Electronic Supplementary Material (ESI) for ChemComm.

Supramolecular nanoprodrug based on chloride channel blocker and glycosylated pillar[5]arene for targeted chemoresistance cancer therapy

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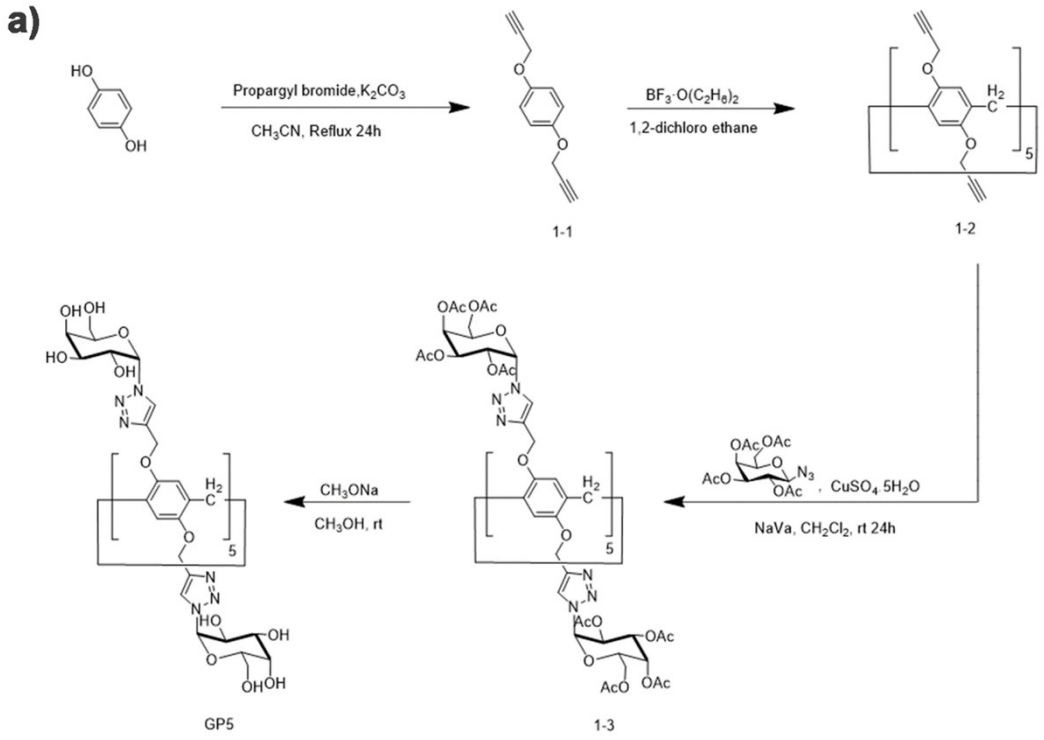
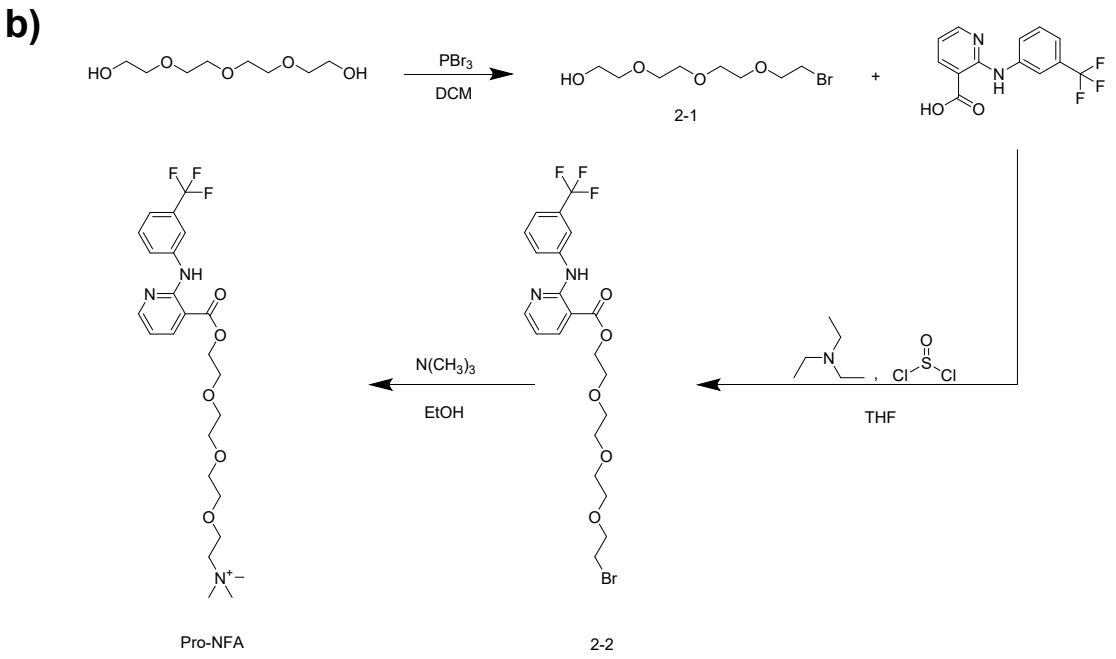
Supporting Information

1. Instrumentation and chemicals

All reagents were purchased from commercial suppliers and used without further purification unless specified. The water used in this work was triple distilled. ^{1}NMR spectra were recorded on a Bruker 500 MHz Spectrometer, with working frequencies of 500 MHz for ^{1}H nuclei (Bruker Corporation, Switzerland). DLS measurements were performed on ZEN3600 NANOPHOX (MALVERN INSTRUMENTS LIMITED, United Kingdom). UV-vis spectra were collected with (Shimadzu UV-2450, Japan) UV-visible spectrophotometer. Scanning electron microscopy (SEM) images were obtained from the Nano SEM-450 instrument (FEI Ltd. U.S.A.). Transmission electron microscopy (TEM) images were obtained from the TECNAI G2 SPIRIT BIO instrument (FEI Ltd. U.S.A.). Cell culture was carried out in an incubator with a humidified atmosphere of 5% CO_2 at 37℃. The confocal laser microscope (CLSM) data were acquired using a Spectroscopic fluorescent lifetime Confocal Laser Scanning Microscopy STELLARIS8 FALCON (Lecia Instrument Co., LTD, Germany). The other fluorescent images were from the DMi8 Inverted fluorescence microscope (Leica MICROSYSTEMS, Germany). Flow cytometry data were obtained from BD FACSAria™ III Flow Cytometer (BD Biosciences, U.S.A.). Intracellular Chloride Assay Kit (Beyotime: BB-481172). Enhanced ATP Assay Kit (Beyotime: S0027).

