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A theranostic agent for cancer therapy and imaging in the second near-infrared window

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Abstract

Theranostic nanoparticles are integrated systems useful for simultaneous diagnosis and imaging guided delivery of therapeutic drugs, with wide ranging potential applications in the clinic. Here we developed a theranostic nanoparticle (~ 24 nm size by dynamic light scattering) p-FE-PTX-FA based on polymeric micelle encapsulating an organic dye (FE) fluorescing in the 1,000–1,700 nm second near-infrared (NIR-II) window and an anti-cancer drug paclitaxel. Folic acid (FA) was conjugated to the nanoparticles to afford specific binding to molecular folate receptors on murine breast cancer 4T1 tumor cells. *In vivo*, the nanoparticles accumulated in 4T1 tumor through both passive and active targeting effect. Under an 808 nm laser excitation, fluorescence detection above 1,300 nm afforded a large Stokes shift, allowing targeted molecular imaging tumor with high signal to background ratios, reaching a high tumor to normal tissue signal ratio (T/NT) of (20.0 ± 2.3). Further, 4T1 tumors on mice were completely eradicated by paclitaxel released from p-FE-PTA-FA within 20 days of the first injection. Pharmacokinetics and histology studies indicated p-FE-PTX-FA had no obvious toxic side effects to major organs. This represented the first NIR-II theranostic agent developed.

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Keywords

theranostic nanoparticles; second near-infrared window; fluorescence imaging; cancer therapy

1. Introduction

Theranostic nanoparticles that combine both diagnostic and therapeutic agents on a single platform have been considered as a promising strategy for cancer detection and treatment [1, 2]. Generally, cancer theranostic nanoparticles accumulate in tumors through passive uptake which utilizes enhanced permeability and retention (EPR) effect [3] as well as molecular targeting that employs ligand binding to specific receptors [4]. The imaging agents help to pinpoint tumor locations by increasing the signal ratio between the tumor and its surrounding normal tissues. Meanwhile, the therapeutic agents are released from the nanocarrier to kill the tumor cells.

Different imaging modalities have been explored with theranostic nanoparticles, including magnetic resonance imaging (MRI) [5, 6], positron emission tomography (PET) [7, 8], and optical imaging [9–11]. Compared to other imaging modalities, fluorescence imaging offers high spatial and temporal resolution [12]. While fluorescence imaging in the visible (400–700 nm) suffers from poor tissue penetration [13], imaging in the conventional near infrared windows (700–900 nm) benefits from low tissue absorption and background fluorescence, allowing for higher penetration depth [14–16]. Recently, fluorescence imaging in the second near-infrared window (NIR-II, 1,000–1,700 nm) has demonstrated significant improvements in spatial resolution and imaging depth, due to further reduced autofluorescence, tissue scattering, and absorption [17–21].

On the other hand, appropriate nanocarriers need to be used to deliver and release aqueous insoluble drug molecules. Although different formulations of chemotherapeutic agents have been developed, most of these formulations could cause significant adverse effects at a high dose [22, 23]. Thus, it is highly desired to explore novel drug delivery vehicles that can afford high efficacy in suppressing tumor growth while exhibiting minimum side effects.

We recently developed a NIR-II nanofluorophore by encapsulating an organic dye (FE) [24] in micelles of an amphiphilic polymer poly(styrene-co-chloromethyl styrene)-graft-poly(ethylene glycol) (PS-g-PEG) [25]. The nanoparticle, named p-FE exhibited good water solubility and biocompatibility while retained the high quantum yield (QY) of the FE dye. Due to its amphiphilic nature, the same polymer could also be employed as a vehicle to trap and deliver hydrophobic anti-cancer drug molecules.

Here, we described the synthesis of a novel theranostic system based on carboxylated poly(styrene-co-chloromethyl styrene)-graft-poly(ethylene glycol) (PS-g-PEG-COOH), the NIR-II dye FE and paclitaxel. We encapsulated FE and the hydrophobic anti-cancer drug paclitaxel (PTX) into the polymer to produce a water-soluble nanoparticle p-FE-PTX. Folic acid (FA) was conjugated to p-FE-PTX to allow targeted molecular imaging and cancer therapy. *In vivo* administration of the complex to murine 4T1 tumor bearing mice was performed. A high tumor to normal tissue signal ratio (T/NT) was achieved, and 4T1 tumors

were completely eliminated. No obvious damage to main organs was observed. The high T/NT in fluorescence imaging, tumor suppression effect at low injection dose, and low toxic side effect made p-FE-PTX-FA a promising system for cancer theranostics.

2 Results

2.1 Synthesis and characterization of p-FE-PTX and p-FE-PTX-FA

p-FE-PTX nanoparticles were prepared using a modified method reported recently [25] (Fig. 1(a) and see Method). In brief, hydrophobic NIR-II dye FE, PTX and amphiphilic polymer PS-g-PEG were dissolved in chloroform. All the substances were reconstituted after the solvent was evaporated. There were absorption peaks at ~ 770 and ~ 230 nm, which corresponded to the absorption of FE [24, 25] and PTX [26, 27], respectively (Figs. 1(b) and 1(c)).

