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A Multi-Functional Polymeric Carrier for Simultaneous Positron Emission Tomography Imaging and Combination Therapy

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Abstract

Multifunctional nanoplateforms offering simultaneous imaging and therapeutic functions have been recognized as a highly promising strategy for personalized nanomedicine. In this work, we synthesized a farnesylthiosalicylate (FTS, a nontoxic Ras antagonist) based triblock copolymer PEOG-*b*-PVBA-*b*-PFTS (POVF) composed of a poly(oligo(ethylene glycol) methacrylate) (POEG) hydrophilic block, a poly(FTS) hydrophobic block, and a poly(4-vinylbenzyl azide) (PVBA) middle block. The POVf polymer itself was active in inhibiting the tumor growth in vitro and in vivo. Besides, it could serve as a carrier to effectively encapsulate paclitaxel (PTX) to form stable PTX/POVF mixed micelles with a diameter around 100 nm. Meanwhile, POVf polymer provides the active azide group for incorporating a positron emission tomography (PET) imaging modality via a facile strategy based on metal-free click chemistry. This nanocarrier system could not only be used for co-delivery of PTX and FTS, but also for PET imaging guided drug delivery. In the 4T1.2 tumor bearing mice, PET imaging showed rapid uptake and slow clearance of radiolabeled PTX/POVF nanomicelles in the tumor tissues. In addition, the FTS-based multifunctional nanocarrier was able to inhibit tumor growth effectively, and the co-delivery of PTX by the carrier further improved the therapeutic effect.

Keywords

PET imaging; farnesyl thiosalicylic acid (FTS); paclitaxel; prodrug micelles; drug delivery

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1. Introduction

Over the years, a variety of nano drug delivery systems, including polymers, micelles, liposomes, dendrimers and inorganic nanoparticles have been developed to improve the therapeutic efficacy of the chemotherapeutic drugs and decrease their systemic toxicities [1–5]. Although many formulations have demonstrated promising outcomes in preclinical studies for cancer treatment, clinical translations of these nanoformulations are still at a slow pace. One of the major hurdles is that different patients in clinic may respond differently to a given formulation with respect to its bioavailability at tumor tissues [6]. Thus, personalized nanomedicine aiming to individualize nanotherapeutic treatment based on in vitro and in vivo disease- and patient-specific information is highly demanded [7, 8]. A promising strategy in the personalized nanomedicine is to incorporate both noninvasive imaging and therapeutic functions into a single formulation, which can provide insights on the individual patient response to therapy and help to adjust the follow-up treatment plans [9–11]. In addition, it may also aid in preselecting the patient groups who benefit maximally from a particular treatment [12].

Polymeric nanoparticles with advantages of small size, flexible structure, and ease of functionalization have attracted increasing attention as dual-functional carriers for simultaneous imaging and drug delivery [13–16]. However, most of these systems only include one single drug which may not meet the clinical need because of the intrinsic heterogeneity of cancer. To achieve better therapeutic outcomes, there is still a need for the construction of multifunctional carriers that can integrate the functionalities of imaging guidance and combination therapy.

As prodrug carriers, amphiphilic polymers attached with hydrophobic drugs via covalent bonds offer an attractive feature for co-delivery of two or more drugs [17–19]. The conjugated multiple hydrophobic drug molecules with proper structures could endow the polymeric carriers with the capabilities to efficiently load other drugs through hydrophobic interaction and π - π stacking effect. The conjugated drug in the carriers can be chosen such that it could counteract the side effects caused by the loaded drug and/or promote synergistic effect with the loaded drug[20,21].

S-trans, trans-farnesylthiosalicylic acid (FTS) is a synthetic farnesylcysteine mimetic that acts as a potent and especially nontoxic Ras antagonist [22]. Constitutively active Ras caused by mutation in the Ras family of proto-oncogenes is present in one-third of human cancers[23,24]. The activated form of Ras constitutively activates its downstream effectors, contributing to cell transformation[25]. FTS inhibits excessively activated Ras proteins, resulting in the inhibition of Ras-dependent tumor growth[26, 27]. FTS causes significant reduction of Ras levels in a wide array of established cancer models and inhibition of tumor growth in animals with no adverse toxicity [28]. In addition to its antitumor activity by itself, FTS can sensitize tumors to other treatments such as chemotherapy and radiation therapy [29]. However, the efficacy of FTS is limited by its poor water solubility and limited oral bioavailability. To improve the delivery efficacy of FTS, we have recently developed a series of FTS-based prodrug carriers that consist of a PEG hydrophilic segment and various FTS-based hydrophobic domains [30, 31]. The carriers could inhibit the tumor growth by

themselves. More importantly, they could self-assemble to form micelles that are effective in formulating a number of hydrophobic agents, including paclitaxel (PTX), doxorubicin (DOX) and curcumin, for combination therapy.

