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Research Article

Synthesis, Radiolabeling, and Biological Evaluation of ^{68}Ga-Mucin1 and Its Folate Hybrid Peptide Conjugates for the Diagnosis of Ovarian Cancer

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Because of our interest in developing new hybrid peptide radioconjugates with suitable biochemical properties for multiple-receptors targeting properties that are overexpressed on many human cancers especially ovarian cancer, we have synthesized ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates using a straightforward and one-step simple reaction. Radiochemical yields were found to be higher than 95% (decay corrected), with a total synthesis time of less than 20 min. Radiochemical purities were always higher than 95% without HPLC purification. *In vitro* studies on KB cancer cells showed that substantial amounts of the radioconjugates were associated with cell fractions and held great affinities and specificities toward the KB cell line. *In vivo* characterization in normal female Balb/c mice revealed rapid blood clearance of these radioconjugates with excretion predominantly by the urinary system. Biodistribution studies in nude mice bearing human KB cell line xenografts demonstrated significant tumor uptake and favorable biodistribution profile for ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate compared to the ^{68}Ga-NODAGA-MUC1 peptide monomeric counterpart. The uptake in the tumors was blocked by the excess injection of hybrid peptide, suggesting a receptor-mediated process. These results demonstrate that ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate may be useful as a molecular probe for early detection and staging of folate and MUC1 receptor-positive cancers such as ovarian cancer and their metastasis as well as monitoring tumor response to treatment.

1. Introduction

Ovarian cancer is considered as the most fatal gynecological cancer worldwide. It is often associated with high mortality and a low survival rate [1]. The high mortality rate is mainly attributed to the late detection of this tumor and, at this stage, the tumor metastasizes to the pelvic cavity and neighboring tissues leading to treatment difficulty and eventually death [2]. Since the early stage of this tumor has no obvious symptoms, the currently used diagnostic tools such as CA-125 and ultrasound are not very successful in the early stage [3]. Peptide-based tumor receptor binding agents have attracted enormous attention as biological vehicles to deliver radioactivity to tumor cells for receptor-targeted imaging and radiotherapy [4–8]. Due to many advantages of peptides which include small molecular weight, rapid tumor uptake, and fast clearance from the blood, several radiolabeled peptides have been developed and are now being used routinely as imaging and therapeutic agents for different cancers [9]. With the increased use of positron emission tomography (PET), there has been a great interest in the development of new positron-emitting peptide radiopharmaceuticals for earlier detection and characterization of cancer, molecular assessment of treatment effects, and a

more fundamental understanding of the disease process [10].

The epithelial mucin coded by the gene mucin1 (MUC1) is a good example of a tumor-specific antigen that has a low expression on normal tissues but it is highly expressed on ovarian cancer and most other cancers [11–16]. MUC1 is a transmembrane glycoprotein of which the extracellular domain is defined by the presence of amino acid sequence PDTRP (Pro-Asp-Thr-Arg-Pro) [17–20]. The malignant phenotype of MUC1 with low glycosylation is located at apical, lateral, and basal surfaces of cancer cells causing disruption of binding between adjacent cells, thus facilitating invasion and metastasis [21]. These characteristics of the malignant phenotype of MUC1 which distinguish it from normal cells make it a suitable ligand for an early ovarian cancer screening [22]. Therefore, the development of small peptide-based agents for targeting MUC1 expressing tumors is more desirable because of their low immunogenic response and favorable biokinetics, together with high affinity and selectivity for target receptors. Moreover, owing to its overexpression in cancerous tissues and limited expression in normal tissue, folate receptor (FR) is another interesting ovarian cancer target [23]. Folic acid (FA) binds with high affinity to folate receptor which ends by endocytosis. The expressions of folate receptors in ovarian cancer cells play crucial roles in cell proliferation and tumor biology [23–25]. Targeting the folate receptor is widely studied as a strategy for cancer diagnosis.

Overexpression of both the MUC1 and FR receptors on ovarian cancer highlights the potential application of the radiolabeled MUC1-conjugated folate hybrid peptide as dual-receptor-targeting imaging probe for ovarian carcinoma imaging. This new class of hybrid peptides may overcome low binding selectivity issues and cancer receptors heterogeneity [26, 27]. We hypothesized that the unique radiolabeled MUC1-conjugated folic acid hybrid peptide targeting both the MUC1 and folate receptors would be superior in ovarian cancer targeting to the radiolabeled MUC1 monomeric peptide. This may represent novel multiple-acting properties to the management and treatment of ovarian cancer disease with unmet medical needs.

In the previous studies, we have described the preclinical evaluation of ^{18}F-labeled Mucin1 as well as its folate conjugate for targeting breast cancer [28, 29]. Based on the encouraging results of these studies, it seems interesting to further explore the potential of this MUC1 and its folate conjugate beyond breast cancer targeting. In this study, we have synthesized by solid-phase synthesis a novel MUC1 peptide based on PDTRP sequence and coupled it to the glutamic acid and lysine residues before hybridization with folic acid. A glutamic acid (Glu) residue acts as a spacer to keep the chelating site away from the receptor-binding region and to increase the hydrophilicity of hybrid molecules, which often resulted in faster renal clearance and improved target to background ratios. Meanwhile, lysine (Lys) amino acid was terminally coupled with Mucin1 peptide to facilitate conjugation with folic acid and to facilitate coupling to a bifunctional chelating agent (BFCA). Owing to the favorable nuclear and chemical characteristics of gallium-68 (^{68}Ga) for PET diagnostic imaging applications and easy availability by generator without a need for a cyclotron [30], we here present the radiolabeling with ^{68}Ga and *in vitro* and *in vivo* evaluation of new MUC1 and its FA hybrid peptides for the diagnosis of breast cancer using PET imaging.

2. Experimental

All reagents and chemicals used in the study were all of analytical reagent grade purchased from commercial sources and were used without further purification. HPLC-grade acetonitrile (ACN) was kept over molecular sieves. Sep-Pak cartridges were purchased from Waters-Millipore (Milford, MA, USA). High-performance liquid chromatography (HPLC) analysis was carried out on Luna, Phenomenex C-18 reversed-phase column (analytical, 250 mm × 4.6 mm) (JASCO, Tokyo, Japan). The solvent system used was a gradient system from 80% solvent A (0.1% TFA in water) and 20% solvent B (0.1% TFA in ACN) (5 min) and ramped to 100% solvent A and 0% solvent B at 25 min. A JASCO chromatographic system equipped with a variable wavelength ultraviolet monitor in tandem with a Canberra flow through radioactivity detector was used (Meriden, CT, USA). Ultraviolet absorption was monitored at 254 nm for FA and 220 nm for MUC1. Chromatograms were acquired and analyzed using the Borwin software (JASCO, Tokyo, Japan). Mass spectroscopy was run on Quattra electrospray mass spectrometer (ES-MS, Perkin Elmer, Waltham, MA, USA).

