Targeted Polypyrrole Nanoparticles for Identification and Treatment of Hepatocellular Carcinoma

Yushen Jin,[a,b] Xin Yang[a,b] and Jie Tian[a,b,c,d*]

[a]CAS Key Laboratory of Molecular Imaging, Institute of Automation, Chinese Academy of Sciences, Beijing 100190, China

[b]Beijing Key Laboratory Molecular Imaging, Beijing 100190, China

[c] The State Key Laboratory of Management and Control for Complex Systems, Institute of Automation, Chinese Academy of Sciences, Beijing 100190, China

[d] School of Artificial Intelligence, University of Chinese Academy of Sciences, Beijing, 100080, China

Author Information

Corresponding author:

*Email: jie.tian@ia.ac.cn

Supplementary Information

Materials and Methods

1.1 Materials

N-hydroxysuccinimide (NHS), N-[3-(dimethylamino)propyl]-N′-ethylcarbodiimide hydrochloride (EDC) and $FeCl_3 \cdot 6H_2O$ were purchased from Aladdin Reagent (Shanghai, China). Fluorescein isothiocyanate (FITC) were obtained from J&K Scientific Ltd (Beijing, China). Bovine serum albumin (BSA), pyrrole monomer and ICG were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM (high glucose), penicillin/streptomycin, trypsin and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Logan, Utah, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was received from Nanjing KeyGEN Biotech (Nanjing, China). SP94 peptide (Sequence: SFSIIHTPILPL) was purchased from ChinaPeptides Co., Ltd (Shanghai, China). Deionized water (DI water) was obtained from a Millipore water purification system with a resistivity of 18.2 $\Omega \cdot cm^{-2}$.

1.2 Synthesis and characterization of SP94 modified PPy-BSA-ICG nanoparticles

The PPy-BSA nanoparticles were prepared via a chemical oxidation polymerization method.[1,2] Briefly, 20 mL of bovine serum albumin (BSA, 37.5 mg/mL) aqueous solution and 230 mmol $FeCl_3 \cdot 6H_2O$ aqueous solution were mixed with vigorous stirring at room temperature to obtain a yellow solution. After one hour to allow equilibration，140 μL pyrrole monomer was introduced into the mixture and

the polymerization proceeded at 4 ^{o}C for 24 h. The obtained PPy-BSA nanoparticles was purified by a PD10 column using DI water as an eluent.

Then, the PPy-BSA nanoparticles were conjugated with SP94 peptide. First, the SP94 peptide were active by EDC and NHS for 20 min. The solution was then mixed with 3.2 mmol of PPy-BSA nanoparticles solution for another 12 h. And then the excess SP94 peptide and impurity were removed through centrifugation (14000 rpm, 10 min) for three times.

Finally, the ICG were loaded into the SP94 modified PPy-BSA nanoparticles. Briefly, ICG solutions (500 μL, 1.0mg/mL) were added to the SP94 modified PPy-BSA nanoparticles (10 mL, 72 mg/mL) and the mixture were shaken for 12 h at 25 oC. The obtained nanoparticles were purified through centrifugation to remove the unadsorbed ICG. The amount of ICG in the supernatant were determined through measuring the fluorescence intensity at 816 nm. The loading ratio of ICG to the final nanoparticles is expressed as:

$$Loading\ Ratio = \frac{M_{ICG} - M_{unadsorbed\ ICG}}{M_{SP94\,\mathrm{mod}ified\ CuS-BSA-ICG}} \times 100\%$$

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where MICG, Munadsorbed ICG and MSP94 modified PPy-BSA-ICG are the mass of ICG, unadsorbed ICG and SP94 modified PPy-BSA-ICG, respectively.

Transmission electron microscopy (TEM) was used to observe and measure the particle size morphology of the SP94 modified PPy-BSA-ICG nanoparticles. The UV-vis absorption spectra and fluorescence spectra were measured with a Perkin-Elmer Lambda 25 spectrophotometer (Perkin Elmer, USA).

