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Universal Molecular Scaffold for Facile Construction of Multivalent and Multimodal Imaging Probes

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Abstract

Multivalent and multimodal imaging probes are rapidly emerging as powerful chemical tools for visualizing various biochemical processes. Herein, we described a bifunctional chelator (BFC)-based scaffold that can be used to construct such promising probes concisely. Compared to other reported similar scaffolds, this new BFC scaffold demonstrated two major advantages: (1) significantly simplified synthesis due to the use of this new BFC that can serve as chelator and linker simultaneously; (2) highly efficient synthesis rendered by using either click chemistry and/or total solid-phase synthesis. In addition, the versatile utility of this molecular scaffold has been demonstrated by constructing several multivalent/multimodal imaging probes labeled with various radioisotopes, and the resulting radiotracers demonstrated substantially improved in vivo performance compared to the two individual monomeric counterparts.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00034. Details on synthesis and characterization of new compounds, peptide conjugation, radiolabeling, in vitro cell staining, saturation binding assay and in vivo PET imaging (PDF)

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ligand(s), dye(s), and other functional moieties. In the future, a library of multivalent probes could be conveniently prepared via our BFC-based scaffold for the high-throughput screening and the following structural optimization after the modularization of moiety A and moiety B.

RESULTS AND DISCUSSION

As a proof-of-principle study, a 1,4,7-triazacyclononane-triacetic acid (NOTA) analog (N_3 - NO^tB_2 , BFC **6**) was prepared as the BFC scaffold. As illustrated in Scheme 1, compound **6** was synthesized in five steps. First, the starting material **1** was treated with $MeCOCl$ and $MeOH$ to obtain its methyl ester **2**. The amino group of **2** was converted to azide through a diazotransfer reaction,¹⁶ and then the resulting compound **3** was tosylated to afford compound **4**. Compound **5** was obtained via alkylation of $NO_2A(tBu)$ (di-*tert*-butyl 2,2'-(1,4,7-triazonane-1,4-diyl)diacetate)¹⁷ using compound **4**. Hydrolysis of methyl ester of **5** resulted in the BFC **6**. By applying this strategy, other similar BFC scaffolds can also be conveniently prepared, such as a cyclen-based scaffold (N_3 - DO^tB_3 , see Scheme S1) for chelating other radiometals or chelating Gd^{3+} for potential MRI applications.

In order to demonstrate the versatile utility of this new BFC scaffold, several multivalent and multimodal probes have been prepared using BFC **6** through two different methods (Scheme 2). In Method 1, BFC **6** was attached to the peptide moiety A via solid-phase peptide synthesis (SPPS), followed by TFA cleavage and ligation with BCN (metal-free click reaction moiety) functionalized moiety B to yield the desired products (**8a–8f** in Table 1 and SI 1.3.3). For instance, BFC **6** was attached to a urokinase receptor (uPAR) targeting ligand AE105¹⁸ to obtain **7b** after TFA cleavage and HPLC purification. Subsequently, **7b** was rapidly conjugated to the BCN functionalized cyclo(RGDyK) (targeting integrin $\alpha_v\beta_3$) to yield heterodimeric probe **8c** (Figure 2). Rendered by the rapid metal-free click chemistry that has been widely applied in the field of radiopharmaceuticals,^{19,20} the ligation between azidofunctionalized moiety A and BCN-attached moiety B proceeded in nearly quantitative yield with greater than 95% purity of the resulting probe, thus avoiding tedious chromatography purification. Alternatively, as shown in Method 2 in Scheme 2, the whole process can be performed completely on the solid phase with a high product purity upon TFA cleavage. Briefly, the azide was reduced to primary amine by PPh_3 , to which the moiety B (another ligand or fluorescent dye) was attached via the standard Fmoc chemistry. The desired products (**9a** and **9b** in Table 1, Figure 2, and SI 1.3.3) were then obtained after the TFA cleavage.

