Mesoporous-SiO_2@pH-Responsive Polypeptides Nanocomposites: Noninterventional Embolization and Ultrasound Imaging Combination Theranostic for Solid Tumors

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Supplementary Information

Materials

All chemicals and materials were from commercial resources and were used as received. γ-benzyl-L-Glutamate (BL-Glu), Cbz-L-Tyrosine (Cbz-L-Tyr), L-threonine (L-Thr) and Cbz-L-cysteine (Cbz-L-Cys), Perfluoropentane(PFP), KH570, cetyltrimethylammonium chloride (CTAC), Pluronic F127 ($EO_{106}PO_{60}EO_{106}$), tetraethyl orthosilicate (TEOS), were purchased from Aladdin in Shanghai of China. Tartaric acid, Hydrochloric acid, Benzyl alcohol, Hydrobromic Acid, Bis(trichloromethyl) Carbonate, Sodium Hydroxide (NaOH) and Lithium Hydroxide

(LiOH) were purchased from Sinopharm Chemical Reagent Co., Ltd in Lanzhou of China. Cy5.5 was purchased from Seebio, Shanghai. Refluxing Triethylamine, Tetrahydrofuran (THF) and Dimethyl Formamide (DMF) by calcium hydride (CaH_2) then got anhydrous Triethylamine, THF and DMF. Pelltobarbitalum Natricum, Xylazine Hydrochloride Injection were purchased from Sinopharm Chemical Reagent Co., Ltd.

Synthesis of pH-responsive polymers (PGTTCs).

Firstly, Cbz-L-Tyrosine N-Carboxyanhydride (Cbz-L-Tyr-NCA), Cbz-L-Threonine N-Carboxyanhydride (Cbz-L-Thr-NCA), Cbz-L-Cysteine N-Carboxyanhydride (Cbz-L-Cys-NCA) and γ-benzyl-L-Glutamate N-Carboxyanhydride (BLG-NCA) were synthesized following a reported procedure. Secondly, using triethylamine (0.1 mmol) as an initiator, γ-benzyl-L-Glutamate N-Carboxyanhydride (BLG-NCA) (1.61g, 6.1mmol) and Cbz-L-Tyrosine N-Carboxyanhydride (Cbz-L-Tyr-NCA)(4.06 g, 11.9 mmol) was dissolved in 5 mL anhydrous DMF and stirred for 72 h under a nitrogen atmosphere at 35 °C. Then L-Threonine N-Carboxyanhydride (L-Thr-NCA) (2.38 g, 16.4 mmol) was added into the reaction system and stirred for another 72 h at 35 °C. Next the Cbz-L-cysteine (Cbz-Cys) (0.59 g, 4.0 mmol) was dissolved in the mixture and stirred for 72 h under a N2 atmosphere at 35 °C, and the primary block copolymers were separated and precipitated out by adding deionized (DI) water (2-3 mL) to the mixture, and centrifuging (7000 rpm, 5 min, 2 times). Then Benzyl was taken out via suspension in LiOH aqueous solution (36.4M) /methyl alcohol (v/v=1/5,

20 mL) for 24 h at 45 °C, and benzyloxycarbonyl was taken out via suspension in trifluoroacetic acid/acetic acid/HBr solution (v/v/v=0.1/1/1, 20 mL) for 12 h at 0 °C. Finally, the block copolymers P(L-Glu-ran-L-Tyr)-*b*-P(L-Thr)-b-P(L-Cys) (PGTTC) were obtained by washed with acid and alkali for 3 times and ethanol for 3 times and then drying 72 h at 40 °C in vacuum. Different pH-responsive PGTTC were obtained by adjusted the monomer ratios during this experiment.

NMR spectra. Prepared chemical compounds were analyzed by nuclear magnetic resonance (NMR) spectroscopy (VARIAN JNMECP 600 MHz instrument), using deuterium dimethyl sulfoxide (DMSO-*d6*) as a solvent.

Molecular mass test. Gel Permeation Chromatography (GPC, Waters 1515) was employed to determine the Molecular Weight and Molecular Weight Distribution of polypeptides, with DMF and LiBr as eluent.

