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Stability Analysis of Glutamic Acid Linked Peptides Coupled to NOTA through Different Chemical Linkages

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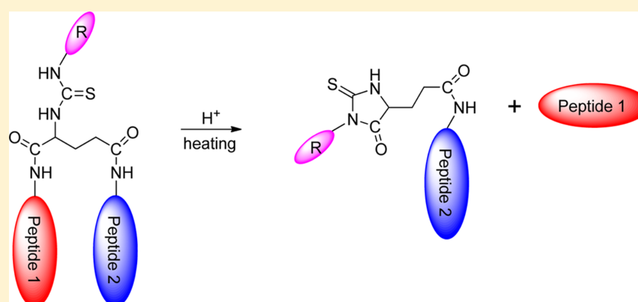
S Supporting Information

ABSTRACT: Glutamic acid is a commonly used linker to form dimeric peptides with enhanced binding affinity than their corresponding monomeric counterparts. We have previously labeled NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$ (NOTA-PRGD2) [**1**] with [^{18}F]AlF and ^{68}Ga for imaging tumor angiogenesis. The p-SCN-Bn-NOTA was attached to E[c(RGDyK)]$_2$ [**2**] through a mini-PEG with a thiourea linkage, and the product [**1**] was stable at radiolabeling condition of 100 °C and pH 4.0 acetate buffer. However, when the same p-SCN-Bn-NOTA was directly attached to the α-amine of E[c(RGDfK)]$_2$ [**3**], the product NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [**4**] became unstable under similar conditions and the release of monomeric c(RGDfK) [**5**] was observed. The purpose of this work was to use HPLC and LC-MS to monitor the decomposition of glutamic acid linked dimeric peptides and their NOTA derivatives. A c(RGDyK) [**6**] and bombesin (BBN) [**7**] heterodimer c(RGDyK)-E-BBN [**8**], and a dimeric bombesin E(BBN)$_2$ [**9**], both with a glutamic acid as the linker, along with a model compound PhSCN-E[c(RGDfK)] [**10**] were also studied. All the compounds were dissolved in 0.5 M pH 4.0 acetate buffer at the concentration of 1 mg/mL, and 0.1 mL of each sample was heated at 100 °C for 10 min and the more stable compounds were heated for another 30 min. The samples at both time points were analyzed with analytical HPLC to monitor the decomposition of the heated samples. The samples with decomposition were further analyzed by LC-MS to determine the mass of products from the decomposition for possible structure elucidation. After 10 min heating, the obvious release of c(RGDfK) [**5**] was observed for NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [**4**] and Ph-SCN-E[c(RGDfK)] [**10**]. Little or no release of monomers was observed for the remaining samples at this time point. After further heating, the release of monomers was clearly observed for E[c(RGDyK)]$_2$ [**2**], E[c(RGDfK)]$_2$ [**3**], c(RGDyK)-E-BBN [**8**], and E(BBN)$_2$ [**9**]. No decomposition or little decomposition was observed for NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$ [**1**], PEG$_3$-E[c(RGDyK)]$_2$ [**11**], NOTA-E[c(RGDyK)]$_2$ [**12**], and NOTA-PEG3-E[c(RGDyK)]$_2$ [**13**]. The glutamic acid linked dimeric peptides with a free α-amine are labile due to the neighboring amine participation in the hydrolysis. The stability of peptides could be increased by converting the free amine into amide. The instability of thiourea derivatives formed from α-amine was caused by participation of thiol group derived from thiourea.



KEYWORDS: *peptide, glutamate linker, thiourea, hydrolysis, Edman degradation*

■ INTRODUCTION

Aluminum [^{18}F]fluoride NOTA complex formation is an appealing method for rapid radiolabeling of peptides under aqueous conditions.[1−3] With the pioneering work of McBride et al.[1] as a guide, we previously reported the synthesis of [^{18}F]AlF-NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$ ([^{18}F]-Alfatide) from NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$ [**1**], a dimeric RGD peptide formed by linking the lysines together with glutamic acid, and the NOTA was attached to amine group through a thiourea linkage.[4] In McBride's subsequent publication, an amide linkage was used to attach the chelators to the peptide.[5] Liu et al. also reported the direct attachment of NOTA to the amine group of RGD peptide through an amide bond.[6] In general, both thiourea and amide linkages are very stable[7,8] and the radioactive aluminum fluoride NOTA complexes are also stable.[1] However, two or more radioactive products can be found due to the formation of isomers of aluminum fluoride NOTA complex as observed by Laverman et al.[2] In our previous study, we showed that our radioactive product was stable in PBS and mouse serum and only a single radioactive peak was detected by analytical RP-HPLC.[4] For the imaging study, we employed a simple C_{18} cartridge trap and release method to formulate the final product. Elution was effected with a small amount of ethanol (0.2−0.3 mL). Dilution with saline provided a suitable vehicle for injection into the

