Supplementary Information

Rational Design of Glycopeptide Probe System based on Reconfigurable Immune Microenvironment

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Contents

Materials

Tentagel resin was obtained from Rapp Polymere (Germany, loading 0.35 mmol/g). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Wang resin, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) were purchased from GL Biochem (China). Trifluoroacetic acid (TFA) and Phenol were purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). DSPE-PEG_{2000}-MAL (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000]) was purchased from Yarebio (China). Doxorubicin (DOX) was purchased from Meryer Chemical Technology (China). Fluorescein isothiocyanate (FITC), Hoechst 33342 and streptavidin-coated magnetic beads (1 μm), were brought from Sigma-Aldrich (USA). N-methyl morpholine (NMM), piperidine, Tips (Triisopropyl silane) and N, N'-dimethylformamide (DMF) were all obtained from Beijing Chemical Plant (China). SPRi gold chips (Nanocapture, 5 mm×75 mm in size, 47.5 nm-thickness of the gold coating) were purchase from Plexera. CD133 protein was obtained from Sino Biological (Beijing, China). Biotin labeling kit was purchased from SoluLink. 1,3-diphenylisobenzofuran (DPBF) was purchased from Beijing Chemical Plant (China). Calcein-AM/PI Kit and CCK-8 Kit were purchased from Solarbio (China). DMEM (Dulbecco's modified Eagle's medium)/high glucose medium and trypsin were purchased from GE Healthcare Life Sciences. Human glioma cell line (U87) and human embryonic kidney cell line (293T) were purchased from Cell Resource Center, Chinese Academy of Medical Sciences (China). All cell lines were supplemented with 10% fetal bovine serum (FBS)(Gibco), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Gibco). All reagents were used as received, and solvents were purified according to general procedures before use.

Method

1. Synthesis of the OBOC peptide library

The OBOC library was synthesized by employing the Fmoc SPPS (solid-phase peptide synthesis) strategy. TentaGel resin (loading 0.35 mmol/g) was used as the solid phase support. The whole synthesis process was carried out in anhydrous DMF. During the coupling steps, TentaGel resin was mixed with HBTU (4 mmol), Fmoc-amino acid (4 mmol), and 4 mL of 0.4 M NMM in DMF solution. The coupling time was 40 min. In the deprotection steps, 20 *v/v*% piperidine (dissolved in DMF) was used to remove the Fmoc group and the deprotection time was 10 min. In the coupling step, solid support beads were split equally, and each portion was then coupled with a different amino acid. The amino acid coupling process was carried out in the "split" step, while the deprotection process was carried out in the "pool" step. Upon completion of the synthesis, a cleavage reagent (95 *v/v*% TFA, 2.5 *v/v*% H_2O, and 2.5 *v/v*% Tips) was introduced to cleave side chain protecting groups on each residue. The cleavage time was 2 h. All the experiments mentioned above were carried out in solid-phase peptide synthesis vessels with sieves. Finally, a cyanogen bromide (CNBr) solution (30 mg/mL) was reacted with the peptide resin overnight to release the synthesized peptides.

2. Solid phase peptide synthesis

The designed peptides were synthesized using the standard solid-phase Fmoc peptide synthesis strategy. The peptide sequences were prolonged from C-termini to N-termini. Wang resin was used as the solid phase support. 20% v/v piperidine (dissolved in anhydrous DMF) was used to remove the Fmoc group of amino acids. The carboxy group of Fmoc protected amino acid was activated by NMM (0.4 mol/L in anhydrous DMF) and HBTU (equal moles as amino acids). The deprotection or coupling efficiency is measured by using Kaiser reagents ((5% ninhydrin ethanol solution (m/v), 80% phenol ethanol solution (m/v), 2% vitamin C/pyridine solution (m/v) 1:1:1 v/v/v). Finally, the peptide is cleaved from the resin in the acid solution (95% v/v TFA, 2.5%

v/v H_20, and 2.5% v/v TIS). Acid solution was removed by vacuum rotary evaporation, and the obtained crude product was precipitated in cold ether. The crude peptides were purified by high performance liquid chromatography (HPLC) and characterized by MALDI-TOF (Matrix assisted laser desorption ionization and time of flight) mass spectra (Bruker Daltonics, Germany)

