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Preparing a 68Ga-labeled Arginine Glycine Aspartate (RGD)-peptide for Angiogenesis

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TITLE:

Preparing a ^{68}Ga-labeled Arginine Glycine Aspartate (RGD)-peptide for Angiogenesis

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KEYWORDS:

Positron emission tomography, arginine-glycine-aspartic acid, ^{68}Ga, radiometal, $\alpha_v\beta_3$ integrin, angiogenesis

SUMMARY:

The $\alpha_v\beta_3$ integrin is a type of adhesion protein that is highly expressed on activated endothelial cells undergoing angiogenesis. Thus, evaluating the integrity of the integrin is of great interest in oncology. Here, we introduce a method to prepare ^{68}Ga-labeled radiopeptides and a method to assess its biological effectiveness.

ABSTRACT:

The $\alpha_v\beta_3$ integrin is a heterodimeric adhesion molecule involved in tumor cell migration and angiogenesis. The integrin is overexpressed in angiogenic tumor endothelial cells, where it typically has a low concentration. This specific expression of $\alpha_v\beta_3$ makes it a valid biomarker for antiangiogenic and imaging drugs. As a functional imaging modality, positron emission tomography (PET) provides information about biochemical and physiological changes *in vivo*, due to its unique high sensitivity at the nanomolar scale. Hence, radiometal-based PET radiopharmaceuticals have received great attention for the non-invasive quantification of tumor angiogenesis. This paper provides a systemic protocol to prepare a new radiometal-labeled peptide for the evaluation of angiogenesis. This protocol contains information about radiochemical reliability, lipophilicity, cell uptake, serum stability, and pharmacokinetic properties. The ^{68}Ga-RGD-peptide is one of the representative PET ligands toward $\alpha_v\beta_3$ integrin. Here, we introduce a protocol to prepare a ^{68}Ga-RGD-peptide and the evaluation of its biological efficacy.

INTRODUCTION:

Angiogenesis is a biological process that is characterized by the development of new blood vessels. Among many angiogenetic factors, $\alpha_v\beta_3$ integrin is associated with invasiveness, because the integrin is highly expressed in angiogenic tumor vessels but is absent in normal tissue[1].

Radiolabeled receptor-binding peptides with the arginine glycine aspartate (RGD) domain, which has a high affinity toward $\alpha_v\beta_3$ integrin receptors, are considered promising angiogenesis imaging agents[2-7]. Several radiopharmaceuticals have been created for PET and its biological properties have been validated in various animal models[8-11]. In terms of a radionuclide, ^{68}Ga has several advantages over other radioisotopes. Firstly, it has a high accessibility for users and is economically advantageous because a cyclotron is not required. Secondly, ^{68}Ga-based radiopharmaceuticals produce high spatial resolution compared with single-photon emission computed tomography (SPECT), allowing more accurate quantification. Lastly, the 67.71 minutes half-life of ^{68}Ga may be sufficient for the preparation of small peptides or proteins.

To produce a stable complex with ^{68}Ga, many chelators have been developed. Representative chelators are 1,4,8,11-tetraazacyclotetradecanetetraacetic acid (TETA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), diethylenetriaminepentaacetic acid (DTPA), and N,N'-di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid (HBED). NOTA has been reported to form a highly stable complex with ^{68}Ga (log stability constant 30.98)[12-14].

The purpose of the present study is to provide a concise protocol for the development of a new radiopeptide (**Figure 1**). As an example, we prepare ^{68}Ga-labeled RGD-peptides and present methods for the biological evaluation of these analogues in a xenograft model.

PROTOCOL:

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals under protocols approved by the Korea Institute of Radiological and Medical Sciences Animal Studies Committee. All reagents and solvents were purchased and used without further purification. NOTA-RGD-peptides were prepared according to literature methods[15].

CAUTION: ^{68}Ga emits both positron and gamma rays. All experiments, including direct or indirect contact with radioactive substances, must be undertaken by trained and permitted personnel only. When handling radioactive materials, proper protective equipment, shielding, radiation dosimeter badge and rings, and a survey meter should be used.

1. Radiolabeling RGD-peptides with $^{68}GaCl_3$

Note: ^{68}Ga ($t_{1/2}$ = 68 min, β^+ = 89%, and EC = 11%) was obtained from the ^{68}Ga/^{68}Ge generator.

