Hybrid Polymeric Nanoprobes for Folate Receptor-targeted Photoacoustic Imaging In Vivo

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Supplementary Information

Materials

Methylene blue (MB) and butyl cyanoacrylate (BCA) was obtained from J&K and Pansine Chemical Co. Ltd., respectively, which were used for synthesis of various PBCA-based agents. Folic acid (FA) was obtained from Sigma. Dextran, acetic acid, MES, PBS, chitosan (CS), paraformaldehyde, penicillin, streptomycin, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS) and deuterated reagents (dimethyl sulfoxide-d_6 and acetonitrile-d_3) were obtained from Aladdin. DMEM, L15 medium and FBS were obtained from Sigma-Aldrich, which were used for cell culture. Isoflurane was purchased from RWD Life Science.

Fabrication of various PBCA-based nanoparticles

The synthetic protocols of PBCA-MB and PBCA-MB@CS were slightly modified as previously reported, starting from BCA monomer emulsification and in-situ encapsulation of MB.[S1,S2] Briefly, methylene blue (0.02 mmol) was dissolved in 100 mL of 0.5% dextran aqueous solution, and pH was adjusted to 2.0 with 1M HCl solution. Then, 1.0 mL of butyl cyanoacrylate was added dropwise into the mixture and magnetic stirring was maintained for 4 h under room temperature to afford PBCA-MB. To fabricate PBCA-MB@CS nanoparticles, 10mL of chitosan solution (80 mg/mL) in 0.3% acetic acid was mixed with PBCA-MB (1.0 mL) and magnetically stirred overnight for chitosan surface-coating. Excessive chitosan and un-embedded methylene blue were removed by washing with deionized water and centrifugation for at least 3 times.

Fabrication of hybrid polymeric nanoprobes PBCA-MB@CS-FA

Folic acid was covalently conjugated to PBCA-MB@CS to afford PBCA-MB@CS-FA via a traditional EDC-NHS activation-conjugation protocol.[S3,S4] Typically, PBCA-MB@CS were concentrated and re-suspended in PBS (pH = 7.4). Then folic acid was reacted with EDC (pH = 5.5) and NHS (pH = 7.2) in MES buffer at a ratio of 1:2:5 at room temperature in darkness. The activated FA-NHS ester was conjugated with PBCA-MB@CS in PBS for overnight stirring to get PBCA-MB@CS-FA. Excessive EDC and NHS were removed by washing with deionized water and repeated centrifugation for at least 3 times. The resulted hybrid FR-targeted probes PBCA-MB@CS-FA were dispersed in PBS and stored at 4℃.

Characterization

The average size and zeta potential of various PBCA-based nanoparticles were measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS, Malvern Instrument Inc., Worcestershire, UK). Three parallel measurements were analyzed to give the average size and zeta potential (Mean ± SD). The absorption spectra of free methylene blue and PBCA-MB@CS-FA were acquired on a UV-Vis spectrometer (Lambda 25, PerkinElmer, USA), and the photoluminescence (PL) emission spectra were obtained on a spectrofluorometer (FluoroMax-4C-L, Horiba Scientific Instrument Inc., USA) with progressively longer excitation wavelengths from 620 nm to 800 nm in 5 nm increments. The determination of FA labeling quantity on the nanoprobes was performed by UV-Vis spectrophotometry at a wavelength of 281 nm, and triplicate OD measurements of lyophilized nanoprobes dissolved in deionized water were obtained by referring to the as-acquired calibration line.[S5,S6] Atomic force microscopy was performed by using an Asylum Research MFP-3D (Oxford Instruments, USA). The confocal laser scanning microscope (CLSM, LSM 880, Carl Zeiss GmbH, Germany) was used for visualizing PBCA-MB and PBCA-MB@CS-FA, and evaluating their in vitro cell targeting capability under excitation at a wavelength of 680 nm. High-resolution images of surface morphology and internal structure of PBCA-MB and PBCA-MB@CS-FA were visualized on a scanning electron microscope (JSM-7610F, JEOL Ltd., Japan) and transmission electron microscope (JEM-2100F, JEOL Ltd., Japan), respectively. And energy dispersive spectroscopy (EDS, Xmax 80, Oxford Instruments, USA) was utilized to analyze their main elemental distribution and chemical composition. Fourier-transformed infrared spectrometry (FT-IR, Bio-Rad FTS-6000, Digilab Division, USA) and nuclear magnetic resonance (NMR, AVANCE III 600, Bruker, Germany) were further applied to identify the chemical structure of PBCA-MB@CS-FA. Different internal standard deuterated reagents (dimethyl sulfoxide-d_6 for folic acid and PBCA-MB@CS-FA, acetonitrile-d_3 for PBCA-MB) were used, and data were analyzed by Bruker Topspin software for the assignments of characteristic peaks (chemical shifts in ppm).

