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^{111}In- and ^{203}Pb-Labeled Cyclic RGD Peptide Conjugate as an $\alpha_v\beta_3$ Integrin-Binding Radiotracer

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Abstract

Methodology for site-specific modification and chelate conjugation of a cyclic RGD (cRGD) peptide for the preparation of a radiotracer molecular imaging agent suitable for detecting $\alpha_v\beta_3$ integrin is described. The method involves functionalizing the peptide with an aldehyde moiety and conjugation to a 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid (DOTA) derivative that possesses an aldehyde reactive aminooxy group. The binding assay of the ^{111}In-labeled peptide conjugate with $\alpha_v\beta_3$ integrin showed 60% bound when four equivalents of the integrin was added, a reasonable binding affinity for a mono-valent modified RGD peptide.

Keywords

Click Chemistry; aminooxy DOTA; RGD peptide; ^{203}Pb

Introduction

Early detection of tumor cells is highly desirable and probably the most effective strategy towards the treatment and cure of cancer. It is well known that the progression and invasiveness of tumors in melanoma, glioma, and breast cancers correlates with $\alpha_v\beta_3$ integrin expression.[1–5] Therefore, inhibition of $\alpha_v\beta_3$ integrin and thus suppression of tumor-induced angiogenesis, which leads to the termination of cancer metastasis, is important for the treatment of various carcinomas. In addition, targeting integrin and visualizing its expression non invasively using a radiotracer by positron emission tomography (PET) or single photon emission computed tomography (SPECT) imaging, would be highly advantageous for early detection of metastatic tumors.

The $\alpha_v\beta_3$ integrin is known to recognize the tripeptide sequence arginine-glycine-aspartic acid (RGD). It is also known that replacing any of the amino acids or changing the spatial conformation results in loss of binding activity, whereas cyclization of the peptide into an optimal conformation results in high affinity and selectivity.[6–8] Over the course of several years, the use of radiolabeled cyclic RGD peptides as radiotracers for tumor imaging by SPECT or PET has been well documented.[9–11] The cyclic RGD peptides have been employed as carrier vectors of metallic radionuclides, which are bound to the peptide through the use of bifunctional chelators, to target the $\alpha_v\beta_3$ integrin that was over-expressed on tumor cells for both imaging and therapy applications.[6,12,13]

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The macrocyclic ligand 1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (DOTA) is employed in a variety of biomedical applications for sequestering metal ions relevant to medical applications. The complex formed with a lanthanide ion such as Gd^{3+} is clinically used as a diagnostic magnetic resonance imaging (MRI) agent, Dotarem.[14] It can also be used as a bifunctional chelator (BFC) when attached to other functional molecules such as a protein or a peptide. It is well documented that DOTA is water soluble and forms highly stable complexes with many transition and rare earth metals (2+ and 3+ charged). This alleviates concerns related to the dissociation of radioactive metal ions or other toxicities, and thus leads to its popularity for *in vivo* sequestration of a variety of radionuclides and lanthanides alike.[15–20] A number of DOTA derivatives have been extensively employed for a variety of metallic radionuclides such as ^{68}Ga, ^{90}Y, and ^{111}In.[21–23]

Conjugation of peptides with DOTA derivatives has been mostly performed by solid phase synthesis using active ester analogs of the chelating agent generally as a tri-*tert*-butyl ester that lends itself to the convenience of this methodology and the associated deprotection protocols.[24] This does however remove one of the carboxylate groups from the donor sphere about the metal ion or changes the character to an amide oxygen donor. In addition, there is the problem associated with the relatively long deprotection times necessary for the removal of the *tert*-butyl groups.[25] Other researchers have reported classic solution based synthetic methods that utilized amino functionalities of peptide or proteins.[23–29] A successful site-specific derivation of peptide or protein via the latter method can be tedious and often involves multi-step syntheses and protection of functional group(s) to avoid random conjugation that may compromise bioactivity of the derivatized compound. To obviate these limitations, click chemistry reactions that proceed efficiently and chemoselectively under physiological conditions were introduced recently as a more promising approach.[30] Chemoselective click reactions between aminooxy derivatized DOTA and aldehyde[31] or recombinant human serum albumin (rHSA)[32] were previously reported. Herein, we extend this conjugation method to site-specifically conjugate the cyclic RGD peptide to aminooxy derivatized DOTA (AOD). The peptide was functionalized with an aldehyde moiety that is known to form a covalent oxime functional group bond with the aminooxy functional group that has been appended onto the DOTA ligand. In this fashion, the full eight coordinate symmetric sphere is preserved for optimal complex stability. The DOTA conjugate was then labeled with ^{111}In and a binding assay was performed to determine the affinity of the peptide to the $\alpha_v\beta_3$ integrin to validate the conjugation and radiolabeling chemistry protocols as being suitable for such applications prior to performing biological experiments.