2.1. MUC1 and MUC1-FA Hybrid Peptide Conjugates. The MUC1 peptide analog was prepared using the method reported previously [28, 31], briefly by solid-phase peptide synthesis (on a CS Bio peptide synthesizer, CA, USA) following standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, using Rink amide methylbenzhydrylamine (MBHA) resin on a 0.2 mmol scale. After incorporating all the desired amino acids, the N-terminal Fmoc-protecting group was removed and the peptide was cleaved from the resin followed by the removal of the other side-chain protecting groups using a mixture of TFA/H_2O/dithiothreitol (DTT) 95 : 2.5 : 2.5 for 2 h at room temperature. The resin was removed by filtration, and the crude peptides were obtained by precipitation with cold diethyl ether (ether) followed by HPLC purification. The identity and purity of the MUC1 peptide analog were characterized by mass spectrometry and HPLC. For the synthesis of MUC1-FA hybrid peptide, the free epsilon (ε) amino group at terminal Lys residue on MUC1 peptide was coupled with FA via the activated gamma (γ) carboxyl moiety. The *N*-succinimidyl folate ester (folate-NHS, 10 μmol) was dissolved in dimethyl sulfoxide (DMSO, 50 μL) and followed by the addition of each peptide (10 μmol) and TEA (10 μmol). The reaction mixture was stirred while being shielded from light for 30 min at 50°C. The MUC1-FA hybrid peptide was precipitated by the addition of ACN (2 mL), centrifuged, and then washed several times with ACN before drying. The

identity and purity of the MUC1-FA hybrid peptide conjugate were characterized by mass spectrometry and HPLC.

2.2. NODAGA-NHS Conjugate. 1,4,7-Triazacyclononane, 1-glutaric acid-4,7-acetic acid (NODAGA) (2 mg, 5.3 μmol) was dissolved in ACN (5 mL) followed by the addition of dicyclohexylcarbodiimide (DCC, 1.64 mg, 7.9 μmol) and *N*-hydroxysuccinimide (NHS, 0.92 mg, 7.9 μmol). The reaction mixture was stirred at ambient temperature for 3 h. The by-product dicyclohexylurea was then removed by filtration and the filtrate was dried by rotary evaporation to yield NODAGA-NHS as an oily product. The identity and purity of NODAGA-NHS were characterized by mass spectrometry and HPLC.

2.3. MUC1-NODAGA Peptide Conjugate. MUC1 (3 mg, 3.6 μmol) was dissolved in dimethyl sulfoxide (DMSO, 3 mL) followed by the addition of TEA (83 μL, 0.6 mmol) and NODAGA-NHS (2 mg, 3.6 μmol). The reaction mixture was stirred while being shielded from light for 2 h at 90°C. The product was precipitated by the addition of ACN (2 mL), centrifuged, and then washed several times with diethyl ether (ether) before drying under vacuum to furnish NODAGA-MUC1 conjugate as a white powder. The identity and purity of the NODAGA-MUC1 peptide analog were characterized by mass spectrometry and HPLC.

2.4. MUC1-FA-NODAGA Hybrid Peptide Conjugate. MUC1-FA (4.3 mg, 3.4 μmol) was dissolved in dimethyl sulfoxide (DMSO, 3 mL) followed by the addition of TEA (3 μL, 0.6 μmol) and NODAGA-NHS (2 mg, 3.4 μmol). Reaction mixture was stirred while being shielded from light for 2 h at 90°C. The product was precipitated by the addition of ACN (2 mL), centrifuged, and then washed several times with diethyl ether (ether) before drying under vacuum to give NODAGA-MUC1 as a yellowish powder. The identity and purity of the NODAGA-MUC1-FA hybrid peptide analog were characterized by mass spectrometry and HPLC.

2.5. Reference Ga^{III}-NODAGA-MUC1 and Ga^{III}-NODAGA-MUC1-FA Hybrid Peptide Conjugates. Reference compounds Ga^{III}-NODAGA-MUC1 and Ga^{III}-NODAGA-MUC1-FA hybrid peptide conjugates were prepared by reacting NODAGA-MUC1 (6 mg, 3.7 μmol) and NODAGA-MUC1-FA hybrid (6 mg, 3.7 μmol) peptide conjugates compounds separately with natural gallium chloride ($GaCl_3$, 11 μmol) in acetic acid (0.1% in EtOH, pH~4.5, 500 μL) at 90°C for 2 h. The reference compound conjugates were precipitated by the addition of ACN (2 mL), centrifuged, and then washed several times with ACN before drying under vacuum into white and yellowish powders. The structures and purities of the reference compound conjugates were characterized by mass spectrometry and HPLC.

2.6. Radiolabeling. $^{68}GaCl_3$ was obtained from $^{68}Ge/^{68}Ga$ generator (ITG GmbH, Munich, Germany). $^{68}Ge/^{68}Ga$ generator was eluted with Suprapur HCl (0.05 M, 6 mL) followed by passing through cation exchange resin column (50 mm, AG 1, Bio-Rad, USA) and subsequent washing with ultrapure water (H_2O, 3 mL) to remove metallic impurities. The concentrated $^{68}GaCl_3$ was then eluted from the column using a small volume of sodium chloride (NaCl, 5 M, 1.5 mL) [32]. The synthetic approaches for the preparation of ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA conjugates were straightforward. Solutions of $^{68}GaCl_3$ (37-300MBq, 100 μL) were added separately in sealed vials containing NODAGA-MUC1 and NODAGA-MUC1-FA hybrid peptide conjugates (50 μg each, in 100 μL DMSO) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, pH = 4–4.5, 100 μL). The reaction mixtures were heated at 90°C for 10 min followed by the addition of H_2O (2 mL) before passing through C18 Sep-Pak cartridge. The radiolabeled peptide conjugates were eluted with ethanol (EtOH, 2 mL) and then dried and resolubilized in sodium chloride (NaCl, 0.9% saline, 1 mL each) before passing through 0.22 μm pore membrane filter for further studies. Radiochemical yields and purities were determined by TLC and confirmed by HPLC.