In this work, we developed a multi-functional FTS-based carrier system by combining a co-delivery function and a positron emission tomography (PET) imaging modality together, which facilitated direct assessment of the biodistribution and efficiency of in vivo delivery. PET imaging is a clinically used noninvasive imaging technique that facilitates quantitative analysis of pharmacokinetics and biodistribution due to its high sensitivity and unlimited penetration depth[32,33]. It is known that high radiolabeling specific activity is important for nanoparticle-based PET imaging system to obtain high-quality images at low dose of radioactivity [34]. In order to tag FTS-based system with PET radioisotopes efficiently, we synthesized a PEOG-*b*-PVBA-*b*-PFTS (POVF) tri-block copolymers composed of a poly(oligo(ethylene glycol) methacrylate) (POEG) hydrophilic block, a poly(FTS) hydrophobic block, and a poly(4-vinylbenzyl azide) (PVBA) middle block via reversible addition fragmentation chain transfer (RAFT) polymerization. As shown in **Scheme 1**, the triblock polymers could form micelles with multiple FTS moieties in the hydrophobic core, which is beneficial for co-loading hydrophobic PTX via π - π stacking effect and hydrophobic interaction. The PEOG hydrophilic shell can stabilize the micelles and protect the loaded drug from premature release in the circulation system [35]. The azide groups in the intermediate layer of micelles allow further reaction with isotope-bearing compounds with alkyne functional groups for radiolabeling via metal-free click chemistry [36, 37]. The resulting radiolabeled PTX/POVF micelles were used for PET imaging of mice bearing 4T1.2 tumor xenografts to monitor the biodistribution in a real-time manner. Additionally, in vitro and in vivo therapeutic efficacies of this multi-functional system were evaluated.

2. Materials and methods

2.1 Materials

4-vinylbenzyl azide (VBA-monomer) was synthesized by reaction of sodium azide with 4-vinylbenzylchloride according to the literature [38, 39]. FTS-monomer and PEOG macroCTA were synthesized as previously reported [31]. 2, 2'-Azobis (2-methylpropionitrile) (AIBN) was recrystallized in anhydrous ethanol before use. Paclitaxel was purchased from AK Scientific Inc. (CA, U. S. A.). Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) and Dulbecco's Modified Eagle's Medium (DMEM) were all bought from Sigma-Aldrich (MO, U. S.A.). Fetal bovine serum (FBS) was purchased from Invitrogen (NY, U. S. A.). Copper-64 was obtained from Washington University (St. Louis, MO) and University of Wisconsin (Madison, WI). Zirconium-89 was obtained from Washington University (St. Louis, MO). Luna C-18 HPLC columns were from Phenomenex (Torrance, CA, USA).

2.2 Characterization

^1H NMR spectrum (400.0 MHz) was recorded on a Varian 400 FT-NMR spectrometer with CDCl_3 as the solvent. Molecular weights (M_n and M_w) and molecular weight distributions (M_w/M_n) of the synthesized polymers were determined by gel permeation chromatography

(GPC) equipped with a Waters 2414 refractive index detector. THF was used as the eluent with a flowing rate of 1.0 mL/min at 35°C. A series of polystyrene standards with narrow molecular weight distribution were applied for calibration. HPLC and FPLC were performed on a Waters 1525 Binary HPLC pump (Milford, MA) with a Waters 2489 UV/visible detector and a model 106 Bioscan radioactivity detector for the analysis of either ^{64}Cu or ^{89}Zr labeled conjugates using either a two-components buffer (0.1 v% TFA in de-ionized water + 0.1 v% TFA in acetonitrile) or PBS as the eluting phase for HPLC and FPLC respectively. PET/CT data were acquired using an Inveon Preclinical Imaging Station (Siemens Medical Solutions).

2.3 Synthesis of POEG-*b*-PVBA polymers

AIBN (1 mg, 0.0062 mmol), POEG macroCTA (233 mg, 0.031 mmol), VBA-monomer (59 mg, 0.372 mmol) and 2 mL dried tetrahydrofuran (THF) were added in a Schlenk tube, and deoxygenated by three freeze-pump-thawing cycles. The mixture was stirred at 70°C under N_2 protection for 3 h, and then the reaction was stopped by immersing the tube into liquid nitrogen. The reaction mixture was precipitated in diethyl ether twice, and dried under vacuum. (32 % conversion, $M_n = 8,450$ g/mol).

2.4 Synthesis of POEG-*b*-PVBA-*b*-PFTS (POVF) triblock polymers

AIBN (2 mg, 0.0124 mmol), POEG-*b*-PVBA (300 mg, 0.0372 mmol), FTS-monomer (279 mg, 0.600 mmol) and 2 mL dried 1, 4-dioxane were added in a Schlenk tube, and deoxygenated by three freeze-pump-thawing cycles. The mixture was stirred at 85°C under N_2 protection for 18 h, and then the reaction was stopped by immersing the tube into liquid nitrogen. The product was purified by precipitating in hexane for 3 times and drying under vacuum. (69 % conversion, $M_n = 12,400$ g/mol).

2.5 Preparation of POVf micelles and evaluation of critical micelle concentration (CMC)

The POVf micelles were prepared via a film hydration method. Briefly, 10 mg of POVf polymers were dissolved in 1 mL of dichloromethane. The solvent was removed by nitrogen flow to form a thin film, which was further dried under vacuum for 1 h. Then 1 mL of PBS buffer was added to hydrate the dry film and the POVf micelles were formed after gentle vortex.