About 10% of the PEG chains in PS-g-PEG were carboxylated, which allowed facile conjugation to targeting ligands. Ethylene diamine was conjugated to the nanoparticles to convert the carboxyl groups to amino groups (see Method), and then FA was conjugated to the nanoparticles through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry to impart targeted molecular imaging and therapy (Fig. 1(a)). There was a peak at ~ 280 nm (Fig. 1(b)) in the absorption spectrum of p-FE-PTX-FA, indicating that the FA was successfully conjugated to the nanoparticles [28, 29]. p-FE-PTX-FA showed an emission peak at ~ 1,010 nm under the excitation of an 808-nm laser (see Fig. 1(c) for absorption and emission spectra of p-FE-PTX-FA), and the relative QY of p-FE-PTX-FA in aqueous solution was ~ 0.8%–8% (based on QY of IR-26 reference was 0.05%–0.5% [30–33]), which was ~ 20 times higher than that of single-walled carbon nanotubes [34, 35] (Fig. 1(c)).

Dynamic light scattering (DLS) analysis revealed that p-FE-PTX and p-FE-PTX-FA nanoparticles had a hydrodynamic size of ~ 15 and ~ 24 nm, respectively (Fig. 1(d) and Fig. S1 in the Electronic Supplementary Material (ESM)). The small size of p-FE-PTX and p-FE-PTX-FA nanoparticles made them suitable for *in vivo* bioimaging and cancer therapy.

2.2 *In vitro* cell imaging and toxicity study

We next investigated p-FE-PTX-FA as a NIR-II probe capable of targeting specific receptors on the cell surface for performing molecular imaging of cancer cells. As-prepared p-FE-PTX and p-FE-PTX-FA were incubated at 4 °C with murine 4T1 breast cancer cells, which expressed folate receptor (FR) [36–39]. The FR-negative HEK-293T cells were used as a negative control. Cells were imaged by a home-built confocal system [25, 40, 41] with a 785-nm excitation laser, and the NIR-II fluorescence signal above 1,050 nm was detected selectively on 4T1 cells treated with p-FE-PTX-FA, but not on the negative HEK-293T cells, or 4T1 cells incubated with p-FE-PTX (Fig. 2(a)). Cell imaging using p-FE-PTX-FA showed a positive/negative ratio of ~ 5.6.

We next tested the *in vitro* efficacy for killing cancer cells of different formulations of PTX. 4T1 cells were incubated with p-FE-PTX or p-FE-PTX-FA at different concentrations at 37 °C for 24 h, and a standard cell viability assay using [3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) [42, 43] was performed. Compared to p-FE-PTX with no FA conjugation, the p-FE-PTX-FA nanoparticles exhibited a lower 50% growth inhibition concentration (IC₅₀) of ~ 15 μ M (Fig. 2(b)), indicating increased cell binding and internalization after ligand conjugation. The IC₅₀ of p-FE-PTX-FA was similar to that of free PTX reported previously [44], suggesting that PTX in this formulation be as an effective chemotherapeutic drug.

2.3 *In vivo* molecular imaging using p-FE-PTX-FA

We performed *in vivo* molecular imaging using p-FE-PTX-FA. C57BL/6 mice ($n = 5$) inoculated with 4T1 tumors were intravenously injected with p-FE-PTX-FA at a dose of 5 mg/kg PTX. Under an excitation of 808 nm, fluorescence signal above 1,300 nm was collected to minimize the influence of tissue scattering and autofluorescence [18, 25]. The fluorescence of tumor could be clearly visualized 2 h post injection (p.i.), and T/NT reached a maximum of (20.0 ± 2.3) at 24 h p.i. (Figs. 3(a) and 3(b)). In comparison, the non-targeted p-FE-PTX showed a lower maximum T/NT of (12.9 ± 1.4) at 24 h p.i. (Figs. S2(a) and S2(b) in the ESM). While p-FE-PTX with no FA conjugation showed a high T/NT due to EPR effect, the uptake of PTX in tumor could be increased by utilizing specific targeting ligands.

2.4 *In vivo* therapy of 4T1 tumor-bearing mice with p-FE-PTX-FA

We next investigated the *in vivo* therapeutic effect of the nanoparticles. The same dose of p-FE-PTX-FA (5 mg/kg PTX) was injected intravenously in 4T1-tumor bearing C57BL/6 mice ($n = 5$) on days 0, 7 and 14 post treatment. The sizes of the subcutaneous tumors were measured over a course of 4 weeks. The size of the tumors started to decrease 3 days after the initialization of the treatment, and the tumors were completely eradicated after 20 days (Figs. 3(c) and 3(d)). Tumors treated with p-FE-PTX were eliminated within 25 days of the first injection (Fig. S2(c) in the ESM). In contrast, p-FE-FA showed no inhibition effect on the growth of 4T1 tumors (Fig. 3(d) and Fig. S3 in the ESM), and the sizes of the tumors increase by (5.9 ± 0.3) fold within 20 days, indicating PS-g-PEG or FE dye alone did not have therapeutic effect. The p-FE-PTX-FA treated group showed a greater inhibition of tumor growth than the p-FE-PTX treated group at an equivalence dose of PTX. This could be explained by an increased uptake of PTX as suggested by the *in vivo* imaging experiments.

