Electronic Supplementary Information

Effects of iRGD conjugation density on the in vitro and in vivo properties of cylindrical polymer brushes

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1. Materials and methods

Materials. Grubbs third-generation catalyst (**G3**) was purchased from Sigma-Aldrich. PEG monomethyl ether monopropargyl ether (2 kDa) and PEG mono(2-aminoethyl) ether monopropargyl ether (2 kDa) were purchased from Ponsure Biological. iRGD (1050 Da) was purchased from GL Biochem Ltd. (Shanghai, China). 4-(Dimethylamino)pyridine (DMAP) and 4,6-diamino-2-phenyl indole (DAPI) was obtained from J&K. Ammonium chloride, hydrochloric acid (HCl), sodium hydroxide (NaOH), and anhydrous sodium sulfate were purchased from Sinopharm Chemical Reagent. Petroleum ether, ethyl acetate, diethyl ether, dichloromethane (DCM), and *N*, *N*-dimethylformamide (DMF) were purchased as regent grade from J&K or Tidea, and used as received unless otherwise noted. Dialysis membranes with the molecular weight cut off (MWCO) of 14000 and 30000 Da were purchased from My BioScience. Human breast cancer cells (MCF-7), mouse colon carcinoma cells (CT26) and murine mammary gland cancer cells (4T1) were obtained from Shanghai Institute of Cell Biology (Shanghai, China).

Characterizations. ^{1}H-NMR spectra were recorded on a Bruker Advance III 400 spectrometer at room temperature. High-Performance Liquid Chromatography (HPLC) analyses were carried out on a Shimadzu HPLC system based on a reversed-phase C18 column. Fluorescence spectra were measured on a Horiba FluoroMax-4 spectrofluorometer. Gel permeation chromatography (GPC) data were measured using a Polymer Laboratories PL-GPC 50 instrument equipped with a 5 μm guard column, a 5 μm mixed-D columns and a refractive index (RI) detector (from Agilent Technology). The calibration was performed using

poly(methyl methacrylate standards) (PMMA) standards. DMF with 0.01 M LiBr was used as the eluent at a flow rate of 1.0 mL/min. Confocal laser scanning microscopy (CLSM) images were recorded on an LSM-710 (Zeiss Inc., Germany). Atomic force microscopy (AFM) images were acquired using a Bruker AFM (Bruker Dimension Icon) with Scan-Asyst-Air tips in Scan Asyst in Air Mode. The samples were prepared by dropping dilute solutions (< 0.01 wt%) in deionized water onto freshly cleaved mica and dried at room temperature. The flow cytometry data were recorded on a Cyto FLEX (Beckman Coulter). In vivo fluorescence imaging was conducted on an IVIS Lumina III imaging station (In Vivo Imaging System, PerkinElmer). Confocal images of the tumor tissue sections were analyzed with the Leica DMi8 Thunder. Dynamic light scattering (DLS) data was recorded by using a Brookhaven NanoBrook Omni.

2. Experimental Procedures

Synthesis of endo, exo-5-norbornene-2-carboxylic acid, 2,3-epoxy-propyl ester (monomer 1). A mixture of *endo* and *exo*-5-norbornene-2-carboxylic acid (1 g, 7.23 mmol), oxalyl chloride (7 mL) and dimethyl formamide (0.1 mL) in dry DCM (15 mL) were stirred at room temperature under N_2 for 6 h. Thereafter, the solvent and excess oxalyl chloride were removed under reduced pressure and 20 mL of DCM was added. To the resulting solution was added dropwise a mixture of glycidol (0.8 g, 10.81 mmol) and triethylamine (1.46 g, 14.46 mmol) at 0 ℃ over 1 h, and then stirred at room temperature for 10 h. The organic phase was washed with 5% HCl, saturated $NaHCO_3$ and deionized water successively, and dried with anhydrous magnesium sulphate. After removal of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel with an eluent of petroleum ether/ethyl

acetate (10/1, v/v) to yield monomer **1** as a colorless liquid (1.12g, 80 % yield).

Synthesis of PNB-epoxy. In a glove box, a solution of monomer **1** (200 mg, 1.03 mmol) in 8 mL of DCM was added to a solution of G-3 (2 mg, 2.26×10^{-3} mmol) in 2 mL of DCM under vigorous stirring. The resulting mixture was stirred at room temperature for 30 min and one drop of ethyl vinyl ether was added to quench the polymerization. Pure product was obtained after precipitating in cold diethyl ether three times and dried under vacuum (196 mg, 98 % conversion).

