiber and 6.4 nm for the cylinder. These sizes were estimated from finite-length fiber and cylinder simulations. Unit cell sizes in the other two directions were set to be large enough to avoid interaction between periodic images of the conjugates.



Figure S4. Two possible packing configurations for self-assembled HCPT-MDP26 molecules: (a) fiber and (b) cylinder micelle (cross section).



Figure S5. Statistical length measurement for nanofiber formed by HCPT-MDP26-PEG



Figure S6. Negatively stained TEM image of FAM-MDP26-PEG

S4 Fluorescence Recovery

FAM-MDP26-PEG was prepared at a concentration of 50 μ M in 20 mM Tris buffer (pH 7.4). The sample was incubated at RT for 1 h before mixing with serum and serum protein. 100 μ l of peptide solution was mixed with 100 μ l of 100% FBS or 80 mg/ml BSA, respectively. Time-dependent fluorescence intensity was collected at $\lambda ex = 494$ and $\lambda em = 521$ nm for overnight.



Figure S7. Fitting of fluorescence recovery data into first-order exchange kinetics. Fluorescence change of FAM-MDP26-PEG (25 μ M) in the presence (A) [FBS]: 50% by weight; and (B) [BSA]: 40 mg/ml. Data was fitted into equation $I(t) = I(\infty)+[I(0)-I(\infty)][fe^{-k}1^t+(1-f) e^{-k}2^t]$. The fast rate constant, k_1 is attributed to the dilution of labeled nanofibers upon the addition of FBS or BSA, leading to fast dissociation of monomers. The slower rate constant, k_2 represents the rate of monomeric desorption from the supramolecular assembly which is trapped by serum proteins and can be used to compare the kinetics stability of supramolecular polymers.

S5 in vitro Cytotoxicity

Human hepatocellular carcinoma HepG2 were cultured in Dulbecco's modified Eagle medium DMEM with 10 % FBS and 1 % antibiotics (penicillin and streptomycin) under 5 % of CO₂ at 37 °C. The culture medium was changed every two days. For cytotoxicity measurement, HepG2 cells were seeded into a 96-well plate with a density of 5000 cells/well and incubated for 24 h. HCPT or HCPT-MDP-PEG conjugates at varying concentrations were added and incubated for 48 h. Cell viability was determined by using CCK-8 assay according to the manufacturer's protocol. The optical density of each well was determined by a microplate reader (Vitor² 1420 Multilabel Counter, PerkinElmer, USA) at the wavelength of 450 nm. The IC₅₀ value of each drug conjugate were obtained by using Igor Pro program and fitted by Hill equation function.



Figure S8. Cell viability of HepG2 cells incubated with MDP26-PEG at varying concentrations.

S6 Cellular Uptake by Confocal Microscopy

HepG2 cell line was used to investigate the cellular uptake of FAM-MDP26-PEG and FAM-MDP24-PEG by confocal microscopy (Leica, SP2). Cells were seeded onto a glass dish and cultured in DMEM for 24 h. After replacing the medium, peptides were then added to reach a final concentration of 20 μ M in the culture medium. Upon 2-hr incubation with peptides, cells were washed with PBS buffer for 3 times every 5 min and 2% of paraformaldehyde was used to fix the cell for 15 min. Images were acquired using confocal laser scanning microscopy.

S7 Flow Cytometry

Hep G2 cells were seeded onto a 24-well plate at a density of 1×10^5 cells/well and cultured for 24 hours. The DMEM medium was replaced and peptide solutions were added. After incubation with peptides for 2 h, cells were washed with PBS buffer for 3 times every 5 min. Thereafter, the cells were harvested with trypsin, washed twice and resuspended in PBS containing 0.1% (w/v). The cell was fixed using 2% paraformaldehyde for 10 min. Cell uptake of MDP26-PEG and MDP24-PEG was quantified using a BD FACSCaliburs flow cytometer. A minimum of 10,000 events per sample was analyzed. The fluorescence signal was analyzed at the FITC-A channel. Data was processed using FlowJo software.



Figure S9. Release kinetics of HCPT from HCPT-MDP26-PEG and HCPT-MDP24-PEG monitored by HPLC. Peptides were diluted in Tris buffer (pH=7.4, 20 mM) at 20 μ M. Similar drug degradation profile was observed for peptides in HEPES (pH=7.4, 10mM) and phosphate buffer (pH=7.4, 10 mM).

References

1 L. Mao, H. Wang, M. Tan, L. Ou, D. Kong and Z. Yang, *Chemical Communications*, 2012, **48**, 395.

2 B. Hess, C. Kutzner, D. van der Spoel and E. Lindahl, *Journal of Chemical Theory and Computation*, 2008, **4**, 435.

3 G. Rossi, P. F. J. Fuchs, J. Barnoud and L. Monticelli, *The Journal of Physical Chemistry B*, 2012, **116**, 14353.

4 K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov and A. D. Mackerell, *Journal of Computational Chemistry*, 2010, **31**, 671.

5 H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, A. DiNola and J. R. Haak, *The Journal of Chemical Physics*, 1984, **81**, 3684.