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Peptide-Conjugated Polymeric Micellar Nanoparticles for Dual SPECT and Optical Imaging of EphB4 Receptors in Prostate Cancer Xenografts

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Abstract

EphB4, a member of the largest family of receptor tyrosine kinases, is overexpressed in numerous tumors. In this study, we developed a new class of multimodal nanopatform for dual single photon emission computed tomography (SPECT) and near-infrared fluorescence imaging of EphB4. EphB4-binding peptide TNYL-FSPNGPIARAW (TNYL-RAW) was conjugated to polyethylene glycol-coated, core-crosslinked polymeric micelles (CCPM) dually labeled with near-infrared fluorescence fluorophores (Cy7) and a radioisotope (indium 111). *In vitro*, TNYL-RAW-CCPM selectively bound to EphB4-positive PC-3M prostate cancer cells, but not to EphB4-negative A549 lung cancer cells. *In vivo*, PC-3M tumors were clearly visualized by both SPECT and near-infrared fluorescence tomography after intravenous administration of ¹¹¹In-labeled TNYL-RAW-CCPM. In contrast, there was little signal in A549 tumors of mice injected with ¹¹¹In-labeled TNYL-RAW-CCPM or in PC-3M tumors of mice injected with ¹¹¹In-labeled CCPM. The high accumulation of ¹¹¹In-labeled TNYL-RAW-CCPM in PC-3M tumor could be significantly reduced after co-injection with an excess amount of TNYL-RAW peptide. Immunohistochemical analysis showed that fluorescence signal from the nanoparticles correlated with their radioactivity count, and co-localized with the EphB4 expressing region. ¹¹¹In-labeled TNYL-RAW-CCPM allowed visualization of cancer cells overexpressing EphB4 by both nuclear and optical techniques. The complementary information acquired with multiple imaging techniques should be advantageous in early detection of cancer.

Keywords

Micelle; Nanoparticle; Fluorescence; Molecular Imaging

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INTRODUCTION

The Eph receptors constitute the largest known family of receptor tyrosine kinases, which regulate a number of biological processes in normal development [1, 2]. The Eph receptors have been reported to correlate with various pathological processes, including tumor progression, chronic pain following tissue damage, pathological forms of angiogenesis, inhibition of nerve regeneration after spinal cord injury, and human congenital malformations [3–10]. EphB4, a key member of this family, is normally expressed on venous endothelial cells and plays a vital role in angiogenesis and vascular network assembly [10, 11]. Overexpression of EphB4 has also been observed in numerous tumor types, including prostate, breast, bladder, lung, colon, gastric, and ovarian cancers [1–4, 10–13]. Knocking down EphB4 can result in inhibition of tumor growth, induction of apoptosis, and reduction in tumor vascularity [13]. Therefore, targeting EphB4 has great potential in cancer diagnosis and therapy.

We have recently reported the first positron emission tomography (PET) imaging agent for detection of EphB4 in cancer cells [14]. This PET imaging agent was developed on the basis of a 14-mer peptide identified by Koolpe et al. by phage display technology [4]. The parent peptide TNYLFSPNGPIARAW (designated as TNYL-RAW) and its derivatives displayed high binding affinity to EphB4, with K_D values of 1.98 to 23 nM [14]. Although μ PET/computed tomography (CT) imaging clearly revealed deposition of ^{64}Cu -DOTA-TNYL-RAW in xenografted tumors overexpressing EphB4, the short circulation time of this peptide might limit its exposure time and penetration into tumors, which would compromise its sensitivity. We hypothesized that optimization of the peptide's pharmacokinetics through the use of long-circulating nanoparticles would enhance the ligand's opportunity to bind to EphB4, improving the likelihood of cancer detection. Towards this end, we conjugated TNYL-RAW to a multifunctional, long-circulating nanocarrier, core-crosslinked polymeric micelles. We show that TNYL-RAW-functionalized micelles dually labeled with a gamma emitter, indium 111 (^{111}In), and a near-infrared fluorescent indocyanine 7 (Cy7)-like dye permitted simultaneous visualization of EphB4-overexpressing tumors by both nuclear and optical imaging.

