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Image-guided and tumor-targeted drug delivery with radiolabeled unimolecular micelles

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Abstract

Unimolecular micelles formed by dendritic amphiphilic block copolymers poly(amidoamine)–poly(L-lactide)–*b*-poly(ethylene glycol) conjugated with anti-CD105 monoclonal antibody (TRC105) and 1,4,7-triazacyclononane-N, N', N-triacetic acid (NOTA, a macrocyclic chelator for ⁶⁴Cu) (abbreviated as PAMAM–PLA–*b*-PEG–TRC105) were synthesized and characterized. Doxorubicin (DOX), a model anti-cancer drug, was loaded into the hydrophobic core of the unimolecular micelles formed by PAMAM and PLA via physical encapsulation. The unimolecular micelles exhibited a uniform size distribution and pH-sensitive drug release behavior. TRC105-conjugated unimolecular micelles showed a CD105-associated cellular uptake in human umbilical vein endothelial cells (HUVEC) compared with non-targeted unimolecular micelles, which was further validated by cellular uptake in CD105-negative MCF-7 cells. In 4T1 murine breast tumor-bearing mice, ⁶⁴Cu-labeled targeted micelles exhibited a much higher level of tumor accumulation than ⁶⁴Cu-labeled non-targeted micelles, measured by serial non-invasive positron emission tomography (PET) imaging and confirmed by biodistribution studies. These unimolecular micelles formed by dendritic amphiphilic block copolymers that synergistically integrate passive and active tumor-targeting abilities with pH-controlled drug release and PET imaging capabilities provide the basis for future cancer theranostics.

Keywords

Unimolecular micelles; Dendritic amphiphilic block copolymer; Nanocarriers; CD105 (endoglin); Molecular imaging; Positron emission tomography (PET)

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1. Introduction

Polymeric micelles with a unique core-shell structure, formed by self-assembly of amphiphilic copolymers, have captured significant attention in diverse biomedical fields such as gene and drug delivery, and especially in tumor therapy [1–5]. The hydrophobic core of the polymeric micelle serves as a container to improve the solubility of water-insoluble agents. The outer hydrophilic shell, which is often composed of poly(ethylene glycol) (PEG), offers the micelles excellent dispersibility in an aqueous solution [6,7]. Polymeric micelles are desirable drug delivery vehicles for targeted cancer therapy due to their unique advantages, such as enhancing the aqueous solubility of hydrophobic drugs, prolonging the circulation time of the drug in the blood, improving the *in vivo* stability of the drug, providing both passive and active tumor-targeting abilities, and reducing nonspecific uptake by the reticuloendothelial system (RES) [8–11]. However, classical multi-molecular polymeric micelles suffer from insufficient stability *in vivo* due to the fact that their *in vivo* stability is sensitive to the surrounding environment, especially the concentration of the amphiphilic block copolymers. Upon dilution in the bloodstream, multi-molecular polymeric micelles disassemble, leading to a burst release of drug and loss of tumor-targeting abilities [12–14].

Unimolecular micelles formed by individual dendritic amphiphilic block copolymers are investigated to overcome this problem. Because of their covalent nature, properly engineered unimolecular micelles can possess excellent *in vitro* and *in vivo* stability [2,15–20]. The size of unimolecular micelles can also be tuned by controlling the number of amphiphilic block copolymer arms and the length of the hydrophobic and hydrophilic segments [21]. Despite these potential advantages, unimolecular micelles incorporating therapeutic agents, molecular targeting, and diagnostic imaging capabilities are rare.

CD105 (i.e., endoglin), which is almost exclusively expressed on proliferating tumor endothelial cells, serves as an ideal vascular target [22]. More importantly, the expression level of CD105 is correlated with poor prognosis in more than 10 solid tumor types [23], which makes it a generally applicable prognostic, diagnostic, and therapeutic vascular target in cancer [24,25]. TRC105, a human/murine chimeric IgG1 monoclonal antibody that binds to both human and murine CD105, was used as the targeting ligand in this work. Due to its high affinity for CD105 and the indispensable role of CD105 in cancer progression, multiple phase II clinical trials with TRC105 are currently ongoing [26]. In our previous studies, TRC105 showed a very high avidity for CD105 in different murine cancer models [27,28]. Positron emission tomography (PET) can provide excellent tissue penetration, higher detection efficiency, non-invasiveness, and superb quantitative accuracy [29], which can serve as an invaluable tool to study the *in vivo* behavior of different nanomaterials [18,30,31]. It was used here for unveiling the *in vivo* behavior of CD105-targeted drug/agent nanocarriers designed in the current study. Rationally designed CD105-targeted nanomedicine can be used for both cancer diagnosis and cancer therapy (i.e., theranostics), which will improve the management of cancer patients in the long run.