1.1. Elute the $^{68}GaCl_3$ from the generator with 4 mL of 0.05 M HCl.

1.2. Purge with nitrogen gas at 80 °C for 30 min to dry $^{68}GaCl_3$ (333 kBq, 1 mL) in a 5 mL reaction

vial.

1.3. Add a solution of RGD-peptide (100 µg) in 1 M sodium acetate (100 µL, pH 5 - 6) to the reaction vial containing $^{68}GaCl_3$ from step 1.2.

1.4. Heat the reaction mixture at 80 °C for 5 min. Then, cool it down to room temperature.

1.5. Purify the crude product with high-performance liquid chromatography (HPLC). Use the following system: a C-18 column, a flow rate of 0.5 mL/min, a gradient slope of acetonitrile of 1.17%/min (5% - 40% in 30 min), and elution components: A = 0.1% trifluoroacetic acid (TFA) in acetonitrile, B = 0.1% TFA in water.

Note: The HPLC is equipped with a photodiode array detector and a radioactivity detector. The ^{68}Ga-RGD-peptide was collected at a retention time of 12.5 min (**Figure 2**).

1.6. Purify the resulting ^{68}Ga-RGD-peptide using a solid phase extraction system.

1.6.1. Pass the solution through a C18 reverse-phase cartridge and wash with 2 mL of saline.

1.6.2. Elute ^{68}Ga-RGD-peptide with 0.7 mL of 95% ethanol. Remove the solvent at 80 °C under nitrogen gas for 20 min and reconstitute with phosphate-buffered saline (PBS) before use.

1.6.3. Filter the radiolabeled product through a 0.22 µm sterile filter and formulate in 1 mL of sterile saline solution.

1.7. Check the radiochemical yield by radio-thin-layer chromatography (TLC).

1.7.1. Spot 1 µL on an instant thin layer chromatography plate (ITLC, 10 cm in length). Develop the plate in a chamber containing the eluent (aqueous 0.1 M citric acid, pH 5.0) until 9 cm away from the spot.

Note: The retention factor for ^{68}Ga-RGD-peptide is 0 and the retention factor for unreacted $^{68}Ga^{3+}$ is 1.

1.8. Calculate the final specific activity from the ratio of radioactivity corresponding to the non-radioactivity as MBq/nmol.

Note: After the injection of 100 µL of the formulated ^{68}Ga-RGD-peptide to HPLC, the amount of non-radioactive component was calculated from the standard calibration curve using nonradioactive Ga-RGD-peptide.

2. *In Vitro* Cellular Uptake

Note: Uppsala 87 Malignant Glioma (U87MG) human glioblastoma cells were grown in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown in 150 mm dishes at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested or split by trypsinization: 0.25% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in PBS at 37 °C for 3 - 5 min.

2.1. Seed U87MG cells into 6-well plates at a density of 1 x 10^6 cells/well.

2.2. Incubate the cells with ^{68}Ga-RGD-peptide (111 kBq) at 37 °C for 30, 60, 90, and 120 min. Prepare samples in triplicate.

2.3. Wash the cells 2x with 2 mL of PBS and harvest by trypsinization. Use 0.25% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in PBS at 37 °C for 3 - 5 min.

2.4. Collect the cell suspension (500 µL) and measure in a γ-counter.

2.5. Calculate the percent uptake of the compound by the cells by % (counts in cells/total counts).

3. *In Vitro* Serum Stability

3.1. Add 500 µL of freshly prepared mouse serum, 500 µL of human serum, and 500 µL of PBS. Incubate the mixture at 37 °C for 2 h.

3.2. Evaluate by ITLC at the specified time intervals (30, 60, 90, and 120 min). Spot 1 - 2 µL aliquot of the mixture to the ITLC plate (mobile phase: 0.1 M citric acid). Develop the plate as in step 1.7.

Note: $^{68}Ga^{3+}$ is expected to move with the solvent front, whereas the labeled compound will remain at the origin.

4. Determination of Lipophilicity

4.1. Add ^{68}Ga-RGD-peptide (3.7 MBq, 3.7 µL) to the octanol-PBS system (1:1, *v*/*v*, total 1 mL).

4.2. Mix the vials vigorously for 5 min at room temperature and centrifuge at 10,000 x *g* for 5 min at room temperature.

4.3. Take 100 µL samples from each layer and measure the radioactivity with a γ-counter. The reported log P value is based on the average of three samples.