3. SPRi analysis of the binding between SC and CD133

SPRi analysis was performed with a Plexera PlexArray HT system (Plexera LLC, Bothell, WA) using a bare gold SPRi chip (a Nanocapture gold chip with a 47.5-nm-thick gold coating). All the purified peptides were printed onto the surface of the gold chip surface through thiol groups on Cysteine residues. The chip was then incubated overnight in a humid box at 4 °C. The SPRi chip was washed and blocked using 5% (*w/v*) skim milk in PBS overnight before use. The SPRi analysis procedure was carried out with injection cycles. And in each cycle, PBST (phosphate buffered saline with 0.1% Tween-20) buffer (baseline stabilization), protein samples (CD133, six different concentrations, binding), PBST buffer (washing), and 0.1 mol/L H_3PO_4 in deionized water (regeneration) were injected in sequence. Protein was diluted with PBST to concentrations of 152 nM, 76 nM, 38 nM and 19 nM. Real-time binding signals were recorded and analyzed by the PlexArray HT software.

4. Confocal laser scanning microscope (CLSM) imaging

For CLSM, about 1×10^5 mL^{-1} of U87/293T cells were seeded into culture dishes and cultured overnight for cell adherence. The cells were added with 200 μL 0.5 mg/mL FITC-labeled polypeptide SR (F-SR) and incubated at 4℃ for 15 min. The F-SR solution was discarded and washed with 1 × PBS for 3 times. Add 100 μL 5×10^{-3}mg/mL Hoechst 33342 and incubate at 4℃ for 15 min. Discard and wash with 1 × PBS for 3 times. The cells were imaged using a laser confocal microscope with a 40-power lens. These cells were incubated with 200 μL of FITC-labeled peptide solution and 1 mM of hoechst 33342 in dark at 4 °C for 30 min. Finally, these cells were washed three times with cold PBS and inspected with a Olympus FV1000-IX81confocal

microscope （Japan）. A 488 nm laser was used as the excitation source for FITC throughout the experiment and a 525 nm laser was used as the emission source. Hoechst 33342 emitted at 461 nm upon excitation at 405 nm.

5. Synthesis and identification of glycopeptides

Glucose (Glu) peptide Glu-SC molecule and Mannose (Man) peptide Man-SC molecule were designed and synthesized by Shanghai Chupeptide Biotechnology Company. Finally, MALDI-TOF-MS was used to identify their molecular weights. Peptide solution and α-Cyano-4-hydroxycinnamic acid (HCCA) supersaturated solution were mixed in a ratio of 1:1, 1 μL was spotted on the mass spectrometry target plate, dried and tested.

6. Preparation and Characterization of Glycopeptide-functionalized Nanomic-elless

1.Preparation of glycopeptide functionalized amphiphilic molecules: functional amphiphiles DSPE-PEG_{2000}-SC, DSPE-PEG_{2000}-SC-Glu and DSPE-PEG_{2000}-SC-Man were synthesized by coupling phosphatidylethanolamine-polyethylene glycol 2000-maleimide (DSPE-PEG_{2000}-MAL) with SC, Glu-SC and Man-SC, respectively, via Michael addition reaction.SC/Glu-SC/Man-SC and DSPE-PEG_{2000}-MAL at a molar ratio of 1:2 were weighed and dissolved in 500 μL of H_2O (DSPE-PEG_{2000}-MAL was first solubilized with 10 μL of acetonitrile), the two were mixed and reacted on a rotary mixer at room temperature for 48 h, the final product was purified using a dialysis bag with a molecular weight cut-off of 3500 Da, and the molecular weight was characterized by MALDI-TOF-MS.

2.Preparation of nanomicelles: nanomicelles SR-M^{DOX}, G-M^{DOX} and M-M^{DOX} are prepared by mixing DSPE-PEG_{2000}SR/DSPE-PEG_{2000}-SR-Glu/DSPE-PEG_{2000}-SR-Man, DSPE-PEG_{2000}-MAL and doxorubicin (DOX) in a mass ratio of 2:8:1 in dichloromethane/methanol (4:1, v/v). The disaccharopeptide functionalized nanomicelles GM-M^{DOX} is prepared by mixing DSPE-PEG_{2000}-SR-Glu, DSPE-

PEG2000-SR-Man, DSPE-PEG_{2000}-MAL and DOX in a mass ratio of 1:1:8:1 in dichloromethane/methanol (4:1, v/v).The mixture was placed in a round-bottomed flask and evaporated on a rotary evaporator to form a uniform film on the bottom of the flask, then the remaining organic solvent in the bottom of the flask was blown to dryness with nitrogen, 5 mL of 1 × PBS was added and hydrated in a water bath at 60℃ for 25 min. Then sonicate with an ultrasonic disperser at 300 W for 15 min to obtain a clear suspension. The obtained suspension was filtered with a filter membrane (0.44 μm sterile filter membrane for 2 times and 0.22 μm sterile filter membrane for 6 times) to finally obtain homogeneous nanomicelles.

