Supporting Information

Targeted delivery of doxorubicin to tumor tissues by a novel legumain sensitive polygonal nanogel

Sen Lin\textsuperscript{a,b}, Tong Li\textsuperscript{a}, Peiling Xie\textsuperscript{a}, Qing Li\textsuperscript{b}, Bailiang Wang\textsuperscript{a}, Lei Wang\textsuperscript{b}, Lingli Li\textsuperscript{a}, Yuqin Wang\textsuperscript{a}, Hao Chen\textsuperscript{a*} and Kaihui Nan\textsuperscript{a*}

\textsuperscript{a} School of Ophthalmology & Optometry and Eye Hospital, Wenzhou Medical University, Wenzhou, 325027, China.

\textsuperscript{b} Wenzhou Institute of Biomaterials and Engineering (in preparation), Chinese Academy of Science, Wenzhou, 325000, China

\textsuperscript{*}Correspondence: Prof. K. Nan, E-mail: nankh@163.com ; Prof. H. Chen, E-mail: chenhaao823@mail.eye.ac.cn

1 The MS spectra of FmocNH-LN(Trt)AA-CONH-DOX
The ESI-MS were obtained using Shimadzu (Kyoto, Japan) LCMS 8050 triple quadrupole MS system equipped with a heated capillary interface and electrospray ionization (ESI) source. Electrospray-ionization was performed both in the positive and negative ion mode. The heated capillary was set at 245 °C, the spray voltage at 3.5 kV. Argon was used as the sheath and auxiliary, and the gas flow for nebulizing, drying, and heating were set at 3, 10, and 10 mL/min, respectively. This purified product has quasi-molecular ion peaks \( m/z \ [M + Na]^+ \) 1400.8 and \( [M-H]^- \) 1376.6 corresponding to a molecular weight of 1377 Da, which matched well with that of DOX-PEP-NHfmc.

![Chemical structure and MS spectra](image)

Figure S1 the chemical structure of FmocNH-LN(Trt)AA-CONH-DOX (A) and its MS spectra (B)

2 NMR spectra of FmocNH-LN(Trt)AA-CONH-DOX
The chemical structure of the purified product was identified by $^1$H-NMR and $^{13}$C-NMR spectra. The NMR spectra were recorded by a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) in deuterated DMSO-d$_6$ at room temperature. $^1$H-NMR (600 MHz, DMSO-d$_6$, $\delta$), 0.83 (d, $J=6.51$ Hz, 6H, H-32 and H-33, CH$_3$$^2$), $\delta$ 1.06 (d, $J=6.4$Hz, 3H, H-27, CH$_3$), $\delta$1.19 (d, $J=7.0$Hz, 6H, H-41 and H-44, CH$_3$$^2$), $\delta$1.37 (m, overlapped, 3H, H-30 and H-31, CH$_2$ and CH), $\delta$ 1.53 and 1.76 (m, 2H, H-23, CH$_2$), $\delta$ 2.10 and 2.18 (m, 2H, H-13, CH$_2$), $\delta$ 2.73 (m, 2H, H-36, CH$_2$), $\delta$ 2.96 (m, 2H, H-11, CH$_2$), $\delta$ 3.90 (m, 1H, H-26, CH), $\delta$ 3.95 (s, 3H, H-1, CH$_3$), $\delta$ 4.06 (m, overlapped, 2H, H-24 and H-25, CH$^2$), $\delta$ 4.24 (m, overlapped, 6H, H-14, H-29, H-35, H-40, H-43, and H-47, CH$^5$), $\delta$ 4.49 (m, 2H, H-46, CH$_2$), $\delta$ 4.58 (d, $J=6.01$Hz, 2H, H-21, CH$_2$), $\delta$ 4.88 (t, 1H, H-22, CH). $^{13}$C-NMR (100 MHz, DMSO-d$_6$, $\delta$), 16.3 (C-27), 18.1(C-41), 18.6 (C-44), 21.5 (C-32), 22.0 (C-33), 24.1 (C-31), 29.7 (C-23), 32.1 (C-11), 35.7 (C-13), 36.6 (C-11), 40.5 (C-30), 44.6 (C-47), 46.6 (C-46) 48.0 (C-40), 49.5 (C-43), 50.0 (C-35), 51.2 (C-29), 56.6 (C-1), 63.6 (C-21), 65.5 (C-26), 66.6 (C-44), 67.8 (C-25), 69.2 (C-37), 69.8 (C-14), 74.9 (C-12), 83.7 (C-38), 100.3 (C-22), 110.6 (C-3), 119.0 (C-5), 120.0 (C-19 and Benzyl corbon), 125.2-128.5 (Benzyl corbons), 134.1 (C-10), 134.1 (C-15), 135.3 (C-4), 136.5 (C-6), 140.6 (Benzyl corbons), 143.8 (Benzyl corbons), 144.6 (Benzyl corbons), 154.5 (C-45), 155.6 (C-9), 156.0 (C-16), 160.8 (C-2), 169.0 (C-37), 170.5 (C-34), 170.9 (C-28), 171.9 (C-39), 172.0 (C-42), 186.5 (C-7), 186.6 (C-18), 213.7 (C-20).
Figure S2 $^1$H-NMR and $^{13}$C-NMR spectra of FmocNH-LN(Trt)AA-CONH-DOX
3 Identification of the multi-overlapped $^1$H-NMR signal

