Original Article

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Development and radiosynthesis of a novel bifunctional tri-γ-glutamic acid polypeptide for gallium-68 labeling

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ABSTRACT

Radiosynthesis of a novel bifunctional chelator for gallium-68 (Ga-68) chelation has been fabricated through peptide synthesis. Using of solid phase peptide synthesizer, linear structure of three repeating unit of glutamic acid was primarily generated. Protecting group of tert-butyl in intermediate was then cleaved and eventually yield as tri-γ-glutamic acid polypeptide (tri-γ-GAP). Mass spectrometry revealed m/z was 406.1378 that well corresponded with proposed structure of three repeating unit of glutamic acids. To label Ga-68 with synthesized tri-γ-GAP, the elution of $^{68}GaCl_3$ was chelated to tri-γ-GAP with high temperature condition. It was subsequently purified through Sep-Pak C18 cartridge. Radio thin-layer chromatography demonstrated that the labeling efficiency of ^{68}Ga-tri-γ-GAP was 3.5–3.8 mCi with radiochemical purity more than 98%. The pH was 4.5–5.0. Conclusively, synthesized tri-γ-GAP comprising three N-dentate and four OH-dentate. One more free OH-dentate of tri-γ-GAP provided more applicability over universal chelator of (*N,N',N"*-1,4,7,-triazacyclononane-1,4,7-triyl)triacetic acid (NOTA) on Ga-68 labeling. It is able to link with pharmacological substances without any further modification whereas NOTA needs to be synthesized a specific linker for an attaching with bioactive pharmacological molecule. Novel small peptide chelator of tri-γ-GAP is a promising molecule for Ga-68 labeling with versatile application in radiopharmaceutical diagnosis.

Keywords: Bifunctional chelator, Ga-68 labeling, gallium-68, glutamic acid polypeptide, radiometal

INTRODUCTION

Positron emission tomography (PET) imaging using radiometal radiopharmaceuticals is gaining an interest in the field of nuclear medicine, especially among PET centers, where cyclotron facility is not established. To date, gallium-68 (Ga-68) has been drawing the attention of many research groups worldwide as PET radiometal of choice due to its nuclear properties in medical application, for example, short half-life of 68 minutes, easy Ga-68 milking using well developed commercially available ^{68}Ge/^{68}Ga generator, high radiochemical yield labeling, and high specific activity product. Furthermore, ^{68}Ga-based radiopharmaceuticals enroll in diagnostic part of theranostics which is already used in clinical practice such as ^{68}Ga-PSMA for prostate cancer imaging, ^{68}Ga-DOTATATE for neuroendocrine imaging.[1-4]

The oxidation state (III) of Ga-68 ion is obtained in acidic aqueous solution by eluting from ^{68}Ge/^{68}Ga generator using hydrochloric acid in 0.05–0.1 M concentration depend on the type of generator. Labeling procedure successfully occurs under pH below 3.3 to form a stable complex with bifunctional chelator which is divided into two groups; (1) non-macrocyclic chelator, for example, DTPA, HBED, and Dedpa and (2) macrocyclic chelator, for example, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and (*N,N',N"*-1,4,7,-triazacyclononane-1,4,7-triyl) triacetic acid (NOTA).[5,6] Amidst chelators, DOTA is the most frequently used in theranostics [Figure 1].[7] In fact, DOTA is