The radionuclide pair, ^{203}Pb and ^{212}Pb, possess favorable properties for use in diagnostic and therapeutic applications, respectively. ^{212} Lead ($t_{1/2}$ = 10.6 h) serves as an '*in vivo* generator' of ^{212}Bi ($t_{1/2}$ = 60 min) to overcome the short half-life of that daughter isotope.[33] When DOTA is used to chelate ^{212}Pb, the complex is adequately stable *in vivo*.[34] Therefore, in addition to ^{111}In, we explored the feasibility of labeling the RGD-DOTA conjugate with ^{203}Pb ($t_{1/2}$ = 52.0 h) which serves not only as a surrogate tracer for ^{212}Pb, but also has potential for use as a SPECT imaging radionuclide (E_γ = 279.2 keV).[34]

Experimental

Materials and Methods

Phosphate buffered saline (PBS) at pH 7.4 was obtained from Digene (Gaithersburg, MD). The Reverse-phase HPLC (RP-HPLC) (method 1) was performed using a Beckman System Gold® (Fullerton, CA) equipped with a Model 126 solvent delivery module and a Model 168 UV-Vis detector with peak detection at 254 and 280 nm, and a C_{18} Ultrasphere™ column (250 × 10 mm; 5 μm). The flow rate was 2.5 mL/min and the mobile phase was

isocratic with 90% A (0.1% TFA in water) and 10% B (0.1% TFA in acetonitrile) at 0 – 5 min, followed by a gradient mobile phase going from 10% B at 5 min to 90% B at 10 min. The mobile phase was then isocratic with 90% B at 10 – 18 min. All water used was purified using a Hydro Ultrapure Water Purification system (Rockville, MD). The cyclic RGD peptide was obtained from Peptide International (Louisville, Kentucky). The radiolabeled compounds were analyzed by RP-HPLC (method 2) on a Waters Breeze System consisting of a Binary HPLC Pump model 1525, an Autosampler model 717plus, monitored on line by a Dual Absorbance Detector model 2487 and a Beckman 170 Radioisotope Detector using a C-18 column (Beckman Coulter Ultrasphere 5 μm, 4.6×250 mm) and a linear gradient running from 100 % solvent A (0.1 M NH_4OAc) to 100 % Solvent B (MeCN) in 30 min.

Succinimidyl-4-formylbenzoate (1)—The title compound was prepared as reported.[35]

2-(4-(3-(aminooxy)-2-oxopropyl)benzyl)1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid (AOD) (2)—Compound 2 was prepared as previously reported.[17,32,36]

1-(2- *cyclo* (Arg-Gly-Asp-d-Phe-Lys)amido)4-formylbenzoate (cRGDf) (3)— Peptide (10 mg; 14.5 μmol), compound **1** (3.6 mg; 14.5 μmol), triethylamine (16.2g; 16.0 μmol) in DMF (400 μL) were stirred at room temperature for 38 h. The title compound was purified by RP-HPLC (method 1, t_R: 16.4 min). ESI m/e: 823 (M+1).

cRGD-AOD (4)—Compound **3** (10 mg; 12.2 μmol) and Compound **2** (8.5 mg; 14.6 μmol) were stirred at room temperature in 0.1M sodium phosphate (0.5 mL; pH 4.5) for 6 h. The title compound was purified by RP-HPLC (method 1, t_R: 4.8 min; 75% yield). ESI m/e: 696 (M+2).

