Supplementary Information Available

Folic Acid-functionalized Two-photon Absorbing Nanoparticles for Targeted MCF-7 Cancer Cell Imaging

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Experimental Section

1. Synthesis and Characterization

1.1 Materials

Multibranched triarylamine end-capped triazine (**T1**) was synthesized according to literature. [1] 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was a gift from Lipoid GmbH (Ludwigshafen, Germany). DSPE-PEG_{5000}-Folate was a commercial product of Avanti Polar Lipids, Inc. Tetrahydrofuran (THF), 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin-streptomycin solution, and trypsin-EDTA solution were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from Gibco (Lige Technologies, Ag, Switzerland). Milli-Q water was supplied

[1] Y. Jiang, Y. C. Wang, J. L. Hua, J. Tang, B. Li, S. X. Qian and H. Tian, *Chem. Commun.*, 2010, **46**, 4689-4691.

by Milli-Q Plus System (Millipore Corporation, Breford, USA). MCF-7 breast cancer cells and NIH/3T3 fibroblast cells were provided by American Type Culture Collection.

1.2 Synthesis of T1 doped NP

A THF solution (0.5 mL) containing 1 mg of **T1** and 3 mg of mixture of DSPE-PEG_{2000} and DSPE-PEG_{5000}-Folate (molar percentage ratio of DSPE-PEG_{5000}-Folate was 0%, 20%, 40%, and 60%, respectively) was poured into 10 mL of 90% (v/v) water/THF solution. This was followed by sonicating the mixture for 60 seconds at 12 W output using a microtip probe sonicator (XL2000, Misonix Incorporated, NY). The emulsion was then stirred at room temperature overnight to evaporate THF. FTNP0, FTNP20, FTNP40, and FTNP60 are assigned to FTNPs prepared with different molar percentages of DSPE-PEG_{5000}-Folate. The preparation of bare **T1** NPs without encapsulation matrix was carried out using the same method for the preparation of FTNPs.

1.3 Characterization Methods

The UV-Vis spectra of FTNP and BTNP aqueous suspensions were recorded on a Shimadzu UV-1700 spectrometer. The fluorescence spectra were measured using a fluorometer (LS-55, Perkin Elmer, USA). Average particle size and size distribution of the NPs were determined by laser light scattering (LLS) with particle size analyzer (90 Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90° at room temperature. The morphology of NPs was also studied by high-resolution transmission electron microscope (HR-TEM, JEM-2010F, JEOL, Japan).

2. Two-photon Absorption Measurements

TPA spectra were measured using two-photon induced fluorescence (TPIF) spectroscopy.[2] The samples were excited with laser pulses of 100 fs produced by the mode-locked Ti:Sapphire laser (Spectraphysics Tsunami) with a repetition rate of 82 MHz, and a

[2] C. Xu and W. W. Webb, *J. Opt. Soc. Am. B*, 1996, **13,** 481-491.

femtosecond optical parametric amplifier (OPA) was used at 800 nm. The emission from FTNP0 and BTNP aqueous suspensions was collected at a 90° angle by a high numerical aperture lens and directed to a spectrometer's entrance slit. The concentration of **T1** in the aqueous suspensions was 10 μM. Rhodamine 6G in methanol was used as a reference. TPA cross section was calculated from equation:[3]

$$\frac{\delta_2}{\delta_1} = \frac{F_2 \eta_1 c_1 n_1}{F_1 \eta_2 c_2 n_2}$$

Where δ_1 and δ_2 are the TPA cross section, F_1 and F_2 are the TPIF intensities, η_1 and η_2 are the fluorescence quantum yields, c_1 and c_2 are the concentrations, n_1 and n_2 are the refractive indexes of solvents (1 corresponds to Rhodamine 6G, 2 is used for FTNP0 or BTNPs).

3. Targeted Cellular Imaging

3.1 Cell Culture

MCF-7 breast cancer cells and NIH/3T3 fibroblast cells were cultured in folate-free Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO_2. Before experiment, the cells were pre-cultured until confluence was reached.