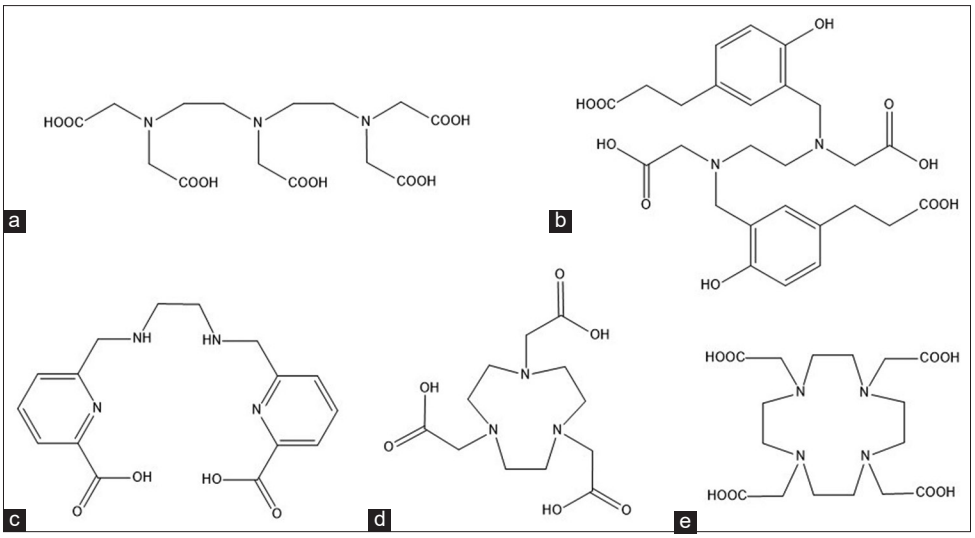

Figure 1: Bifunctional Ga-68 chelators; (a) DTPA, (b) HBED, (c) Dedpa, (d) NOTA, (e) DOTA

not the most suitable chelator for Ga-68 because Ga-68 ion is too small to fit in the DOTA cavity with its low stability constant (log K_{cal} = 21.3) while it is more suitable to chelate to bigger radiometal radii of lutetium-177 or actinium-225. However, it is optimal chelator to serve both diagnostic and therapeutic purpose under theranostic concept. On the other hand, NOTA is the most applicable for Ga-68 labeling due to its smaller cyclic cavity with higher stability constant (log K_{cal} = 31.0).

Recent reports revealed that some peptide-based molecules have a potential to develop as a new bifunctional chelator which is able to chelate Ga-68.[8,9] The excitatory glutamic acid is a potent neurotransmitter in the central nervous system. Glutamic acid polypeptide (GAP) exerts its action through binding to glutamate receptors. GAP has been commonly employed as a targeted carrier, which conjugates to an anti-cancer agent, a chemotherapeutic agent through peptide or ester linkage. Moreover, its acid residues could chelate to radiometal isotopes such as Tc-99m and Ga-68 for imaging application. The previous study reported that a commercially available of poly-L-glutamate (MW. 750–40,000) is not suitable for imaging needs because high molecular weight (MW) leads to occasionally difficult explanation of experiment results. Moreover, the heterogeneity of MW of commercially available poly-L-glutamate has usually been found that most eventually to the inconsistent formation of the chelated products. Herein, we rationally designed a new GAP chelator to avoid complicated debates by limiting peptide size to three glutamic acid moieties. Therefore, tri-γ-GAP is a mimic chemical structure of NOTA to serve an assessment of precise Ga-68 chelation.

MATERIALS AND METHODS

Materials

All solvents and reagents were purchased and used without further purification unless otherwise noted. Fmoc-Glu(*tBu*)-OH was purchased from Merck. 4-(4-hydroxymethyl-3-methoxyphenoxy) butyric acid (HMPB) resin, polymer-bound to ChemMatrix® resin, triisopropylsilane (TIS), carbodiimide (DIC), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich. N,N'-dimethylformamide (DMF), dichloromethane (DCM), diethylether (Et_2O), and trifluoroacetic acid (TFA) were purchased from Tokyo Chemical Industry, Japan. Sep-Pak C18 cartridge was purchased from Waters Co., Ltd., USA. $^{68}GaCl_3$ was eluted using 0.05 N HCl from $^{68}Ge/^{68}Ga$ generator (ITG). Hydrochloric acid (Sigma-Aldrich, USA) 99.999% trace metals basis was used to elute the ITG $^{68}Ge/^{68}Ge$ Generator. MQuant® pH-indicator strips were purchased from Merck.

Synthesis of Tri-γ-GAP

Linear tri-γ-GAP was assembled on solid support using solid-phase peptide synthesis (SPPS) (CS Bio, CS136X, USA). Synthetic step regarding tripeptide formation was shown as following:

- Step 1: Initiation of an attachment of the first glutamic acid residue on the resin support through C-terminus
- Step 2: Fmoc deprotection of the N-terminus of first glutamic acid
- Step 3: Peptide bonding of the second glutamic acid in conjunction with Fmoc deprotection
- Step 4: Final attachment of the third glutamic acid by peptide formation
- Step 5: Cocktail cleavage of synthesized tripeptide

In depth detail, the synthesis began with resin swelling preparation described hereinafter.