2. Synthesis and Characterization of the compounds





Scheme S1. Synthetic route of GP5(a) and Pro-NFA(b)

GP5 and synthesized according to the previous reporters.[1]

Compound 2-1: Dissolve tetraethylene glycol (4 g, 20 mmol) in 50 mL of dry dichloromethane, add phosphorus tribromide (1.79 g, 6.6 mmol) dropwise under ice-cooling, react for 2 h, add saturated Sodium bicarbonate aqueous solution, extract the organic phase, wash with water, combine the organic layers, dry over sodium sulfate, and spin dry the organic solution to obtain a brown mixture, separate on a silica gel column (ethyl acetate: petroleum ether=1:1 v/v), and obtain shallow yellow oily liquid 2-1 (1.68 g, 30%),^{1}HNMR (500 MHz, $CDCl_3$) δ (ppm) : 3.73 (t, *J* = 5, 2H), 3.67 (s, 8H), 3.62-3.59 (m, 4H), 3.52 (dd. *J*= 10, 2H).

Compound 2-2: Thionyl chloride 5 mL was slowly added to niflumic acid (1 g, 3.54 mmol) with stirring, and the suspension was stirred for 5 minutes. The mixture was heated to reflux at 60°C for 180 minutes until a clear solution was obtained. Excess thionyl chloride was spin-dried, 10 mL of anhydrous benzene was added to the residue and spin-dried, and the remaining solid was dissolved in 0.5 mL (3.54 mmol) tris add 2-1 (1 g, 3.6 mmol) to the dry tetrahydrofuran solution of ethylamine, keep the temperature at 20°C, stir the mixture for 5 hours, spin dry the organic solvent, add dichloromethane, wash with water, and dry to obtain dark red oily mixture was separated on a silica gel column (petroleum ether: ethyl acetate=1:2 v/v) to obtain light yellow oily compound 2-2 (1.5 g, 78%). ^{1}HNMR(500 MHz, $CDCl_3$) δ (ppm) : 10.31 (s, 1H), 8.38 (d, *J*= 4, 1H), 8.28 (d, *J*= 7.5, 1H), 8.11 (s, 1H), 7.87 (d, J= 8, 1H), 7.42 (t, *J*=8, 1H), 7.26 (d, *J*= 8, 1H), 6.78 (dd, *J*= 4.5, 1H), 4.48 (t, *J*= 4.5, m 1H), 3.84 (d, *J*= 4.5, 2H), 3.78 (t, *J*= 6, 2H), 3.69 (s, 2H), 3.68 (s, 2H), 3.63 (s, 4H), 3.45 (t , *J*=4.5, 2H).^{13}C NMR (126 MHZ, DMSO-d_6) *δ* (ppm) : 167.3, 155.6, 153.0, 140.4, 129.2, 123.4, 118.9, 116.9, 114.0, 107.5, 77.5, 77.2, 71.2. 70.1, 70.69, 70.6, 70.5, 69.0, 64.5. MALDI-TOF-MS m/z calcd for $[M+H]^+$ $C_{21}H_{25}BrF_3N_2O_5$, 520.0821, found 520.0866.

Pro-NFA: Take 2-2 (0.5 g, 1.39 mmol) and add 3 mL of trimethylamine (33% ethanol solution), then add 15 mL of ethanol solution, stir for 12 hours, spin dry the reactant at room temperature, and obtain a dark yellow oily crude product, Add ethyl acetate to dissolve, wash with water, use ethanol to break the emulsification, collect the organic phase, and spin dry the organic solution to obtain a light yellow oily crude product, add a little 1 mL of ethyl acetate to dissolve, add 5 mL of petroleum

ether to the system, The insolubles were collected to obtain a light yellow oily product (0.2 g, 44%). ^{1}H NMR (500 MHz, $CDCl_3$): 10.29 (s, 1H), 8.42 (d, *J* = 5, 1H), 8.31 (d, *J* = 10, 1H), 8.08(s, 1H), 7.89 (d, *J* = 5, 1H), 7.45 (t, *J* = 10, 1H), 7.29 (s, 1H), 6.84 (t, *J* = 10, 1H), 4.51 (s, 2H), 3.95 (s, 2H), 3.93 (s, 2H), 3.84 (s, 2H), 3.68-3.62 (m, 10H), 3.48 (s, 10H).^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 167.0, 155.57, 153.16, 140.53, 140.34, 131.07, 129.41, 123.50, 119.16, 116.93, 114.20, 107.3, 77.36, 77.10, 76.85, 70.67, 70.41, 70.29, 65.65, 64.33, 54.34. Maldi-TOF-MS for $[M+H]^+C_{24}H_{33}F_3N_3O_5^+$, 500.2367, found 500.2339.

3. Preparation and characterization of GP5⊃Pro-NFA vesicles

To the pure water (1 mL) was added GP5 (6 mg) and Pro-NFA (1 mg). Then the mixture was subjected to ultrasonication for 30 min and left to stand still overnight to obtain GP5⊃Pro-NFA vesicles (1.84 mM, 1 mL). Accordingly, DOX (1 mg, 1.84 mM) were added to the mixture, repeating the steps above to obtain DOX-loaded GP5⊃Pro-NFA vesicles (DOX@GP5⊃Pro-NFA). The vesicles above were characterized by SEM, TEM and DLS.

4. DOX loading and release profile of vesicles

The prepared DOX-loaded GP5⊃Pro-NFA vesicles solution was dialysed in distilled water for 24 h, and the amount of unloaded DOX in the dialysate was quantitatively measured by Ultraviolet and visible spectrophotometry (UV-Vis). According to the formula below, the DOX encapsulation efficiency was calculated to be 30%. The loading rate has been calculated to be 12%.

$$\text{Encapsulation efficiency (\%)} = \frac{m_{loaded\ DOX}}{m_{total\ DOX}} \times 100\%$$

$$\text{Loading capability (\%)} = \frac{m_{loaded\ DOX}}{m_{DOX@GP5\supset Pro-NFA}} \times 100\%$$

The prepared DOX-loaded GP5⊃Pro-NFA vesicles solution (1.84 mM, 1 mL) was dialysed in H_2O, and aqueous solution containing esterase (100 U/L), away from light at r.t. At specified time intervals, the concentration of released DOX was determined by UV-vis.

