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Macrocyclic chelator assembled RGD multimers for tumor targeting

Xiaofen Zhang^{a,b}, Hongguang Liu^a, Zheng Miao^a, Richard Kimura^a, Feiyue Fan^b, Zhen Cheng^{a,*}^a Molecular Imaging Program at Stanford (MIPS), Department of Radiology, Stanford University Medical Center, California 94305, USA^b Institute of Radiation Medicine, Chinese Academy of Medical Science, Peking Union Medical College, Tsinghua University, Tianjin 300192, China

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ABSTRACT

Macrocyclic chelators have been extensively used for complexation of metal ions. A widely used chelator, DOTA, has been explored as a molecular platform to assemble multiple bioactive peptides in this paper. The multivalent DOTA-peptide bioconjugates demonstrate promising tumor targeting ability.

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Multivalent biomolecules such as peptides have been explored as a useful strategy to construct molecular imaging probes and drug delivery carriers. It is generally accepted that multivalency has advantages over monovalency for improving binding affinities and even activity.¹ Compared to the monomeric peptides, many multivalent peptides could show improved binding affinities in vitro and tumor targeting and imaging ability in vivo.² Multivalent biomolecules thus have been constructed by using a variety of molecular platforms including small molecules such as amino acids (glutamine) and a fluorescent dye,^{2,3} multiple antigen peptides (MAP),⁴ proteins,⁵ dendrimers and polymers,⁶ and nanoparticles.⁷

Macrocyclic chelators are generally used for complexation of metal ions, and they have been widely used in molecular imaging applications when they complex with radioactive, paramagnetic or fluorescent metal ions.⁸ Various macrocyclic chelators such as polyamino polycarboxylate-type macrocycles have been successfully and extensively used as bifunctional chelators (BFCA) for labeling biomolecules.⁹ Recently, a polyamino polycarboxylate-type chelator, CB-TE2A, was explored as a scaffold to build a dimeric peptide while simultaneously forming a stable complex with the positron emission tomography (PET) radiometal, ⁶⁴Cu ($t_{1/2} = 12.7$ h, E_{β^+} max = 656 keV, 19%). The resulting divalent positron emission tomography (PET) probe exhibits excellent tumor imaging quality which highlights the advantages of this novel approach for designing multivalent probes using BFCA as a molecular scaffold.¹⁰ But CB-TE2A can only be used to construct divalent probes,

and the availability of the chelator is limited and synthesis of the compound requires significant effort.

Herein, we present the use of a widely available macrocyclic chelator, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), for the assembly of multiple peptides in tumor targeting. DOTA is an excellent choice for attaching metal ions to peptides, antibodies, and many other biologically active molecules, because it forms stable complexes with a variety of metal ions [⁶⁴Cu, ⁶⁷/₆₈Ga, ¹¹¹In, ^{86/90}Y, ¹⁷⁷Lu, ¹⁶⁶Ho, Gd(III), Eu(III), Tb(III), Yb(III), etc.] with high thermodynamic stability and kinetic inertness.⁸ Traditionally only one arm of DOTA is used for conjugation with one biomolecule. Considering that DOTA has four acetic acid side-arms, they could all be easily activated, functionalized and used for preparation of mono, di, tri, tetra-valent biomolecules. For the proof of concept, a small cyclic arginine–glycine–aspartic acid (RGD) peptide [c(RGDyK)] which is a well known ligand for tumor integrin receptors was selected for construction of multivalent molecules (Fig. 1).^{6,11} The resulting bioconjugates were radiolabeled with ⁶⁴Cu and their in vivo performance was then studied using microPET.

The synthesis of mono or multi-meric DOTA-RGD peptides (DOTA-P1, DOTA-P2, DOTA-P3, and DOTA-P4, Fig. 1) was achieved through a simple one pot synthesis reaction which involves coupling of in situ prepared DOTA-OSSu esters with the ε-amino group of the lysine residue in the large excess of c(RGDyK) (Supplementary data). The desired multi-meric DOTA-RGD peptides could be easily separated and purified by an analytic HPLC, and the purity of target compounds was generally obtained with over 95% purity. But the yields for each peptide are quite different, which DOTA-P2 shows highest, DOTA-P1 and DOTA-P3 shows moderate and

* Corresponding author. Tel.: +1 650 723 7866; fax: +1 650 736 7925.