Nile red was used as a fluorescence probe to determine the CMC of POVf micelles [40]. In brief, 0.05 mg/mL solution of Nile red in dichloromethane was added to each tube. After complete removal of solvent, PBS solution (150 μL) with various POVf concentrations was added to each tube. After incubation for 8 h, the solutions were transferred into a 96-well plate for a fluorescence measurement. The emission spectra were scanned from 570 to 720 nm at a fixed excitation wavelength of 550 nm.

2.6 Preparation of PTX-loaded micelles

POVF polymers (10 mg) and PTX (1 mg) were dissolved in 1 mL of dichloromethane. The solvent was removed to form a dry film, which was then hydrated with PBS buffer to generate PTX/POVF micelles. PTX loading efficiency was measured by high performance

liquid chromatography (HPLC). Drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated as follows:

$$\text{DLC (\%)} = [\text{weight of drug loaded}/(\text{weight of polymer} + \text{drug used})] \times 100$$

$$\text{DLE (\%)} = (\text{weight of loaded drug}/\text{weight of input drug}) \times 100$$

The size distribution and morphology of PTX-free and PTX-loaded POVf micelles were measured by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

2.7 In vitro PTX release

In vitro release profile of PTX from PTX/POVF micelles was examined by a dialysis method [31]. PTX/POVF micelles or Taxol (1 mL) at a PTX concentration of 1 mg/mL was placed into a dialysis bag (MWCO = 3.5 kDa), and dialyzed against 50 mL PBS solution containing 0.5% (w/v) Tween 80 at 37 °C with gentle shaking. At predetermined time intervals, the PTX concentration in the dialysis bag was analyzed by HPLC at 227 nm wavelength.

2.8 Synthesis of DBCO-DFO

DBCO-NHS (2 mg, 5 μmole) was added to DFO-NH₂ (1.1 mg, 2 μmole) dissolved in 0.5 ml DMF. Twenty μmole DIEA was subsequently added, and the reaction mixture was allowed to stir at room temperature for 2 h. DMF was removed by the lyophilizer. The residue was then dissolved in acetonitrile: H₂O = 1: 1 and purified by HPLC to give 0.9 mg product (yield, 56 %). ESI-MS: [M+H]⁺ found 849.06, calculated 849.02.

2.9 Synthesis of DBCO-LNETA

DBCO-NHS (2 mg, 5 μmole) was added to LNETA-NH₂ (0.95 mg, 2 μmole) dissolved in 0.5 ml DMF. Twenty μmole DIEA was subsequently added, and the reaction mixture was allowed to stir at room temperature for 2 h. DMF was removed by the lyophilizer. The residue was then dissolved in Acetonitrile: H₂O = 1:1 and purified by HPLC to give 0.8 mg product (yield, 53%). ESI-MS: [M+H]⁺ found 763.89, calculated 763.87.

2.10 ⁸⁹Zr labeling of PTX/POVF micelles

⁸⁹Zr-labeled micelles were prepared following the post-radiolabeling conjugation method (Fig. 2). Briefly, DBCO-DFO was radiolabeled with ⁸⁹Zr in 1.0M HEPES (pH = 7.0) at 95°C for 30min. The resulting DBCO-(⁸⁹Zr)DFO with a specific activity of 0.5mCi/nmole was then mixed with the PTX-loaded micelles (0.1 mCi of DBCO-(⁸⁹Zr)DFO per mg of micelles) in PBS buffer. The reaction mixture was incubated at 37 °C for 60 min, and the unconjugated DBCO-(⁸⁹Zr)DFO was removed by the zeba spin desalting column to give ⁸⁹Zr-labeled micelles.

2.11 ⁶⁴Cu labeling of POVf and PTX/POVF micelles

⁶⁴Cu-labeled micelles were prepared following a similar post-radiolabeling conjugation method (Fig. 2) as applied in the ⁸⁹Zr labeling but using L-NETA as the chelator instead. Briefly, DBCO-L-NETA was first radiolabeled with ⁶⁴Cu in 0.1M NH₄OAc (pH = 8.0) at 95°C. The resulting DBCO-(⁶⁴Cu)L-NETA with a specific activity of 0.5 mCi/nmole was

then mixed with the PTX-loaded micelles (0.1 mCi of DBCO- ^{64}Cu -L- NETA per mg of micelles) in PBS buffer. The reaction mixture was incubated at 37 °C for 60 min, and the unconjugated DBCO- ^{64}Cu -L- NETA was removed by the zeba spin desalting column to give ^{64}Cu -labeled micelles.

2.12 Serum stability study

The serum stability was assessed by measuring the disassociation of ^{64}Cu from the complex in mouse serum using radio-ITLC. Briefly, ^{64}Cu -labeled POVf (~100 μCi) was mixed with 0.5 mL mouse serum, and the resulting mixture was incubated at 37 °C for 4h and 24h, respectively. After incubation, plasma proteins were precipitated with 0.5 mL acetone and centrifuged at 14000 RPM for 5 min, and then the percentage of dissociated $^{64}\text{Cu}^{2+}$ was determined by ITLC: dissociated $^{64}\text{Cu}^{2+}$ moved to the front solvent, while the intact ($^{64}\text{Cu}^{2+}$)- POVf stayed on the baseline.

