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Gallium labeled NOTA-based conjugates for peptide receptor-mediated medical imaging

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ABSTRACT

We report a straightforward and efficient synthetic strategy for the synthesis of three model glycine-arginine-glycine-aspartic acid-glycine (GRGDG) conjugates based on derivatives of NOTA and of their Ga(III) complexes targeted to the integrin $\alpha_v\beta_3$ receptor. ^{71}Ga NMR spectroscopy showed that the Ga(III)-labeled conjugates are highly stable in aqueous solution. The ^{67}Ga-labeled conjugates proved to have high kinetic stability and showed a weak but specific binding to the receptors in a U87MG-glioblastoma cell line.

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Gallium has three radioisotopes of interest for medical imaging, ^{67}Ga ($t_{1/2}$ = 3.25 days), ^{66}Ga ($t_{1/2}$ = 9.5 h) and ^{68}Ga ($t_{1/2}$ = 68 min). The first one, a γ emitter, can be used in γ-scintigraphy, while the others, being β^+ emitters, are appropriate for positron emission tomography (PET). ^{68}Ga is a very attractive radionuclide for PET, as it can be produced in situ from a ^{68}Ge generator, allowing easy routine manufacture in the hospital facilities comparable to what happens with the ^{99}Mo/^{99m}Tc-generator. Gallium-based radiopharmaceuticals preparation is easy and fast, contrarily to the preparation of ^{18}F or ^{11}C covalently labeled PET agents, leading to a minimum loss of activity.[1,2]

The most common chelators for Ga(III) are hexadentate[1,3–17] and, among them, triaza macrocycles are specially suitable due to their high conformational and size selectivities, allowing a good fit of the relatively small cation in the macrocyclic cavity. Such is the case of 1,4,7-triazacyclononane-*N,N′,N″*-triacetic acid (NOTA),[5] which forms highly stable chelates and allows a faster incorporation of Ga(III) at lower temperatures than 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid (DOTA), its tetraaza analog.[18]

Recently we reported the synthesis and study of micelles forming Ga(III) chelates of NOTA-based ligands with a variable size α-alkyl chain in one of the acetate arms.[4] The synthetic route of these ligands is compatible with their conjugation to amine-containing biomolecules, rendering them bifunctional chelators. Bifunctional chelators are molecules displaying a targeting vector with high affinity and selectivity for a specific receptor and a metal ion that can be used for diagnosis or therapy.[19] Amongst biomolecules, peptides have shown to be the most effective targeting moieties known for cellular receptors, drug delivery, molecular imaging and radiotherapeutic applications.[20–23] The compatibility with peptide synthesis requires that bifunctional chelators, often bearing carboxylic acid groups, offer the possibility of orthogonal protection.[24]

The arginine-glycine-aspartic acid (RGD) peptide sequence has emerged as one of the most efficient epitopes for targeting the integrin $\alpha_v\beta_3$ receptor. Integrin $\alpha_v\beta_3$ receptor is a transmembrane protein that acts as a receptor for extracellular matrix proteins with RGD tripeptide sequence.[25,26] This integrin is highly expressed in active endothelial cells of the neovasculature of various tumors and it has been demonstrated that it is overexpressed in both endothelial cells and tumor cells in human breast cancer xenografts.[27] The integrin $\alpha_v\beta_3$ receptor expression correlates well with tumor progression and invasiveness of melanoma, glioma, ovarian and breast cancers.[25,27] Due to the restricted expression of $\alpha_v\beta_3$ in tumors, this integrin is considered an adequate molecular target for tumor targeting.[28–30]

In this Letter we report an efficient synthetic route for coupling NOTA-based pro-chelators to peptides. As proof-of-concept we have used as a model peptide a linear glycine-arginine-glycine-aspartic acid-glycine (GRGDG) pentapeptide. Three conjugates

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were obtained (Fig. 1) and their radiolabeling with ^{67}Ga has been characterized; stability and hydrophilicity studies have been performed. In vitro studies with a human glioblastoma cell line complemented this work.

