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Preparation and preliminary biological evaluation of ^{177}Lu-labeled GluDTPA-cyclo (RGDfK) for integrin $\alpha_v\beta_3$ receptor-positive tumor targeting

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Integrin $\alpha_v\beta_3$ is a receptor and is highly expressed on activated and proliferating endothelial cells during the growth and metastasis of solid tumors but not on resting endothelial cells and normal organs. Because RGD peptide binds to integrin $\alpha_v\beta_3$ receptor, a variety of radiolabeled RGD peptides have been evaluated for non-invasive imaging of integrin $\alpha_v\beta_3$-positive tumors.

In an attempt to develop RGD-based radiopharmaceuticals, a novel GluDTPA-cyclo arginine-glycine-aspartic acid-D-phenylalanine-lysine (GluDTPA-cycloRGDfK) was simply synthesized and radiolabeled with ^{177}Lu. Also, tumor targeting and retention of the radiolabeled complex were evaluated in U87MG glioma-bearing mice.

The ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was formulated with a high radiolabeling yield (>98%) under mild condition, and the radiochemical purity was sustained in both saline and serum for over 4 days at 37°C. The radiolabeled compounds were rapidly cleared from the blood pool and non-target tissue. Tumor-to-blood ratio was 12.09 at 2 h post injection and increased to 134.67 at 24 h, while tumor to liver ratio was 2.01 at 24 h similar to that of 2 h.

Though it is inappropriate for targeted therapy due to its low uptake in tumor (~ 1 %ID/g), the acceptable results on radiochemistry and biodistribution propose to take a further assessment for non-invasive imaging and detection of integrin $\alpha_v\beta_3$-positive tumors by applying diagnostic radionuclides.

Keywords: Lutetium-177 (^{177}Lu); DTPA; RGD; integrin; tumor targeting; angiogenesis

Introduction

Integrins that are composed of non-covalently associated α and β subunits are involved in a wide range of interaction between cell–cell as well as cell–extracellular matrixes.[1,2] Among the integrin family members, it is well-known that the integrin $\alpha_v\beta_3$ is highly expressed on activated endothelial cells but not on resting vessels during angiogenesis. Additionally, it plays an important role in the regulation for tumor growth, local invasiveness, and metastatic potential.[2] Therefore, intensive investigation of integrin $\alpha_v\beta_3$ receptor targeted anti-angiogenic strategies for cancer treatment, and the prevention of cancer recurrence or metastasis has been underway. Also, non-invasive imaging of integrin $\alpha_v\beta_3$ expression by molecular imaging techniques would be of great help for early detection of malignancy, evaluation of tumor progress, and monitoring treatment efficacy of anti-angiogenic drugs.[3] Actually, in recent years, RGD (Arg-Gly-Asp) peptide-based multimodality probes were developed for non-invasive imaging of integrin $\alpha_v\beta_3$ expression.[4–10] Several positron emission tomography probes using ^{18}F have been used in clinical investigations.[11–16] In particular, ^{18}F-labeled galacto-RGD derivatives showed rapid and predominant excretion, resulting in a low concentration in most of the non-target organs and visualization of integrin $\alpha_v\beta_3$ expressing tumor.[5,11]

In designing radiolabeled peptides for labeling with diagnostic radioisotopes such as ^{99m}Tc, ^{111}In, ^{68}Ga, and ^{64}Cu for imaging of tumor sites and with particle emitters such as ^{177}Lu, ^{90}Y, ^{188}Re, ^{153}Sm, and ^{213}Bi for targeted radionuclide therapy, bifunctional chelating agents (BFCAs) have received increasing interest because of their important role in developing radiolabeled bioactive molecules.[17,18] Among many BFCA series such as ethylenediamine tetraacetic acid (EDTA); 1,4,7,10-tetraazacyclododecome-1,4,7,10-tetraacetic acid (DOTA); and diethylenetriamine pentaacetic acid (DTPA), DTPA is good for its higher affinity and reasonable stability to almost all kinds of radionuclides used in clinical applications.[19] However, when DTPA is directly used for the preparation of a DTPA conjugate,

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the compound possesses only seven coordination sites that results in reduced kinetic stability in vivo. The typical example is the ^{111}In-DTPA-*D*Phe-octreotide for detecting of tumors expressing somatostatin receptors. When the DTPA-*D*Phe-octreotide was labeled with ^{90}Y, yttrium was detached from its DTPA complex and presented in serum because of its deficiency of the coordination demand.[20]

In particular, the DTPA-conjugated biomolecule should have eight coordination sites to form a stable complex with therapeutic radionuclide, such as ^{90}Y, ^{177}Lu, ^{153}Sm, and ^{188}Re. To synthesize DTPA-conjugated peptide for using radiotracer and radionuclide therapy, a DTPA chelator containing six carboxylates is needed. Because peptides can be easily produced by using solid phase peptide synthesis method, the DTPA chelator system should possess monocarboxylic acid and penta-protected carboxylate in the structure directly applicable for the peptide synthesis method, which forms up to eight bonds with radionuclides even though it was conjugated to peptides.

Because ^{177}Lu decays by a half-life of 6.71 days with emission of beta particles and lower gamma photons, it came into spotlight for imaging and therapy of tumor among many radionuclides. So, ^{177}Lu can monitor *in vivo* localization of the injected therapeutic radiopharmaceutical during treatment performing dosimetric evaluation.[21]

In this study, a glutamic acid-based DTPA was prepared by applying the synthetic method for lysine-DTPA previously reported.[22] The synthesized GluDTPA was easily incorporated into cyclo arginine-glycine-aspartic acid-D-phenylalanine-lysine (cycloRGDfK) for targeting the $\alpha_v\beta_3$ integrin receptor using a conventional peptide synthesis strategy. The prepared GluDTPA-cyclo(RGDfK) has eight coordination sites to form a stable complex with a variety of radionuclides. Sequentially, GluDTPA-cyclo(RGDfK) was labeled with ^{177}Lu and was evaluated for its radiochemical stability. The biological behavior of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) in $\alpha_v\beta_3$ integrin receptor-positive U87MG xenografted mice was performed not only to determine the aspect of tumor uptake, retention, and excretion but also to investigate its correlation between tumor uptake and tumor size.