Great amount of $^1$H-NMR signal was present as overlapped peak at $\delta$ 4.0-4.3 and $\delta$ 7.0-8.0 ppm. In order to further identify this overlapped signal, the heteronuclear multiple bond correlation (HMBC) experiment was conducted. The data present in Figure S3 further confirmed the successful synthesized of FmocNH-LN(Trt)AA-CONH-DOX.

Figure S3. The HMBC spectra overlapped $^1$H-NMR signal
4 UPLC-MS-MS analysis of the *in vitro* drug release

The *in vitro* drug release from HA-PEP-DOX nanogel with or without enzyme treatment (control) was analyzed using a UPLC-MS-MS system. To quantify the released DOX-Leu, single ion monitoring scan for $m/z$ 679 was conducted, and the peak area was determined by the LabSolutions LCMS Version 5.60 (Shimadzu, Kyoto, Japan). As shown in Figure S4, the peak area was increased as the function of time with the presence of legumain. On the contrary, there is no DOX-Leu observed without presence of legumain. These results indicating the release of DOX-Leu was indeed triggered by legumain.
Figure S4 The release DOX-Leu form HA-PEP-DOX nanogel with the present of legumain.
5 Asparaginyl endopeptidase activity examinations

The tissues from tumour bearing mice were dissected. After cleaning, they were cut into small pieces and treated with a lysis buffer (Beyotime® Biotechnology Co. Ltd, Jiangsu, China). After centrifugation (14000 rpm, 5min), the amount of protein in the supernatant was determined by micro-bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). After appropriate diluted, the asparaginyl endopeptidase activity of these extracted proteins were determined by theirs ability to cleave the fluorogenic peptide substrate, N-carbobenzyloxy-Ala-Ala-Asn-7-amido-4-methylcoumarin (Z-AAN-AMC) according to manufacturers’ instructions of legumain (Novoprotein Scientific CO., New Jersey, US). In briefly, Z-AAN-AMC and the extracted proteins were added into a 96-well plate. The emission fluorescence intensity at 460 nm with the excitation wavelength at 380 nm was recorded at 37 °C in kinetic mode for 30 minutes.

Fig S5 Asparaginyl endopeptidase activity of the extracted protein from tumour tissue
The system toxicity of HA-PEP-DOX nanogel

The toxicity of HA-PEP-DOX nanogel to normal tissues including liver, heart, kidney, and spleen were evaluated using H&E staining and TUNEL assay. When compared to free DOX treated group, the HA-PEP-DOX nanogel treated group exhibited much less cell apoptosis in liver and kidney, possibly due to much less DOX was distributed in liver and kidney. However, no obviously difference of cell apoptosis was observed in heart and spleen.
Figure S6. System toxicity evaluation of HA-PEP-DOX nanogel (the microscopies at 200 × magnification)