In this work, we report a type of unimolecular micelle (Fig. 1) formed by dendritic amphiphilic block copolymers poly(amidoamine)-poly(L-lactide)-*b*-poly(ethylene glycol) conjugated with an anti-CD105 monoclonal antibody (TRC105) and 1,4,7-triaza cyclononane-N, N', N-triacetic acid (NOTA, a macrocyclic chelator for ⁶⁴Cu) (abbreviated as PAMAM-PLA-*b*-PEG-TRC105) for targeted cancer therapy and imaging. The effects of the TRC105 targeting ligands on the cellular uptake in cell lines with different CD105 expression levels (e.g., human umbilical vein endothelial cells [HUVEC] or MCF-7 human breast cancer cell line) were studied *in vitro*. The effects of TRC105 as the targeting ligand

on the tumor accumulation of the unimolecular micelles in 4T1 murine breast tumor-bearing mice were studied *in vivo*.

2. Materials and methods

2.1. Materials

Poly(amidoamine) (Core:1,4-diaminobutane; {*dendri*-poly(amidoamine)-(OH)₆₄}; (*G* = 4)) dendrimer was purchased from NanoSynthons LLC (Mt. Pleasant, MI, USA). L-lactide (LA) (Sigma–Aldrich, Milwaukee, WI, USA) was recrystallized twice from ethyl acetate (Sigma–Aldrich) before use. The heterobifunctional poly(ethylene glycol) (PEG) derivatives, HOOC–PEG–maleimide (HOOC–PEG–Mal) (*M_w* = 5000) and HOOC–PEG–OCH₃ (*M_w* = 5000) were acquired from JenKem Technology (Allen, TX, USA). 4-Dimethylamino pyridine (DMAP), 1,3-dicyclohexylcarbodiimide (DCC), and amino-ethanethiol hydrochloride (AET·HCl) were purchased from ACROS and used without further purification. Stannous (II) octoate (Sn(Oct)₂), (Sigma–Aldrich) was used as received. Tetrahydrofuran (THF), triethylamine (TEA), dimethyl sulfoxide (DMSO), anhydrous dimethylformamide (DMF), anhydrous dichloromethane (DCM), and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma–Aldrich. 5,5'-Dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) and 2-iminothiolane (Traut's reagent) were purchased from Thermo Scientific (Rockford, IL, USA). All other chemicals and reagents used were of analytical reagent grade. The anti-cancer drug doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Mesochem Technology Co., Ltd. (S)-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was purchased from Macrocyclics, Inc. (Dallas, TX, USA). TRC105 was kindly provided by TRACON Pharmaceuticals, Inc. (San Diego, CA, USA). All organic solvents were of analytic grade. Phosphate buffered solutions (PBS, pH 7.4) and acetate buffered solutions (ABS, pH5.3) were prepared fresh in our laboratory. Ultrapure deionized water (DI water, Milli-QWater Systems) was used for all buffer solutions and experiments. The buffers were of Millipore grade and pretreated with Chelex 100 resin (50–100 mesh, Sigma–Aldrich, St. Louis, MO, USA) to ensure that the aqueous solution was free of heavy metals.

2.2. Synthesis of PAMAM–PLA

PAMAM–PLA was prepared by the ring-opening polymerization of LA monomer using PAMAM-OH as the micro-initiator (cf. Scheme 1). A 50 ml two-neck flask equipped with an argon gas inlet was charged with PAMAM-OH (40 mg, 2.80×10^{-6} mol) and placed in an oil bath. A predetermined amount of LA (645.6 mg, 4.48 mmol) was slowly introduced and a catalytic amount of Sn(Oct)₂ ([Sn(Oct)₂]/[LA] = 1/1000 mol/mol) was added subsequently. The reaction was stirred at 120 °C for 24 h. THF was added to stop the reaction. The resulting reaction mixture was added drop-wise into the methanol (three times) to obtain the final polymer product via precipitation. The products were dried under vacuum.

2.3. Synthesis of PAMAM–PLA-b-PEG–OCH₃/Mal

The PAMAM–PLA (40 mg), HOOC–PEG–Mal (Maleimide content: 70% in molar, 72.9 mg, 14.6×10^{-6} mol), HOOC–PEG–OCH₃ (76.6 mg, 15.32×10^{-6} mol), DCC (5.27 mg, 25.54×10^{-6} mol), and DMAP (0.31 mg, 2.54×10^{-6} mol) were added in a 50 ml two-neck flask. The mixture was dried under vacuum for 1 h. Afterwards, 10 ml anhydrous DCM was added via a syringe. The mixture was stirred under argon gas at room temperature for 20 h and the by-product, dicyclohexylurea, was removed by filtration. Subsequently, the solution was added into cold diethyl ether to collect the crude product. The impurities and unreacted PEG were removed by dialysis against DI water using a cellulose dialysis membrane (molecular weight cutoff, 15 kDa). After 48 h dialysis, the product was dried by lyophilization.