MATERIALS AND METHODS

Reagents

Common reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Acros (Geel, Belgium) and were used as received unless otherwise specified. *p*-Isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (DTPA-Bz-NCS) was purchased from Macrocylics (Dallas, TX). Indium-111 chloride ($^{111}\text{InCl}_3$) was obtained from Perkin-Elmer (Waltham, MA).

CCPM Nanoparticles

Core-crosslinked polymeric micelle (CCPM) nanoparticles were a kind gift of Carestream Health, Inc. (Rochester, NY). Each CCPM nanoparticle contained approximately 21 Cy7-like dye molecules and 180 amino (NH_2) groups. DTPA loading was estimated to be approximately 19 DTPA molecules per nanoparticle. The detailed procedure for synthesis of CCPM nanoparticles was described in a previous report [15].

TNYL-RAW-Functionalized CCPM

The reaction scheme for the conjugation of TNYL-RAW to the surface of CCPM is shown in Figure 1A. To introduce the maleimide group to CCPM, an aliquot of N-[γ -maleimidobutyryloxy]-succinimide ester (GMBS) in dimethylformamide (22.5 μL , 7 μmol /

mL, 0.16 μ mol) was added into CCPM (250 μ L, 2.6 nmol nanoparticles) in 1.2 mL of phosphate-buffered saline (PBS, pH 8). The mixture was stirred for 3 h at 37°C. The product was purified using a PD-10 column to remove unreacted GMBS. TNYL-RAW peptide, Tyr-Asn-Tyr-Leu-Phe-Ser-Pro-Asn-Gly-Pro-Ile-Ala-Arg-Ala-Trp-NH₂, was synthesized manually using Rink amide resin (Novabiochem, NJ). To introduce a sulfhydryl group to TNYL-RAW, amino acid cysteine was conjugated to the C-terminal amine of the TNYL-RAW peptide. The resulting sulfhydryl-containing TNYL-RAW peptide was mixed with 2 mL of a solution of CCPM-maleimide (0.16 μ mol equivalent maleimide) in PBS with a molar ratio of TNYL-RAW to maleimide groups (CCPM) of 2:1. The solution was stirred for 12 h at 4°C and then purified using a fast protein liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with a G200 column and an ultraviolet (UV) light detector (254 nm). The column was eluted with PBS to remove unreacted TNYL-RAW peptide. The unreacted TNYL-RAW peptide was quantified using the Agilent 1100 Series LC/MSD-TOF instrument equipped with a Vydac C18 column (Agilent Tech, CA), and the data were used to calculate the molar ratio of TNYL-RAW bound to CCPM in TNYL-RAW-CCPM.

Characterization of TNYL-RAW-CCPM

For transmission electron microscopic examination, a drop of aqueous sample solution was placed on a 400-mesh copper grid coated with 0.5% poly(vinyl formal) aqueous solution (w/w). Negative staining was performed using a droplet of 1% uranyl acetate solution. The sample was air-dried and examined with a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA). Fluorescence spectra were recorded using a Fluorolog fluorometer (Horiba, Edison, NJ).

Radiolabeling

Aliquots of TNYL-RAW-CCPM in 0.1 M sodium acetate solution (pH 5.2) were mixed with an aqueous solution of ¹¹¹InCl₃ at room temperature for 30 min. Radiolabeled nanoparticles were analyzed using an instant thin-layer chromatography system. The instant thin-layer chromatography strips were developed with PBS (pH 7.4) containing 4 mM EDTA and quantified using a Bioscan IAR-2000 TLC Imaging Scanner (Washington, DC). Free ¹¹¹In³⁺ moved to the solvent front (R_f = 0.9), and the nanoparticles remained at the original spot (R_f = 0.0).

Fluorescence Microscopy

PC-3M human prostate cancer cells were obtained from Professor Dominic Fan (The University of Texas MD Anderson Cancer Center, Houston, TX). A549 human lung adenocarcinoma epithelial cells were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum and a mixture of antibiotics (100 units mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin; Biochrom AG, Holliston, MA). Cells were grown at 37°C in a humidified atmosphere and 5% CO₂. PC-3M and A549 cells were incubated with TNYL-RAW-CCPM in RPMI-1640 medium for 15 min at 37°C at a final concentration of 10 nM nanoparticles. In the blocking group, PC-3M cells were incubated with 10 nM TNYL-RAW-CCPM plus 60 μ M TNYL-RAW peptide as a control. After incubation, cells were washed 3 times with PBS and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, Saint Louis, MO). The cell samples were transferred onto Lab-Tek II chambered cover glass and visualized under a Zeiss Axio Observer.Z1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Thornwood, NY) equipped with a UV filter for DAPI and a Cy7 filter (wavelength 710/810 nm).

