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Synthesis and evaluation of new iRGD peptide analogs for tumor optical imaging

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ABSTRACT

Recently, a disulfide-based cyclic RGD peptide called iRGD, that is, c(CRGDKGPDC), has been reported to interact with both integrin and neuropilin-1 receptors for cellular and deep tissue penetration to improve the imaging sensitivity and therapeutic efficacy. In this study, two new near-infrared fluorescent iRGD conjugates, that is, Ac-Cys(IRDye®800CW)-iRGD (**1**), and its dual labeling analog DOTA-Cys(IRDye®800CW)-iRGD (**2**) were synthesized via the specific mercapto–maleimide reaction for tumor imaging. Both **1** and **2** showed significant tumor localization in optical imaging of MDA-MB-435 tumor-bearing mice. The potential of such iRGD compounds in tumor-targeted imaging and drug delivery deserves further exploration.

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Integrins are a family of heterodimeric cell surface receptors that bind extracellular matrix proteins to mediate cell attachment and signaling. Currently, 24 integrin subtypes have been reported.[1–3] Among them, some integrins such as αvβ3, αvβ5, and α5β1 have served as attractive targets for studying cancer pathology, imaging, and targeted therapy due to their over-expression on different types of tumors and related neovasculature for mediating tumor growth, angiogenesis, and metastasis.[4–10] Importantly, various tumor imaging agents have been discovered and developed based on integrin receptors because integrin-targeted tumor imaging holds great promise to improve early detection, diagnosis, and therapy as well as discovery and development of novel targeted anticancer agents.

For a long time, RGD peptides are known for molecular recognition of integrin receptors.[2] Diverse RGD peptides especially cyclic pentapeptide, that is, c(RGDfK) analogs exhibit remarkable binding affinity and selectivity with integrin αvβ3 and αvβ5. They have been applied widely to integrin targeting for cancer pathology, molecular imaging and drug delivery.[11–19] It has recently been reported that a disulfide-based cyclic RGD called iRGD, that is, c(CRGDKGPDC) discovered from phage display can interact with both integrin and neuropilin-1 receptors to mediate cellular internalization and extravasation as well as facilitate deep tissue penetration for improved imaging sensitivity and therapeutic efficacy.[20,21] These studies have inspired us to explore new strategies for integrin targeting and tumor imaging based on such iRGD peptides. Optical imaging has emerged as a powerful modality for studying molecular recognitions and molecular imaging in a non-invasive, sensitive, and real-time way. Some advantages of optical imaging include cost-effectiveness, convenience, and non-ionization safety as well as complementation with other imaging modalities such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Therefore, we have been interested in further exploring some novel iRGD analogs for tumor-targeted optical imaging. Herein, we report two new near-infrared (NIR) fluorescent Cys-containing iRGD conjugates, that is, Ac-Cys(IRDye®800CW)-iRGD (**1**), and its dual labeling analog DOTA-Cys(IRDye®800CW)-iRGD (**2**) (Fig. 1). Both were synthesized via the specific mercapto–maleimide reaction and showed significant tumor localization in MDA-MB-435 tumor xenograft-bearing nude mice as revealed by optical imaging.

Based on the structure of iRGD peptide motif, we suggest it should be feasible to perform some N and C terminal modifications on iRGD first. Both N-acetylation and C-amidation of protein and peptide termini have been used as effective approaches to improve the stability and biological activities for some peptides.[22,23] Although N-terminal acetylation and/or C-terminal amidation reduce the overall charge and the solubility of the peptide, they can increase the permeability of the peptides to cells for intracellular, in vivo assay or in vitro functional studies. They can also increase the metabolic stability of the peptide toward degradation by some enzymes such as aminopetidases, exopeptidases or synthetase. To explore molecular design of novel iRGD peptide analogs for integrin targeting and tumor imaging, we first focused on some new iRGD analogs derived from N-terminal acetylation and C-terminal amidation based on the cyclic structure of iRGD. Therefore, we designed a new iRGD analog (**3**) containing one Cys residue

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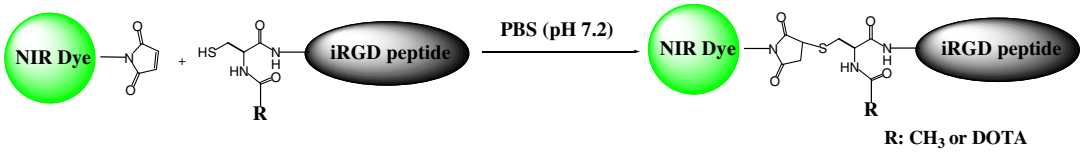


Figure 1. The two NIR fluorescent iRGD conjugates **1** (R: CH_3) or **2** (R: DOTA) designed and synthesized for tumor optical imaging.

at N-terminus, allowing for some specific chemical modifications on its mercapto group for exploring various biomedical applications as shown in Figure 2. For example, a near-infrared fluorescent iRGD conjugate (**1**) based on the reaction of mercapto group with a commercially available IRDye®800CW maleimide[24] was designed.