2.7. Partition Coefficient. 100 μL of ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates were added to test tubes containing 1 mL of each n-octanol and buffered H_2O (pH = 7.3). The tubes were shaken for 1 min. After partial separation of the phases by gravity, 0.7 mL of each phase was transferred to separate tubes and centrifuged at 5000 rpm for 5 min. Duplicate 0.2 mL aliquots of each phase were taken for radioactivity measurement and the partition coefficient was determined by the function: partition coefficient = Log_{10} (counts in *n*-octanol layer/counts in the aqueous layer) [28, 29].

2.8. Stability in Plasma. For stability in plasma, the purified ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates (100 μL, 20 μCi each) were incubated with human plasma (500 μL) in duplicate at 37°C for 2 h. This was followed by precipitation using a mixture of ACN/EtOH (400 μL, 1 : 1 v/v) and centrifugation at 5000 rpm for 5 min [28, 29]. The supernatant layer was then analyzed by HPLC under the conditions described above.

2.9. In Vitro Cell Binding. The cell-binding activity of the ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates was measured on KB human oral carcinoma cell line (ATCC, Rockville, MD). KB cell line was grown in RPMI-1640 culture media with 10% fetal bovine serum (FBS) in tissue culture flasks. 24 h before conducting the cell-binding assay, media was replaced with RPMI-1640 without further addition of FBS. Confluent cultures were harvested by trypsinization, and 6×10^6 cells were suspended in 1.8 mL of sterile saline for binding assay [28, 29]. Approximately 300,000 cells (in 0.3 mL of sterile saline) were incubated with various amounts of the purified ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates ranging from 0.3 to 18 nM in duplicate

for 60 min at room temperature. Incubation was terminated by dilution with cold saline (0.3 mL) and cells were pelleted by centrifugation. The cell pellets were then washed with cold saline to remove unbound radioactivity and centrifuged to collect supernatants. Radioactivity in the cell pellets (total bound) and washings (unbound) were measured in a γ-well counter. Nonspecific binding was determined in the presence of approximately 100-fold excess of unlabeled MUC1 and MUC1-FA-hybrid peptide. Specific binding was calculated by subtracting the nonspecific bound radioactivity from that of the total binding. The data were analyzed by a nonlinear regression analysis program (GraphPad Software Inc., San Diego, CA, USA) using a one-site binding equation. All binding data were corrected for nonspecific binding and presented as the mean ± SD.

2.10. In Vivo Biodistribution. Approval for the animal protocol used in this study was obtained from the Institutional Animal Care and Use Committee. Animal biodistribution experiments were performed according to the international regulations governing the safe and humane use of laboratory animals in research [31]. The biodistribution was performed in normal female Balb/c mice (body mass 20-25 g) to ascertain the *in vivo* distribution profile of the ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates as described previously [28, 29]. Briefly, mice were injected via the lateral tail vein with 100 μL of the radiotracers formulated in saline. Each dose contained 740 kBq of radioactivity. Animals were sacrificed at different time intervals and tissues of interest were dissected, weighed, and assayed for radioactivity. The percentage of the injected dose per gram (% ID/g) was then calculated by counting all tissues in a γ-well counter using a stored sample of the injection solution as a standard to estimate the total dose injected per mouse.

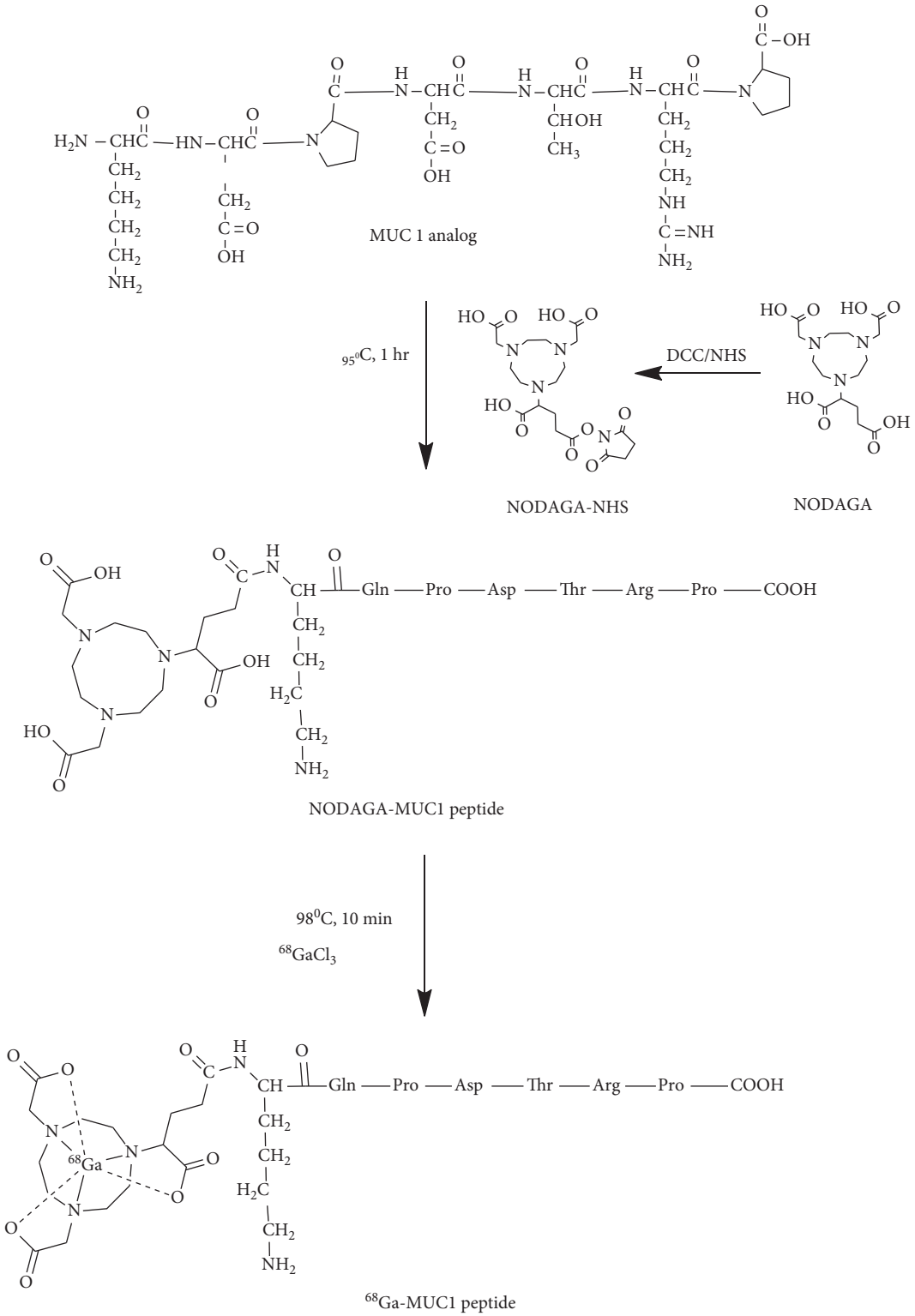
2.11. In Vivo Tumor Targeting. Human KB cell line xenografts in nude female mouse models were used for *in vivo* tumor targeting experiments. For the implantation of tumor xenografts, approximately 3×10^6 KB cancer cells in suspension of 100 μL sterile saline were injected subcutaneously into the right thigh of each mouse. Tumors were allowed to grow for 2-3 weeks by which tumors had reached weights of ~500 mg. Animals were injected with 740 kBq of the radiotracers. For the blocking studies, each animal was intravenously injected with the excess cold of MUC1 and MUC1-FA hybrid peptide (~100 μg) 30 min before the radiotracers injection. The animals ($n = 4$ per group) were sacrificed at 60 min after radiotracers injection (p.i.) and the % ID/g for the tumor and major organs were calculated as described above.