Phantom photoacoustic imaging

A series of PBCA-MB@CS-FA samples with different embedded MB concentrations (3-78 mM) were put in 1.5 mL Eppendorf tubes. These tubes were located in a photoacoustic imaging system (Nexus128, Endra, USA) with water immersed at the bottom and excited by a laser at the wavelength of 680 nm. An ultrasound transducer was fixed over the samples to receive the photoacoustic signals. PA images were then reconstructed, and PA signal intensity was recorded and analyzed by a built-in software to fit a linear correlation.

Cell cultures

Human breast cancer cells MDA-MB231 (FR-positive) and human glioblastoma cells U87MG (FR-negative) were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. MDA-MB231 and U87MG cells were cultured with L15 and DMEM media supplemented with 10% FBS, 50 IU/mL penicillin and 50 μg/mL streptomycin, respectively. All of the cells were cultured at 37℃ in a humidified environment of 5% CO_2.

In vitro cellular studies

MDA-MB231 cells were seeded in 60 mm cell tissue-culture plate at a density of 3×10^5 per well. After 24 h incubation, the plate was washed twice to remove the non-adherent cells and replaced with fresh medium containing targeted PBCA-MB@CS-FA (100 μL) and non-targeted PBCA-MB (100 μL) at an identical MB concentration (50 mg/L) respectively. For FA block experiment, the plate was given 1.0 mL FA (0.05 mM) solution before PBCA-MB@CS-FA was introduced. After further incubation for 4 h, all the cells were washed with PBS for 3 times after incubation with nanoprobes so as to remove the free nanoprobes in the solution. Each group of cells was fixed with 4% paraformaldehyde. Confocal laser scanning microscopy (excitation: 640 nm; emission: 670 nm) and triplicate flow cytometry (FC500-MPL, Beckman Coulter, USA) measurements (excitation: 633 nm; emission: 670 nm) were performed to evaluate the cell targeting capability. The U87MG cell experiments were completed following a similar operational protocol as above-mentioned MDA-MB231.

In vivo photoacoustic imaging

Female tumor-bearing nude mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. To generate the MDA-MB231/U87MG tumor model, 1.0×10^6 tumor cells (MDA-MB231/U87MG) in a 100 μL solution were subcutaneously injected into the flank area of each nude mouse. All animal experimental procedures have been approved by the Administrative Panel of Wenzhou Institute of Biomaterials and Engineering, Wenzhou Medical University, and these methods were carried out in accordance with the animal experimental guidelines of Wenzhou Medical University. Animals were used for in vivo experiments when the tumors size reached approximately 100 mm^3 after tumor-cell inoculation. For MDA-MB231 tumor-bearing nude mice, 100 μL of freshly prepared non-targeted PBCA-MB and targeted

PBCA-MB@CS-FA at an identical MB concentration (100 mg/L) was subcutaneously injected close the tumors, respectively. The mice were fully anesthetized with isoflurane (1.5% mixed with oxygen). An in vivo photoacoustic small-animal imaging system (LAZR, Visualsonics, Canada) with a transducer (LZ400, Visualsonics, Canada) frequency of 30 MHz at laser wavelength of 680 nm was conducted to capture merged PA and ultrasound images at different time points post-injection. A defined tumor area of 28 mm^2 was selected as the region of interest (ROI) for PA imaging and data analysis. PA signal intensity was analyzed with a built-in software (VevoLAB, Visualsonics, Canada) to display the intensity-time curve for each probe. The U87MG tumor-xenografted nude mice experiments were completed following a similar operational protocol as above-mentioned MDA-MB231.