As shown in Scheme 1, the protected linear peptide Cys(Acm)-Arg(Pbf)-Gly-Asp(OBu^t)-Lys(Boc)-Gly-Pro-Asp(OBu^t)-Cys(Acm) was first assembled on Rink amide resin using the conventional Fmoc chemistry. The disulfide formation was realized on solid support by using a solution of thallium trifluoroacetate in DMF.[25,26] A Fmoc–Cys(Trt) residue was further introduced at the N-terminus of the resin-bound protected iRGD peptide using Fmoc chemistry. Finally, the acetylated Cys-containing iRGD analog (**3**) was obtained by TFA cleavage (TFA/thioanisole/TIS).

As shown in Scheme 2, compound **3** was conjugated with IRDye®800CW maleimide in PBS buffer (pH 7.2) to give **1** for optical imaging. As monitored by analytical HPLC, the conjugation progressed quickly and was complete within 5–10 min.

Multimodal molecular imaging has emerged as a powerful tool for cancer diagnosis, therapy. Because each imaging modality has its own unique strengths and weaknesses, the combination of different imaging modalities has the potential to overcome the respective limitations. Dual-modality optical/PET imaging agents may be attractive for coupling the high-resolution of optical imaging and the sensitivity of nuclear imaging to improve the cancer detection and diagnosis. Based on the above design and synthesis of **1**, we further designed a NIR fluorescent iRGD analog (**2**) containing a metal chelator DOTA (1,4,7,10-tetra-azacyclododecane-N,N′,N″,N‴-tetraacetic acid). As shown in Figure 3, DOTA chelator was similarly introduced at the N-terminus of Cys(IRDye®800CW)-iRGD peptide instead of acetylation to give **2**. Such an agent might be very useful as DOTA can be labeled with radioactive metals such as ^{64}Cu to serve as a dual labeling imaging agent for dual-modality optical/PET imaging and for potential radiotherapy as well.

As shown in Scheme 3 **2** can be synthesized similarly via a precursor DOTA-Cys-iRGD (**4**). DOTA chelator was introduced at the N-terminus of iRGD peptide in the presence of HBTU/HOBT/DIEA instead of acetylation. TFA cleavage afforded the iRGD analog (**4**) containing both DOTA and Cys motifs. Similarly, **4** was reacted with IRDye®800CW maleimide in PBS (pH 7.2) to give the dual labeling analog containing both IRDye®800CW and DOTA (**2**).

The purity and identity of all the compounds were fully identified by both analytical HPLC and LC–MS. The two NIR fluorescent compounds **1** and **2** showed similar UV–vis and emission spectra with the dye material IRDye®800CW maleimide in PBS buffer (pH 7.2) (λ_{max}: UV 774 nm, emission 789 nm).

We tested the ability of the two near-infrared fluorescent compounds **1** and **2** to target tumors in nude mice to evaluate their potential as in vivo molecular imaging agents. Both **1** and **2** were dissolved in PBS buffer (100 μL, 10 μM) and injected via tail vein into MDA-MB-435 tumor xenografts-bearing nude mice.[31–33] The mice were monitored by a dynamic data acquisition for 60 min, followed by static acquisitions at 1, 2, and 4 h postinjection. Figure 4 showed some representative optical images of both **1** and **2**. Both were found to preferentially localize in the tumor within 4 h post injection. Based on the time dependent tumor uptake curve, the tumor uptake of both **1** and **2** peaked at 10 min after probe injection. However, the tumor/muscle ratio kept increasing and reached the maximum level at 1 h post injection. Based on