2.5 Pharmacokinetics and biodistribution of p-FE-PTX-FA

To investigate the pharmacokinetics of p-FE-PTX-FA, C57BL/6 mice ($n = 5$ each group) were injected with p-FE-PTX or p-FE-PTX-FA at the same dose as used for *in vivo* imaging. The fluorescence signal in blood vessels was plotted as a function of time (Fig. 4(a)), and blood half-time was determined based on a previous method [41, 45]. The p-FE-PTX and p-FE-PTX-FA nanoparticles demonstrated a blood half-time of 16.6 ± 2.6 and 13.1 ± 1.8 h, respectively, which was similar to that of previously reported p-FE nanoparticles [25]. The long circulation time of the particles facilitate uptake into tumors. Biodistribution at 24 h p.i. indicated that other than the tumor, liver was the main organ for accumulation of both p-FE-PTX and p-FE-PTX-FA. Compared with the nontargeted particles, p-FE-PTX-FA showed a significantly increased accumulation from 1.5 %ID/g to 9.0 %ID/g in tumors at 24 h p.i. (Fig. 4(b)). The fluorescence in liver decreased gradually in long-term imaging experiments

(Figs. S4 and S5 in the ESM), suggesting p-FE-PTX-FA could be cleared from the body through the hepatobiliary pathway [25].

To evaluate the toxicity of p-FE-PTX and p-FE-PTX-FA formulations, we measured body weight of the mice ($n = 5$ each group) in the process of cancer treatment. Mice in both groups showed a steady body weight during treatment (Fig. 4(c)), suggesting low toxicity of the formulations. Furthermore, histological studies of hematoxylin and eosin (H&E) stained sections of major organs from the mice ($n = 5$) treated with p-FE-PTX-FA were examined, and no obvious damage in major organs was observed (Fig. 4(d) and Fig. S6 in the ESM). Especially, organs with high uptake of p-FE-PTX-FA, such as liver and spleen, showed no detectable injury during the procedure (Fig. 4(d)).

3 Discussion

Here we developed the first NIR-II theranostic system for simultaneous tumor imaging in the 1,000–1,700 nm range and drug delivery. Fluorescence imaging benefits from micro-scale spatial resolution and high frame rate (> 25 frames per second), which would be difficult to achieve by other medical imaging modalities [5–8]. U.S. Food and Drug Administration (FDA)-approved fluorescent molecules in the conventional near infrared window (NIR-I window, 700–900 nm), such as indocyanine green (ICG) and methylene blue, have been used for tumor imaging and therapy [15, 16]. However, the NIR-I fluorescence-based theranostic nanoparticles usually suffer from high background signal and low T/NT ratio ~ 5 [14, 46], much lower than T/NT ~ 20 achieved here with p-FE-PTX-FA in NIR-II. Compared to fluorescence imaging in the NIR-I window, *in vivo* imaging in the NIR-II window exhibits improved imaging clarity and spatial resolution, owing to reduced photon scattering in biological tissues, which scales with $\lambda^{-\alpha}$ (λ is the wavelength, $\alpha = 0.2–4$) [47]. The FE dye showed an absorption peak at ~ 760 nm, while the main peak of the emission spectrum was $\sim 1,010$ nm. The large Stokes shift of the NIR-II fluorescent dyes helped to reduce the background signal caused by autofluorescence. Furthermore, molecular targeting by folic acid facilitated the specific accumulation of the nanoparticles in the folate expressing 4T1 murine breast tumor. Hence, an impressive T/NT ratio ~ 20 was achieved by p-FE-PTX-FA.

It should be noted that p-FE-PTX without FA conjugation also showed high uptake in 4T1 tumors and good therapeutic efficacy, although the cell experiments suggested it did not bind to 4T1 cells specifically *ex vivo* at 4°C and the IC₅₀ was much higher than that of p-FE-PTX-FA due to the lack of strong nanoparticle-cell interaction without the specific ligands. The discrepancy between the *in vitro* cell experiment and the *in vivo* animal experiment could be attributed to the long blood circulation of the nanoparticles *in vivo* and the EPR effect. *In vivo*, PEGylation could reduce the uptake of the nanoparticles by the reticuloendothelial system (RES), and increase the blood circulation half time [48]. The long blood circulation of p-FE-PTX facilitated its accumulation in tumors through the EPR effect, and thus a high T/NT could be achieved without targeting ligands. What's more, the long retention time in tumors allowed cellular uptake of the nanoparticles through endocytosis pathways [49]. While p-FE-PTX-FA could be internalized by receptor-mediated endocytosis pathway, p-FE-PTX without FA conjugation could utilize other non-specific pathways.

Without a targeting ligand, long circulating p-FE-PTX could still work as a theranostic agent *in vivo*, and its tumor homing effect can be further enhanced by conjugation to targeting ligands.

Although our current study focused on imaging and treating 4T1 breast tumors with FA conjugated theranostic nanoparticles, similar ideas can be applied to other tumor models by using different targeting ligands/antibodies and therapeutic molecules that are poorly water soluble. Visible and NIR-I fluorescent probes have been conjugated to targeting ligands, such as antibodies [50, 51], peptides [52–54], and nucleic acid aptamers [55, 56] for *in vivo* molecular imaging of tumors. It is possible to employ these ligands to p-FE-PTX through bioconjugation chemistry, such as EDC chemistry demonstrated in this work, and higher T/NT ratios achievable than in NIR-I. Besides PTX, other hydrophobic anti-cancer drugs can also be delivered using the system. The poor water solubility of many anti-cancer molecules interferes the bioactivity and therefore hinders their clinical applications [22, 57]. The amphiphilic polymer PS-g-PEG helps to deliver the hydrophobic molecules in aqueous environment, and the therapeutic efficacy could be retained.