2.12. In Vivo Nano-PET/CT Imaging. PET/CT scans were performed using a preclinical Nano-PET/CT scanner (Mediso, Hungary) on KB tumor-bearing nude female mice (8 weeks old). ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate (5.5 MBq/100 μL) was injected into each mouse through tail vein and placed in the Nano-PET/CT scanner with continuous O_2 and 2% isoflurane supply. The mice were imaged 30 and 60 min after tail vein injection of the radiotracers. CT scan was performed using the following parameters: X-ray voltage = 50 kVp and exposure time = 300 ms. A total projection of 288 projects over 360° of rotation was acquired and reconstructed using a cosine filter. This was followed by a PET data acquisition with the following parameters: 5 ns coincidence window and 400-600 keV energy window in 1–5 coincidence mode. Crystal efficiency correction was also applied, with a ring difference of 8, and the images were reconstructed by three-dimensional ordered subsets. Pixel size was 0.3 mm. The acquired data in these studies were analyzed by InterView FUSION software developed by Mediso.

2.13. Statistical Analysis. Data are expressed as mean ± SD where appropriate. For data comparisons, a Student's t-test of the mean values was performed using GraphPad Software (GraphPad Software Inc., San Diego, CA, USA). A probability value of $P < 0.5$ was considered statistically significant.