E-mail address: zcheng@stanford.edu (Z. Cheng).

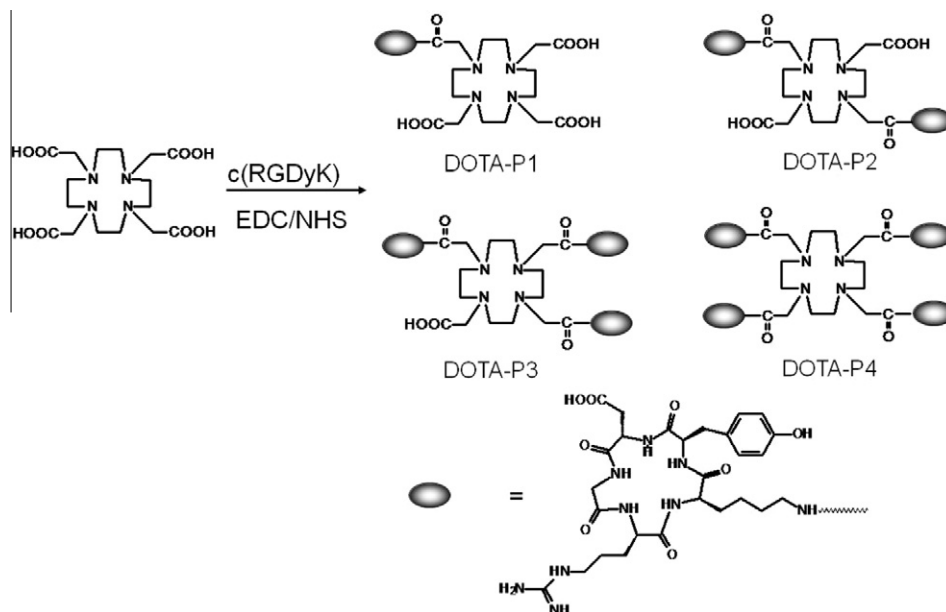


Figure 1. The structure of multivalent DOTA-RGD peptides (monomer: DOTA-P1; dimer: DOTA-P2; trimer: DOTA-P3; Tetramer: DOTA-P4).

DOTA-P4 has very low yield. The retention time on the analytical HPLC for these bioconjugates was found to be 16.8, 21.2, 24.1 and 26.6 min (Table 1 and Supplementary data). The purified DOTA-RGD peptides were characterized by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The measured molecular weights (MW) of the compounds were consistent with the expected MWs (Table 1).

The relative binding affinities of DOTA-RGD peptides to cell surface integrin receptors were determined through a competitive binding assay with ^{125}I -echistatin.¹¹ All the DOTA-RGD peptides inhibited the binding of ^{125}I -echistatin to integrin-expressing U87MG cells in a concentration-dependent manner. The mean \pm standard deviation IC_{50} values of DOTA-P1, DOTA-P2, and DOTA-P3 were 380 ± 190 nM, 37.1 ± 7.8 nM, and 13.6 ± 1.4 nM, respectively ($n = 3$). The biological evaluation of DOTA-P4 was not performed because of its low yield and potential low in vivo stability when it is complexed with ^{64}Cu .¹² The receptor binding study clearly indicates that conjugation of multiple RGD peptides to the DOTA molecular platform can improve the binding affinity and achieve multivalency effects.

DOTA-P1, DOTA-P2 and DOTA-P3 conjugates were then successfully radiolabeled with ^{64}Cu by a 1 h incubation at 50°C . The purification of radiolabeling solution using RP-HPLC afforded ^{64}Cu -DOTA-peptides with $>95\%$ radiochemical purity, and modest specific activities of $0.28\text{--}0.33$ Ci/ μmol ($10.3\text{--}12.3$ MBq/nmol) ^{64}Cu -DOTA-peptides were obtained as estimated by analytical HPLC (decay corrected). In vitro serum stability of the radiolabeled peptides was also tested by incubation of the ^{64}Cu -DOTA-peptide with mouse serum for 1 h at 37°C . All the radiolabeled peptides

were stable and over 90% of peptides were still intact after 1 h incubation.