2.13 Cell culture and animals models

4T1.2 (mouse metastatic breast cancer cell line) and HCT116 (human colon carcinoma cell line) were cultured in DMEM medium at 37 °C in a 5% CO_2 atmosphere.

Female BALB/c mice (4–6 weeks old) were used for in vivo experiments, which was approved by the Animal Use and Care Administrative Advisory Committee at University of Pittsburgh.

2.14 In vitro cytotoxicity assay

The cytotoxicity of POVf blank micelles and PTX/POVf mixed micelles in both 4T1.2 and HCT116 cells were determined by MTT assay. Briefly, cells were seeded into 96-well plates at a density of 2000 cells/well and incubated at 37 °C overnight. Then, various formulations (POVf blank micelles, free FTS, PTX/POVf micelles and PTX) were added to the cells. After incubation for 72 h, cell viability was determined by MTT assay as previously reported [41].

2.15 In vivo PET imaging

Either ^{64}Cu or ^{89}Zr -labeled POVf or PTX/POVf micelles were injected into mice via tail vein at 200 $\mu\text{Ci}/\text{mouse}$ ($n = 3$). For mice injected with ^{64}Cu labeled polymer, whole body PET scan was performed at 1h, 4h, and 8h post-injection time points while for mice injected with ^{89}Zr labeled polymer, PET scan was performed at 24h, 48h, 72h and 96h post-injection time points. The resulting PET images were reconstructed using the 3-dimensional ordered-subsets expectation maximization algorithm. The PET images were co-registered with CT images using Inveon Research Workstation (IRW) software. Regions of interest (ROI) were drawn over tumor and other major organs using the CT scan on the decay-corrected whole-body coronal images, and the associated PET activities were calculated using the IRW software and expressed as percentage injected dose per gram of tissue (% ID/g).

2.16 In vivo therapeutic study

4T1.2 cells were inoculated into the right flank of female BALB/c mice. When the tumor size grew to around 50 mm^3 , the mice were treated with PBS, PTX-free POVf micelles,

Taxol and PTX/POVF micelles (PTX dosage: 10 mg/kg) via intravenous injection. The injections were performed every three days for five times. Changes in tumor sizes and body weights were measured over time. The tumor volumes (V) were calculated based on the formula: $V = (\text{length of tumor}) \times (\text{width of tumor})^2 / 2$. The mice were sacrificed after the completion of the experiment. Tumor tissues and major organs including heart, liver, spleen, lung and kidney were excised, and further studied by a Zeiss Axiostar plus Microscope (PA, USA) after H&E staining.

The apoptotic cells in tumor tissues were also evaluated using the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay with a commercial apoptosis detection kit (Roche, Indianapolis, IN). Tissue sections were incubated with proteinase K, and then treated with TUNEL reaction mixture. Apoptotic cells were observed under fluorescence microscope. The percentage of TUNEL-positive cells was calculated using ImageJ software.

2.17 Statistical analysis

Two-tailed Student's T test was used to compare two groups, and one-way ANOVA was used to analyze multiple groups. Newman-Keuls test was performed if the overall p-value is <0.05 . In all statistical analyses, $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Synthesis and characterization of the POEG-*b*-PVBA-*b*-PFTS polymers

The POVf triblock polymer was synthesized by continuous RAFT polymerization according to the procedure shown in **Fig. 1**. First, 4-vinylbenzyl azide (VBA-monomer) was synthesized by reaction of sodium azide with 4-vinylbenzylchloride according to the literatures [38, 39]. Then, FTS-monomer and POEG macro-chain transfer agent (macroCTA) were synthesized as previously reported [31]. In the next step, POEG-*b*-PVBA block copolymers were synthesized by POEG macroCTA-mediated polymerization of VBA-monomer. Compared to other azide-containing compounds, VBA-monomer is relatively stable, and can be polymerized at high temperature [38, 39, 42]. It has been reported that the thermal decomposition of the azide groups in the VBA-based polymers happens above 150 °C [38, 42]. Thus, the azide group is stable at the polymerization temperature in this work. The obtained POEG-*b*-PVBA polymer was then used to further initiate the RAFT polymerization of FTS-monomer to afford the POEG-*b*-PVBA-*b*-PFTS triblock polymer. The polymer structures were confirmed by ^1H NMR (**Fig. S1&S2**). The average degree of polymerization (DP) of the VBA-monomer was calculated to be 4 by comparing the intensities of I_a and I_d (**Fig. S1**). The average DP of the FTS monomer was calculated to be 11 by comparing the intensities of I_a and I_e (**Fig. S2**). The molecular weights and polydispersity index (PDI) of these polymers were determined by gel permeation chromatography (GPC). As shown in **Table 1**, all of the synthesized polymers showed narrow polydispersities (M_w/M_n), further suggesting the successful synthesis of the well-defined POEG-*b*-PVBA-*b*-PFTS (denoted as POVf) triblock polymer.

