Supporting Information

Tumor Microenvironment Responsive Supramolecular Glyco-Nanovesicles Based on Diselenium-Bridged Pillar[5]arene Dimer for Targeting Chemotherapy

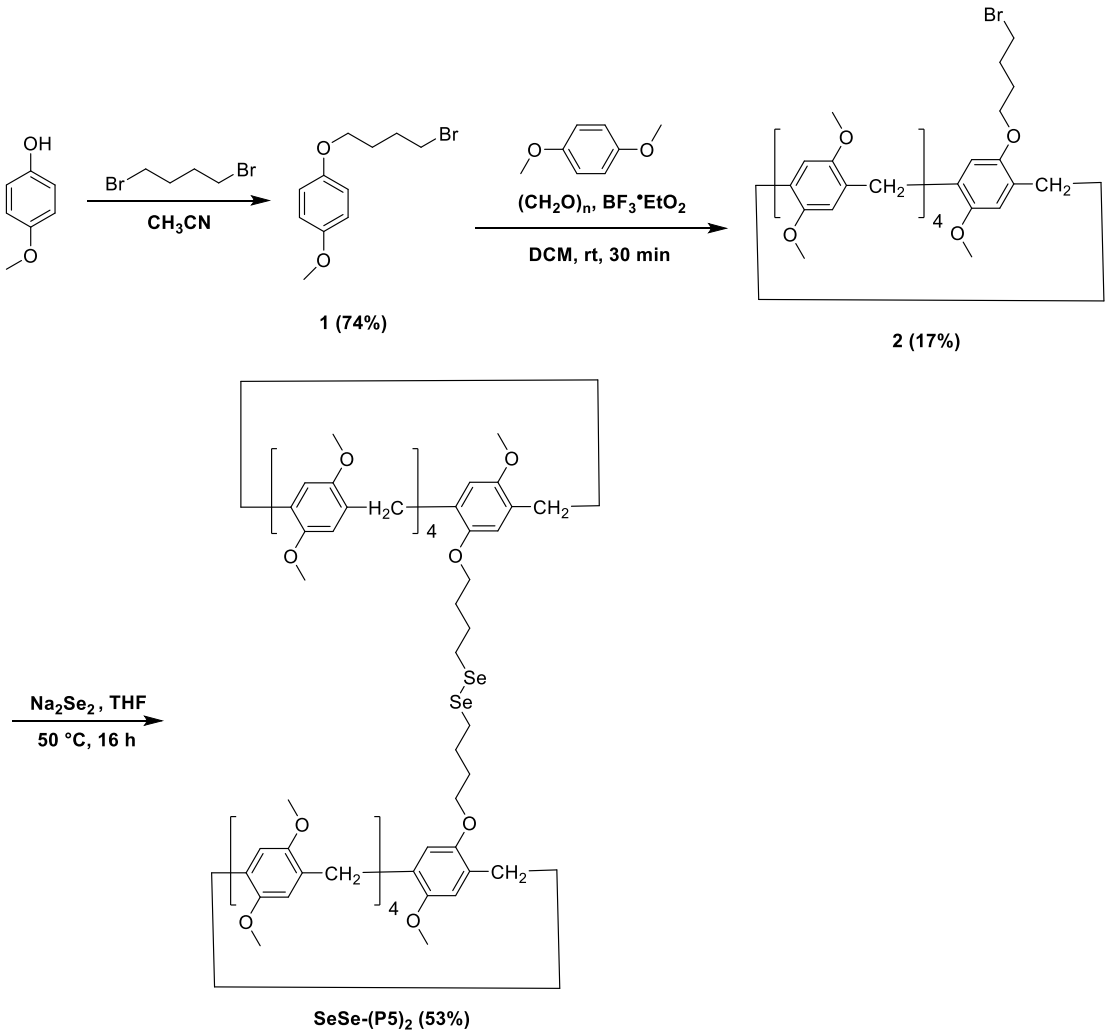
Yang Wang, Ming Jin, Zelong Chen, Xianjun Hu, Liang Pu, Zhichao Pei and Yuxin Pei*

Shaanxi Key Laboratory of Natural Products & Chemical Biology, College of Chemistry & Pharmacy, Northwest A&F University, Yangling 712100, P. R. China.

1. Instrumentation and chemicals

All reagents were purchased from commercial suppliers and used without further purification unless specified. Water used in this work was triple distilled. ^{1}H NMR spectra were recorded on a Bruker 500 MHz Spectrometer, with working frequencies of 500 MHz for ^{1}H nuclei. Scanning electron microscopy (SEM) images were obtained using an S-4800 instrument (Hitachi Ltd.) with an accelerating voltage of 10.0 kV. Transmission electron microscopy (TEM) images were obtained using a TEANCI G2 SPIRIT BIO instrument (FEI Ltd. U.S.A.). DLS measurements were performed on a Malvern Zen3600 instrument (Malvern Instruments Limited, U.K.). UV-vis spectra were recorded with Shimadzu 1750 UV-visible spectrophotometer (Japan) at 298K. Water surface tension was recorded with BZY-3B surface tension measurer (China). Cell culture was carried out in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. Cell toxicity was tested by microplate reader (KHB ST-360). The confocal laser microscope (CLSM) data were acquired using a CLSM (Andor REVOLUTION WD). The flow cytometry (FCM) was performed on a BD FACSAria™ III flow cytometer (BD Biosciences, U.S.A.).

2. Synthesis and characterizations of the compounds



Scheme S1. Synthetic route of **SeSe-(P5)$_2$**.[S1]

Compound **1**: 4-Methoxyphenol (3.1 g, 0.025 mol) and K_2CO_3 (5.5 g, 0.04 mol) were added into acetonitrile (50 mL) and the mixture were stirred at room temperature for 30 min. Then, KI (0.05 g, 2.5 mmol) and 1,4-dibromobutane (8.875 mL, 0.075 mol) were added to the solution. The mixture was heated to reflux for 16 h. Finally, the mixture was concentrated under vacuum, and subjected to silica gel chromatography (petroleum ether (PE):ethyl acetate (EA) = 10:1) to give the compound **1** as white powder (4.82 g, yield: 73.8%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.83 (s, 4H), 3.95 (t, J = 6.0 Hz, 2H), 3.77 (s, 3H), 3.49 (t, J = 6.5 Hz, 2H), 2.09 – 2.04 (m, 2H), 1.95 – 1.89 (m, 2H) ppm.