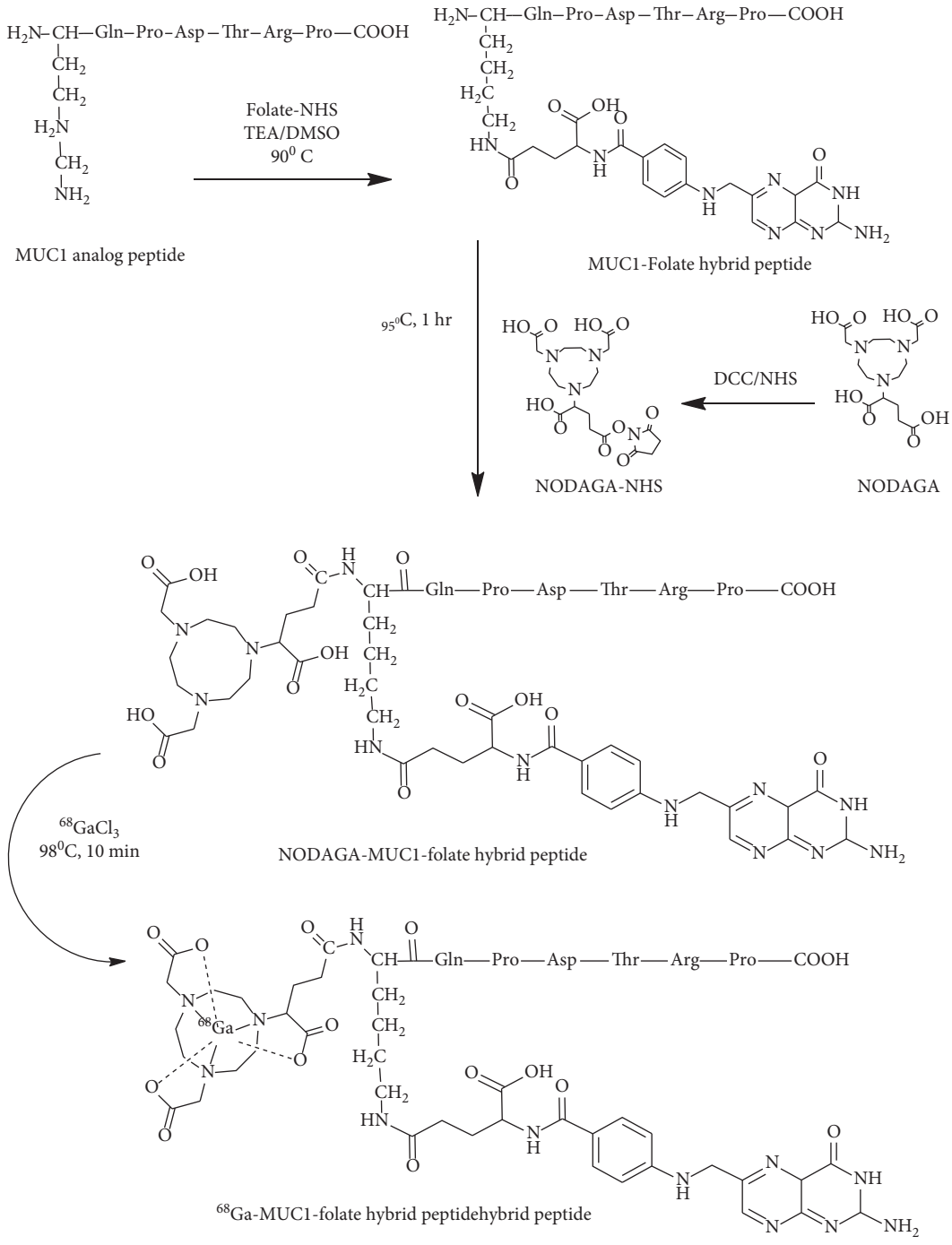
3. Results and Discussion

3.1. Organic Chemistry. The MUC1 peptide analog was successfully synthesized by solid-phase peptide synthesis according to standard Fmoc-chemistry in satisfactory yield (~30%). Lysine and glutamic acid residues were introduced to the MUC1 peptide analog sequence as a spacer function and to facilitate conjugation with the activated folate-NHS. The conjugation between MUC1 and the activated folate-NHS was carried out by amide bond formation between free ε amino group on lysine terminus of MUC1 peptide and activated γ-folate carboxylate to furnish MUC1-FA hybrid peptide in 85% yield (Scheme 1). The chemical purities of the MUC1 and MUC1-FA hybrid peptide analogs were found to be greater than 97% as assessed by HPLC and the calculated molecular masses for MUC1 and MUC1-FA hybrid peptides were in agreement with the experimentally found ES-MS $[M + 1]^+$ values of 841 and 1264, respectively. In addition, the NODAGA conjugates of MUC1 and MUC1-FA hybrid peptides were synthesized by coupling the primary amine group at the N-terminal of lysine amino acid residue of the MUC1 and MUC1-FA hybrid peptides to the NODAGA-NHS through amide bond (Scheme 2 and 1). As confirmed by HPLC, the overall yields and chemical purities were greater than 70% and 95%, respectively. The calculated molecular masses for NODAGA-MUC1and NODAGA-MUC1-FA hybrid peptide conjugates were in agreement with the experimentally found ES-MS $[M + 1]^+$ values of 1227 and 1651, respectively. The reference Ga^{III}-NODAGA-MUC1 and Ga^{III}-NODAGA-MUC-FA hybrid peptide conjugates were obtained with overall chemical yields of 75 and 80%, respectively, and chemical purities were greater than 95% as determined by HPLC (Figure 1). The calculated molecular masses for Ga^{III}-NODAGA-MUC1and Ga^{III}-NODAGA-MUC1-FA hybrid peptide conjugates were in agreement with the experimentally found ES-MS $[M + 1]^+$ values of 1291 and 1690, respectively.



Scheme 1: Synthetic and radiosynthetic approaches for the ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate.

3.2. Radiochemistry. Mucin1 and folate receptors can be used as a tumor-associated molecular target, since they are overexpressed in ovarian carcinomas [31]. Thus, we designed and developed ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA radiotracers utilizing a ^{68}Ga generator to facilitate the production of some PET radiopharmaceuticals in PET centers lacking cyclotrons. The radiolabeling reaction entailed a straightforward and simple one-step reaction (Schemes 2 and 1). The complexation reaction of $^{68}GaCl_3$ with NODAGA-MUC1 and NODAGA-



Scheme 2: Synthetic and radiosynthetic approaches for the ^{68}Ga-NODAGA-MUC1 peptide conjugate.

MUC1-FA hybrid peptide conjugates was carried out using preconcentered and purified $^{68}GaCl_3$ eluate at 95°C for 10 min. As shown in Figure 1, radiochemical purities of ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates were always greater than 98%, as determined by HPLC with retention times of 11.2 and 12.8 min, respectively. The MUC1-folate hybrid peptide and MUC1 peptide bind to ^{68}Ga through available denticity 6 in NODAGA forming complex as shown in Schemes 2 and 1 [30]. In addition, the measured partition coefficients (log D) for ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates were found to be −2.05 and −1.69, respectively, representing a low lipophilic characteristic for both conjugates.

3.3. Stability in Plasma. The proteolytic degradation of the ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates were determined in human plasma *in vitro*. HPLC analysis of the plasma revealed that

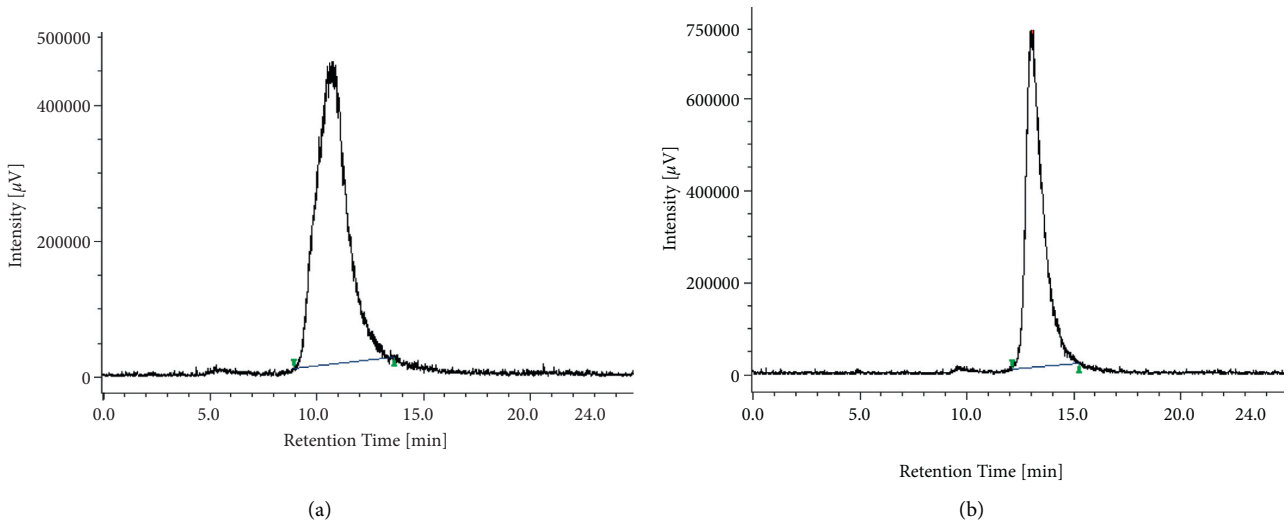


Figure 1: HPLC chromatograms of ^{68}Ga-NODAGA-MUC1 peptide (a) and ^{68}Ga-NODAGA-MUC1-folate hybrid peptide (b) conjugates.

both radioconjugates remained sufficiently stable (>99%) during incubation at 37°C for at least 2 h, demonstrating a high *in vitro* stability of these radioconjugates.