Synthesis of PNB-N_3. To a solution of PNB-epoxy (20 mg, 0.10 mmol) in dry DMF (3 mL) were added ammonium chloride (22.06 mg, 0.41 mmol) and sodium azide (26.80 mg, 0.41 mmol). The resulting mixture was stirred at 45 ℃ for 48 h. After removal of the salts by centrifugation, the product was obtained by precipitating into deionized water three times and dried under vacuum at room temperature. The conversion efficiency of the epoxy group into azido group is demonstrated to be close to 100% by ^{1}H NMR.

Synthesis of PEG 1. PEG **1** was synthesized through maleimide–thiol coupling chemistry of the maleimide group in PEG with thiol group in iRGD. In brief, alk-PEG-mal (100 mg, 0.05 mmol) and iRGD (63 mg, 0.06 mmol) were dissolved in 5 mL of DMF and stirred at 35 ℃ for 10 h. Thereafter, the product was obtained after dialysis against deionized water in a 2 kDa MWCO membrane for two days and lyophilization.

Synthesis of CPBs-1. PNB-N_3 (11.85 mg, 0.05 mmol), PEG **2** (200 mg, 0.1 mmol), PEG **3** (20 mg, 0.01 mmol), and ascorbic acid (44 mg, 0.25 mmol) were dissolved in DMF (5 mL). The mixture was degassed by two freeze-evacuate thaw cycles and backfilled with N_2. After the addition of $CuSO_4 \cdot 5H_2O$ (6.25 mg, 0.025 mmol), the mixture was degassed again by one freeze-evacuate thaw cycle and backfilled with N_2. The resulting mixture was stirred at 50 °C for 48 h and then the solvent was removed under reduced pressure. The product was obtained after dialysis against deionized water in a 14 kDa MWCO membrane for two days and lyophilization. CPBs-2 and CPBs-3 were synthesized by a similar procedure.

Labeling of the CPBs with FITC or RBITC. FITC or RBITC was added to a solution of CPBs (10 mg) and trimethylamine (5 μL, 0.036 mmol) in 1 mL of dry DMF. The reaction solution was gently stirred at room temperature under dark for 12 h. The product was obtained after dialysis against deionized water in a 14 kDa MWCO membrane for two days and lyophilization.

Cellular uptake. The cellular uptake of the FITC-labeled CPBs was examined by using CT26, 4T1 and MCF-7 cell lines. The cells were seeded in a 6-well plate at a density of 2×10^5 cells and incubated in DMEM medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. After 4 h incubation with FITC-labeled CPBs, the cells were washed with PBS three times and then the cell nuclei were stained with DAPI. The cells were observed on a confocal laser scanning microscope.

Flow cytometry measurements. For the quantitative analysis of cellular uptake, the cells were seeded in a 24-well plate at a density of 10^6 cells and incubated with the FITC-labeled CPBs at 37 °C for 4 h. The cells were then harvested and washed twice with cold PBS, and 100 μL of binding buffer was added while gently mixing to prepare cell suspension. The suspension of the cells was quantified by a flow cytometer. Flow cytometry measurements were performed in triplicate and analyzed by using FlowJo software.

Western Blotting. MCF-7, 4T1 and CT26 cells were seeded in a 6-well plate at a density of 2 $\times 10^5$ cells and incubated in DMEM medium for 24 h. Thereafter, the medium was removed and the cells was wished with PBS twice. The cells were then lysed with RIPA buffer and the lysate was separated by SDS-PAGE protein gel electrophoresis, followed by transferred to polyvinylidene fluoride (PVDF) membranes. After blocked with 5% skimmed milk powder, the membranes were incubated with anti-GAPDH, anti-αvβ3 and anti NRP-1, respectively, at 37 ℃ for 1 h. After washing with TBS buffer containing 0.5% tween 20 for three times, the membranes were incubated with corresponding horseradish peroxidase (HRP)–conjugated secondary antibody at 37 ℃ for 1 h. Proteins on the membranes were imaged by Electro-Chemi-Luminescence (ECL) instrument (Tanon-5200, Tanon Science & Technology Co., Ltd. Shanghai, China).

Penetration in MCs. 3D spheroids of CT26 cells with a diameter of about 300 μm were used in vitro to evaluate the tumor permeability of CPBs. The tumor spheroids were incubated with the FITC-labeled CPBs at 37 °C for different time period. The incubated spheroids were rinsed

with PBS three times and transferred to a glass bottom Petri dish before the observation on confocal laser scanning microscopy. Z-stack images were obtained by scanning the tumor spheroids from the top to the equatorial plane step by step. Each scanning layer was 10 μm in thickness, and the total scan was 80 μm in depth.

