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Dual-Functional, Receptor-Targeted Fluorogenic Probe for In Vivo Imaging of Extracellular Protease Expressions

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Abstract

Herein we report a new type of *in vivo* fluorogenic probe that enables simultaneous and active targeting of overexpressed receptors, $\alpha_v\beta_3$ integrins, and extracellular proteases, matrix metalloproteinases (MMPs), in the tumor regions. This c(RGDyK)-conjugated MMP fluorogenic probe efficiently targets the tumor regions with high retention time while maintaining receptor binding affinity and substrate activity. The probe minimizes nonspecific accumulation, and thus demonstrating improved tumor-to-background signal ratio (T/N) in both $\alpha_v\beta_3$ integrin- and MMP-overexpressing U87MG tumor-bearing mouse model. This strategy can be easily tuned for a wide array of applications targeting various receptors and extracellular proteases *in vivo*.

Fluorogenic substrates, consisting of protease substrates labeled with a fluorescent reporter and a quencher dye, are highly sensitive and sequence specific that have long been used in *in vitro* diagnostic assays. Recent advances in optical imaging instrumentation and development of various near-infrared (NIR) fluorescent dyes and NIR quenchers enabled the use of conventional fluorogenic substrates for *in vivo* imaging applications. Any reported fluorogenic substrate with known sequences can be easily modified to NIR fluorogenic probes, so called peptide-based activatable probes,^{1–6} for *in vivo* imaging by replacing conventional dye molecules to a NIR fluorescent dye/quencher pair. Although reported NIR fluorogenic probes showed potential in *in vivo* imaging, short half-life, poor pharmacokinetic profiles, instability, and high background induced by a nonspecific degradation of fluorogenic substrates still hamper its application.

Development of novel techniques for noninvasive imaging of specific protease's activity is critical and urgently needed. Proteases are known as extremely important signaling molecules that are involved in numerous pathological processes including cancer, inflammatory, neurological, and cardiovascular diseases.^{7–9} Successful protease imaging techniques can be used for studying the role of protease expressions in protease-associated disease animal models or monitoring therapeutic efficacy of a number of newly developed protease inhibitors *in vivo*. Various unique forms of protease-activatable probes have been

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ASSOCIATED CONTENT

Supporting Information. Details of synthesis and experimental methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

designed in combination with fluorogenic substrates and materials including targeting ligands, polymers, and inorganic metals such as gold nanoparticles and quantum dots.^{1–6, 10–18} Although the use of activatable probes seems to be popular, surprisingly, very limited platforms of activatable probes are available which can clearly image proteases' activity *in vivo* after systemic administration. Unfortunately, the majority of reported systems demonstrate their proof-of-concept only *in vitro* or in *ex vivo* conditions such as after intratumoral injection in tumor models.

Ideal activatable probes can be designed by increasing *in vivo* stability and/or target (i.e. tumor) specificity of the fluorogenic probe while maintaining biological activity against specific proteases. Chemically, these improvements can be achieved by modifying fluorogenic peptides with water-soluble biomacromolecules such as PEGylated poly-L-lysine^{11, 19} or tumor-homing polymeric nanoparticles¹⁰ since both polymers can increase the circulation half-life and efficiently deliver fluorogenic probes to the tumor mainly by the enhanced permeability retention (EPR) effect.²⁰ Indeed, these concepts showed promising results in many *in vivo* models, however, there are some drawbacks. For example, such probes take a long time for full activation due to the decreased substrate sensitivity from the conjugated high molecular weight polymer backbone and more importantly, their EPR effect may cause nonspecific uptake causing false fluorescence signals *in vivo*.

In this study, we report a fluorogenic probe with an active targeting ligand that can be used as an efficient optical imaging probe to monitor extracellular protease expressions. We hypothesize that a targeting ligand will efficiently increase the retention time of the probe in the target region and maintain substrate activity, minimizing nonspecific accumulation and enhancing tumor-to-background signal ratio. To test the rationale of our strategy, we adopted a cyclic Arg-Gly-Asp peptide, c(RGDyK), a well-known $\alpha_v\beta_3$ integrin ligand that is overexpressed on the surface of angiogenic endothelial cells that mediates cell adhesion.^{21–23} We synthesized c(RGDyK)-labeled matrix metalloproteinases (MMPs) specific fluorogenic probes, confirmed their enzyme and receptor activity *in vitro* and demonstrated enhanced imaging of MMPs in both an $\alpha_v\beta_3$ integrin- and MMP-overexpressing tumor-bearing mouse model. MMPs are a large family of zinc-dependent proteases and play a crucial role in a variety of pathological disorders including cancer and inflammation diseases.²⁴ Accordingly, MMPs are important pharmaceutical and diagnostic targets for treatment of these diseases.

