Importance of the physicochemical properties of fluorescent dyes for obtaining target-specific *in vivo* images by membrane-permeable macromolecular imaging probes

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Abstract

Background: Membrane-permeable macromolecular (MPM) probes are designed to deliver functional proteins to disease sites and into cells by using protein transduction domain (PTD). To visualize intracellular molecular events in tumor sites, we previously developed MPM imaging probes conjugated with near-infrared fluorescence (NIRF) dyes. However, the factors which influence target-specific *in vivo* fluorescence images of MPM imaging probes are still unclear. We studied whether the physicochemical properties of NIRF dye significantly affect the *in vivo* images of MPM imaging probes.

Methods: We constructed three MPM imaging probes conjugated with NIRF dyes including IRDye800, AlexaFluor750 and the newly synthesized PBI3921 and compared their biodistribution in tumor-bearing mice using *in vivo* optical imaging. In addition, we addressed to reveal relationships between biodistribution of MPM imaging probes and conjugated NIRF properties.

Results: *In vivo* images obtained by three MPM probes were spatiotemporally different each other. These probes (~46 kDa) are different only in their NIRF dyes (~2 kDa), which have different physicochemical properties including surface charge and hydrophobicity based on chromatograph analyses. Moreover, the results of clearance efficiency obtained by using cancer cells were not necessarily correlated with the ones obtained by using mouse models.

Conclusions: We concluded that physicochemical properties of NIRF dyes do affect the *in vivo* off-target images after injection of MPM imaging probes and that clearance of non-specific image may be predicted by the analysis of the property of a fluorescent dye conjugated to MPMs. These results would provide useful information to design MPM imaging probes with high target specificity *in vivo*.

Keywords: Membrane-permeable macromolecule, *In vivo* imaging probe, Biodistribution, Near-infrared fluorescence dye, Protein transduction domain

Introduction

In vivo imaging probes have been developed extensively for preclinical studies and diagnosis of cancers [1,2]. Engineered antibodies are successful macromolecular probes to image cancer cells *in vivo* [3,4]. However, their targets are limited on the cell-surface components because of poor cell-membrane penetration of macromolecules. Intracellular components contain strong candidates of diagnosis and therapeutic targets, motivating us to develop membrane-permeable macromolecules (MPMs) for imaging intracellular molecules. Recent studies demonstrate that protein transduction domains (PTDs), also known as cell-penetrating peptides, can deliver conjugated macromolecular cargos by penetrating the cell membrane, and they have been utilized as transport vectors in biomedical applications [5-7]. At present, *in vivo*

studies of PTDs are being conducted to deliver proteins, oligonucleotides, siRNA, liposomes, and nanoparticles inside cells where they target disease-specific molecules [8-12]. Therefore, PTD-conjugated macromolecules are promising materials targeting intracellular molecules.

In small animal studies, near-infrared fluorescence (NIRF) technologies have attracted much attention because of the almost complete elimination of background noise caused by tissue autofluorescence and the reduced scattered light from the excitation source [13]. Various long-wavelength fluorescent dyes have been synthesized, with fluorescence maxima ranging from 700 to approximately 850 nm [14]. We recently developed a functional MPM, PTD-ODD-HaloTag (POH) fusion protein (46 kDa) comprising of 3 domains: a PTD, an oxygen-dependent degradation domain (ODD) corresponding to human hypoxia-inducible factor (HIF)

-1 α (548-603), and a HaloTag (HT) that is easily conjugated with any chemical through covalent binding of HT and its ligand. The PTD of POH efficiently enables it to enter cells and the ODD allows it to be selectively stabilized in HIF-active cells [15], which are responsible for treatment resistance and malignant progression of tumor hypoxia [16]. POH-N, a NIRF dye-conjugated POH successfully imaged HIF-active cells *in vitro* and *in vivo*. However, we found that the *in vivo* biodistribution of POH-N was significantly influenced by the NIRF dye component.

For *in vivo* imaging probes, one of the most important factors to achieve target-specific imaging in whole-body is quick clearance of the off-target signals from the excretory organs such as the kidneys and liver. Furthermore, MPM imaging probes have to be quickly cleared from the non-targeted tissue cells by the cellular excretion system after entering cells. Thus, both excretion systems must be studied to optimize the *in vivo* distribution of MPM imaging probes. Although there are some *in vivo* biodistribution studies for PTD-containing therapeutic probes indicating that a cargo of probes influences the excretion process of the probes *in vivo* [17,18], no study has been reported for MPM imaging probes.