3.Encapsulation efficiency test: prepare DOX standard solutions with different concentrations, and use a fluorescence spectrometer (excitation wavelength:488 nm), and the fluorescence intensity-concentration standard curve of DOX was drawn. Take 1 mL of the prepared DOX-loaded nanomicelles and place it in a dialysis bag, dialyze it for 24 h with 1 × PBS as dialysate, take the dialysate for fluorescence test, substitute it into the above standard curve, calculate the DOX content and calculate the entrapment efficiency by the following formula:

Encapsulation efficiency EE (%) = micelle-loaded drug mass/total administered mass × 100%

7. Study on in vitro release of glycopeptide-functionalized nanomicelles

The nanomicelles (0.1 mg/mL, 1 mL) were placed in a dialysis bag and dialyzed against 1 × PBS solution containing 0.5% Tween-80 at 37℃ on a horizontal shaker. At different time points (1 h, 3 h, 5 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h), 0.2 mL dialysate was taken into EP tube, and the fluorescence intensity of DOX was measured by fluorescence spectrometer (Hitachi, Japan). According to the fluorescence intensity-concentration standard curve of DOX, the DOX amount released by the nanomicelles were calculated.

8. Atomic force microscopy (AFM) imaging:

AFM images were obtained in air conditions using scanning probe microscope (Bruker,

dimension fast-scan). For morphology characterization, the sample was dropped onto the mica sheet surface for 10 min, then the excess solution was washed off with water and blown dry with nitrogen. Scanning mode: ScanAsyst; probe type: ScanAsyst-AIR; resolution: 512×512; scanning speed; 1.0 Hz.

9. Cell culture

Human glioma cell line U87 were employed as CD133 positive cells. Human kidney epithelial cell line (293T) was used as CD133 negative cells. U87 cells were cultured in DMEM medium, while 293T cells were cultured in DMEM medium. All cells were incubated under humidified atmosphere containing 5% CO_2 at 37 ℃.

10.Cellular level imaging of polypeptide probes

1. Human glioma cell line U87 (with high CD133 expression) was used as the experimental cell model, and human embryonic kidney cell line 293T (with low CD133 expression) was used as the control. Cell culture and inoculation were carried out according to Method 9.

2. Cell incubation and imaging: 200 μL 0.5 mg/mL FITC-labeled polypeptide SC (F-SC) was added to the two kinds of cells, and incubated at 4℃ for 15 min. The F-SC solution was discarded and washed with 1 × PBS for 3 times. Add 100 μL 5×10^{-3} mg/mL Hoechst 33342 and incubate at 4℃ for 15 min. Discard and wash with 1 × PBS for 3 times. The cells were imaged using a laser confocal microscope with a 40-power lens. The excitation wavelength of Hoechst 33342 is 405 nm, and that of FITC is 488 nm.

11. Transmission electron microscopy (TEM) imaging

10 μL peptide solution (500 μM) was dropped onto the TEM copper mesh coated with a carbon support film for 6 min and then the excess solution was removed by filter paper. 2% uranyl acetate was used for the negative staining for 8 min. Finally, the samples were imaged on a JEM-2100 (JEOL Ltd., Japan) electron microscope with an

accelerating voltage of 120 kV.

12. Transport study in the constructed in vitro BBB monolayer cell model

1.Establishment of BBB model in vitro: seeding of bEnd.3 cells in nested 24-well plates (6.5 mm in diameter, 3.0 μm in pore size, purchased from Corning, United States), with a density of 10^5 cells per well, were cultured in DMEM medium containing 10% FBS, 1% penicillin and streptomycin in a 37℃ thermostatic incubator (5% CO_2). 200 μL and 500 μL of medium were added to the upper and lower chambers, respectively, and the medium was changed every day for 9 days until a dense monolayer was formed.

2.BBB compactness test: Before the experiment, the compactness of BBB model was verified by fluorescein sodium (FLS) permeation test. Firstly, FLS standard solutions with different concentrations were prepared, the fluorescence intensity was measured by fluorescence spectrometer (excitation wavelength was 480 nm), and the FLS fluorescence intensity-concentration standard curve was drawn. Then, prepare 20 μg/mL FLS in 1 × PBS, aspirate the culture medium in the upper chamber and well plate, add 200 μL of 20 μg/mL FLS solution in the upper chamber and 500 μL of 1 × PBS in the lower chamber. At various time points, 10 μL of the lower chamber solution was diluted to 200 μL with 1 × PBS and immediately replenished to the lower chamber with an equal volume of fresh 1 × PBS. The fluorescence values of the solutions taken were measured using a fluorescence spectrometer and the FLS content was calculated from the fluorescence intensity-concentration standard curve of FLS. The apparent permeability coefficient of FLS was calculated using the following formula:

$$P_{app} = dQ / (C_0 \times A \times dT)$$

where dQ is the amount of FLS passing through the BBB into the lower chamber, C_0 is the initial concentration of FLS in the upper chamber (μg/mL), A is the surface area of the chamber (cm^2), and dT is the incubation time (s).