Cell Binding Study

To study cell uptake of ^{111}In -labeled TNYL-RAW-CCPM, PC-3M and A549 cells were grown in 6-cm petri dishes to subconfluent densities in RPMI-1640 medium containing 10% fetal bovine serum 1 day before experiments. The medium was replaced with 2 mL of fresh medium containing 10 nM ^{111}In -labeled TNYL-RAW-CCPM nanoparticles (~ 2.0 MBq/mL), and cells were incubated for 15 min. In the blocking group, PC-3M cells were co-incubated with 10 nM ^{111}In -labeled TNYL-RAW-CCPM nanoparticles (~ 2.0 MBq/mL) and 60 μM unlabeled TNYL-RAW peptide for 15 min. After incubation, the cell monolayers were scraped and transferred into 5-mL tubes, and the tubes were briefly subjected to vortexing. Aliquots of PC-3M or A549 cell suspension (100 μL) were transferred into microcentrifuge tubes containing 500 μL of a 75:25 mixture of silicon oil (density 1.05, Aldrich) and mineral oil (density 0.872, Acros). The mixture was subjected to centrifugation at 14,000 rpm for 5 min. After the tubes were frozen with liquid nitrogen, the bottom tips containing the cell pellet were cut off. The cell pellets and the supernatants were counted with a gamma counter (Perkin-Elmer). The protein content in 100 μL of cell suspension was quantified in a separate experiment using a Bio-Rad protein assay kit according to the manufacturer's protocol. Activity ratios of the cell pellet to medium ([cpm/ μg of protein in pellet]/[cpm/ μg of medium]) were calculated and plotted against time. The experiments were performed in pentaplicate.

Surface Plasmon Resonance Assay

Binding assays were performed at 25°C in HBSEP running buffer (0.01 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid [EDTA], and 0.005% [v/v] surfactant P20 solution). The TNYL-RAW-CCPM and CCPM were diluted in HBSEP buffer, filtered, degassed, and injected as a series of 8 two-fold concentrations ranging from 0.16 nM to 20 nM at a flow rate of 30 $\mu\text{L}/\text{min}$. The injection time of peptides in the HBSEP buffer was 7 min, followed by a 3-min dissociation period. The chips were regenerated using a 30-sec pulse of 10 mM glycine (pH 2.2) after each binding cycle. All analyzed samples were additionally injected onto an uncoated reference surface, including a sample of the running buffer, which was also tested on a EphB4-coated flow cell. Data were evaluated with the BIAevaluation software (version 3.1). The obtained sensorgrams were fitted globally over the whole range of injected concentrations and simultaneously over the association and dissociation phases. Equilibrium dissociation constants were then calculated from the rate constants ($K_D = k_{\text{off}}/k_{\text{on}}$).

Pharmacokinetic Study

All animal studies were carried out in the Small Animal Imaging Facility at MD Anderson Cancer Center in accordance with institutional guidelines. For the pharmacokinetic study, healthy male Swiss mice (22–25 g; Charles River Laboratories, Wilmington, MA) ($n = 5$) were injected intravenously at a dose of 2×10^{13} ^{111}In -labeled TNYL-RAW-CCPM or 5 μg TNYL-RAW peptide per mouse (1.6 MBq/mouse). At predetermined intervals, blood samples (10 μL) were taken from the tail vein, and the radioactivity of each sample was measured with a Cobra Autogamma counter (Packard, Downers Grove, IL). The blood pharmacokinetic parameters for the radiotracer were analyzed using a noncompartmental model with WinNonlin 5.0.1 software (Pharsight Corp., Palo Alto, CA).