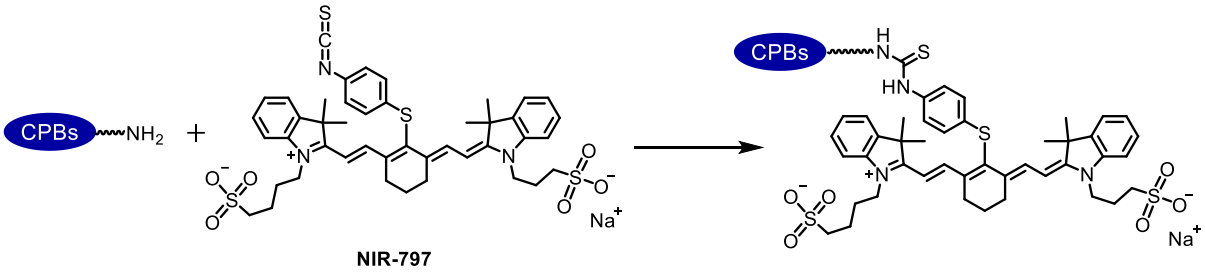
In vivo experiments. All procedures involving animals were conducted in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University and approved by the Medical Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University (Nanjing, China). To establish the subcutaneous murine hepatic 4T1 tumor model, 5×10^5 4T1 cells in 100 μL of PBS were injected subcutaneously at the left front leg of Balb/c mice. When the tumors reached 150 mm^3, the mice were divided into three groups randomly and administrated respectively with dye-labeled CPBs-1, CPBs-2 and CPBs-3 via tail vein at a dosage of 5 mg/kg body weight, respectively, with 3 mice one sample, respectively.

Tumor slices imaging. To establish the subcutaneous murine hepatic 4T1 tumor model, 5×10^5 4T1 cells in 100 μL of PBS were injected subcutaneously at the left front leg of Balb/c mice. When the tumors reached 150 mm^3, the mice were divided into three groups randomly and administrated respectively with RBITC-labeled CPBs-1, CPBs-2 and CPBs-3 via tail vein at a dosage of 5 mg/kg body weight, with 3 mice one sample, respectively. The tumors were excised at 72 h postinjection and processed to 10 μm frozen tissue sections by a cryotome. Blood

vessels were stained immunochemically with anti-CD31, and cell nuclei were stained with DAPI. Then the tumor sections were observed by CLSM.

Statistical analysis. Statistical analyses were performed by Student's t-test and *p* values less than 0.05 were statistically significant.

3. Supplementary Schemes, Figures and Tables



Scheme S1. Modification of the CPBs with NIR-797.

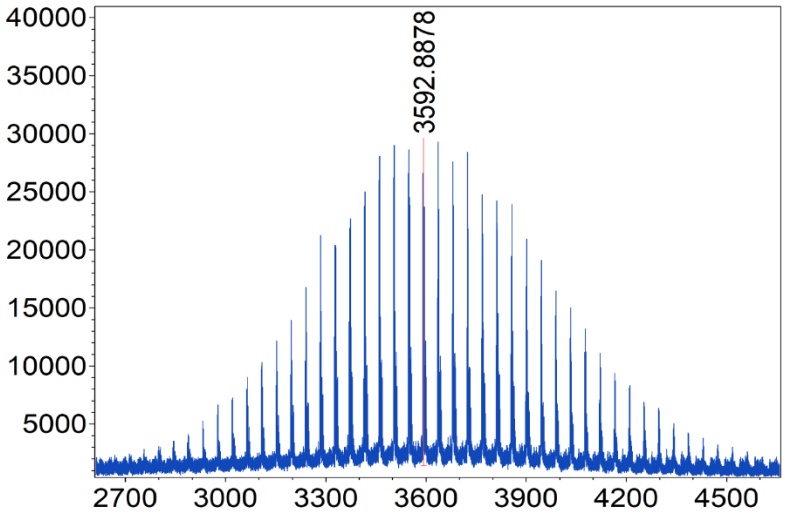


Fig. S1 MALDI-TOF mass spectrum of PEG **1**.

