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REVIEW

Chelators for ^{68}Ga radiopharmaceuticals

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ABSTRACT

^{68}Ga is a promising radionuclide for positron emission tomography (PET). It is a generator-produced (^{68}Ge/^{68}Ga-generator) radionuclide with a half-life of 68 min. The employment of ^{68}Ga for basic research and clinical applications is growing exponentially. Bifunctional chelators (BFCs) that can be efficiently radiolabeled with ^{68}Ga to yield complexes with good in vivo stability are needed. Given the practical advantages of ^{68}Ga in PET applications, gallium complexes are gaining increasing attention in biomedical imaging. However, new ^{68}Ga-labeled radiopharmaceuticals that can replace ^{18}F-labeled agents like [^{18}F]fluorodeoxyglucose (FDG) are needed. The majority of ^{68}Ga-labeled derivatives currently in use consist of peptide agents, but the development of other agents, such as amino acid or nitroimidazole derivatives and glycosylated human serum albumin, is being actively pursued in many laboratories. Thus, the availability of new ^{68}Ga-labeled radiopharmaceuticals with high impact is expected in the near future. Here, we present an overview of the different new classes of chelators for application in molecular imaging using ^{68}Ga PET.
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Key Words: ^{68}Ga, Radiochemistry, Coordination chemistry, Bifunctional chelators, Cyclic and acyclic chelators

Introduction

Positron emission tomography (PET) is a nuclear imaging technique applied for diagnosis of different types of cancer such as colorectal cancer, melanoma, head and neck cancer, lung cancer, breast cancer, and prostate cancer, due to its wide scope and high sensitivity (1-7). ^{68}Ga ($t_{1/2}$ = 68 min, 89% β+, $E_{\beta+max}$ = 1.92 MeV, 11% EC) is a generator-produced radionuclide that offers excellent coordination chemistry with a wide range of bifunctional chelating agents. It also provides rapid radiolabeling in various pH ranges (8-15).

Recent studies have shown that some ^{68}Ga-labeled peptides provide distinctly better images than their ^{111}In-labeled analogues (16-19) and ^{18}F-based radiotracers (20). Unlike other PET radioisotopes, like ^{18}F or ^{11}C, ionic Ga^{3+} cannot be bound covalently to targeting vectors but must be conjugated to a target vector using bifunctional chelators (BFCs). Nevertheless, labeling can be performed just prior to diagnostic examinations, with minimum loss of radioactivity. The only stable chemical form of Ga in solution under physiological conditions is Ga^{3+}, and it can form stable complexes with chelators that are either free or conjugated with macromolecules or small organic molecules (10,21).

The principle requirement for such chelators is that they are able to form stable complexes with Ga^{3+}, preferably of octahedral geometry. Association kinetics must be fast and the reaction has to desirably take place at room temperature; on the other hand, dissociation kinetics must be slow (22). Charge

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and lipophilicity can be adjusted depending on the application. Two types of chelators, macrocyclic and open chain chelators, have been considered. Although the latter commonly form unstable complexes, they still generate great interest due to the need for fast complexation kinetics at room temperature. However, newly formed gallium complexes should be resistant to hydrolysis and more stable than Ga^{3+}-transferrin complexes. The high-value formation constant of Ga^{3+}-transferrin (log K= 20.3) (23) and the high plasma concentration of the protein (0.25 g/100 mL) favor the thermodynamic exchange of Ga^{3+} complexes with transferrin *in vivo*. Therefore, the majority of radioactive gallium complexes used as radiopharmaceuticals have high thermodynamic and kinetic stabilities.

Several ^{68}Ga-based radiopharmaceuticals have been developed conjugating peptides, proteins, or small biological molecules to BFCs through active esters, isothiocyanates, maleimides, hydrazides, or haloamides (Table 1). ^{68}Ga-1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetraacetic acid-Tyr3-octreotide (DOTA-TOC), ^{68}Ga-DOTA-1-Nal-octreotide (DOTA-NOC), ^{68}Ga-DOTA-bombesin, ^{68}Ga-1,4,7-triazacyclononanetriacetic acid (NOTA)-RGD, ^{68}Ga-DOTAalbumin, and ^{68}Ga-DOTA-human epidermal growth factor (hEGF) are examples of such agents (9,21,23-29). Similarly, some agents, such as ^{68}Ga-[$(4,6\text{-}MeO_2sal)_2$-BAPEN]+ and ^{68}Ga-N_2S_2, are chelates of radioactive gallium and used for myocardial imaging (15,30-32). NOTA provide the possibility for kit type preparations of radiopharmaceuticals and labeling of temperature-labile molecules (15,25).