2.4. Synthesis of the NOTA-SH

NOTA-SH was synthesized via the reaction between the SCN group of p-SCN-Bn-NOTA (2.8 mg, 0.005 mmol) and the amino group of AET·HCl (0.57 mg, 0.005 mmol) in the presence of TEA in PBS (pretreated with Chelex 100 resin to prevent oxidation of the thiol) for 2 h at room temperature under an N₂ atmosphere [18].

2.5. Synthesis of TRC105-SH

TRC105-SH was prepared by reacting TRC105 with Traut's reagent at a molar ratio of 1:20 in PBS (pretreated with Chelex 100 resin to prevent oxidation of the thiol) at a pH of 8.0. After 2 h of incubation at room temperature, TRC105-SH was purified by size-exclusive chromatography using PBS as the mobile phase. Based on our previous experience, there were about 5 thiol groups per TRC105-SH molecule based on Ellman's reagent titration [30].

2.6. Synthesis of the dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA block copolymers

A predetermined amount of NOTA-SH PBS solution and TRC105-SH PBS solution were added into the PAMAM-PLA-*b*-PEG-OCH₃/Mal PBS suspension with a feed molar ratio of TRC105:NOTA:PAMAM-PLA-*b*-PEG-OCH₃/Mal at 5:7:1. The reaction was carried out at a pH of 7.5 in the presence of TCEP (1.6 mmol/L) to prevent disulfide formation among TRC105-SH and NOTA-SH. The product was purified by centrifugation filtration using 30 kDa cut-off Amicon filters and the TCEP was removed. The resulting final product—i.e., PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA, which was abbreviated as PAMAM-PLA-*b*-PEG-TRC105—will be used to prepare targeted unimolecular micelles. Dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/NOTA block copolymers (without TRC105 conjugation) were prepared following a similar procedure. The PAMAM-PLA-*b*-PEG-OCH₃/NOTA copolymers were used to prepare non-targeted unimolecular micelles.

2.7. Preparation of DOX-loaded unimolecular micelles

The polymer PAMAM-PLA-*b*-PEG (50 mg) and DOX·HCl (20 mg) treated with 2 mol excess of TEA were dissolved in 8 mL of anhydrous DMF. Next, 24 mL of DI water were added dropwise to the solution under stirring. Thereafter, the mixture was purified by centrifugation filtration using 30 kDa cut-off Amicon filters. The product was obtained by lyophilization.

2.8. Characterization

¹H NMR spectra of all intermediates and final polymer samples were recorded on a Varian Mercury Plus 300 spectrometer using CDCl₃ as a solvent at 25 °C. Molecular weights and polydispersity indices of the polymers were determined by gel permeation chromatography (GPC) equipped with a refractive index detector, a viscometer detector, and a light scattering detector (Viscotek, USA). DMF was used as a mobile phase with a flow rate of 1 mL/min.

The hydrodynamic size and size distribution of the unimolecular micelles were determined by dynamic light scattering (DLS) (ZetaSizer Nano ZS90, Malvern Instruments, USA), at a polymer concentration of 0.05 mg/mL. The morphology and size of the dried unimolecular micelles were measured using a transmission electron microscope (TEM) (FEI Tecnai G² F30 TWIN 300 kV, E.A. Fischione Instruments, Inc., USA). To prepare the TEM sample, a drop of copolymer solution (0.05 mg/mL) containing 1 wt% of phosphotungstic acid was deposited onto a 200 mesh copper grid coated with carbon and dried at room temperature. The DOX loading content, defined as the weight percentage of DOX in the DOX-loaded unimolecular micelles, was quantified by a Varian Cary 300 Bio UV-Vis

spectrophotometer. The absorbance of DOX at 485 nm was measured to determine the drug loading content in the DOX-loaded unimolecular micelle solution based on an established calibration curve obtained using different concentrations of DOX at the same wavelength.

2.9. In vitro drug release study

Drug release studies were performed in a glass apparatus at 37 °C in acetate buffer (pH 5.3) and phosphate buffer (pH 7.4) solutions. 10 mg of DOX-loaded unimolecular micelles were dispersed uniformly in 5 ml of medium and placed in a dialysis bag with a molecular weight cut-off of 2 kDa. The dialysis bag was immersed in 50 ml of the release medium and kept in a horizontal laboratory shaker (100 rpm) under constant temperature 37 °C. At certain time intervals, 3 ml of release media were collected and replaced by the same volume of fresh media. The amount of released DOX was analyzed by a UV-Vis spectrophotometer at 485 nm.