The POVf triblock polymer has VBA units in the middle block between POEG and PFTS blocks, which allow further reaction with isotope-bearing compounds with alkyne functional groups for micelle radiolabeling via click chemistry in solution. The strategy that places the azide group in the intermediate layer of the micelles might improve the efficiency of the click reaction by overcoming the difficulties for isotope-bearing compounds to reach the hydrophobic core layer. Besides, dissociation of water-soluble isotope ion will be minimized due to the fact that the isotope complex is hidden and wrapped in the intermediate layer.

3.2 Physicochemical characterization of POVf and PTX/POVf micelles

Blank and PTX-loaded POVf micelles were prepared by a simple solvent evaporation method. The size and morphology of POVf micelles were measured by dynamic light scattering (DLS) and transmission electron microscopy (TEM). As shown in **Fig. 2A**, POVf polymer could form micelles in aqueous PBS solution with a diameter of 104 nm. TEM image revealed the spherical shape of POVf micelles (**Fig. 2B**). The critical micelle concentration (CMC) of POVf was determined using Nile red as a probe. As shown in **Fig. 2C**, POVf had a low CMC value of 0.0071 mg/mL in PBS solution, which shall minimize the dissociation of POVf micelle upon dilution in blood circulation. Besides, the FTS loading capacity of the POVf prodrug micelles was calculated to be 29.4% (**Table 2**).

POVf prodrug micelles could serve as a carrier to encapsulate PTX at a drug/carrier ratio as high as 1/10 (mg/mg) to form PTX/POVf nanoparticles. At this ratio, PTX loading capacity was measured to be 8.6% by HPLC (**Table 2**). DLS (**Fig. 2D**) and TEM (**Fig. 2E**) showed that there were no significant changes in particle size and morphology of the POVf micelles after loading of PTX.

Dialysis method was used to evaluate the in vitro PTX release from PTX/POVf micelles. As shown in **Fig. 2F**, PTX/POVf micelles showed significantly slower PTX release compared to Taxol. Less than 20% of PTX was released from the PTX/POVf micelles in the first 4 hours and then a slow and sustained release of PTX over 60 hours was revealed. The sustained PTX release might be attributed to the strong hydrophobic interaction and π - π stacking effect between POVf carriers and PTX. FTS release was also evaluated in vitro, and no FTS was released from POVf micelles in PBS solution due to the covalent conjugation of FTS in the carrier. In addition, FTS release from POVf micelles was also studied in 50% serum. Less than 1% FTS was released from the POVf micelles at 48 h following incubation in serum, indicating that FTS moieties were tightly packed in the hydrophobic core of POVf carrier, which would provide excellent stability to prevent the esterase digestion in the circulation system.

3.3 Radiolabeling of POVf and PTX/POVf micelles

A universal strategy based on SPAAC (strain-promoted [3+2] azide-alkyne cycloaddition) was developed to incorporate PET radioisotope into the PTX/POVf micelles through the azide groups in the intermediate layer of micelles. As shown in **Fig. 3**, we synthesized two bifunctional chelators DBCO-DFO and DBCO-LNETA for the chelation of radioisotope ^{89}Zr and ^{64}Cu , respectively. DBCO (dibenzocyclooctyne) is a metal-free click moiety which can react with azide group in aqueous solution without metal catalyst [43, 44]. In particular,

DBCO-DFO was successfully labeled with ^{89}Zr at a specific activity of 0.5mCi/nmole with more than 95% labeling yield, which ensured the following post-labeling conjugation with N_3 functionalized micelles to be conducted smoothly. The resulting radiolabeled DBCO- (^{89}Zr)DFO was then directly conjugated to the pre-formed micelles via SPAAC. During the post-labeling conjugation, the amount of azide groups in PVBA intermediate layer was ~100- fold excess compared to input DBCO- (^{89}Zr)DFO, which was expected to yield a high efficiency of ^{89}Zr incorporation. The radio-incorporation yield was determined by fast protein liquid chromatography with SUPEROSE 12 10/300 GL size exclusion column (**Fig. S3**). DBCO- (^{89}Zr)DFO was successfully conjugated to the polymer based on the peak area at retention time of around 13 min, respectively, giving a labeling yield above 90%. Besides, there are no obvious free ^{89}Zr ions peaks at around 26 min on FPLC, indicating essentially the absence of free ^{89}Zr ions in the radio-labeled micelles. Similar results were obtained for ^{64}Cu labeling via DBCO-LNETA (**Fig. S3**). The resulting PET radiotracer showed excellent serum stability with less than 5% disassociation of labeled ^{64}Cu following incubation with serum over 24 h at 37 °C (**Fig. S4**). These data suggest that our method represents a facial and universal approach for the radiolabeling of the azide-containing, drug-loaded micelles.

3.4 Cytotoxicity of POVFN-Cu²⁺nanomicelles alone and PTX/POVF-N-Cu²⁺nanomicelles

In order to conveniently evaluate the therapeutic effect of the system in vitro and in vivo, nonradioactive isotope (Cu^{2+})-labeled POEG-*b*-PVBA-*b*-PFTS (denoted as POVFN-Cu²⁺) and PTX/POEG-*b*-PVBA-*b*-PFTS (denoted as PTX/POVF-N-Cu²⁺) micelles were also similarly prepared except that the radioisotope (^{64}Cu) was replaced by a nonradioactive-isotope (Cu^{2+}).