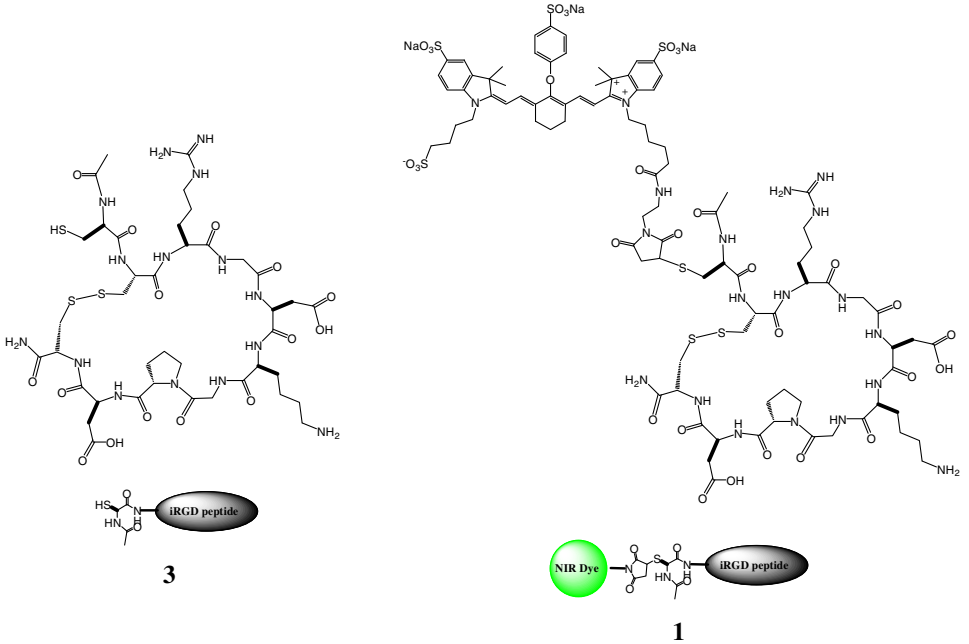
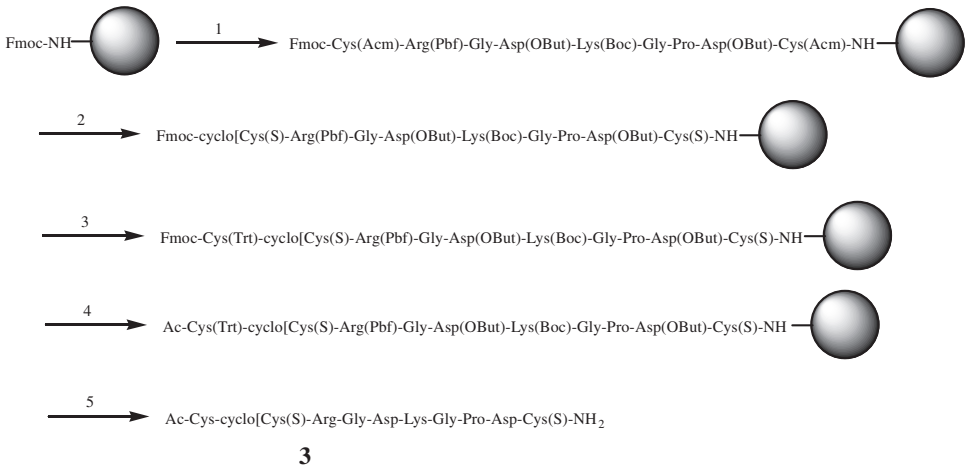
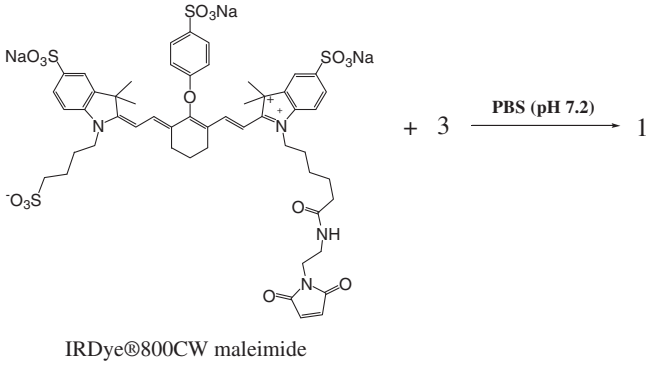


Figure 2. The structures of two new iRGD analogs Ac-Cys(IRDye800)-iRGD (**1**) and Ac-Cys-iRGD (**3**).



Scheme 1. Synthesis of Ac-Cys-iRGD (**3**). Reagents and conditions: (1) Fmoc chemistry; (2) $Tl(CF_3COO)_3$/DMF; (3) (a) piperidine/DMF (20%); (b) Fmoc-Cys(Trt)/HOBT/HBTU/DIEA/DMF; (4) (a) piperidine/DMF (20%); (c) Ac_2O/DIEA/DMF; (5) TFA/water/TIS/thioanisole (85:5:5:5).



Scheme 2. Conjugation of IRDye800-maleimide and Ac-Cys-iRGD (**3**) to form Ac-Cys(IRDye800)-iRGD (**1**).