Experimental

All chemicals were analytical grade purchased from chemical company and used without further purification. The preparation of peptide was accomplished by the use of an automated Multiple Biomolecular Synthesizer (Peptron, Daejeon, Korea). Analytical and preparative reverse-phase high performance liquid chromatography (RP-HPLC) for the prepared peptide was performed on a Shimadzu prominence HPLC (Kyoto, Japan) by using Shiseido capcell pak C-18 column. The wavelength used for UV detection was 220 nm for analytical RP-HPLC. The liquid chromatography-mass spectrometer was performed by using HP 1100 series. ^{177}Lu was produced at the Hanaro research reactor (30 MW) installed in Korea Atomic Energy Research Institute (KAERI). The radioactivity was measured by using an ionizing chamber (Atomlab 200, Biodex, Shirley, NY, USA). The radiolabeling yield and the radiochemical purity were determined by using Cyclone Storage Phosphor System (Perkin Elmer, Wellesley, MA, USA). Radioactivity for biodistribution experiments was determined with a Wallac 1470 automated gamma counter (Perkin Elmer).

Preparation of chelator conjugated peptide; GluDTPA-cyclo (RGDfK)

Synthesis of glutamic acid DTPA (tBu)$_5$

Synthetic glutamic acid DTPA penta-*tert*-butyl-ester was prepared as described in Figure 1 according to the previous reported procedure.[23]

Step (1) *5-benzyl 1-tert-butyl glutamic acid*

γ-benzyl glutamate-*tert*-butyl-ester (14.82 g, 62.4 mmol) was dissolved at *tert*-butylacetate (219 ml, 26 eq, 1.624 mol) in a 500-ml round-bottom flask with a magnetic bar. 70% $HClO_4$ (6.13 ml, 1.62 eq, 101 mmol) was added slowly and reacted for 24 h at room temperature. 10% Na_2CO_3 (188 ml, 2.83 eq) was poured to neutralize the solution and filtrated. The pH of filtered solution was adjusted to 10 by adding 10 M NaOH and dried with $MgSO_4$. The solvent was concentrated to afford a residue as yellowish oil. Purification was performed with flash column chromatography to yield an oily compound (**1**) (ethylacetate : hexane = 0:10–3:7).

Infrared (neat) 3371 (w), 2977 (w), 1728 (s), 1455 (w), 1367 (w), 1152 (m), 1024 (w); 1H NMR ($CDCl_3$, 400 MHz) δ 1.45 (s, 9H), 1.79–1.86 (q, 1H), 2.01–2.10 (q, 1H), 2.51 (t, 2H), 3.33 (t, 1H), 5.10 (s, 2H), 7.28–7.38 (m, 5H).

Step (2) *5-benzyl-1-tert-2-(bis(2-(bis(2-tert-butoxy-2-oxoethyl) amino)ethyl)amino)pentanedioate*

To solution of γ-benzyl-glutamate-*tert* butyl-ester (2.36 g, 8.03 mmol) in 100 ml of acetonitrile, *tert*-butyl 2,2′-(2-bromoethylazanediyl) diacetate (6.50 g, 2.3 eq, 18.46 mmol) and 100 ml of 2 M phosphate buffer (pH = 8) were added, and the mixture was reacted for 48 h at room temperature. Then, acetonitrile layer was concentrated and extracted with diethylether–distilled water. The organic layer was separated, dried with $MgSO_4$, and evaporated *in vacuo*. An oily crude was purified using flash column chromatography (ethylacetate : hexane = 1:9 ~ 1:4) to give a clear oily compound (**2**).

Infrared (neat) 2978 (w), 1731 (s), 1367 (m), 1218 (m), 1144 (s), 846 (w); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.44 (s, 45H), 1.80–1.89 (m, 1H), 1.95–2.01 (m, 1H), 2.44 (m, 2H), 2.67–2.78 (m, 8H), 3.33 (m, 1H), 3.40 (s, 8H), 5.10 (s, 2H), 7.30–7.36 (m, 5H).

Step (3) *Glutamic acid-DTPA penta-tert-butyl-ester; [GluDTPA-(tBu)$_5$]*

To a solution of compound **2** (4.18 g, 5.00 mmol) in methanol (100 ml), 10% Pd/C powder (0.42 g, 10 wt%) was added portionwise followed by the addition of 3–4 drops of distilled water. After filling with H_2 gas, it was reacted for 3 h at room temperature. After the solid residue was filtered off, the filtrated solution was concentrated and purified with flash column chromatography (ethylacetate : hexane = 1:4–1:2) to give a colorless oily compound (**3**).

Infrared (neat) 2976 (w), 1725 (s), 1367 (m), 1219 (m), 1147 (s), 848 (w); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.45 (s, 45H), 1.88–2.02 (m, 2H), 2.65 (m, 2H), 2.80–2.94 (m, 8H), 3.42 (s, 8H), 3.88 (dd, 1H), LC/MS: calculated 745.7; found 746.4 (M + 1).