Our lead compound, MMP-P-RGD conjugate **4**, is composed of: i) Cy5.5 as a NIR dye (ex/em; 675/695 nm), ii) the MMP-cleavable substrate *GPLGVRGKGG* as a recognition site indicated by italics with the cleavage site between Gly and Val, iii) NIR dark quencher BHQ-3²⁵ (abs. 650 nm), and iv) c(RGDyK) as a targeting ligand. Two Gly residues were incorporated as a spacer between the MMPs-activatable fluorogenic peptide and the targeting ligand. Figure 1 shows the chemical structure of **4**. The multi-step synthetic route involves three parts: i) conjugation of c(RGDyK) to NHS ester activated C-terminal carboxyl group in fully protected MMP substrate **1**, Fmoc-*GPLGVR(Pbf)GK(tboc)GG*; ii) labeling NHS ester of NIR Cy5.5 dye to the Fmoc-deprotected N-terminal α -amino group of **2**, *GPLGVR(Pbf)GK(tboc)GG-c(RGDyK)*; iii) conjugation of NHS ester of NIR dark quencher BHQ-3 to the ϵ -amino group of lysine followed by TFA cleavage of **3**, Cy5.5-*GPLGVRGKGG-c(RGDyK)*. The peptide sequence of **1** was synthesized by standard solid-phase Fmoc peptide chemistry. In addition, only Cy5.5-labeled analog, Cy5.5-MMP-RGD **5** was prepared for cellular imaging. As controls, an MMP fluorogenic peptide without c(RGDyK) ligand, MMP-P **6** and CW800 dye (ex/em; 775/790 nm)-labeled c(RGDyK), CW800-RGD **7**, were also synthesized. Serial conjugation and deprotection of the peptide was performed in solution followed by preparative HPLC purifications. Purified products

were confirmed by analytical HPLC and LC/MS (see the Supporting Information for details).

Firstly, the MMP specificity of **4** was compared to that of **6** to confirm whether conjugation of the targeting ligand affects its enzyme specificity. *In vitro* enzyme specificity was measured in the reaction buffer (100 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.1% Brij, pH 7.5) containing activated MMP-2 with and without a homophenylalanine-hydroxamine acid based broad spectrum MMP inhibitor (EMD Bioscience) using a spectrofluorometer. The MMP-2 was activated by incubation of 2.5 mM of *p*-aminophenyl mercuric acid (APMA) in the reaction buffer for 2 h at 37 °C prior to use. As shown in Figure 2a, MMP-2 (40 nM) treatment of **4** and **6** (15 nM) resulted in an increased recovery of the NIR fluorescence signal and showed greater than 20-fold increase relative to the control sample without MMP-2. The fluorescence recovery of **4** was inhibited in the presence of the MMP inhibitor indicating probe selectivity. Similar experiments were performed by incubating **4** in the presence of different concentrations of MMP-2 (0, 2, 5, 10, 20, 40 nM) and the recovery of fluorescence was dependent on the MMP-2 concentration (Figure 2b). The overall MMP specificity of **4** against MMP-2 was similar to that of **6**. These results indicate that conjugation of the targeting ligand c(RGDyK) did not alter the MMP specificity of the probe.

Secondly, the effect of a bulky fluorogenic peptide on the $\alpha_v\beta_3$ binding affinity of c(RGDyK) was measured by a competitive cell-binding assay in U87MG cells. ¹²⁵I-eschistatin was used as a specific radio-ligand for competitive displacement. The U87MG cell is known to have high $\alpha_v\beta_3$ integrin density on the cell surface. All analogs containing c(RGDyK) ligand maintained reasonable binding affinities to its receptor. The IC₅₀ values of c(RGDyK), **4**, **5**, and **7** were 592, 267, 184, and 456 nM, respectively (Figure 3a). Next, receptor specificity of the probes in cell culture was verified by fluorescent microscopic studies. The probes were incubated in fixed U87MG cells with and without a blocking dose of c(RGDyK) (10 μ M). Since **5**, the non-quenched form of **4**, showed similar $\alpha_v\beta_3$ binding to **4**, **5** was used for *in vitro* cellular imaging studies. As shown in Figure 3b, **5** showed strong positive fluorescent signals on the cell membranes after 30 min of incubation at 37 °C. Since receptor blocking with an excess c(RGDyK) significantly decreased binding of **5**, the probe exhibits significant receptor specificity. Taken together, a NIR fluorogenic probe containing c(RGDyK) ligand shows specific activities against both MMP-2 and $\alpha_v\beta_3$ signifying **4** could be used as an $\alpha_v\beta_3$ receptor-targeted MMP-specific molecular beacon.