Tumor Models

Six- to 8-week-old male nude mice were inoculated subcutaneously in the right flank with PC-3M or A549 cells (1.0×10^6). Three weeks after inoculation, when tumors reached approximately 5–6 mm in diameter, mice were divided into 4 groups. Mice in group 1 ($n=6$)

bearing a PC-3M tumor and mice in group 3 (n=5) bearing a A549 tumor received an intravenous injection of ^{111}In -labeled TNYL-RAW-CCPM. Mice in group 2 and group 4 (n=6) bearing a PC-3M tumor were injected intravenously with ^{111}In -labeled TNYL-RAW-CCPM plus excess unlabeled TNYL-RAW peptide (100-fold, blocking group) or ^{111}In -labeled plain CCPM (control group), respectively.

Imaging and Biodistribution Studies

As described in the preceding section, ^{111}In -labeled TNYL-RAW-CCPM or ^{111}In -labeled CCPM (1×10^{14} particles/mouse, 9.0 MBq/mouse) was injected intravenously via the tail vein into tumor-bearing mice. At 24 h after administration of those particles, SPECT and CT images were acquired using an xSPECT-CT scanner (Gamma Medica, Northridge, CA). SPECT scans (radius of rotation 3 cm, 32 projections, 20 s per projection) and CT scans (512 projections, 75 kv, 500 mA) were acquired and co-registered for image fusion and presentation of 3-dimensional anatomical localization of the tracer signal. Acquired SPECT and CT data sets were processed with AMIRA 5.1 (San Diego, CA). The whole-body optical imaging was performed using fluorescent molecular tomography (Visen, Bedford, MA) equipped with a 760/790 nm excitation/emission wavelength filter. During each imaging session, mice were anesthetized with 2% isoflurane gas (Iso-Thesia, Rockville, NY) in oxygen.

At the end of imaging section, mice in all 4 groups were killed immediately. Various tissues were removed, weighed, and counted for radioactivity with a Cobra Autogamma counter (Packard). Uptake of the nanoparticles was calculated as the percentage of the injected dose per gram of tissue (% ID/g). Student's *t* test was used to compare differences in tissue uptake between different groups, and *P*-values less than 0.01 were considered highly significant.

Autoradiography and Optical Imaging

Tumors harvested at the end of the imaging session were snap-frozen and sectioned into 5- μm sections. The sections were photographed and exposed on BAS-SR 2025 Fuji phosphorus films. The film was scanned with a FLA5100 Multifunctional Imaging System (Fujifilm Medical Systems USA, Stamford, CT).

After autoradiographic study, optical images of the sections were acquired by scanning at 800 nm using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Immunohistochemistry

Following the imaging studies, 1 slice of each tumor was fixed in 4% paraformaldehyde solution for 20 min at room temperature and washed with PBS 3 times. Tumor sections were blocked with 10% goat serum for 30 min at 37°C, and the slides were then incubated with rabbit anti-human EphB4 antibody (Santa Cruz, CA) in PBS at 37°C overnight. After incubation with primary antibody, slides were washed with PBS 3 times and incubated with Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G (Invitrogen, CA). Then, the stained slides were washed again in PBS and counterstained with DAPI. Images were recorded using a Zeiss Axio Observer.Z1 fluorescence microscope equipped with UV filter for DAPI, fluorescein isothiocyanate (FITC) filter (wavelength 488/520 nm), and Cy7 filter (wavelength 710/810 nm). Images of the whole sections were also scanned with a FLA5100 Multifunctional Imaging System equipped with FITC filter (wavelength 488/520 nm).

RESULTS

Characterization of TNYL-RAW-CCPM

The reaction scheme for the conjugation of TNYL-RAW peptide to the surface of CCPM is shown in Figure 1A. Figure 1B is a transmission electron microscopic image of TNYL-RAW-CCPM. The average diameter of the nanoparticles was 23 nm on the basis of transmission electron microscopy images. Calculated on the basis of the molar feed ratio, each TNYL-RAW-CCPM nanoparticle contained approximately 60 TNYL-RAW molecules on the surface. The excitation and emission light intensities for TNYL-RAW-CCPM nanoparticles peaked at 755 nm and 781 nm, respectively (Fig. 1C). As indicated by instant thin-layer chromatography, the radiolabeling efficiency of TNYL-RAW-CCPM was greater than 98% without further purification. The specific activity was 180 MBq/nmol nanoparticles.