Conjugation of glutamic acid DTPA with cyclo(RGDfK): GluDTPA-cyclo(RGDfK)

GluDTPA-cyclo(RGDfK) was synthesized by applying standard Fmoc strategy as detailed in Figure 2. Briefly, Wang resin

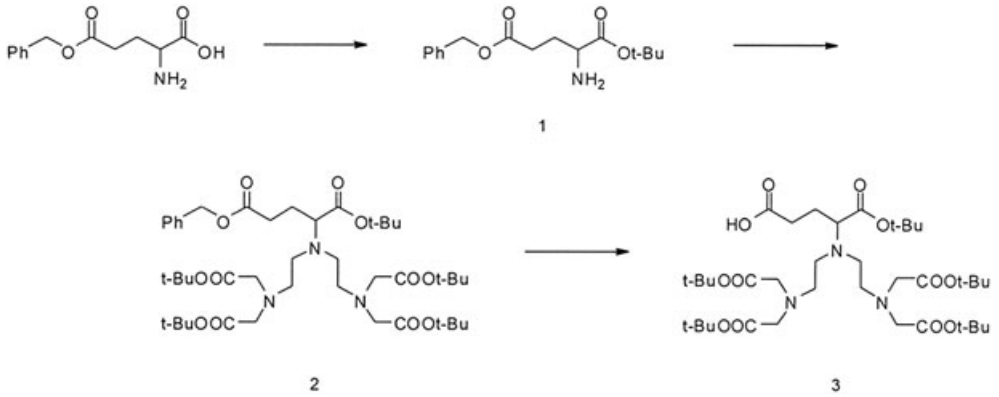

Figure 1. Reaction route for glutamic acid-based DTPA-penta-*tert*-butyl ester, GluDTPA-$(tBu)_5$.

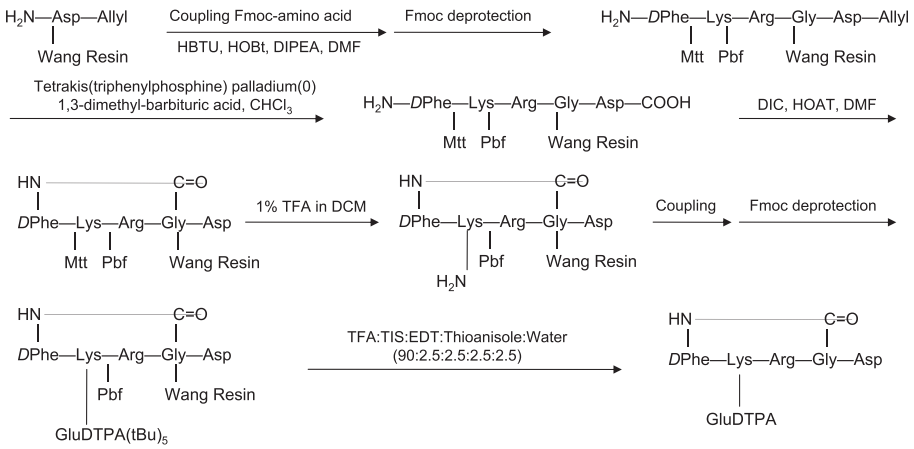

Figure 2. Solid phase synthesis of GluDTPA-cyclo(RGDfK).

conjugated Fmoc–Aspartic acid allylester was used as a solid support the reaction. After the Fmoc protecting group was removed from resin bounded Fmoc–Aspartic acid allylester under standard condition (20% piperidine in N,N-dimethylformamide), the linear sequence peptide was prepared by the sequential coupling of Fmoc-Gly-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Lys (Mtt)-OH, Fmoc-D-Phe-OH in which Fmoc group was deprotected after every coupling step. *O*-benzotriazole-*N*,*N*,*N*′*N*′-tetramethyl-uronium-hexafluoro-phosphate and *N*-hydroxybenzotriazole were applied as an activating reagent to ensure efficient coupling. Cyclization of RGDfK was accomplished by the reaction between an amine of phenylalanine and a carboxylic acid of aspartic acid under a conventional amide coupling procedure after deprotection of an allyl group of aspartic acid using tetrakis (triphenylphosphine) (0) palladium and 1,3-dimethyl-barbituric acid in dichloromethane. To introduce the DTPA chelators, resin bounded cyclo(RGDfK) compounds were elongated by the coupling of GluDTPA-$(tBu)_5$. After the last assembly step, the resulting peptide of GluDTPA-cyclo(RGDfK) was cleaved from the polymeric support by treatment with the mixture solvent of 90% trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane (TIS), 2.5% ethanedithiol (EDT), 2.5% thioanisole, and 2.5% deionized water. (TFA : TIS : EDT : thioanisole : H_2O = 90:2.5:2.5:2.5:2.5). The crude GluDTPA-cyclo (RGDfK) product was purified by Shimadzu HPLC equipped with capcell pak C18 column, using mobile phase with 0.1% TFA/water (A) and 0.1% TFA/acetonitrile (B) and under gradient conditions of 0–10% B in 2 min, 10–40% B in 10 min, 40–70% B in 2 min at a flow rate of 1 ml/min. The purified GluDTPA-cyclo(RGDfK) was analyzed by LC/MS (HP1100).

Radiochemistry of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK)

Preparation of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK)

Radiolabeling of the GluDTPA-cyclo(RGDfK) was accomplished by simply mixing with ^{177}Lu solution at room temperature as previously described.[21]

GluDTPA-cyclo(RGDfK) was dissolved in distilled water to give a concentration of 10^{-6} mole/ml and ^{177}Lu was diluted in 50 mM sodium acetate buffer (pH = 5.5) to give a concentration of 37 MBq/ml. ^{177}Lu solution was injected to GluDTPA-cyclo (RGDfK) solution vial and stood for 15 min at room temperature. Saline was added to the solution to give a radiotracer concentration of 370 KBq/10^{-9} mol/200 μl (^{177}Lu/ligand/volume). The radiolabeling yield and radiochemical purity of radiolabeled compound was analyzed using silica TLC plate with methanol/water (3:1, v/v).

Serum stability assay

^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was added to 200 μl of 25% human serum in phosphate buffered saline (PBS) and incubated at 37°C for 4 days. After incubation, serum samples were analyzed by using silica TLC at given time intervals. The stability of radiolabeled compound was determined by Cyclone Storage Phosphor System.

Determination of log p value

37 KBq of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was dissolved in an equal volume mixture of 1-octanol and PBS buffer (1 ml : 1 ml). After stirring vigorously for 20 min, the mixture was centrifuged at a speed of 8000 rpm for 5 min. One hundred microliters of samples from both 1-octanol and PBS layers were transferred and measured the radioactivity using a Wallac 1470 Wizard automated gamma counter. Partition coefficients were measured three different times. The log *p* values were reported as the average of three independent measurements.