5. Cell Culture

HL7702 cells (normal human liver), HepG2 cells (DOX-sensitive), HepG2-ADR cells (DOX-resistant), Hela cells and Pig ST cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an atmosphere with 5% CO_2 at 37℃.

6. Cell Uptake observed by Confocal laser scanning microscopy (CLSM)

For the details of the uptake characterization experiment, HepG2-ADR cells were cultured in two 35 mm confocal dishes at a density of 2×10^5 cells/well and co-cultured with 10 μM DOX@GP5⊃Pro-NFA. After 24 h, discarded the old medium and washed with PBS and cells were immobilized with 4% paraformaldehyde for 10-15 min. Finally, the cells were washed with PBS twice and the fluorescence images were captured by a confocal microscope with different channels under a Laser Scanning Confocal Microscope (Andor REVOLUTION WD).

7. Target Ability Assay

Cells were cultured with a density of 8000 cells/well in 96-well plates. HL7702 cells were used as control, and HepG2-ADR cells were untreated or pre-treated with lactose (2 mg/mL) for 4 h. After 4 h, all of the cells were co-cultured with DOX@GP5⊃Pro-NFA for another 4 h. DOX fluorescence intensity was detected using flow cytometry. Cells (5000 well) including HL7702 HepG2 (DOX-sensitive) Hela and Pig ST were inoculated in 96-well plates and co-cultured with the cells using a supramolecular drug delivery system loaded with the anti-cancer drug DOX(4μM) for 4 hours, the culture medium was discarded, washed with PBS buffer, excited under a fluorescent inverted microscope and photographed.

8. Cytotoxicity Evaluation

The relative cytotoxicities of different corresponding compounds (DOX, GP5⊃Pro-NFA and DOX@GP5⊃Pro-NFA) were evaluated *in vitro* by MTT assay. The cells (HL7702 cells normal human liver, HepG2 cells DOX-sensitive, HepG2-ADR cells DOX-resistant, Hela cells and Pig ST cells) were cultured with complete DMEM for 24 h with a density of 8000 cells/well in 96-well plates (100 μL medium/well). The compounds above in different concentrations were added and co-cultured for 24 h and 48 h, cells were washed and the fresh medium containing MTT (0.5 mg/mL) was added into each plate. The cells were incubated for another 4 h. After that, the medium containing MTT was removed and dimethyl sulfoxide (100 μL) was added to each well to dissolve the formazan crystals. Finally, the plate was gently shaken for 10 min and the absorbance at 490 nm was recorded with a microplate reader.

9. Cell Scratch Test

Cell scratch test was used to evaluate cell migration capacity. HepG2 cells were seeded in a 6-well plate with a density of 5×10^5 cells/well. When the cells had reached 90% fusion, lines were made vertically in the cell monolayer with 200 μL tip, the cells were washed with PBS for 3 times. Then, the experimental group was treated with GP5⊃Pro-NFA (10 μM) the control group was cultured in serum-free medium. The relative distance of cell migration was observed and photographed at 0 and 24 h with the Inverted Fluorescence Microscope.

10. Cellular drug efflux assay

Tumour resistant cells HepG2/ADR cells were inoculated on 96-well plates using DMEM medium, maintained at a DOX concentration of 2μM using free DOX, GP5@DOX and supramolecular drug loading system DOX@GP5⊃Pro-NFA (GP5 content as in the previous group), incubated with the cells for 4 hours, changed to drug-free medium and continued to incubate for 2h, washed with PBS and observed under a fluorescent inverted microscope and photographed.

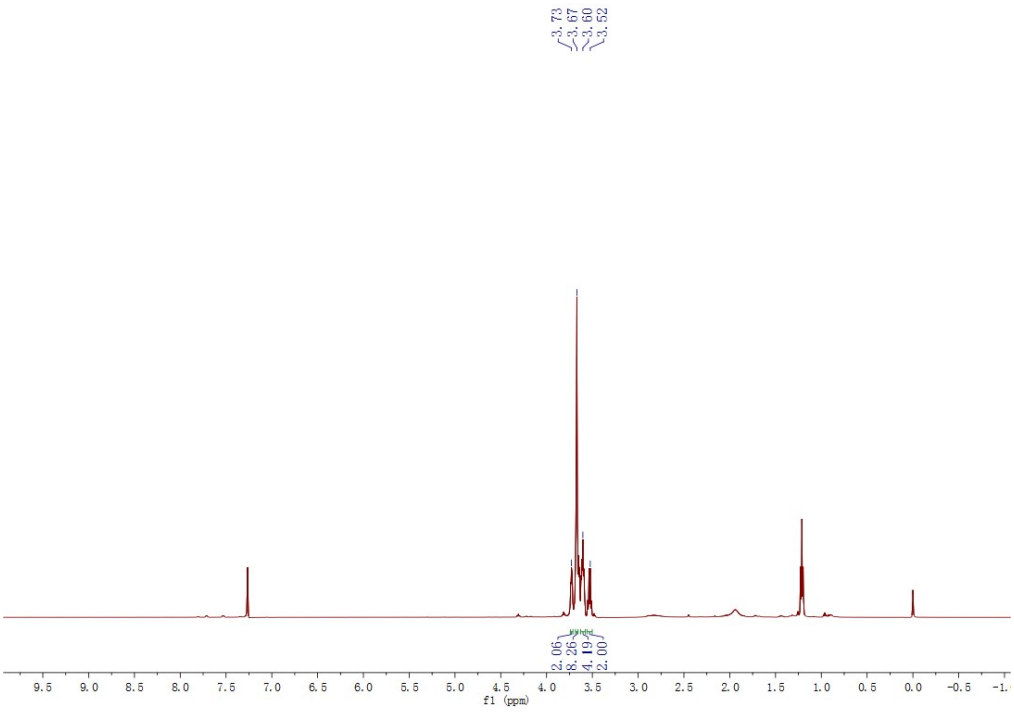


Fig S1: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of Compound 2-1.