(1) DMF 15 mL was added to HMPB resin 0.347 g. After heating at 70°C for 20 min, the solvent was drained to waste. Subsequently, the resin was deprotected Fmoc using 20% piperidine. To the deported resin vessel, (2) Fmoc-Glu(*tBu*)-OH 1.73 g., DIC 2.0 mL, and DMAP 0.2 mL were added, reacted 1 h at room temperature for bonding the first glutamic acid to resin. Drained the solvent and washed with DMF 4.5 mL 3 times, DCM 4.5 mL 3 times, and DMF 4.5 mL 3 times, deprotected Fmoc using 20% piperidine. (3) To the reaction vessel, Fmoc-Glu(*tBu*)-OH, DIC 2.0 mL, and

0.5M Oxyma 0.2 mL were added, reacted at 75°C 15 min for bonding the second glutamic acid. Drained the solvent and washed with DMF 4.0 mL 4 times, deprotected Fmoc using 20% piperidine. (4) Repeated step (3) for bonding the third glutamic acid. Drained the solvent and washed with DMF 4.0 mL 4 times, deprotected Fmoc using 20% piperidine. (5) Crude peptide with resin was washed with DCM 4.5 mL 6 times, dried 1 h at room temperature. (6) Cleavage of peptide from resin and *tBu* group deprotection was performed by adding TFA:TIS:H_2O (95:2.5:2.5) 5 mL, reacted 3 h at room temperature. The solution was removed to 50 mL falcon tube, rinsed to wash the reaction vessel with TFA 1.5 mL 3 times. (7) Cold Et_2O (–20°C) was added to the falcon tube to 50 mL and vortexed well. (8) Centrifuged at 4000G for 5 min at below –4°C. Repeating the step (6) and (7) for 2 times. (9) Collected the product of tri-γ-GAP and dried it overnight at room temperature.

Radiosynthesis of ^{68}Ga-tri-γ-GAP

$^{68}GaCl_3$ was eluted with 0.05 N HCl from $^{68}Ge/^{68}Ga$ generator (ITG). Pre-treatment of Sep-Pak C18 cartridge was performed by eluting ethanol (EtOH) 2.0 mL. To the solution of tri-γ-GAP 50 µg in 100 µl H_2O and acetate buffer 1.5 mL, elution of $^{68}GaCl_3$ 4.0 mL 7.0 mCi was added, the solution was mixed and heated at 90°C for 15 min. After cooling, the crude labeled product was trapped in Sep-Pak C18 cartridge and later washed with 5 mL of normal saline solution. Sep-Pak C18 cartridge was then eluted with EtOH: H_2O (1:1) 2 mL. The labeling efficiency was determined by Radio thin-layer chromatography (RTLC) using 1 M NH_4OAc: MeOH (1:1) and radioactivity was measured by gamma well counter. The pH of ^{68}Ga-tri-γ-GAP was also measured using pH-indicator strips.

RESULTS

Chemistry

The protected tri-γ-GAP was effectively synthesized through SPPS using a standard Fmoc protocol in peptide synthesizer. Well-established SPPS methodology rapidly provided the Fmoc protected tri-γ-GAP through employing 3–5 equivalents of reagents. Subsequently, manual cleavage of the remaining *tBu*-protecting groups using cocktail of TFA:TIS:H_2O to give tri-γ-GAP. Total yield of synthesized product was 24%. Due to the simple and short linear peptide structure, carefully washing the final product after centrifuge as mention in protocol was performed as purification step. The physical appearance of synthesized product was found to be a light to pale yellow powder. The identity of tri-γ-GAP was confirmed by mass spectroscopy. The tri-γ-GAP was examined by MS without further purification. HRMS (ESI) calculated for $C_{15}H_{23}N_3O_{10}$ 405.1383, found: 406.1378 $[M+H]^+$.

Radiochemistry

Preparation of Ga-68 labeling of tri-γ-GAP was adapted from the standard Ga-68 labeling of PSMA-HBED-CC. $^{68}GaCl_3$ was eluted with 0.05N HCl from a $^{68}Ge/^{68}Ga$ generator (ITG, Germany), in highly acidic Ga-68 solution. To obtain quantitative ^{68}Ga-tri-γ-GAP, 50 µg of tri-γ-GAP was dissolved in 100 µL H_2O, mixed with acetate buffer and heated at 90°C for 15 min. After to cool down to room temperature, the crude product was purified by Sep-Pak C-18 cartridge resulted in radiochemical purity >98% [Figure 2]. Radiochemical yield was 77%. RTLC was employed to confirm radiochemical purity using NH_4OAc:H_2O (1:1), Rf = 0.75. The radioactivity of ^{68}Ga-tri-γ-GAP was found to be 3.5–3.8 mCi (n = 5), resulting in specific activity of ^{68}Ga-tri-γ-GAP in the range of 29.17–31.67 mCi/µmol. In addition, the pH of the radiolabeled product was in the range of 4.5–5.0.