Biological evaluation of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK)

Cell culture

U87MG cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in 100-mm culture dishes (Corning, Lowell, MA, USA). The cells were cultured in Eagle's minimum essential medium (Lonza, Walkersville, MD, USA), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin in an atmosphere of 5% CO_2 in air at 37°C up to approximately 90% confluence.

Animal models

Female BALB/c nu/nu mice were purchased at 6 weeks of age from Oriental, Inc. (Seoul, Korea) and were allowed 1 week for quarantine and acclimatization. For the induction of tumor xenografts, U87MG cells were subcutaneously injected in the right upper flank at a concentration of 1×10^6 cells/mouse with 200 μl of 1:1 mixture of RPMI1640 culture medium and Matrigel. The animals were housed in a room that was maintained at 23 ± 2°C and 50 ± 5% relative humidity, with artificial lighting from 08:00 h to 20:00 h and 13–18 air changes per hour. They housed four animals per cage and given tap water and commercial rodent chow (Samyang Feed, Seoul, Korea) *ad libitum*. The Institutional Animal Care and Use Committee at KAERI approved the protocols used in this study, and the animals were cared for in accordance with the Guidelines for Animal Experiments.

Biodistribution of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) in U87MG xenograft-bearing mice

Biodistribution studies were carried out using the athymic nude mice bearing U87MG human glioma xenograft. U87MG tumor-bearing mice with 200 ~ 400 mg tumors were randomly divided into three groups ($n = 4$). The ^{177}Lu-labeled GluDTPA-cyclo (RGDfK) (370 KBq) in 0.2 ml of saline was administered via tail vein. After 2 and 24 h post injection (p.i.), all animals were sacrificed by using 60 ~ 70% CO_2 chamber. Blood samples were withdrawn from the heart. The tumor and normal organs (liver, kidney, spleen, heart, intestine, lung, stomach) were excised, washed with saline, dried with absorbent tissue, weighed, and counted on a Wallac 1470 Wizard automated gamma counter. The organ uptake was calculated as a percentage of the injected dose per gram of tissue (%ID/g). In parallel, for the blocking experiment, four animals were pre-administered 2×10^{-7} mole of cyclo(RGDyK) in 0.2 ml of saline via tail vein 30 min before the injection of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK), and after 2 h p.i., biodistribution experiment was performed.

Statistical analysis

The results are expressed as the mean ± standard deviation. All the experiments were tested with an analysis of variance. In some experiments, means were compared with the control using the Bonferroni–Dunn test. A p value < 0.05 was considered significant.

Results and discussion

The glutamic acid DTPA penta-*tert*-butyl-ester and the novel glutamic acid DTPA-conjugated RGD peptide via the solid phase synthetic method were easily prepared as described in Figures **1** and **2**, and their final structures were shown in Figure **3**. The major peak counterpart for GluDTPA-cyclo(RGDfK) was purified using HPLC. As shown in Figure **4**, the retention time on analytical HPLC for GluDTPA-cyclo(RGDfK) was found at 7.35 min, and the MS data were consistent with their proposed formula. The measured molecular weight was 1051.5 for GluDTPA-cyclo (RGDfK) (calculated molecular weight = 1051.06).

The ^{177}Lu radiolabeling was accomplished by simple mixing of DTPA-conjugated RGD peptide with ^{177}Lu solution. Because the separation of radiolabeled peptide using HPLC with various solvent systems was not achieved successfully, the instant TLC impregnated with silica gel using a solvent mixture of methanol/water (3:1, v/v) was applied to discriminate the radiolabeled peptide from un-bounded ^{177}Lu and ^{177}Lu-colloids. ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was migrated to the solvent front, whereas unbounded ^{177}Lu solution and colloids were retained at the starting point (Figure 5). A high labeling yield (>98%) was achieved and used directly without any further purification.

^{177}Lu-labeled GluDTPA-cyclo(RGDfK) showed excellent *in vitro* stability in saline and serum at 37°C for 4 days (>98%) (Figure **6**). Decomposition was not observed more than 4 days of incubation, and only about 10% of the radiopeptide was decomposed after 7 days. The log *p* value of ^{177}Lu-labeled GluDTPA-cyclo (RGDfK) was −4.8, which indicates a relative high hydrophilicity of the radiolabeled compound.

In an attempt to evaluate the correlation between tumor mass and ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) uptake, the results of two different U87MG biodistribution experiments were analyzed. The %ID of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) within each tumor was plotted against the respective tumor mass. As shown in Figure **7**, the %ID of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) in tumors was correlated ($R^2 = 0.909$) with tumor weight. Although further study is needed, it was assumed from this analysis that as the tumor size increases, tumor-associated ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) uptake remains readily accessible to blood-borne RGD conjugates.

The results of biodistribution studies were summarized in Figure 6, when U87MG xenografted mice were injected ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) with or without RGD blocking. By blocking studies, it was confirmed that ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) uptake in U87MG tumors was specifically mediated by integrin $\alpha_v\beta_3$ receptor. As shown in Figure 8, the uptake of the radiolabeled compounds was the highest in tumor and kidney, in turn. In other organs, such as blood, heart, lung, and stomach, the accumulation kept in

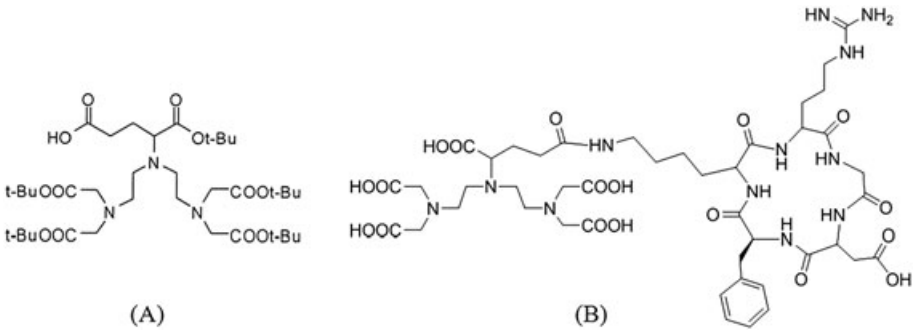

Figure 3. Structure of glutamic acid-based DTPA derivative, GluDTPA-$(tBu)_5$ (A) and GluDTPA-Cyclo(RGDfK) (B).