4 Methods

4.1 Synthesis of PS-g-PEG-COOH

The poly(styrene-co-chloromethyl styrene) (PS) backbone was synthesized using a reversible addition-fragmentation chain transfer (RAFT) reaction described previously [25]. To prepare carboxylated polyethylene glycol (PEG-COOH), $\text{Zn}(\text{OAc})_2$ (0.045 g, 0.25 mmol) and polyethylene glycol ($M_n \sim 1,000$, 2.5 g, 2.5 mmol) were dissolved in 10 mL dichloromethane (DCM) and di-tert-butyl decarbonate (0.55 g, 2.5 mmol) was added, and the mixture was stirred for 17 h at 40 °C under nitrogen. The crude product was purified by silica-gel column chromatography. To graft both poly(ethylene glycol) methyl ether (mPEG) and PEG-COOH onto the PS backbone, the copolymer (0.05 g), mPEG ($M_n \sim 1,000$, 0.31 mmol), PEG-COOH (0.04 g, 0.04 mmol) and NaOH (0.02 g) were added to dry THF (3 mL), and the mixture was stirred for 18 h at room temperature under nitrogen. The reaction mixture was filtered and the filtrate was concentrated under vacuum. Three droplets of trifluoroacetic acid were added and the solution was stirred at room temperature for 2 h to remove the tert-butyl protection group. The crude product was dialyzed against water using a 10k membrane for 2 days and lyophilized to remove the solvent.

4.2 Synthesis of p-FE-PTX-FA

First, p-FE-PTX was prepared. 28 μg FE dye (synthesized according to our previous protocol [24, 25]), 100 μg paclitaxel and 10 mg PS-g-PEG-COOH were dissolved in 100 μL chloroform. The solvent was evaporated at room temperature overnight. All the substances were reconstituted with water under sonication for 10 min. Precipitates were removed by centrifugation at 4,400 rpm for 30 min.

To synthesize p-FE-PTX-FA, as-prepared p-FE-PTX was dissolved in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) solution at a concentration of 10 mg/mL PS-g-PEG-COOH. In a typical reaction, 50 μL ethylene diamine was added to 200 μL p-FE-PTX

solution. 15 mg EDC was added to the solution, and the solution was stirred at room temperature overnight. Excess ethylene diamine was removed by washing the solution with 10 mM MES solution using an 100k centrifugal filter (4,400 rpm, 20 min) for 6 times. The sample was diluted with 10 mM MES to 2.85 mL, and mixed with 150 μ L FA solution (11.76 mg/mL in dimethyl sulfoxide (DMSO)). 15 mg EDC was added to the solution, and the solution was stirred at room temperature overnight. To remove excess folic acid, 200 μ L NaOH (1 M) was added to the solution, and the solution was washed with phosphate buffered saline (PBS) buffer using an 100k centrifugal filter (4,400 rpm for 20 min) for 6 times.

4.3 Cell staining and confocal imaging

4T1 cells and HEK-293T cells were cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) high glucose medium, respectively. All the cells were seeded in chamber slides, and cultured at 37 °C with 5% CO₂ for 24 h before staining. Cells were then incubated with p-FE-PTX or p-FE-PTX-FA (contained 20 μ M FE) at 4 °C for 30 min. Cells were washed with PBS buffer for 3 times to remove unbounded fluorophores, and fixed by 4% paraformaldehyde (PFA) at room temperature for 20 min. To label the nuclei, the fixed cells were incubated with DRAQ5 (5 μ M) at room temperature for 30 min.

A home-built galvo mirror scanning confocal microscope system was used for cell imaging in both NIR-I and NIR-II windows. An 100 $\times$ objective (Olympus, oil immersion, NA 0.8) was used to focus the excitation light onto the sample, and the emission light went through a dichroic and emission filters before being detected by PMTs (Hamamatsu H7422–50 for NIR-I imaging and Hamamatsu H12397–75 for NIR-II imaging). The scanning rate of the galvo mirror was 2.5 ms/pixel. An 150- μ m pinhole was used to block the out-of-focus signals. For DRAQ5 channel, a 658-nm laser diode (Thorlabs) was used as the excitation source, and a 699-nm long-pass and an 850-nm short-pass filter were used as the emission filters. For FE channel, a 785-nm laser (RPMC lasers) was used as the excitation source, and an 1,050-nm long-pass filter was used as the emission filter. The images were analyzed by ImageJ.

4.4 Cytotoxicity of p-FE-PTX and p-FE-PTX-FA

Cytotoxicity of p-FE-PTX and p-FE-PTX-FA was determined with a MTS assay using a CellTiter96 kit (Promega). About 5000 4T1 cells were cultured in each well with 100 μ L RPMI-1640 medium, and incubated with serially diluted p-FE-PTX or p-FE-PTX-FA solution ($n = 6$ for each concentration). The cells were kept at 37 °C with 5% CO₂ for 24 h. The cell culture medium was removed, and replaced with fresh medium before addition of 15 μ L CellTiter96 in each well. The cells were incubated for 1 h, and the absorbance at 490 nm was recorded using a 96-well plate reader. The absorbance of wells without adding p-FE-PTX or p-FE-PTX-FA was used as control. Cell viability was calculated as a fraction of the absorbance of the control wells.

4.5 Mouse handling

All animal experiments were approved by Stanford Institutional Animal Care and Use Committee (IACUC). C57BL/6 mice were obtained from Charles River. 4T1 tumors were

generated by subcutaneous injection of 2×10^6 cells in 50 μ L PBS into the right hindlimb. The mice were used for imaging and treatment when the volume of the tumor reached 50 mm^3 (about 5 days post inoculation). For imaging and treatment, p-FE-PTX or p-FE-PTX-FA was dispersed in PBS buffer, and injected intravenously into the mice ($n = 5$ each group). The injection dose of PTX was 5 mg/kg for each mice. The same dose of p-FE-PTX or p-FE-PTX-FA was injected on days 0, 7 and 14 after the treatment was initialized. The size of the tumor was measured every other day using a caliper, and the volume of the tumor was calculated using the following formula: $\text{Volume} = (\text{tumor length}) \times (\text{tumor width})^2/2$. Relative tumor size was calculated as the V/V_0 (V_0 is the volume of the tumor at the beginning of the treatment). Body weight of the mice was measured every other day, and normalized to the initial weight.