In the present study, we have attempted to elucidate the decisive factors in NIRF dyes influencing the *in vivo* images of MPM imaging probes by using three different POH-Ns. The results indicate that the physicochemical property of NIRF dyes such as electrical charge distribution and relative hydrophobicity strongly influences the rates of membrane-penetration and *in vivo* off-target images after injection of POH-Ns.

Materials and methods

Preparation of the POH protein

POH protein (46 kDa) was prepared as described previously [15]. Briefly, the plasmid POH/pGEX-6P-3 encodes PTD-ODD-HaloTag7 protein (POH), which consists of polypeptides indicated in (Supplement figure S1). The POH protein was expressed in BL21-CodonPlus cells (Stratagene, La Jolla, CA, USA) as a GST-tagged protein. The GST-tagged protein was purified with a GST-column and then digested with precision protease (GE healthcare Bio-Science Corp., Piscataway, NJ, USA) to remove GST-tag from the fusion protein. The final product was equilibrated in Mg²⁺-/-Ca²⁺-free PBS (pH 8.0).

Preparation of the POH-N probes

The HaloTag ligand-near infrared fluorescence dye (HL-N), HaloTag ligand-PBI3920 (HL-P) (Molecular weight: 1350.09) and HaloTag ligand-IR800 (HL-I) (Molecular weight: 1297.01), were provided by Promega Corporation (Madison, WI, USA). HaloTag ligand-Alexa Fluor 750 (HL-A) (Molecular weight: ~1800) was prepared as described previously [15]. Synthesis procedure of HL-P was indicated in the supporting information. HL-N (100 nmol) was dissolved in 20 μ L of DMF.

POH protein (20 nmol) was mixed with HL-N (40 nmol) in 10 mL of PBS (pH 8.0) containing 100 mM Tris-HCl (pH 8.0) and 3 M (NH₄)₂SO₄ for 3 h. Then, the resultant POH labeled with NIRF dye (POH-N) was purified with a PD-10 gel filtration column (GE Healthcare, Waukesha, WI, USA) and an Amicon-10 centrifugation column (Millipore, Milford, MA, USA). The purified POH-N (2 nmol) was finally dissolved in 100 μ L of PBS (pH 8.0). Labeling of POH-Ns was confirmed by SDS-PAGE fluorescence imaging and the labeling rate was calculated as described by the manufacturer's protocol. The labeling rate was constantly > 0.7.

Animals

Male Balb/c nu/nu was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). All mice underwent experiments at 6-10 weeks of age. All animal experiments were performed with approval of the Animal Ethics Committees of Tokyo Institute of Technology (No. 2010008) and in accordance with the Ethical Guidelines for Animal Experimentation of the Tokyo Institute of Technology.

Cell culture conditions

Human pancreatic cancer SUIT-2 cells were maintained in 5% FCS-DMEM (Nacalai Tesque, Kyoto, Japan) containing the supplements of penicillin (100 units/mL) and streptomycin (100 μ g/mL). All cells were maintained at 37°C in 5% CO₂ culture incubator. Mouse renal and liver primary cells were prepared from excreted the kidney and the liver from a FVB mouse purchased from Oriental Yeast Co., Ltd. Primary cells harvested after crashing excreted organs were maintained in 10% FCS-RPMI (Nacalai Tesque) containing supplements of penicillin (100 units/mL) and streptomycin (100 μ g/mL).

Transplantation of subcutaneous cancer xenografts

SUIT-2 cells suspended in PBS (1.0 $\times$ 10⁶ cells/20 μ L) were mixed with an equal volume of Geltrex (Invitrogen) and injected total volume into both forelegs of 7-week-old male nude mice. Mice with subcutaneous tumors of 5–10 mm in diameter were used for experiments.

In vivo fluorescence imaging

Two nmol of POH-N in 100 μ L of PBS (pH 8.0) was intravenously injected into tumor-bearing mice. Fluorescence images were acquired at the indicated times. All of the fluorescence images were acquired with IVIS[®] Spectrum (Caliper Life Sciences, Alameda, CA, USA) using the excitation filter (710 $\pm$ 15 nm), emission filter (800 $\pm$ 10 nm) for POH-P, POH-I or emission filter (780 $\pm$ 10 nm) for POH-A, exposure time = 1 s, binning = medium (8), field of view = 19 $\times$ 19 cm, f/stop = 1. Fluorescence intensity was analyzed with Living Image 4.0 (Caliper Life Sciences).

