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Supporting Information for

A CD326 Monoclonal Antibody Modified Core Cross-linked Curcumin-polyphosphoester Prodrug for Targeted Delivery and Cancer Treatment

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Materials and Methods

Materials and Characterization

The following agents were purchased and used as received: 2,2'-Dithiodiethanol (HO-*ss*-OH, Sigma-Aldrich), α-bromoisobutyryl bromide (98%, Sigma-Aldrich), sodium azide (NaN_3, Sinopharm Chemical Reagent), curcumin (CUR, 98%, Adamas), triphosgene (99%, J&K Chemical), 1,8-Diazabicycloundec-7-ene (DBU, 98%, TCI), CuBr (98%, J&K Chemical), *N*,*N*,*N*′,*N*″,*N*″-pentamethyl diethylenetriamine (PMDETA, 98%, Sigma-Aldrich), CD326 mouse monoclonal antibody (mAb-CD326, Proteintech, Catalog 66316-1-lg), 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (photoinitiator 2959, 98%, Aladdin), L-glutathione (reduced) (GSH, 99%, Shanghai Yuanye Biotechnology), methyl thiazolyl tetrazolium (MTT, 98%, Sigma-Aldrich), PE anti-human CD326 antibody (Biolegend, Catalog 324206), Hoechst 33342 Solution (Mesgen Biotechnology, Catalog MG1790), Tri-ethylamine (TEA, A.R., Enox). Tetrahydrofuran (THF, A.R., Enox) and *N*, *N*-dimethylformamide (DMF, A.R., Enox) were distilled before use. dichloromethane (CH_2Cl_2, A.R., Enox) was dried over CaH_2 for at least 24 h and distilled before use. Milli-Q water (18.2 MΩ·cm at 25 °C) was produced through a water purification system (Simplicity UV, Millipore). Other reagents for biological experiments were analytical reagents and used as received unless otherwise mentioned.

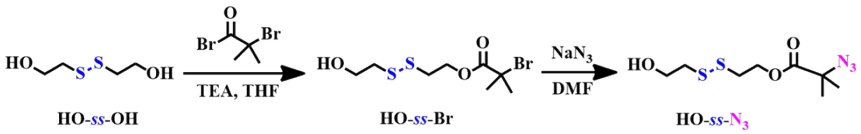
Synthesis of CUR Derivative (N_3-*ss*-CUR-*ss*-N_3)

The core crosslinker N_3-*ss*-CUR-*ss*-N_3 was prepared by three steps as follows. In the first step, 2,2'-dithiobis[1-(2-bromo-2-methylpropionyloxy) ethane] (HO-*ss*-Br) was synthesized. Specifically, to a 150 mL branched flask, 60 mL of THF, HO-*ss*-OH (8.02 g,

51.99 mmol), and TEA (3.85 g, 38.13 mmol) were added under a nitrogen atmosphere. α-Bromoisobutyryl bromide (7.97 g, 34.65 mmol) was dissolved in 20 mL of THF and transferred to a constant pressure drip funnel. Then, the branched flask was placed in an alcohol bath at −10 °C and α-bromoisobutyryl bromide/THF solution was added dropwise with stirring. The reaction was continued for 1 h after dropping, and then the flask was transferred to an oil bath at 25 °C and stirred for 24 h. Subsequently, the reaction mixture was filtered for desalination. After rotary evaporating the solvent, 80 mL of CH_2Cl_2 was added for dissolution. The crude product was extracted using 1 mol L^{-1} HCl solution, saturated $NaHCO_3$ solution, and saturated NaCl solution, respectively. The organic phase was collected, dried with anhydrous Na_2SO_4 for 4 h, filtered for desalination, and rotary evaporated to remove solvent CH_2Cl_2. The product was purified by silica gel column chromatography (200−300 mesh), in which dichloromethane: petroleum ether (4:1 by volume) and dichloromethane: ethyl acetate (4:1 by volume) were used as eluents, respectively. Finally, the solvent was removed and a pale-yellow viscous liquid was obtained. (HO-*ss*-Br, 6.46 g, yield: 61.48%).