1.3 Flow cytometry assay

Hep3B cells were seed onto 6-well plate (4×10^5 cells/well) and incubated in the cell culture incubator overnight. The next day, the cells were treated with the different concentration of SP94 modified PPy-BSA-FITC nanoparticles or PPy-BSA-FITC nanoparticles and incubated for another 4 h. Cells were next digested with 0.25% trypsin-EDTA, and washed with PBS buffer solutions for three times. Finally, the cells were analyzed by flow cytometry (BD Accuri C6, USA).

Confocal microscopy

Hep3B cells were seed onto confocal dish (4×10^5 cells/well). After overnight, the cells were treated with SP94 modified PPy-BSA-FITC nanoparticles or PPy-BSA-FITC nanoparticles (5 μg/mL) and incubated for an additional 4 h. Cells were next fixed in 4% paraformaldehyde followed by rinsed in PBS (3×for 5 min each). And further the cell membrane and nucleus were stained with DiI and DAPI, respectively. Finally, the targeted ability and distribution of SP94 modified PPy-BSA-FITC nanoparticles were observed through a Zeiss LSM710 laser scanning confocal microscope (Carl Zeiss, Germany) equipped with a variety of laser.

In vitro photothermal effect

To evaluate the photothermal behavior induced by SP94 modified PPy-BSA-FITC nanoparticles, 3mL aqueous solution of SP94 modified PPy-BSA-FITC nanoparticles at different concentrations was irradiated using a NIR laser (808 nm, 0.5 W/cm^2) for 5 min. The temperature was measured using a fiber-optic temperature measuring system submerged in the solution. The temperature evaluation of a solution of 17.4 μg/mL SP94 modified PPy-BSA-FITC nanoparticles over four laser ON/OFF

cycles of NIR irradiation (808 nm, 0.5 W/cm^2) was measured to assess the photothermal stability of the SP94 modified PPy-BSA-FITC nanoparticles.

1.4 In vitro biocompatibility

To evaluate the biocompatibility at the cellular level of the nanoparticles, HUVEC cells (1×10^4) were incubated with different concentrations of SP94 modified PPy-BSA-FITC nanoparticles for 24 hours, and then washed with PBS twice. The cell viability was evaluated using standard MTT assay.

In vitro photothermal cytotoxicity

To qualitatively evaluate the local photothermal cytotoxicity, Hep3B cells (4×10^5 cells/well, 6-well plate) were incubated with SP94 modified PPy-BSA-FITC nanoparticles or PPy-BSA-FITC nanoparticles for 4 hours, and then washed with PBS buffer solutions (10 mM, pH 7.4) for three times, followed by NIR laser irradiation for 10 minutes (808 nm, 0.5 W/cm^2). After 30 minutes, the cells were stained with calcein-AM and propidium iodide (PI), then observed with a fluorescence microscope to evaluate the phothermal effect of the nanoparticles on cancer cells.[1,3,4]

To further quantitatively evaluate the local photothermal cytotoxicity, Hep3B cells ($1\times$104 cells/well, 96-well plate) were incubated with different concentrations of SP94 modified PPy-BSA-FITC nanoparticles or PPy-BSA-FITC nanoparticles for 4 hours, and then washed with PBS for three times, followed by laser irradiation for 10 minutes (808 nm, 0.5 W/cm^2). After 4 hours, the cell viability was evaluated using standard MTT assay.

1.5 Animal models

Male Balb/c nude mice at 6-8 weeks old were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. To establish HCC tumor model, Hep3B cells (5×10^6) were injected into the hips or shoulders of the mice subcutaneously. All the animal studies in our experiments were all in accordance with the institutional animal care and use committee of Peking University approved protocols.