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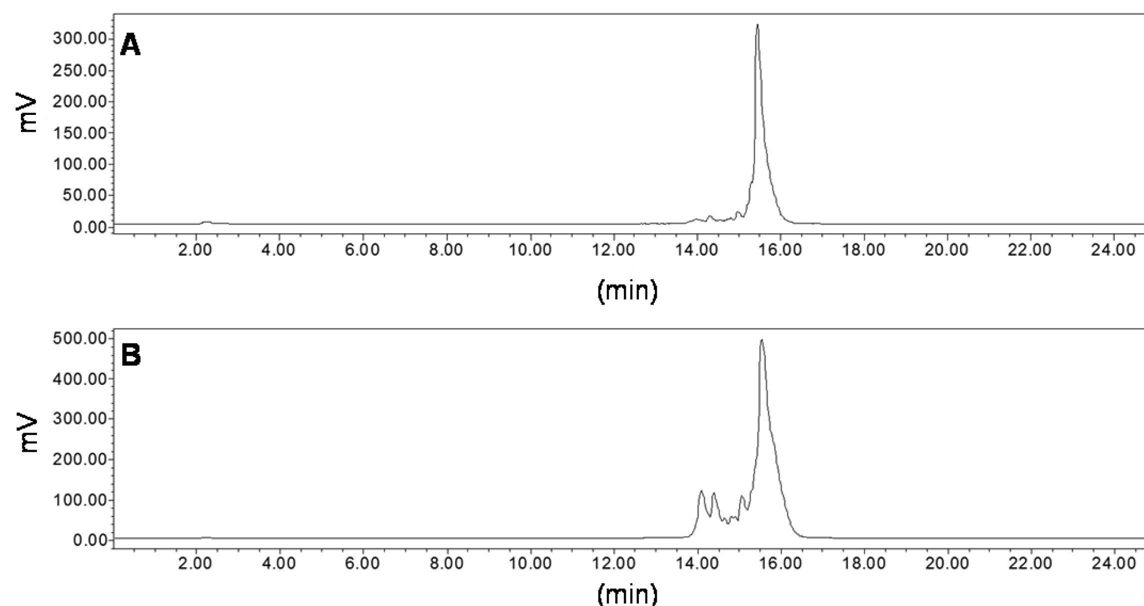

Figure 1. HPLC chromatograms of [^{18}F]AlF-NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$ ([^{18}F]AlF-**1**) before (A) and after (B) evaporation of ethanol.

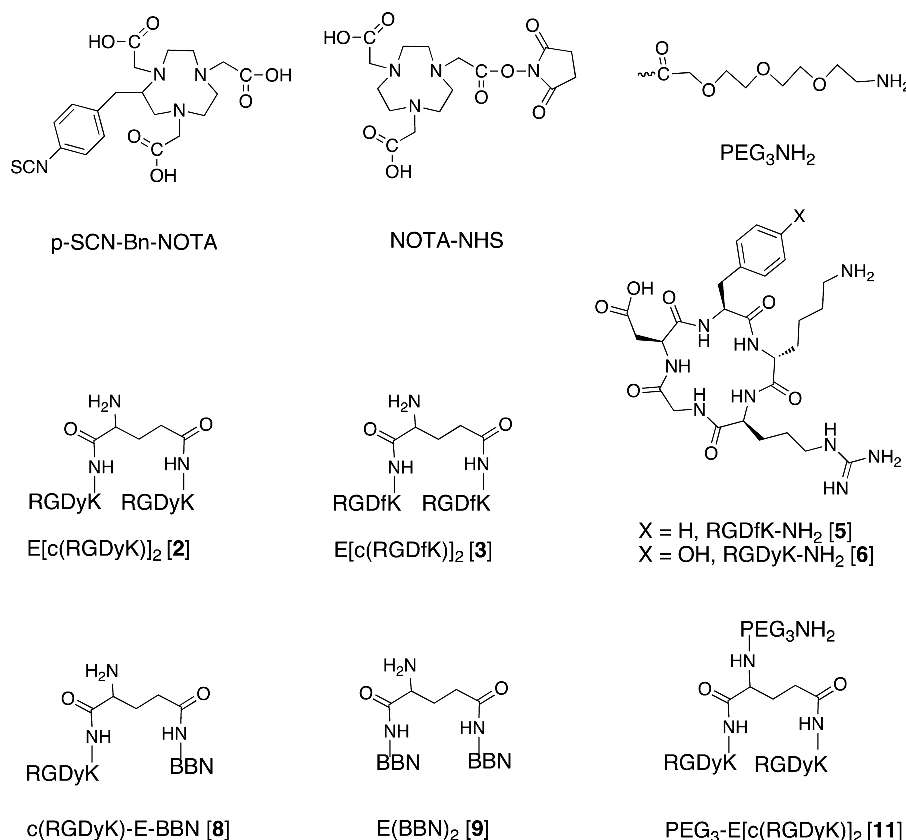

Figure 2. Structures of starting materials and RGD and BBN peptides. To simplify the structure representation, two monomeric RGD peptides c(RGDfK) [**5**] and c(RGDyK) [**6**] are designated as RGDfK-NH$_2$ and RGDyK-NH$_2$ with NH$_2$ representing amino group from lysine.

animals. In considering the clinical translation,[9] we realized that this formulation might not be compatible with those patients who are alcohol intolerant. To produce alcohol-free product, the ethanol needs to be removed before adding saline. The ethanol was easily removed through evaporation with a stream of argon at room temperature. However, after evaporating the solvent and adding saline, the final product contained multiple radioactive components when analyzed with RP-HPLC (Figure 1). Because these radioactive peaks cannot be explained by the formation of isomers, the product will fail QC procedures. In this study, we evaluated the influence of the peptide structure and the functional group attachment of NOTA to the peptide on the stability of the final construct. HPLC and LC-MS methods were used to evaluate the stability and identify decomposition products.