2.10. Cell uptake assays

The cellular uptake behavior and intracellular distribution of the DOX-loaded unimolecular micelles were analyzed using both flow cytometry and a fluorescence microscope. For flow cytometry, human umbilical vein endothelial cells (HUVEC, CD105-positive [27,30]) were seeded in 6-well culture plates and cultured in M-200 overnight. After digestion with 0.05% trypsin, the cells (5×10^6 per ml) were treated with DOX-loaded PAMAM-PLA-PEG (non-targeted unimolecular micelles), or DOX-loaded PAMAM-PLA-PEG-TRC105 (targeted unimolecular micelles) for 15 and 120 min (DOX concentration: 20 µg/ml). DOX uptake was analyzed by a BD FACS Calibur four-color analysis cytometer (Becton Dickinson, San Jose, CA, USA) and FlowJo analysis software (Tree Star, Inc., Ashland, OR, USA). A minimum of 1×10^4 cells were analyzed from each sample with the DOX fluorescence intensity displayed on a four-decade log scale.

For fluorescent microscopy studies, HUVEC or MCF-7 (1×10^6 per well) cells were seeded onto 24-well cell culture plates and cultured overnight. Cells (HUVEC were fixed by methanol/water/acetic acid (5:4:1) before micelle treatment) were treated with DOX-loaded targeted or non-targeted unimolecular micelles, or DOX-loaded targeted unimolecular micelles, with TRC105 blocking (0.5 mg/ml TRC105) for 120 min (DOX concentration: 20 µg/ml). After incubation, the cells were washed with PBS. DOX uptake was analyzed using a Nikon Eclipse Ti-U inverted microscope (Nikon, Melville, NY). Fluorescent images were acquired using NIS-Elements BR Software.

2.11. Animal model

All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. 4T1 cells were used for tumor inoculation when they reached ~80% confluence. Four- to five-week-old female Balb/c mice were purchased from Harlan (Indianapolis, IN) and tumors were established by subcutaneously injecting 2×10^6 cells, suspended in 100 µl of PBS, into the front flank of the mice. The tumor sizes were monitored every other day and the animals were subjected to *in vivo* experiments when the diameter of the tumors reached 6–8 mm (typically 2 weeks after inoculation).

2.12. ^{64}Cu -labeling of the unimolecular micelles

In brief, $^{64}\text{CuCl}_2$ (74 MBq) was diluted in 300 ml of 0.1 M sodium acetate buffer (pH 6.5) and added to the targeted or non-targeted unimolecular micelle solution (~2 mg/ml) with a ratio of 30 µg per 37 MBq of $^{64}\text{CuCl}_2$. The reaction mixture was incubated for 30 min at 37 °C with constant shaking. The PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA- ^{64}Cu

(denoted as ^{64}Cu -labeled targeted micelles) or PAMAM-PLA-*b*-PEG-OCH₃/NOTA- ^{64}Cu (denoted as ^{64}Cu -labeled non-targeted micelles) were purified using PD-10 columns with PBS as the mobile phase before the animal studies.

2.13. PET imaging and biodistribution studies

PET scans at various time points post-injection (p.i.), image reconstruction (maximum a posteriori (MAP) algorithm, with no attenuation or scatter correction), and region-of-interest (ROI) analysis were performed using a microPET/microCT Inveon rodent model scanner (Siemens Medical Solutions USA, Inc.) as described previously [18,27,30]. Each tumor-bearing mouse was injected with 5–10 MBq of ^{64}Cu -labeled targeted or non-targeted micelles via the tail vein and 3–15 min static PET scans were performed. Quantitative data were presented as percent injected dose per gram of tissue (%ID/g). Four 4T1 tumor-bearing mice were pre-injected with 1 mg of TRC105 1 h before ^{64}Cu -labeled targeted micelles were administered to evaluate the target specificity *in vivo*. Biodistribution studies were carried out after the last PET scans to validate the PET results. Radioactivity in the tissue was measured using a gamma-counter (Perkin Elmer) and presented as %ID/g (mean $\pm$ SD).

3. Results and discussion

3.1. Synthesis and characterization of the dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA block copolymer

The synthesis of dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA block copolymers was carried out following Scheme 1. First, PAMAM-PLA polymers were synthesized by ring-opening polymerization of LA, using dendrimer PAMAM-OH (G4 with 64 terminal hydroxyl groups) as the micro-initiator, and Sn(Oct)₂ as the catalyst. The reaction was conducted under rigorously anhydrous conditions to avoid initiation of ring-opening polymerization by water. The structure of the copolymer was confirmed by ¹H NMR and FTIR (Fig. 2). The ¹H NMR spectrum of PAMAM-PLA is shown in Fig. 2(A). The peaks at 5.16 (a) and 1.54 ppm (b) are ascribed to the protons of methine and methyl groups in the PLA main chains, respectively. The signal at 4.35 ppm (c) corresponding to the terminal methine protons of PLA [HOCH(CH₃)–] can also be identified. According to the ¹H NMR spectrum, the repeat units of [–C(O)–CH(CH₃)–O–] in PLA arms were determined to be 21. This calculation was based on the relative intensity ratio of the peaks at 5.16 ppm (a) and 4.35 ppm (c) corresponding to the methine groups in the PLA main chains and the methine group adjacent to the hydroxyl end group, respectively. Due to the low content of PAMAM in the PAMAM-PLA copolymer, the H NMR peaks of PAMAM in PAMAM-PLA did not show up clearly compared to the absorption peaks of the PLA arms [32].