3.4. In Vitro Cell Binding. The binding affinities (K_d) for ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates for both MUC1 and folate receptors were evaluated using the KB human carcinoma cell line. The K_d values of these bioconjugates were determined by saturation assays (Figure 2). The results demonstrate that the conjugate ^{68}Ga-NODAGA-MUC1-FA hybrid peptide has more than twofold higher binding affinities toward the KB cancer cell line than ^{68}Ga-NODAGA-MUC1 conjugate (11.56 ± 2.05 nM and 26.18 ± 5.78 nM). This result indicates that binding affinity for the new bioconjugates is similar to the same pattern of their corresponding fluorine-18 (^{18}F) labeled conjugates when evaluated using MCF7 breast cancer cell line [31]. It is generally expected that hybrid molecules, capable of targeting dual receptor systems, may improve the tumor-targeting efficacy of the compound by increasing the accumulation of radioactivity in the tumors. This is because more tumor cells would be targeted with hybrid radioligand than would be possible with only a single radioligand. The same trend was true for ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate, where the affinity increased twofold after the conjugation of MUC1 to folic acid. Additional studies are required to get a better insight into this binding variation.

3.5. In Vivo Biodistribution and Tumor Uptake. The results of biodistribution studies in normal Balb/c mice for both ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates at 15, 60, and 120 min after injection (p.i.) are presented in Table 1. The results of biodistribution for both ^{68}Ga-NODAGA-MUC1 peptide conjugate and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate are comparable, and both peptide conjugates generally demonstrate fast and efficient clearance from the blood and most of the organs and tissues. Significant radioactivity accumulation in the kidneys for both conjugates was observed, suggesting that the route of elimination was predominantly urinary system. These results are in agreement with the partition coefficient measurements performed previously.

Biodistribution studies in female nude mice bearing human KB cell line xenografts for ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates at 15, 60, and 120 min p.i. are presented in Table 2. Both conjugates demonstrated fast and efficient clearance from the blood as observed in normal Balb/c mice. Decent tumor uptake was observed for ^{68}Ga-NODAGA-MUC1 peptide conjugate (2.75 ± 0.23% ID/g) at 15 min p.i. and this uptake was significantly reduced at 60 and 120 min p.i. to 0.71 ± 0.52 and 0.35 ± 0.1% ID/g, respectively. It is worth mentioning that the biodistribution and tumor uptake profiles of ^{68}Ga-NODAGA-MUC1 peptide conjugate are comparable to the ^{99m}Tc-MAG$_3$-MUC1 peptide reported previously in T47D and MCF7 tumor xenografts [21].

The hybrid ^{68}Ga-NODAGA-MUC1-FA peptide conjugate displayed an excellent tumor uptake of 12.92 ± 4.38 and 4.85 ± 0.17% ID/g at 15 and 60 min p.i., respectively. This uptake appeared to be more than fourfold higher than uptake for its corresponding monomeric counterpart ^{68}Ga-NODAGa-MUC1 peptide conjugate and mainly attributed to the dual receptor targeting action of this hybrid peptide conjugate. The uptake values in the tumor were always higher than the radioactivity in other organs except for kidneys. The higher radioactivity uptake in kidneys is likely a result of the presence of folate receptors in the proximal tubules. Tumor-to-blood and tumor-to-muscle ratios obtained at 15 and 60 min p.i. were 2.07, 10.34, and 3.19, 8.37, and 6.38, respectively. The high uptake by the tumors combined with good tumor to background uptake ratios indicating the potential of ^{68}Ga-NODAGA-MUC1-FA hybrid peptide over ^{68}Ga-NODAGA-MUC1 peptide conjugate for targeting human ovarian cancer.

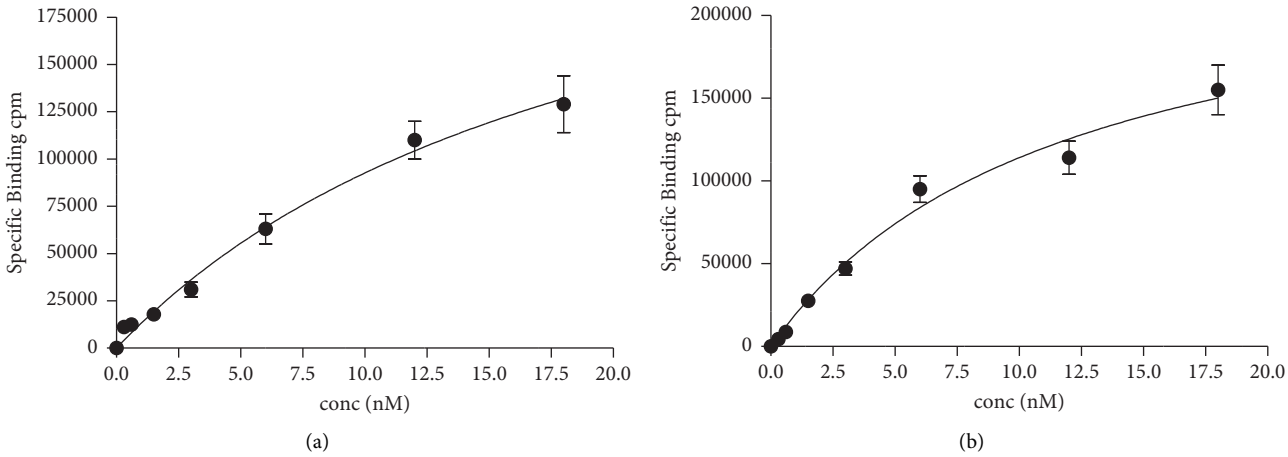


Figure 2: Determination of binding affinity (K_d) values of ^{68}Ga-NODAGA-MUC1 peptide (a), ^{68}Ga-NODAGA-MUC1-folate hybrid peptide (b), and conjugates in the KB human cancer cell line.

Table 1: Biodistribution of ^{68}Ga-NODAGA-MUC1-FA hybrid and ^{68}Ga-NODAGA-MUC1 peptide conjugates in normal Balb/c mice.

	^{68}Ga-labeled MUC1-folate hybrid peptide			^{68}Ga-labeled MUC1 peptide		
	15 min	60 min	120 min	15 min	60 min	120 min
Blood	3.78 ± 1.16	1.34 ± 0.13	0.82 ± 0.31	3.43 ± 0.66	0.79 ± 0.40	0.18 ± 0.07
Liver	1.32 ± 1.10	1.58 ± 1.32	0.92 ± 0.12	2.22 ± 0.34	1.06 ± 0.30	0.46 ± 0.14
Lung	1.34 ± 1.02	1.94 ± 1.19	1.38 ± 0.34	2.61 ± 0.59	0.98 ± 0.18	0.23 ± 0.08
Kidney	13.00 ± 1.21	5.34 ± 1.99	1.19 ± 0.40	16.16 ± 0.75	8.07 ± 0.36	5.56 ± 0.74
Intestine	0.76 ± 0.39	1.39 ± 0.66	0.66 ± 0.34	2.07 ± 0.52	1.24 ± 0.69	0.52 ± 0.06
Heart	1.35 ± 0.83	0.64 ± 0.54	0.54 ± 0.33	2.58 ± 0.38	0.63 ± 0.23	0.14 ± 0.04
Muscle	0.82 ± 0.43	0.31 ± 0.59	0.59 ± 0.46	1.73 ± 0.27	0.94 ± 0.66	0.15 ± 0.05
Bone	0.78 ± 0.34	0.25 ± 0.52	0.52 ± 0.12	0.70 ± 0.26	0.99 ± 0.57	0.42 ± 0.15
Spleen	1.47 ± 0.94	1.13 ± 0.49	0.66 ± 0.07	1.75 ± 0.38	1.31 ± 0.12	0.67 ± 0.21

The values are average of % injected dose/gram ± SD for $n = 4$.