Reference

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Tables and Figures

Table S1 Dynamic light scattering (DLS) analysis of size and zeta potential of as-prepared PBCA, PBCA-MB and PBCA-MB@CS-FA.

Probe	Diameter (nm, Mean ± SD)	Zeta Potential (mV, Mean ± SD)
PBCA	290 ± 0.3	-13 ± 0.3
PBCA-MB	530 ± 0.2	-40 ± 0.2
PBCA-MB@CS	778 ± 0.1	3.0 ± 0.6
PBCA-MB@CS-FA	700 ± 0.2	2.0 ± 0.2

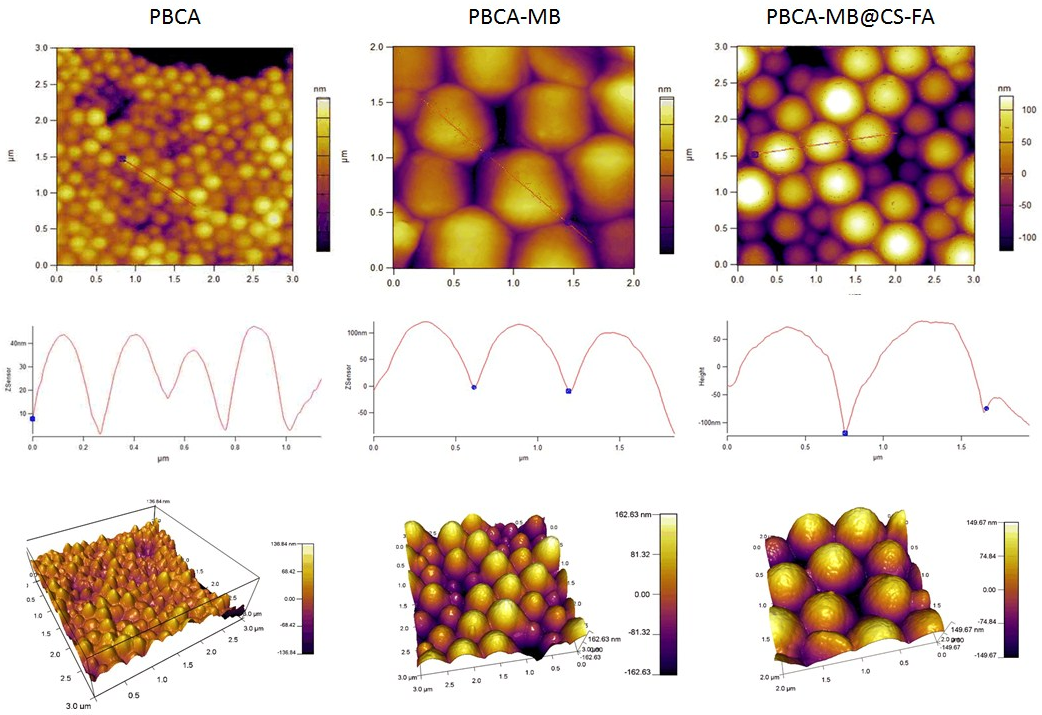
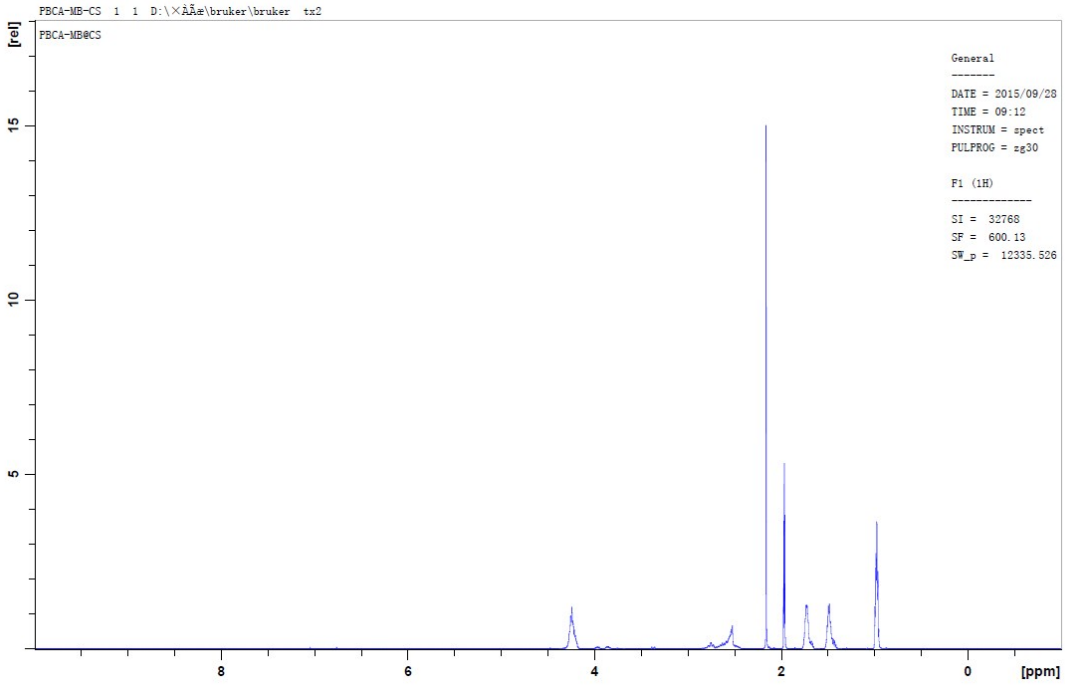