The FTIR spectrum of PAMAM-PLA is shown in Fig. 2(B). The peaks at 1667 cm^{–1} and 1539 cm^{–1} are ascribed to C=O stretching and N–H bending of the amide groups of PAMAM. The appearance of these peaks confirmed the presence of dendritic structural units (PAMAM) in the polymer product [33]. The peaks at 1751 cm^{–1} and 1085 cm^{–1} corresponding to the C=O and –C–O– stretching vibrations of the ester groups in PLA can also be identified. The combined NMR and FTIR analyses confirmed the structure of PAMAM-PLA.

The average number of arms per dendritic PAMAM-PLA molecule was estimated by comparing the molecular weights of PAMAM-OH and PAMAM-PLA determined by GPC, as shown in Table 1. The average number of arms per PAMAM-PLA molecule was estimated to be 33 according to the following equation:

$$\begin{aligned}\text{Number of arms} &= (M_n \text{ of PAMAM-PLA} - M_n \text{ of PAMAM}) / M_n \text{ of PLA segments} \times (21 \text{ repeat units der} \\ &= (63,409 - 14,277) / (72 \times 21) = 33.\end{aligned}$$

In the next step, two types of PEG (HOOC-PEG-OCH₃ and HOOC-PEG-Mal) were conjugated onto the dendritic PAMAM-PLA polymers via ester linkages to form dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/Mal block copolymers. The feed molar ratio of HOOC-PEG-OCH₃ and HOOC-PEG-maleimide was set at 3:2 (corresponding to a 60% PEG-OCH₃ and 40% PEG-Mal molar ratio). The maleimide terminal groups present in PEG-Mal were used for TRC105 and NOTA conjugation as described in the Materials and methods, while the -OCH₃ groups present in PEG-OCH₃ were used to control the molar ratio of maleimide at the surface of the unimolecular micelle, which subsequently controls the number of TRC105 and NOTA conjugations per unimolecular micelle. The esterification reaction was carried out in anhydrous dichloromethane at room temperature in the presence of DMAP as the catalyst and DCC as the activating agent for the carboxylic group. The product was purified by a dialysis method to remove the unreacted HOOC-PEG-OCH₃ and HOOC-PEG-Mal. In the ¹H NMR spectrum of PAMAM-PLA-*b*-PEG-OCH₃/Mal (Fig. 3), in addition to the peaks from PLA blocks, the peaks at 3.67 ppm and 3.37 ppm were observed due to the methylene protons of oxyethylene units and methoxyl protons of PEG, respectively. The peak at 6.67 ppm was ascribed to the proton of the maleimide group. The molar ratio of the maleimide group was calculated to be 35%. In other words, the molar ratio of PLA-PEG-MAL:PLA-PEG-OCH₃ was 35:65. This calculation was based on the relative intensity ratio of the peaks at 6.68 ppm and 3.67 ppm corresponding to the proton of the maleimide group and the methylene protons in the PEG main chains. Thus, the NMR spectra clearly demonstrated the formation of the dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/Mal block copolymer, which is further supported by the molecular weight measurements shown in Table 1. Similarly, the average number of arms per dendritic PAMAM-PLA-*b*-PEG-OCH₃/Mal molecule was estimated to be 33 using the following equation:

$$\begin{aligned}\text{Number of arms} &= (M_n \text{ of PAMAM-PLA-}b\text{-PEG-OCH}_3/\text{Mal} - M_n \text{ of PAMAM-PLA}) / (M_n \text{ of PEG}) \\ &= (230,005 - 63,409) / (5000) = 33.\end{aligned}$$

This is consistent with the number of PLA arms estimated for PAMAM-PLA calculated earlier. This is also consistent with a previous study reported by Zhao Y. et al. [34]. In that study, the number of arms for the dendritic PAMAM-PLLA polymer synthesized by ring-opening polymerization of L-lactide using a 4th generation PAMAM-OH as the initiator was 32.