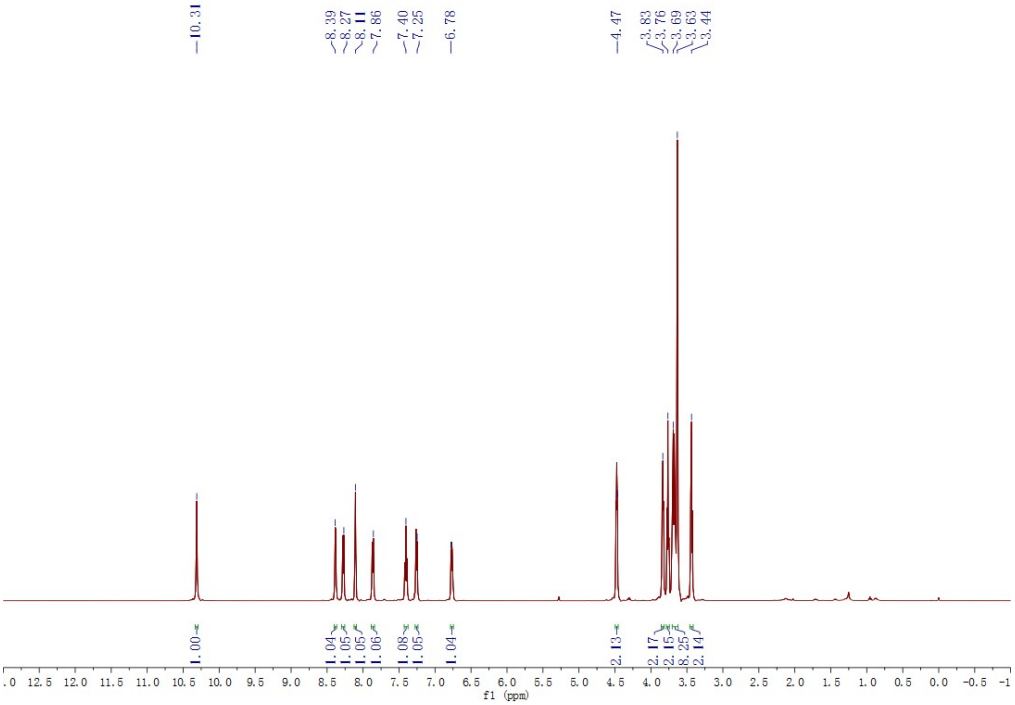


Fig S2: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of Compound 2-2

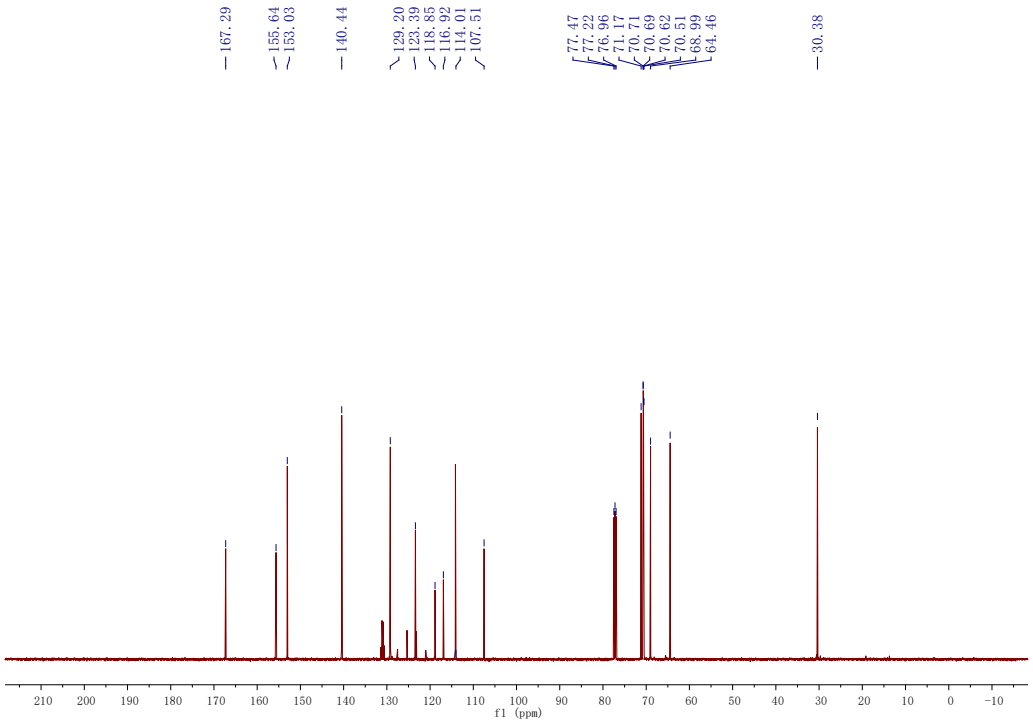


Fig S3: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of Compound 2-2

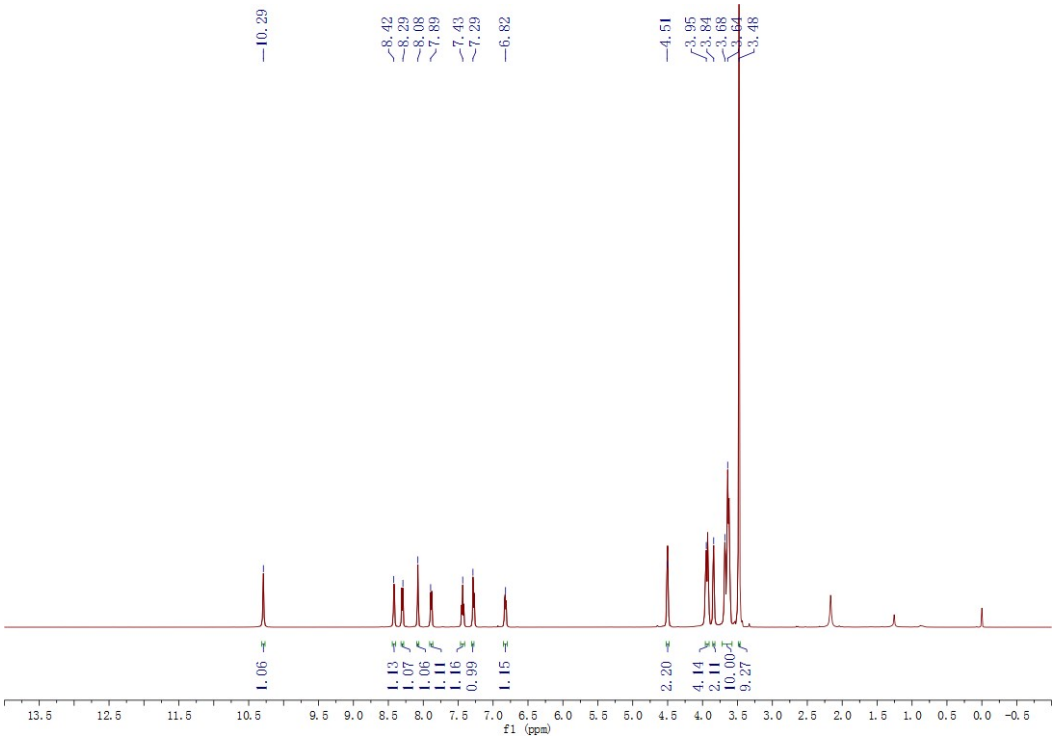