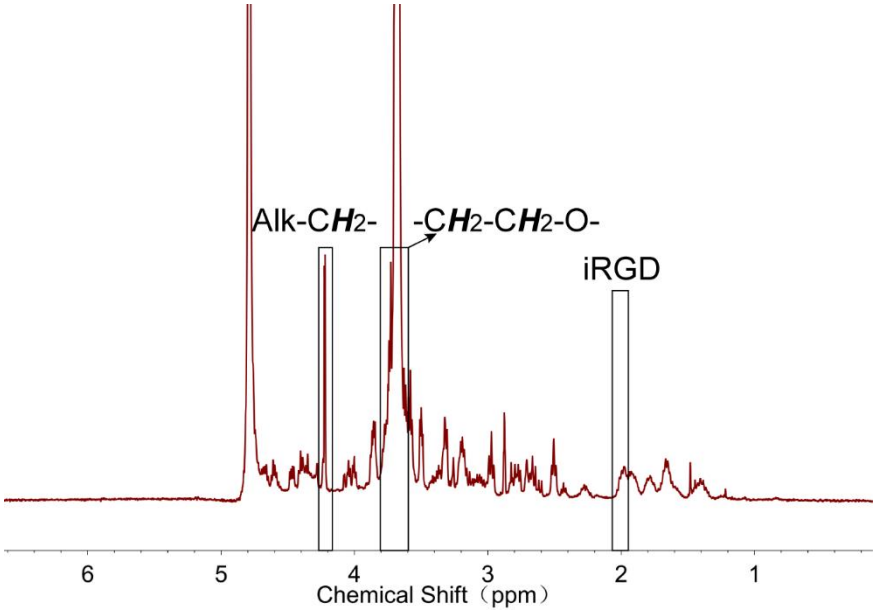


Fig. S2 ^{1}H NMR spectrum of PEG **1** in D_2O.

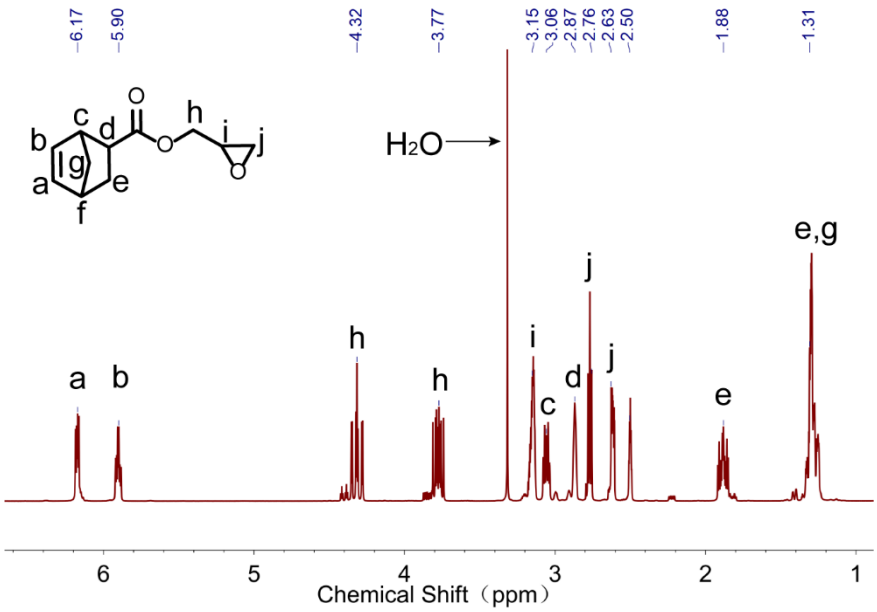


Fig. S3 ^{1}H NMR spectrum of monomer **1** in DMSO-d_6.