To investigate whether the receptor-targeting ligand can enhance the visualization of extracellular protease expression *in vivo*, 3 nmol of **4** was intravenously injected into U87MG tumor-bearing mice treated with and without an MMP inhibitor. In order to inhibit the activity of MMPs in U87MG tumor, MMP inhibitor was intratumorally injected 30 min before the injection of **4**. To demonstrate the superiority of this new receptor-targeted fluorogenic probe over existing NIR dye-labeled receptor-targeted peptides in tumor imaging, **5** and **7** (0.5 nmol) were each also injected into an U87MG tumor model. It should be noted that only a limited dose of dye-labeled peptide probe like **5** and **7** can be injected for whole body imaging because of its high fluorescent background from the dye. In contrast, since fluorogenic probes are strongly quenched and a majority of the probe is activated at the target site, higher doses of the probe can be injected to enhance the ratio of region-of-interest (ROI) signal of tumor to normal tissue, muscle, (T/N) at very early times. After injection, *in vivo* optical imaging was performed for 4 h using a small animal imaging system (Maestro 2) equipped with appropriate filters for Cy5.5 and CW800. Figure 4a shows representative serial images of mice at selected time points. Both **4** and **6** enabled visualization of MMP expression in an MMP-positive tumor but **4** provided brighter NIR fluorescent images compared to **6** from 1 h post-injection. As expected, when MMP

expression was inhibited by MMP inhibitors, the NIR fluorescence signal from the tumor was significantly reduced. In addition, tumor uptake was effectively blocked at 30 min post-injection with excess amount of unlabeled c(RGDyK) (200 nmol) with **4** demonstrating the c(RGDyK)-mediated specific binding of **4** *in vivo* (see the Supporting Information, Figure S3). Like other conventional types of NIR dye-labeled peptide probes, **5** and **7** showed gradually increased fluorescent signals in the tumor after the probe was slowly cleared from the blood and other organs. T/N ratios are illustrated in Figure 4b. **4** showed enhanced T/N and were up to 2.3, 1.5, 2.0, 3.4-fold higher than **5**, **6**, **7** and **4** treated with an inhibitor, respectively. After whole-mouse imaging at 4 h time point, tumors and organs were carefully removed and the biodistribution of **4** and **6** were evaluated (Figure 4c). *Ex vivo* biodistribution studies also indicated that **4** was predominantly activated in the tumors among other organs and compared to other groups. Selective tumor tissues were also analyzed by immunohistochemistry and NIR fluorescent microscopy to confirm the expression of biomarkers and accumulation of the probe. As shown in Figure 4d, $\alpha_v\beta_3$ integrin and MMP-2 antibody staining revealed strong expression of biomarkers in U87MG tumors and NIR fluorescence microscopy images showed a strong Cy5.5 signal, consistent with *in vivo* imaging. Overall, our *in vivo* results markedly supported the role of c(RGDyK) as a receptor-targeting ligand.

Tumor-targeting fluorogenic probes and dye-labeled peptide ligands have different targets; fluorogenic probes target overexpressed enzymes around the tumor site and peptide ligands target overexpressed receptors in the tumor. Yet, both probes can be used in combination to target the entire tumor area. Our results demonstrate the advantages of receptor-targeted fluorogenic probes for *in vivo* imaging applications. We anticipate that the combination of NIR fluorogenic probes and peptide ligands becomes applicable to a wide variety of target extracellular proteases and receptors, offering a series of receptor-targeted fluorogenic probes as novel imaging probes with improved efficacy *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Probes	Structures
4	R ₁ = c(RGDyK), R ₂ = Cy5.5, R ₃ = BHQ-3
5	R ₁ = c(RGDyK), R ₂ = Cy5.5, R ₃ = H
6	R ₁ = H, R ₂ = Cy5.5, R ₃ = BHQ-3
7	CW800-c(RGDyK)

Figure 1.
Chemical structures of relevant compounds.

Figure 2.

In vitro MMP specificity of MMP-P-RGD **4** and MMP-P **6**. (a) Fluorescence emission kinetic spectra of the probes in the presence of MMP-2 with and without a broad spectrum MMP inhibitor. Inset: Fluorescence emission spectra of the probes at 80 min. (b) Fluorescence intensities of the probes in the presence of various concentrations of activated MMP-2 (0, 2, 5, 10, 20, and 40 nM) following an 80 min incubation. Means $\pm$ SD ($n = 3$).

Figure 3.

(a) Competitive $\alpha_v\beta_3$ integrin receptor assay of c(RGDyK), **4**, **5**, and **7** to U87MG human glioma cells. Means $\pm$ SD. ($n = 3$). (b) Specific $\alpha_v\beta_3$ integrin receptor binding of **4**. NIR fluorescence images of U87MG cells were taken after 30 min incubation at 37 °C in the presence of **4** with and without a blocking dose of c(RGDyK) (10 μ M).

Figure 4.

(a) Representative serial in vivo NIR fluorescence images of MMP- and $\alpha_v\beta_3$ integrin receptor-positive U77MG tumor-bearing mice injected intravenously with **4**, **5**, **6**, **4** with MMP inhibitor, and **7**. Images were acquired at the indicated time points and were normalized by the maximum average value. The color bar indicates radiant efficiency (upper bar; for Cy5.5, low 0, high 0.111, lower bar; for CW800, low 0, high 0.03 scaled counts/sec). (b) T/N ratio analysis of NIR fluorescence intensity of U77MG tumor *in vivo*. (c) Biodistribution of **4** and **6**. Means $\pm$ SD. (n = 3), * $P < 0.05$. (d) Tumor section counterstained with FITC-labeled antibody for $\alpha_v\beta_3$ integrin receptor (upper) and MMP-2 (middle). NIR fluorescence microscopy of tumor section injected with **4** (lower).