Fig. S1 AFM analysis of PBCA, PBCA-MB and PBCA-MB@CS-FA, respectively. 2D Planar images (first row), cross-section analysis (second row) and 3D images (third row) were represented to show the size and morphology of various PBCA-based nanoparticles.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2
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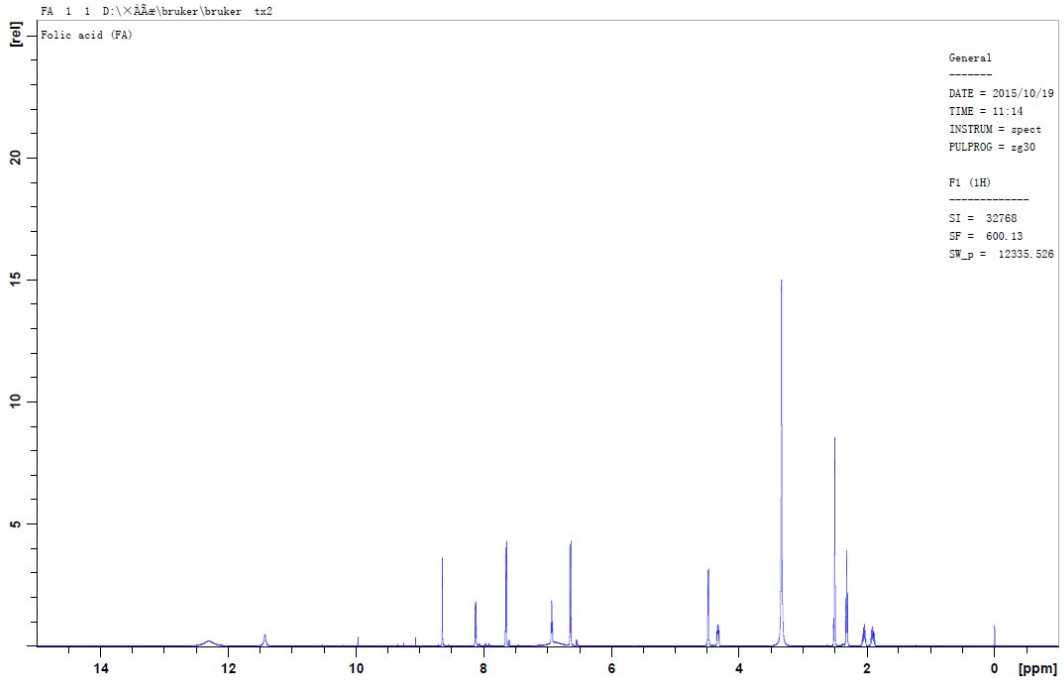
(a)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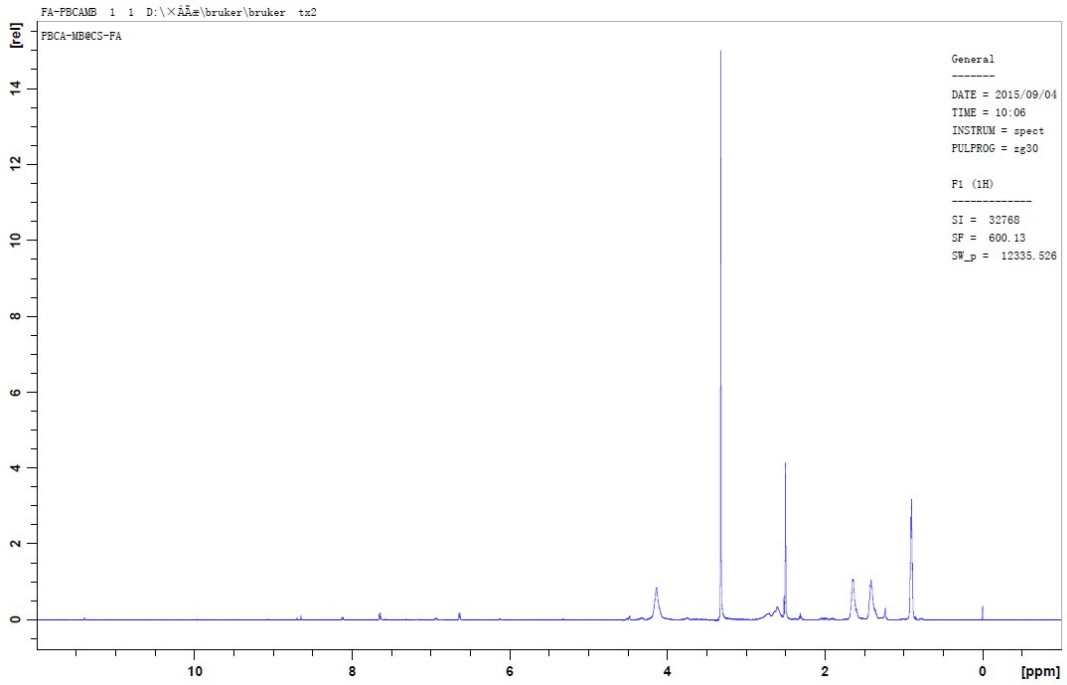
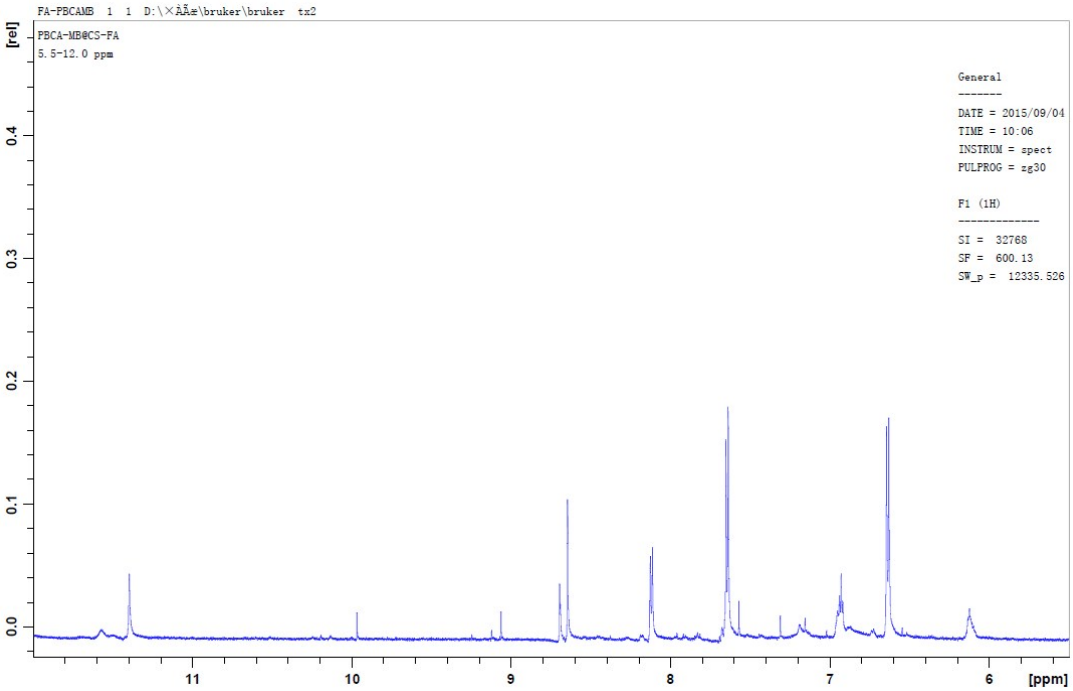
(b)