5. Tumor Model

Note: BALB/c nude mice (6 - 8 weeks old, female, *n* = 23) were used for this study. The mice were subsequently used for PET studies (*n* = 3) and biodistribution (*n* = 20) when the tumor volumes reached 200 - 300 mm^3 (1 - 2 weeks after implantation).

5.1. Load tumor cells into 28 G, 1/2 inch insulin syringes.

5.2. Inject U87MG cells (5 x 10^6) in 100 µL of PBS into the left arm region.

5.3. Anesthetize the mouse with 2% isoflurane in oxygen gas during cell injection.

5.3.1. Ensure that the mouse has been anesthetized by the loss of the pedal withdrawal reflex following pinching with forceps between the toes of the right hind foot. Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.

6. *In Vivo* Quantification of $\alpha_v\beta_3$ Integrin Using PET

6.1. Anesthetize the mice with 2% isoflurane in oxygen.

6.1.1. Ensure that the mouse has been anesthetized by the loss of the pedal withdrawal reflex following pinching with forceps between the toes of the right hind foot. Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.

6.2. Place the head in the center of the PET gantry.

6.3. Intravenously administer the ^{68}Ga-RGD-peptide solution (7.4 MBq, 200 µL) to the xenograft mouse model *via* the tail vein for 1 min.

6.4. At the same time, perform a PET scan in list mode (dynamic scan) for 150 min.

Note: The raw PET data were reconstructed by a user-defined time frame (*i.e.*, every 30 min). After the PET scan, a micro-computed tomography (CT) scan (50 kVp of X-ray, 0.16 mA) was conducted for attenuation correction.

7. *Ex Vivo* Biodistribution

7.1. Inject ^{68}Ga-RGD-peptide (0.37 MBq, 200 µL) into the tail vein of the xenograft mouse model. Anesthetize the mouse with 2% isoflurane in oxygen gas during the injections.

Note: BALB/c nude mice, as described in section 5, were divided into four groups and sacrificed at different time points (n = 5 per group).

7.2. Wake the mice immediately after the administration of ^{68}Ga-RGD-peptide and sacrifice them at 30, 60, 90, and 120 min postinjection with carbon dioxide euthanasia.

Note: The tissues of interest were extracted. Selected targets were the blood, muscle, heart, lung, liver, spleen, stomach, intestine, kidneys, bone, and tumor.

7.3. Weigh the tissue and measure the radioactivity with a γ-counter.

Note: Results were expressed as the percentage injected dose per gram of tissue (% ID/g).

REPRESENTATIVE RESULTS:

The chelation of $^{68}GaCl_3$ with the NOTA-RGD-peptide was straightforward, and the radiolabeling yield was 99%. Reaction impurities were successfully removed as shown in **Figure 2**. The radiochemical purity of ^{68}Ga-RGD-peptide was greater than 99%, and specific activity at the end of the synthesis was 90 - 130 MBq/nmol (**Figure 3**).

The cell uptake values for ^{68}Ga-RGD-peptide were 1.49%, 0.85%, 0.36%, and 0.39% at 30, 60, 90, and 120 min, respectively. Serum stability showed that ^{68}Ga-RGD-peptide remained almost intact after 2 h of incubation with human or mouse serum as well as PBS (> 92% stability at 2 h). The partition coefficient (log P) was 2.96, indicating high lipophilicity. PET showed an initial high uptake in the major organs, including the liver, kidney, heart, muscle, and tumor. However, in the late period (90 - 150 min), the tumor region was clearly visualized. The tumor-to-muscle ratio at 90 min was 17.57 and remained unchanged, indicating kinetic stability. The *ex vivo* biodistribution showed that the accumulated radioactivity in the tumor was 6.19, 4.96, 4.44, and 4.39 (% ID/g) at 30, 60, 90, and 120 min, respectively. The results of the *ex vivo* experiment were in accordance with the *in vivo* PET findings (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Flow diagram of the experimental procedures. This figure shows a schematic overview of the development of radiopharmaceutical.

Figure 2: Purification of ^{68}Ga-RGD-peptide by HPLC. Blue is radioactivity signal and black is ultraviolet (UV) signal. The UV wavelength is 314 nm. The X-axis is time and the Y-axis is absorbance unit (AU). The ^{68}Ga-RGD-peptide has 12.4 min of retention time.