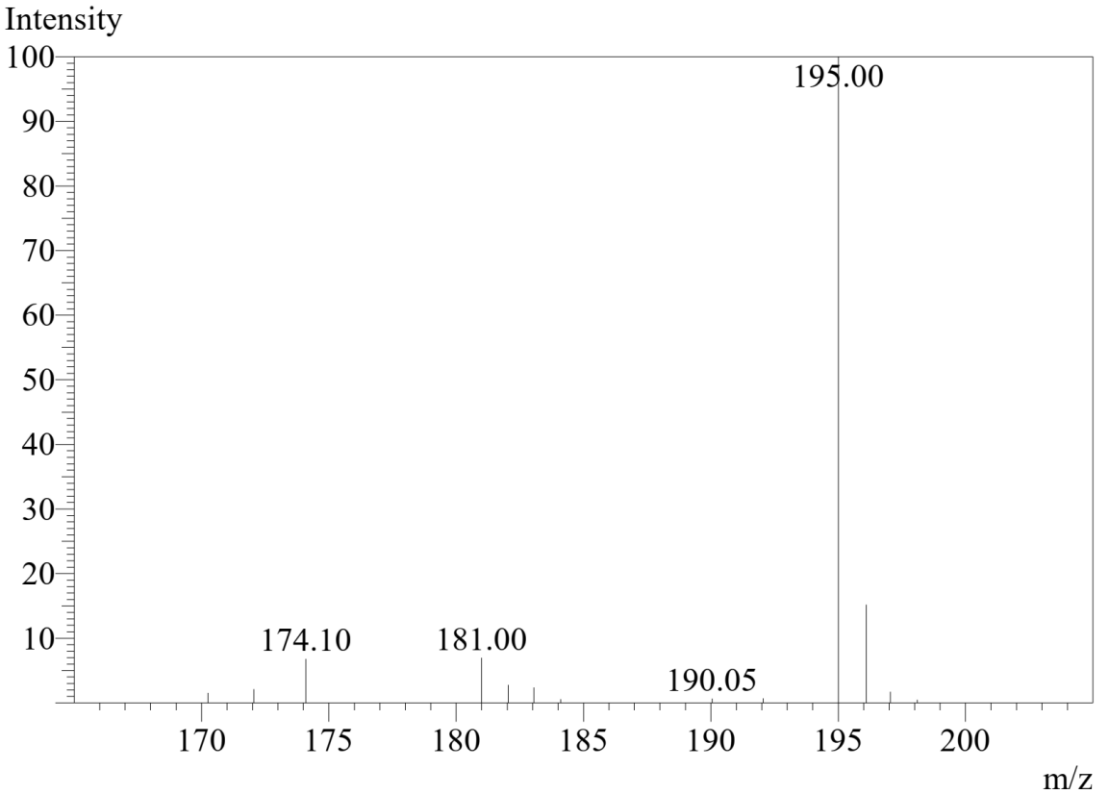


Fig. S4 ESI-MS spectrum of monomer **1**.

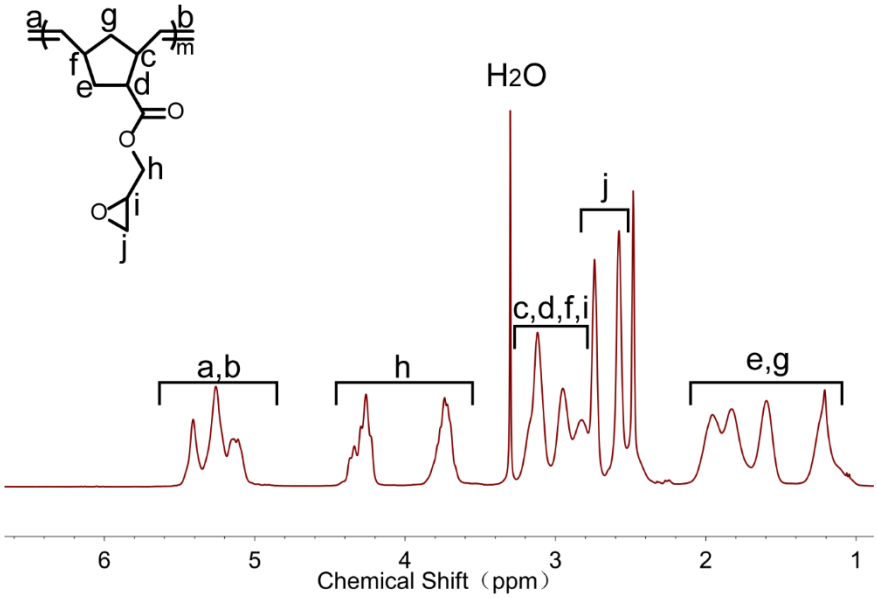


Fig. S5 ^{1}H NMR spectrum of PNB-epoxy in DMSO-d_6.

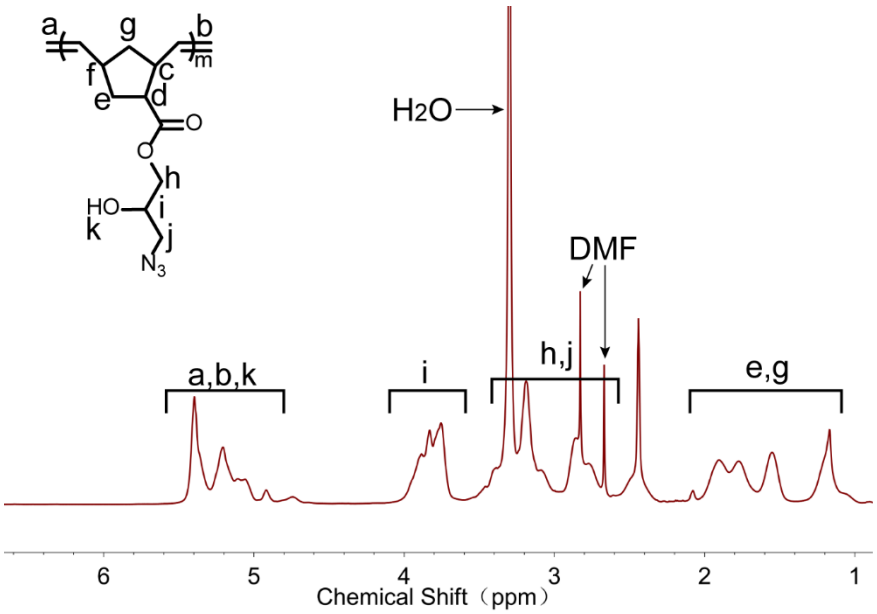


Fig. S6 ^{1}H NMR spectrum of PNB-N_3 in DMSO-d_6.