The NOTA conjugates were prepared according to the strategy presented in Scheme 1. NOTA-based pro-chelators (**1a**–**c**) display one acetate pendant arm with a α-alkyl chain of variable size [1,4,7-triazacyclononane-*N*-hexanamide-(glycine-arginine-glycine-aspartic acid-glycine)-*N*′,*N*″-diacetic acid (NOTAC6-GRGDG) **1a**, 1,4,7-triazacyclononane-*N*-octanamide-(glycine-arginine-glycine-aspartic acid-glycine)-*N*′,*N*″-diacetic acid (NOTAC8-GRGDG) **1b**, 1,4,7-triazacyclononane-*N*-decanamide-(glycine-arginine-glycine-aspartic acid-glycine)-*N*′,*N*″-diacetic acid (NOTAC10-GRGDG) **1c**] and were synthesized following a procedure previously developed by us which involves an orthogonal protection strategy for the carboxylic moieties.[4,31] The protecting groups used were the benzhydryl ester, cleaved by Pd catalyzed hydrogenolysis, and the *tert*-butyl ester, cleaved in acid. After cleavage of the single benzhydryl ester, the pro-chelators **2a**–**c** were ready for coupling to the GRGDG peptide. The model GRGDG peptide was prepared by standard solid phase peptide synthesis (SPPS) using a fluorenylmethoxycarbonyl (Fmoc) protocol and a 2-chlorotrityl chloride resin. For side-chain protection the 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl (Pbf) group for arginine and the *tert*-butyl (^{t}Bu) group for aspartic acid were used. Coupling reactions were performed with diisopropylcarbodiimida (DIC) and 1-hydroxybenzotriazole (HOBt). The coupling of the NOTA pro-chelators (**2a**–**c**) to the GRGDG peptide were carried out in solid phase using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) and HOBt (Scheme 1). After cleavage from the resin and removal of the protecting groups, the peptide conjugates NOTAC6-GRGDG (**3a**), NOTAC8-GRGDG (**3b**), NOTAC10-GRGDG (**3c**) were obtained with good yields (Scheme 1).[32]

The ^{71}Ga NMR spectrum of the Ga(III) complex of NOTAC6-GRGDG in aqueous solution (pH 7) shows a signal at 166 ppm ($\Delta\nu_{1/2}$ = 454 Hz) in conformity with an octahedral or pseudo-octahedral coordination geometry, comparable to what has been previously observed by us for [Ga(NOTAC6)].[4] It is interesting to verify that the replacement of a carboxylate oxygen by an amide carbonyl oxygen in the conjugate does not affect the coordination geometry of Ga(III). The ^{71}Ga NMR signal was observed unchanged over a

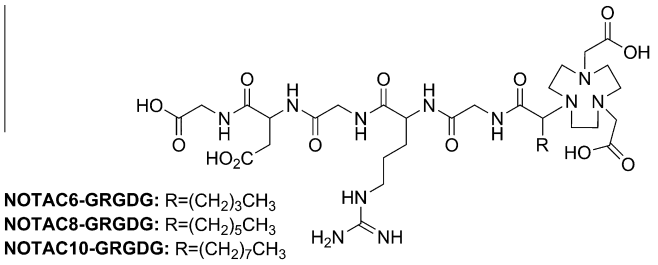
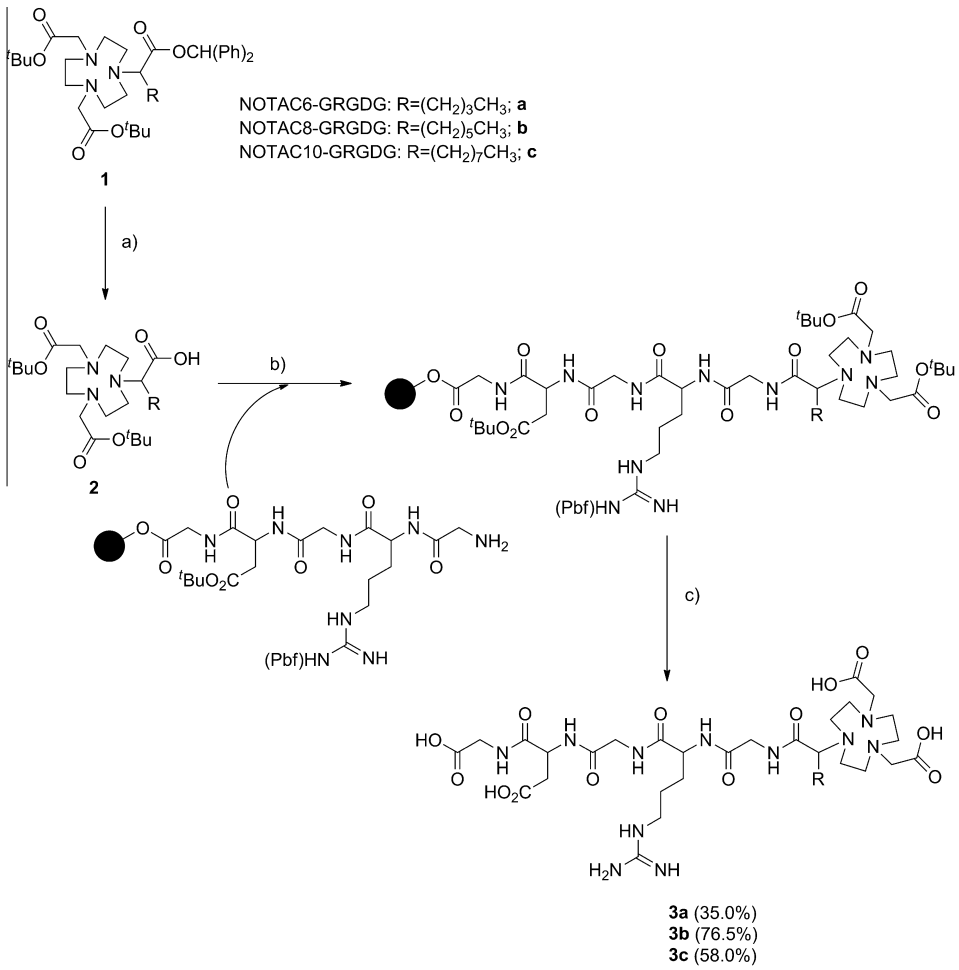