Figure 3: Structure of ^{68}Ga-RGD-peptide and its radiochemical purity. The ITLC of ^{68}Ga-RGD-peptide showed high radiochemical purity.

Figure 4: PET imaging (upper) and *ex vivo* biodistribution data for ^{68}Ga-RGD-peptide (lower). PET data were expressed on the SUV scale from 0 to 5. Biodistribution data shown are the mean ± the standard deviation from five mice at each time point.

DISCUSSION:

In the present study, we introduced a protocol to prepare a radiopeptide targeting $\alpha_v\beta_3$ integrin and its biological evaluation. Traditional drug development involves a complicated procedure. It requires a large quantity of reference material and a relatively long evaluation time. Although the suggested methodology cannot replace the delicate evaluation process, this system can be used for screening purposes. This proposed system would considerably reduce the time and cost.

Over the past decade, many radiolabeled RGD-peptides have been extensively studied as radiotracers for imaging tumors[16]. To obtain promising radiopharmaceuticals for clinical trials, systemic approaches for drug development should be provided. Radiochemical feasibility, high selectivity-affinity to the target, metabolic stability, and proper pharmacokinetics are four major concerns. For a routine PET study, a reasonable radiochemical yield ensures the reliability of the radiopharmaceuticals. The issues of high affinity (> nM) and selectivity (> 100x) to the target protein are also satisfied. In terms of pharmacokinetics, the candidate PET tracer is rapidly excreted from the non-target tissue and has a long retention time in the tumor, allowing a high target-to-reference ratio. Candidate radiopharmaceuticals should not have troublesome metabolites *in vivo* that could increase non-specific binding and provide low contrast imaging. It is important to assess the comprehensive characteristics because each term influences the other properties, which are not independent.

The radiopeptide introduced in this research has suitable drug-like properties. The ^{68}Ga-RGD-peptide has a high radiochemical yield of 99%, metabolic stability, and proper lipophilicity. In the *in vivo* experiment, the radiopeptide exhibited high selectivity (tumor-to-reference ratio = 17.57), and the *ex vivo* biodistribution data also showed significant tumor uptake (up to 6.19% ID/g).