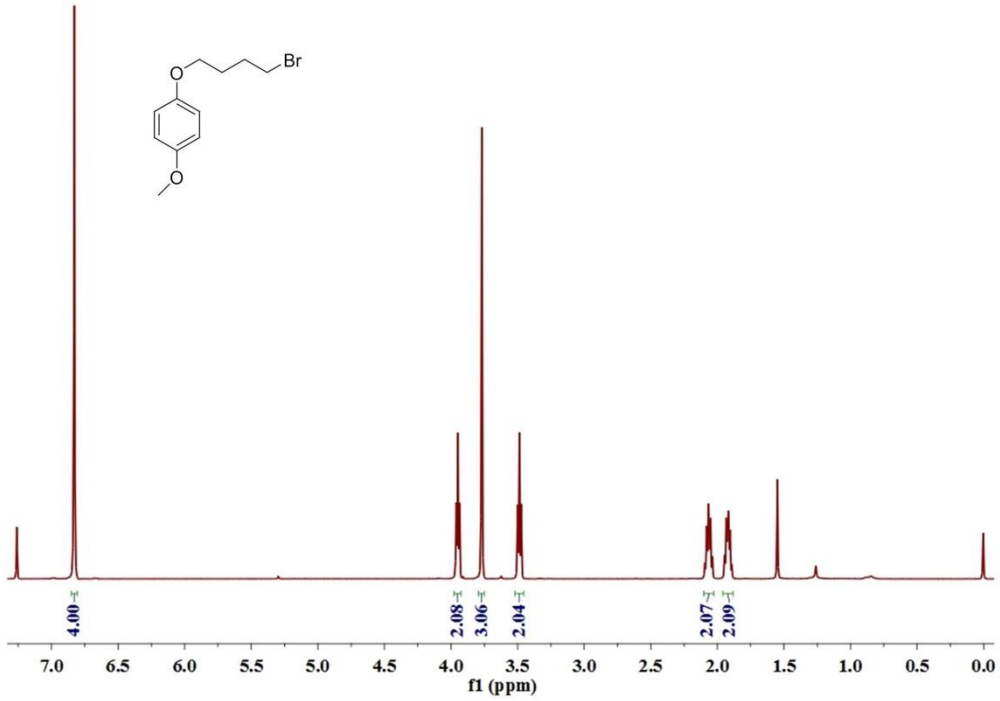


Fig. S1. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **1**.

Compound **2**: Compound **1** (1.04 g, 4 mmol), 1,4-dimethoxybenzene (2.75 g, 20 mmol) and $(CH_2O)_n$ (2.52 g, 84 mmol) was added into dichloromethane (180 mL) under ice-water bath and stirred for 30 min. Next, $BF_3{\cdot}Et_2O$ (3.6 mL) was added and then removed the ice-water bath to reaction for 30 min. After the color of mixture changed to dark-green, water (300 mL) was poured into solution to quench the reaction. The pure compound **2** was obtained as white powder (609 mg, yield: 17%) over silicon gel column chromatography (PE:DCM:EA = 90:30:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.81 – 6.70 (m, 10H), 3.78 (s, 12H), 3.66 – 3.63 (m, 27H), 3.25 – 3.12 (m, 2H), 1.76 – 1.71 (m, 4H) ppm.

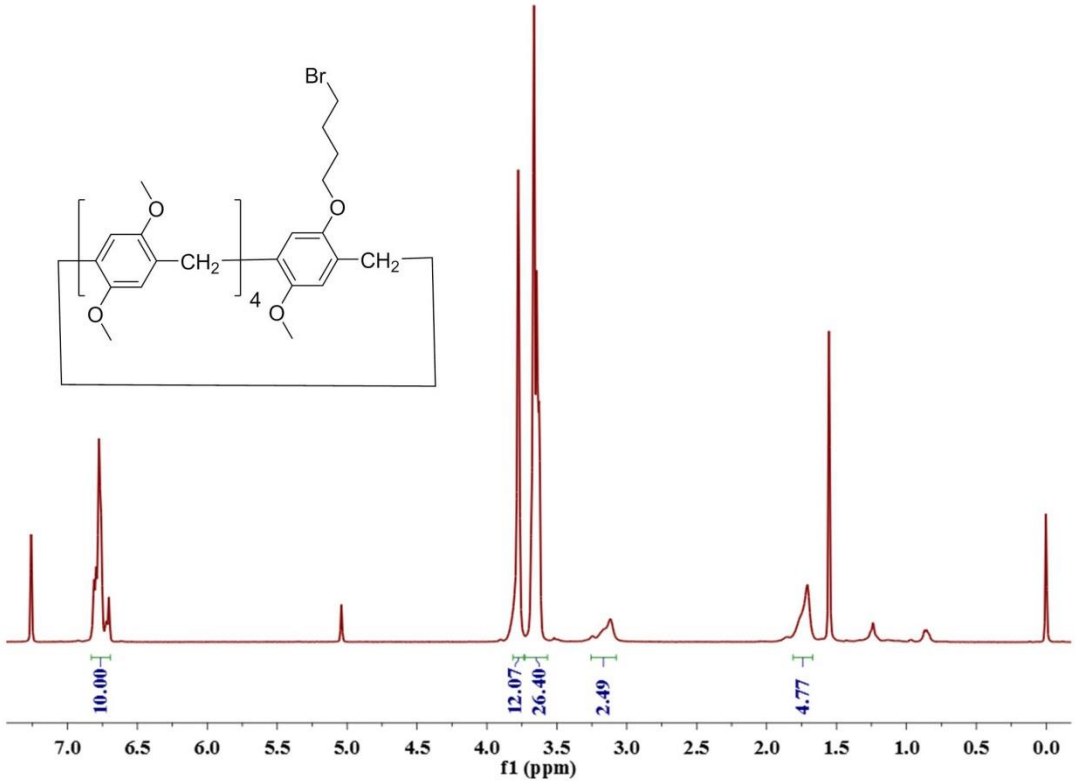


Fig. S2. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **2**.

SeSe-(P5)$_2$: First, $NaBH_4$ (18.94 mg, 0.5 mmol) and Se (39.48 mg, 0.5 mmol) were added into H_2O (1.5 mL), and then stirred for 15 minutes to obtain Na_2Se_2 solution. After that, compound **2** (0.255 g, 0.3 mmol) and THF (15 mL) were added into the Na_2Se_2 solution and the mixture was stirred for 10 h at 50°C. During this period, the solution changes from crimson to yellow. After silicon gel column chromatography (PE:EA = 15:1), the final **SeSe-(P5)$_2$** was obtained as yellow powder (134 mg, yield: 53%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.81 – 6.70 (m, 20H), 3.86 – 3.82 (m, 4H), 3.80 – 3.74 (m, 20H), 3.68 – 3.55 (m, 54H), 2.89 (t, J = 6.5 Hz, 4H), 2.00 – 1.72 (m, 8H) ppm.