In Vitro Binding to Cancer Cells

Figure 2 shows representative sensorgrams obtained from surface plasmon resonance (SPR) analyses of TNYL-RAW-CCPM and plain CCPM. When TNYL-RAW-CCPM was incubated with a chip coated with EphB4, increasing amounts of the nanoparticles bound to the chip in a dose-dependent manner. In contrast, no binding to EphB4 was detected with CCPM. When the binding curves of TNYL-RAW-CCPM were analyzed by conventional (1:1 or 1:2) mass transfer models, the fit was poor, indicating that the binding kinetics of TNYL-RAW-CCPM to EphB4 is complex and likely involves multivalency interaction.

While PC-3M cells expressed EphB4, EphB4 was undetectable in A549 cells [14]. Figure 3A shows fluorescence microscopic images of PC-3M human prostate cancer cells and A549 human lung adenocarcinoma epithelial cells incubated with TNYL-RAW-CCPM. Uptake of TNYL-RAW-CCPM was high in EphB4-positive PC-3M cells, but not in EphB4-negative A549 cells. Moreover, uptake of TNYL-RAW-CCPM in PC-3M cells could be efficiently blocked by an excessive amount of TNYL-RAW peptide.

Cell uptake of TNYL-RAW-CCPM was also analyzed by the radioactivity count method, and the result was consistent with the one obtained by fluorescence microscopy. PC-3M cells showed significantly higher uptake of ^{111}In -labeled TNYL-RAW-CCPM than A549 cells (Fig. 3B). The uptake of ^{111}In -labeled TNYL-RAW-CCPM in PC-3M cells was reduced 95% by an excess amount of unlabeled TNYL-RAW peptide (Fig. 3B). Taken together, both cell binding studies confirmed that ^{111}In -labeled TNYL-RAW-CCPM selectively bound to EphB4 on the surface of cancer cells.

Pharmacokinetics

Figure 4 compares the activity-time profiles of ^{111}In -labeled TNYL-RAW-CCPM and TNYL-RAW peptide. Blood pharmacokinetic characteristics of TNYL-RAW and TNYL-RAW-CCPM were determined by noncompartmental analysis. The TNYL-RAW-CCPM formula had a significantly (4-fold) higher systemic exposure than TNYL-RAW (AUC=138%ID h/mL versus 29.1%ID h/mL), which resulted mainly from a significantly slower mean systemic clearance (CL= 0.77 mL/h for TNYL-RAW-CCPM versus 3.52 mL/h for TNYL-RAW). The data indicate that the TNYL-RAW-CCPM formula provided longer circulation of the drug in the blood. On the other hand, the mean volume of distribution at steady-state for the TNYL-RAW-CCPM formula was much smaller (V_{ss} =6.57 mL) than that of TNYL-RAW (V_{ss} =64.9 mL), suggesting that the widely distributed nature of the TNYL-RAW peptide was significantly altered after micelle formulation. The smaller volume of distribution of TNYL-RAW-CCPM was also attributed to the observed higher concentrations of TNYL-RAW-CCPM in the blood.

Nuclear and Optical Imaging

Figure 5 compares μ SPECT and fluorescent molecular tomography optical images of mice bearing a PC-3M or A549 tumor 24 h after administration of ^{111}In -labeled TNYL-RAW-CCPM or plain CCPM. The EphB4-positive PC-3M tumor was clearly visualized after intravenous injection of ^{111}In -labeled TNYL-RAW-CCPM, while there was little signal in EphB4-negative A549 tumors of mice injected with ^{111}In -labeled TNYL-RAW-CCPM or in PC-3M tumors of mice co-injected with ^{111}In -labeled TNYL-RAW-CCPM and excess cold TNYL-RAW peptide. There was no detectable uptake of ^{111}In -labeled CCPM in PC-3M tumors, either. Similar findings were observed in optical images (Fig. 5B).

Biodistribution

The biodistribution data described in Figure 6A are consistent with the imaging results. At 24 h after injection, uptake of ^{111}In -labeled TNYL-RAW-CCPM was significantly higher in PC-3M tumors (2.87% ID/g) than in A549 tumors (1.37% ID/g) ($P < 0.01$). The accumulation of substantial amounts of ^{111}In -labeled TNYL-RAW-CCPM was efficiently blocked by an excess amount of unlabeled TNYL-RAW peptide in group 3 (1.42% ID/g). The tumor-to-blood ratio was 6.5 in the mice bearing a PC-3M tumor after administration of ^{111}In -labeled TNYL-RAW-CCPM, while it was 2.3 in the mice bearing an A549 tumor after injection of ^{111}In -labeled TNYL-RAW-CCPM and 2.6 in mice bearing a PC-3M tumor after co-injection of ^{111}In -labeled TNYL-RAW-CCPM and excess TNYL-RAW peptide. In PC-3M tumors, furthermore, uptake of ^{111}In -labeled TNYL-RAW-CCPM (2.87% ID/g) was significantly higher than uptake of ^{111}In -labeled plain CCPM (1.61 % ID/g) or ^{64}Cu -labeled TNYL-RAW (0.84 % ID/g) ($P < 0.01$) (Fig. 6B).