The cytotoxicity of different POVFN-Cu²⁺ nanomicelles alone was examined by MTT assay in 4T1.2 mouse breast cancer cells and HCT116 human colon cancer cells with free FTS as a control. As shown in **Fig. 4**, free FTS exhibited tumor cell killing effect in a concentration-dependent manner. Compared to free FTS, the POVFN-Cu²⁺ nanomicelles showed less cytotoxicity in both 4T1.2 and HCT116 tumor cells (**Fig. 4A, B**), which might be attributed to the limited FTS cleavage from the prodrug polymeric micelle during a relative short time of treatment.

The cytotoxicity of PTX/POVF-N-Cu²⁺ nanomicelles was also examined by MTT assay with clinical formulation Taxol as a control. As presented in **Fig. 5A & B**, PTX/POVF-N-Cu²⁺ micelles showed similar tumor cell killing effect in 4T1.2 and HCT116 cell lines compared to Taxol control. A maximal of ~80% inhibition of tumor cell proliferation was achieved. The inability to kill all tumor cells might be due to the intrinsic resistance mechanism in 4T1.2 and HCT116 cells.

3.5 PET imaging studies

The tumor uptake of radiolabeled PTX/POVF nanomicelles was visualized by PET imaging in a real-time fashion in an aggressive 4T1.2 mouse tumor model (**Fig. 6A&B**). For early time points such as 1h, 4h and 8h, PET imaging was conducted with ^{64}Cu labeled micelles while for late time points such as 24h, 48h, 72h and 96h, PET imaging was conducted with

^{89}Zr labeled micelles in another group of mice. The radioactive signals in the tumors were calculated through ROI (a region of interest) analysis of the PET images. The PTX/POVF micelles started to accumulate in tumors as early as 1 h post injection, and showed increased tumor accumulation over time from 1 h to 24 h post injection. At 24 h p.i., radiolabeled PTX/POVF micelles showed the highest accumulation in the tumor ($13.6 \pm 2.3\%$ ID/g), and then a slow decrease in the tumor accumulation was observed over time. However, large amounts of radioactive signals remained in the tumors even at 96 h ($7.9 \pm 1.1\%$ ID/g). These results demonstrated that radiolabeled PTX/POVF nanomicelles showed rapid uptake and slow clearance in the tumor tissues. Furthermore, tumor uptake was found to be above 10%ID/g after 8h post injection. According to the PTX cumulative release curve (**Fig. 2F**), most PTX still remained in the micelles at the 8h time point, indicating an efficient PTX delivery to the tumor site by this newly developed polymeric carrier.

The biodistribution of PTX/POVF micelles was also similarly investigated by PET imaging with ^{64}Cu (1 h, 4 h, and 8 h) (**Fig. S5A**) and ^{89}Zr (24 h, 48 h, 72 h and 96 h) (**Fig. 7A**). Results of ROI quantitative analysis are shown in **Fig. S5A** and **Fig. 7B** for ^{64}Cu and ^{89}Zr , respectively. At early time points, majority of radiolabeled PTX/POVF micelles were found in liver, kidney, and blood with highest uptake values of $27.1 \pm 2.5\%$ ID/g, $19.6 \pm 3.1\%$ ID/g and $14.4 \pm 1.3\%$ ID/g respectively. At late time points, the accumulation in blood and liver decreased while the accumulation in tumor increased. Specifically, majority of radiolabeled PTX/POVF micelles were detected in the tumor ($13.6 \pm 2.3\%$ ID/g), liver ($12.4 \pm 3.4\%$ ID/g) and kidney ($16.7 \pm 2.7\%$ ID/g) at 24 h post injection. Uptake in blood dropped to $7.8 \pm 2.1\%$ ID/g, and only a very small amount of micelles were detected in the muscle ($3.9 \pm 0.9\%$ ID/g) and bone ($3.4 \pm 0.8\%$ ID/g). Then, the signals of micelles in all of the organs decreased over time. Uptake in tumor decreased to $10.3 \pm 1.2\%$ ID/g at 48 h, followed by $9.1 \pm 1.2\%$ ID/g at 72 h and $7.9 \pm 1.1\%$ ID/g at 96 h. Uptake in liver decreased to $8.8 \pm 1.5\%$ ID/g at 48 h, followed by $8.8 \pm 1.3\%$ ID/g at 72 h and $6.0 \pm 0.8\%$ ID/g at 96 h. Uptake in kidney decreased to $11.6 \pm 1.5\%$ ID/g at 48 h, followed by $9.1 \pm 2.3\%$ ID/g at 72 h and $8.6 \pm 1.4\%$ ID/g at 96 h. Uptakes in blood, muscle and bone decreased to $2.8 \pm 0.6\%$ ID/g, $0.9 \pm 0.3\%$ ID/g and $1.0 \pm 0.2\%$ ID/g at 96 h, respectively. The high accumulation of PTX/POVF micelles in the tumor might be attributed to the enhanced permeability and retention (EPR) effect which allowed micelles to effectively accumulate at the tumor site [45, 46]. In addition to accumulation in the tumor, substantial amounts of signals were found in liver and kidney. This is a general issue for most of intravenously injected micelles-based nanocarriers [47–49], due to the nonspecific uptake by reticuloendothelial systems (RES) and renal elimination [50, 51]. Despite the very low CMC value of POVf (~ 0.0071 mg/mL), POVf micelles are still subjected to the disassociation of the polymer molecules from the assembled particles, resulting in the accumulation of radiolabeled polymer in kidney [52]. In the future, the micelles will be further optimized to improve their stability in the blood and tumor targeting efficiency via a strategy of cross-linking and the use of a targeting ligand.