1.6 Photoacoustic imaging

The in vivo photoacoustic imaging was carried out on a commercial MOST InVision128 PA tomography system (iThera Medical, Germany). The system houses 128 unfocused ultrasound transducers (with 5 MHz center frequency and 3 mm diameter) arranged in a hemispherical bowl filled with water and a temperature monitor of the water bath. We optimized the imaging protocol to suit our experiments using 200 views and 100 pulses per view, which would take 5 in 30 s to acquire one data set. Before intravenously administration of SP94 modified PPy-BSA-ICG nanoparticles, we first obtained pre-contrast data with the spectra of the nanoparticles. Then, post-contrast data were acquired at 12 h and 24 h after intravenously administration of SP94 modified PPy-BSA-ICG nanoparticles with the spectra of the nanoparticles (n = 4). The photoacoustic images were reconstructed off-line using data acquired from all 128 transducers at each view through a modified back-projection algorithm, and the algorithm corrects for pulse to pulse variations in laser intensity and small changes in the temperature that affect acoustic velocity in the water.

1.7 Fluorescence detection

To confirm the specific localization of the SP94 modified PPy-BSA-ICG nanoparticles, animals with xenografts of approximately 2-5 mm in length were injected intravenously with SP94 modified PPy-BSA-ICG nanoparticles and the fluorescence images of the mice were obtained at different time point post-injection using an IVIS spectrum imaging system. All experiments were repeated at least four times.

1.8 In vivo photothermal efficacy

To examine the chemo-thermal treatment efficacy, SP94 modified PPy-BSA-ICG nanoparticles were injected into the mice bearing Hep3B tumor (approximately 500 mm^3) by intravenous administration (10 mg/kg) (n = 7). After 24 hours, tumors were irradiated with NIR laser (808 nm, 0.5 W/cm^2) for 5 min, and simultaneously images were taken using an infrared camera (FlukeTi25, USA) before and after the laser irradiation. In addition, bioluminescent images of the tumor-bearing mice were obtained, and the bioluminescent intensity and the tumor volume were calculated to evaluate the photothermal efficacy on day 0, 3, 6, 9, 12, 15, 18 and 21.

$$\text{Tumor volume} = \frac{\text{tumor length} \times (\text{tumor width})^2}{2}$$

The percentage of the tumor growth inhibition (TGI) was calculated from the relative tumor volume on day 15 according to the formulation:

$$\text{TGI}\ (\%) = \left(1 - \frac{\text{mean tumor volume in experimental groups}}{\text{mean tumor volume in untreated groups}}\right) \times 100\%$$

1.9 Histopathological analysis

In the photothermal efficacy experiments, the mice were sacrificed by carbon dioxide euthanasia on day 21 or when the tumor volume measured over 2000 mm^3.

1.10 Statistical analysis

All data are presented as the mean ± SD. All the tests were repeated at least three times. Statistical analyses were performed using one-way ANOVA or the Student's test. A P value <0.05 was considered significant (SPSS 13.0).

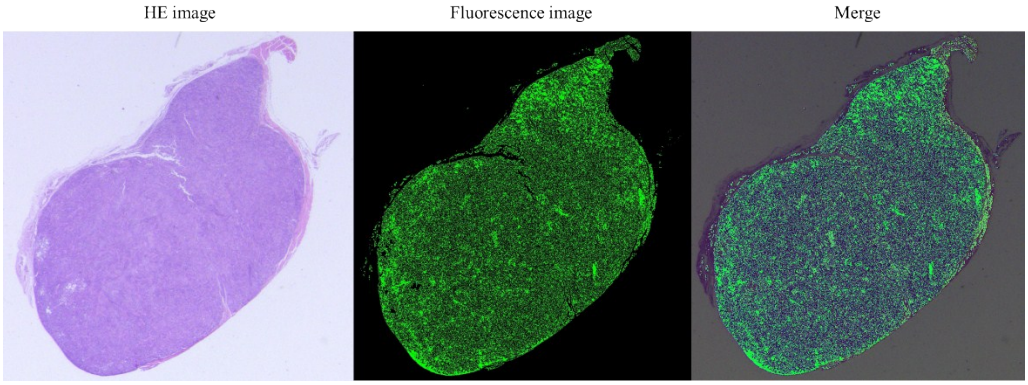

Figure S1. HE staining, NIR-fluorescence images and the merged images of the tumor specimen encompassed the border regions between normal and tumor tissues. Experiments were run in triplicate.

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