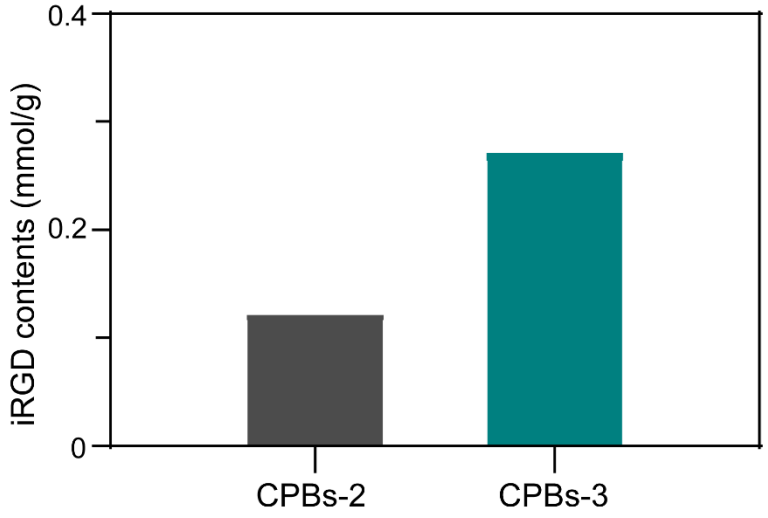


Fig. S7 iRGD contents of CPBs-2 and CPBs-3 measured by ninhydrin assay.

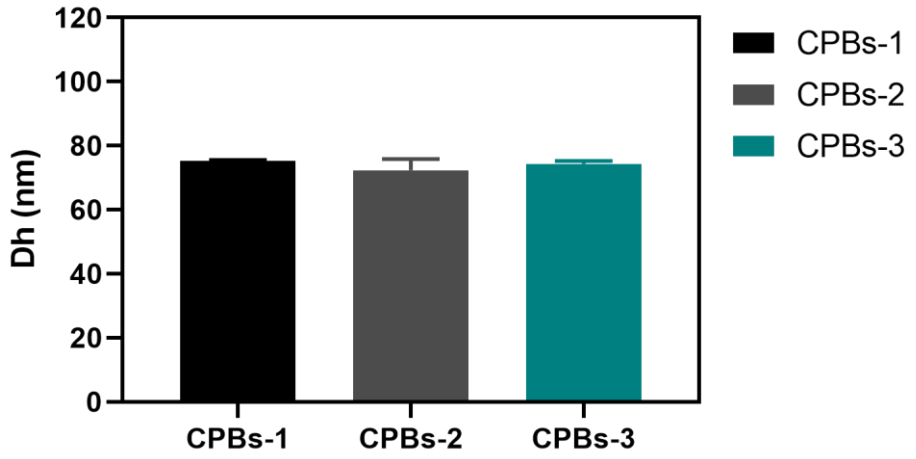


Fig. S8 Mean hydrodynamic diameters of CPBs-1, CPBs-2 and CPBs-3 in water. Dh = hydrodynamic diameter.

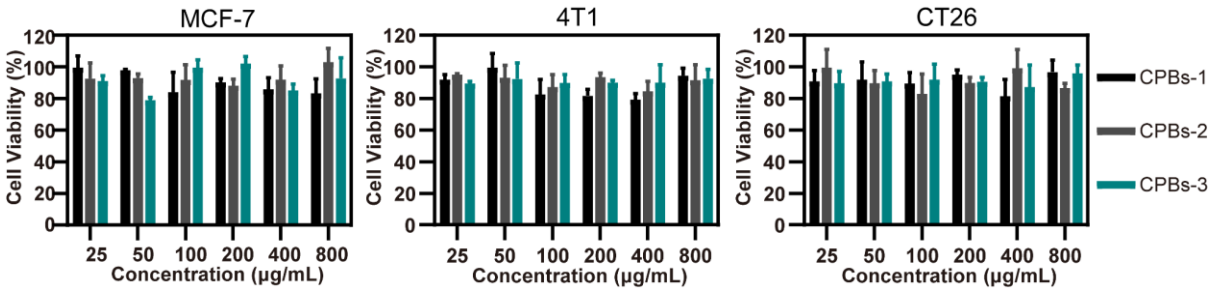


Fig. S9 In vitro cytotoxicities of CPBs-1, CPBs-2 and CPBs-3 against MCF-7, 4T1 and CT26 cells after 24 h incubation. Data as mean values ± SD (n = 3).