DISCUSSION

The rational design of tri-γ-GAP was mimic the chemical structure of NOTA as the current gold standard for Ga complexation forming N_3O_3 hexadentate at ambient temperature [Figure 3]. To expand the clinical application of NOTA, some NOTA derivatives are modified to provide a branch structure from its carbon skeleton with specific functional group to bond other bioactive pharmaceutical molecule or monoclonal antibodies, for example, p-SCN-Bn-NOTA (C-NOTA),[10,11] NODAGA.[12] Moreover, some NOTA derivatives were designed to increase possessing faster kinetics in labeling or to chelate other bigger radionuclides with $^{86}/^{90}$Y to support theranostic concept, for example, NETA[13-18] and TACN-TM.[19-22]

In spite of versatile chelating complex of NOTA, tri-γ-GAP was intentionally designed to contain three N-dentate

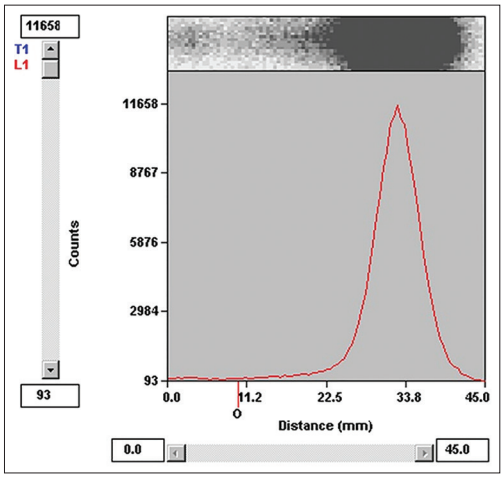

Figure 2: Radio Thin Layer Chromatography of ^{68}Ga-tri-γ-GAP

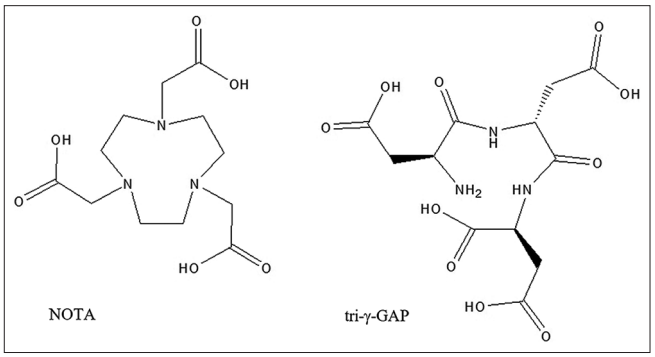

Figure 3: Chemical structure of NOTA and mimic configuration of chemical structure of tri-γ-GAP

and four OH-dentate. It also forms N_3O_3 hexadentate at ambient temperature in the same manner to NOTA, the other one tri-γ-GAP residue together with one OH dentate is available to conjugate to bioactive pharmacological molecules, for example, anti-cancers, through peptide linkage or ester linkage [Figure 4]. Moreover, tri-γ-GAP is a small peptide that enhanced its conjugated molecule to uptake into targeted cell through glutamic acid transporter on cell membrane. The proposed molecular assembly between Ga-68 in conjunction with synthesized tri-γ-GAP was subsequently illustrated in Figure 5. According to the proposed configurational structure of tri-γ-GAP, it has been simulated as shown in Figure 4. It could be seen that the in and out of amide plane of a chiral carbon from each glutamic acid unit had been proposed. Naturally, glutamic acid containing one chiral carbon that eventually resulting in a L-(+)-form configuration. When two or more glutamic acids had been linked together through peptide linkage, tripeptide should be appeared with alternative configuration peptide chain. It was due to the stearic hindrance effect. To maintain its stability, an optimized configurational structure of tri-γ-GAP should be illustrated as Figure 4. Thus, tri-γ-GAP revealed the potential on conjugation to theranostic ligand such as PSMA for prostate cancer and TATE, TOC, and NOC for neuroendocrine tumor. In advance, tri-γ-GAP can also be expected to utilize as a novel small peptide chelator in future clinical application.