Figure 1. Structure of the GRGDG conjugates.



Scheme 1. Synthesis of the NOTA-GRGDG conjugates. Reagents and conditions: (a) EtOH, $CHO_2H{\cdot}NH_3$, 10% Pd/C, N_2, Δ; (b) (i) DMF, HBTU, HOBt; (ii) Et_3N; (c) (i) AcOH/TFE/DCM (1:1:3); (ii) TFA.

time period of 2 months, reflecting the high stability of the chelate in aqueous solution.

NOTAC6-GRGDG and NOTAC8-GRGDG were efficiently labeled with ^{67}Ga(III) using HEPES buffer (pH 3.6, 0.1 M) and heating at 95 °C for 1 h. The ^{67}Ga source was gallium citrate. The radiochemical purity control was performed by radio-HPLC showing a single peak with rt = 10.1 min for [^{67}Ga]NOTAC6-GRGDG and a single peak with rt = 10.5 min for [^{67}Ga]NOTAC8-GRGDG. In both cases the labeling yields were >80%.

Stability studies have demonstrated that [^{67}Ga]NOTAC8-GRGDG does not release the radiometal, even under extreme conditions (a 10^4 fold molar excess of DTPA) at least during the period of time evaluated, 24 h. The HPLC results have shown that no radiolysis breakdown products were formed. The stability observed for [^{67}Ga]NOTAC8-GRGDG is in agreement with the thermodynamic stability of most of the Ga(III) triaza complexes.[3,5,9,13,15,16,33]

The lipophilicity of the chelates (log *P* values) was determined measuring the octanol/water partition coefficients of [^{67}Ga]NOTAC6-GRGDG and [^{67}Ga]NOTAC8-GRGDG which demonstrated that these chelates are moderately hydrophilic (log $P = -2.66$ and log $P = -1.30$, respectively). These results are in accordance with our previous studies and correlate with the different lengths of the α-alkyl side chain.[4]

The human glioblastoma (U87MG) cell line is known to overexpress the $\alpha_v\beta_3$ receptor.[34] In this experiment, we evaluated the interaction of [^{67}Ga]NOTAC6-GRGDG with the $\alpha_v\beta_3$ receptors. Echistatin, known as the most potent integrin receptor blocker,[35] was added to evaluate the specificity of the process. The cells were incubated with [^{67}Ga]NOTAC6-GRGDG for 60 min at 37 °C. After 60 min the cells were washed and treated with an acid buffer to remove the tracer bounded to the cell surface, and then detached from the plates. The radioactivity inside the cells, together with the activity associated to the cell membranes (removed by acid wash), were measured in a γ well counter. The results of this experiment are the average of three independent measurements and are expressed as percentage of added activity per million of cells (Table 1).

After 1 h, the activity retained in the cells was somewhat low, 0.24 ± 0.05 (% added activity per million of cells), suggesting a low binding affinity of the [^{67}Ga]NOTAC6-GRGDG conjugate towards the integrin receptor, in accordance to what is known for linear RGD peptides.[29] This value was reduced to 0.14 ± 0.01 when the cells were treated with echistatin indicating that, despite the low affinity of the bioconjugate, we are facing a specific process.