3.BBB Penetration Test: The nanomicelles (0.1 mg/mL, 1 mL) were placed in a dialysis bag and dialyzed against a 1 × PBS solution containing 0.5% Tween-80 at 37℃ on a horizontal shaker. At different time points (1 h, 3 h, 5 h, 12 h, 24 h, 36 h, 48 h, 60 h and

72 h), 0.2 mL dialysate was taken into EP tube, and the fluorescence intensity of DOX was measured by fluorescence spectrometer. According to the fluorescence intensity-concentration standard curve of DOX, the DOX amount released by the nano-micelle is calculated. Therefore, the calculation formula of Transport efficiency (TE) of each sample in BBB model is:

BBB penetration rate TE (%) = (The fluorescence intensity at each time point from the basolateral side medium containing different samples)/(The initial fluorescence intensity at apical side)× 100%.

13. Classification of Macrophages by Flow Cytometry

1.Raw264.7 cells (M0 type macrophages) were cultured in DMEM medium containing 10% FBS, 1% penicillin and streptavidin in a 37℃ incubator (5% CO_2). M1 type macrophages were obtained by adding 1 μg/mL lipopolysaccharide (LPS) to M0 type macrophages and culturing for 48 h. 50 ng/mL interleukin-4 (IL-4) was added to the other M0 type macrophages, and M2 type macrophages were obtained by culturing for 24 h.

2.SR-M, G-M, M-M and GM-M nanomicelles (0.5 mg/mL, without DOX) were added to different groups of M2 cells, respectively, and incubated overnight. After discarding, the cells were washed 3 times with 1 × PBS. The above micelle-treated M2 cells and untreated M0, M1 and M2 cells were incubated with 2.5 μg/mL PE dye-labeled CD86 antibody (PE-CD86, purchased from Beijing Solaibao) and 2.5 μg/mL FITC dye-labeled CD206 antibody (FITC-CD206, purchased from Beijing Solaibao) for 10 min, discard the dye, wash the cells with 1 × PBS for 3 times, digest and centrifuge, discard the supernatant, blow the cells with 1% paraformaldehyde solution, and allow to stand at 37℃ for 10 min to fix the cells. The fixed cells were centrifuged and the supernatant was discarded. The cells were blown away with 1 × PBS to prepare cell suspension. The fluorescence intensity of FITC and PE was detected at 488 nm and 561 nm excitation wavelengths, respectively. Finally, the experimental results were analyzed by Flowjo software.

14. Enzyme-linked immunosorbent assay

The SR-M, G-M, M-M, GM-M nanomicelles were added into different groups of M2 cells (0.5 mg/mL) for 4 h. After discarding, M2 cells treated with the above micelles and untreated M0, M1 and M2 cells were added with DMEM medium without FBS, penicillin and streptomycin for culture for 24 h. The supernatant of cells was collected and analyzed by enzyme-linked immunosorbent assay.IL-10 and IL-12 were measured by Enzyme linked immunosorbent assay (ELISA).