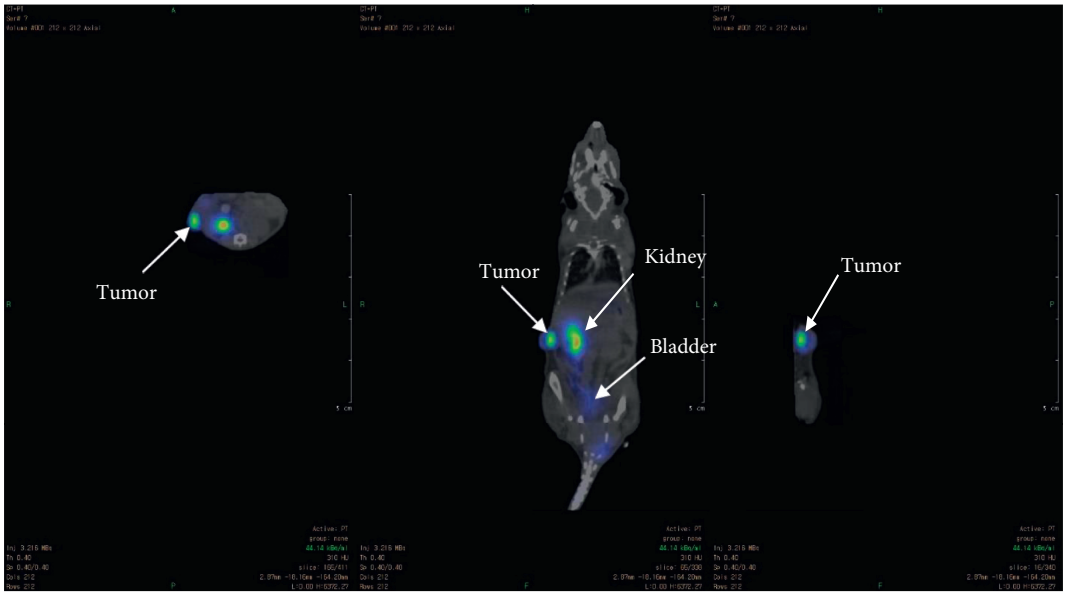
Table 2: Biodistribution of ^{68}Ga-NODAGA-MUC1-FA hybrid and ^{68}Ga-NODAGA-MUC1 peptide conjugates in KB tumor-bearing nude mice.

	^{68}Ga-labeled MUC1-folate hybrid peptide			^{68}Ga-labeled MUC1 peptide		
	15 min	60 min	120 min	15 min	60 min	120 min
Blood	6.24 ± 1.16	1.52 ± 0.27	0.47 ± 0.13	4.63 ± 1.29	1.03 ± 0.91	0.18 ± 0.01
Liver	1.95 ± 0.09	1.72 ± 0.20	1.18 ± 0.20	3.10 ± 0.47	2.53 ± 0.94	0.55 ± 0.01
Lung	2.52 ± 0.85	0.91 ± 0.11	0.76 ± 0.21	3.21 ± 0.77	1.11 ± 1.15	0.34 ± 0.09
Kidney	18.40 ± 5.55	7.58 ± 0.69	6.55 ± 0.43	16.76 ± 1.85	7.58 ± 5.58	4.98 ± 0.44
Intestine	1.58 ± 1.07	1.99 ± 0.43	1.50 ± 0.55	1.34 ± 0.45	1.44 ± 1.82	0.85 ± 0.99
Heart	1.30 ± 0.62	0.51 ± 0.16	0.56 ± 0.22	2.26 ± 0.02	0.53 ± 0.42	0.53 ± 0.05
Muscle	1.25 ± 0.15	0.76 ± 0.45	0.36 ± 0.07	1.19 ± 0.10	0.39 ± 0.24	0.25 ± 0.07
Bone	1.50 ± 0.35	0.37 ± 0.14	0.17 ± 0.05	1.53 ± 0.38	1.72 ± 0.15	0.45 ± 0.15
Spleen	1.32 ± 0.33	0.76 ± 0.05	0.53 ± 0.16	1.37 ± 0.41	1.16 ± 0.66	1.12 ± 0.47
Tumor	12.92 ± 4.38	4.85 ± 0.17	0.89 ± 0.03	2.75 ± 0.23	0.71 ± 0.52	0.35 ± 0.01

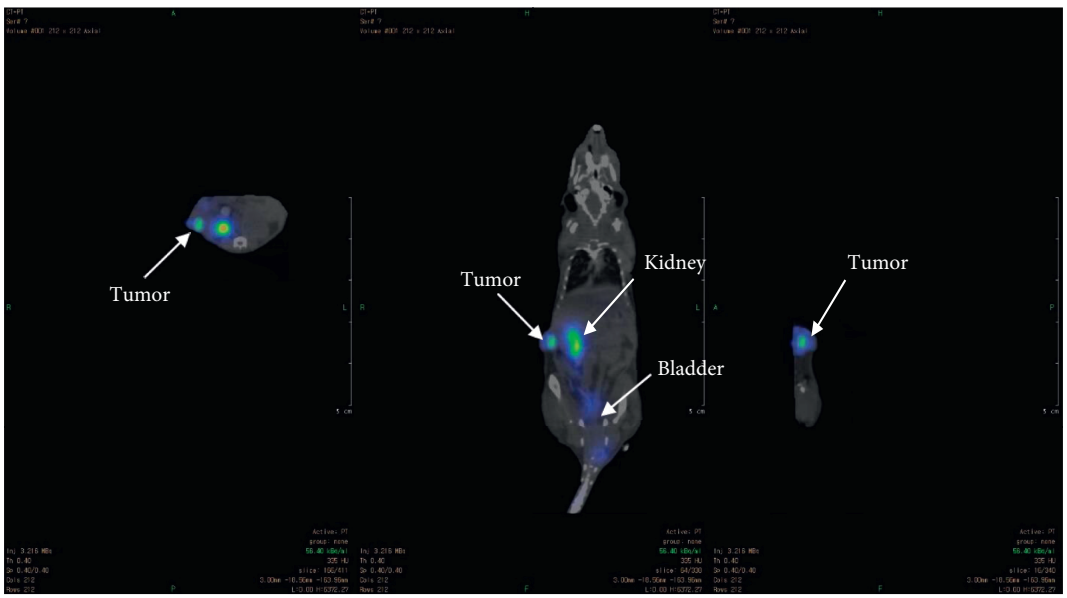
The values are average of % injected dose/gram ± SD for $n = 4$.