The TRC105 antibody and NOTA for ⁶⁴Cu chelation were conjugated onto the surface of the dendritic PAMAM-PLA-*b*-PEG-OCH₃/Mal copolymer through the reaction between the maleimide groups of PAMAM-PLA-*b*-PEG-OCH₃/Mal and the thiol groups of TRC105-SH and NOTA-SH. To conduct the reaction, TRC105-SH was first prepared by reacting TRC105 with Traut's reagent and then purified by size-exclusive chromatography using PBS as a mobile phase, resulting in about 5 thiol groups per TRC105-SH. At the same time, NOTA-SH was synthesized by reacting p-SCN-Bn-NOTA with AET·HCl in the presence of TEA. Thereafter, a predetermined amount of NOTA-SH and TRC105-SH, both in PBS solution, were added to the PAMAM-PLA-*b*-PEG-OCH₃/Mal PBS solution with a feed molar ratio of TRC105:NOTA:PAMAM-PLA-*b*-PEG-OCH₃/Mal at 5:7:1. The PAMAM-PLA-*b*-PEG-OCH₃/NOTA copolymer used to prepare non-targeted (without TRC105) unimolecular micelles was synthesized using a similar method.

3.2. The properties of the unimolecular micelles

The dendritic amphiphilic block copolymers synthesized formed stable unimolecular micelles in an aqueous solution due to the large number of amphiphilic block copolymer arms (~33) with proper hydrophilic (PEG) to hydrophobic (PLA) ratios as well as their globular architecture [17,35]. The size and morphology of the unimolecular micelles were studied by DLS and TEM. Fig. 4 shows the size distribution histogram of the unimolecular micelles obtained by DLS. The diameter of the unimolecular micelles ranged from 16 to 89 nm with an average diameter of 37 nm. Fig. 5 shows a representative TEM image of the unimolecular micelles. As expected, the unimolecular micelles exhibited a spherical morphology and the average size of the dried unimolecular micelles was approximately 25 nm.

3.3. In vitro drug release study

The DOX loading level in the unimolecular micelles determined by UV-Vis spectroscopy was 13.8 wt%. The *in vitro* drug release study was conducted both at a pH of 7.4 and 5.3. As shown in Fig. 6, the pH value of the drug release medium had a strong influence on the DOX release profile of the unimolecular micelles. The DOX release profiles exhibited a biphasic release pattern under both pH values, with a slight burst release during the first stage, followed by a period of sustained release. At a pH of 7.4, the amount of DOX released during the first 4 h was 11.3%. However, the amount of drug released after 82 h was only 17.3%, suggesting that the DOX encapsulated in the unimolecular micelles was stable under physiological conditions. At a pH of 5.3, nearly 46% of the drug was released during the first 10 h, and the amount of drug released after 82 h was 60%, indicating that the DOX encapsulated in the unimolecular micelles released much faster in an acidic environment (e.g., in endosomes/lysosomes) than under physiological conditions (e.g., the bloodstream). Faster DOX release from such unimolecular micelles may be attributed to the protonation of the DOX amino group and the faster degradation of the hydrophobic polyester micelle core at lower pH levels [36].

This pH-dependent drug release behavior is very desirable for targeted cancer therapy. Most of the drug encapsulated in the unimolecular micelles will remain in the cores of the nanoparticles for a considerable amount of time under physiological conditions when the micelles are intravenously injected into the body, but relatively rapid drug release occurs once the drug-loaded micelles are taken up by the tumor cells into the endosomes/lysosomes via an endocytosis process. This will greatly increase the efficacy of cancer therapy while minimizing the undesirable side effects [37,38].

3.4. Cell uptake studies

To investigate the effects of TRC105 conjugation on the cellular uptake of the unimolecular micelle, fluorescent microscopic analyses of HUVEC (CD105 positive [27,28]) and MCF-7 (CD105 negative [27,28]) were performed to examine the intracellular localization of DOX. HUVEC treated with targeted unimolecular micelles showed a high level of cellular DOX fluorescence. In contrast, cells treated with non-targeted micelles only showed a low-level cytoplasmic DOX fluorescence. Blocking with 0.5 mg/ml of TRC105 significantly reduced the cyto-fluorescence intensity of targeted micelles, which was at a level comparable to that of non-targeted micelles (Fig. 7(A)). Compared with those results, both targeted and non-targeted micelles demonstrated very low cellular uptake in MCF-7 and did not reveal any difference, which further confirmed that cellular uptake of targeted micelles in HUVEC was CD105-mediated.

Fig. 7(B) shows the quantitative flow cytometry analysis results of HUVEC cellular uptake of targeted micelles and non-targeted micelles at an early (15 min) and late (120 min) time

point. Cells without any treatment were used as a negative control, which showed only negligible levels of autofluorescence at both time points. As early as 15 min post-treatment, the cellular uptake of targeted micelles in HUVEC was significantly higher (>50%) than that of non-targeted micelles. At 120 min, all treatment groups showed a marked increase in the cellular uptake of DOX. Cells treated with targeted micelles exhibited a significantly higher level of cellular uptake than cells treated with non-targeted micelles. Taken together, fluorescent microscopic studies confirmed that TRC105 conjugation markedly increased the cellular uptake of PAMAM-based micelles in a CD105 specific manner.