Autoradiography and Immunohistochemistry

Intratumoral distribution of ^{111}In -labeled TNYL-RAW-CCPM is shown in both autoradiographic images and fluorescence optical images of tumor sections (Fig. 5 C&D). Sections of PC-3M tumors from mice that received a single administration of ^{111}In -labeled TNYL-RAW-CCPM exhibited higher radioactivity and fluorescent signal intensity than tumor sections from the other 3 groups, which was generally in concordance with the imaging and biodistribution results. The localization of both radioactivity and fluorescence signal from ^{111}In -labeled TNYL-RAW-CCPM correlated with expression of EphB4 stained with anti-EphB4 antibody (Fig. 5C–E). Moreover, immunohistochemical analysis confirmed that EphB4 is overexpressed in PC-3M tumor cells, but not in A549 tumor cells. Fluorescence signal from TNYL-RAW-CCPM co-localized with EphB4-expressing areas (Figure 7).

DISCUSSION

EphB4 is overexpressed in many solid tumors [1–4, 10–13]. To date, however, there have been few reports about development of tracers based on EphB4 [14]. In this study, we developed a dual-labeled TNYL-RAW-CCPM nanoparticle that allowed ready visualization of EphB4-overexpressing tumors with both SPECT and near-infrared fluorescence imaging in mice. Moreover, TNYL-RAW-CCPM permitted detection of these cancer cells at the microscopic level by optical imaging.

TNYL-RAW peptide has a short blood half-life in mice, which could lead to limited exposure time at tumor sites and thus lower tumor uptake. To improve imaging quality, we hypothesized that modulation of the pharmacokinetics of TNYL-RAW peptide through introduction of long-circulating nanoparticles would make TNYL-RAW more readily available for receptor binding. In this study, TNYL-RAW was conjugated to the surface of polyethylene glycol-coated CCPM nanoparticles. As expected, the pharmacokinetic data

show clearly that the micelle formula had significantly reduced clearance and reduced volume of distribution, which resulted in higher drug concentration in the blood (AUC) for better tumor-targeting efficiency. It is noted that the tumor uptake value and tumor-to-blood ratio for TNYL-RAW-CCPM in PC-3M tumors were 2.87% ID/g and 6.51, respectively, which were 2 times higher than the corresponding values for TNYL-RAW peptide (0.84% ID/g and 2.68, respectively), indicating that prolonging the blood half-life of TNYL-RAW could lead to increased uptake of the radiotracer in tumors. The biodistribution study also demonstrated that CCPM uptake was only 1.61% ID/g in PC-3M tumor (Fig. 6B), suggesting that the increased tumor uptake of TNYL-RAW-CCPM was not a result of the increased permeability and retention effects of the nanoparticles. Taken together, our data support the hypothesis that prolonging the half-life of TNYL-RAW improves the level of signal in tumors and is superior for noninvasive molecular imaging of cancer.

The introduction of TNYL-RAW to CCPM nanoparticles may increase the avidity of the resulting nano-imaging probe to EphB4 through a multivalent effect. Using multivalent targeting systems, including dimers, tetramers, and multimers, has been reported as a successful strategy for enhancing the avidity and/or biological functions of peptides, proteins, and antibodies [16–20]. This approach is particularly useful for ligands that have a weak affinity to their target receptors in their monomer form. For example, trastuzumab, an engineered antibody for Her2+ breast cancer therapy, was increased in potency up to 25 times through conjugation to liposomes [21]. In our study, each TNYL-RAW-CCPM nanoparticle contained approximately 60 TNYL-RAW molecules on the surface. Thus, the repertoire of TNYL-RAW was greatly expanded by multiple, simultaneous interactions between the surface of the nanoparticle and multiple receptors of cancer cells. While SPR curves of the TNYL-RAW peptide fit smoothly with a 1:1 binding model, the SPR sensorgrams of TNYL-RAW-CCPM failed to fit into the standard 1:1 or 1:2 binding models, indicating that the binding kinetics between EphB4 and TNYL-RAW-CCPM were complex. The complex binding profile probably was induced by the multivalent nature of TNYL-RAW-CCPM (Fig. 2).