Gel Permeation Chromatography-Eighteen Angles Laser Light Scattering Method (DAWN EOS, Laser wavelength: 690.0 nm) was used to determine the Molecular Weight and Molecular Weight Distribution of polypeptides. Gel Permeation Chromatography equipped with TSKPXwl column and TSK-G4000 PXwl column, an Optilab refractometer was simultaneously connected. Solvent: water. Refractive index: 1.330. Flow rate: 1.0 $mL \bullet min^{-1}$.

Degradability test. The degradation process of PGTTCs by dialysis at 37°C, the specification of the dialysis-membrane was 3000. The polymer was split into two groups and dissolved in 50 mL PBS buffer solution until 18 days. Protamex was added

to only one group. After scheduled times, samples were washed three times with 4 mL of double distilled water, freeze-dried for 3 days and weighed. It was calculated according to the following formula:(w_0: the initial mass; w_t: the remaining mass at different time points)

$$\text{Mass Loss}(\%) = \frac{w_0 - w_t}{w_0} \times 100\%$$

Cytotoxicity Evaluation. The cytotoxicity of the *m*-SiO_2@PGTTCs samples was evaluated using the ISO10993-5 standard test method. 578 cells (normal healthy mammalian cell line) were cultured in 96-well plates at a density of 105 cells per well containing 10% fetal bovine serum, 1.0% penicillin−streptomycin, and 1.2% glutamine at 37 °C in a 5% CO_2 incubator. The polymers of varying concentration (10μL, in PBS) were then added; after 24, 48, and 72 h of incubation, the CCK-8 solution (20μL) was added to each well, and the dissolved solution was swirled homogeneously for 10 min in a shaker. The optical density of the solution was detected by a microplate reader at 540 nm. Results were reported as the percentage of cell viability (percentage of control) after subtracting the average absorbance of the medium (without cells) as follows according to equation 2:

$$cell\ viability = \frac{absorbance\ of\ the\ test\ samples}{absorbance\ of\ the\ control\ samples} \times 100\%$$

Blood sample collection. The hemocompatibility of the *m*-SiO_2@PGTTCs was determined using hemolysis and coagulation studies. Blood samples were collected

from healthy mice. Blood was collected in evacuated glass tubes containing potassium oxalate as an anticoagulant, dilute with saline for experiment.

Hemolysis study. *M*-SiO_2@PGTTC-6.2 and *m*-SiO_2@PGTTC-4.3 were formulated into 1 mg/mL, 5 mg/mL, 10 mg/mL with physiological saline; the positive control was distilled water, and the negative control was 0.9% aqueous sodium chloride solution and preheated in a 37 °C water bath for 1h. The negative control, the positive control and the solution of different concentration gradient *m*-SiO_2@PGTTCs were separately added to the test tube (2 mL) of a certain amount of the above mouse blood, and then preheated in 37℃ water bath for 1 h, and the solutions in all the test tubes were separately transferred to a centrifuge tube for centrifugation. (1500 rpm, 10 min), the supernatant was taken out and its absorbance at 540 nm was measured. The three groups were averaged in parallel, and the degree of hemolysis was calculated according to the formula: (A: absorbance of the sample, A_1: absorbance of the negative control, A_2: absorbance of the positive control)

$$\text{Hemolysis (\%)} = \frac{A - A_1}{A_2 - A_1} \times 100\%$$

Coagulation study. Blood of healthy mice was collected in evacuated glass tubes containing sodium citrate as an anticoagulant. The activated partial thromboplastin time (APTT) was recorded and the prothrombin time (PT) was compared to normal values. Pipette 50μL of different concentration gradient *m*-SiO_2@PGTTCs solution, aspirin solution and *m*-SiO_2@PGTTCs solution (10mg/mL) with protamine sulfate separately

with a volume ratio of 4:1 into 200μL of platelet-poor plasma (PPP) as the prepared plasma, and the PBS solution as a control. two tests for in vitro anticoagulant activity (APTT, PT) were performed separately. The detection methods are as follows: pipetted 50 μL of the prepared plasma, pre-warmed at 37℃ for 3 minutes, then added 50μL of APTT, PT, reagents separately. Two coagulation indicators (APTT, PT) were determined using an automatic blood coagulation analyzer (CS-5100). Furthermore, an equal amount (50μL) of the PBS solution with the PPP was prepared as the control plasma, and the above operation was repeated to obtain APTT, PT of the control group.