Ex vivo fluorescence imaging

Randomly selected mice were sacrificed after the *in vivo* imaging and organs were harvested. Fluorescence images

Figure 1. Construction of POH-N imaging probes. (A) Schematic diagram of a POH-N imaging probe. (B) Near-infrared fluorescence dye PBI3920 and IRDye800. Details of the synthetic methodology of PBI3920 can be found in the supporting information. (C) Excitation (blue) and fluorescence (red) spectrum of HL-Ns. Maximum wavelength of absorption (Abs max) and emission (Em max) were indicated.

from these organs were obtained with the IVIS Spectrum using the same conditions as *in vivo* imaging except field of view = 12.9 x 12.9 cm. Fluorescence intensity was analyzed with Living Image 4.0.

Liquid chromatography analysis

To analyze the surface charge of HL-Ns, chromatography was performed on an ion-exchange column HiTrap Q HP (GE Healthcare) with a linear gradient from 0 to 2 M NaCl in Tris-HCl (pH 7.5) in 40 min at a flow rate of 1.0 mL/min. Hydrophobicity analysis was performed with reverse-phase column HiTrap Phenyl HP (GE Healthcare) with a linear gradient from 2 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in 20 mM HEPES NaOH (pH 7.5) in 40 min at a flow rate of 1.0 mL/min. Retention time of HL-N was monitored with absorbance of wavelength 700 nm.

In vitro cellular uptake and excretion assay

SUIT-2 cells (2×10^5 cells/well), mouse renal and liver primary cells (2×10^6 cells/well) were seeded with 2 mL volume in 6-well plates. The cells were preincubated under aerobic condition at 37°C for 16 h. After the addition of 500 nM POH-N or 5 μM HL-N, the cells were incubated for 1 h. The cells were then washed with fresh medium and suspended in 200 μL of radio-immunoprecipitation assay (RIPA) buffer for uptake assessment. Fluorescence measurement was performed with 150 μL of cell lysate. For the excretion assay, the cells were washed with fresh growth medium after incubation with 500 nM POH-N or 5 mM HL-N for 1 h. The cells were then incubated for 1 h and suspended in 200 μL RIPA buffer. Fluorescence measurement was performed for 150 μL of suspension

in a 96-well plate by using Infinite® 500 plate reader (Tecan, Männedorf, Switzerland) with 740 nm excitation filter and 780 nm emission filter. For excretion assay, data were shown as relative NIRF intensity to 0 h NIRF intensity of each probes.

Statistical analysis

Data were shown as the mean value plus or minus the standard deviation of the mean. Statistical analyses were carried out with a Student's t test. Values of $p < 0.05$ were considered statistically significant.

Results

Construction of POH-N imaging probes

We previously constructed POH proteins labeled with IRDye800 (I) or Alexa Fluor750 (A), namely POH-I, or POH-A [15]. NIRF dyes are covalently bound to POH through HT and its ligand (HL) reaction, that is each POH-N contains one NIRF dye at the specific site (Figure 1A). The *in vivo* biodistribution and clearance were significantly different between them in tumor-bearing mice, indicating that a small chemical (~2 kDa) influenced the *in vivo* biodistribution of a large macromolecule (~46 kDa). We hypothesized that in NIRF dyes there would be a decisive factor that influenced the *in vivo* behavior of MPMs. Because precise structure of Alexa Fluor 750 is publically unknown, in order to explore the influence of a NIRF dye on the *in vivo* images after injection of a POH-N, we newly synthesized a NIRF dye, PBI3920, which has very similar chemical structure and size to IRDye800 (Figure 1B). First, HL was conjugated to NIRF dyes. The resultant HL-NIRF dyes (i.e., HL-P, HL-I, and HL-A) had almost identical excitation and emission wavelengths (Supplement figure S3A and 1C). Then HL-Ns were covalently bound to POH and the resultant POH-Ns (i.e., POH-P, POH-I and POH-A) were analyzed by SDS-PAGE, confirming that the POH-N preparations did not contain any obvious side products (Supplement figure S3B) and that the absorbance and fluorescence spectra of POH-Ns were almost identical to those of corresponding HL-Ns.

Differential *in vivo* fluorescent image of POH-Ns in tumor-bearing mice

POH-Ns (i.e., POH-P, POH-I, and POH-A) were intravenously administrated to mice carrying subcutaneous SUIT-2 xenografts, and time-course analysis of *in vivo* imaging was performed. In spite of structural similarities, *in vivo* images of POH-P and POH-I was significantly different first 12 h after administration: POH-I showed a faster clearance than POH-P; POH-A was circulating for longer period of time and cleared slower than others (Figure 2A). Although all of the POH-Ns were slowly cleared from tumors, the relative fluorescence intensity of the tumor to muscle (T/M) ratio was significantly different among them 24 h after administration (Figure 2B). *In vitro* assay using SUIT-2 cells revealed that POH-P entered cells at the lowest rate and was quickly released from cells (Figure 2C). On the other hand, POH-A showed the highest rates of entering the cells and slowest cellular clearance

among the three POH-Ns (Figure 2C).