Radiolabeling of 4 (^{111}In-4 and ^{203}Pb-4)

To a solution of 0.15 M NH_4OAc (100 μL, pH = 7.0) was added 50 μL (1 mg/mL) of cRGDf-AOD and 2 μL of $^{111}InCl_3$ solution (~500 μCi) in 0.05 N HCl. The reaction mixture was then incubated at 75 °C for 30 min. After heating, the vial was allowed to stand at room temperature for ~5 min. For ^{203}Pb-**4**, radiolabeling could be completed in low pH (pH = 5.5) and by allowing the reaction mixture to incubate at 75 °C for 60 min. the resulting solution was analyzed and purified by HPLC (method 2, Figure 2)

Binding assay with integrin $\alpha_v\beta_3$ receptor

The purified ^{111}In-**4** (2×10^6 cpm, 0.3 μM) was incubated with 0, 0.3, 0.6 and 1.2 μM of purified human $\alpha_v\beta_3$ integrin (Millipore, Temecula, CA) in a total volume of 25 μL PBS for 3 h at 37°C. For non-specific binding, excess **4** (to a final concentration of 20 μM) was added to reaction mixture. The reaction mixture was then separated on a 10 mL Sephadex G50 (Sigma, St. Louis, MO) column using PBS as eluent. Fractions (0.5 mL) were collected and counted on a γ-counter (Wallac, 1480 Wizard 3, Waltham, MA). The percent of the radioactivity bound to the receptor was then calculated (Table 1).

Result and Discussion

We chose to modify the peptide with an aldehyde rather than a ketone because aldehydes show much higher reactivity toward aminooxy groups or hydroxylamines than ketones.[31] The reaction conditions reported here were optimal for modifying the peptide with an aldehyde group. The product formed was a 1:1 conjugate of the DOTA at the primary amine of the lysine residue, which is the only reactive amino site available under these conditions. In a previous study, the cyclic RGD peptide was functionalized with a hydroxylamine

moiety and reacted with an ^{18}F-labeled aldehyde to obtain a radiotracer that was used to study the $\alpha_v\beta_3$ integrin binding of the peptide.[6,37,38] Here, we reversed the process by functionalizing the peptide with the aldehyde functionality in a single step and then reacted that product with a DOTA-hydroxylamine derivative to produce the ligand conjugate used to generate the ^{111}In- and ^{203}Pb-labeled radiotracer. The results showed that this synthetic route is quite convenient and efficient with moderate yields. Using the bifunctional DOTA ligand provides for the possibility of radiolabeling with a wide range of radionuclides, which is highly advantageous with a single precursor being suitable for multiple applications by just varying the radiolabel. Additionally, it is equally clear that the analogous chemistry would also support the incorporation and use of other bifunctional chelating agents and thus open up the range of possible radionuclides that might be attractive for targeting the $\alpha_v\beta_3$ integrin receptor.

The peptide conjugate was successfully radiolabeled with ^{111}In and ^{203}Pb (85% yield, Figure 2), while the reactive binding fraction of the resulting radiolabeled ^{111}In product was measured as 35.7 and 60.5% when incubated with 0.6 and 1.2 μM of integrin. The receptor binding was completely blocked by 20 μM (~7-fold excess) of cold peptide conjugate (Table 1). Thus, we demonstrate that the radiolabeled products of this strategy provide products comparable to those prepared by other means.[30–32,39]

Conclusions

In summary, a new peptide-bifunctional chelate conjugate was synthesized with a moderate to high yield through a “click” oxime formation strategy. The bifunctional chelate was successfully radiolabeled with ^{111}In and ^{203}Pb, affording radiolabeled products suitable for SPECT imaging or γ-scintigraphy with reasonable reactivity and binding toward the target $\alpha_v\beta_3$ integrin. These results indicate that it is potentially possible to label this peptide conjugate with a large variety of radionuclides, the resulting radiotracer being suitable for *in vivo* tumor targeting. Further studies, such as biodistribution, *in vivo* tumor targeting and imaging, along with labeling with other radionuclides, are underway and will be reported in due course. We conclude that preservation of the full chelating potential of the DOTA chelating agent will lead to well defined stable products which, combined with the target binding ability of the peptide, will also make this conjugate attractive for potentially generating radiotherapeutic agents.