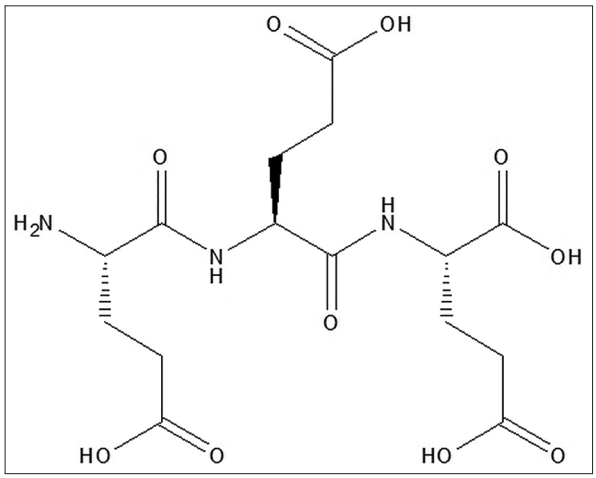

Figure 4: Design of tri-γ-GAP

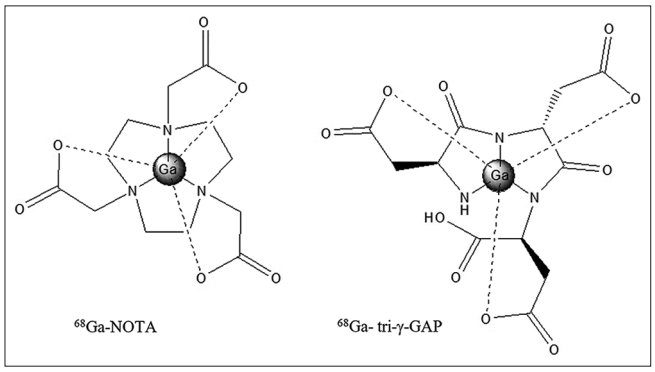

Figure 5: Proposed complexation of ^{68}Ga-NOTA and ^{68}Ga- tri-γ-GAP

CONCLUSION

Tri-γ-GAP was successfully developed as a novel bifunctional peptide-based chelator for Ga-68 which was able to conjugate with bioactive pharmaceutical molecule through either peptide linkage or ester linkage. The complexation of ^{68}Ga-tri-γ-GAP was formed with facile condition of 90°C for 15 min. In addition, the confirmation method of this radiolabeling is also simply detected using RTLC. Therefore, tri-γ-GAP showed a high potential to apply for the theranostic approach. Furthermore, chelation between anticancer drugs with tri-γ-GAP has now been propositioned to employ as targeted drug delivery system for novel oncological therapy. Another application of tri-γ-GAP is essentially appeared in neurological system. Most of neurological therapeutic agents showed the high lipophilicity that was an important obstacle on the delivery pathway to the target site in the brain. If they can be increased their hydrophilicity by chelating with hydrophilic chelator such as tri-γ-GAP, the new complex will be formulated and passing through the brain efficiently. Even a high hydrophilic compound generally shows a less ability to come across the blood–brain barrier, tri-γ-GAP-neurological drug complex in which determined as a more hydrophilic should better expressed a sufficient neuronal transportation. It should pronounce a promising on the brain transport because of glutamic residue through amino acid carrier-mediated active transport.

ACKNOWLEDGMENTS

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REFERENCES

1. Al-nahhas A, Win Z, Szyszko T, Singh A, Nanni C, Fanti S, *et al*. Gallium-68 PET: A new frontier in receptor cancer imaging. Anticancer Res 2007;27:4087-94.
2. Lenzo NP, Meyrick D, Turner JH. Review of Gallium-68 PSMA PET/CT imaging in the management of prostate cancer. Diagnostics 2018;8:16-32.
3. Martiniova L, De Palatis L, Etchebehere E, Ravizzini G. Gallium-68 in medical imaging. Curr Radiopharm 2016;9:187-207.
4. Velikyan I. Prospective of ^{68}Ga-radiopharmaceutical development. Theranostics 2014;4:47-80.
5. Spang P, Herrmann C, Roesch F. Bifunctional gallium-68 chelators: Past, present and future. Semin Nucl Med 2016;46:373-94.
6. Martell AE, Motekaitis RJ, Clarke ET, Delgado R, Sun R, Ma R. Stability constants of metal complexes of macrocyclic ligands with pendant donor groups. Supramol Chem 1996;6:353-63.
7. Price EW, Orvig C. Matching chelators to radiometals for radiopharmaceuticals. Chem Soc Rev 2014;43:260-90.
8. Theeraladanon C, Takahashi N, Shiina M, Hamada K, Takada Y, Endo H, *et al*. Rational approach to the synthesis, evaluation, and ^{68}Ga labeling of a novel 4-anilinoquinoline epidermal growth factor receptor inhibitor as a new imaging agent that selectively targets the epidermal growth factor receptor tyrosine kinase. Cancer Biother Radiopharm 2010;25:479-85.
9. Takahashi N, Yang DJ, Kohanim S, Oh CS, Yu DF, Azhdarinia A, *et al*. Targeted functional imaging of estrogen receptors with ^{99m}Tc-GAP-EDL. Eur J Nucl Med Mol Imaging 2007;34:354-62.
10. McMurry TJ, Brechbiel M, Kumar K, Gansow OA. Convenient synthesis of bifunctional tetraza macrocycles. Bioconjugate Chem 1992;3:108-17.
11. McMurry TJ, Brechbiel M, Wu C, Gansow OA. Synthesis of 2-(p-thiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic