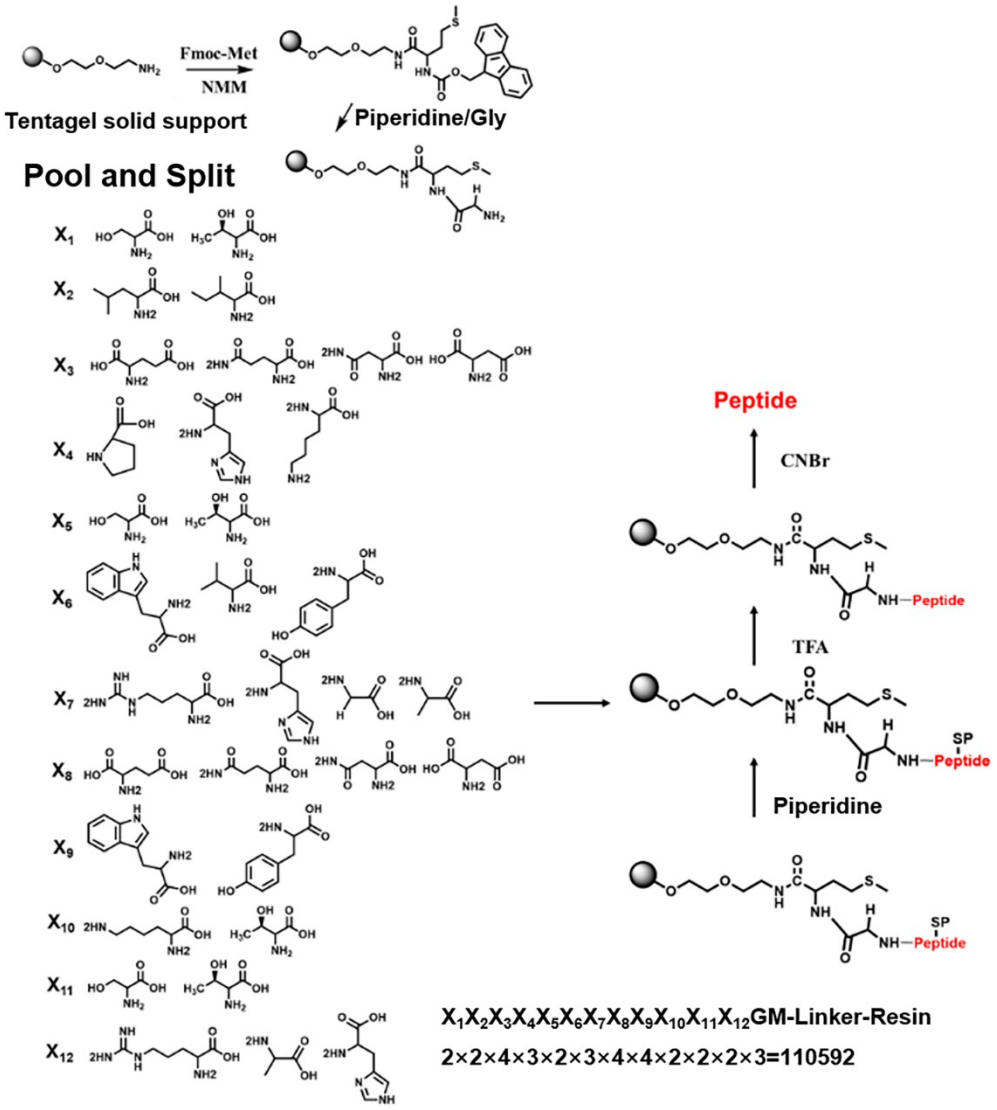


Figure S1. The schematic of the synthesis process of 'OBOC' peptide library towards CD133 through combinatorial chemistry strategy.