In the second step, 2'-azidoisobutyryloxy-2-hydroxyethyl disulfide (HO-*ss*-N_3) was prepared by substitution reaction. 20 mL of DMF, HO-*ss*-Br (2.08 g, 6.86 mmol), and NaN_3 (1.02 g, 15.69 mmol) were added to a 50 mL flask, and reacted in an oil bath at 35 °C for 24 h. After the reaction was completed, the undissolved NaN_3 was removed by centrifugation, and DMF was removed by rotary evaporation. 80 mL of CH_2Cl_2 was added for dissolution, and the mixture was extracted with sodium chloride (NaCl) aqueous solution three times. The organic layer was dried with anhydrous Na_2SO_4. and vacuum drying was carried out for 24 h to obtain a light-yellow viscous liquid (HO-*ss*-

N_3, 1.62 g, yield: 88.99%). The synthetic routes for the first two reaction steps are shown in Scheme S1.



Scheme S1. Synthetic routes of OH-ss-N_3.

Finally, to a 150 mL dry branch flask, HO-*ss*-N_3 (1.00 g, 3.77 mmol), DMAP (0.92 g, 7.53 mmol) and triphosgene (0.37 g, 1.26 mmol) in anhydrous THF (50 mL) were added and stirred under a nitrogen atmosphere at 25 °C for 1 h. Then, CUR (0.46 g, 1.25 mmol) in anhydrous THF (20 mL) was instilled into the flask, which was further stirred for 12 h. Then, the mixture was extracted with 1 mol L^{-1} HCl solution, saturated $NaHCO_3$ solution, and saturated NaCl solution, respectively. The organic layer was dried with anhydrous Na_2SO_4. The filtrate was concentrated and further purified by silica gel column chromatography (200–300 mesh) using dichloromethane: ethyl acetate (1:1 by volume) as the eluent. Finally, yellow viscous liquid was obtained. (N_3-*ss*-CUR-*ss*-N_3, 0.51 g, yield: 42.9 %).

Synthesis of Amphiphilic Polymer (APEG-*b*-PBYP)

The copolymer APEG-*b*-PBYP was prepared by ring opening polymerization of BYP. The reaction was carried out as follows: Firstly, 10 mL of CH_2Cl_2, pre-dried APEG-OH (0.30 g, 0.13 mmol), DBU (57.00 mg, 0.37 mmol) and BYP (0.36 g, 2.05 mmol) was added to a 50 mL branched flask under a nitrogen atmosphere. The reaction was carried out at 30 °C for 1 h and was terminated by 2 drops of glacial acetic acid. The mixed product was concentrated and precipitated three times in a mixture of ice anhydrous ether and anhydrous methanol (10:1, by volume). The precipitated solution was aspirated

and the product was vacuum dried for 24 h to obtain a kind of white viscous solid. (APEG-*b*-PBYP, 0.34 g, yield: 52.1 %).

Synthesis of Polymer Prodrug (APEG-*b*-PBYP-*ss*-CUR)

The cross-linked polymer prodrug APEG-*b*-PBYP-*ss*-CUR was synthesized via CuAAC reaction. Briefly, to a flask, a mixture of APEG-*b*-PBYP (59.20 mg, 0.01 mmol), N_3-*ss*-CUR-*ss*-N_3 (57.00 mg, 0.06 mmol) and PMDETA (47.00 mg, 0.27 mmol) was dissolved in 8 mL DMF, and then CuBr (20.00 mg, 0.14 mmol) was also added into the flask. The reaction mixture was stirred magnetically at 35 °C under a nitrogen atmosphere. A dialysis bag (MWCO 3500) was used to dialyze in DMF and deionized water for 24 h, respectively. The copper absorbing resin was added to the dialysate to remove copper ions and the dialysate was changed every 6 h. This was followed by freeze-drying to obtain a tan solid powder (APEG-*b*-PBYP-*ss*-CUR, 54.10 mg, yield: 46.6%).

Preparation of Core Cross-linked Nanoparticles (CCL NPs)

The core cross-linked nanoparticles were prepared by solvent replacement method. 10.0 mg of APEG-*b*-PBYP-*ss*-CUR was dissolved in 1.0 mL DMF, 15 mL of solution such as deionized water or PBS was added using a microinjection pump at a rate of 2 mL h^{-1}. Then, the dialysis bag (MWCO 3500) was used to dialyze for 24 h in the same solution for addition, and the dialysis solution was changed every 4 h. Finally, the solution was diluted to 20 mL to obtain the targeted core cross-linked nanoparticles (CCL NPs) of desired concentration (0.5 mg mL^{-1}).