Establishment of mouse tumor model and rabbit liver tumor model. We performed animal experiments and in vivo experiments following the protocols approved by the institutional committee for animal care and the policy of the National Ministry of Health. All animal experiments for wound closure were performed following the guidelines of the National Regulations on Experimental Animals and with the approval of the Regional Ethics Committee for Animal Experiments established by the College of Life Sciences of Northwest Normal University. The New Zealand white rabbits (3-3.5 Kg in weight, n = 3 per group) and mice (female, weighing 15-18 g) were purchased from the Model Animal Research Center of The Cancer Hospital of Gansu province and Southeast University. All animals experimental procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of The Cancer Hospital of Gansu and Southeast University (license NO. SYXK-2016-0013).

Establishment of mouse tumor model. H22 cells (mouse hepatoma cells) were cultured under standard conditions. The H22 tumor models were generated by subcutaneous injection of 20000 cells in 200 uL of 1640 medium into the front right arm of the female mice. The 4 weeks old mice (18−22g,) were subcutaneously injected with 5×10^6 H22 cells/mouse in the front right arm. The tumor nodules had a volume of 0.6 cm^3 at 2 weeks post-injection.

The rabbit models of liver tumors were established by using VX2. five healthy New Zealand white rabbits (2.5-3 kg in weight) were injected with VX2 cells (1×108 cells/L, 500 μL) in PBS into the inner muscles of the left hind legs. After half a month, the tumor-bearing rabbit models were made, and a 2-3 cm mass could be felt at the injected site. Then, the muscle layer was cut to remove the tumor mass, and the fresh tissue was washed with normal saline to remove the necrotic part. The tumor masses were cut into 1 mm^3 for use. Randomly select 9 healthy New Zealand white rabbits (2.5-3 kg in weight, n = 3 per group), weigh them first, anesthetize them, fix them in the supine position and sterilize them, and implant a prepared tumor mass (1 mm^3) into each of the rabbit's liver lobes using surgical methods. After 14 days, use MRI to check the establishment of rabbit liver tumor models. Calculate the volume of the tumor from the measurement data of the MRI image (the largest diameter and the smallest diameter perpendicular to it).

SPECT/CT Imaging and MRI Imaging. SPECT/CT scans was carried out on day 13 after establishing VX2 tumor-bearing rabbit models. First, ^{131}I-*m*-

SiO_2@PGTTCs (150 mg/kg) was injected through tumor-bearing rabbits' ear vein and then to get SPECT/CT imaging of tumor-bearing rabbit models, the tomographic acquisitions were done on a combined SPECT/CT scanner (Discovery NM/CT 670PRO, Gansu Provincial Hospital) according to a previously described method 92. Each VX2-bearing New Zealand white rabbit was injected via ear vein with 37 MBq (1 mCi) of 131I-*m*-SiO_2@PGTTCs (n = 3/group), rabbits were anesthetized by intraperitoneal injection of veterinary anaesthetic Xylazine Hydochloride Injection (10% (v/v), 0.1 mL/kg) and placed in the prone position at the center of the field of view (FOV) of the scanner, and imaged using the SPECT/CT camera (8 h, 24 h). The following agreement is for SPECT Acquisition: 128 × 128 matrix, 360 ° SPECT with 6 ° steps, 30 s / frame, and a variable radius of rotation adapting to the closest possible distance from the phantom surface. For CT image acquisition, the scan parameters were 80 mA, 120 kV, 3.75 mm slice thickness, and a 4.12 mGy ctdivol. Both SPECT and CT data were reconstructed and displayed as transaxial and coronal slices using an Astonish bone application package (Discovery NM/CT 670PRO). The SPECT and CT fusion images were obtained using the Syntegra software (Discovery NM/CT 670PRO). Before getting DSA imaging the tumor-bearing rabbits MRI (Siemens MAGNETOM Skyra 3.0 T, Gansu Provincial Hospital) imaging were carried out to determine the exact location of the liver tumor. T2-SPACE-3D weighted MR images of rabbits with intraperitoneal tumor were acquired using a compact 3.0 T MRI system with a 2D-multislice sequence. The parameters were as follows:Resolution maxtrix =

384×376, Repetition time = 2400 ms, Echo time = 698 ms, Slice thickness =1.2 mm, and number of averages = 2.