■ MATERIALS AND METHODS

Cyclic RGD peptides, E[c(RGDyK)]$_2$ [**2**], c(RGDfK) [**5**], and PEG$_3$-E[c(RGDyK)]$_2$ (PRGD2) [**11**], were purchased from Peptides International (Louisville, KY), and c(RGDyK) [**6**] was obtained from AnaSpec Inc. (Fremont, CA). Bombesin (BBN, Aca-QWAVGHLM) [**7**], c(RGDyK)-bombesin heterodimer c(RGDyK)-E-BBN [**8**][10] and bombesin dimer E(BBN)$_2$ [**9**] were also purchased from Peptides International. E[c-(RGDfK)]$_2$ [**3**] was synthesized by CS Bio Inc. (Menlo Park, CA). The *S*-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was purchased from Macrocyclics (Dallas, TX), and NOTA-NHS ester was obtained from CheMatech (Dijon, France). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Mass spectra (MS) were acquired from a Waters Acquity UPLC system coupled with Waters Qtof Premier MS (LC-MS). For peptide purification, preparative reversed-phase HPLC was performed on Waters 600 gradient system with a Waters 996 photodiode array (PDA) detector using a Higgins PROTO 300 C-18 column (5 μm, 250 mm × 20 mm). Analytical reversed-phase HPLC was performed on a Perkin-Elmer series 200 LC gradient system with a Waters 2784 dual absorbance UV detector plus a Bioscan radioisotope detector using a Waters Symmetry C18 column (5 μm, 150 mm × 3.9 mm). The flow rate was 12 mL/min for preparative column and 1 mL/min for analytical column running the same linear gradient starting from 5% A (0.1% TFA in acetonitrile) and 95% B (0.1% TFA in water) for 5 min and increasing A to 65% at 2% per min. Varian BOND ELUT C 18 cartridge (100 mg) was used for solid phase extraction of labeled peptides. [^{18}F]Fluoride was obtained from the NIH cyclotron facility. The structures of starting material are shown in Figure 2.

Preparation of NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [4]. To a 4 mL glass vial containing 13.2 mg (10.0 μmol) of E[c-(RGDfK)]$_2$ [**3**] and 10 μL of diisopropylethylamine (DIPEA) in 0.4 mL of dimethyl sulfoxide (DMSO) was added 4.5 mg of p-SCN-Bn-NOTA (10.0 μmol). After standing at room temperature for 2 h, the reaction was quenched with 15 μL of acetic acid (HOAc) in 1 mL of water. The reaction mixture was purified with a preparative HPLC running a linear gradient as described earlier. The fraction containing the desired product (R_t = 21.5 min) was collected and lyophilized to give 8.4 mg of white powder (47% yield). The purity of the product was >97% by analytical HPLC (R_t = 16.7 min). LC-MS: [MH]$^+$ = 1769.1952 (*m/z*), calcd 1767.8151 ($C_{79}H_{113}N_{23}O_{22}S$).

Preparation of Ph-NCS-E[c(RGDfK)]$_2$ [10]. To a 4 mL glass vial containing 14.0 mg (10.6 μmol) of E[c(RGDfK)]$_2$ [**3**] and 1.7 mg (12.7 μmol) of phenyl isothiocyanate in 200 μL of DMSO was added 20 μL of DIPEA and stirred at room temperature for 2 h. The reaction was quenched with 25 μL of acetic acid and the reaction mixture injected onto HPLC for purification. Preparative RP HPLC purification gave 10.0 mg of desired product after lyophilization overnight (R_t = 22.5 min, yield 66%). LC-MS: [MH]$^+$ = 1437.8139 (*m/z*), calcd 1436.6949 ($C_{66}H_{92}N_{20}O_{17}$).

Preparation of NOTA-E[c(RGDyK)]$_2$ [12] and NOTA-PEG$_3$-E[c(RGDyK)]$_2$ [13]. PEG$_3$-E[c(RGDyK)]$_2$ ((PRGD2) [**7**], 10.4 mg), 20 μL of DIPEA, and 15.0 mg of NOTA-NHS ester (~5 equiv) were dissolved in 0.5 mL of DMF. The reaction mixture was stirred at room temperature and monitored with the analytical HPLC. After 30 min, another 20 μL of DIPEA and 15.0 mg of NOTA-NHS ester were added and repeated one more time until all the starting peptide was consumed. The reaction was then quenched with 70 μL of acetic acid in 1 mL of water and purified with the preparative HPLC. The fractions containing the desired product (R_t = 20.5 min) were collected and lyophilized to give 8.0 mg of NOTA-PEG$_3$-E[c(RGDyK)]$_2$ [**13**] as white powder in 65% yield. The purity of the product was >97% by analytical HPLC (R_t = 14.2 min). LC-MS: [MH]$^+$ = 1825.0124 (*m/z*), calc: 1823.8802 ($C_{79}H_{121}N_{23}O_{27}$). The couplings of NOTA-NHS ester to the amines of E[c(RGDyK)]$_2$ [**2**] was performed with similar procedure to give NOTA-E[c(RGDyK)]$_2$ [**12**] in 50% yield. LC-MS: [MH]$^+$ = 1635.9032 (*m/z*), calcd 1634.7801 ($C_{79}H_{121}N_{23}O_{27}$).

Preparation of Al-NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [14] and Ga-NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [15]. To a 1 mL V-vial containing 3.6 mg (2.0 μmol) of NOTA-Bn-NCS-E[c-(RGDfK)]$_2$ [**4**] in 0.3 mL of 0.5 M pH 4.0 sodium acetate buffer was added 0.27 mg (2.0 μmol) of aluminum chloride in 25 μL of 0.5 M pH 4.0 sodium acetate buffer. The vial was sealed, and the mixture was heated at 90 °C for 10 min. After cooling, the reaction mixture was analyzed with LC-MS and purified with preparative RP HPLC. The UV peaks were collected and analyzed by LC-MS again. The desired product [**14**] was lyophilized overnight to give 1.0 mg of while powder (27% yield). LC-MS: [MH]$^+$ = 1793.1354 (*m/z*), calcd 1791.7732 ($C_{79}H_{110}AlN_{23}O_{22}S$). Ga-NOTA-Bn-NCS-E[c-(RGDyK)]$_2$ [**15**] was prepared with the same procedure in 34% yield. LC-MS: [MH]$^+$ = 1835.0818 (*m/z*), calcd 1833.7172 ($C_{79}H_{110}GaN_{23}O_{22}S$).

