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An ^{125}I-labeled octavalent peptide fluorescent nanoprobe for tumor-homing imaging *in vivo*

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ABSTRACT

Targeting radiopeptides are promising agents for radio-theranostics. However, *in vivo* evaluation of their targeting specificity is often obscured by their short biologic half-lives and low binding affinities. Here, we report an approach to efficiently examine targeting radiopeptides with a new class of octavalent peptide fluorescent nanoprobe (Octa-FNP) platform, which is composed of candidate targeting peptides and a tetrameric far-red fluorescent protein (tfRFP) scaffold. To shed light on this process, ^{125}I-Octa-FNP, ^{125}I-tfRFP and ^{125}I-peptide were synthesized, and their targeting functionalities were compared. Both fluorescence imaging and radioactive quantification results confirmed that ^{125}I-Octa-FNP had a significantly higher cellular binding capability than ^{125}I-tfRFP. *In vivo* biodistribution studies show that at 6 h post-injection, ^{125}I-Octa-FNP had 2-fold and 30-fold higher tumor uptake than that of ^{125}I-tfRFP and ^{125}I-peptide, respectively. Moreover, γ-imaging at 24 h post-injection revealed a remarkable accumulation of ^{125}I-Octa-FNP in the tumor while maintaining an extremely low background contrast, which was further confirmed by immunofluorescence analysis. These data suggested that, as an engineered and multivalent platform, Octa-FNP could enhance the tumor targeting of a designed peptide and provide excellent contrast radioimaging, making it a valuable tool for the evaluation of the targeting ability of specifically designed radiopeptides for cancer theranostics.

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1. Introduction

Positron emission tomography (PET)-based molecular imaging provides a sensitive way to diagnose and monitoring the therapeutic response of many cancers [1]. The successful development of a target-specific PET-based imaging probe is considered to be as a prerequisite to better understand the molecular mechanisms of cancer [2,3]. Although monoclonal antibodies (mAbs) exhibit high tumor uptake and retention, the prolonged half-life times (7–12d) of radiolabeled intact mAbs lead to high background signals in the blood and normal organs, making them a suboptimal choice for radioimaging [4,5]. Compared to mAbs, small targeting radiopeptides are currently the agents of choice for diagnostic imaging and radiotherapy due to their fast clearance and rapid tissue penetration [6], which cause an early occurrence of adequate tumor/blood ratios [7,8]. However, their rapid proteolysis in plasma and extremely low molecular weight lead to short biologic half-lives and weak binding affinities, which often result in low tumor uptake and rapid tumor washout, thus hindering the evaluation and validation of their tumor targeting abilities for radiotheranostics [7,9]. To overcome these limitations, polyvalent and polymeric approaches have been used to develop advanced peptide-based radioprobes with high tumor binding affinities [10,11]. The use of multimers [12], peptidomimetics [13], affibodies [14], nanomaterials such as quantum dots (QDs) [15], and lipoprotein-inspired nanoparticles [16,17] as scaffolds has recently been adopted to facilitate tumor uptake and retention. However, most of these material preparations suffer from sophisticated bioconjugation processes, low radiolabeling yields [18], or predominant renal excretion and liver accumulation [10], which reduce the detection sensitivity and limit the maximum tolerated dose in therapeutic applications.

KatushkaS158A [19], a tetrameric far-red fluorescent protein (tfRFP), may be an ideal candidate scaffold for improving the

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binding affinity and pharmacokinetics of target-specific peptides. tfRFP not only has the advantages of high brightness, photostability and deep detection for *in vivo* [20], but it can also increase the polyvalent effect of targeting peptides through its self-tetramerization. In our previous study, the tetrameric property of tfRFP was used as a scaffold to engineer an octavalent peptide fluorescent nanoprobe (Octa-FNP) [21]. The multivalent effect of Octa-FNP was validated by the highly specific uptake in human nasopharyngeal cells *in vitro*. Moreover, its specific targeting ability was confirmed *in vivo* with the observation of a remarkable accumulation of Octa-FNP in tumor tissues and cells and almost no preferential accumulation in the kidney and liver by optical imaging and Western blot analysis, resulting in high-contrast tumor imaging [21]. Due to these advantages, it is hypothesized that the self-assembled Octa-FNP is an excellent candidate for target-specific delivery of radionucleotides and may offer an opportunity for the accurate assessment of specifically designed radiolabeled peptides in diagnostic imaging and radiotherapy.