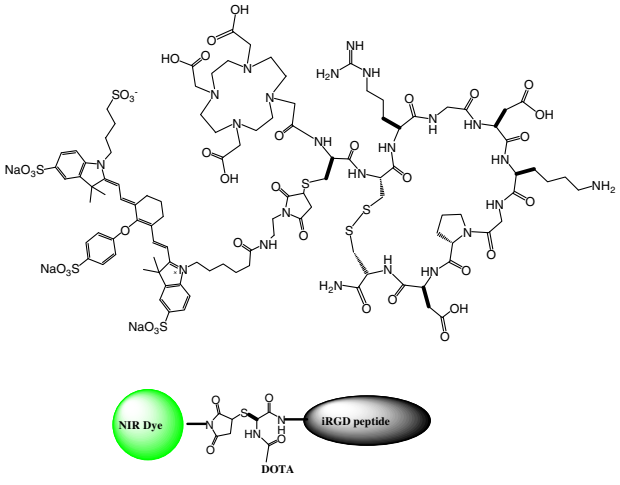


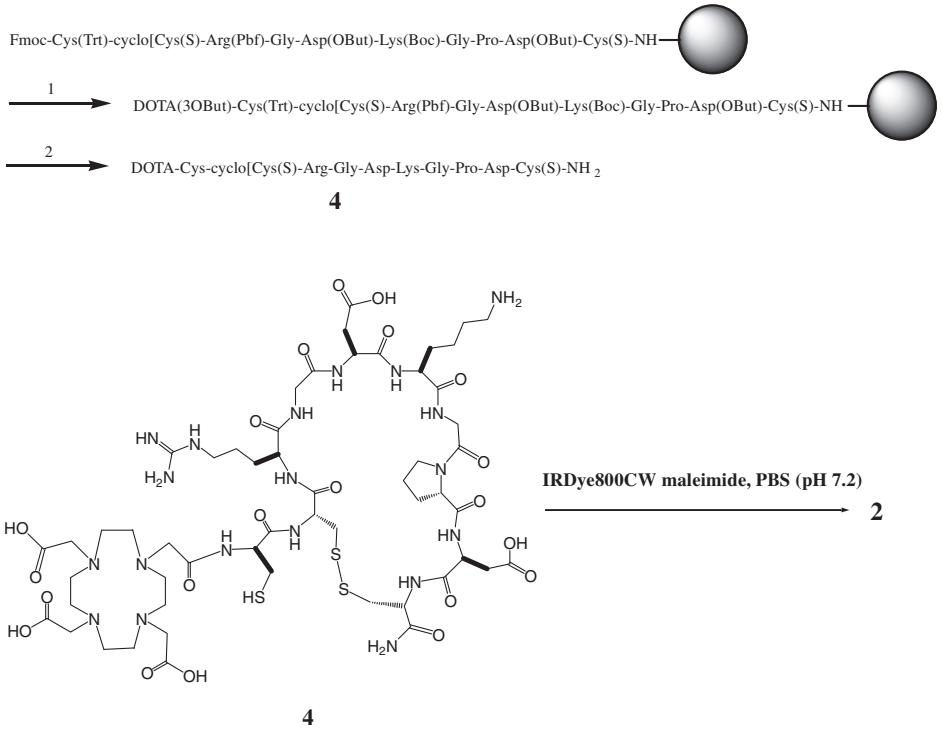
Figure 3. The structure of DOTA-Cys(IRDye®800CW)-iRGD (**2**) designed.

the fluorescence intensity over bladder region, we deduced that the probes were mainly excreted through kidneys.

The complexity and diversity of integrin receptors in their structures and functions suggest that it is important to discover diverse novel ligands for targeting integrins.[2,5,9,16–37] Diverse tumor imaging agents have been discovered based on the targeting of integrin and some other receptors related to tumor angiogenesis, growth, and metastasis. Nevertheless, integrin-targeted imaging agents of deep tumor penetration should be attractive. As described above, we have successfully explored the molecular design, synthesis, and evaluation of some novel iRGD peptide analogs. The iRGD motif provides at least two sites at N- and C-termini for chemical modifications. Our results have clearly demonstrated significant tumor localization in vivo and the potential of such iRGD peptides as represented by compounds **1** and **2** in tumor targeting and optical imaging, especially **2** with potential in dual-modality imaging. In addition, the free mercapto groups of compounds **3** and **4** allow site-specific reactions for constructing novel diverse iRGD conjugates to further explore their potential in receptor targeting, tumor imaging, and drug delivery. For example, we have recently explored its applications for PET imaging using the ^{64}Cu-DOTA-containing analogs and ^{18}FBEM labeling. All the compounds showed remarkable tumor accumulation and retention in orthotopic MDA-MB-435 xenograft model as revealed by both optical and PET imaging modalities.