Stability Analysis of RGD Peptides with HPLC and LC-MS. The stability of glutamic acid coupled dimeric peptides was tested under the conditions used for radiolabeling. In general, all peptides were dissolved in 0.5 M pH 4.0 sodium acetate buffer at the concentration of 1 mg/mL and about 0.1 mL of each sample was heated at 100 °C for 10 min in a sealed plastic tube. After cooling, 5 μL of each sample was analyzed with analytical HPLC. For those samples with decomposition, another 5 μL of sample was analyzed with a separate LC-MS. For those samples with no or little decomposition, additional 30 min heating was applied and the analyses were repeated.

Radiosyntheses and Stability Analyses of ^{18}F-Radiolabeled Products. The ^{18}F labeling of NOTA coupled RGD peptides followed a previously published procedure with some modifications.[1] Briefly, 3 μL of 2 mM aluminum chloride (6 nmol) in 0.5 M pH 4 sodium acetate buffer was added to a 1 mL polypropylene tube containing 6 μL of 2 mM NOTA coupled RGD peptide (12 nmol). Then, 0.15 mL of acetonitrile and 0.05 mL of aqueous [^{18}F]fluoride (0.37–0.74 GBq) were added to the vial. The vial was sealed and heated at 100 °C for 10 min to form the radioactive aluminum-fluoride NOTA complex. The vial was cooled and diluted with 10 mL of water and trapped on a Varian Bond Elut C_{18} cartridge (100 mg). The radioactivity trapped on the C_{18} cartridge was eluted with 0.3 mL of ethanol containing 1 mM HCl, the ethanol solution was evaporated with argon flow, and the final product was dissolved in PBS. The radioactive products were analyzed with radio HPLC before and after evaporating the ethanol.

Stability of [^{68}Ga]Ga-NOTA-Bn-NCS-E[c(RGDfK)]$_2$. The ^{68}Ga labeling was also performed according to the previously published procedure.[4] The ^{68}Ga activity was eluted from the ^{68}Ge/^{68}Ga generator with 0.6 M HCl at 0.5 mL per fraction into the 1.5 mL polypropylene tubes. The fraction containing

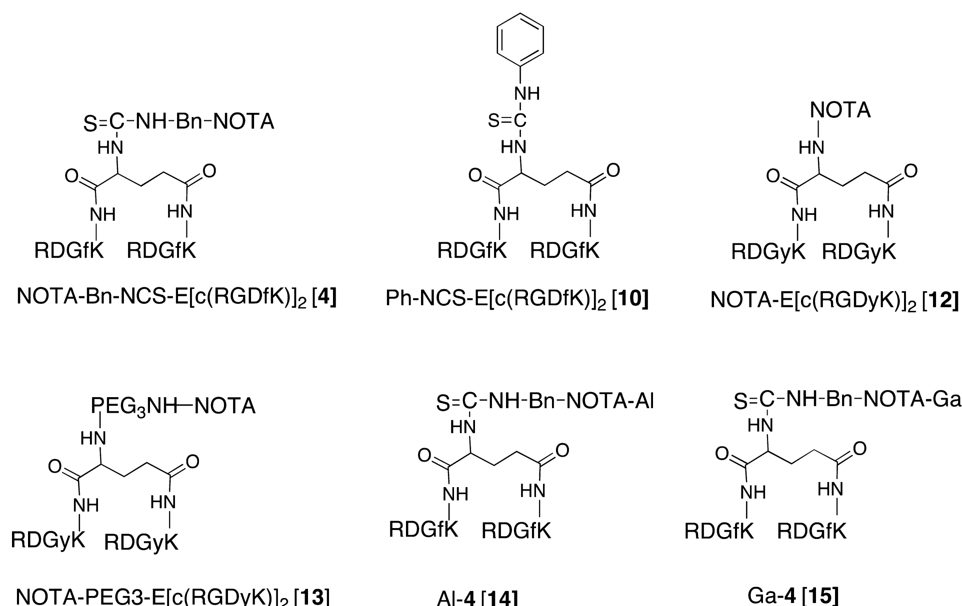

Figure 3. Structures of RGD peptides prepared for the study.

the most radioactivity (~0.15 GBq) was added to 0.45 mL of 1 M HEPES buffer and 14 μg (8 nmol) of NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [**4**] in 4 μL of 0.5 M pH 4 sodium acetate buffer. The mixture was heated at 80 °C for 10 min. At the end of the reaction, the reaction mixture was analyzed with radioHPLC.

In Vitro Serum Stability of [^{18}F] Radiotracers. About 37 kBq of [^{18}F]AlF-NOTA-E[c(RGDyK)]$_2$ (or [^{18}F]AlF-NOTA-PEG$_3$-E[c(RGDyK)]$_2$) in 50 μL of PBS was added to 450 μL of mouse serum and incubated at 37 °C. About 50 μL of this serum sample was taken out at 1, 30, 60, and 120 min and put into a plastic tube. An equal volume of acetonitrile was added to each tube and centrifuged at 6000 rpm for 10 min. The supernatant was separated from the pellet, and the supernatant was diluted with 300 μL of water and injected onto an analytical radio HPLC.