3.2 Cell imaging

MCF-7 breast cancer cells were cultured in the confocal imaging chambers (LAB-TEK, Chambered Coverglass System) at 37 °C. After 80% confluence, the medium was removed and the adherent cells were washed twice with 1× PBS buffer. The BTNPs, FTNP0, FTNP20, FTNP40, and FTNP60 in FBS-free DMEM medium at 1 μM **T1** were then added to the chambers, respectively. After incubation for 2 h, the cells were washed

[3] D. A. Oulianov, I. V. Tomov, A. S. Dvornikov and P. M. Rentzepis, *Opt. Commun.*, 2001, **191**, 235-243.

three times with 1× PBS buffer and then fixed by 75% ethanol for 20 minutes, which were further washed twice with 1× PBS buffer and stained by DAPI for 10 min. The cell monolayer was then washed twice with 1× PBS buffer and imaged by confocal laser scanning microscope (CLSM, Zeiss LSM 410, Jena, Germany) with imaging software (Olympus Fluoview FV1000) under the same experimental condition. The fluorescence signal from FTNPs was collected at 488 nm excitation (2.5 mW) with a 505 nm longpass barrier filter. NIH/3T3 fibroblast cells incubated with BTNPs, FTNP0, and FTNP40 were also studied following the same procedures. Two-photon fluorescence images of MCF-7 cancer cells after incubation with FTNP0 and FTNP40 were studied by laser scanning microscope (Olympus Fluoview FV300) equipped with two-photon Chameleon, respectively. The detection of TPA fluorescence is achieved by excitation at 800 nm with a 505 nm longpass barrier filter.

3.3 Cytotoxicity of FTNP40

Methylthiazolyldiphenyl-tetrazolium (MTT) assays were performed to assess the metabolic activity of MCF-7 cancer cells. MCF-7 cells were seeded in 96-well plates (Costar, IL, USA) at an intensity of 4×10^4 cells/mL. After 24 h incubation, the medium was replaced by the FTNP40 suspension at different **T1** concentration, and the cells were then incubated for 24, 48, and 72 h, respectively. After the designated time intervals, the wells were washed twice with 1×PBS buffer and 100 μL of freshly prepared MTT (0.5 mg/mL) solution in culture medium was added into each well. The MTT medium solution was carefully removed after 3 h incubation in the incubator. DMSO (100 μL) was then added into each well and the plate was gently shaken for 10 minutes at room temperature to dissolve all the precipitates formed. The absorbance of MTT at 570 nm was monitored by the microplate reader (Genios Tecan). Cell viability was expressed by the ratio of

absorbance of the cells incubated with FTNP40 suspension to that of the cells incubated with culture medium only.

Tables and Figures

Table S1. Characteristics of the FTNPs and BTNPs in water.

	FTNP0	FTNP20	FTNP40	FTNP60	BTNPs
Size (nm)	87 ± 2	83 ± 2	85 ± 3	88 ± 2	132 ± 4
Quantum yield	0.24	0.24	0.25	0.24	0.25
λ_{abs} (nm)	353	353	354	352	352
λ_{em} (nm)	601	600	602	600	599

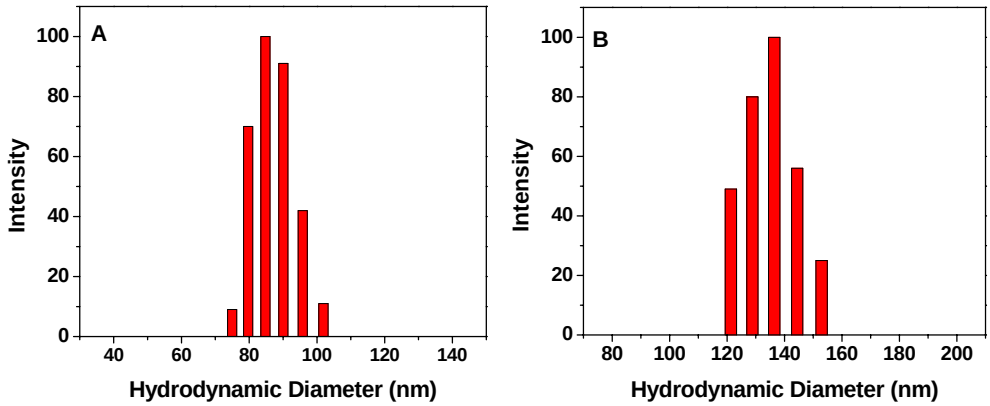

Figure S1. Particle size distribution of FTNP0 and BTNPs in water.

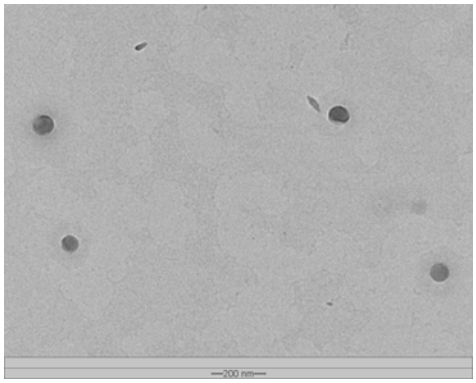

Figure S2. HR-TEM image of BTNPs.