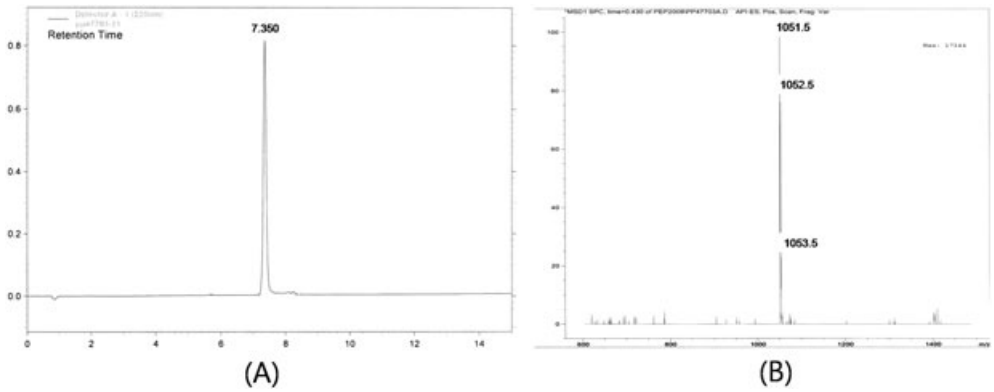

Figure 4. HPLC analysis (A) and LC/MS profile (B) of GluDTPA-cyclo(RGDfK).

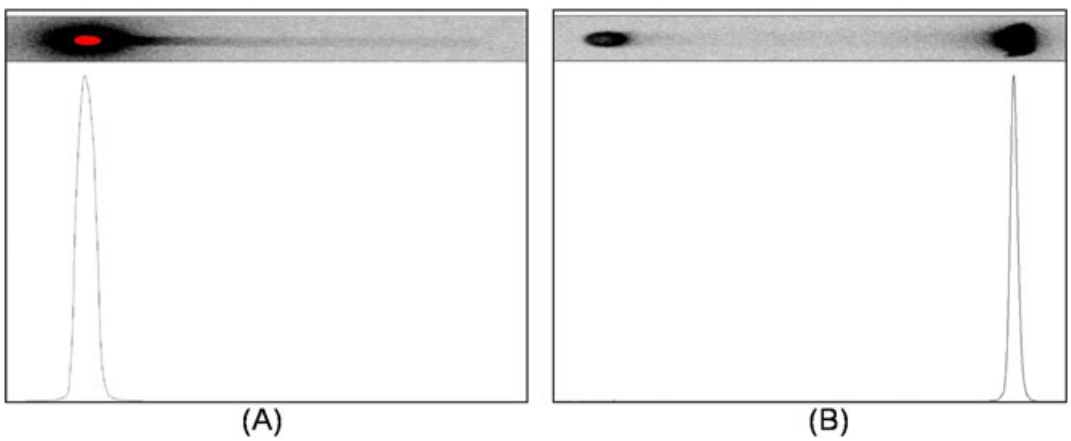

Figure 5. Typical instant thin layer chromatography profiles of ^{177}Lu solution (A) and ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) (B) determined by Cyclone storage phosphor system using instant thin layer chromatography impregnated with silica gel with methanol/water (3:1, v/v) as a mobile phase.

a low level. ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) had the tumor uptake of 1.05 ± 0.03 %ID/g at 2 h and 0.70 ± 0.13 %ID/g at 24 h p.i. with very fast blood clearance (0.088 ± 0.017 %ID/g at 2 h and 0.009 ± 0.007 %ID/g at 24 h). A relatively high radioactivity in the kidney was found (0.95 ± 0.04 %ID/g at 2 h and 0.69 ± 0.03 %ID/g at 24 h). The tumor-to-blood ratios increased from 12.09 at 2 h to 134.67 at 24 h, whereas the tumor-to-liver (T/L) and the tumor-to-kidney (T/K) ratios were kept at certain proportion. The T/L ratios were 2.39 at 2 h and 2.01 at 24 h, and the T/K ratios were 1.11 at 2 h and 1.08 at 24 h. Those data revealed that the tumor uptake of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was the highest compared with the other tissues.

Radiolabeled cyclic RGD (Arg-Gly-Asp) peptides represent a new class of radiotracers that target the integrin $\alpha_v\beta_3$ receptor overexpressed on both tumor cells and activated endothelial cells of the neovasculature during tumor growth, invasion, and metastasis.[24] In addition to specific expression on the activated tumor vasculature, integrin $\alpha_v\beta_3$ receptor also overexpressed on some tumor cells, including late-stage glioblastoma, breast