Compared to the traditional NOTA, which possesses three carboxylic acids available for Cu^{2+} coordination, there are only two carboxylic acids available for Cu^{2+} coordination in our BFC. We anticipated that the triazole formed by click reaction would promote the formation of a stable Cu^{2+} complex and subsequently enhance its stability, similar to results in previous reports.^{21,22} Due to the absence of crystal structures of triazole- (Cu^{2+}) NOTA, a density functional theory (DFT) calculation was performed to generate the optimized geometry of the triazole- (Cu^{2+}) NOTA complex (see SI for computational details).²³ Due to the formation of a coordinating bond between the triazole group and $Cu(II)$, the energy of model B (Figure 3) was 10.2 kcal/mol lower than that of model A (Figure 3), indicating that

the participant of a triazole group to this coordination system may lead to a more stable complex with Cu(II). DFT calculation was also performed on a triazole-(Ga³⁺)NOTA complex, and consistent results were obtained that the triazole could form a coordinate bond with Ga³⁺ and enhance its stability (Figure S1).

Selected compounds, **8a**, **8c**, and **9b**, representing homodimeric, heterodimeric, and dual-modal imaging probes, respectively, were labeled with ⁶⁸Ga and ⁶⁴Cu. Good specific activities (1.0–1.50 mCi/nmol) and high radiopurity (>98%) were obtained. As shown in Figure S2, the resulting PET tracers exhibited good human serum stability (no or less than 5% disassociation) after 24 h (⁶⁴Cu-labeled tracers) and 2 h (⁶⁸Ga-labeled tracers) incubation at 37 °C, which was consistent with our DFT calculation. In addition, metabolism studies were performed on **8c** to further investigate the in vivo stability of triazole-(Cu²⁺)NOTA chelate using the reported method.²⁴ The FPLC (fast protein liquid chromatography) result (Figure S4) indicated that the liver extracts contained over 80% intact (⁶⁴Cu)**8c**. Compared to the commonly used NODAGA chelator containing 70–80% intact (⁶⁴Cu)NODAGA-AE105 in liver extracts, the triazole-(Cu²⁺)NOTA chelate demonstrated comparable or slight higher in vivo stability.

Biological evaluation of the prepared probes was then conducted. First, the multimodal imaging probe **9b** was used in the immunofluorescent staining of human U87MG glioblastoma cells. Strong staining and good blocking were observed (Figure 4), which confirmed the strong overexpression of uPAR on the U87MG human glioblastoma cell line and demonstrated its utility as a good optical imaging probe. Then the ⁶⁴Cu labeled heterodimer (⁶⁴Cu)**8c** was used to measure the specificity and avidity on the U87MG human glioblastoma cell line using cell saturation binding assay. Good binding affinity (9.9 ± 4.2 nM) and high *B*_{max} (488 ± 73 fmol/mg) were obtained (Figure S3).

Encouraged by the saturation binding assay results, (⁶⁴Cu)**8c** was further evaluated in a mouse xenograft model. Briefly, nude mice (6–7 weeks old) bearing U87MG xenografts in the right front shoulder were injected with ~150 μCi of (⁶⁴Cu)**8c** for PET/CT imaging after the tumor size reached 100–200 mm³. As shown in Figure 5, all xenografted tumors were clearly visible at both 1 and 4 h p.i. with good tumor-to-background contrasts. Tumor uptake values were (3.13 ± 0.49) %ID/g and (3.27 ± 0.25) %ID/g at 1 and 4 h, respectively, determined by quantitative analysis of the PET images. High intestine uptakes were observed, which could be attributed to high expression of the targeted receptors in the intestine in young mice, since similar observations have been reported with other AE105 and RGD PET tracers labeled with ⁶⁴Cu using BFCs, DOTA, and CB-TE2A (“gold standard” for ⁶⁴Cu) respectively.^{13,17} In a blocking study performed by coinjecting unlabeled cyclo-(RGDyK) and AE105, the tumor uptake of (⁶⁴Cu)**8c** was significantly reduced to (1.01 ± 0.18) %ID/g (*p* < 0.001), which further confirmed the specificity of the heterodimer for targeting α_vβ₃ integrin and uPAR.