In this study, Octa-FNP and tfRFP were prepared, and their targeting abilities were evaluated in cells with a fluorescence spectroscopy assay as the proof of concept. After labeling with ^{125}I, Octa-FNP was compared with tfRFP for several parameters, such as labeling yield, binding ability, serum stability and targeting specificity. Subsequently, a comparison of the *in vivo* behavior of ^{125}I-Octa-FNP, ^{125}I-tfRFP and ^{125}I-peptide, including pharmacokinetics, tissue bio-distribution, immuno-fluorescence analysis and γ-imaging, was performed to demonstrate the *in vivo* targeting capability of ^{125}I-Octa-FNP. The objective of this study was to investigate the target-specific characteristics of ^{125}I-Octa-FNP *in vitro* and *in vivo*, thereby providing valuable information for the future design of radiolabeled tumor-homing peptide agents based on the engineered Octa-FNP platform and contributing to potential evaluation of radiopeptides in location targeting and diagnostic imaging.

2. Experimental section

2.1. Materials

Free peptide (LTVSPWYLTVSPWY) was purchased from Shanghai Apeptide Co., Ltd. (Shanghai, China). Proteins were purified by the Akta fast protein liquid chromatography (FPLC) system (Purifier 10; GE Healthcare) equipped with 5 ml HisTrap FF crude. The mouse anti-His mAb was purchased by Gen-Script (Piscataway, NJ, USA). Cell culture media and supplements were obtained from Life Technologies (Carlsbad, CA, USA). Human nasopharyngeal cancer 5–8F cells were gifts from Prof. Yi-Xin Zeng and Prof. Mu-Sheng Zeng (Sun Yat-sen University Cancer Center, Guangzhou, China). Five-week-old female BLAB/c nu/nu mice were purchased from Hunan Slack King of Laboratory Animal Co., Ltd. (Changsha, China). Iodogen (Sigma, St. Louis, MO), Na^{125}I (Beijing Atom High Tech., Beijing, China) and other chemicals and solvents were used as received.

2.2. Purification and labeling with ^{125}I

The pRSET plasmids encoding Octa-FNP and tfRFP were constructed and purified using a procedure similar to that described previously [21]. In brief, the plasmids encoding Octa-FNP and tfRFP were transformed into *Escherichia coli* BL21(DE3) cells. Single colonies were expanded to culture and induced with 1 mM isopropyl-β-d-thiogalactoside to grow for 16 h at 26 °C. After centrifugation, the cell extracts were prepared for sonication and centrifugation to obtain the supernatants, which were purified by 5 ml HisTrap FF crude (GE Healthcare, USA). After dialyzing against PBS, purified Octa-FNP and tfRFP probes were concentrated by concentrator tubes with a 30-kD molecular weight cutoff (Millipore, USA) and quantified by the Lowry Assay Kit (Pierce,USA). Absorption and fluorescence spectra for Octa-FNP and tfRFP were measured with spectrophotometer (Lambda 35, PerkinElmer, USA). For radiolabeling, equimolar concentrations of Octa-FNP, tfRFP and free peptide (1.76 nmol) in 100 μL phosphate buffered saline (PBS; 0.1 M, pH = 7.4) was combined with 1.50 mCi ^{125}I. The mixture was transferred to an Iodogen pre-coated tube (50 μg/tube). After 10 min at room temperature, all radiolabeled preparations were purified by a PD MiniTrap G-25 column (17-0851-01, GE Healthcare) using PBS as the eluent. For all preparations, the radiochemical purity (RCP) of the radiotracer was determined by instant thin-layer chromatography (ITLC) using ITLC silica gel strips (Gelman-Sciences, Inc.) with acetone as themobile phase.

2.3. Confocal microscopy

5–8F cells were seeded into 8-well Lab-TekII chambered cover glass plates (2×10^4/well; ThermoScientific, USA) for confocal microscopy and cultured for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Equimolar concentrations (1 μM) of Octa-FNP and tfRFP in RPMI1640 solution containing 10% fetal bovine serum (FBS; Life Technologies) were incubated with 5–8F cells at 37 °C or 4 °C for 3 h. In the blocking group, 5–8F cells were co-incubated with 1 μM Octa-FNP plus 50 μM free peptide. The cells were washed 3 times with PBS and imaged with the Olympus FV1000 laser confocal scanning microscope (Olympus, Tokyo, Japan) with a 60 × water objective and 543 nm excitation wavelength.