■ RESULTS AND DISCUSSION

Chemistry. We prepared a series of peptide derivatives to evaluate their relative stability (Figure 3) along with some other dimeric peptides obtained from commercial sources (Figure 2). All the peptides used in the study are listed in Table 1 along with possible side products formed from decomposition. The dimeric peptides analyzed in this study can be divided into four categories: (category I) peptides with the free α-amine of glutamic acid linker (**2**, **3**, **8**, and **9**), (category II) peptides with a thiourea linkage directly attached to the α-amine of glutamic acid (**4**, **10**, **14**, **15**), (category III) peptides with an amide linkage on the α-amine of glutamic acid (**11**, **12**, and **13**), and (category IV) peptide with a PEG$_3$ amine linker arm with NOTA at the N-terminus attached via a thiourea (**1**). The NOTA chelate was conjugated to the dimeric RGD peptides using a simple one-step coupling of the amine group of the peptides to the NOTA derivatives through a thiourea linkage or an amide linkage. While only 1 equiv of p-SCN-Bn-NOTA was required to react with the peptides to convert most of starting materials to final products based on the analytical HPLC analysis, the coupling of peptides to NOTA-NHS required 10−15 equiv of active ester to drive the reaction to completion. All

Table 1. Peptides Used in the Study with Their Corresponding Number, Name, and Category

compd no.	compd name	compd category	side products
1	NOTA-Bn-NCS-PEG$_3$-E[c(RGDyK)]$_2$	IV	stable[a]
2	E[c(RGDyK)]$_2$	I	**6**, **18**
3	E[c(RGDfK)]$_2$	I	**5**, **19**
4	NOTA-Bn-NCS-E[c(RGDfK)]$_2$	II	**5**, **17**
5	c(RGDfK)	n/a	n/a
6	c(RGDyK)	n/a	n/a
7	bombesin (BBN)	n/a	n/a
8	c(RGDyK)-E-BBN	I	**6**
9	E(BBN)$_2$	I	**7**
10	Ph-NCS-E[c(RGDfK)]$_2$	II	**5**, **20**, **21**
11	PEG$_3$-E[c(RGDyK)]$_2$	III	stable
12	NOTA-E[c(RGDyK)]$_2$	III	stable
13	NOTA-PEG$_3$-E[c(RGDyK)]$_2$	III	stable
14	Al-NOTA-Bn-NCS-E[c(RGDfK)]$_2$	II	**5**
15	Ga-NOTA-Bn-NCS-E[c(RGDfK)]$_2$	II	**5**, **16**

[a]This compound is stable, but multiple radioactive peaks were formed after removal of ethanol.

products were purified by preparative HPLC and structures confirmed by high resolution LC-MS; the purity was confirmed by analytical HPLC.

In preparing the nonradioactive NOTA complexes **14** and **15**, the yields were much lower than those of Al-NOTA-PRGD2 and Ga-NOTA-PRGD2.[4] The yield of Al-NOTA-PRGD2 and Ga-NOTA-PRGD2 was almost quantitative, while that for **14** and **15** was only around 30% based on the amount of peptide used.

Stability of Dimeric Peptides. The stability of the peptides was evaluated under the conditions used for radiolabeling (pH 4 at 100 °C), with analysis by HPLC. The peptides in categories I and II were labile under the testing conditions, with the category II peptides exhibiting more extensive decomposition. The representative analytical HPLC chromatograms are shown in Figure 4.