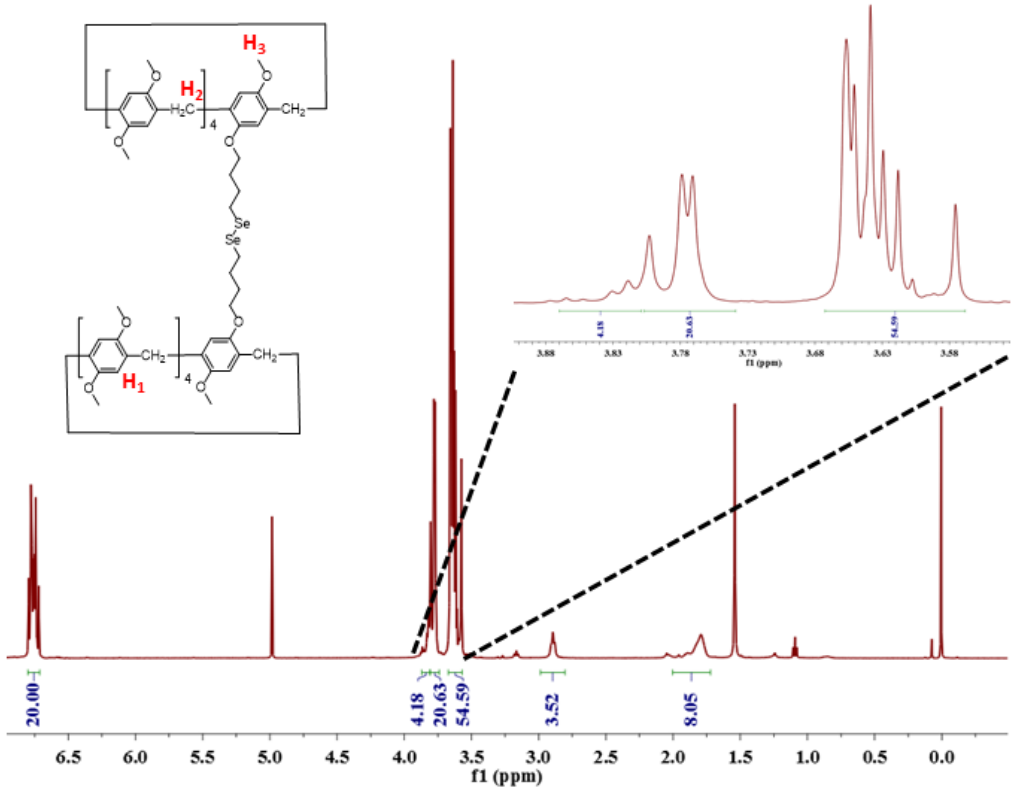
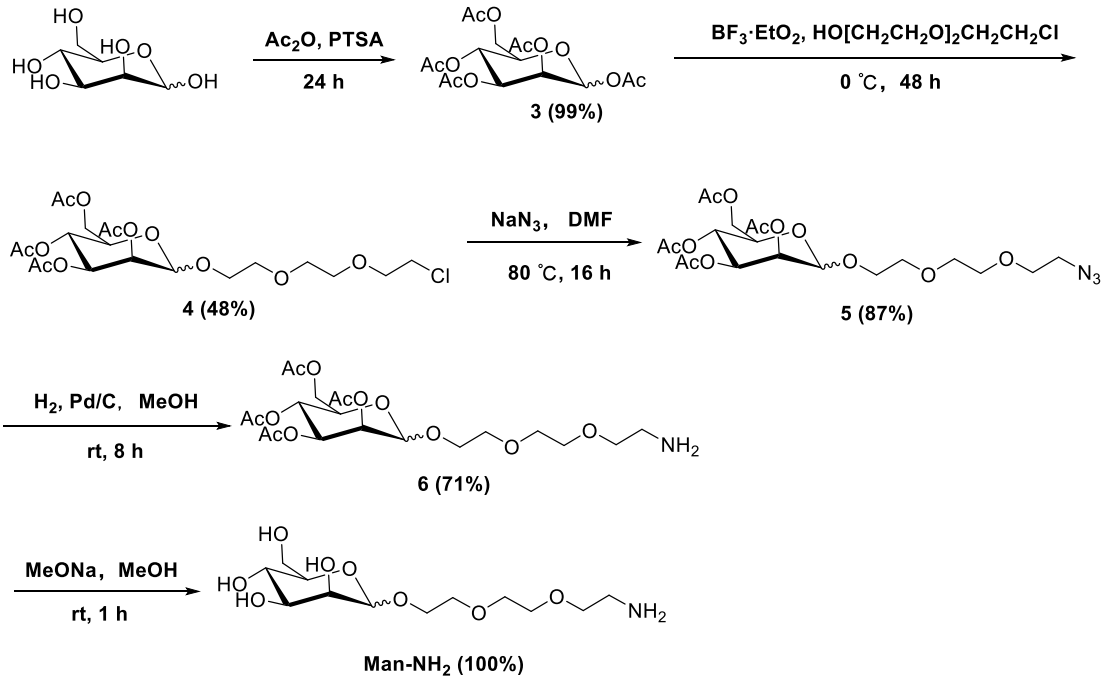


Fig. S3. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **SeSe-(P5)$_2$**.



Scheme S2. Synthetic route of compound **Man-NH$_2$**.[S2]

Compound **3**: PTSA (4.82 g, 1.1 mmol) was added to a stirred mixture of acetic anhydride (30 mL) and D-mannopyranoside (5.0 g, 27.8 mmol). Then, the mixture

was stirred at room temperature for 24 h. After the reaction completed, the mixture was diluted with dichloromethane (100 mL) and was washed against saturated Na_2CO_3 aqueous solution. The solvent was evaporated under reduced pressure to afford compound **3** (10.7g, 99%).

Compound **4**: 2-[2-(2-chloroethoxy)ethoxy]ethanol (3.1 mL, 21.3 mmol) and compound **3** (5.0 g, 12.8 mmol) were mixed in dry dichloromethane (30 mL). $BF_3{\cdot}OEt_2$ (5 mL) was slowly added into the mixture under ice bath. Then the mixture was stirred at room temperature for 48 h. After the reaction completed, the resulting mixture was washed with saturated Na_2CO_3 aqueous solution. Evaporation of the solvent under reduced pressure and further purification was carried out by silicon gel column chromatography (PE:EA = 3:2) to afford compound **4** (3.1 g, 48%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.36–5.25 (m, 3H), 4.86 (d, J = 1.0 Hz, 1H), 4.28 (dd, J_1 = 5 Hz, J_2 = 12.0 Hz, 1H), 4.11 – 4.04 (m, 2H), 3.83 – 3.78 (m, 1H), 3.75 (t, J = 6.0 Hz, 2H), 3.69 – 3.62(m, 9H), 2.14 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H) ppm.