Fig S4: ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of Pro-NFA

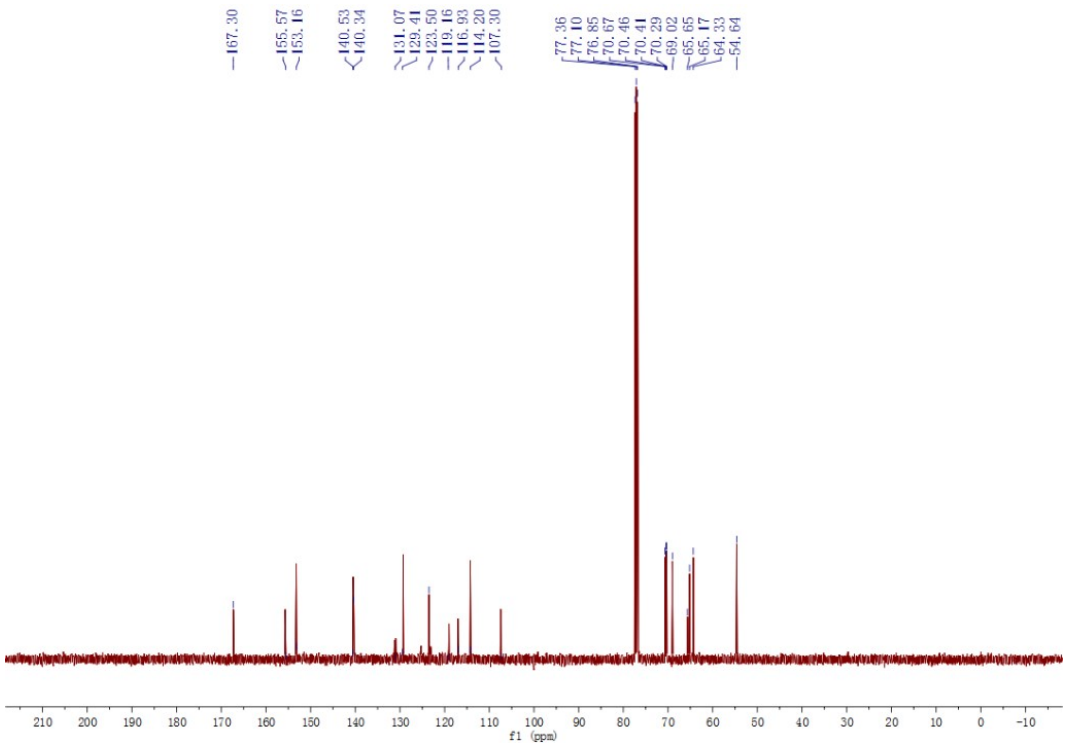


Fig S5: ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of Pro-NFA

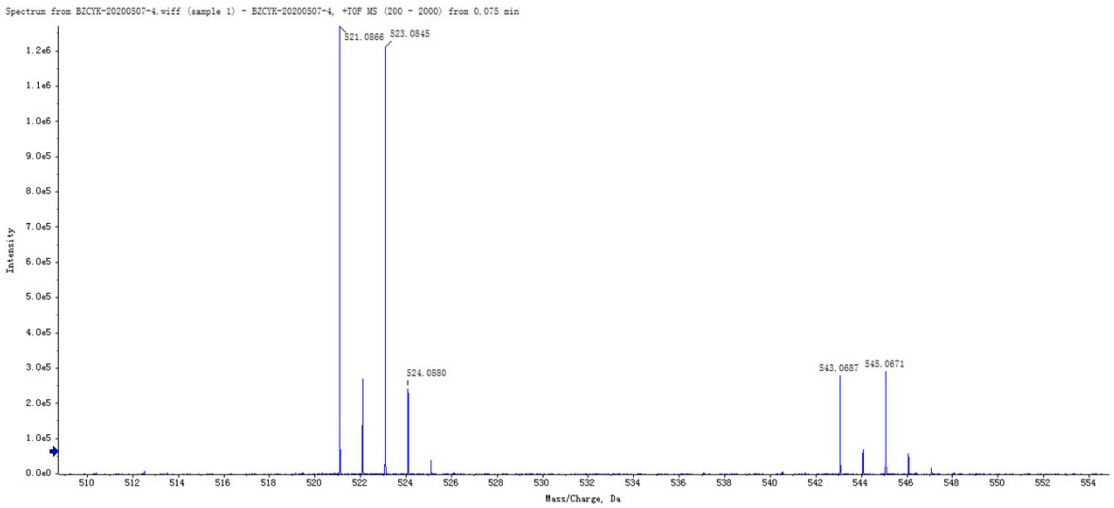


Fig S6: HRMS of Compound 2-2.