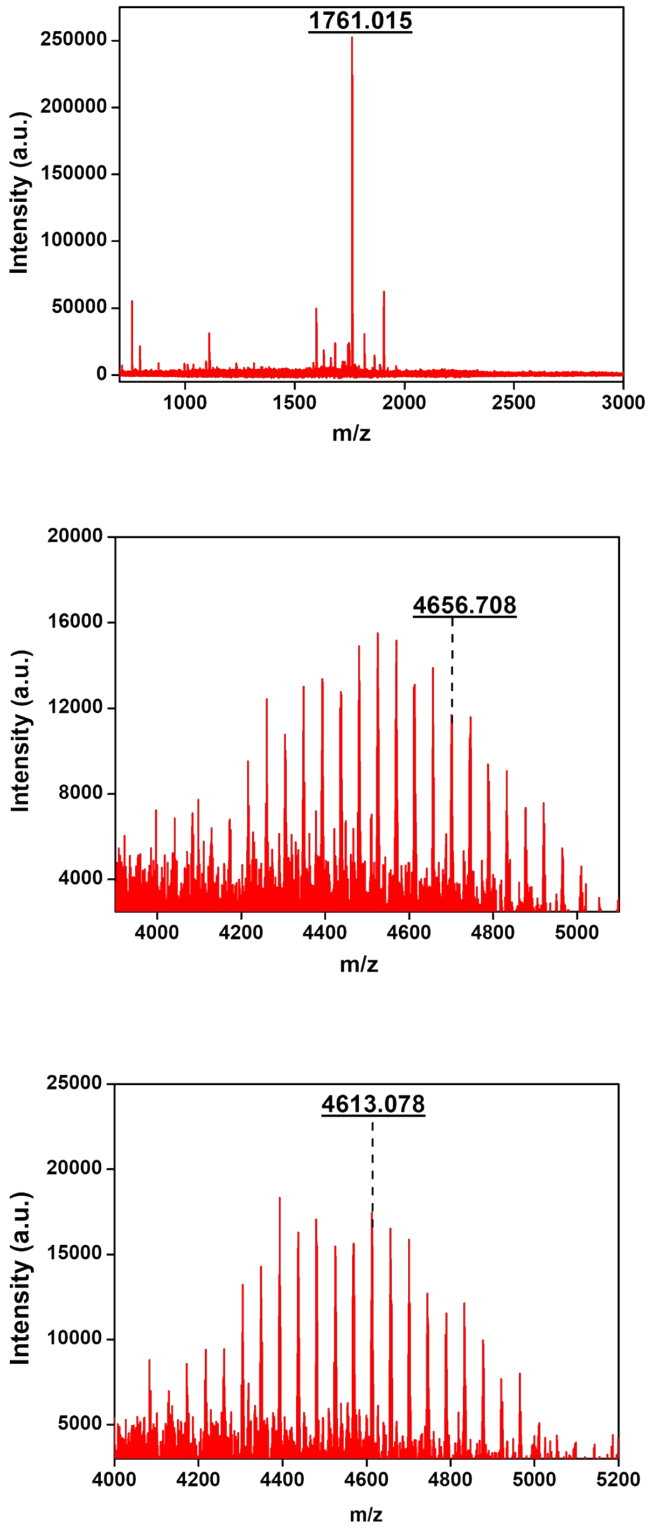
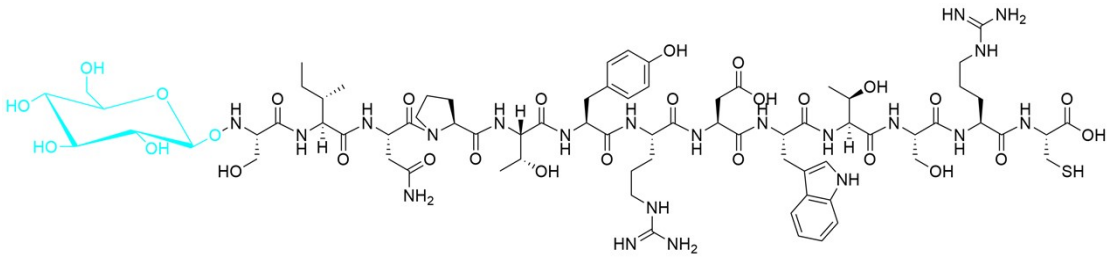
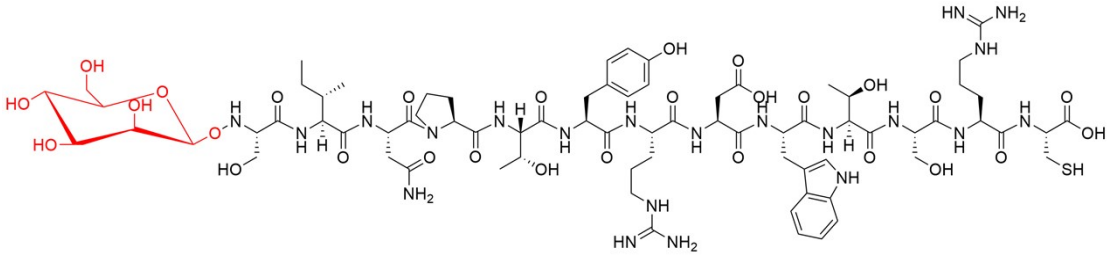


Figure S2. Mass spectrum of Glu-SR, Glu-SR-DSPE-PEG_{2000} and Man-SR-DSPE-PEG_{2000}, respectively.