Near-infrared fluorescence (NIRF) optical imaging offers several advantages over conventional imaging modalities in that it is extremely sensitive, inexpensive, robust, involves no harmful radiation, and allows real-time visualization. Although this technique has shown promise in several small animal studies, the utility of targeted NIRF optical imaging in the clinic has yet to be demonstrated. The major barriers to implementing NIRF imaging in detection of pancreatic cancer in the clinical setting include the absence of well-validated molecular imaging agents that are specific to pancreatic cancer, the limit of penetration depth for optical signal, and the lack of a miniature NIRF camera system that can be used endoscopically. On the other hand, nuclear imaging offers high detection sensitivity, which makes it especially suited for tracking radiotracers used in *in vivo* molecular imaging. However, neither PET nor SPECT can localize radiotracers at the cellular level because of low spatial resolution. An effective and economical imaging strategy is to develop multimodality imaging probes that allow extraction of as much diagnostic information as possible from each examination. The combination of two or more imaging techniques can therefore offer synergistic advantages over a single modality alone in providing valuable diagnostic information. The complementary information acquired with multiple imaging techniques should be advantageous in diagnosis of cancer.

To date, a number of imaging agents labeled with radioisotopes or fluorescent dyes have been developed for diagnosis of cancer [22–29]. However, it is highly desirable that an imaging probe combining a radioisotope and a near-infrared fluorescent dye be available for dual nuclear and optical imaging. Several nanoparticle-based multimodal imaging probes have been developed and applied for multimodal imaging in living animals [30–33]. These

multimodal imaging systems have shown great promise in preclinical drug development and biomedical research. In this study, Cy-7 dye was entrapped in the core of CCPM and radioisotope chelator DTPA was conjugated on the surface of CCPM. Each micellar nanoparticle was loaded with multiple Cy7 dye molecules and ^{111}In ions, providing a huge boost in signal intensity, which probably explains why the signal intensity of a fluorescent image scanned at 800 nm was much higher than that obtained at 520 nm. As shown in both nuclear and optical imaging (Fig. 5 A&B), ^{111}In -labeled TNYL-RAW-CCPM potentially can be used to locate EphB4-overexpressing tumor by whole-body nuclear and optical imaging. Histopathologically, ^{111}In -labeled TNYL-RAW-CCPM revealed the region of EphB4 expression in tumor xenografts consistent with autoradiographic and fluorescent findings in tumor sections (Fig. 7).

CONCLUSIONS

In this study, we evaluated the potential application of ^{111}In -labeled TNYL-RAW-functionalized core-crosslinked polymeric micelles for multimodal detection of EphB4-overexpressing cancer cells. EphB4-positive PC-3M tumor was clearly visualized with ^{111}In -labeled TNYL-RAW-CCPM by both SPECT and optical imaging. Moreover, TNYL-RAW-CCPM permitted detection of PC-3M cells at the microscopic level by near-infrared fluorescence imaging. Successful translation of ^{111}In -labeled TNYL-RAW-CCPM and other multimodal imaging probes into the clinic should improve the efficacy of cancer diagnosis.

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Fig. 1. Synthesis and characterization of ^{111}In -labeled TNYL-RAW-CCPM nanoparticles. (A) Reaction scheme for the conjugation of TNYL-RAW to CCPM. (B) Transmission electron micrograph of TNYL-RAW-CCPM nanoparticles. (C) Absorbance and emission spectra of TNYL-RAW-CCPM nanoparticles.

Fig. 2. SPR sensorgrams of binding between EphB4 and TNYL-RAW-CCPM or CCPM. TNYL-RAW-CCPM (left) and CCPM (right) nanoparticles were injected as a series of 8 two-fold concentration increases ranging from 0.16 to 20 nM. The various resonance units, which are shown on the vertical axis, represent the difference in binding affinity of each concentration of nanoparticles to immobilized receptors.