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

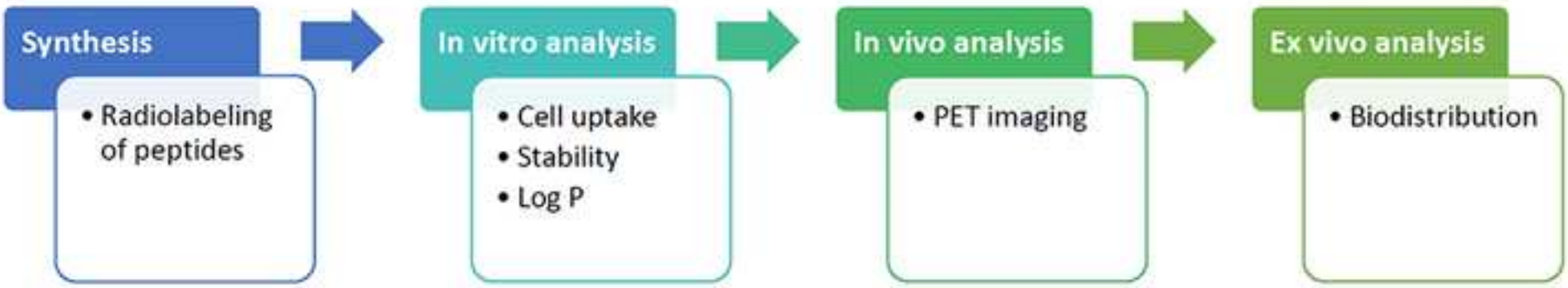

Figure2

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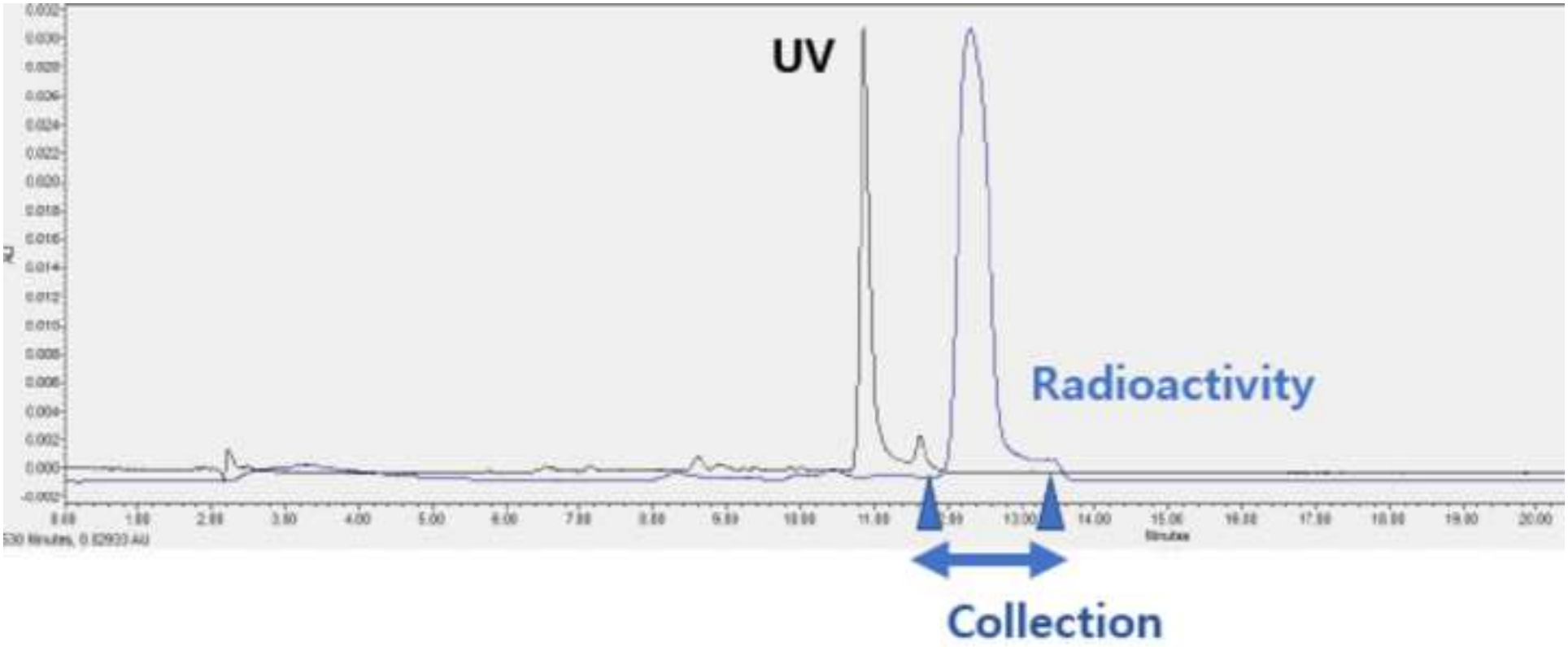