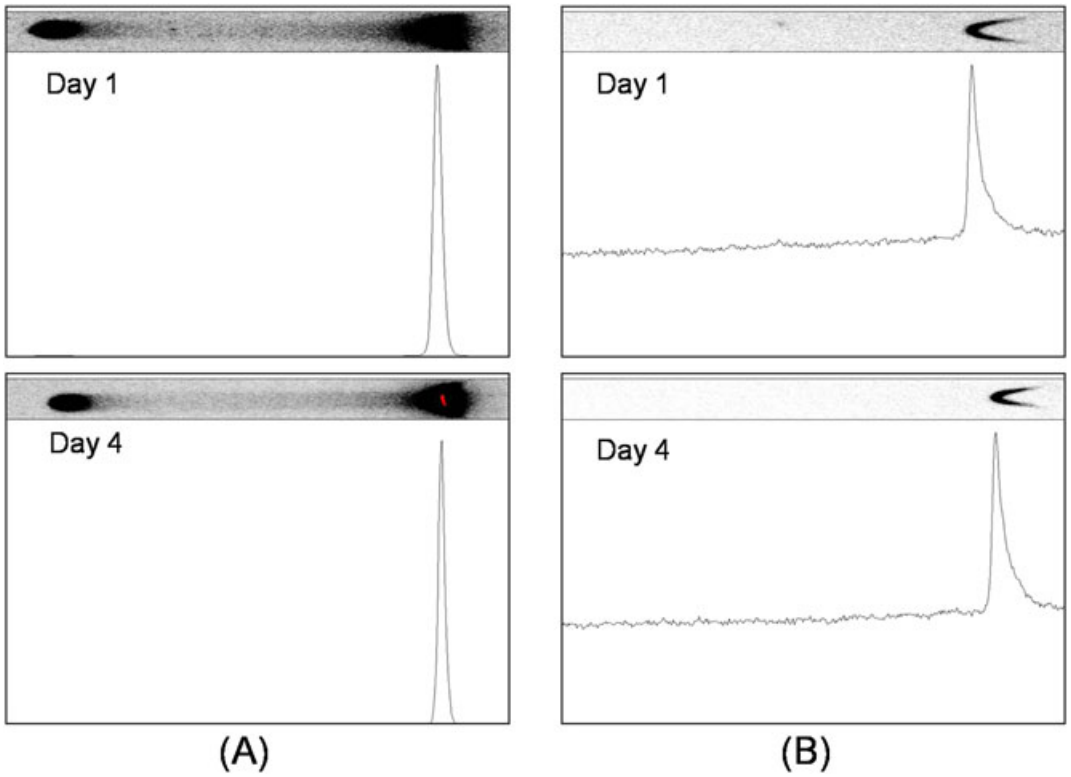

Figure 6. Stability of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) for 4 days in saline (A) and human serum (B).

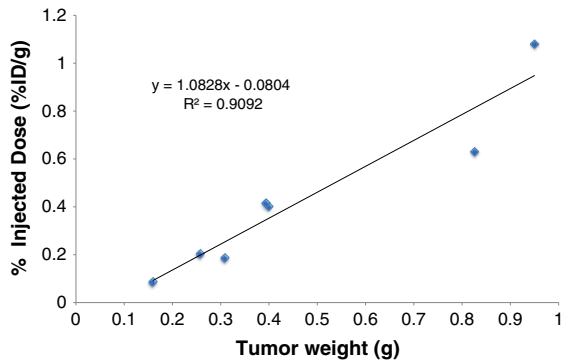

Figure 7. ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) uptake in U87MG tumors varying on tumor weight.

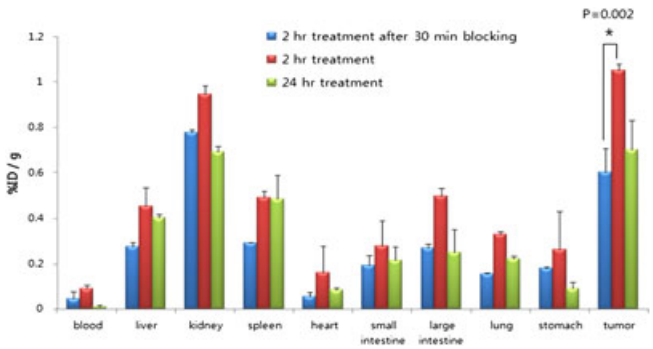

Figure 8. Biodistribution of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) in BALB/c nude mice bearing U87MG tumor.

and prostate cancers, malignant melanoma, and ovarian carcinoma.[25,26] It was previously reported that U87MG cells are one of the tumor cells that express a very high level of human integrin $\alpha_v\beta_3$ receptor.[3] In this study, we synthesized a GluDTPA-conjugated RGD peptide, labeled with ^{177}Lu and examined its affinity for U87MG xenografted mice.

A variety of BFCAs have been prepared and conjugated to tumor-specific biomolecules such as peptides and antibodies for the development of radiotracers and magnetic resonance imaging contrast-enhancing agents. Among many BFCAs, DTPA is reasonable for their high complexity with metal cation in which DTPA forms potentially an octadentate ligand. So, the formation constant of metal-DTPA complex is about 100 times greater than that of EDTA.[27] Also, contrary to DOTA, which demands on incubation an elevated temperature to form a metal complex, DTPA can complex with cationic metal ions at room temperature.[21,27–38]

Accordingly, a glutamic acid-based DTPA derivative can bind with various radionuclides, such as ^{213}Bi, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{64}Gd, ^{166}Ho, ^{111}In, ^{177}Lu, ^{188}Re, ^{153}Sm, and ^{99m}Tc. In particular, the prepared GluDTPA-penta-*tert*-butyl-ester can be applicable to various fields including targeting diagnosis and therapy using metal ions for computed tomography/magnetic resonance imaging contrast-enhanced imaging and radionuclide imaging/therapy because of its easiness for the conjugation with biomolecules.

To prepare the RGD derivative containing DTPA chelator, an cyclo(RGDfK) peptide was conjugated with GluDTPA using a solid phase synthesis where the DTPA served as a metal chelator

to be radiolabeled with ^{177}Lu that forms an eight-coordinate complex. Because the DTPA was attached apart from the targeting site of the peptide, the binding affinity may not be affected by the radiolabeling process. After the preparation of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) at ambient temperature by simple mixing of reactants, HPLC analysis was performed but failed its discrimination from unbounded ^{177}Lu. This phenomenon has shown most of the radiolabeled DTPA-containing compounds. The aspect that radiolabeled DTPA-conjugated compounds were not separated from impurities using HPLC might be caused by its anionic charge and its higher hydrophilicity. When the reaction solution was differentiated with TLC, the labeling yield of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was greater than 98% and its radiochemical stability was maintained over 4 days even in human serum at 37°C without any degradation, which is sufficient for further investigation.