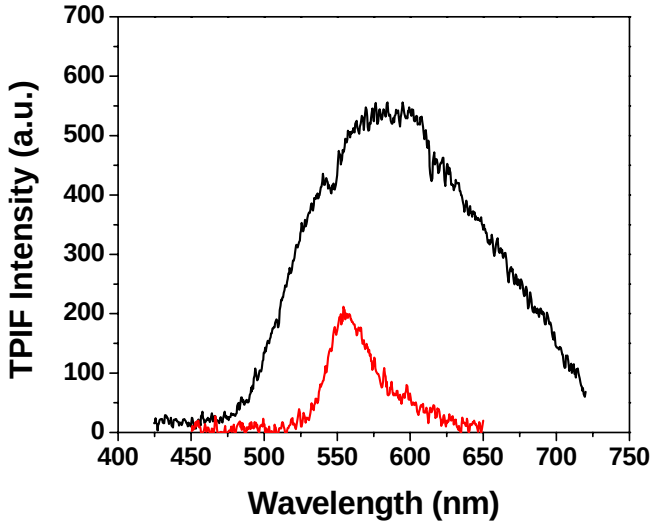

Figure S3. Two-photon induced fluorescence spectrum of FTNP0 in aqueous suspension (black) and Rhodamine 6G in methanol (red) excited at 800 nm, [**T1] = [Rhodamine]** = 10 μM.

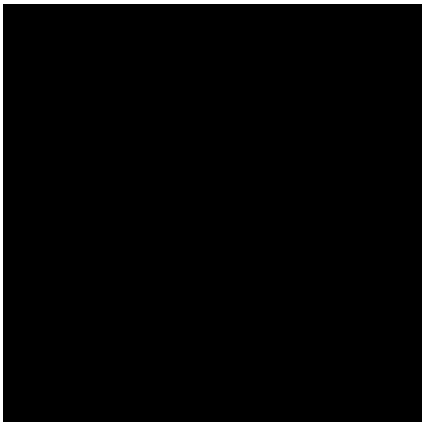

Figure S4. CLSM image the MCF-7 cancer cells without FTNP incubation.

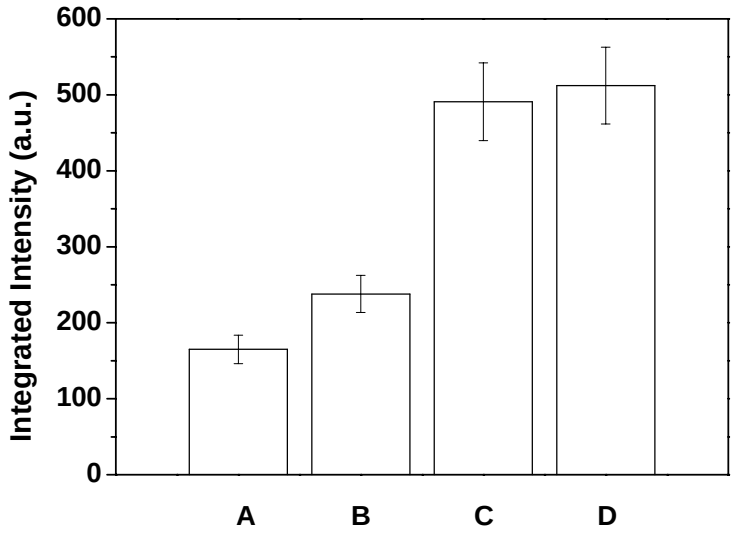

Figure S5. Integrated intensity of individual MCF-7 cancer cell after incubation with (A) FTNP0, (B) FTNP20, (C) FTNP40 and (D) FTNP60, respectively. Confocal images were processed by ImageJ and ten cells with similar size were analyzed individually for each sample.

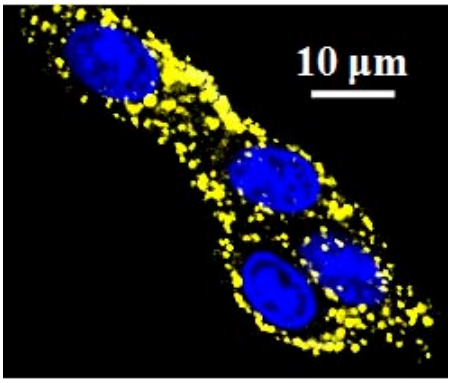

Figure S6. Enlarged CLSM image of the MCF-7 cancer cells after incubation with FTNP40 for 2 h at 37 °C ([**T1**] = 1 µM, λ_{max} = 488 nm).