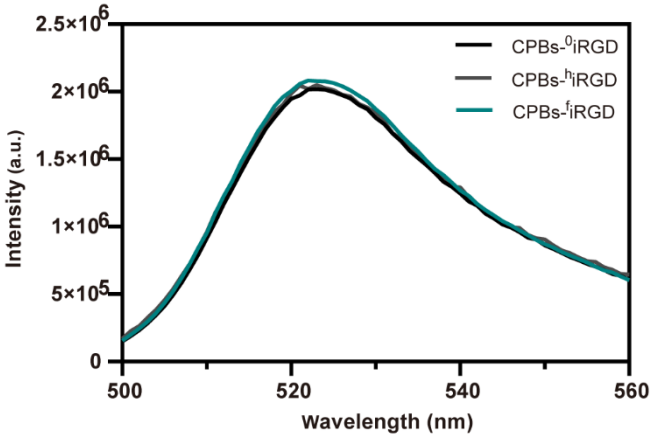


Fig. S10 Fluorescence spectra of the FITC-labeled CPBs-1, CPBs-2 and CPBs-3 at the same concentration.

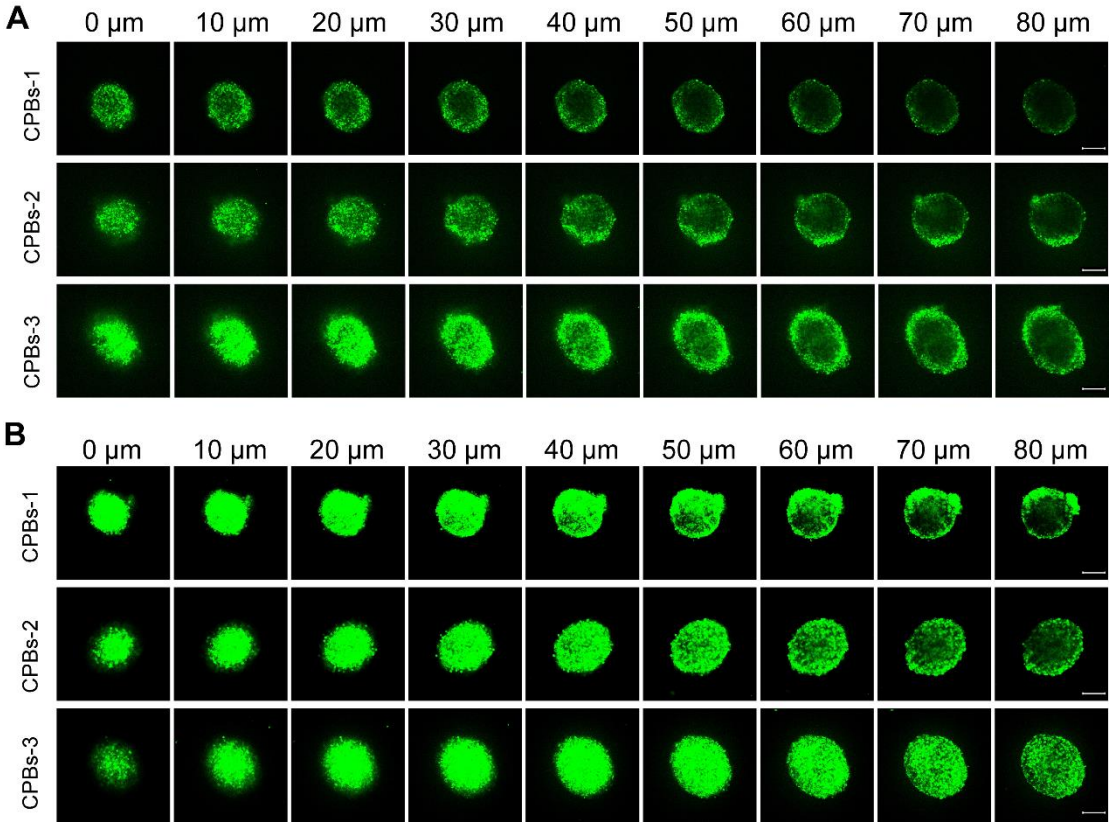


Fig. S11 CLSM examination of the CPBs-1, CPBs-2 and CPBs-3 distributions in 3D MCs after 6 h (A) and 24 h (B) incubation at 100 μg/mL, respectively. The z-stack images were scanned from the top to the middle of the spheroids per 10 μm. Scale bars = 100 μm.