4.6 *In vivo* fluorescence imaging in the NIR-II window

NIR-II fluorescence images were recorded by a liquid-nitrogen-cooled two-dimensional InGaAs array (Princeton Instrument). The excitation light was provided by an 808 nm fiber-coupled diode laser (RPMC lasers) at a power density of 70 mW/cm^2 . The excitation light was filtered through an 850-nm short-pass and an 1,000-nm short-pass filter (Thorlabs). Mice were placed on a stage beneath the laser. The emission light was filtered using a 900-nm long-pass and an 1,300-nm long-pass filter (Thorlabs), and focused by a lens set onto the camera. All images were analyzed by MATLAB.

4.7 Pharmacokinetics and biodistribution studies

4T1 tumor-bearing C57BL/6 mice ($n = 5$ each group) were used for pharmacokinetics and biodistribution studies. All mice were intravenously injected with p-FE-PTX or p-FE-PTX-FA at the same dose for *in vivo* imaging. The mice were imaged using an 808-nm diode laser (RPMC lasers), and the concentration of the particles in blood was estimated based on the NIR-II fluorescence intensity (filtered by an 1,300 nm long-pass filter) of the vessels. Biodistribution was performed 24 h post injection. Major organs including heart, liver, spleen, lung, kidney, testis and tumor were collected. Fluorescence signal in tissue homogenate was used to quantify the amount of nanoparticles.

For pathology experiments, 4T1 tumor-bearing C57BL/6 mice ($n = 5$ each group) were sacrificed 28 days after the initialization of the cancer treatment. Main organs including liver, spleen, heart lung, kidney, brain, stomach, and intestine were collected. The organs were fixed in 10% formalin for 48 h, and made into paraffin sections. The sections were treated according to a standard H&E protocol, and was examined by an optical microscope (Olympus).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Janib SM; Moses AS; MacKay JA Imaging and drug delivery using theranostic nanoparticles. *Adv. Drug Deliv. Rev.* 2010, 62, 1052–1063. [PubMed: 20709124]
- [2]. Wicki A; Witzigmann D; Balasubramanian V; Huwyler J Nanomedicine in cancer therapy: Challenges, opportunities, and clinical applications. *J. Control. Release* 2015, 200, 138–157. [PubMed: 25545217]
- [3]. Maeda H; Nakamura H; Fang J The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo. *Adv. Drug Deliv. Rev.* 2013, 65, 71–79. [PubMed: 23088862]
- [4]. Cole AJ; Yang VC; David AE Cancer theranostics: The rise of targeted magnetic nanoparticles. *Trends Biotechnol.* 2011, 29, 323–332. [PubMed: 21489647]
- [5]. Lee GY; Qian WP; Wang LY; Wang YA; Staley CA; Satpathy M; Nie SM; Mao H; Yang L Theranostic nanoparticles with controlled release of gemcitabine for targeted therapy and MRI of pancreatic cancer. *ACS Nano* 2013, 7, 2078–2089. [PubMed: 23402593]
- [6]. Liu T; Wu GY; Cheng JJ; Lu Q; Yao YJ; Liu ZJ; Zhu DC; Zhou J; Xu JR; Zhu J et al. Multifunctional lymph-targeted platform based on Mn@mSiO₂ nanocomposites: Combining PFOB for dual-mode imaging and DOX for cancer diagnose and treatment. *Nano Res.* 2016, 9, 473–489.
- [7]. Zhou M; Song SL; Zhao J; Tian M; Li C Theranostic CuS nano-particles targeting folate receptors for PET image-guided photothermal therapy. *J. Mater. Chem. B* 2015, 3, 8939–8948. [PubMed: 27725882]
- [8]. Baum RP; Kulkarni HR THERANOSTICS: From molecular imaging using Ga-68 labeled tracers and PET/CT to personalized radionuclide therapy—The Bad Berka experience. *Theranostics* 2012, 2, 437–447. [PubMed: 22768024]
- [9]. Nurunnabi M; Cho KJ; Choi JS; Huh KM; Lee YK Targeted near-IR QDs-loaded micelles for cancer therapy and imaging. *Biomaterials* 2010, 37, 5436–5444.
- [10]. Ge JC; Jia QY; Liu WM; Guo L; Liu QY; Lan MH; Zhang HY; Meng XM; Wang PF Red-emissive carbon dots for fluorescent, photoacoustic, and thermal theranostics in living mice. *Adv. Mater.* 2015, 27, 4169–4177. [PubMed: 26045099]
- [11]. Wu XM; Sun XR; Guo ZQ; Tang JB; Shen YQ; James TD; Tian H; Zhu WH In vivo and in situ tracking cancer chemotherapy by highly photostable NIR fluorescent theranostic prodrug. *J. Am. Chem. Soc.* 2014, 136, 3579–3588. [PubMed: 24524232]
- [12]. Smith BR; Gambhir SS Nanomaterials for in vivo imaging. *Chem. Rev.* 2017, 117, 901–986. [PubMed: 28045253]
- [13]. Stolik S; Delgado JA; Pérez A; Anasagasti L Measurement of the penetration depths of red and near infrared light in human “ex vivo” tissues. *J. Photochem. Photobiol. B* 2000, 57, 90–93. [PubMed: 11154088]
- [14]. Frangioni JV In vivo near-infrared fluorescence imaging. *Curr. Opin. Chem. Biol.* 2003, 7, 626–634. [PubMed: 14580568]
- [15]. Tagaya N; Yamazaki R; Nakagawa A; Abe A; Hamada K; Kubota K; Oyama T Intraoperative identification of sentinel lymph nodes by near-infrared fluorescence imaging in patients with breast cancer. *Am. J. Surg.* 2008, 195, 850–853. [PubMed: 18353274]
- [16]. He XX; Wu X; Wang KM; Shi BH; Hai L Methylene blue-encapsulated phosphonate-terminated silica nanoparticles for simultaneous in vivo imaging and photodynamic therapy. *Biomaterials* 2009, 30, 5601–5609. [PubMed: 19595455]
- [17]. Smith AM; Mancini MC; Nie SM Bioimaging: Second window for in vivo imaging. *Nat. Nanotechnol.* 2009, 4, 710–711. [PubMed: 19898521]
- [18]. Hong GS; Diao S; Chang JL; Antaris AL; Chen CX; Zhang B; Zhao S; Atochin DN; Huang PL; Andreasson KI et al. Through-skull fluorescence imaging of the brain in a new near-infrared window. *Nat. Photonics* 2014, 8, 723–730. [PubMed: 27642366]
- [19]. Diao S; Blackburn JL; Hong GS; Antaris AL; Chang JL; Wu JZ; Zhang B; Cheng K; Kuo CJ; Dai HJ Fluorescence imaging in vivo at wavelengths beyond 1500 nm. *Angew. Chem.* 2015, 127, 14971–14975.