DSA Imaging. Digital subtraction angiography (DSA, Siemens Bicor Top., Germany) imaging was carried out on day 14 after rabbit's VX2-tumor implantation. First, rabbit was anesthetized by intraperitoneal injection of veterinary anaesthetic Xylazine Hydrochloride Injection (10% (v/v), 0.1 mL/kg), and a 4F vascular sheath was then placed into the femoral artery (Terumo, Tokyo, Japan). Selective catheterization to the hepatic artery feeding carcinoma was achieved by utilizing a 2.7 F microcatheter (Terumo, Tokyo, Japan) under Siemens Bicor Top Digital Subtraction Angiography System (DSA), which can guarantee tumor targeted embolization and spared arterial flow through the hepatic artery into the liver. Subsequently, a mixture containing 30 mg MMs (100−150 μm) and moderate contrast agent (loversol, Yangzijiang Pharmaceutical Co., China), and then the pH-responsive composites *m*-SiO_2@PGTTCs (150 mg/kg) was carefully injected into tumor-bearing rabbits' ear vein after 8 h, 12 h use the same way to get DSA imaging of tumor-bearing rabbit models, Finally, the experiment carried out DSA imaging (control 0 h, 12 h, 24 h).

In vivo antitumor effect of rabbit. The embolization therapy experiment was carried out on day 14 after rabbit's VX2-tumor implantation, the rabbits were divided at random into two groups (n=3), the *m*-SiO_2@PGTTC-6.2 was administered at a dose of 150mg/kg in a physiological saline solution at a concentration of 10 mg/mL through the rabbit ear vein inject, and set a blank control group to inject the same volume of

normal saline. Experiments were carried out on 3 T MRI scanner (Siemens MAGNETOM Skyra 3.0 T, Gansu Provincial Hospital). In vivo MRI scans were performed on rabbit's VX2-tumor mode in the control and *m*-SiO_2@PGTTCs-treated groups during the treatment. T2-SPACE-3D weighted MR images of rabbits with intraperitoneal tumor were acquired using a compact 3.0 T MRI system with a 2D-multislice sequence. The parameters were as follows: Resolution maxtrix = 384×376, Repetition time = 2400ms, Echo time = 698ms, Slice thickness =1.2 mm, and number of averages = 2. Images from pre- and post-inject administration as well as those from control rabbits and/or therapy rabbits were compared to evaluate the evaluate the size of liver tumor.

Histology Analysis. After the animal experiment, the mice and rabbits were sacrificed to acquire the tumor tissues and main organs (heart, liver, spleen, lung and kidney), which were fixed with 4% paraformaldehyde. Then the fixed specimens were washed, dehydrated, embedded in paraffin, and sectioned with H&E for historical analysis. The Ki-67 kit was used to apoptosis of tumor cells, the CD31 assay kit was used to detect the tumor angiogenesis and tumor growth.

Table S1. The molecular mass and monomeric unit ratio of polypeptides.

Polymer	M_n [a] (kDa)	M_w [a] (kDa)	PDI [a]	M_n [b](kDa)	M_w [b](kDa)	PDI [b]	I:G:T:T:C [c]	I:G:T:T:C [d]	M_c [e] (kDa)
PGTTC-(4.3)	9.48	22.67	2.38	20.53	25.24	1.48	1:80:120:160:40	1:53:138:42:14	32.71
PGTTC-(6.2)	9.83	25.74	1.91	18.68	23.37	1.61	1:60:120:160:40	1:41:127:37:11	28.83

[a]Molecular mass and PDI were calculated via light scattering; [b]Molecular mass and PDI were calculated via GPC;[c] The feed ratio of monomers. [d] Polymer ratios of monomers observed by ^{1}H NMR. [e] Molecular mass determined based on the ratio of monomers observed by ^{1}H NMR.