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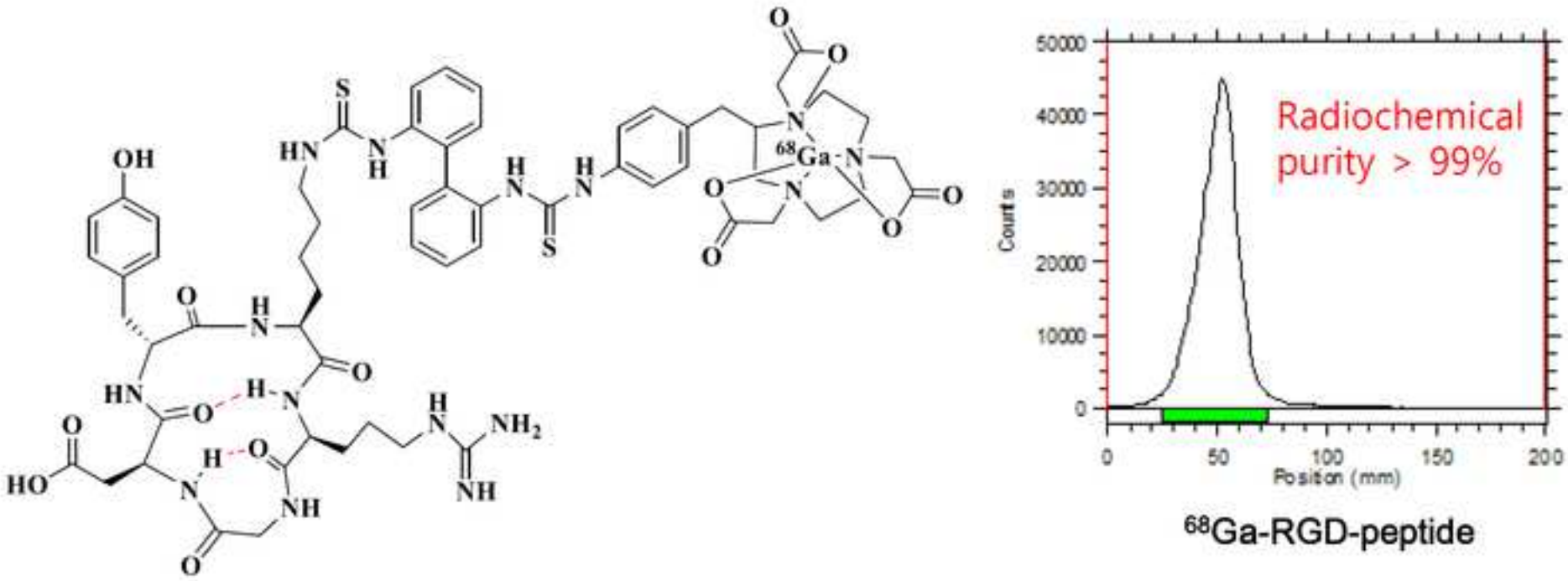
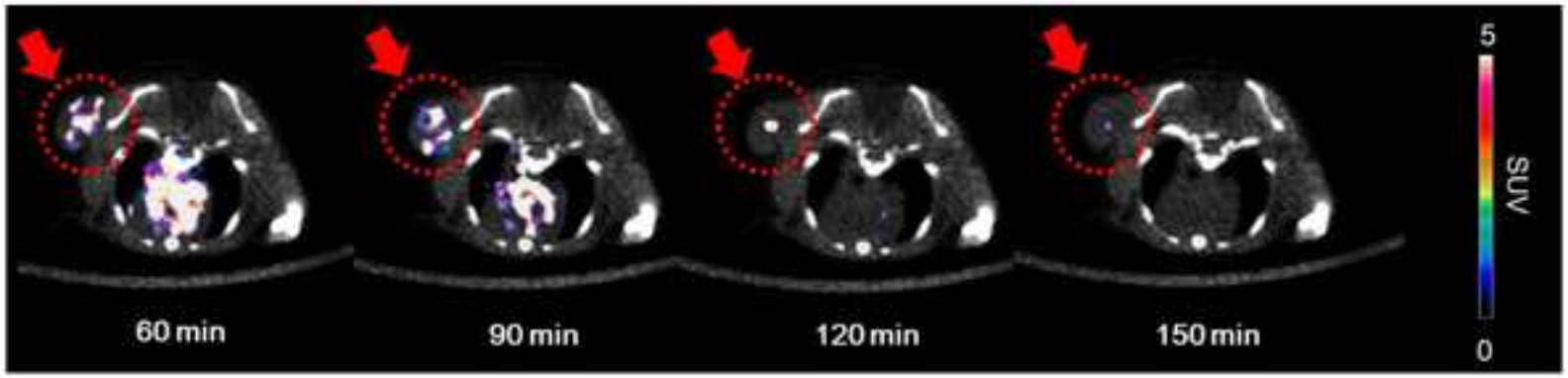