Static microPET scans were performed on integrin receptors positive U87MG tumor-bearing mice and the representative decay-corrected coronal images at different time points after tail vein injection of ^{64}Cu -DOTA-P1, ^{64}Cu -DOTA-P2, or ^{64}Cu -DOTA-P3 are shown in Figure 2 (A, B and C; $n = 3$ for each group), from which the U87MG tumors were clearly visualized with good tumor-to-background contrasts for all the probes. Interestingly, under the same scale it can be easily seen that the tumor uptake of these three radiocomplexes ranks as ^{64}Cu -DOTA-P2 $>$ ^{64}Cu -DOTA-P3 $>$ ^{64}Cu -DOTA-P1. This result proves the advantage of multimerization of RGD peptide for in vivo tumor targeting. It is also consistent with previous reports.^{2,10} The microPET quantification analysis of tumors and other organs was obtained from the region of interest (ROI) analysis and the results at these time points are shown in Figure 2 D, E and F. ^{64}Cu -labeled dimeric RGD peptide (^{64}Cu -DOTA-P2) indeed displayed significant higher tumor uptake than that of monomer and trimer, while trimer showed significant higher tumor uptake than that of monomer ($P < 0.05$). At 1 h post-injection (p.i.), the tumor uptake was 2.32 ± 0.55 , 7.20 ± 0.82 , and $3.80 \pm 0.46\%$ ID/g for ^{64}Cu -DOTA-P1, ^{64}Cu -DOTA-P2, ^{64}Cu -DOTA-P3, respectively. Furthermore, it was found multimerization caused elevated kidney accumulation, both dimer and trimer showed relatively higher kidney uptakes than that of monomer (at 1 h p.i., 4.84 ± 0.72 and 5.96 ± 0.36 vs $4.00 \pm 0.32\%$ ID/g), which is also in consistence with the study of the other multimeric RGD peptides.² Of note, the liver uptake of ^{64}Cu -DOTA-P2 was also significantly higher than of ^{64}Cu -DOTA-P1 and ^{64}Cu -DOTA-P3.

In order to further demonstrate the in vivo integrin targeting specificity of the multivalent probe, microPET imaging of ^{64}Cu -DOTA-P2 was performed in MDA-MB-435 subcutaneous tumor model, which expresses much lower integrin receptors than that of U87MG.¹¹ ^{64}Cu -DOTA-P2 displayed significant lower uptake in MDA-MB-435 tumor than in U87MG at 2 h p.i. (7.27 ± 0.76 vs 1.47 ± 0.67 , $P < 0.05$), suggesting the specificity of the probe (Fig. 3).

Here, we demonstrate a simple strategy for developing multimeric biomolecules. Compared to many other molecular platforms, the metal chelator, DOTA, is cheap and can be easily modified with up to four biomolecules. Meanwhile, the in vivo performance of the

Table 1
Retention time, molecular weight and IC_{50} of multivalent DOTA-RGD peptides.

	Retention time (min)	MW Expected	MW Measured	IC_{50} (nM)
DOTA-P1	16.8	1006.49	1006.46	380 ± 190
DOTA-P2	21.2	1607.69	1607.70	37.1 ± 7.8
DOTA-P3	24.1	2209.08	2209.46	13.6 ± 1.4
DOTA-P4	26.6	2810.38	2810.47	n/a

n/a: not applicable.