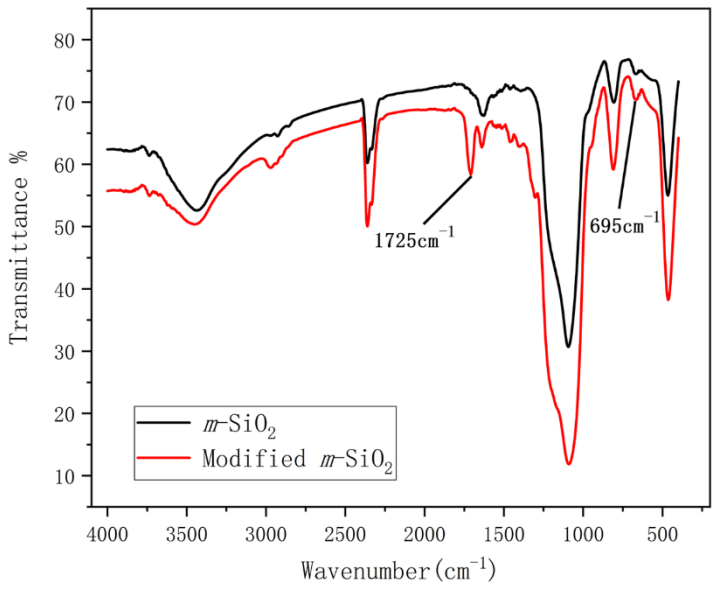

Figure S1 FTIR spectra of *m*-SiO_2 and modification *m*-SiO_2

In the spectrum, the peaks at 1100 cm^{-1} is attributed to the Si—O—Si band. Figure 3 displays the C=O band at 1725 cm^{-1} and the Si—C band at 695 cm^{-1}, which was the functional group of KH570, after modification with the KH570.