Preparation of Core Cross-linked Nanoparticles Modified by CD326 Monoclonal Antibody (R-mAb-CD326@CCL NPs)

Firstly, the reduction reaction was performed on CD326 monoclonal antibody to increase the thiol group contents. Specifically, 10 μg of CD326 monoclonal antibody was dissolved in 500 μl of PBS solution, and then 10 μg of TCEP hydrochloride was added. The reaction system was placed in a thermostatic shaker (37 °C, 160 rpm) for 1 h, and the reducing agent TCEP was removed by ultra-filtration and centrifugation (4 °C, 12000 rpm, 20 min) after the reaction. The product (R-mAb-CD326) in the ultrafiltration tube was collected and tested for sulfhydryl content using the DTNB kit.

Next, through Michael addition reaction between thiol groups and alkenyl groups, we coupled the monoclonal antibody at the hydrophilic end of nanoparticles. The specific steps are as follows: R-mAb-CD326 was added to the PBS solution of CCL NPs, and then the photoinitiator 2959 was added into the mixture. The whole solution was stirred for 1 h with 365 nm UV light initiation. After the reaction, the mixture was transferred to a dialysis bag (MWCO 3500) and dialyzed in PBS for 6 h to remove small molecule impurities. At last, the solution was diluted to obtain CD326 monoclonal antibody modified core cross-linked nanoparticles (R-mAb-CD326@CCL NPs) of desired concentration (0.5 mg mL^{-1}).

Stability and *In Vitro* Drug Release

To study the stability of core cross-linked nanoparticles (CCL NPs), they were dispersed with different concentrations or in different media. The changes of particle size were measured and recorded at different points of time. The samples were also prepared in PB 7.4 solution containing 10 mM GSH or without GSH to study the *in vitro* drug release behavior. The samples were transferred to the dialysis bag (MWCO 3500) and

placed in 40 mL of the corresponding release solution, then they were incubated in a thermostatic shaker (37 °C, 160 rpm), and 5 mL of buffer outside the dialysis bag were collected at predetermined intervals and an equal amount of buffer was replenished. The UV-Vis spectrophotometer was used to measure the release amount of CUR at different GSH concentrations within 144 h.

In Vitro Enzymatic Degradation

To verify the degradability of the main chain of phosphate esters, ^{1}H NMR was used to test the degradation products at different times in the presence of phosphodiesterase I (PDE I). Firstly, 7.5 mg PDE I and 75 mg $MgCl_2 \cdot 6H_2O$ were dissolved in 15 mL PB 7.4 solution to obtain the degradation solution. Then, 30.00 mg of APEG-*b*-PBYP was dissolved in the degradation solution, and the mixture was divided into three centrifuge tubes which was subsequently placed in a thermostatic shaker (160 rpm, 37 °C). Each portion was freeze-dried at a predetermined time point and then subjected to ^{1}H NMR analysis.

In Vitro Hemolysis Activity

The degree of red blood cell fragmentation after incubation with the drug was measured by spectrophotometry. The specific steps are as follows: the mouse eyeballs were removed, and the blood flowed into the centrifuge tube which had been pretreated with heparin sodium to prevent coagulation. 10 mL of PBS was added and the solution was resuspended 3 times (3000 rpm, 6 min) until there is no obvious color in the supernatant. Then, 10 mL of PBS was added and mixed for later use.