3.5. In vivo investigation

To investigate both the short-term and long-term behaviors of these two types of unimolecular micelles, the time points of 0.5, 2, 5, 16, 24, and 48 h p.i. were chosen for PET scans after intravenous injection of ^{64}Cu -labeled targeted micelles or ^{64}Cu -labeled non-targeted micelles, based on our previous experience [18,30,31,39]. The reason 4T1 was chosen as the tumor model in this study was due to the fact that it is a fast-growing tumor type and has significant amounts of angiogenesis and CD105 expression during tumor growth, even though 4T1 cells themselves are CD105-negative. The coronal slices that contained the 4T1 tumors are shown in Fig. 8. The radioactivity fluctuation in the blood pool indicated that the blood half-lives of both targeted and non-targeted micelles were several hours (^{64}Cu -labeled targeted micelles have a longer circulation time based on PET data). The prolonged circulation time will also facilitate *in vivo* tumor accumulation. Besides the blood, ^{64}Cu -labeled targeted micelles primarily accumulated in the liver, tumor, spleen, kidneys, and intestines, but not in most normal tissues (e.g., muscle, bone, brain, etc.), demonstrating good tumor-targeting efficacy and good tumor contrast. In contrast, tumor accumulation of ^{64}Cu -labeled non-targeted micelles, as well as ^{64}Cu -labeled targeted micelles with a blocking dose of TRC105 (1 mg per mouse), was quite low (Fig. 8), which was further confirmed by quantitative analysis as discussed below.

Quantitative data obtained from ROI analyses are shown in Fig. 9. Uptake of ^{64}Cu -labeled targeted micelles in the liver was prominent at early time points and gradually declined over time. The liver uptake of ^{64}Cu -labeled targeted micelles was 16.2 ± 1.4 , 16.0 ± 0.7 , 11.8 ± 0.7 , 8.5 ± 0.6 , 8.1 ± 0.4 , and $6.7 \pm 0.3\%$ ID/g at 0.5, 2, 5, 16, 24, and 48 h p.i., respectively. The 4T1 tumor uptake of ^{64}Cu -labeled targeted micelles was clearly visible at 0.5 h p.i. and dropped slowly after 5 h p.i. (4.5 ± 0.6 , 5.5 ± 0.3 , 5.7 ± 0.3 , 4.5 ± 0.3 , 4.3 ± 0.1 , and $3.0 \pm 0.1\%$ ID/g at 0.5, 2, 5, 16, 24, and 48 h p.i., respectively; $n = 4$; Fig. 9(A)). A tumor/muscle ratio of 14.0 ± 0.8 was achieved for ^{64}Cu -labeled targeted micelles at as early as 2 h p.i. ($n = 4$), which gave excellent tumor contrast. Administering a blocking dose of TRC105 (1 mg per mouse) along with ^{64}Cu -labeled targeted micelles reduced the tumor uptake significantly (Fig. 9(B)), which confirmed the CD105 specificity of ^{64}Cu -labeled targeted micelles *in vivo*. Liver uptake in the blocking group was also significantly higher than ^{64}Cu -labeled non-targeted micelles at early time points (0.5 h and 2 h p.i.). Radioactivity in the blood was 10.0 ± 1.5 , 7.8 ± 1.0 , 6.3 ± 0.3 , 3.8 ± 0.2 , 3.5 ± 0.3 , and $2.8 \pm 0.3\%$ ID/g at 0.5, 2, 5, 16, 24, and 48 h p.i., respectively ($n = 4$, Fig. 9(A)).

^{64}Cu -labeled non-targeted micelles served as a control for investigating the uptake of unimolecular micelles in 4T1 tumors due to passive targeting only (i.e., via the enhanced permeability and retention effect). As can be seen in Fig. 9(C), despite its long circulation time, the tumor uptake of ^{64}Cu -labeled non-targeted micelles (2.7 ± 0.4 , 3.1 ± 0.4 , 3.1 ± 0.7 , 2.4 ± 0.5 , 2.0 ± 0.4 , and $2.2 \pm 0.7\%$ ID/g at 0.5, 2, 5, 16, 24, and 48 h p.i., respectively; $n = 3$; Fig. 9(B) and (D)) was significantly lower than that of ^{64}Cu -labeled targeted micelles at time points before 48 h p.i. ($P < 0.05$; $n = 4$), which further confirmed the CD105 specificity of ^{64}Cu -labeled targeted micelles *in vivo*.