- [20]. Zebibula A; Alifu N; Xia LQ; Sun CW; Yu XM; Xue DW; Liu LW; Li G H.; Qian, J. Ultrastable and biocompatible NIR-II quantum dots for functional bioimaging. *Adv. Funct. Mater.* 2018, 28, 1703451.
- [21]. Naczynski DJ; Tan MC; Zevon M; Wall B; Kohl J; Kulesa A; Chen S; Roth CM; Riman RE; Moghe PV Rare-earth-doped biological composites as in vivo shortwave infrared reporters. *Nat. Commun.* 2013, 4, 2199. [PubMed: 23873342]
- [22]. Singla AK; Garg A; Aggarwal D Paclitaxel and its formulations. *Int. J. Pharm.* 2002, 235, 179–192. [PubMed: 11879753]
- [23]. Kim SC; Kim DW; Shim YH; Bang JS; Oh HS; Kim SW; Seo MH In vivo evaluation of polymeric micellar paclitaxel formulation: Toxicity and efficacy. *J. Control. Release* 2001, 72, 191–202. [PubMed: 11389998]
- [24]. Yang QL; Ma ZR; Wang HS; Zhou B; Zhu SJ; Zhong YT; Wang JY; Wan H; Antaris A; Ma R et al. Rational design of molecular fluorophores for biological imaging in the NIR-II window. *Adv. Mater.* 2017, 29, 1605497.
- [25]. Wan H; Yue JY; Zhu SJ; Uno T; Zhang XD; Yang QL; Yu K; Hong GS; Wang JY; Li LL et al. A bright organic NIR-II nanofluorophore for three-dimensional imaging into biological tissues. *Nat. Commun.* 2018, 9, 1171. [PubMed: 29563581]
- [26]. Liu Z; Chen K; Davis C; Sherlock S; Cao QZ; Chen XY; Dai HJ Drug delivery with carbon nanotubes for in vivo cancer treatment. *Cancer Res.* 2008, 68, 6652–6660. [PubMed: 18701489]
- [27]. Chen LX; Zhang YM; Cao Y; Zhang HY; Liu Y Bridged bis(β -cyclodextrin)-s-based polysaccharide nanoparticles for controlled paclitaxel delivery. *RSC Adv.* 2016, 6, 28593–28598.
- [28]. Song YC; Shi W; Chen W; Li XH; Ma HM Fluorescent carbon nanodots conjugated with folic acid for distinguishing folate-receptor-positive cancer cells from normal cells. *J. Mater. Chem.* 2012, 22, 12568–12573.
- [29]. Biabanikhankahdani R; Bayat S; Ho KL; Alitheen NBM; Tan WS A simple add-and-display method for immobilisation of cancer drug on his-tagged virus-like nanoparticles for controlled drug delivery. *Sci. Rep.* 2017, 7, 5303. [PubMed: 28706267]
- [30]. Murphy JE; Beard MC; Norman AG; Ahrenkiel SP; Johnson JC; Yu PR; Mi i OI; Ellingson RJ; Nozik AJ PbTe colloidal nanocrystals: Synthesis, characterization, and multiple exciton generation. *J. Am. Chem. Soc.* 2006, 128, 3241–3247. [PubMed: 16522105]
- [31]. Sun Y; Qu CR; Chen H; He MM; Tang C; Shou KQ; Hong S; Yang M; Jiang YX; Ding BB et al. Novel benzo-bis(1,2,5-thiadiazole) fluorophores for in vivo NIR-II imaging of cancer. *Chem. Sci.* 2016, 7, 6203–6207. [PubMed: 30034761]
- [32]. Semonin OE; Johnson JC; Luther JM; Midgett A G; Nozik, A. J.; Beard, M. C. Absolute photoluminescence quantum yields of IR-26 dye, PbS, and PbSe quantum dots. *J. Phys. Chem. Lett.* 2010, 1, 2445–2450.
- [33]. Hatami S; Würth C; Kaiser M; Leubner S; Gabriel S; Bahrig L; Lesnyak V; Pauli J; Gaponik N; Eychemüller A et al. Absolute photo-luminescence quantum yields of IR26 and IR-emissive Cd_{1-x}Hg_x Te and PbS quantum dots—Method- and material-inherent challenges. *Nanoscale* 2015, 7, 133–143. [PubMed: 25407424]
- [34]. Welsher K; Liu Z; Sherlock SP; Robinson JT; Chen Z; Daranciang D; Dai HJ A route to brightly fluorescent carbon nanotubes for near-infrared imaging in mice. *Nat. Nanotechnol.* 2009, 4, 773–780. [PubMed: 19893526]
- [35]. Hong GS; Lee JC; Robinson JT; Raaz U; Xie LM; Huang NF; Cooke JP; Dai HJ Multifunctional in vivo vascular imaging using near-infrared II fluorescence. *Nat. Med.* 2012, 18, 1841–1846. [PubMed: 23160236]
- [36]. Alibolandi M; Abnous K; Sadeghi F; Hosseinkhani H; Ramezani M; Hadizadeh F Folate receptor-targeted multimodal polymersomes for delivery of quantum dots and doxorubicin to breast adenocarcinoma: In vitro and in vivo evaluation. *Int. J. Pharm.* 2016, 500, 162–178. [PubMed: 26802496]
- [37]. Karimi Shervedani R; Yaghoobi F; Torabi M; Samiei Foroushani M Nanobioconjugated system formed of folic acid–deferrioxamine–Ga(III) on gold surface: Preparation, characterization, and activities for capturing of mouse breast cancer cells 4T1. *J. Phys. Chem. C* 2016, 120, 23212–23220.