In conclusion, we synthesized three NOTA-based conjugates of a model GRGDG peptide adequate for radiolabeling with Ga(III), which are targeted to the integrin $\alpha_v\beta_3$ receptor. The synthetic route that was used makes use of an orthogonal protection strategy that may also be efficiently applied to DOTA-based chelators[36] and to other peptides.[37] Potentially, the presence of a pendant α-alkyl chain in the chelator moiety has an effect on the pharmacokinetic properties of the correspondent Ga(III) labeled conjugates, and these might be tuned in accordance to the number of carbon atoms of the chain. Despite the fact that one of the triaza carboxylic groups is used for conjugation to the peptide, through amide bond formation, the Ga(III) complex in the conjugate remains hexacoordinated with an octahedral or pseudo-octahedral geometry, presenting high stability in aqueous solution as shown by ^{71}Ga NMR and competition experiments with DTPA.

Table 1
[^{67}Ga]NOTAC6-GRGDG internalization at 1 h in the U87MG cell line with and without receptor blocker (echistatin). The results are express as percentage of added activity per million of cells

Condition	Cell surface	Internalized
[^{67}Ga]NOTAC6-GRGDG	2.43 ± 0.16	0.24 ± 0.05
[^{67}Ga]NOTAC6-GRGDG and echistatin	1.03 ± 0.03	0.14 ± 0.01

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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.2010.10.059.

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32. *Synthesis of compound* **3b**: the GRGDG peptide was prepared by solid phase synthesis. *N*-Fluorenylmethoxycarbonylglycine (Fmoc-Gly-OH) (1.2 equiv) and diisopropylethylamine (DIPEA) (4 equiv relative to the amino acid) in dry dichloromethane (DCM) were added to the 2-chlorotrityl chloride resin (1.00 g). The loading amount was 0.525 mmol g^{-1}. After cleavage of the Fmoc group with a solution of 20% piperidine in DMF, couplings were carried out using an excess of the Fmoc-amino acid (4 equiv, 2.10 mmol) with diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt) in DMF. Coupling of compound **2b** (0.089 g, 0.189 mmol) to 0.197 g of resin was carried out using HBTU (0.286 g, 0.754 mmol), HOBt (0.203 g, 1.50 mmol) and triethylamine (0.026 mL, 0.188 mmol). After 48 h additional amounts of HBTU and HOBt were added. Cleavage from the resin and removal of the protecting groups afforded compound **3b** as a white solid (0.043 g, 0.052 mmol), 76.5% yield. ^{1}H NMR (400 MHz, DMSO, TMS, δ (ppm)): 0.84

(3H, t, J = 6.8 Hz, $(CH_2)_4CH_3$), 1.21–1.26 (8H, m, $(CH_2)_4CH_3$), 1.40–1.75 (6H, m, β-CH_2+γ-CH_2 Arg and CH_2 *ABX*), 2.64–3.80 (28H, m, β-CH_2 Asp, N*H*, δ-CH_2 Arg, 3×CH_2 Gly, *en*, 2×CH_2CO and *CH* AB*X*), 4.28–4.35 (1H, m, α-C*H* Arg), 4.57–4.62 (1H, m, α-C*H* Asp), 7.20 (2H, br s, NH_2), 7.59–8.44 (6H, br s, N*H* 3×Gly, Asp, 2×Arg). ^{13}C NMR (100.613 MHz, DMSO, TMS, δ (ppm)): 13.94 [$(CH_2)_5CH_3$], 16.72–31.13 (($CH_2)_5CH_3$ and 2×CH_2 Arg), 36.32 (CH_2 Asp), 38.88–40.86 (*en*), 41.79 (2×CH_2 Arg + Gly), 43.71 (2×CH_2 Gly), 49.30 (*C*H Asp), 52.30 (*C*H Arg), 53.54 (CH_2CO), 55.10 (*C*H AB*X*), 156.76 (*C*═N), 161.94 to 171.41 (*C*═O). HRMS (ESI+) calcd for $C_{34}H_{60}N_{11}O_{13}$ $(M+H)^+$ 830.43721. Found 830.43467. HPLC (retention time): 7.09 min (LiChrospher® 100 RP-18 (5 μm), water/acetonitrile (9:1) with 0.1% TFA, 0.8 mL min^{-1}).

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37. Results not published.