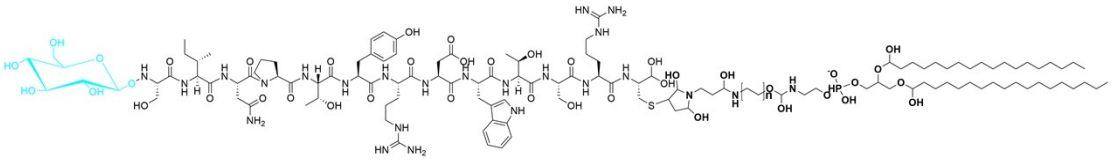
Glu-SR



Man-SR



Glu-SR-DSPE-PEG$_{2000}$-MAL



Man-SR-DSPE-PEG$_{2000}$-MAL

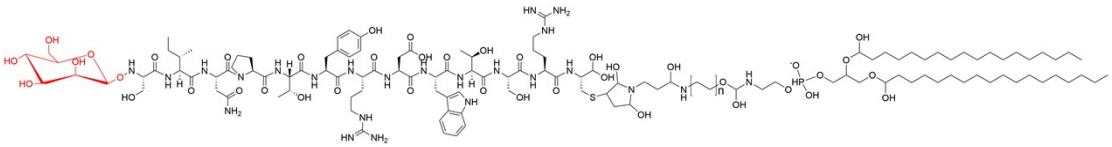


Figure S3. Molecular structure of Glu-SR, Man-SR, Glu-SR-DSPE-PEG$_{2000}$ and Man-SR-DSPE-PEG$_{2000}$.

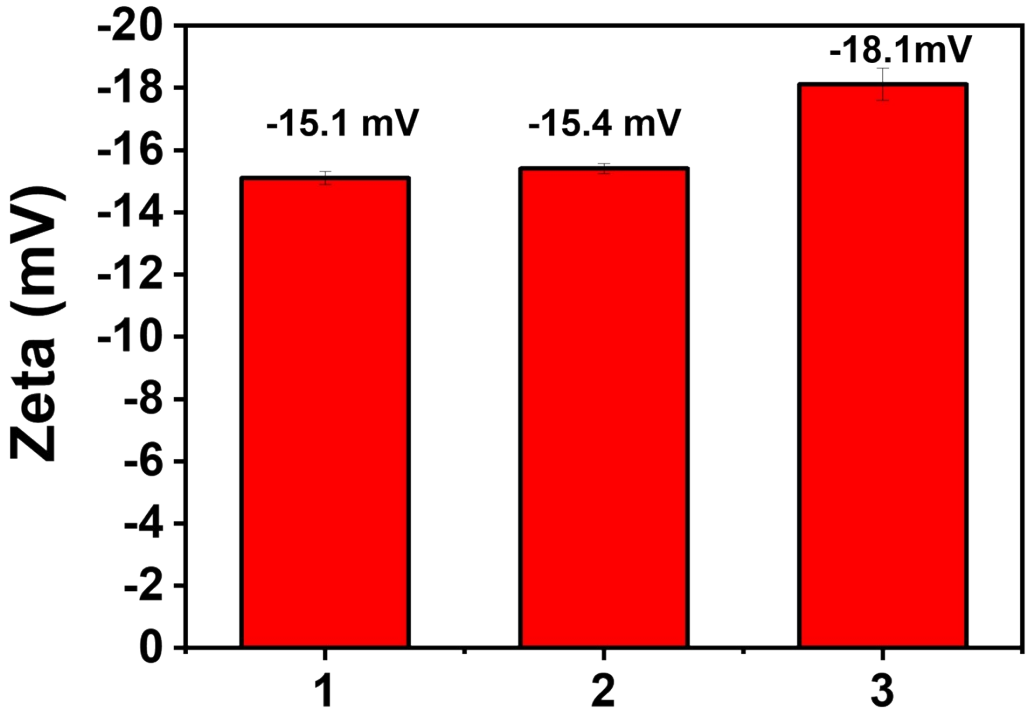


Figure S4. Zeta potential of Glu-M, Man-M and GM-M, respectively.