- [38]. Gao ZG; Tian L; Hu J; Park IS; Bae YH Prevention of metastasis in a 4T1 murine breast cancer model by doxorubicin carried by folate conjugated pH sensitive polymeric micelles. *J. Control. Release* 2011, 152, 84–89. [PubMed: 21295088]
- [39]. Liu SQ; Wiradharma N; Gao SJ; Tong YW; Yang YY Bio-functional micelles self-assembled from a folate-conjugated block copolymer for targeted intracellular delivery of anticancer drugs. *Biomaterials* 2007, 28, 1423–1433. [PubMed: 17141308]
- [40]. Zhu SJ; Yang QL; Antaris AL; Yue JY; Ma ZR; Wang HS; Huang W; Wan H; Wang J; Diao S et al. Molecular imaging of biological systems with a clickable dye in the broad 800- to 1,700-nm near-infrared window. *Proc. Natl. Acad. Sci. USA* 2017, 114, 962–967. [PubMed: 28096386]
- [41]. Wang WZ; Ma ZR; Zhu SJ; Wan H; Yue JY; Ma HL; Ma R; Yang QL; Wang ZH; Li Q et al. Molecular cancer imaging in the second near-infrared window using a renal-excreted NIR-II fluorophore-peptide probe. *Adv. Mater.* 2018, 30, 1800106.
- [42]. O'Toole SA; Sheppard BL; McGuinness EPJ; Gleeson NC; Yoneda M; Bonnar J The MTS assay as an indicator of chemosensitivity/resistance in malignant gynaecological tumours. *Cancer Detect. Prev.* 2003, 27, 47–54. [PubMed: 12600417]
- [43]. Zubris KAV; Liu R; Colby A; Schulz MD; Colson YL; Grinstaff MW In vitro activity of paclitaxel-loaded polymeric expansile nanoparticles in breast cancer cells. *Biomacromolecules* 2013, 14, 2074–2082. [PubMed: 23617223]
- [44]. Yi XL; Lian XH; Dong JX; Wan ZY; Xia CY; Song X; Fu Y; Gong T; Zhang ZR Co-delivery of pirarubicin and paclitaxel by human serum albumin nanoparticles to enhance antitumor effect and reduce systemic toxicity in breast cancers. *Mol. Pharmaceutics* 2015, 12, 4085–4098.
- [45]. Zhang XD; Wang HS; Antaris AL; Li LL; Diao S; Ma R; Nguyen A; Hong G S.; Ma, Z. R.; Wang, J. et al. Traumatic brain injury imaging in the second near-infrared window with a molecular fluorophore. *Adv. Mater.* 2016, 28, 6872–6879. [PubMed: 27253071]
- [46]. Onda N; Kimura M; Yoshida T; Shibutani M Preferential tumor cellular uptake and retention of indocyanine green for in vivo tumor imaging. *Int. J. Cancer* 2016, 139, 673–682. [PubMed: 27006261]
- [47]. Hong GS; Antaris AL; Dai HJ Near-infrared fluorophores for biomedical imaging. *Nat. Biomed. Eng.* 2017, 1, 0010.
- [48]. Fang J; Nakamura H; Maeda H The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv. Drug Deliv. Rev.* 2011, 63, 136–151. [PubMed: 20441782]
- [49]. Oh N; Park JH Endocytosis and exocytosis of nanoparticles in mammalian cells. *Int. J. Nanomedicine* 2014, 9, 51–63. [PubMed: 24872703]
- [50]. Day KE; Sweeny L; Kulbersh B; Zinn KR; Rosenthal EL Preclinical comparison of near-infrared-labeled cetuximab and panitumumab for optical imaging of head and neck squamous cell carcinoma. *Mol. Imaging Biol.* 2013, 15, 722–729. [PubMed: 23715932]
- [51]. Knutson S; Raja E; Bomgarden R; Nlend M; Chen A; Kalyanasundaram R; Desai S Development and evaluation of a fluorescent antibody-drug conjugate for molecular imaging and targeted therapy of pancreatic cancer. *PLoS One* 2016, 11, e0157762.
- [52]. Cheng Z; Wu Y; Xiong ZM; Gambhir SS; Chen XY Near-infrared fluorescent RGD peptides for optical imaging of integrin $\alpha_v\beta_3$ expression in living mice. *Bioconjug. Chem.* 2005, 16, 1433–1441. [PubMed: 16287239]
- [53]. Choi HS; Gibbs SL; Lee JH; Kim SH; Ashitate Y; Liu FB; Hyun H; Park G; Xie Y; Bae S et al. Targeted zwitterionic near-infrared fluorophores for improved optical imaging. *Nat. Biotechnol.* 2013, 31, 148–153. [PubMed: 23292608]
- [54]. Peng MY; Qin SY; Jia HZ; Zheng DW; Rong L; Zhang XZ Self-delivery of a peptide-based prodrug for tumor-targeting therapy. *Nano Res.* 2016, 9, 663–673.
- [55]. Wang ZG; Fu BS; Zou SW; Duan B; Chang CY; Yang B; Zhou X; Zhang L Facile construction of carbon dots via acid catalytic hydrothermal method and their application for target imaging of cancer cells. *Nano Res.* 2016, 9, 214–223.
- [56]. Wang DL; Liu B; Ma Y; Wu CW; Mou QB; Deng HP; Wang RB; Yan DY; Zhang C; Zhu XY A molecular recognition approach to synthesize nucleoside analogue based multifunctional