CCL NPs of different concentrations in PBS solutions were configured according to the above preparation method. 0.5 mL of red blood cell suspension was added to

each of the 12 clean centrifuge tubes, and 0.5 mL CCL NPs of different concentrations was also added into five tubes. To the other five tubes, 0.5 mL of CUR with different concentrations was added, and the contents of CUR in the two groups was the same. In addition, the last two tubes were each filled with PBS and Milli-Q water as the negative control group and the positive control group, respectively. Then they were incubated in a thermostatic shaker (37 °C, 160 rpm) for 4 h, and the intact red blood cells were sedimented through centrifugation (4 °C, 12000 rpm, 5 min). 100 μL of supernatant from each centrifuge tube were removed to the 96-well plate. An enzyme-linked immunosorbent assay was used to test the absorbance of hemoglobin in the supernatant at 540 nm. the percentage of hemolysis was calculated using formula according to **Equation (1)**:

$$\text{Hemolysis (\%)} = \frac{OD_{\text{sample}} - OD_{(-)\text{ control}}}{OD_{(+)\text{ control}} - OD_{(-)\text{ control}}} \times 100 \quad (1)$$

where OD_{sample} is the absorbance of the sample, $OD_{(-)\text{ control}}$ is the absorbance of the negative control group, and $OD_{(+)\text{ control}}$ is the absorbance of the positive control group.

In Vitro Free Radical Scavenging

1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) is a stable free radical with purple color in ethanolic solution whose maximum absorption is at the wavelength of 518 nm, and has a linear relationship between absorbance and concentration. Based on these two points, an *in vitro* radical scavenging experiment was simulated here as follows: 3.94 mg of 1,1-diphenyl-2-bitter hydrazine (DPPH) was weighed and dissolved in 100 mL of ethanol (95%) to obtain DPPH solution. 16 centrifuge tubes were prepared and 1 mL of DPPH solution was added to each tube. The remaining 15 tubes were divided into 5 groups, and 1 mg of APEG-*b*-PBYP, 0.11 mg CUR, 3.07 mg GSH, 1

mg CCL NPs and 1 mg CCL NPs + 3.07 mg GSH were added respectively. The free radical scavenging rate DPPH Seavening (%) was calculated from the absorbance values of the control group according to **Equation (2)** after the configuration was stirred for 30 min and tested at 518 nm:

$$\text{DPPH Seavening } (\%) = \frac{A_i}{A_0} \times 100 \quad (2)$$

where A_i represents the absorbance of the sample, and A_0 represents the absorbance of the control group.

Cell Culture

Human umbilical vein endothelial cells (HUVEC cells), Human breast cancer cells (MCF-7 cells) were obtained from American Type Culture Collection (ATCC) and cultured in high glucose DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. Both cell lines were passaged once every 2 days and incubated at 37 °C in an atmosphere containing 5% CO_2 and certain humidity.

***In Vitro* Cytotoxicity**

The cytotoxicity was measured using the MTT assay. HUVEC cells and MCF-7 cells were seeded into 96 well plates respectively at a density of 5×10^3 per well to allow them attached to the plate bottom. Cells were then incubated with corresponding samples at different concentrations for 48 h (CUR concentration was set to 0.19 to 25 mg L^{-1} for a total of 7 groups, n=3). Then, MTT was added into the samples and incubated for 4 h. The solution was removed and replaced by 150 μL of DMSO, and the results were obtained by testing OD_{570} with an enzyme-linked immunosorbent assay.

Cellular Uptake

The cellular uptake and intracellular release in MCF-7 cells of free CUR, CCL NPs and R-mAb-CD326@CCL NPs were investigated by the confocal laser scanning microscope (Zeiss, CLSM 800) and flow cytometry (BD, FACVERSE).

The cells were diluted and inoculated into confocal dishes (Φ=20 mm), and 1 mL of cell suspension was added to each dish at a density of 1×10^5 cells/mL, which was incubated in a cell culture incubator (37 °C, 5% CO_2) for 12 h. Then, the culture medium was removed and replaced by 1 mL of 10 μg mL^{-1} Hoechst 33342 staining agent in PBS solution and stained for 20 min in the incubator protected from light, followed by aspiration of the stain, and washing with PBS three times. After that, PBS solution of free CUR, CCL NPs and R-mAb-CD326@CCL NPs which had been diluted 10 times by complete medium (the final concentration of CUR was 12.5 mg L^{-1}) was added into the dishes, and incubated in cell culture incubator. Then, captured images in the DAPI (blue) and FITC (green) channels within 8 h using the confocal laser scanning microscope.