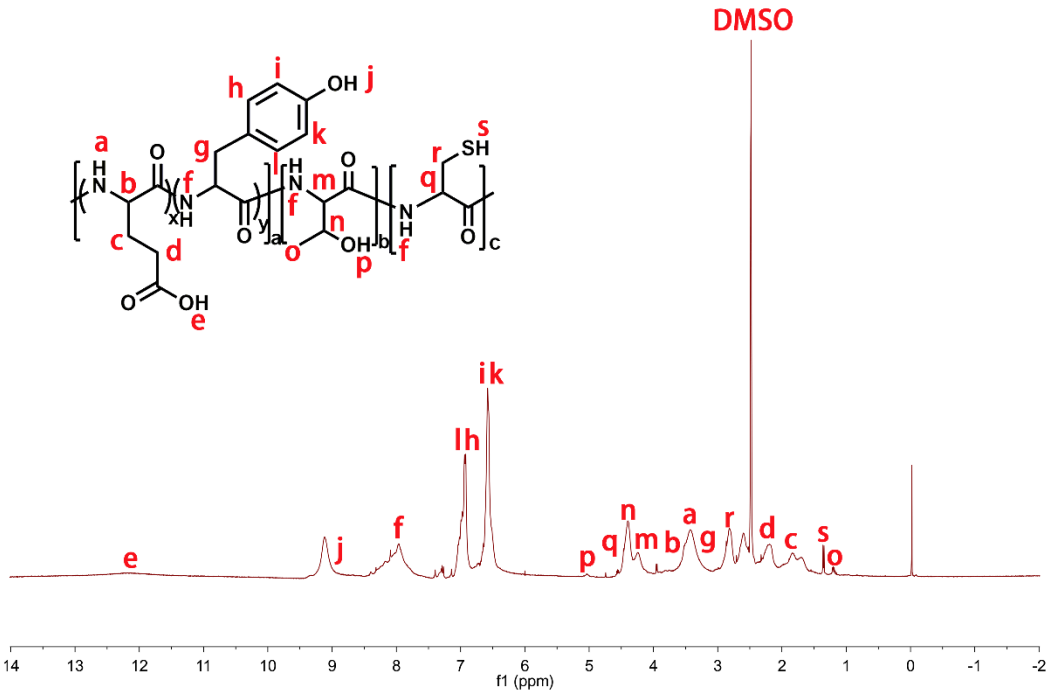

Figure S2 ^{1}H NMR of PGTTC-4.3

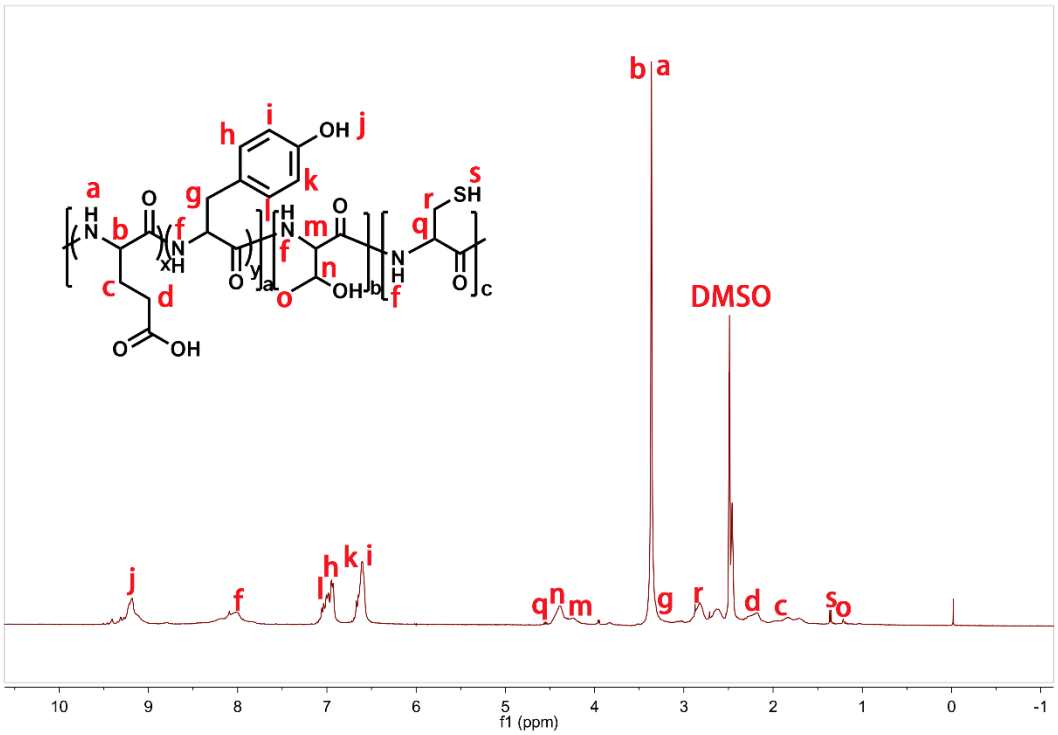

Figure S3 ^{1}H NMR of PGTTC-6.2

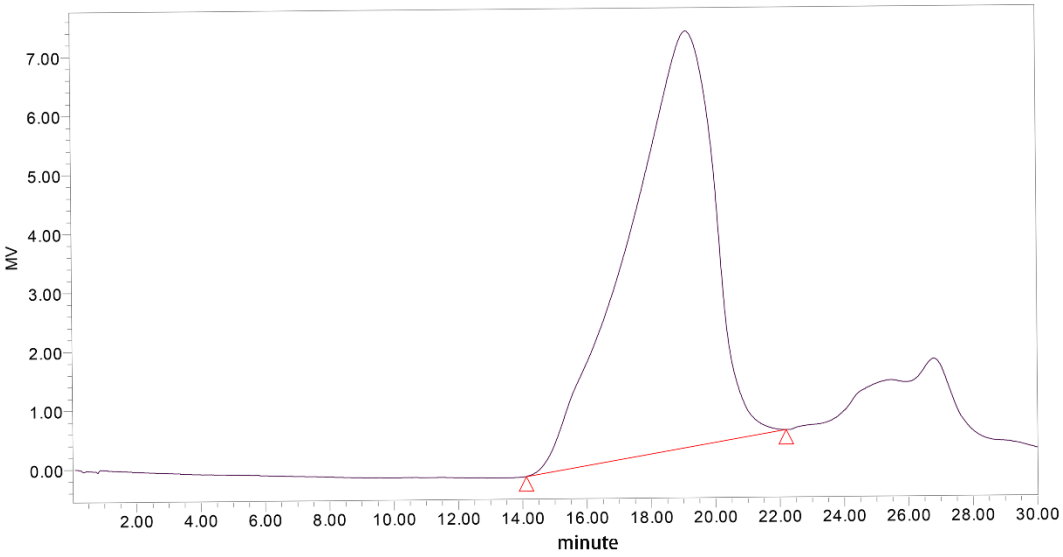

Figure S4 GPC of PGTTC-4.3

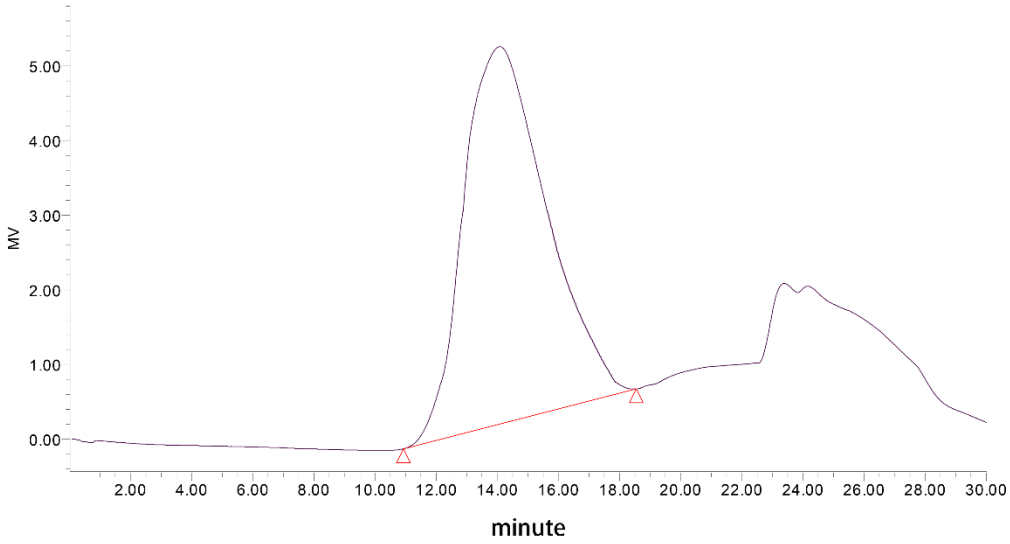

Figure S5 GPC of PGTTC-6.2

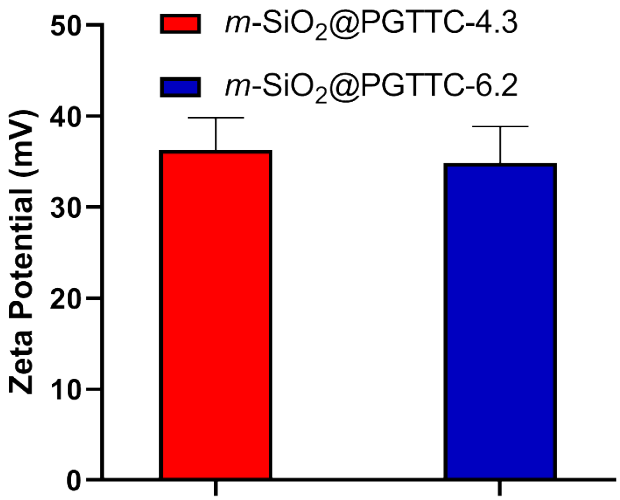

Figure S6. The Zeta Potentia of *m*-SiO_2@PGTTCs

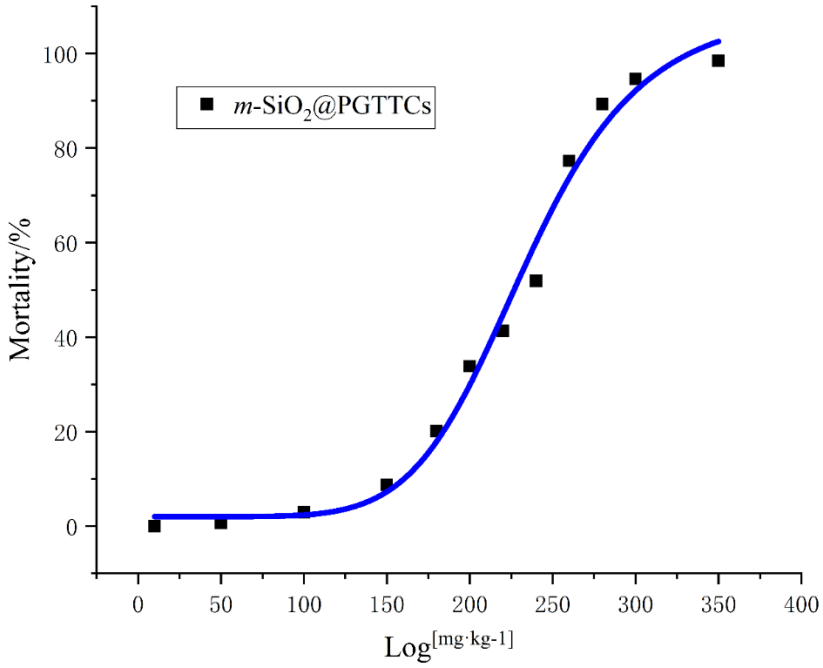

Figure S7. Dose acute toxicity evaluation (LD_{50}) of *m*-SiO_2@PGTTCs

Dose acute toxicity evaluation (LD_{50}) of *m*-SiO_2@PGTTCs，Nontumor-bearing mice were administered *m*-SiO_2@PGTTCs (10 animals per dose group) at 10, 50, 100, 150, 180, 200, 220, 240, 260, 280, 300, 350 mg/kg, the drugs were administered continuously through the tail vein for 5 days. Mortality data from tumor-free mice were plotted against dosage and curve-fit using Origin software.