It is important to compare iRGD with the conventional cyclic RGD peptide c(RGDfK) in vitro and in vivo for their receptor targeting as well as tumor penetration in future work. The iRGD peptide containing nine amino acid residues in its ring has very flexible structural conformations, which may not compete with the conventional lactam-based cyclic RGD peptide, that is, pentapeptide c(RGDfK) in integrin αvβ3 binding affinity and selectivity. Nevertheless, the novel structure and significant tumor localization suggest that iRGD compounds might exhibit some unique features for tumor imaging and other potential applications. In addition, the mechanism of iRGD for tumor targeting involves its binding with integrin αvβ3 first, followed by enzymatic hydrolysis to form an active CendR peptide that binds to neuropilin-1 and mediates an active transport system for extravasation and deep tumor penetration as reported.[20,21] Therefore, it is also important to study the biodegradation of our new iRGD compounds and further elucidate the mechanism of action for tumor-targeted imaging.

In conclusion, optical imaging has demonstrated the potential of iRGD for tumor imaging in mouse models. Further structural modification for improving tumor-targeted imaging and elucidating its mechanism is currently underway and the results will be reported separately. All these should facilitate the discovery and development of novel tumor-targeted imaging and therapeutic agents.



Scheme 3. Synthesis of DOTA-Cys-iRGD (**4**) and DOTA-Cys(IRDye800)-iRGD (**2**). Reagents and conditions: (1) (a) piperidine/DMF (20%); (b) DOTA(3OBut)-COOH/DIEA/HBTU/HOBT/DMF; (2)TFA/water/TIS/thioanisole (85:5:5:5).

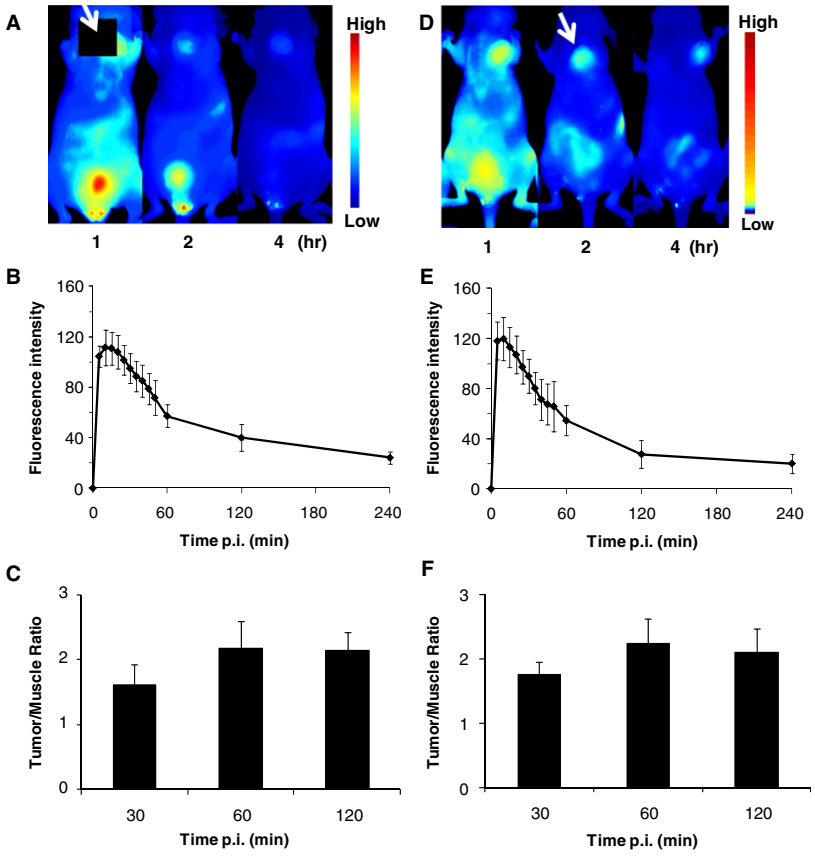


Figure 4. Optical imaging of MDA-MB-435 tumors with **1** (A, B, C) and **2** (D, E, F). A. In vivo optical tumor imaging with **1**; B. Time dependent tumor uptake of **1**; C. Tumor muscle ration of **1**; D. In vivo optical tumor imaging with **2**; E. Time dependent tumor uptake of **2**; F. Tumor muscle ration of **2**.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.112.

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