In another experiment, MCF-7 cells were plated at a density of 5×10^5 cells per well and incubated in a cell culture incubator (37 °C, 5% CO_2) for 12 h. Then, the medium was replaced by PBS solution of free CUR, CCL NPs and R-mAb-CD326@CCL NPs which had been diluted 10 times by complete medium (the final concentration of CUR was 12.5 mg L^{-1}), and continued to incubate for a certain time. Finally, the cells were collected and washed by PBS three times and resuspended in 500 μl of PBS. Then, intracellular fluorescence intensity was tested using flow cytometry.

In Vitro Apoptosis

MCF-7 cells were arranged at a density of 5×10^5 cells per well and cultured in a cell

culture incubator (37 °C, 5% CO_2) for 12 h. The medium was then replaced with PBS solution of free CUR, CCL NPs and R-mAb-CD326@CCL NPs diluted 10 times by complete medium (the final concentration of CUR was 6.25 mg L^{-1}), and the incubation was continued for 48 h. Finally, the cells were collected and washed three times with PBS. Cells were resuspended in Annexin V binding buffer with staining solutions and incubated at room temperature for 5 min before testing by flow cytometry.

Animal Models

6–8 weeks-old balb/c nude mice were purchased from Hangzhou Ziyuan Laboratory Ani-mal Technology Co., Ltd. Firstly, mice needed to be administered the long-acting estrogen estradiol cypionate (dose: 2 mg/kg) to ensure the estrogen level in the body. Two days later, MCF-7 tumor model was established by subcutaneous injection of 5×10^6 MCF-7 cells into the right fat pad, and we had to wait 10 days for tumors to grow.

***In Vivo* Anti-cancer Efficacy**

To assess the in vivo antitumor efficacy of R-mAb-CD163@CCL NPs, 24 balb/c nude mice bearing MCF-7 tumors were randomly divided into four groups (n=6). When the average tumor volume reached approximately 100 mm^3, these mice received different types of treatment. In four control groups, mice were injected intraperitoneally with PBS, free CUR, CCL NPs and R-mAb-CD163@CCL NPs at a dose of 20 mg kg^{-1} of CUR equivalent every two days, respectively. All mice were injected seven times consecutively over two weeks. Tumor diameter and mouse body weight were recorded before each injection. Tumor volume was calculated according to the formula: $V=0.5\times L\times W^2$ (where L is the longest diameter and W is the shortest

diameter). After 14 days, mice were recorded and sacrificed. Tumors and major organs were collected and washed with PBS for further analysis.

***In Vivo* Biodistribution and Fluorescence Imaging.**

In vivo biodistribution experiments were performed after completion of treatment. All mice (balb/c) were sacrificed. Their tumors and major organs (heart, liver, spleen, lung and kidney) were removed and fluorescent images of these organs and tumors were obtained. Meanwhile, they were also weighed and ground into homogenates. Methanol was used to extract the drug contained therein, and the fluorescence absorption intensity of the drug CUR was tested using an enzymatic standard after 12 h in a 4 °C refrigerator. The distribution of drugs is expressed as a percentage (% ID/g) of the total injected drug per gram of tissue, calculated according to **Equation (3)**:

$$\text{Drug level (\%ID / g)} = \frac{I_x - I_0}{m \times (I_s \times V)} \times 100$$

where, I_x is the fluorescence intensity of extracts from different tissues, I_0 is the fluorescence intensity of tissue extracts from the PBS group, m is the tissue mass (g), I_s is the fluorescence intensity of injected sample solution diluted 1000 times, and V is the injection volume (μL).

Histopathology and Immunohistochemical Analysis

After anti-tumor experiments, tumor tissues were obtained by dissecting each group of mice and then processed for paraffin embedding. After dewaxing with xylene, the tumor tissues were cut into 5 μm sections. Tissue sections were stained with H&E. Finally, tissue changes and apoptosis were observed by microscopy.

Immunohistochemical analysis was based on the operating guidelines of the

immunohistochemistry assay kit to immunohistochemical stain of TNF-α, IFN-γ, Bcl-2 and NF-κBp65 in tumor sections of each group, and the expression of various cytokines were observed using a microscope.