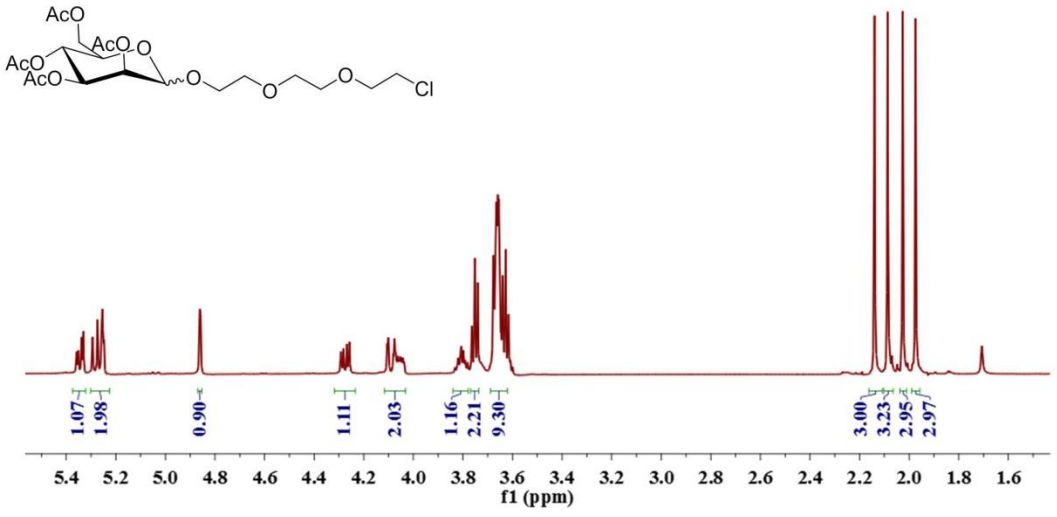


Fig. S4. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **4**.

Compound **5**: Compound **4** (2.49 g, 5 mmol) and sodium azide (0.975 g, 15 mmol) were mixed in dry DMF (15 mL), the mixture was stirred at 80°C for 16 h. After the

reaction completed, the mixture was poured into ice water, and the aqueous phase was extracted with dichloromethane (3 × 60 mL). The combined organic phases were washed with brine (50 mL) and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure and further purification was carried out by silicon gel column chromatography (PE:EA = 3:2) to afford compound **5** (1.96 g, 87.4%) (The configuration of mixture was composed of α:β = 5:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.35 – 5.25 (m, 3H), 4.85 (d, *J* = 1.0 Hz, 1H), 4.28 – 4.24 (m, 1H), 4.10 – 4.02 (m, 2H), 3.82 – 3.77 (m, 1H), 3.67 – 3.64 (m, 9H), 3.38 (t, *J* = 5.0 Hz, 2H), 2.13 (s, 3H), 2.08 (s, 3H), 2.02 (s, 3H), 1.97 (s, 3H) ppm.

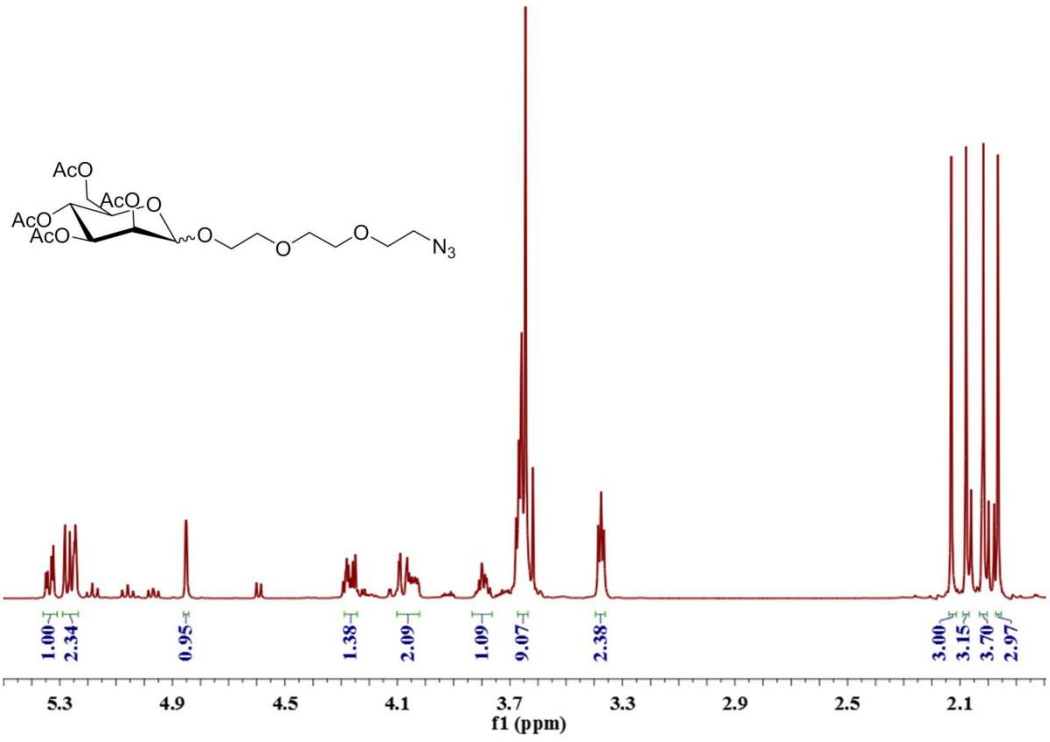


Fig. S5. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **5**.

Compound **6**: Compound **5** (1.9g, 3.75 mmol) was dissolved in methol (25 mL) and the mixture was hydrogenated under hydrogen pressure (10 atm) in the presence of 10% Pd/C (850 mg). Then the mixture was stirred at room temperature for 12 h. After the reaction completed, the mixture was filtrated and concentrated to afford compound **6** (1.28 g, 71%) (The configuration of mixture was composed of α:β = 5:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.39 – 5.38 (m, 1H), 5.35 – 5.16 (m, 2H), 4.93 (s, 1H),

4.36 – 4.29 (m, 1H), 4.21 – 4.06 (m, 2H), 3.85 – 3.81(m, 2H), 3.73 – 3.54(m, 10H), 2.22 (s, 3H), 2.13 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H) ppm.