Moreover, PET images obtained from the heterodimeric (⁶⁴Cu)**8c** were compared to the ones obtained from the two ⁶⁴Cu incorporated individual monomers: (⁶⁴Cu)AE105 ((⁶⁴Cu)NODAGA-AE105) and (⁶⁴Cu)RGD ((⁶⁴Cu)-NODAGA-RGD). The monomers were radiolabeled with Cu-64 through the attached BFC NODAGA (1,4,7-triazacyclononane, 1-

glutaric acid-4,7-acetic acid). As shown in Figure 6, (^{64}Cu)8c prepared via the developed BFC scaffold demonstrated significantly increased tumor uptake ($p < 0.01$) (1 h $3.13 \pm 0.49\% \text{ID/g}$, 4 h $3.27 \pm 0.25\% \text{ID/g}$) compared to the other two monomer tracers ((^{64}Cu)AE105 (1 h $1.45 \pm 0.15\% \text{ID/g}$, 4 h $1.73 \pm 0.31\% \text{ID/g}$) and (^{64}Cu)RGD (1 h $1.50 \pm 0.42\% \text{ID/g}$, 4 h $1.55 \pm 0.51\% \text{ID/g}$)).

CONCLUSIONS

In summary, a BFC-based molecular scaffold has been successfully developed as a reliable and universal platform for the facile construction of multivalent and multimodal imaging probes targeting any interested disease related biomarker for routine preclinical/clinical applications. Compared to other reported small-molecule scaffolds, this new BFC scaffold demonstrated several advantages, including simplified synthesis, ease of purification, higher synthetic yields, and even the possibility of conducting a semiautomatic preparation. This developed molecular scaffold has greatly facilitated our ongoing structural optimization of multivalent/multimodal imaging probes by varying the length of PEG-linker, and more comprehensive evaluation of potent heterodimeric PET tracers will be performed as a part of our future study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Diagram of multivalent and multimodal imaging probe.

Scheme 1.
Synthesis of BFC 6 (N₃-NO^tB₂)

Scheme 2.

Synthesis of Multivalent and Multimodal Imaging Probes Using scaffold N_3 - NO^tB_2 via Metal-Free Click Chemistry (Method 1) or SPPS (Method 2) Approaches

Figure 2.
Structures of selected peptidic heterodimer **8c** and multimodal probe **9b**.

Figure 3. DFT optimized geometry of the triazole-(Cu²⁺)NOTA complex. Model A, acetic acid coordinated complex; model B, triazole coordinated complex.

Figure 4.

(A) Fluorescence images of U87MG cells incubated with compound **9b** (10 nM) for 2 h at 37 °C. (B) Fluorescence images of U87MG cells pretreated with 10 μ g AE105 for 1 h at 37 °C and further incubated with compound **9b** (10 nM) for 2 h at 37 °C.

Figure 5. PET/CT images of U87MG tumor-bearing mice post injection of $(^{64}\text{Cu})\mathbf{8c}$ (100–150 μCi) with/without blocking agents (100 μg AE105 and 100 μg c(RGDyK)). Arrows indicate tumor.

Figure 6. PET/CT images of U87MG tumor-bearing mice at 1 and 4 h post injection of 100–150 μ Ci $(^{64}\text{Cu})\text{NODAGA-AE105}$, $(^{64}\text{Cu})\text{NODAGA-RGD}$, and $(^{64}\text{Cu})\mathbf{8c}$. Arrows indicate tumor.

Table 1

Construction of Multivalent or Multimodal Molecular Probes Using BFC Scaffold 6 via Two Approaches

moiety A	moiety B	product	method	purity ^a
AE105	AE105	8a	1	>95
AE105	Cy5	8b	1	>95
AE105-PEG ₈	RGDyK	8c	1	>95
AE105	DAPTA	8d	1	>95
AE105	LLP2A	8e	1	>95
AE105	AE105	9a	2	~70 ^b
AE105	Cy3	9b	2	~85 ^b

^aPurity of probes prepared via method 1 was measured by HPLC after incubation for 2–4 h at room temperature.^bPurity of cleaved peptide was determined by HPLC.