Statistical Analysis

All experiments were conducted with at least three or more parallel samples, and the statistical data were evaluated via GraphPad Prism 7.0 software. The data were presented in terms of mean ± standard deviation (mean ± SD). In addition, single factor variance analysis (ANOVA) was used to test the significant difference between the data, which were reflected by using *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

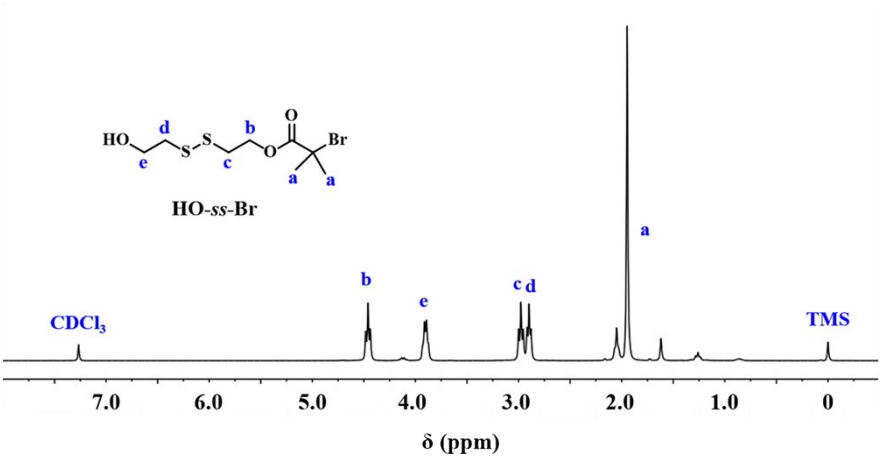


Figure S1. ^{1}H NMR spectrum of HO-*ss*-Br (Solvent: $CDCl_3$).

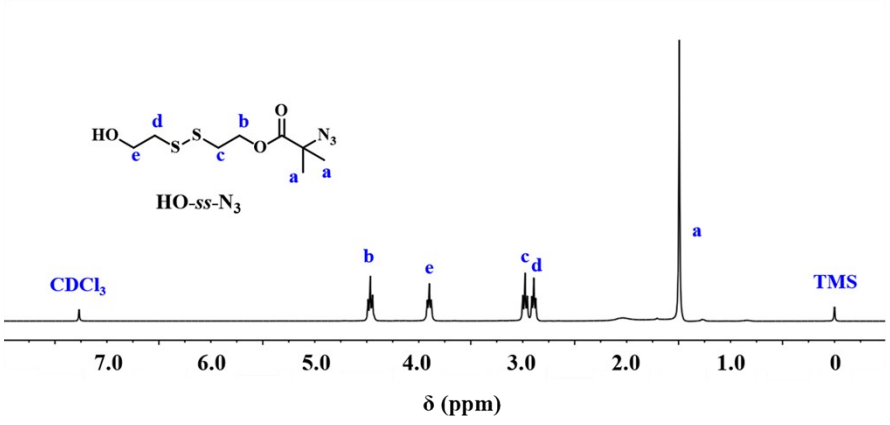


Figure S2. ^{1}H NMR spectrum of HO-*ss*-N_3 (Solvent: $CDCl_3$).

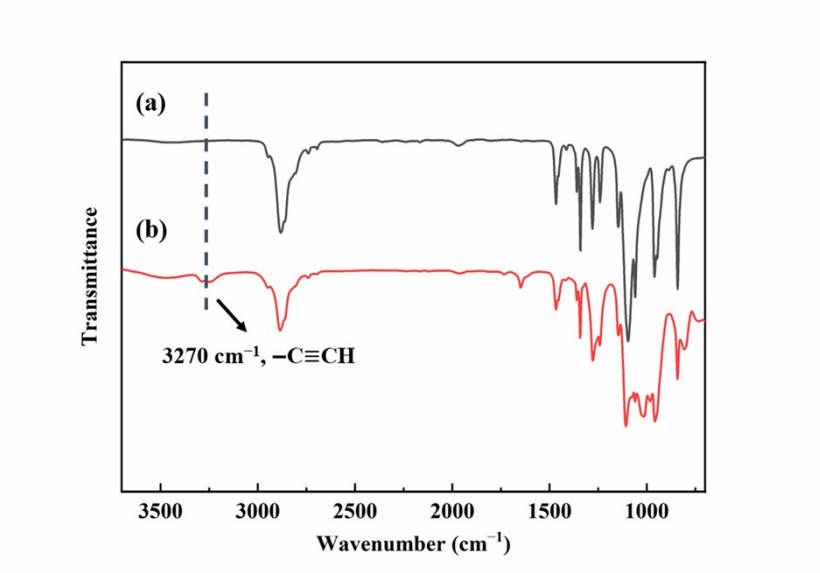


Figure S3. FT-IR spectra of (a) APEG-OH and (b) APEG-*b*-PBYP.