2.4. Cell binding assay

To verify the cell-binding capability of ^{125}I-labeled Octa-FNP and tfRFP, 5–8F cells were seeded into 96-well plates at a density of 1×10^5 cells/well and incubated overnight at 37 °C to allow a firm adherence. The *in vitro* cell binding affinities of the two probes were assessed by incubating the cells with ^{125}I-labeled Octa-FNP and tfRFP at 4 °C for 3 h. In the blocking group, 5–8F cells were co-incubated with 5 nM ^{125}I-labeled Octa-FNP (18.5 MBq/L) and 60 mM unlabeled free peptide at 4 °C for 3 h. The total incubation volume was adjusted to 200 μL. After incubation, the cells were rinsed 3 times with cold PBS buffer and then lysed with 2 M NaOH at room temperature. The cell-associated radioactivity was determined using a γ-counter (Wallac 1470-002, Perkin–Elmer). All experiments were repeated twice with quadruplicate samples.

2.5. Tumor-bearing nude mouse model

Female BALB/c nude mice were purchased from the Department of Experimental Animals, Hunan Slack King Laboratory Animal Co., Ltd. (Changsha, China). 5–8F cells were used to prepare the tumor-bearing nude mouse model in compliance with the animal experiment guidelines of the Animal Experimentation Ethics Committee of Huazhong University of Science and Technology. The protocol for these studies was approved by the Hubei Provincial Animal Care and Use Committee. 5–8F cells (2×10^6 cells) in 200 μL PBS were injected subcutaneously into the right upper flank. When tumors reached a mean diameter of ~0.8 cm, the tumor-bearing mice were used for both biodistribution and imaging studies.

2.6. Pharmacokinetics

For the pharmacokinetics studies, seven normal BALB/c mice were used as one group for the experiment of the blood clearance of ^{125}I-labeled Octa-FNP, tfRFP and free peptide. The ^{125}I radiotracers (10 μCi in 200 μL 0.9% saline) were administered intravenously to each mouse. Blood was harvested from orbital sinus at 10 min, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 12 h and 24 h post-injection for ^{125}I-labeled Octa-FNP and tfRFP, and 1 min, 5 min, 10 min, 20 min, 0.5 h, 1 h, 2 h, 4 h, 12 h and 24 h for ^{125}I-labeled free peptide. The radioactivity was measured using a γ-counter (Wallac 1470-002, Finland).

2.7. Biodistribution and histological assays

The tumor-bearing mice were used for both biodistribution and imaging studies. Sixteen tumor-bearing mice were randomly divided into 4 groups, each of which had 4 animals. The ^{125}I radiotracer (10 μCi in 100 μL PBS) was administered intravenously to each tumor-bearing mouse via the tail vein. For the blocking experiments, 4 tumor-bearing nude mice (20–25 g) were used, and each animal was administered ~10 μCi of ^{125}I-Octa-FNP (0.01 nmol) and a 1000-fold excess of free peptide. Animals were killed by cervical dislocation at 3, 6, 12 and 24 h post-injection. Organ samples (tumor, blood, heart, liver, lungs, spleen, kidneys, stomach, intestine, muscle and bone) were collected and weighed, and the radioactivity was counted in a γ-counter. The data of the blood and distribution were expressed as the percent of injected dose per gram of tissue (% ID/g). The biodistribution data and target-to-background (T/B) ratios were reported as the mean plus the standard deviation. A comparison between the two different radiotracers was also made using the one-way ANOVA test (GraphPad Prim 5.0, San Diego, CA). The level of significance was set at $P = 0.05$.

Octa-FNP and tfRFP in tumor, liver and kidney tissues were detected by immuno-fluorescent staining. Briefly, organs from 5–8F tumor-bearing nude mice were fixed in 4% paraformaldehyde for paraffin slicing. One slice of each tumor was cut into 5-μm thick sections which were rinsed in PBS and blocked with 3% BSA in PBS for 30 min. The sections were then incubated with mouse anti-His mAb (Gen-Script, Piscataway, NJ, USA) at 4 °C for 1 h. After rinsing with PBS, the sections were incubated with an FITC-conjugated anti-rat antibody (Invitrogen, San Diego, CA) for 2 h at room temperature. The stained slices were then washed in PBS and counterstained with DAPI (BIOS, Hubei, China). Images were recorded using FV1000

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