After the last PET scans at 48 h p.i., all mice were euthanized. All major organs were collected for biodistribution studies to further validate *in vivo* PET data (Fig. 10). Another separate group of four mice injected with ^{64}Cu -labeled targeted micelles were used for biodistribution studies at 5 h p.i. (when the tumor uptake peaks, based on PET imaging). The results indicated that the ^{64}Cu -labeled targeted micelles had dominant uptake in the 4T1 tumor (only lower than the liver/spleen/intestine/kidneys) (Fig. 10). Overall, the quantification results obtained from the biodistribution studies and PET scans matched very well, confirming that the quantitative ROI analysis of non-invasive microPET scans truly reflected the distribution of ^{64}Cu -labeled targeted micelles and ^{64}Cu -labeled non-targeted micelles *in vivo*.

4. Conclusions

Multifunctional unimolecular micelles made of dendritic amphiphilic block copolymers poly(amidoamine)-poly(L-lactide)-*b*-poly(ethylene glycol) conjugated with anti-CD105 monoclonal antibody (TRC105) and NOTA was synthesized and characterized for tumor-targeted drug delivery and PET imaging. Stable unimolecular micelles formed by the dendritic amphiphilic block copolymers exhibited a spherical morphology with an average hydrodynamic diameter of 37 nm. The DOX encapsulated in the unimolecular micelles showed a pH-sensitive drug release behavior due to the protonation of the DOX amino group and faster degradation of the hydrophobic polyester micelle core at lower pH levels. Tumor targeting efficacy of the unimolecular micelles was investigated both *in vitro* via flow cytometry and CLSM and *in vivo* via serial non-invasive PET imaging and biodistribution studies. It was found that TRC105-conjugated unimolecular micelles could be specifically directed to tumor neovasculature *in vivo* via targeting of CD105, a vascular marker for tumor angiogenesis. Thus, TRC105-conjugated unimolecular micelles can potentially enhance the therapeutic efficacy significantly. The versatile chemistry and multifunctionality of these unimolecular micelles make them a desirable nanoplatform for future biomedical research.

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Fig. 1.

A schematic illustration of the multifunctional PAMAM-PLA-*b*-PEG-OCH₃/TRCI05/NOTA unimolecular micelles for tumor-targeted drug delivery and PET imaging.

Scheme 1.

Synthesis scheme of the dendritic amphiphilic PAMAM-PLA-*b*-PEG-OCH₃/TRC105/NOTA block copolymers.

Fig. 2.
(A) ^1H NMR and (B) FTIR spectra of the PAMAM-PLA polymer.

Fig. 3.
 ^1H NMR spectrum of the PAMAM-PLA-*b*-PEG- OCH_3 /Mal.

Fig. 4.
DLS histogram of unimolecular micelles.

Fig. 5.
TEM image of unimolecular micelles.

Fig. 6.

In vitro drug release profiles of DOX-loaded unimolecular micelles at pH = 7.4 (●) and pH = 5.3 (■).

Fig. 7. (A) Fluorescence microscopy images of HUVEC and MCF-7 incubated with targeted micelles, non-targeted micelles, or targeted micelles with a blocking dose of TRC105 at 37 °C for 2 h. All samples have a DOX concentration of 20 µg/ml. (B) Flow cytometry analysis of HUVECs treated with targeted or non-targeted micelles (all with equivalent DOX concentration of 20 µg/ml) for 15 min and 120 min at 37 °C.

Fig. 8. Serial coronal PET images of 4T1 tumor-bearing mice at different time points post-injection of ^{64}Cu -labeled targeted micelles, non-targeted micelles, or targeted micelles with a blocking dose of TRC105.

Fig. 9.

Region-of-interest analysis of PET. (A) Time-activity curves of 4T1 tumor, liver, blood, and muscle upon intravenous injection of ⁶⁴Cu-labeled targeted micelles ($n = 4$). (B) Time-activity curves of 4T1 tumor, liver, blood, and muscle upon intravenous injection of ⁶⁴Cu-labeled targeted micelles with a blocking dose of TRC105 ($n = 4$). (C) Time-activity curves of 4T1 tumor, liver, blood, and muscle upon intravenous injection of ⁶⁴Cu-labeled non-targeted micelles ($n = 4$). (D) Comparison of 4T1 tumor uptake for ⁶⁴Cu-labeled targeted micelles, non-targeted micelles, and targeted micelles with TRC105 blocking at different time points post-injection. * $P < 0.05$.

Fig. 10. (A) Biodistribution of ^{64}Cu -labeled targeted micelles at 5 h post-injection. (B) Biodistribution of ^{64}Cu -labeled targeted micelles, non-targeted micelles, and targeted micelles with TRC105 blocking at 48 h post-injection.

Table 1Molecular weights of PAMAM-OH, PAMAM-PLA, and PAMAM-PLA-*b*-PEG-OCH₃/Mal.

Sample	M_n	M_w	M_w/M_n
PAMAM-OH	14,277		
PAMAM-PLA	63,409	110,861	1.748
PAMAM-PLA- <i>b</i> -PEG-OCH ₃ /Mal	230,005	423,899	1.843