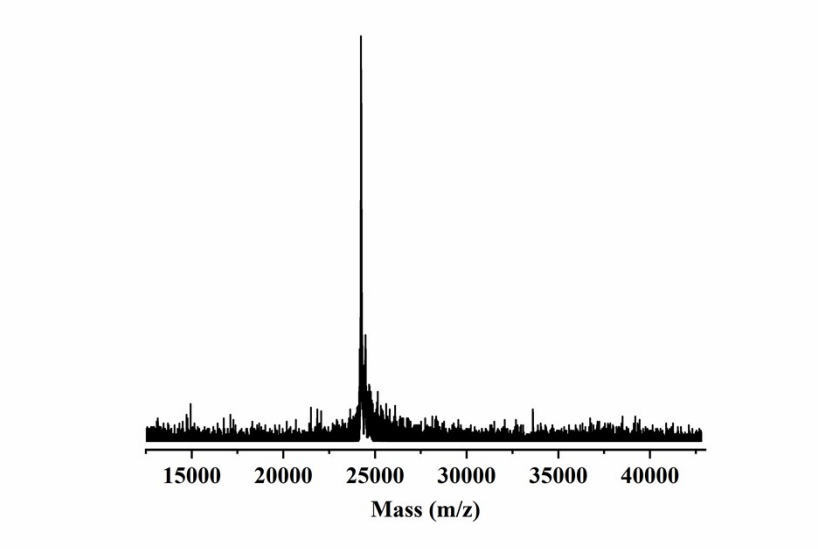


Figure S4. MALDI-TOF MS spectrum of R-mAb-CD326.

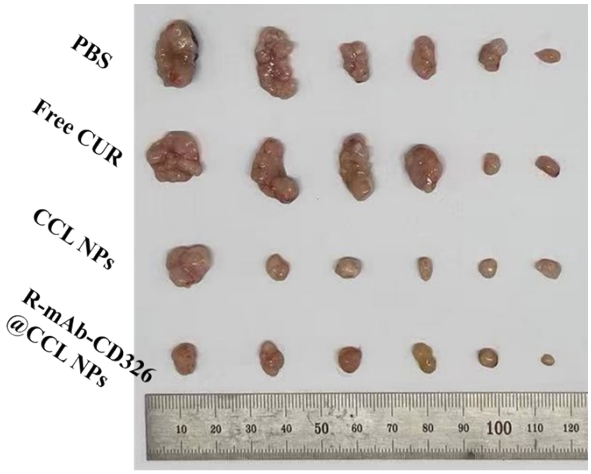


Figure S5. Representative image of tumors after treatments on 14th day.

Table S1. Molecular weights and dispersity (*Đ*) of APEG-*b*-PBYP.

Samples	Feed ratio (m_1 : m_2) [a]	$\overline{M}_n$ [b] (g mol^{-1})	$\overline{M}_w$ [b] (g mol^{-1})	*Đ* [b]
APEG-*b*-PBYP-1	1 : 0.8	5700	6700	1.19
APEG-*b*-PBYP-2	1 : 1	5700	6500	1.14
APEG-*b*-PBYP-3	1 : 1.2	5600	6500	1.15
APEG-*b*-PBYP-4	1 : 1.4	5500	6400	1.16
APEG-*b*-PBYP-5	1 : 1.6	6100	7300	1.19
APEG-*b*-PBYP-6	1 : 1.8	7500	9700	1.29

[a] m_1 : m_2 is the mass ratio of APEG-OH and BYP.

[b] Calculated by GPC (mobile phase: DMF; standard sample: PS).