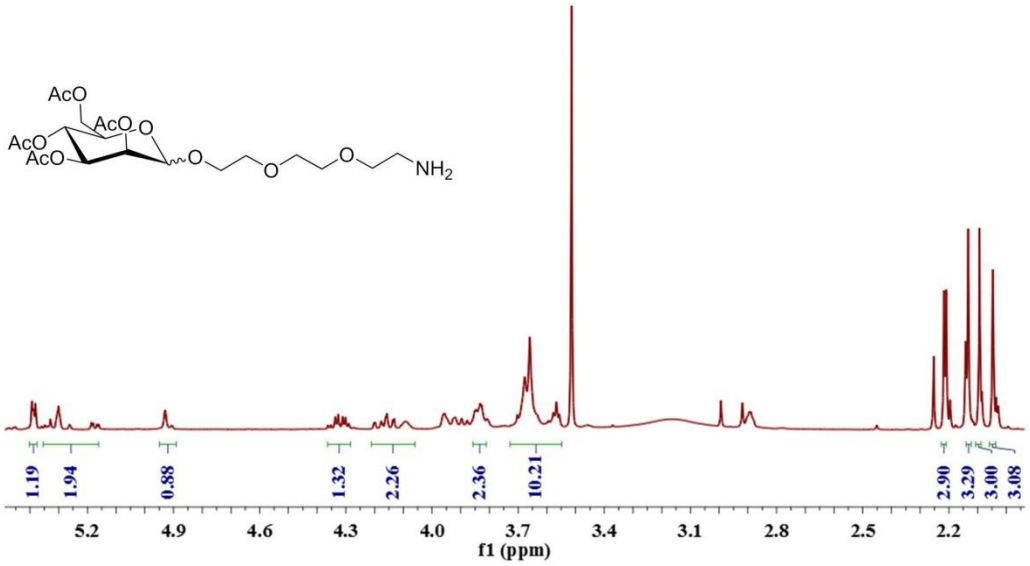


Fig. S6. The ^{1}H NMR (500 MHz, 298K, $CDCl_3$) spectrum of **6**.

Man-NH$_2$: Compound **6** (90 mg, 0.18 mmol) and sodium methoxide (50.7 mg, 0.93 mmol) were dissolved in methol (8 mL). The mixture was stirred at room temperature for 12 h. Then the mixture was neutralized by addition of ion-exchange resin (Amberlite IR 120 H^+) until pH = 7, filtrated, and the solvent was removed under reduced pressure to give the **Man-NH$_2$** (82.4 mg, 100%). ^{1}H NMR (500 MHz, D_2O): δ 4.90 (s, 1H), 3.97 – 3.88 (m, 3H), 3.84 – 3.63 (m, 13H), 3.39 (t, J = 5.5 Hz, 1.5H), 3.23 (t, J = 5.0 Hz, 0.5H) ppm.

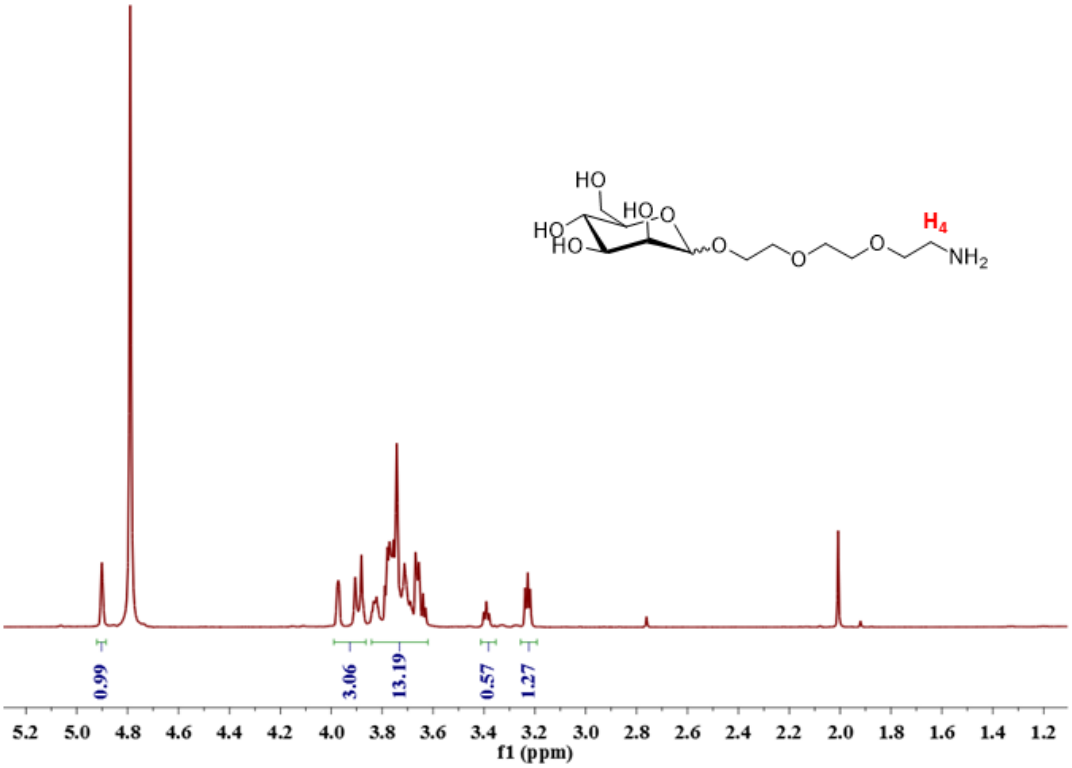


Fig. S7. The ^{1}H NMR (500 MHz, 298K, D_2O) spectrum of **Man-NH_2**.

3. The preparation and characterization of the SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanoglavesicles

Man-NH_2 (1.25 mg, 4 μmol) and HCl (4 μL, 1 M) were dissolved in PBS (4 mL) and stirred at room temperature for 30 min, SeSe-$(P5)_2$ (3.5 mg, 2 μmol) was dissolved in THF (300 μL). Then the THF solution was slowly added into the PBS solution. After ultrasonicated for 1 h and stood overnight, the SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles were obtained, which were characterized by SEM, TEM and DLS.

4. DOX loading and release of SeSe-$(P5)_2$⊃ Man-NH_3^+ glyco-nanovesicles

Man-NH_2 (1.25 mg, 4 μmol) and HCl (4 μL, 1 M) were dissolved in PBS (4 mL) and stirred at room temperature for 30 min, SeSe-$(P5)_2$ (3.5 mg, 2 μmol) was dissolved in THF (300 μL). Then the THF solution was slowly added into the PBS solution, and DOX (1.16 mg, 2 μmol) was added. After ultrasonic for 1 h in the ice bath and staying overnight, the solution was transferred to a dialysis bag (molecular

weight cutoff: 1000) and dialyzed to the outside of the dialysis bag without DOX fluorescence, thereby obtaining an aqueous solution of DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles. The amount of unloaded DOX in the dialysate was quantitatively measured by ultraviolet spectrophotometry. Finally, the drug encapsulation efficiency (DEE) and drug loading capacity (DLC) of DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles were calculated by total dose of DOX added and unloaded DOX, respectively.

The DOX encapsulation was calculated by the following equations:

DEE (%) = ($m_{DOX\text{-}loaded}/m_{DOX}$) 100%

DLC (%) = ($m_{DOX\text{-}loaded}/m_{DOX\text{-}loaded\ glyco\text{-}nanovesicles}$) 100%

$m_{DOX\text{-}loaded}$ and m_{DOX} are the mass of DOX encapsulated in the vesicles and DOX added, respectively. $m_{DOX\text{-}loaded\ glyco\text{-}nanovesicles}$ is the mass of the DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles.