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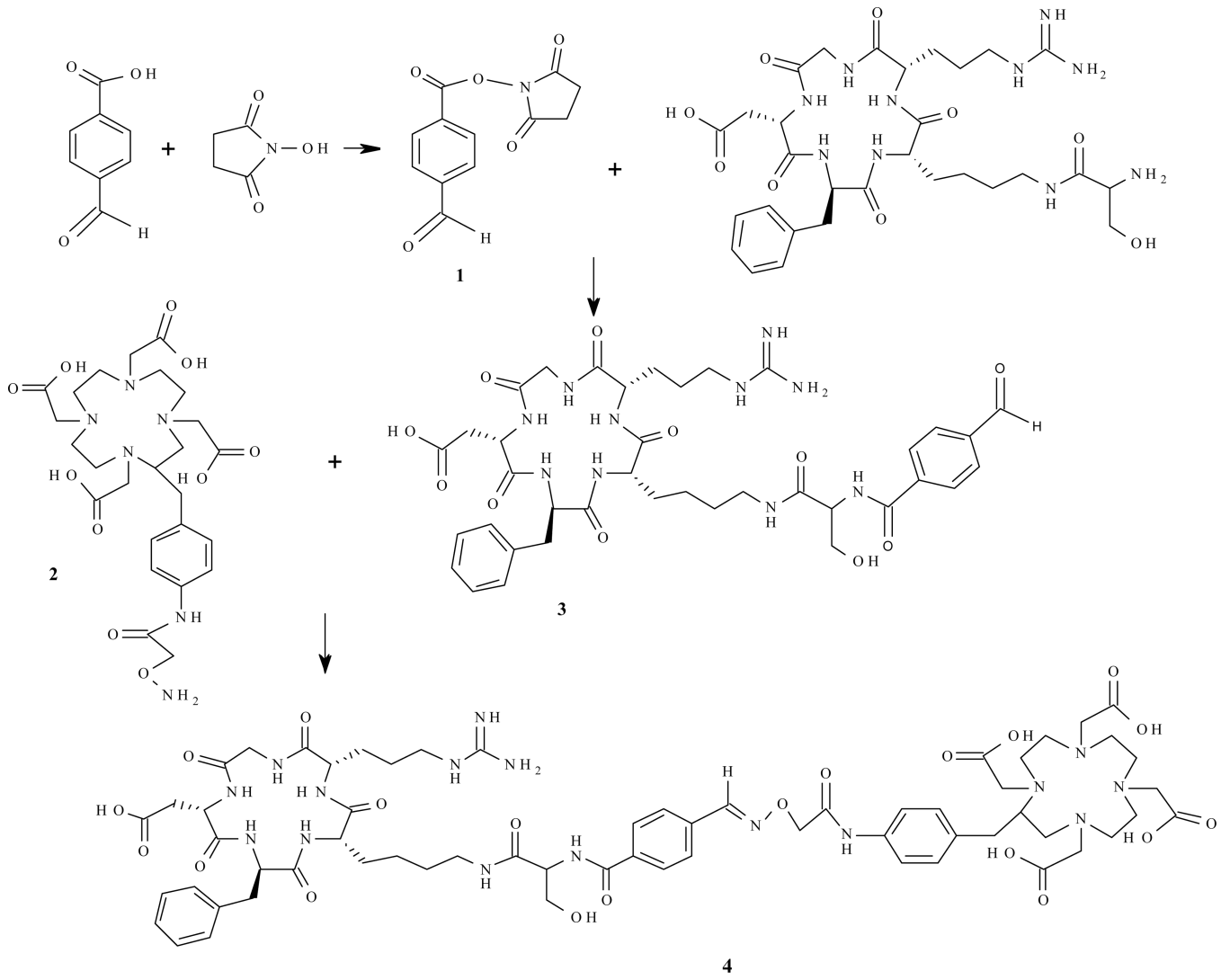

Figure 1.
Reaction scheme for preparation of the peptide conjugate.