(c)



(d)

Fig. S2 NMR spectra of PBCA-MB@CS (a), free folic acid (FA) (b) and PBCA-MB@CS-FA (c and d for magnified region of 5.5-12.0 ppm).

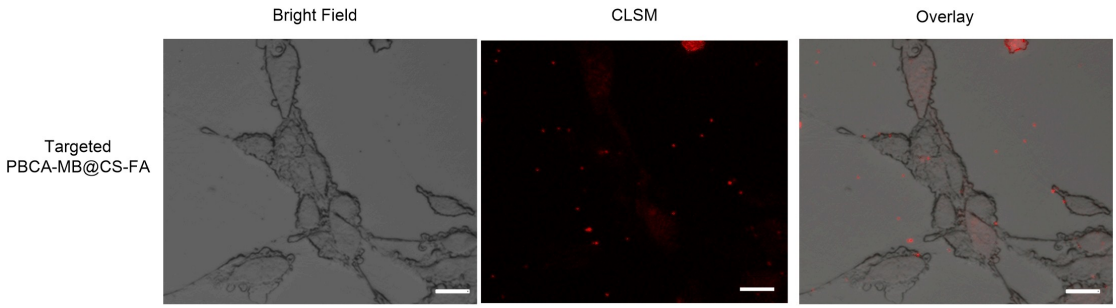


Fig. S3 Evaluation of hybrid nanoprobes PBCA-MB@CS-FA for U87MG cell targeting in vitro and characterization by confocal laser scanning microscopy. (Scale bar: 20 μm)

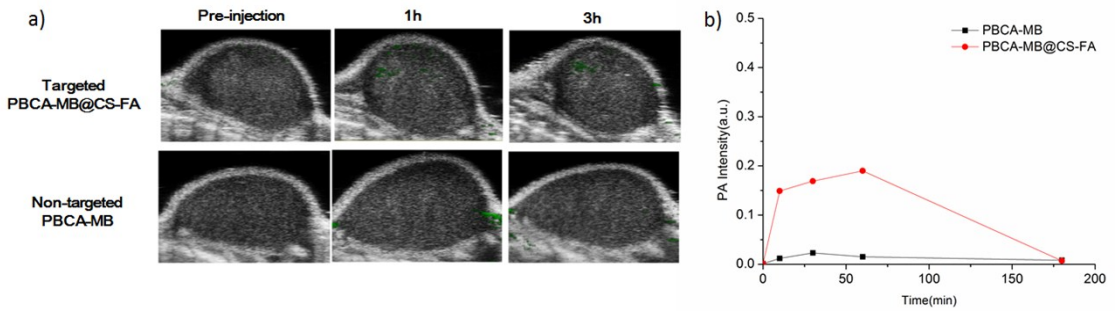


Fig. S4 In vivo PA imaging for breast cancer targeting using U87MG tumor-xenografted nude mice. (a) Merged PA and ultrasound images displayed tumor targeting and accumulation at time points of 1 h and 3 h after subcutaneous injection of targeted PBCA-MB@CS-FA and non-targeted PBCA-MB; (b) The quantitative analysis of PA signal intensity in the tumors at different time points post-injection.