The DOX release from DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles was studied at buffer solutions with or without GSH. 1 mL of DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles in a dialysis bag was added into 4 mL of corresponding release medium at room temperature and in dark conditions. At specified time intervals, 0.1 mL of the release medium was taken out for measuring the DOX released DOX concentration with a microplate reader. The concentration of DOX was determined by measurement of absorbance by using a standard absorbance vs. concentration curve constructed for DOX in the corresponding release medium.

5. Cell culture

MCF-7, 293T cells were cultured in DMEM medium containing 10% FBS, 1% penicillin/streptomycin (complete DMEM medium) in 5% CO_2 at 37°C. HepG2 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin (complete RPMI 1640 medium) in 5% CO_2 at 37°C.

6. Confocal laser scanning microscopy (CLSM)

HepG2 and 293T cells were seeded in 20 mm plastic bottomed μ-dishes for 24 h. The medium was replaced with a fresh one. The HepG2 and 293T cells were then incubated with DOX-loaded glyco-nanovesicles (DOX concentration: 5 μM) for 2, 4, 12 h. In contrast, one group of HepG2 cells was pretreated with mannose (2 mg/mL) for 4 h and then incubated with DOX-loaded glyco-nanovesicles for 4 h. The dishes were washed with PBS three times and fixed with 4.0% paraformaldehyde at room temperature for 15 min. After washing with PBS (2 mL × 2), the cells were all stained with Hoechst 33258 and DAPI for 5 min, respectively. Finally, the cells were washed with PBS and then observed under a confocal fluorescence microscope.

7. Flow cytometry

HepG2 cells and MCF-7 cells were seeded in 6-well plates (1×10^5 cells/well) and cultured in complete medium for 24 h. The fresh medium containing 5 μM free DOX, DOX-loaded glyco-nanovesicles were added, respectively. In contrast, one group was pretreated with mannose (2 mg/mL) for 4 h before the incubation with DOX-loaded glyco-nanovesicles. After 4 h, the cells were harvested and washed for two times with cold PBS and resuspended in 500 μL PBS. Finally, cells were analyzed by a flow cytometer.

8. Cytotoxicity evaluation

The relative cytotoxicities of blank and DOX-loaded glyco-nanovesicles were evaluated *in vitro* by MTT assay, respectively. The cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 μL complete medium and grew for 24 h at 37°C. Subsequently, cells were incubated with blank and DOX-loaded glyco-nanovesicles at different concentrations for 24, 48, or 72 h. The cells were washed and the fresh medium containing MTT was added into each plate. The cells were incubated for another 4 h. After that, the medium containing MTT was removed, dimethyl sulfoxide (100 μL) was added to each well, and the plates were gently shaken for 10 min to dissolve the formazan crystals. Finally, the absorbance at 490 nm was recorded with a microplate reader.

9. Fig. S8-S18

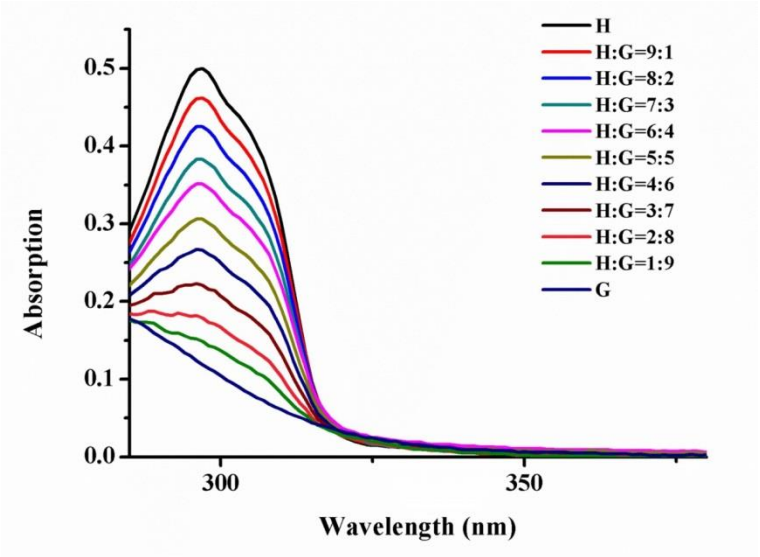


Fig. S8. UV-vis spectra of SeSe-$(P5)_2$ in aqueous solution at room temperature with different concentrations of Man-NH_3^+.

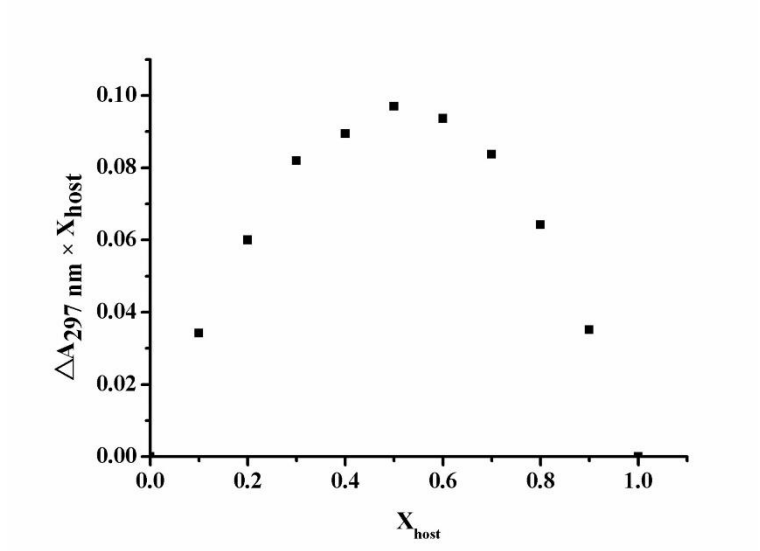


Fig. S9. Binding stoichiometry of SeSe-$(P5)_2$ and Man-NH_3^+.

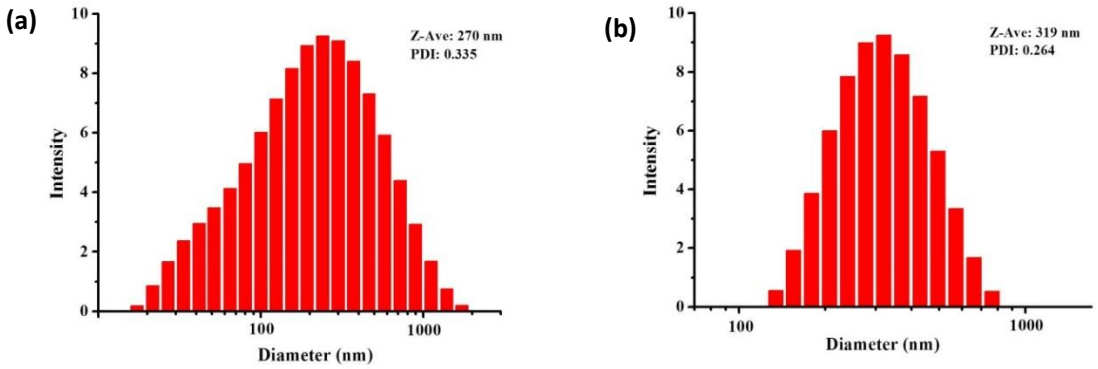


Fig. S10. DLS histogram of (a) SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles and (b) DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles.