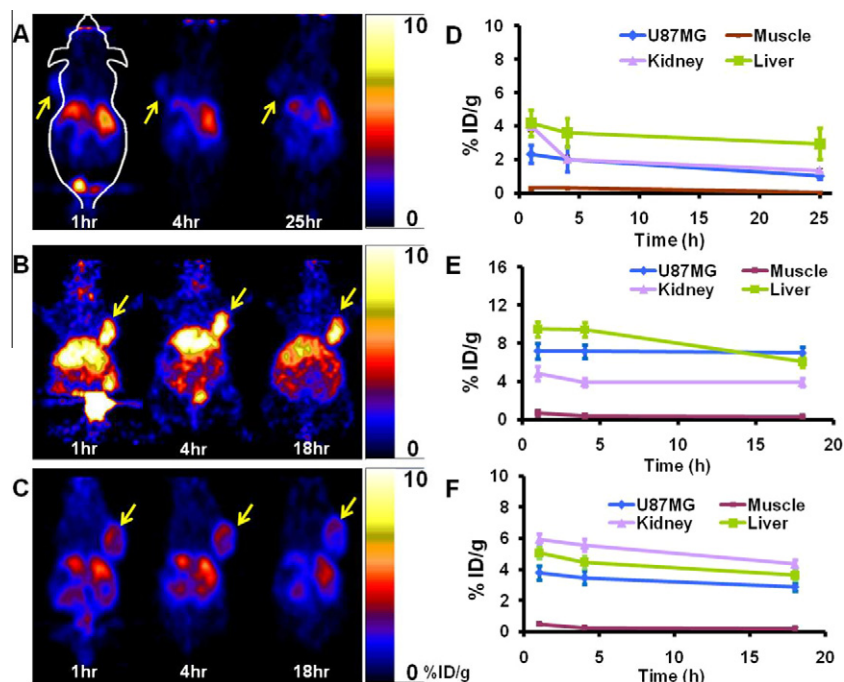


Figure 2. A, B and C: Representative coronal microPET images of the U87MG tumor-bearing mice at different time points (1, 4, 18 or 25 h) post-injection of ^{64}Cu -DOTA-P1, ^{64}Cu -DOTA-P2, and ^{64}Cu -DOTA-P3, respectively (arrows indicate tumors). D, E, and F: MicroPET quantification results, expressed as percentage of injected dose per gram (%ID/g), in different organs and tumor of subcutaneous U87MG glioblastoma xenograft model after intravenous injection of ^{64}Cu -DOTA-P1, ^{64}Cu -DOTA-P2, and ^{64}Cu -DOTA-P3, respectively.

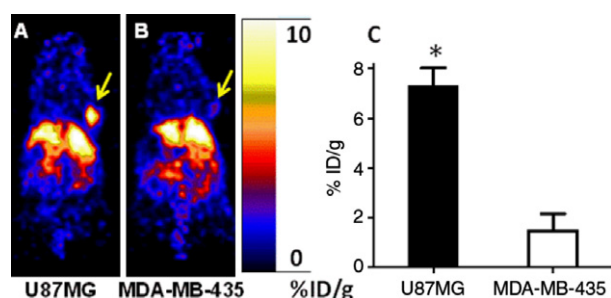


Figure 3. Coronal microPET images of mice bearing U87MG (A) and MDA-MB-435 (B) tumors at 2 h after injection of ^{64}Cu -DOTA-P2 (arrows indicate tumors). Quantification of microPET images was performed and shown in (C). (*p < 0.05)

resulting bioconjugates could be easily studied when DOTA is labeled with a radiocopper such as ^{64}Cu . The ^{64}Cu or other copper radioisotope labeled multimeric DOTA-bioconjugates could potentially find broad applications in tumor imaging and therapy.⁸ It also should be noted that for RGD dimer system, it is possible to form isomeric constructs (cis or trans). Further research on separation and characterization of these two isomers and studying their tumor imaging properties is important for understanding their structure–activity relationship. Moreover, DOTA-RGD bioconjugates could have very different copper coordination chemistry depending on the number of RGD ligands. More studies such as crystallography could be helpful to reveal the coordination number and fashion of multimeric probes. Finally, it is interesting that RGD dimer shows better in vivo properties than that of trimer. The in vivo performance of a probe depends on many factors including charge, size, hydrophilicity, stability of the label and more. The overall effects of these parameters may contribute to the excellent in vivo imaging properties of the dimeric RGD probe.

In conclusion, multivalent RGD peptides could be assembled using a macrocyclic chelator, DOTA, as a molecular scaffold. The

four arms of DOTA provide an opportunity to prepare monomeric, dimeric, trimeric and tetrameric peptides by a one pot chemical synthesis. The resulting multimeric peptides show good tumor imaging quality. Especially, it was found that the radiolabeled multimeric DOTA-RGD peptides (^{64}Cu -DOTA-P2 and ^{64}Cu -DOTA-P3) displayed higher tumor uptakes than that of monomeric peptide. Taken together, the macrocyclic chelator DOTA is a promising molecular scaffold for construction of multimeric biomolecules, and the the RGD multimeric peptides reported here are promising tumor targeting agents for clinical translation.

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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.2011.03.110](https://doi.org/10.1016/j.bmcl.2011.03.110).

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