3.6 In vivo therapeutic efficacy

Finally, we investigated the therapeutic effect of the new multi-functional system in 4T1.2 tumor bearing mice. As shown in **Fig. 8A**, PTX-free micelle POVf-N- Cu^{2+} was active in

inhibiting the tumor growth and the anti-tumor activity was similar to that of commercial formulation Taxol. This is in contrast to in vitro cytotoxicity assay (**Fig. 4**) in which the carrier alone showed much less effect in inhibiting the proliferation of tumor cells. This is likely attributed to a more efficient release of FTS from POVf-N-Cu²⁺ micelle in vivo due to the exposure of the carrier to the esterase in tumor tissues over a relatively prolonged period of time. It is also noted that PTX/POVF-N-Cu²⁺ exhibited significantly higher anti-tumor activity compared to POVf-N-Cu²⁺ alone ($P<0.05$). The therapeutic effect of PTX/POVF-N-Cu²⁺ was further investigated via histological examination of tumor tissues. As shown in **Fig. 8C**, compared to other groups, tumors treated with PTX/POVF-N-Cu²⁺ demonstrated a widespread area of cancer cell necrosis/apoptosis. The TUNEL apoptosis assay also demonstrated that among all the groups, PTX/POVF-N-Cu²⁺ treatment led to the largest area of apoptotic cells, further supporting the excellent therapeutic effect of the new multifunctional system (**Fig. 8D**). The superior therapeutic effect of PTX/POVF-N-Cu²⁺ might be attributed to the combination or synergistic effect between cleaved FTS and co-delivered PTX.

To evaluate the in vivo toxicity of PTX/POVF-N-Cu²⁺ micelles, changes in body weights of treated mice were monitored over the duration of the experiment. As shown in **Fig. 8B**, there were no obvious changes in body weights for all of the treated mice, suggesting little toxicity with all the formulations. This is further supported by histological examination of several major organs/tissues (liver, heart, lung, kidney, and spleen) that were collected from the mice at day 17 post treatment (**Fig. S6**). Compared to the control group, no apparent changes were observed in all of the major organs/tissues from the mice treated with PTX/POVF-N-Cu²⁺ micelles.

The above results suggest a potential of PTX/POVF multi-functional system in biomedical applications with real-time imaging function, superior antitumor efficacy and little adverse effect. This new system provides a possibility to facilitate the real-time evaluation of biodistribution and prediction of therapeutic efficacy and toxicity profiles. This may pave the way for personalized nanomedicine through identifying the patients who will likely respond to the therapy. Additionally, the radiolabeling strategy developed in this study is universal and can be applied to other azide-containing polymeric micellar systems. It can also be adapted to incorporate other radioisotopes such as ¹⁷⁷Lu and ⁹⁰Y, two isotopes that are currently used in the clinic for radiotherapy. This will lead to the future development of a new type of chemoradiotherapy that is based on POVf-mediated codelivery of FTS, PTX and a therapeutic radioisotope.

4. Conclusions

In summary, we developed a FTS-based triblock polymer POVf as a multi-functional nanocarrier which could not only be used for co-delivery of PTX and FTS, but also for PET imaging-guided drug delivery. POVf carrier was effective in formulating PTX with high drug loading capacity and a slow kinetics of drug release. Meanwhile, a simple and universal approach was developed to incorporate a PET radioisotope into the azide-containing PTX/POVF micelles via metal-free click chemistry in aqueous solution. The radiolabeled PTX/POVF micelles exhibited excellent serum stability, rapid tumor uptake and slow clearance,

which validated the feasibility of the PET image-guided delivery of PTX/POVF micelles. The POVf carrier itself was active in inhibiting the tumor growth in vitro and in vivo. Formulation of PTX into POVf system further enhanced the tumor inhibition effect, which was significantly higher than that of the commercial formulation Taxol. Our work not only provides an effective strategy to radiolabel polymeric micelles for PET imaging, but also provides a potential multi-functional system that integrates imaging and co-delivery functions for future personalized combination therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of significance

Due to the intrinsic heterogeneity of cancer and variability in individual patient response, personalized nanomedicine based on multi-functional carriers that integrate the functionalities of combination therapy and imaging guidance is highly demanded. Here we developed a multi-functional nanocarrier based on triblock copolymer POEG-b-PVBA-b- PFTS (POVF), which could not only be used for co-delivery of anticancer drugs PTX and Ras inhibitor FTS, but also for PET imaging guided drug delivery. The POVf carrier itself was active in inhibiting the tumor growth in vitro and in vivo. Besides, it was effective in formulating PTX with high drug loading capacity, which further enhanced the tumor inhibition effect. Meanwhile, we developed a simple and universal approach to incorporate a PET radioisotope (Zr-89 and Cu-64) into the azide-containing PTX/POVF micelles via metal- free click chemistry in aqueous solution. The radiolabeled PTX/POVF micelles exhibited excellent serum stability, rapid tumor uptake and slow clearance, which validated the feasibility of the PET image-guided delivery of PTX/POVF micelles.