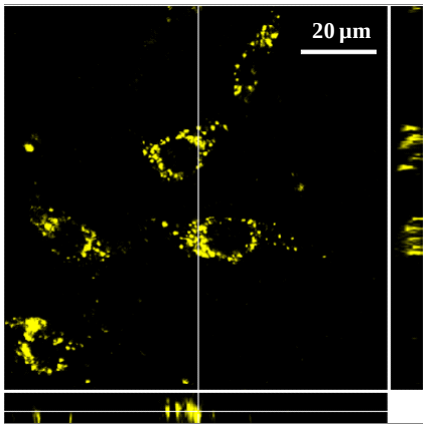

Figure S7. 3D CLSM image the MCF-7 cancer cells after incubation with FTNP40 for 2 h at 37 °C ([**T1**] = 1 µM, λ_{max} = 488 nm).

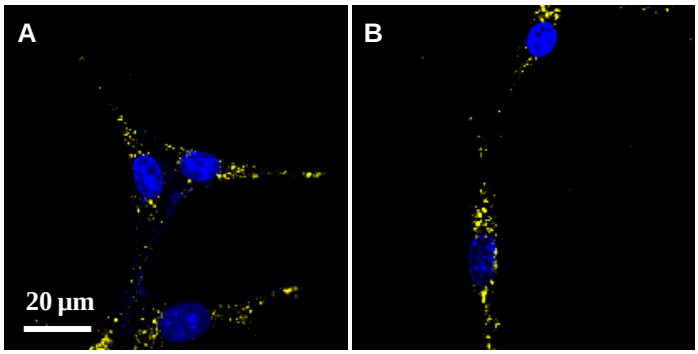

Figure S8. CLSM images of NIH/3T3 fibroblast cells after incubation with FTNP0 (A) and FTNP40 (B) for 2 h at 37 °C ([**T1**] = 1 μM). The fluorescence of FTNPs was recorded under excitation at 488 nm with a 505 nm longpass barrier filter. The blue signal indicates cell nuclei stained by DAPI. The scale bar is the same for images A and B.

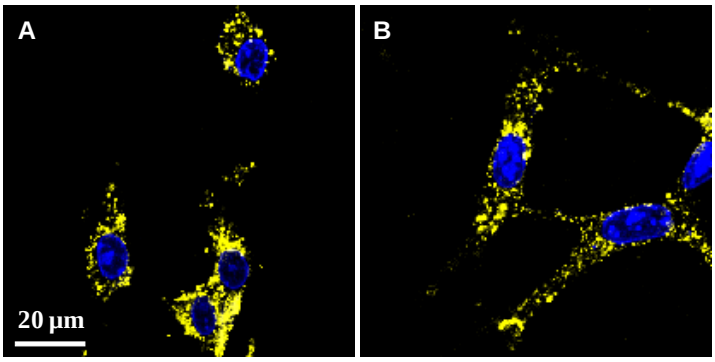

Figure S9. CLSM images of MCF-7 breast cancer cells (A) and NIH/3T3 fibroblast cells (B) after incubation with BTNPs for 2 h at 37 °C ([**T1**] = 1 μM). The fluorescence of BTNPs was recorded under excitation at 488 nm with a 505 nm longpass barrier filter. The blue fluorescence indicates cell nuclei stained by DAPI. The scale bars of images A and B are the same.

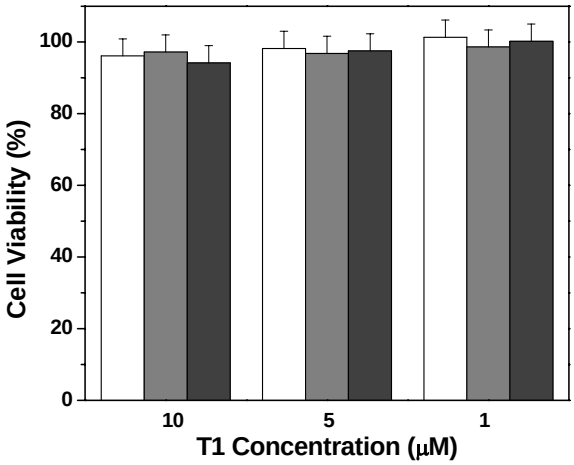

Figure S10. Metabolic viability of MCF-7 breast cancer cells after incubation with FTNP40 suspension at different **T1** concentrations for 24 (blank), 48 (gray), or 72 h (dark gray), respectively.