Ex vivo imaging analysis of excretory organs such as the liver, kidneys, and intestines was performed 24 h after POH-Ns administration because strong signals were observed in the abdominal region of the POH-A-injected mice (Figure 2A). Semiquantitative analysis of the relative fluorescence intensity in each organ revealed that a significant amount of POH-A was located in the liver 24 h after administration (Figure 3A and B). More detailed *in vivo* imaging analyses indicated that although all of the POH-Ns accumulated in the liver 1 h after administration, the clearance speed of POH-A was the slowest; the clearance speed of POH-P was relatively slower than that of POH-I (Figure 4A and Supplement figure S4A). POH-I was cleared

from the kidneys faster than both POH-P and POH-A (Figure 4B and Supplement figure S4B). These data indicate that fluorescence dyes significantly influence the *in vivo* images after injection of POH-Ns.

Influence of POH-N degradation on the *in vivo* image of POH-N probes

A previous study indicates that most POH protein is degraded in and excreted from non-target cells 3 h after entering the cells [15] and that PTD-ODD fusion protein probe quickly disappeared from blood flow (only 15% in blood flow 1 h after injection and then half-life in blood was approximately 2 h) [19]. Therefore, we investigated whether the differential off-target images after injection of POH-Ns *in vivo* are caused by intact POH-Ns or HL-Ns derived from degraded POH-Ns. HL-N exhibited clearly different *in vivo* images from the corresponding POH-N; HL-I and HL-A did not accumulate in the liver or kidneys soon after injection; however, the clearance speed of HL-P was relatively slower

Table 1. Retention time of chromatograph analyses of HL-Ns. IEC: ion-exchange chromatograph, RPC: reverse-phase chromatograph.

HL-N	Ion-exchange chromatograph (min)	Reverse-phase chromatograph (min)	Ratio of IEC to RPC
HL-P	16.5	34.8	0.474
HL-I	14.3	34.6	0.413
HL-A	13.1	32.4	0.404

than that of HL-I and HL-A in both the liver and kidneys (Figure 5A, 5B and Supplement figure S5). All POH-Ns were stable after 9 h-incubation with serum at 37°C (Figure 5C). These findings, together with our previous study, indicate that the behavior of HL-Ns detached from POH would cause off-target fluorescent image in the excretory organs.

Chromatographic analysis and cell penetration efficiency of HL-Ns

To elucidate a possible factor in HL-Ns influencing the *in vivo* biodistribution of POH-Ns, we analyzed the surface charge and hydrophobicity of HL-Ns by ion-exchange and reverse-phase liquid chromatography. Even though HL-P and HL-I have the same net charge of -3, long retention time of HL-P in ion-exchange liquid chromatograph indicates that HL-P possesses a larger effective charge moment induced by the distribution of negative charge than either HL-I or HL-A. Short retention time of HL-A in reverse-phase liquid chromatograph indicates that HL-A is overall more hydrophilic than either HL-P or HL-I (Table 1).

Next, we assessed HL-Ns in the rate of uptake and cellular clearance with primary mouse renal and liver cells (Figure 6A and 6B). Larger amounts of HL-P entered the primary cells than HL-I (Figure 6A), while HL-P was released from cells more slowly than HL-I (Figure 6B). Furthermore, HL-A had significantly less cell membrane permeability and a slower diffusion rate than both HL-P and HL-I (Figure 6A and 6B). These results suggest that the physicochemical properties of fluorescence dyes such as electrical charge distribution and relative hydrophobicity strongly influence the rate of entering and diffusing from cells. Furthermore, fluorescent PTD probes must take into account the influence of the properties of dyes released

from the probes with respect to the proper evaluation of the *in vivo* biodistribution of the probes.