Scheme 1.

Schematic illustration of multi-functional POEG-*b*-PVBA-*b*-PFTS polymeric carrier for real-time PET imaging and co-delivery of PTX/FTS.

Figure 1.

The schemes for the synthesis of POEG-*b*-PVBA-*b*-PFTS copolymers via RAFT polymerization.

Figure 2.

(A) The particle size distribution of prodrug micelle measured by DLS. (B) TEM image of PTX-free micelle using negative staining. Scale bar is 100 nm. (C) Plots of fluorescence intensity ratio at 650 nm versus concentrations of prodrug micelles. (D) The particle size distribution of PTX/POVF mixed micelle measured by DLS. (E) TEM image of PTX/POVF mixed micelle using negative staining. Scale bar is 100 nm. (F) The *in vitro* release profiles of PTX from the PTX/POVF mixed micelle in PBS containing 0.5% (w/v) Tween 80 at 37 °C.

Figure 3.

(A) Preparation of ^{89}Zr labeled POVf micelles; and (B) Preparation of ^{64}Cu labeled POVf micelles.

Figure 4. MTT cytotoxicity of POV-F-N-Cu²⁺ prodrug micelles in 4T1.2 mouse breast cancer cell line (A) and HCT116 human colon cancer cell line (B) with free FTS as the control. Cells were treated with different micelles for 72 h and values reported are the means $\pm$ SD for triplicate samples.

Figure 5. MTT cytotoxicity assay of PTX/POVF-N-Cu²⁺ micelles in 4T1.2 (A) and HCT116 cell line (B) after 72 h treatment.

Figure 6.

(A) Monitoring of tumor uptakes at early time points (1h, 4h, and 8h) using ^{64}Cu labeled PTX/POVF micelles; (B) Monitoring of tumor uptakes at late time points (24h, 48h, 72h, and 96h) using ^{89}Zr labeled PTX/POVF micelles.

Figure 7.

(A) PET/CT images using ^{89}Zr labeled PTX/POVF micelles. (B) Biodistribution of PTX/POVF- ^{89}Zr micelles in 4T1.2 tumor-bearing mice at different post injection time points.

Figure 8.

Antitumor activity of taxol, POVf prodrug micelles and PTX/POVF micelles in a syngeneic murine breast cancer model (4T1.2). (A) Tumor volume change. (B) Body weight changes in mice receiving different treatments. Five injections were given on days 1, 3, 5, 8 and 11 and each point represents the mean of tumor size (n = 5). **P < 0.01 (vs control); &P < 0.01 (vs POVf-N-Cu²⁺). (C) Typical H&E staining images of tumors in the mice treated with various formulations, scale bar: 200 μ m. (D) TUNEL staining of tumors in the mice treated

with various formulations, scale bar: 50 μm . Image J was used to quantitatively analyze the TUNEL-positive cells, and the results are shown as mean $\pm$ S.D.

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Table 1.

Synthetic results of the POEG₁₅ Macro-RAFT agent and POEG₁₅-*b*-PVBA₄, POEG₁₅-*b*-PVBA₄-*b*-PFTS₁₁ block copolymers.

Block copolymer	M_n^a (NMR)	M_n^b (GPC) ^b	M_w^b (GPC)	M_w/M_n^b (GPC)
POEG ₁₅	7500	8220	8710	1.06
POEG ₁₅ - <i>b</i> -PVBA ₄	8130	8450	9360	1.11
POEG ₁₅ - <i>b</i> -PVBA ₄ - <i>b</i> -PFTS ₁₁	13340	12400	15700	1.27

Notes:

^a Calculated by NMR results.

^b Measured by GPC with THF as the eluent, and the molecular weights and their distributions were calculated with polystyrene standards.

Table 2.

Physicochemical characterization of blank and PTX-loaded POEG₁₅-*b*-PVBA₄-*b*-PFTS₁₁ micelles.

Micelles	Size (nm) ^a	PDI ^b	CMC (mg/mL)	DLC _{FTS} (%) ^c	DLC _{PTX} (%) ^d
POEG ₁₅ - <i>b</i> -PVBA ₄ - <i>b</i> -PFTS ₁₁	104.2	0.23	0.0071	29.4	0
PTX/POEG ₁₅ - <i>b</i> -PVBA ₄ - <i>b</i> -PFTS ₁₁	108.5	0.24	--	29.4	8.6

^a Measured by dynamic light scattering particle sizer.

^b PDI = polydispersity index.

^c FTS loading capacity.

^d PTX loading capacity.