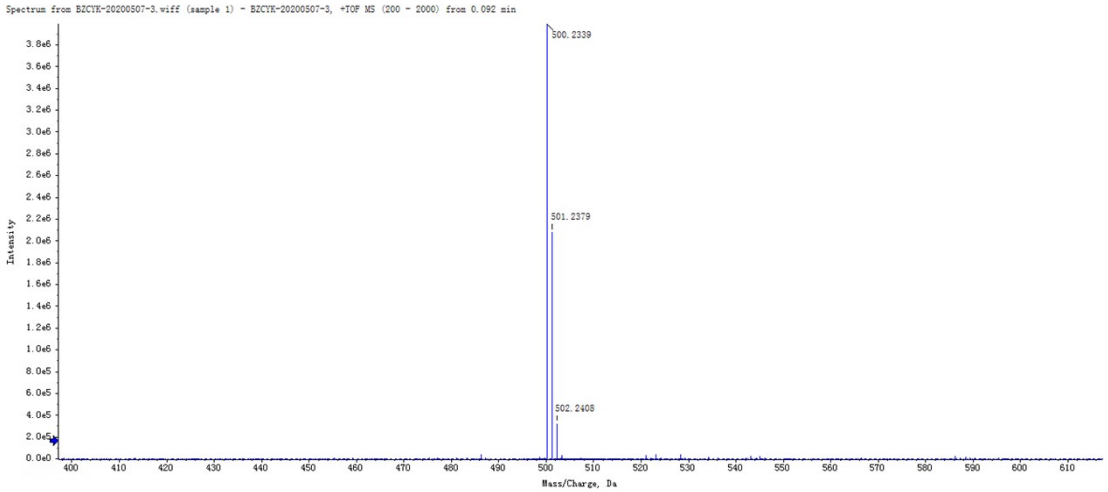


Fig S7: HRMS of Pro-NFA

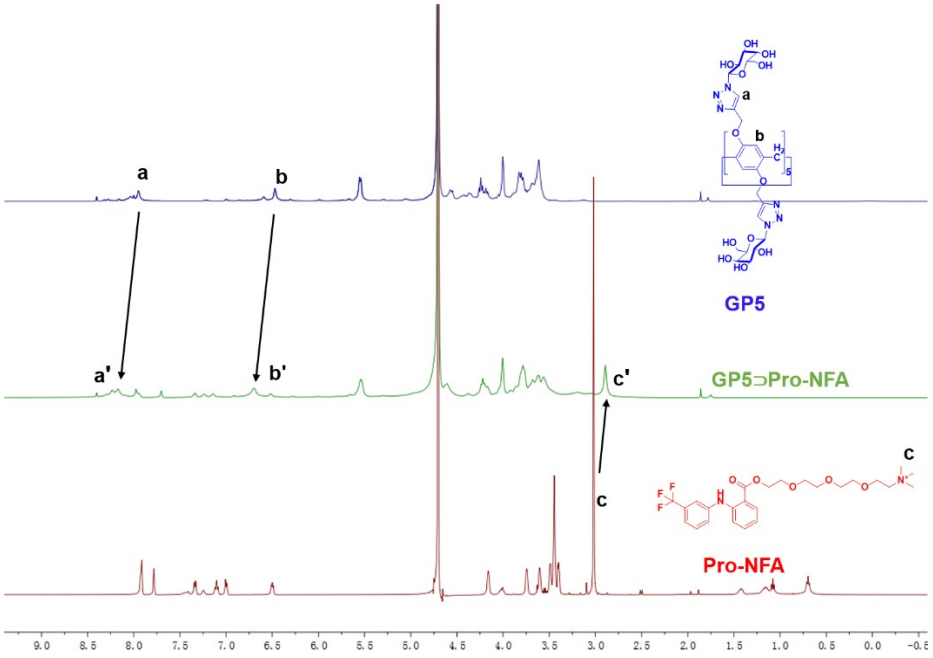


Fig S8. ^{1}H NMR spectra (500 MHz, D_2O): (top) GP5 (1.84 mM); (middle) GP5 : Pro-NFA = 1:1; (bottom) Pro-NFA (1.84 mM).

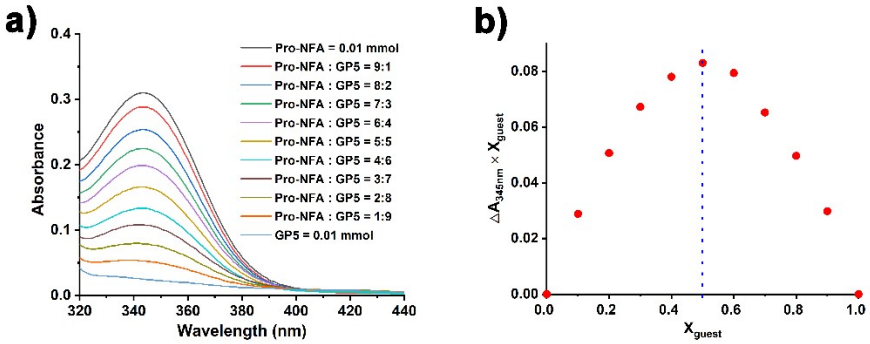


Fig S9. UV-Vis absorption of the mixture of GP5 and Pro-NFA in water at different molar ratios while GP5 + Pro-NFA = 1.0×10^{-4} M. Binding stoichiometry of GP5 and Pro-NFA.

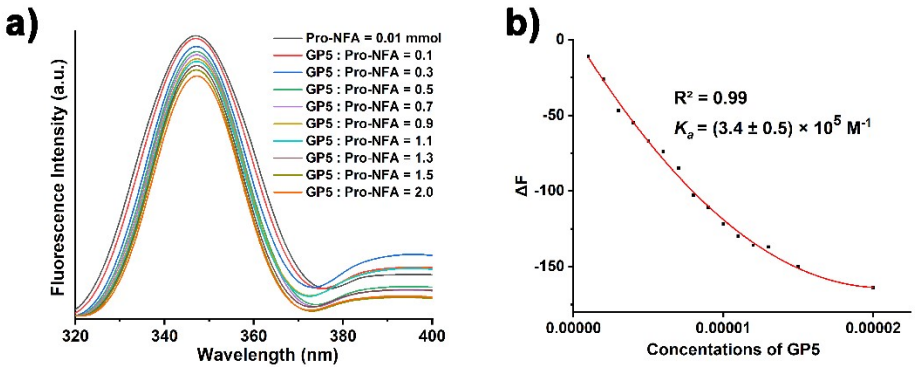


Fig. S10 Determination of the association constants between GP5 and Pro-NFA: UV−vis launch changes of GP5 with varied concentrations of Pro-NFA (a); Dependence of the UV−vis launch at 340 nm on GP5 with varied concentrations of Pro-NFA (b). The non-linear curve-fittings were based on the equation:

$\Delta F = (\Delta F_\infty/[H]_0)\ (0.5[G]_0 + 0.5([H]_0 + 1/K_a) - (0.5\ ([G]_0^2 + (2[G]_0(1/K_a - [H]_0)) + (1/K_a + [H]_0)^2)^{0.5}))$; Where ΔF is the UV-vis launch changes at 340 nm at $[H]_0$, ΔF_∞ is the UV-vis launch changes at 340 nm when Pro-NFA is completely complex, $[G]_0$ is the initial concentration of Pro-NFA, and $[H]_0$ is the fixed initial concentration of GP5

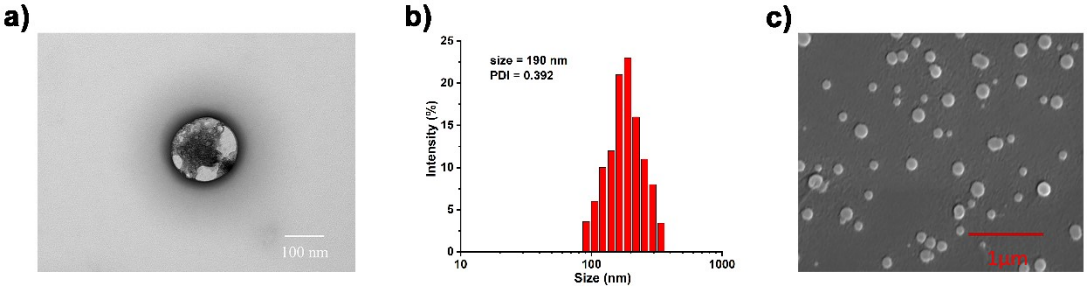


Fig S11 (a) TEM image of GP5⊃Pro-NFA (b) DLS of DOX@GP5⊃Pro-NFA; (c) SEM image of DOX@GP5⊃Pro-NFA.

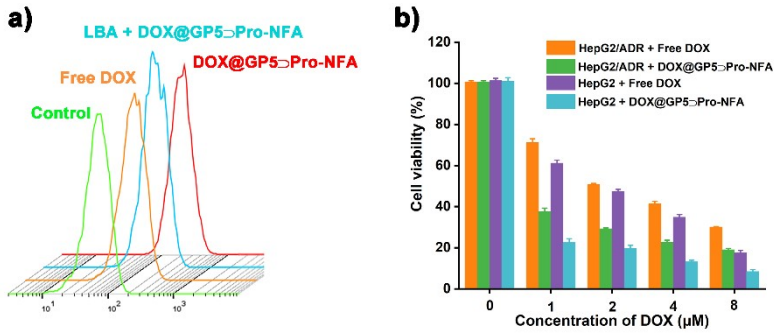


Fig S12 (a) Flow cytometry analyses of HepG2/ADR cells. (b) Cell viability of HepG2/ADR and HepG2 cells incubated with DOX@GP5⊃Pro-NFA and free DOX for 48 h.

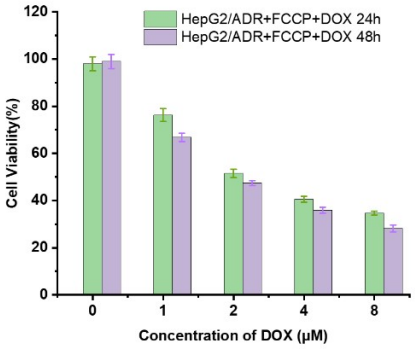


Fig S13 Cell viability of HepG2/ADR cells incubated with DOX and FCCP for 24 h and 48h.

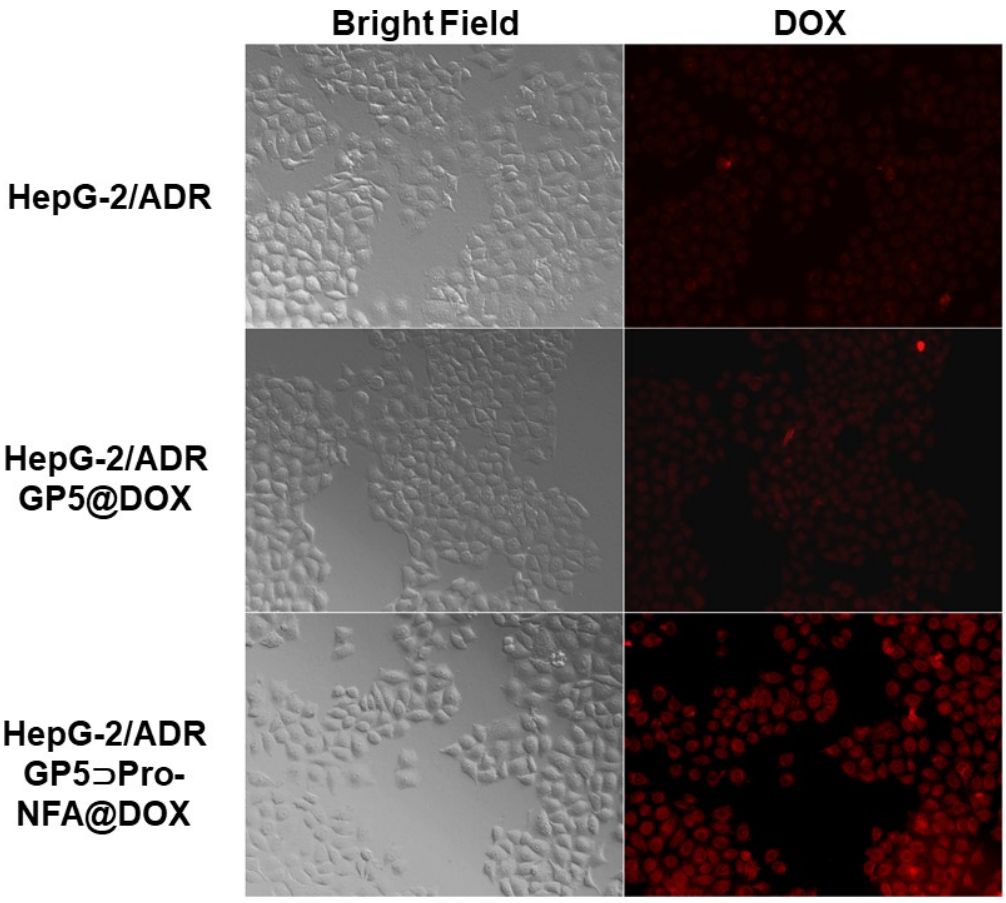


Fig S14 Fluorescent inverted micrograph of HepG-2/ADR cells, taken after HepG-2/ADR cells were co-cultured with free DOX and DOX+NFA for 4 hours respectively, and incubated for 2 hours using drug-free medium, (Scar bars 20 μm).