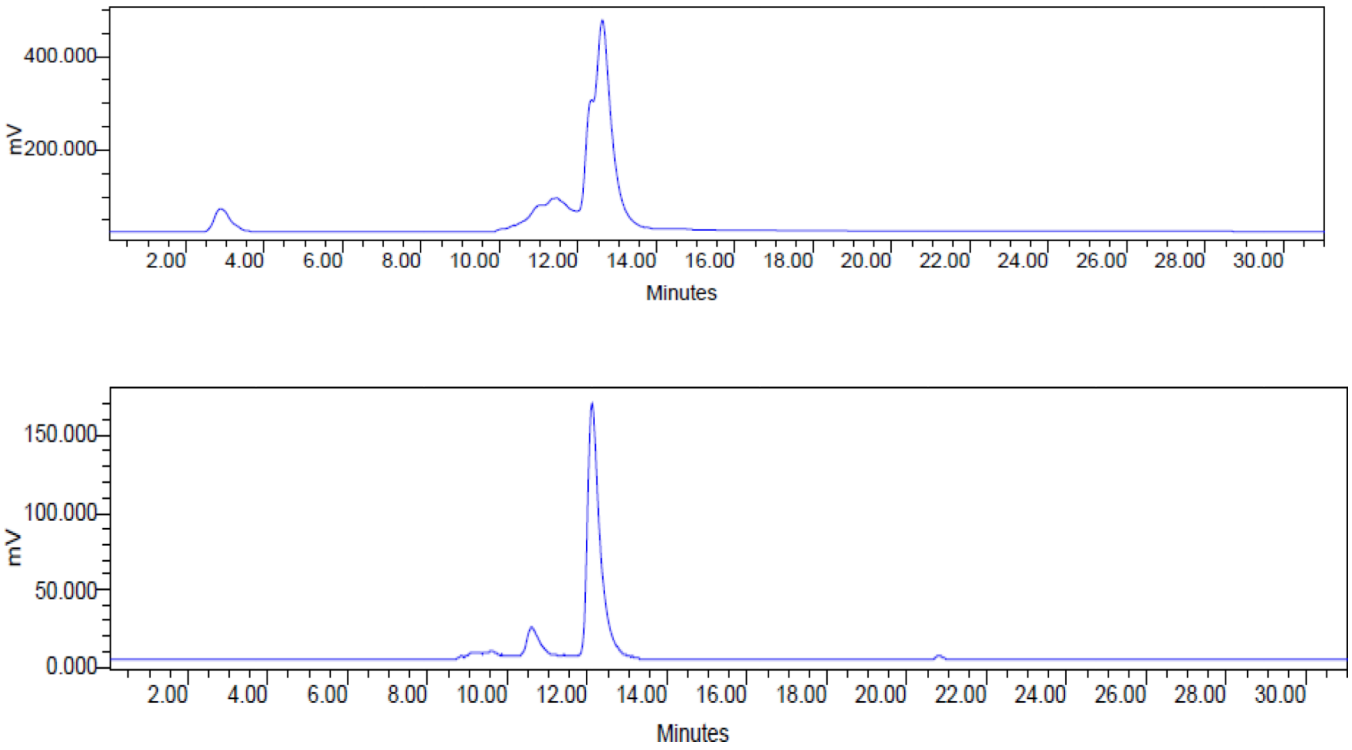

Figure 2.
Radio-HPLC profiles of ^{111}In-**4** (top) and ^{203}Pb-**4** (bottom)

Table 1

Binding of ^{111}In-**4** (0.3 μM) to a purified $\alpha_v\beta_3$ integrin.

	% Bound
^{111}In-**4**	2.4
^{111}In-**4** + 0.3 μM Integrin	10.5
^{111}In-**4** + 0.6 μM Integrin	35.7
^{111}In-**4** + 1.2 μM Integrin	60.5
^{111}In-**4** + 1.2 μM Integrin + **4**	2.9