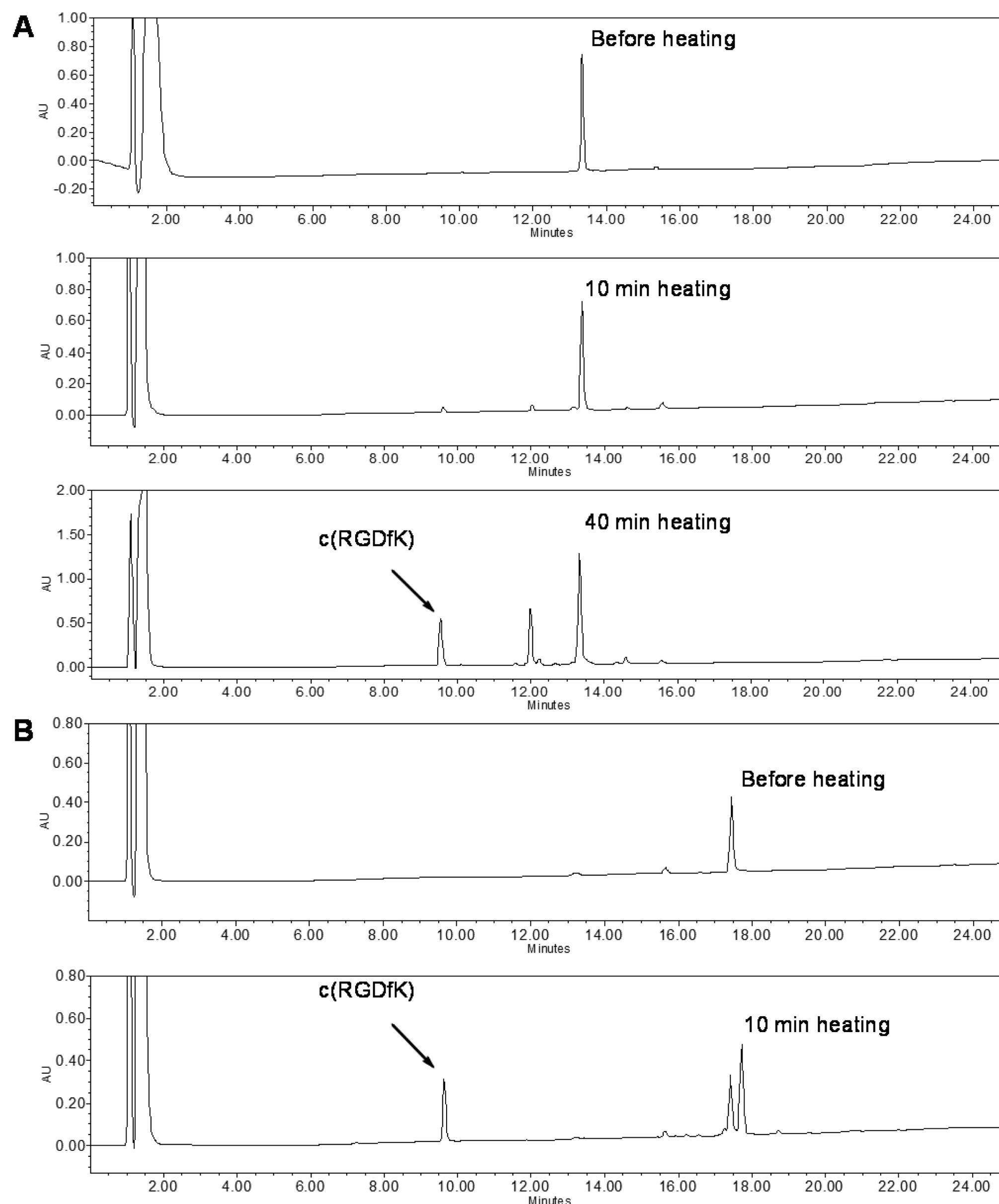

Figure 4. (A) Decomposition of dimeric peptide E[c(RGDfK)]$_2$ in category I. (B) Decomposition of dimeric peptide PhSCN-E[c(RGDfK)] in category II.

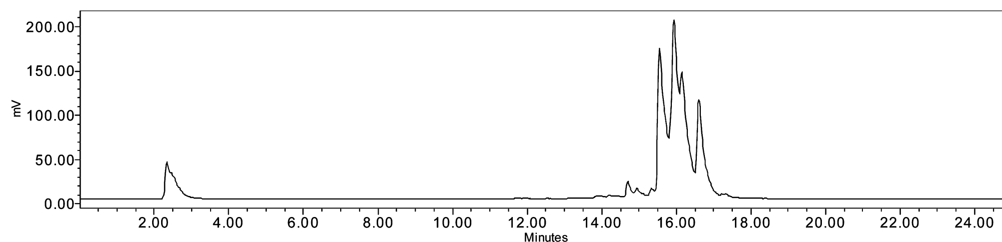

Figure 5. HPLC profile of the reaction mixture after ^{68}Ga labeling of NOTA-Bn-NCS-E[c(RGDfK)]$_2$.

The HPLC traces showed three major components. One was the parent compound and the other two were decomposition products. LC-MS analysis of the components suggested one decomposition product was a monomeric RGD and the other being RGD plus glutamic acid with the loss of water. The peptides in the category III and IV were most stable without any detectable formation of monomeric RGD peptide.

Radiochemistry and Stability of Radiolabeled Peptides. The NOTA conjugated peptides included in category II, III, and IV were used for ^{18}F radiolabeling. The radiolabeling yields of these compounds were 40–60% based on the radioactivity retained on C_{18} SPE column. The total synthesis time was about 20–25 min. Although there was little difference in the radiolabeling yields, the radiochemical stabilities were

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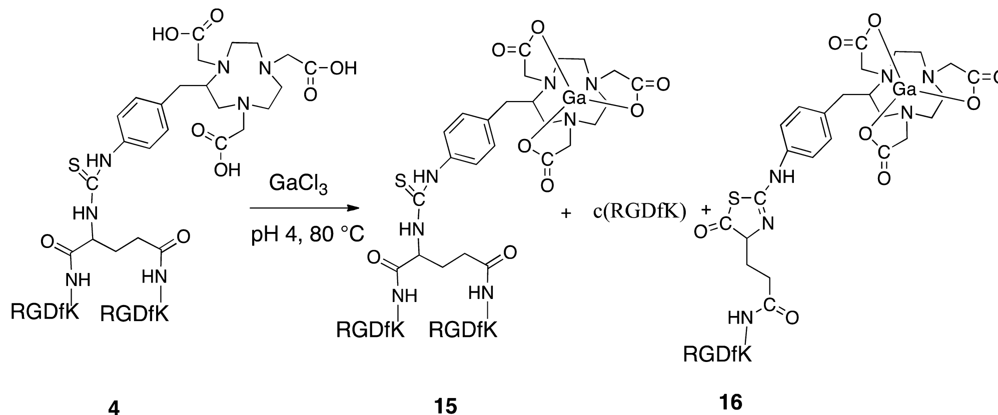

Figure 6. Possible side products of Ga-NOTA-Bn-NCS-E[c(RGDfK)]$_2$ hydrolysis based on LC-MS analysis (Figure S1 in Supporting Information).

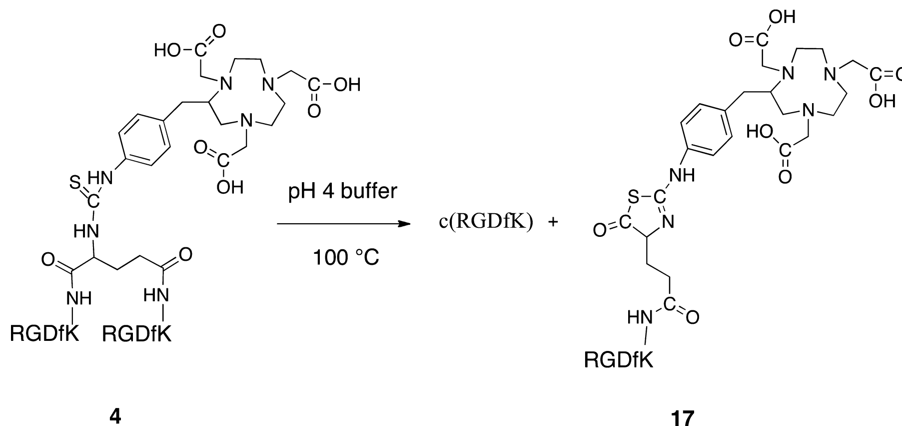

Figure 7. Hydrolysis of NOTA-Bn-NCS-E[c(RGDfK)]$_2$.