^{68}Ga-RGD-peptide

Figure4



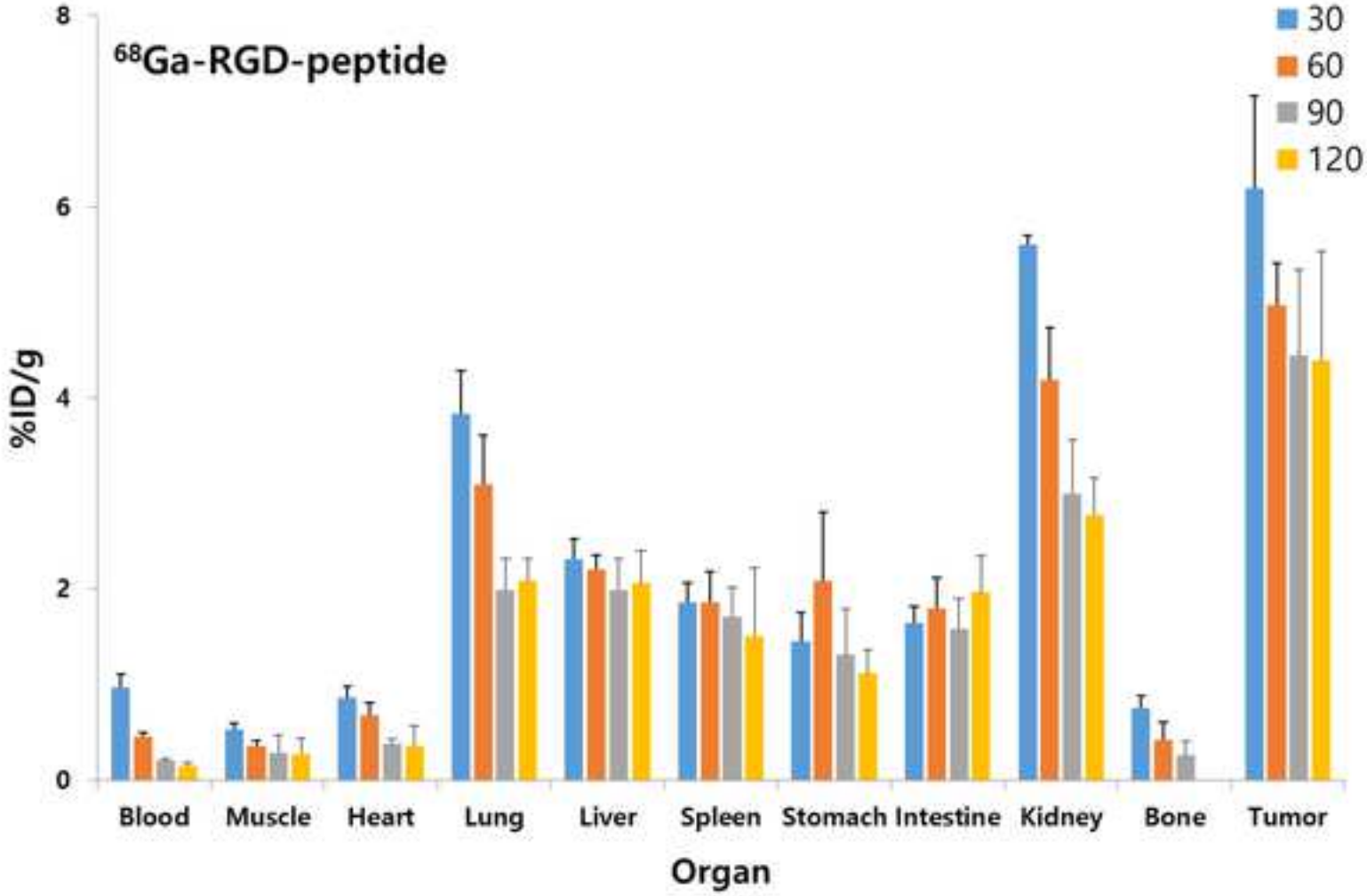


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
$^{68}Ga/^{68}Ge$ generator	ITG Company	-	10 mCi
Hydrogen chloride solution	Sigma-aldrich	84429	
Sodium acetate	Sigma-aldrich	S2889	
C18 reverse-phase cartridge	Waters	WAT020515	
0.22-μm sterile filter	Milllipore	SLGV033RS	
Radio-TLC scanner	Bioscan	AR2000	
ITLC paper	Agilent	SGI001	
Citric acid	Sigma-aldrich	251275	
HPLC	Waters	-	Waters 1525 system containing binary pump, photo diode array (Waters 2998), radioactivity detector (Raytest, Gabi)
C-18 colunm	Develosil	HG31546150W	ODS-HG-5, 150 x 4.6 mm, 5μm
Acetonitrile	J.T. Baker	14-650-359	
Trifluoroacetic acid	Sigma-aldrich	302031	
Dulbecco's modified Eagle media	Thermo fisher scientific	11965092	
fetal bovine serum	Thermo fisher scientific	16000044	
T175 flasks	Corning	CLS431080	
Trypsin-EDTA (0.25%)	Thermo fisher scientific	25200072	
penicillin-streptomycin	Thermo fisher scientific	15240112	
γ-counter	Perkin Elmer	-	1480 Wizard 3
Insunlin syringe	Becton Dickinson	326105	
Synringe pump	Harvard Apparatus	70-4500	
micro-PET/CT	Siemens Inveon	-	

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