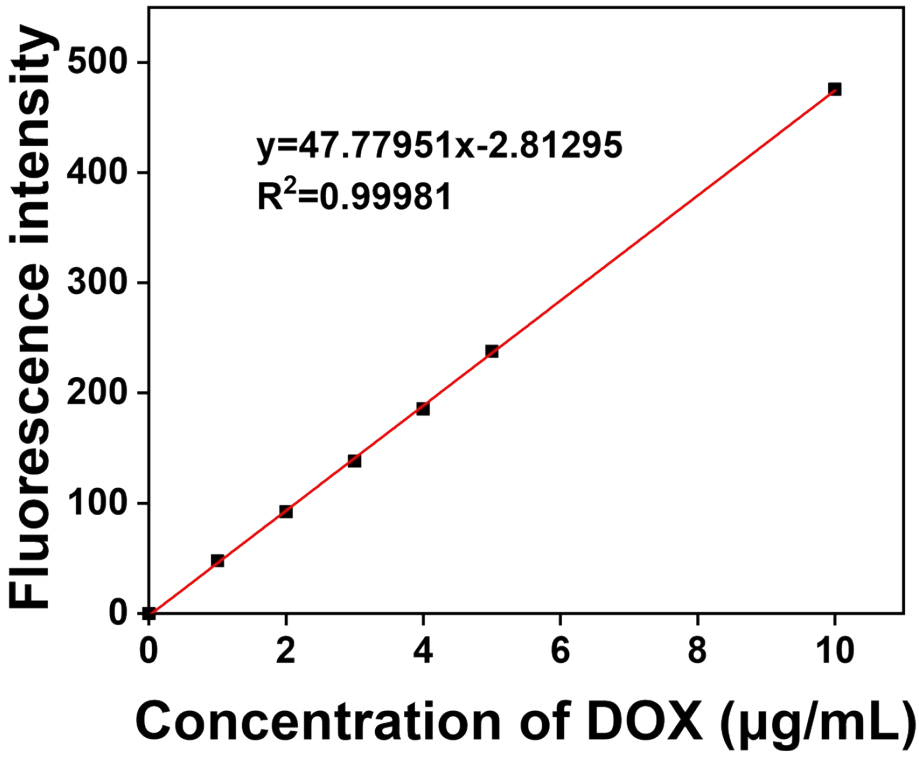


Figure S5. Encapsulation efficiency of the G-M^{DOX}, M-M^{DOX}, GM-M^{DOX} was 74.66%, 75.01%, 78.39%, respectively.

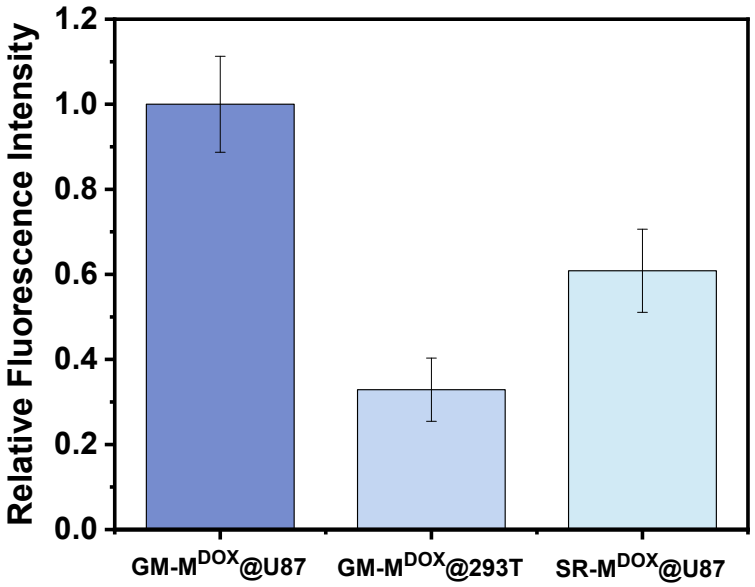


Figure S6. Statistical analysis of fluorescence intensity of different cells in different environments. They were U87 cells in GM-M^{DOX}, 293T cells in GM-M^{DOX} and U87 cells in SR-M^{DOX}. Data are means ± SD (n = 4).

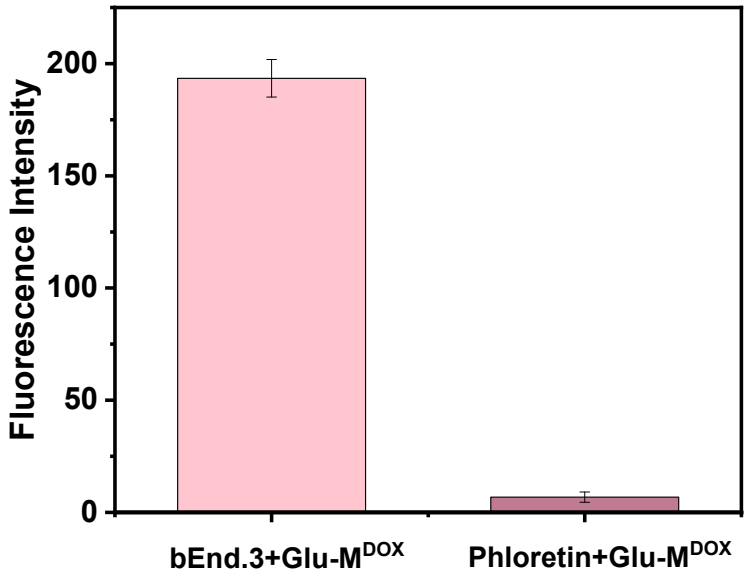


Figure S7. Quantitative analysis of fluorescence intensity of Glu-M^{DOX} uptake by Bend.3 with phloretin or not. Data are means ± SD (n = 3).