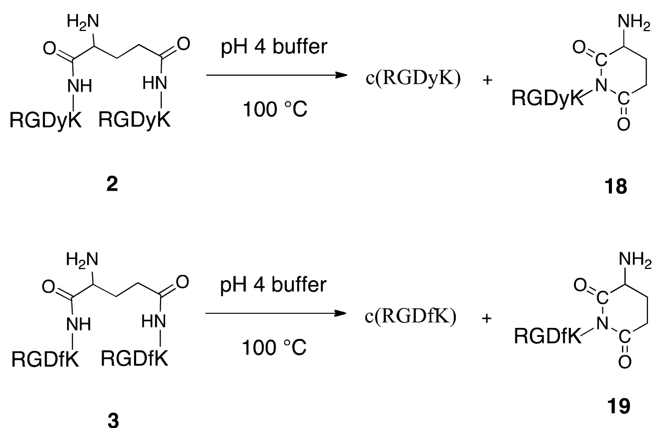

Figure 8. Hydrolysis of E[c(RGDfK)]$_2$ and E[c(RGDyK)]$_2$.

quite different. For the compounds in the category II, multiple radioactive peaks besides free [^{18}F]F$^-$ were detected in the reaction mixture before the C$_{18}$ trapping. The compounds in categories III and IV gave a single radioactive product after C$_{18}$ trapping. However, multiple radioactive peaks were formed after evaporating the solvent for compounds with a thiourea linkage. The compounds in category III with only amide linkage were much more stable with radiochemical purity unchanged after evaporating the solvent. The radiolabeled compounds from categories III and IV were also stable in mouse serum as demonstrated by observation of only parent peak following 2 h incubation with mouse serum at 37 °C.

To evaluate the effect of the metal ion on peptide stability, we also radiolabeled compound **4** with ^{68}Ga. The labeling was conducted at a slightly higher pH. The crude reaction mixture showed a small portion of free ^{68}Ga and a number of other radiolabeled components (Figure 5). Compound **4** belongs to category II and ^{68}Ga labeling led to multiple peaks even before ethanol evaporation, indicating decomposition of the radioactive compound. We thus sought to understand the factors determining the stability of this class of radiotracers.

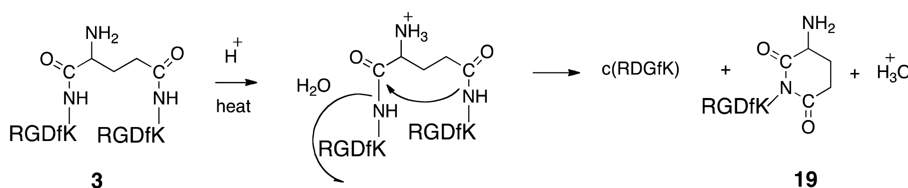

Figure 9. The mechanism of amide bond hydrolysis with the neighboring amine participation.

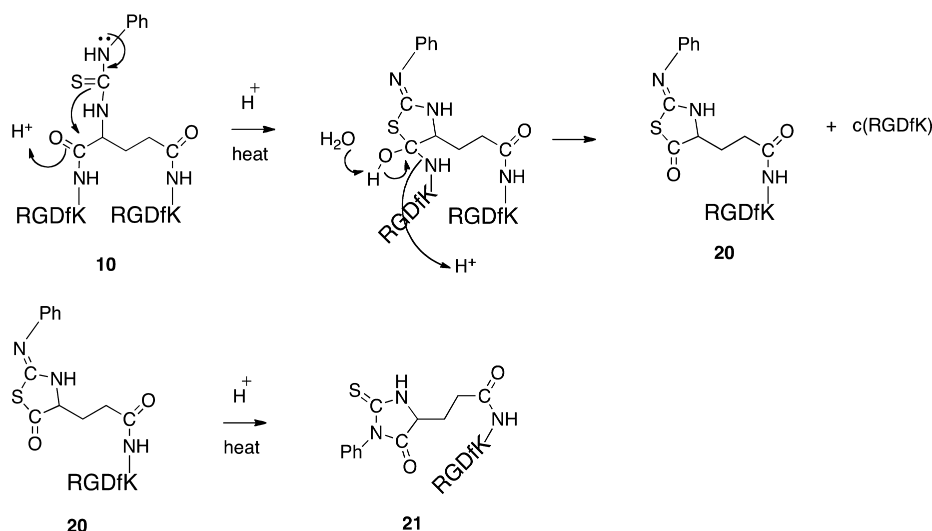

Figure 10. The mechanism of amide bond hydrolysis with the neighboring thiourea group participation.

Mechanism Studies. We previously reported the synthesis of [^{18}F]AlF-**1**, a dimeric RGD formed by linking the lysines together with glutamic acid.[4] The α-amine of the glutamic acid was functionalized with a mini-PEG containing a terminal amine to which the NOTA chelator was attached via a thiourea moiety. Our initial animal studies were conducted with a formulation that contained ethanol. Our attempts to remove the ethanol in order to produce an alcohol free formulation resulted in the decomposition of our desired product. In this study, the major effort was focused on the determination of the factors affecting the stability of this radiotracer.