In a blocking study where 100 μg of the full-sequence MUC1-FA peptide was administered 30 min before the injection of ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate, the high tumor uptakes at 15 and 60 min p.i. were dramatically decreased (12.92 ± 4.38% versus 1.37 ± 0.22% and 4.85 ± 0.17 versus 0.63 ± 0.06 ID/g, $P = 0.02$) indicating the specificity of the ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate for the folate- and MUC1-positive cancer. A marked influence of the blocking dose was observed also in kidneys which is a result of the masking of folate receptors in the proximal tubules.

The rapid clearance from the blood and the excretion mainly by the renal pathway for ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1 hybrid peptide conjugates is

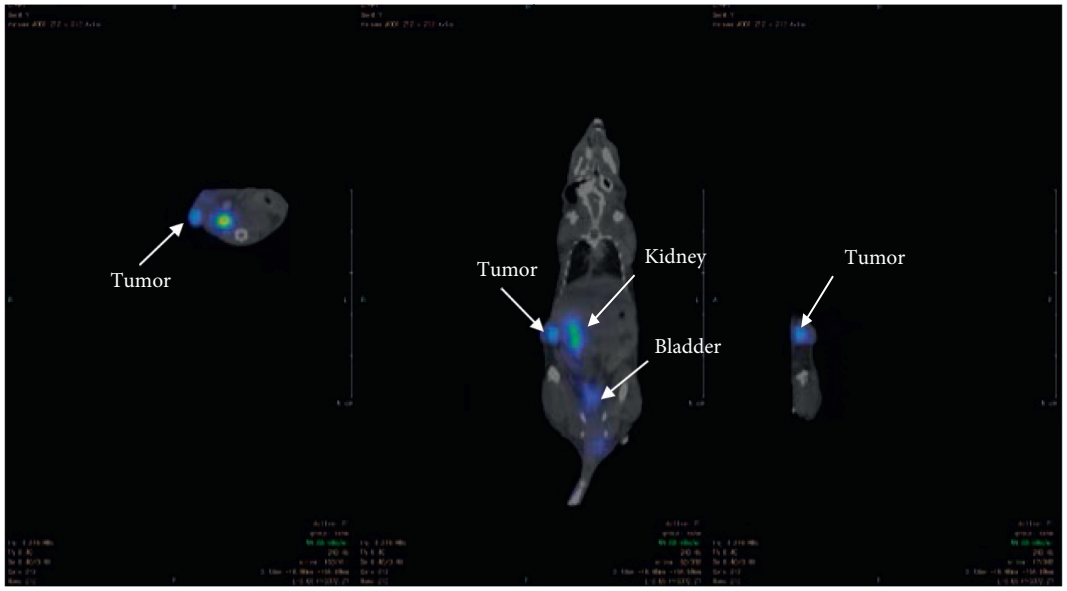


(a)



(b)

Figure 3: Continued.



(c)

Figure 3: Coronal, sagittal, and axial images of tumor-bearing mouse after 0.5 (a), 1 (b), and 2 h (c) after injection using 5 MBq ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate.

attributed to the hydrophilic characteristic of these conjugates. The amounts of the radioactivity of all the conjugates excreted into the urine at the time of sacrifice (60 and 120 min p.i.) were collected and examined by HPLC to determine the *in vivo* stability of ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1 hybrid peptide conjugates. Radio-HPLC analysis of the urine samples showed that a significant amount of radioactivity (>95%) was still associated with the radioconjugates. These findings demonstrate that these radiolabeled conjugates are not prone to *in vivo* rapid degradation and correlate well with the findings of high metabolic stability in human plasma *in vitro*.

3.6. In Vivo Nano-PET/CT Imaging. Due to the excellent tumor uptake of the ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate, the initial tumor-targeting efficacy and pharmacokinetic pattern of this peptide conjugate were evaluated in nude mice bearing subcutaneous KB cancer cell line xenografts at 30, 60, and 120 min with static scans. The uptake of tumors and major organs was quantified based on the analysis of Nano-PET/CT images. The tumor uptake image of ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate after 30 min p.i. was visible and uptake starts to wash out at 60 min p.i. (Figure 3). These images are per the finding obtained in quantitative biodistribution data reported in Table 2. The favorable biodistribution profile of ^{68}Ga labeled MUC1-folate hybrid peptide conjugate warrants further evaluation and may tempt one to infer that this PET hybrid radiotracer may be useful as a dual receptor-targeting PET imaging molecular probe for ovarian cancer detection and monitoring tumor response to the treatment.

4. Conclusion

The synthesis of ^{68}Ga-NODAGA-MUC1 and ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugates was performed using a simple one-step reaction. The overall radiochemical yields and purities for these radioconjugates were quantitative with a total synthesis time of less than 20 min. *In vitro* binding studies on human KB cell lines showed superior affinity of ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate over only ^{68}Ga-NODAGA-MUC1 peptide conjugate MUC1 and folate receptors. Biodistribution studies in normal mice revealed rapid blood clearance of these radioconjugates with excretion primarily by the urinary system. In nude mice model bearing human KB cell line xenografts, ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate demonstrated significant tumor uptake and favorable pharmacokinetics over ^{68}Ga-NODAGA-MUC1 peptide conjugate. These observations were confirmed by initial Nano-PET/CT imaging with a high accumulation of radioactivity in the tumor. These results demonstrate that ^{68}Ga-NODAGA-MUC1-FA hybrid peptide conjugate may be useful as a dual receptor-targeting PET imaging probe for ovarian cancer detection and monitoring tumor response to the treatment; however, further evaluation is warranted.

Data Availability

The HPLC analytical data and mass spectrometry data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare no conflicts of interest regarding the publication of this article.

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