Discussion

Information for *in vivo* biodistribution of MPMs is valuable for developing novel diagnostic and therapeutic agents specific to disease-related molecules inside cells. To our knowledge, this is the first report describing the physicochemical properties of small chemicals that significantly influence the *in vivo* images of MPMs. For MPM imaging probes, quick clearance of the off-target signals from the non-targeted tissue cells as well as the excretory organs is important to achieve high target-specific imaging in living animals. POH-P showed the highest T/M ratio 24h after its intravenous administration. The results of *in vitro* assay of POH-N using cancer cells were not necessarily correlated with the T/M ratio, which is one of the parameters of target specificity (Figure 2B and 2C). One of the reasons of this discrepancy is probably that tumors are composed of wide variety of cells such as endothelial cells, stromal cells and blood cells and that the rates of uptake and cellular clearance are different among the cell types. In other words, results obtained by *in vitro* assays using cancer cells do not always provide proper information of *in vivo* target-specificity of MPMs.

Off-target fluorescence signal is common problem for *in vivo* optical imaging. Reduction of off-target signal of imaging probes based on a nanoparticle and antibody mostly depends on their excretion pathway [20,21]. Renal excretion is generally promoted when an MPM can go through size and charge filtration in the kidneys [22,23]. Meanwhile, large or lipophilic molecules tend to be trapped in the liver [24,25]. Although all of the POH-Ns accumulated in the kidneys and liver soon after administration, their clearance speed from these organs differed (Figure 2A). The accumulation of POH-Ns in the kidneys is consistent with the results of previous studies in which the permeable nature of the cell membranes promoted the accumulation of imaging probes in the kidneys due to their absorption into proximal tubules [26,27]. Furthermore, POH-P, in which HL-P has a larger effective charge moment, exhibited relatively slow renal clearance rate compared with POH-I or POH-A (Figure 4B). This result compliments those of a recent study in which NIRF dyes with strong surface charges were nonspecifically absorbed in normal tissues [28]. In fact, overall effective charge as measured by ion-exchange chromatography correlates with observations made *in vivo*. POH-Ns differed more drastically with respect to hepatic clearance than renal clearance (Figure 4). POH-P exhibited relatively slower clearance than POH-I. Unfortunately the structure of Alexa Fluor 750 in POH-A is publicly unknown, but the observed chromatography trends for HL-A indicate it is more hydrophilic but less charged. However we observed *in vivo* that both POH-A and HL-A tended to accumulate in the liver.

Many studies with primary hepatocytes indicate that the physicochemical properties of small molecule xenobiotics

greatly influence the process of hepatic clearance [29–31]. For instance, indocyanine green is a lipophilic and anionic NIRF dye that accumulates in the liver. An experiment with multidrug-resistant-2 (MDR2)-knockout mice shows that indocyanine green is mostly excreted from hepatocytes by MDR2 transporter protein. However, the clearance of some organic anions with different hydrophobicity does not absolutely depend on MDR2 [32]. These results suggest that hydrophobicity influences the hepatic clearance processes, resulting in different clearance rates from the liver among xenobiotic compounds. In our experiment, even though HL-A was less lipophilic than HL-P or HL-I, it has a smaller effective charge (Table 1) and the charge to hydrophobicity ratio would help explain the slower clearance of POH-A from the liver (Figure 4A). Conversely, although there were no significant differences between HL-P and HL-I with respect to their physical property of hydrophobicity (Table 1) and *in vitro* stability (Figure 5C), their cell membrane permeability (Figure 6A) and systemic clearance speed (Figure 6B) were very different. Again this can be explained by the overall ratio of effective charge to hydrophobicity as measured by simple chromatography methods, where HL-P has the largest such value (Table 1).

Although the number of NIRF dyes investigated here was limited and although there would be more factors that influence *in vivo* biodistribution of MPMs, here we demonstrate that a subtle difference in a small chemical component influences *in vivo* biodistribution of a large MPM. This study might be useful to establish general methodology of rational design of MPM imaging probes. However, to explain detail mechanism of differential images among POH-Ns, we might have to consider other physicochemical properties besides surface charge and hydrophobicity. Further studies of the correlation between the physicochemical properties of a fluorescent dye and *in vivo* biodistribution of MPM will be needed to give a generality for designing MPM imaging probes.

Conclusions

We demonstrated the significance of the physicochemical properties of fluorescence dye components for obtaining target-specific images by MPM imaging probes. Although MPM imaging probes are very promising for advancing the *in vivo* imaging of molecular targets of various diseases, no reports indicate decisive factors for improving target-specific *in vivo* images of such probes. This study provides insights into more rational design of MPM imaging probes possessing high target-specificity *in vivo*.

Additional files

Supplement figures S1-S5

Competing interests

Authors T. Kuchimaru, T. Kadonosono, S. Takahashi, T.

Mori, Y. Okahata and S. Kizaka-Kondoh have no conflict of interest. Authors C. Corona, S. J. Dwight and M. McDougall research was funded by Promega Biosciences LLC.

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