Decomposition of radioactive compounds is often an oxidative process. Because [^{18}F]AlF-**1** has two tyrosine residues, we hypothesized that phenolic oxidation had occurred. However, substitution of the tyrosine of NOTA-Bn-NCS -PEG$_3$-E[c(RGDyK)]$_2$ [**1**] with phenylalanine and removal of PEG to provide NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [**4**] showed a more enhanced decomposition with multiple radioactive peaks detectable before ethanol evaporation.

To ascertain whether the $AlCl_3$ reagent was responsible for this decomposition, we tried ^{68}Ga labeling of the NOTA complex. ^{68}Ga labeling was conducted with much lower amount of Ga. However, the reaction also showed multiple radioactive components. We also attempted the synthesis of nonradioactive Ga and Al complexes of **4**. In preparing Ga-NOTA-Bn-NCS-E[c(RGDfK)]$_2$ [**15**] standard, we observed the formation of three major products. LC-MS provided m/z ions that were consistent with the desired Ga complex, c(RGDfK) monomer, and a new compound with the mass of desired product minus the mass of c(RGDfK) and a water molecule, indicating an internal cyclization and the possible structure [**16**] was given in Figure 6.

No stability issues had been observed for previously reported Ga-**1**, and the difference is that the current version has no mini-PEG between NOTA and glutamic acid. To explain the difference, we initially proposed intermolecular Lewis acid catalyzed hydrolysis as the reaction mechanism. Without the mini-PEG, we assumed that the Ga atom is closer to the amide bond and hence can participate in the hydrolysis. However, this hypothesis was soon proven false as monomer formation and a cyclized compound with possible structure of **17** was observed simply by heating the compound **4** in pH 4 buffer without the presence of Al or Ga ions (Figure 7). In addition, compounds E[c(RGDfK)]$_2$ and E[c(RGDyK)]$_2$ slowly released monomers and cyclized compounds **18** and **19** upon heating (Figure 8). Thus, the structural proximity between NOTA and NOTA-Ga is not required to initiate the decomposition of the dimeric peptide.

The observations above prompted us to further study the stability of these dimeric peptides and the use of LC-MS to elucidate the possible structures of hydrolysis products. To simplify structural data analysis, compound **10** was prepared which contained a thiourea linkage on the α-amine but without the NOTA functional group. Because the glutamic acid is asymmetrical, another unanswered question is from which side the monomer is released during the hydrolysis. This problem was solved with the analysis of c(RGDyK)-E-BBN [**8**] and E(BBN)$_2$ [**9**]. For E(BBN)$_2$, the release of BBN was observed. For E(RGD-BBN), only the release of RGD but not BBN was observed, indicating that the cleavage happened to the amide that is closer to the α-amine. The reaction mechanism using **3** as an example is proposed as the hydrolysis of amide bond by neighboring participation of amine group and internal cyclization to form a six-membered ring glutarimide derivative [**18**] (Figure 9). This internal cyclization mechanism is supported by literature reports that asparaginyl peptides and glutaminyl peptides can undergo deamidation to form cyclic imides such as five-membered ring succinimides and six-membered ring glutarimides.[11−13]

When thiourea linkage was attached to α-amine of glutamic acid such as in compound **10**, there was no free amine group to participate in the amide hydrolysis, however, the hydrolysis proceeded with a much faster rate due to thiourea group participation. LC-MS analysis showed three major components with the mass units corresponding to parent compound **10**, RGD monomer **5**, and a cyclized product **20**, respectively. The reaction mechanism is thus proposed in Figure 10, which is virtually the same as Edman degradation[14,15] except that the thiourea linkage is on the α-amine of glutamic acid instead of N-terminal amine group, and product **20** should rearrange to form the more stable compound **21** (Figure 10). The rearrangement reactions should also occur for compounds **16** and **17**.

At this point, it is clear that thiourea linkage on α-amines including those at the N-terminals are the least desirable under acidic labeling conditions at elevated temperature. When the thiourea linkage is not directly attached to the α-amines, such as compound **1** in category IV, the conjugates become much more stable under the similar conditions. However, they are still sensitive toward radiolysis especially at more concentrated conditions during the removal of ethanol. Because under acidic condition, thiourea can exist in both thione form and thiol form and the thiol form would be more easily oxidized and susceptible to radiolysis. Ethanol has some protective effect against radiolysis. When the ethanol was removed, the solution became more concentrated and radiolysis could be accelerated without protection.

On the basis of these findings, two NOTA conjugated RGD peptides, NOTA-E[c(RGDyK)]$_2$ **12** and NOTA-PEG3-E[c-(RGDyK)]$_2$ **13** in category III with only amide linkages were prepared and these compounds are much more stable with radiochemical purity unchanged after evaporating the solvent. These compounds from category III were also stable in mouse serum as demonstrated by observation of only parent peak following 2 h incubation with mouse serum at 37 °C.

■ CONCLUSIONS

In this study, we investigated the stability of a series of glutamic acid linked dimeric RGD peptides in order to produce more stable ligands that are suitable for labeling peptides with NOTA-[^{18}F]AlF complexes. The glutamic acid linked dimeric RGD peptides with a free α-amine are labile due to the neighboring amine participation of the hydrolysis and the stability of peptides can be increased by converting their free amines into the amides. The instability of thiourea formed from α-amine is caused by participation of thione group from thiourea and the formation of thiourea linkages should be avoided for labeling peptides with metal complexes at elevated temperature and acidic conditions.

■ ASSOCIATED CONTENT

Supporting Information

LC-MS analysis of possible side products of Ga-NOTA-Bn-NCSE[c(RGDfK)]$_2$ (**15**) hydrolysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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