acid: Application of the 4-methoxy-2,3,6-trimethylbenzene sulfonamide protecting group in the synthesis of macrocyclic polyamines. Bioconjugate Chem 1993;4:236-45.
12. Eisenwiener KP, Prata MI, Buschmann I, Zhang HW, Santos AC, Wenger S, *et al*. NODAGATOC, A new chelator-coupled somatostatin analogue labeled with [$^{67/68}$Ga] and [^{111}In] for SPECT, PET, and targeted therapeutic applications of somatostatin receptor (hsst2) expressing tumors. Bioconjugate Chem 2002;13:530-41.
13. Kang CS, Sun X, Jia F, Song HA, Chen Y, Lewis M, *et al*. Synthesis and preclinical evaluation of bifunctional ligands for improved chelation chemistry of^{90}Y and^{177}Lu for targeted radioimmunotherapy. Bioconjugate Chem 2012;23:1775-82.
14. Chong HS, Milenic DE, Garmestani K, Brady ED, Arora H, Pfiester C, *et al*. In vitro and in vivo evaluation of novel ligands for radioimmunotherapy. Nucl Med Biol 2006;33:459-67.
15. Chong HS, Song HA, Birch N, Le T, Lim S, Ma X. Efficient synthesis and evaluation of bimodal ligand NETA. Bioorg Med Chem Lett 2008;18:3436-9.
16. Chong HS, Song HA, Ma X, Milenic DE, Brady ED, Lim S, *et al*. Novel bimodal bifunctional ligands for radioimmunotherapy and targeted MRI. Bioconjugate Chem 2008;19:1439-47.
17. Chong HS, Garmestani K, Ma D, Milenic DE, Overstreet T, Brechbiel MW. Synthesis and biological evaluation of novel macrocyclic ligands with pendent donor groups as potential Yttrium chelators for radioimmunotherapy with improved complex formation kinetics. J Med Chem 2002;45:3458-64.
18. Kang CS, Song HA, Milenic DE, Baidoo KE, Brechbiel MW, Chong HS. Preclinical evaluation of NETA-based bifunctional ligand for radioimmunotherapy applications using^{212} Bi and^{213} Bi: Radiolabeling, serum stability, and biodistribution and tumor uptake studies. Nucl Med Biol 2013;40:600-5.
19. Chappell LL, Deal KA, Dadachova E, Brechbiel MW. Synthesis, conjugation, and radiolabeling of a novel bifunctional chelating agent for^{225}Ac radioimmunotherapy applications. Bioconjugate Chem 2000;11:510-9.
20. Boswell CA, Regino CA, Baidoo KE, Wong KJ, Bumb A, Xu H, *et al*. Synthesis of a cross-bridged cyclam derivative for peptide conjugation and ^{64}Cu radiolabeling. Bioconjugate Chem 2008;19:1476-84.
21. Stigers DJ, Ferdani R, Weisman GR, Wong EH, Anderson CJ, Golen JA, *et al*. A new phosphonate pendant-armed cross-bridged tetraaminechelator accelerates copper (II) binding for radiopharmaceutical applications. Dalton Trans 2010;39:1699-701.
22. Velikyan I, Maecke H, Langstrom B. Convenient preparation of^{68}Ga-based PET-radiopharmaceuticals at room temperature. Bioconjugate Chem 2008;19:569-73.