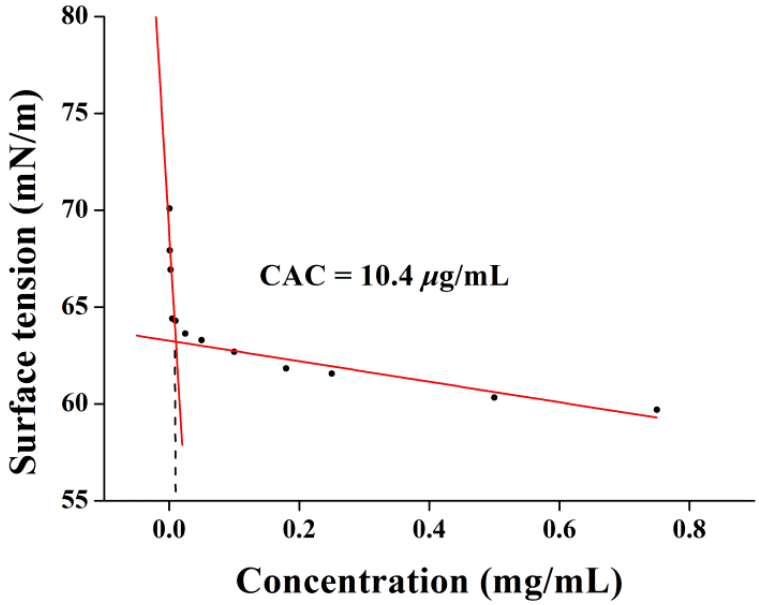


Fig. S11. The critical aggregation concentration of SeSe-$(P5)_2$⊃Man-NH_3^+ complex.

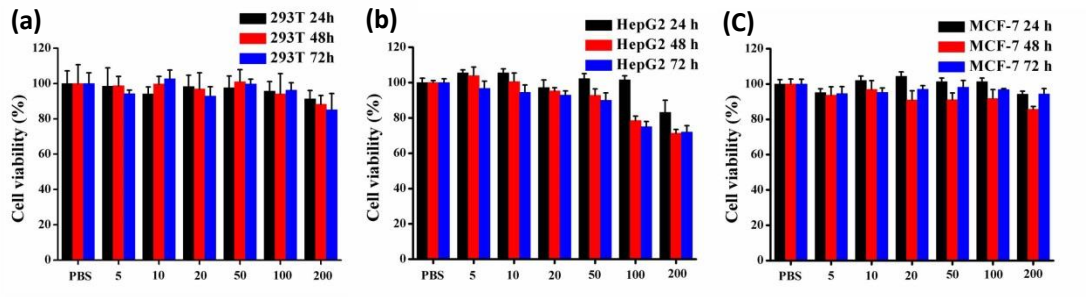


Fig. S12. Cell viability of 293T cells (a), HepG2 cells (b), and MCF-7 cells (c) incubated with SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles at different concentration (μM) for 24,

48 and 72 h.

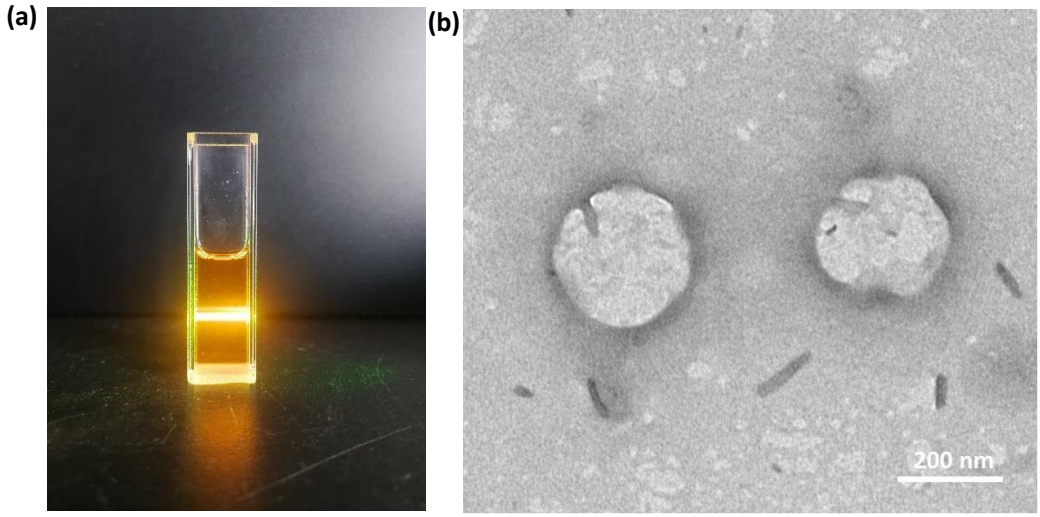


Fig. S13. Tyndall effect (a) and the TEM images (b) of vesicles formed by pristine DOX (100 μM)

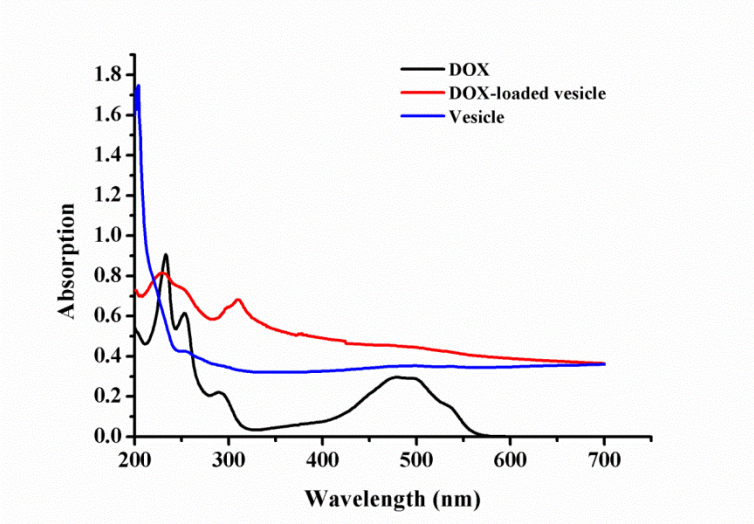


Fig. S14. UV-Vis spectra of DOX, SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles, and DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles in H_2O.