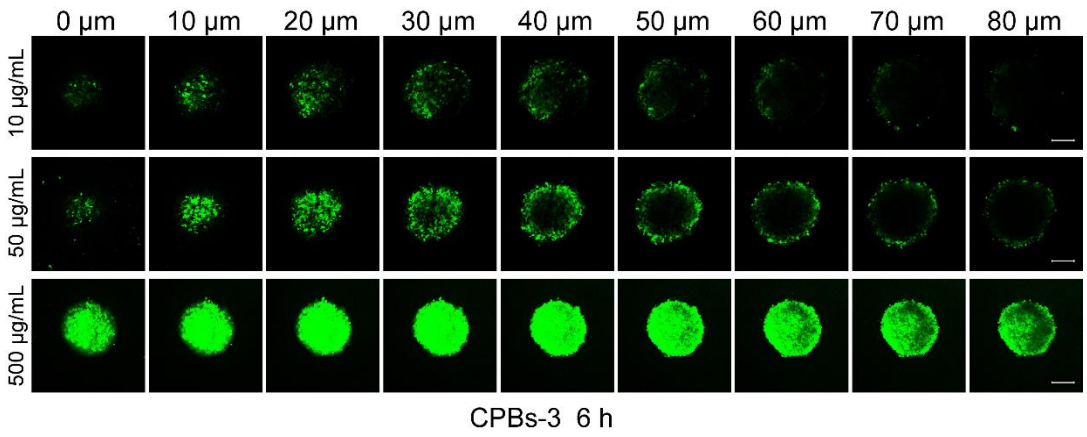


Fig. S12 CLSM examination of CPBs-3 distributions in 3D MCs after 6 h incubation at 10 μg/mL, 50 μg/mL, and 500 μg/mL, respectively. The z-stack images were scanned from the top to the middle of the spheroids per 10 μm. Scale bars = 100 μm.

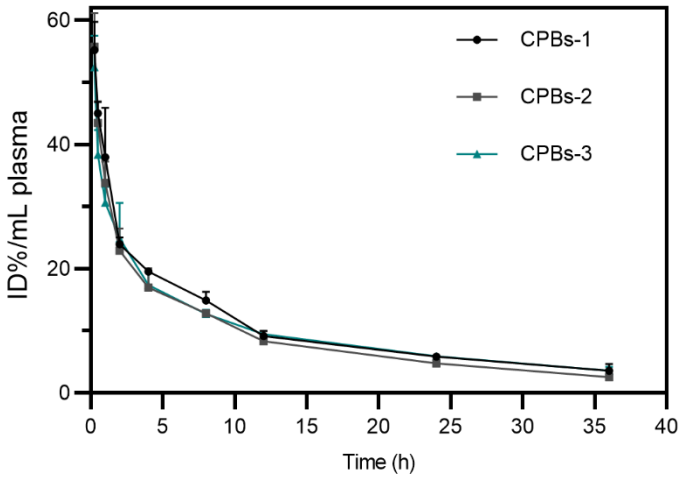


Fig. S13 Plasma concentration-time profiles of the FITC-labeled CPBs-1, CPBs-2 and CPBs-3 at a dose 5 mg kg^{-1}. Data as mean values ± SD (n = 3).

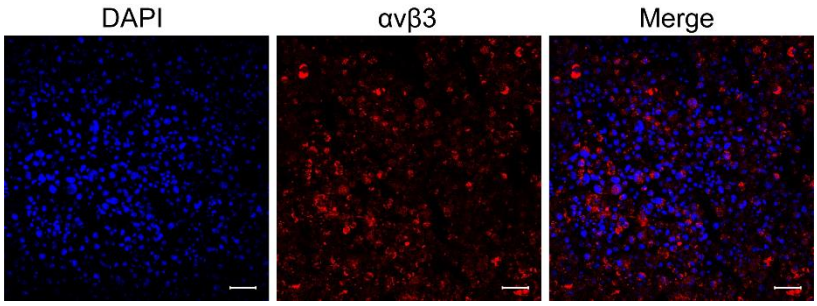


Fig. S14 CLSM images of the 4T1 tumor frozen section. αvβ3 were detected with rabbit anti-αvβ3 antibody, followed by staining with Alexa Fluor 594-AffiniPure goat anti-rabbit (red). Nuclei were stained with DAPI (blue). Scale bars = 50 μm.