Fig. 3.

Fluorescence microphotographs and uptake study of EphB4-positive PC-3M and EphB4-negative A549 cancer cells after exposure to TNYL-RAW-CCPM. (A) Representative fluorescence microphotographic images. PC-3M cells were incubated with TNYL-RAW-CCPM for 30 min (left); PC-3M cells were co-incubated with TNYL-RAW-CCPM and TNYL-RAW (100-fold excess) for 30 min (middle); A549 cells were incubated with TNYL-RAW-CCPM for 30 min (right). Signal from Cy7-loaded CCPM is pseudocolored green. Cell nuclei were counterstained with DAPI (blue). Bar, 50 μ m. (B) Uptake study of cells incubated with ^{111}In -labeled TNYL-RAW-CCPM. PC-3M cells were incubated with ^{111}In -labeled TNYL-RAW-CCPM (black); PC-3M cells were co-incubated with ^{111}In -labeled TNYL-RAW-CCPM and TNYL-RAW (100-fold excess) (gray); A549 cells were incubated with ^{111}In -labeled TNYL-RAW-CCPM (white). The data are expressed as cpm/ μ g protein in cell pellet over cpm/ μ g medium and presented as mean $\pm$ standard deviation ($n = 5$). * Indicates statistically significance with $P < 0.01$.

Fig. 4. Blood activity-time profiles of ^{111}In -labeled TNYL-RAW-CCPM and TNYL-RAW. The open circles represent the mean radioactivity expressed as a percentage of the injected dose per gram of blood from 5 mice.

Fig. 5.

Dual SPECT/CT and near-infrared fluorescence optical imaging of mice bearing a PC-3M or A549 tumor after intravenous administration of ^{111}In -labeled TNYL-RAW-CCPM or CCPM. The mice bearing a PC-3M tumor received ^{111}In -labeled TNYL-RAW-CCPM, ^{111}In -labeled TNYL-RAW-CCPM plus excess cold TNYL-RAW peptide, or plain CCPM. The mice bearing an A549 tumor received ^{111}In -labeled TNYL-RAW-CCPM. (A) Representative SPECT/CT images. (B) Representative fluorescence molecular tomographic images. (C) Representative autoradiographs of excised tumors. (D) Fluorescence images of the same slides in autoradiographic studies scanned at 800 nm. (E) Fluorescence image of the same slide in autoradiographic studies scanned at 520 nm after immunostaining. The section was stained with rabbit anti-human EphB4 antibody and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G. Arrows indicate tumors.

Fig. 6. Biodistribution and tumor uptake of ^{111}In -labeled TNYL-RAW-CCPM 24 h after intravenous injection. (A) Biodistribution of ^{111}In -labeled TNYL-RAW-CCPM in mice bearing a PC-3M or A549 tumor, 24 h after intravenous injection. The mice bearing a PC-3M tumor received ^{111}In -labeled TNYL-RAW-CCPM (black), or ^{111}In -labeled TNYL-RAW-CCPM plus an excess of cold TNYL-RAW peptide (gray). The mice bearing an A549 tumor received ^{111}In -labeled TNYL-RAW-CCPM (white). (B) PC-3M tumor uptake of TNYL-RAW-CCPM (black), TNYL-RAW (gray), or CCPM (white) 24 h after intravenous administration. Data were plotted as percentage of injected dose per gram of tissue (% ID/g). All data are expressed as mean $\pm$ standard deviation ($n=5-6$). * $P < 0.01$.

Fig. 7.

Fluorescence microscopy of PC-3M and A549 tumors excised after imaging studies. The mice bearing a PC-3M tumor were injected intravenously with ¹¹¹In-labeled TNYL-RAW-CCPM, ¹¹¹In-labeled CCPM, or ¹¹¹In-labeled TNYL-RAW-CCPM plus an excess of cold TNYL-RAW peptide. The mice bearing an A549 tumor received an intravenous injection of ¹¹¹In-labeled TNYL-RAW-CCPM. Signal from Cy7-loaded TNYL-RAW-CCPM was pseudocolored green. EphB4 (red) was stained with rabbit anti-human EphB4 antibody and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G. Cell nuclei were counterstained with DAPI (blue). Bar, 50 μ m.