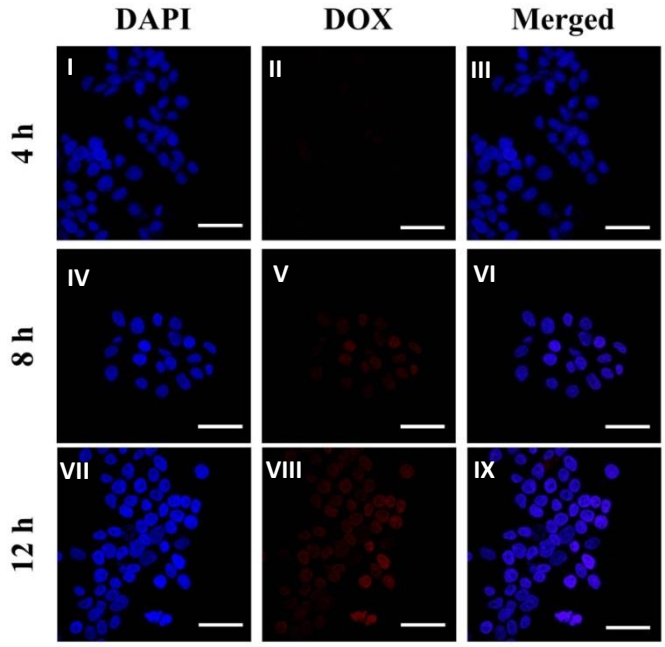


Fig. S15. (a) CLSM images of 293T cells incubated with DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles for 2, 4, 12 h (DOX concentration: 5 μM), respectively (scale bar: 50 μm).

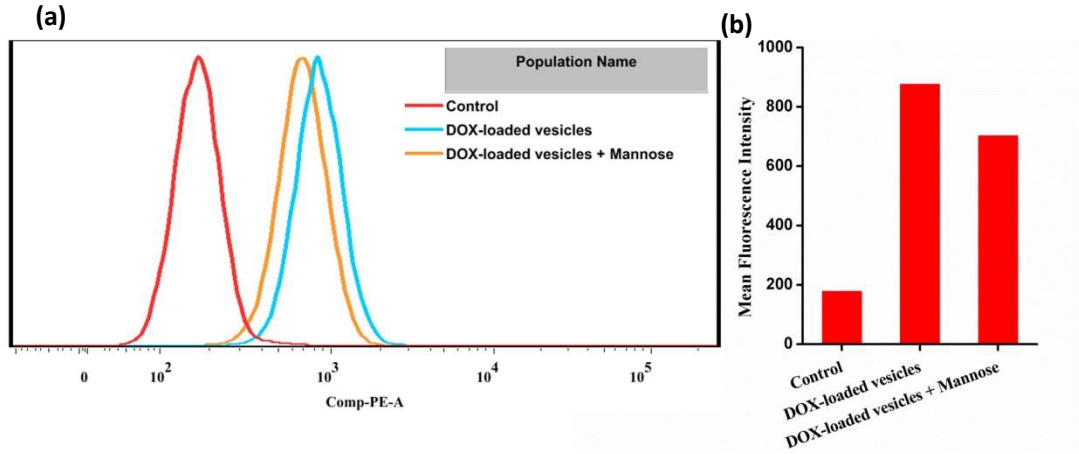


Fig. S16. Flow cytometry analysis of (a, b) MCF-7 cells after incubation with DOX, DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles, and DOX-loaded SeSe-$(P5)_2$⊃Man-NH_3^+ glyco-nanovesicles after pre-incubation with Mannose for 4 h, respectively for 4 h (DOX concentration: 5 μM).

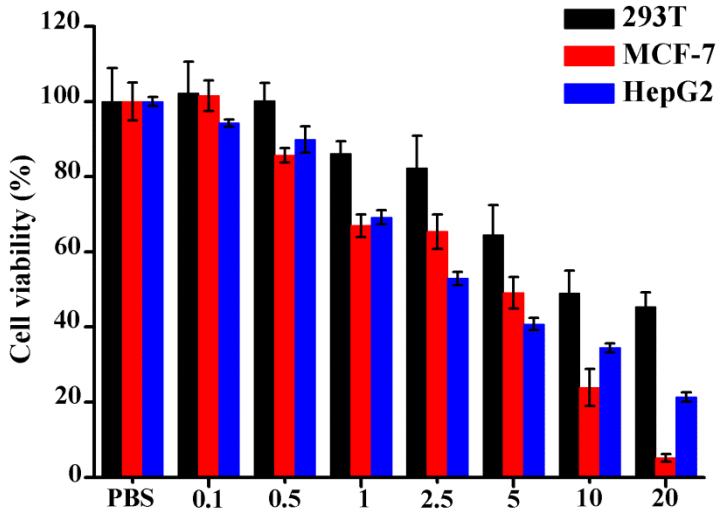


Fig. S17. Cell viability of 293T cells, MCF-7 cells and HepG2 cells incubated with DOX-loaded glyco-nanovesicles at different concentrations of DOX (μM) for 72 h.

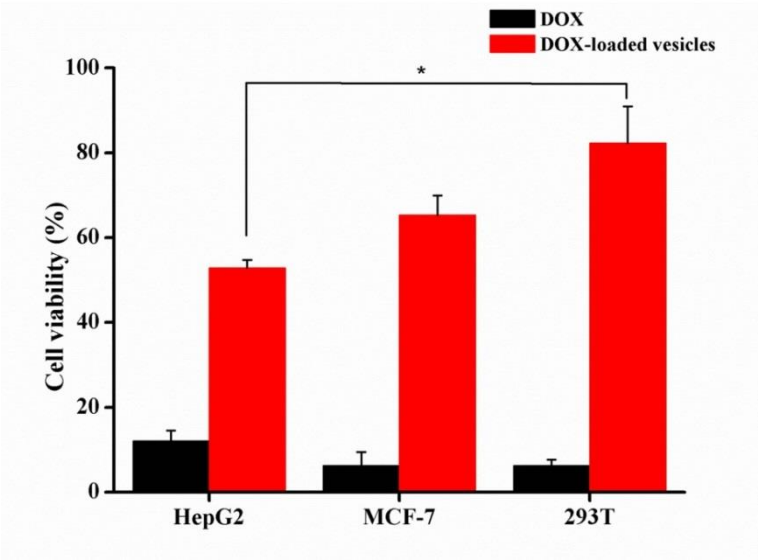


Fig. S18. Cell viability of 293T cells, MCF-7 cells and HepG2 cells incubated with DOX, DOX-loaded glyco-nanovesicles for 72 h (DOX concentration: 2.5 μM) (n = 6).

10. References

S1. Y. Zhou, K. Jie, B. Shi and Y. Yao, *Chem. Commun.*, 2015, **51**, 11112-11114.

S2. K. Shang, S. Song, Y. Cheng, L. Guo, Y. Pei, X. Lv, T. Aastrup and Z. Pei, *Polymers*, 2018, **10**, 1275.