The therapeutic efficacy of drugs can be decreased by a large tumor volume via various mechanisms. Actually, a solid tumor is likely to form vasculatures poorly with intermittent blood flow and large distances between functional blood vessels. Additionally, increased interstitial pressure in the center of large tumors causes radial fluid flow from the center to the periphery. The drugs must penetrate through multiple layers of solid tissue to reach all of the tumor cells, and this requirement is a formidable barrier to many therapeutic drugs. For ^{177}Lu-labeled GluDTPA-cyclo(RGDfK), it was found that its tumor uptake was not significantly decreased by an increase of tumor volume. There was a consistent increase in the relative amount of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) in the tumor as a function of tumor weight over the range of 100–1000 mg in U87MG xenografted mice. These results correspond with the report by Chakraborty *et al.* that there are linear relationships between the tumor volume and %ID of ^{111}In-labeled DOTA-3 G-RGD$_2$ uptake.[24] Taken together, we suggest that the RGD conjugate can readily penetrate deeply into solid RGD-positive tumors, and the net uptake is apparently proportional to the tumor volume. Therefore, radiolabeled GluDTPA-cyclo(RGDfK) might have the potential not only for detecting tumors and monitoring tumor growth or shrinkage after treatment but also for treating the primary and metastatic tumor.

Several studies have reported the uptake using cyclo(RGDfK) as a tumor targeting moiety. By injection of ^{111}In-labeled DOTA-E-c(RGDfK) to SK-RC-52 tumor-bearing mice, the tumor-to-blood (T/B) ratio was 67.5 and T/K ratio was 1.45; however, intestine uptake was higher (tumor-to-intestine ratio = 0.85) at 2 h p.i.[9,10] Also, by injection of ^{99m}Tc-HYNIC-c(RGDfK) to OVCAR-3 tumor-bearing mice, T/B ratio was 15 and T/K ratio was 1.0 at 2 h p.i.[39] In addition, multimeric cyclic RGD peptides have been used to maximize the integrin $\alpha_v\beta_3$ binding affinity and improve the radiotracer tumor uptake and retention time.[24,39,40] In this study, T/B ratio was 12.1, T/K ratio was 1.11, and other tissue uptake was much lower than that of tumor. Most important, in spite of the result that the tumor uptake of ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was lower than those of previous studies using RGD peptide as targeting moiety, its tumor-to-tissue ratios were predominant.

In addition, ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) cleared significantly through the renal pathway as evidenced by relatively high kidney uptake (0.95 ± 0.04 %ID/g at 2 h and 0.69 ± 0.03 % ID/g at 24 h p.i., respectively). These results might be because of its relative low log *p* value (−4.8). Blood clearance rate was excellent in that T/B ratios were increased with the course of time (T/B ratios = 12.09 at 2 h, 134.67 at 24 h p.i., respectively). Moreover, T/L ratios (2.39 at 2 h, 2.01 at 24 h p.i., respectively) and T/K ratios (1.11 at 2 h, 1.08 at 24 h p.i., respectively) were desirable.

The results of blocking experiments demonstrated that the tumor uptake was derived by receptor-mediated specific binding. The pre-injection of excess cyclo(RGDyK) decreased considerably the tumor uptake of ^{177}Lu-labeled GluDTPA-cyclo (RGDfK), in which tumor uptake was reduced to 0.60 ± 0.10 % ID/g with blockage, compared with 1.05 ± 0.03 %ID/g without blockage ($p < 0.05$).

Conclusions

In this study, a glutamic acid-based DTPA (GluDTPA) was prepared and conjugated with cyclo(RGDfK) by applying a conventional solid phase synthesis method. The ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) was formulated by simple mixing of the peptide with ^{177}Lu at mild condition and confirmed its biological affinity as a targeting agent for integrin $\alpha_v\beta_3$-positive tumor. The results for ^{177}Lu-labeled GluDTPA-cyclo(RGDfK) suggest that further assessments of the peptide of GluDTPA-cyclo(RGDfK) with a different radionuclide such as ^{68}Ga, ^{111}In, and ^{99m}Tc for non-invasive imaging of integrin $\alpha_v\beta_3$-positive tumor.

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Conflict of interest

The authors did not report any conflict of interest.

References

[1] P. C. Brooks, R. A. Clark, D. A. Cheresh, *Science* **1994**, *264*, 569.
[2] J. D. Hood, D. A. Cheresh, *Nat. Rev. Cancer* **2002**, *2*, 91.
[3] Z. Liu, B. Jia, J. Shi, X. Jin, H. Zhao, F. Li, S. Liu, F. Wang, *Bioconjug. Chem.* **2010**, *21*, 548.
[4] R. Haubner, H. J. Wester, F. Burkhart, R. Seekowitsch-Schmidtke, W. Weber, S. L. Goodman, H. Kessler, M. Schwaiger, *J. Nucl. Med.* **2001**, *42*, 326.
[5] R. Haubner, H. J. Wester, W. Weber, C. Mang, S. I. Ziegler, S. L. Goodman, R. Seekowitsch-Schmidtke, H. Kessler, M. Schwaiger, *Cancer Res.* **2001**, *61*, 1781.
[6] X. Chen, *Mini Rev. Med. Chem.* **2006**, *6*, 227.
[7] S. Liu, *Mol. Pharm.* **2006**, *3*, 472.
[8] A. J. Beer, M. Schwaiger, *Cancer Metastasis Rev.* **2008**, *27*, 631.
[9] I. Dijkgraaf, J. A. Kruijtzer, S. Liu, A. C. Soede, W. J. Oyen, F. H. Corstens, R. M. Liskamp, O. C. Boerman, *Eur. J. Nucl. Med. Mol. Imaging* **2007**, *34*, 267.
[10] I. Dijkgraaf, A. J. Beer, H. J. Wester, *Front. Biosci.* **2009**, *14*, 887.
[11] A. J. Beer, A. L. Grosu, J. Carlsen, A. Kolk, M. Sarbia, I. Stangier, P. Watzlowik, H. J. Wester, R. Haubner, M. Schwaiger, *Clin. Cancer Res.* **2007**, *13*, 6610.
[12] A. J. Beer, S. Lorenzen, S. Metz, K. Herrmann, P. Watzlowik, H. J. Wester, C. Peschel, F. Lordick, M. Schwaiger, *J. Nucl. Med.* **2008**, *49*, 22.
[13] A. J. Beer, M. Niemeyer, J. Carlsen, M. Sarbia, J. Nahrig, P. Watzlowik, H. J. Wester, N. Harbeck, M. Schwaiger, *J. Nucl. Med.* **2008**, *49*, 255.
[14] L. M. Kenny, R. C. Coombes, I. Oulie, K. B. Contractor, M. Miller, T. J. Spinks, B. McParland, P. S. Cohen, A. M. Hui, C. Palmieri, S. Osman, M. Glaser, D. Turton, A. Al-Nahhas, E. O. Aboagye, *J. Nucl. Med.* **2008**, *49*, 879.
[15] D. R. K. A. Mohan, Q. Y. Jian, *J. Nucl. Med.* **2009**, *50*(Supplement 2), 447.
[16] O. Schnell, B. Krebs, J. Carlsen, I. Miederer, C. Goetz, R. H. Goldbrunner, H. J. Wester, R. Haubner, G. Popperl, M. Holtmannspotter,