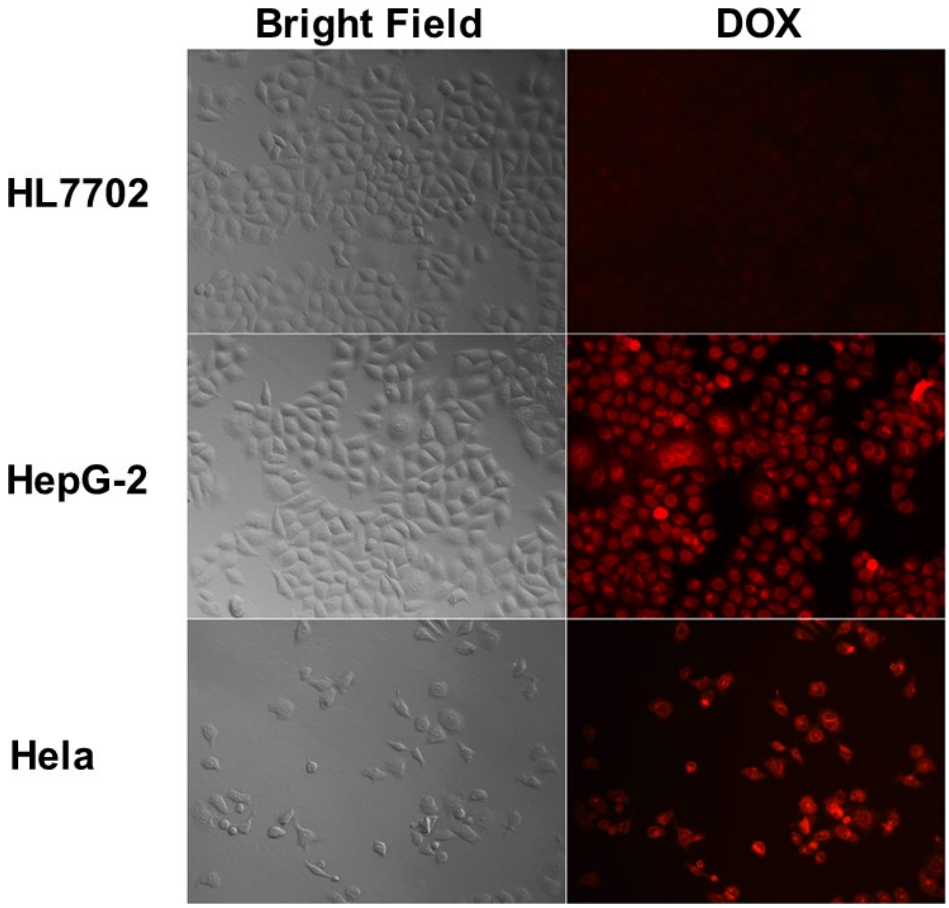


Fig S15 Fluorescent inverted micrographs of Hl7702 and HepG-2 cells after co-culture with DOX@ GP5⊃Pro-NFA(DOX concentration of 4μM) for 4 hours, (Scar bars 20 μm).

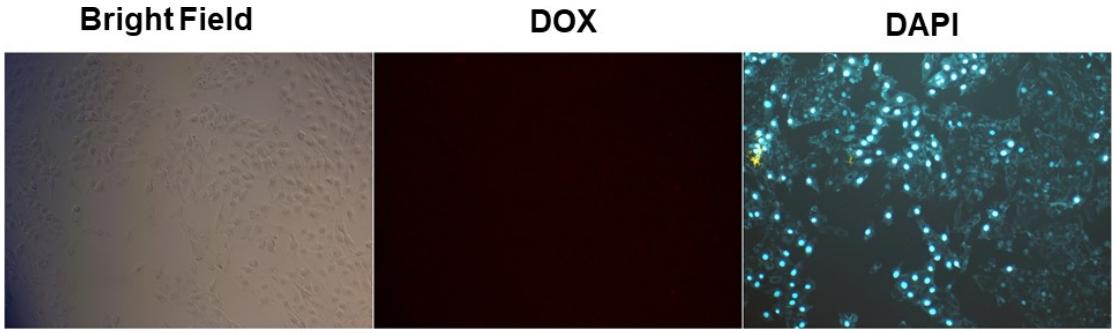


Fig S16 Fluorescent inverted micrographs of ST cells after co-culture with DOX@ GP5⊃Pro-NFA(DOX concentration of 4μM) for 4 hours, (Scar bars 20 μm).

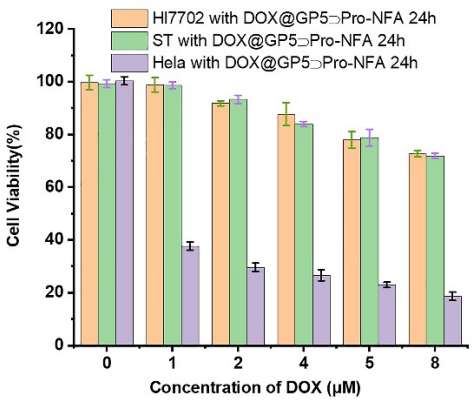


Fig S17 Toxicity of drug-loaded vesicles loaded with the anticancer drug DOX to Hl7702 and ST cells and Hela at 24 hours

10.References

1. X. Wu, Y. Zhang, Y. Lu, S. Pang, K. Yang, Z. Tian, Y. Pei, Y. Qu, F. Wang and Z. Pei, *J Mater Chem B*, **2017**, **5**, 3483-3487.