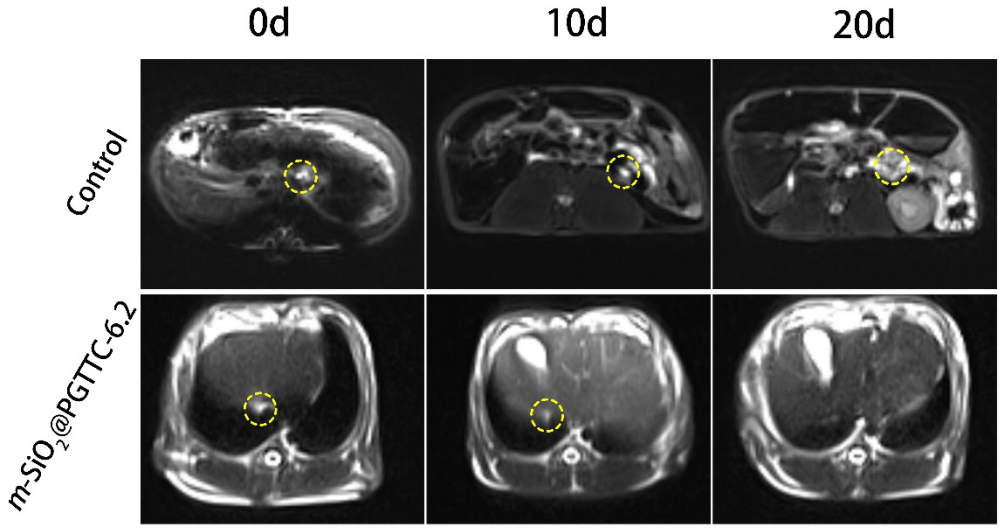

Figure S8. Higher resolution Figure 5E

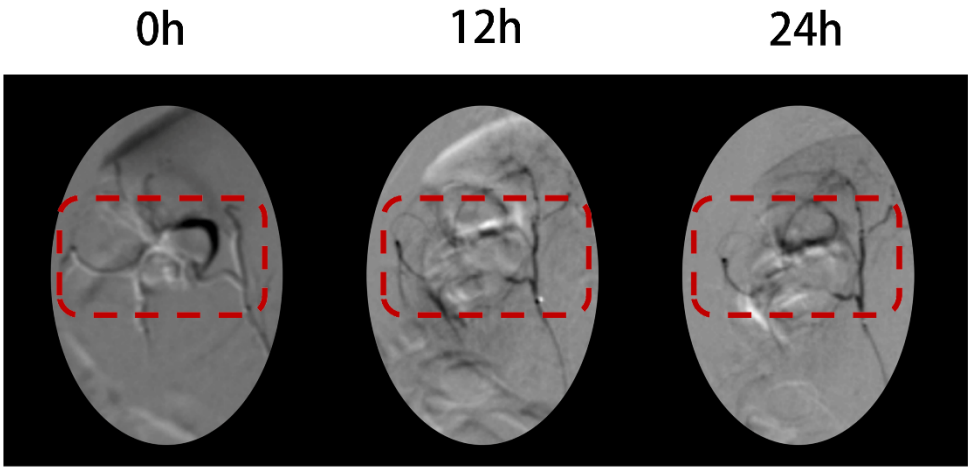

Figure S9. DSA imaging of the control group

DSA imaging of the liver-tumor of rabbits before and after embolization with *m*-SiO_2@PGTTC-4.3. It can be clearly seen that the shadow at the end of the vessel did not disappear after the injection of SiO_2@PGTTC-4.3, and when the angiography was performed again, it was clearly seen that the contrast agent could spread into the tumor, indicating that no embolism was formed in the control group.