H. A. Kretzschmar, H. Kessler, J. C. Tonn, M. Schwaiger, A. J. Beer, *Neuro Oncol.* **2009**, *11*, 861.
[17] S. Mundwiler, R. Waibel, B. Spingler, S. Kunze, R. Alberto, *Nucl. Med. Biol.* **2005**, *32*, 473.
[18] R. Schibli, P. A. Schubiger, *Eur. J. Nucl. Med. Mol. Imaging* **2002**, *29*, 1529.
[19] K. H. Choi, Y. D. Hong, M. S. Pyun, S. J. Choi, *Bull. Korean. Chem. Soc.* **2006**, *27*, 1194.
[20] W. A. P. Breeman, M. de Jong, D. J. Kwekkeboom, R. Valkema, W. H. Bakker, P. P. M. Kooij, T. J. Visser, E. P. Krenning, *Eur. J. Nucl. Med.* **2001**, *28*, 1421.
[21] S. Y. Lee, Y. D. Hong, P. M. Felipe, M. S. Pyun, S. J. Choi, *Appl. Radiat. Isot.* **2009**, *67*, 1366.
[22] M. S. Pyun, K. H. Choi, Y. D. Hong, S. J. Choi, *Bull. Korean. Chem. Soc.* **2009**, *30*, 1187.
[23] F. F. Anelli, O. Gazzotti, L. Lattauada, G. Lux, F. Rebasti, *Bioconjug. Chem.* **1999**, *10*, 137.
[24] S. Chakraborty, J. Shi, Y. S. Kim, Y. Zhou, B. Jia, F. Wang, S. Liu, *Bioconjug. Chem.* **2010**, *21*, 969.
[25] R. O. Hynes, *Cell* **1992**, *69*, 11.
[26] R. E. Seftor, E. A. Seftor, K. R. Gehlsen, W. G. Stetler-Stevenson, P. D. Brown, E. Ruoslahti, M. J. Hendrix, *Proc. Natl. Acad. Sci.* **1992**, *89*, 1557.
[27] J. R. Hart, Ethylenediaminetetraacetic acid and related chelating agents. In *Ullmann's Encyclopedia of Industrial Chemistry* Wiley-VCH, Weinheim, **2005**.
[28] M. K. Dewanjee, D. J. Hnatowich, R. Beh, *J. Nucl. Med.* **1976**, *17*, 1003.
[29] B. Maziere, O. Stulzaft, J. M. Verret, D. Comar, A. Syrota, *Int. J. Appl. Radiat. Isot.* **1983**, *34*, 595.
[30] C. J. Anderson, P. A. Rocque, C. J. Weinheimer, M. J. Welch, *Nucl. Med. Biol.* **1993**, *20*, 461.
[31] Y. D. Hong, K. B. Park, B. S. Jang, S. J. Choi, S. M. Choi, Y. M. Kim, *Nucl. Med. Biol.* **2002**, *29*, 833.
[32] L. Correa-Gonzalez, C. Arteaga de Murphy, G. Ferro-Flores, M. Pedraza-Lopez, E. Murphy-Stack, D. Mino-Leon, G. Perez-Villasenor, Y. Diaz-Torres, R. Munoz-Olvera, *Nucl. Med. Biol.* **2003**, *30*, 135.
[33] M. P. Kelly, F. T. Lee, K. Tahtis, F. E. Smyth, M. W. Brechbiel, A. M. Scott, *Clin. Cancer Res.* **2007**, *13*, 5604s.
[34] S. Shanehsazzadeh, A. R. Jalilian, H. R. Sadeghi, M. Allahverdi, *Radiat. Prot. Dosimetry* **2009**, *134*, 79.
[35] A. Yurt, F. Z. Muftuler, P. Unak, S. Yolcular, C. Acar, H. Enginar, *Cancer Biother. Radiopharm.* **2009**, *24*, 707.
[36] Y. Chen, Q. F. Xiong, X. Q. Yang, L. He, Z. W. Huang, *Am. J. Roentgenol.* **2010**, *194*, 761.
[37] P. A. Erba, A. G. Cataldi, C. Tascini, A. Leonildi, C. Manfredi, G. Mariani, E. Lazzeri, *Nucl. Med. Commun.* **2010**, *31*, 994.
[38] J. Shi, Z. Liu, B. Jia, Z. Yu, H. Zhao, F. Wang, *Amino Acids* **2010**, *39*, 111.
[39] M. Janssen, W. J. Oyen, L. F. Massuger, C. Frielink, I. Dijkgraaf, D. S. Edwards, M. Radjopadhye, F. H. Corstens, O. C. Boerman, *Cancer Biother. Radiopharm.* **2002**, *17*, 641.
[40] G. Thumshirn, U. Hersel, S. L. Goodman, H. Kessler, *Chemistry* **2003**, *9*, 2717.