- nanoparticles for targeted cancer therapy. J. Am. Chem. Soc. 2017, 139, 14021–14024. [PubMed: 28945366]
- [57]. Gelderblom H; Verweij J; Nooter K; Sparreboom A Cremophor EL: The drawbacks and advantages of vehicle selection for drug formulation. Eur. J. Cancer 2001, 37, 1590–1598. [PubMed: 11527683]

Figure 1.

Synthesis and characterization of p-FE-PTX and p-FE-PTX-FA. (a) Scheme of synthesis of p-FE-PTX and p-FE-PTX-FA. (b) Absorption spectra of p-FE-PTX, p-FE-PTX-FA and PTX (200–500 nm). p-FE-PTX and p-FE-PTX-FA were dissolved in PBS buffer, and PTX was dissolved in methanol. The peak of the absorption spectrum was normalized to 1. (c) The absorption (600–900 nm) and emission (900–1,500 nm) spectra of p-FE-PTX and p-FE-PTX-FA. The peak of the absorption spectrum was normalized to 1. The excitation was provided by an 808 nm laser diode, and a 900 nm long-pass filter was used as the emission filter. The emission spectra of p-FE-PTX and p-FE-PTX-FA were normalized by the absorption at 808 nm. (d) Dynamic light scattering analysis of p-FE-PTX and p-FE-PTX-FA in PBS buffer.

Figure 2.

In vitro cell imaging and toxicity study. (a) Molecular imaging of 4T1 cells which overexpress folate receptor, and HEK-293T cells (folate receptor negative) with p-FE-PTX or p-FE-PTX-FA. The cells were imaged by a home-built confocal microscope system. FE was excited by a 785-nm laser, and detected with an 1,050-nm long-pass filter (red channel on the images). QRAQ5 was excited by a 658-nm laser, and detected with a 699-nm long-pass filter and an 850-nm short-pass filter (blue channel on the images). (b) Relative viabilities of 4T1 cells after being treated with p-FE-PTX or p-FE-PTX-FA at different concentrations for 24 h ($n = 6$ for each condition).

Figure 3.

In vivo fluorescence imaging and treatment of 4T1 tumor bearing mice with p-FE-PTX-FA.

(a) Wide-field NIR-II fluorescence imaging of mice inoculated with 4T1 tumors after different time points post intravenous injection. C57BL/6 mice ($n = 5$) were injected with p-FE-PTX-FA at a dose of 5 mg/kg PTX. The mice were excited by an 808 nm laser at a power density of 70 mW/cm^2 , and the fluorescence signal above 1,300 nm was collected. (b) Time course variation of T/NT of the mice ($n = 5$) injected with p-FE-PTX-FA. (c) Representative photographs of 4T1-bearing C57BL/6 mice treated with p-FE-PTX-FA. (d) Tumor growth curves of 4T1-bearing mice ($n = 5$) treated with p-FE-PTX-FA or p-FE-FA ($n = 5$ each group). The same dose of PTX (5 mg/kg) was injected on days 0, 7 and 14 (marked by arrow) for the p-FE-PTX-FA group.

Figure 4.

Pharmacokinetics, biodistribution and toxicity of p-FE-PTX and p-FE-PTX-FA. (a) Normalized fluorescence intensity of p-FE-PTX and p-FE-PTX-FA in blood over the course of 72 h. (b) Biodistribution of p-FE-PTX and p-FE-PTX-FA in 4T1 tumor bearing mice at 24 h post intravenous injection. (c) Change of body weight of mice treated with p-FE-PTX and p-FE-PTX-FA. No obvious loss of body weight was observed in both groups. (d) H&E stained histological sections of the liver and spleen from healthy mice and the mice treated with p-FE-PTX-FA.