Acyclic BFCs, such as diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), desferrioxamine (DFO), N,N'-di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid (HBED), and their derivatives, have been used for the labeling of macromolecules with ^{111}In, $^{67/68}$Ga, or ^{90}Y for tumor imaging and therapy (33-36). The similarity in coordination chemistry between Fe^{3+} and Ga^{3+} suggests the use of siderophore-like chelators for gallium. Desferrioxamine-B (DFO-B) has already been used as a bifunctional chelator for ^{68}Ga, however it displays disadvantages like low blood clearance of ^{68}Ga-DFO-octreotide. The acyclic ligand DTPA complexes gallium with high affinity but weak kinetic stability (37). Derivatizing the carbon backbone of DTPA can increase the stability of ^{68}Ga complexes. Nevertheless, these ^{68}Ga complexes undergo significant dissociation in serum (38). However, the majority of these complexes have low *in vivo* and *in vitro* stability due to their tendency to undergo acid- or cation-promoted dissociation (39,40). However, ^{67}Ga-labeled tripodal 3-hydroxy-4-pyridinone [NTP $(PrHP)_3$, Figure 7] showed *in vivo* stability and fast renal excretion in healthy rats (41). These, limitations are overcome using macrocyclic BFCs, such as NOTA, DOTA, or 1,4,8,11-tetraazacyclotetradecanetetraacetic acid (TETA), which form highly stable complexes with these radiometals (39,42).

This review looks at recent advances in ligand design to fulfill these criteria and developments in radiolabeling methodologies.

Table 1. Reported conditions of gallium chelating agents

Chelator	pH	Temp (°C)	Time (min)	Radiochemical yield (%)	Log K (Ga^{3+})	Reference
NOTA	~4	25	10	99	30.9	(45)
DOTA	2.3 - 4.6	95	10		21.3	(102)
PCTA	4 - 4.5	RT	5	95-98		(62, 64)
TRAP	3 - 5	95	5	99	35.6	(51, 103)
FSC	4.5 - 5.0	25	15	>94		(77)
DATA	3.7 - 7	25	3	>96		(14) (75)
DEDPA	~4.5	25	10	>97	28.1	(89) (90) (104)
HBED	~4.5	25	5	>80	38.5	(87) (105) (106)
CP256	5.5 - 6.5	95	5	>95		(85)
$(NH_2)_2$sar		85	30	>95		(68)
Bis-(thiosemicarbazones)	~3.9	90	30	>70		(78)
6SS		RT	15		41.0	(107)
NS3-Bn	7.0	RT	15	>90	20.5	(65) (108)

Preparation and purification of $^{68}Ga^{3+}$

Although various methods for the elution of ^{68}Ga from the generator, its purification, and the labeling of radiopharmaceuticals with ^{68}Ga have been established, these approaches are primarily based on four different ^{68}Ga eluate concentration and purification procedures (Figure 1). Among these, a potentially effective method uses the generator eluate directly from a fractional elution from the ^{68}Ge/^{68}Ga generator. In this method, the fraction with the highest volume activity is collected and buffered to the appropriate pH for radiolabeling (e.g., in NaOAc, pH ~4.5) (10-13,43). However, this method has the drawback that only a fraction of the eluted ^{68}Ga is employed, thus reducing the achievable specific activity of the final product (Figure 1, route 3). Recently, a sodium chloride (NaCl)-based ^{68}Ga eluate concentration and labeling method that enables rapid, high-efficiency labeling of DOTA-conjugated peptides in high radiochemical purity has been described. Briefly, the ^{68}Ga generator eluate is passed through a strong cation exchange cartridge, and subsequently ^{68}Ga is eluted from the cation exchange cartridge with 0.5 mL of a 5 M NaCl solution containing a small amount of 5.5 M HCl to achieve 98% of the total activity (44).

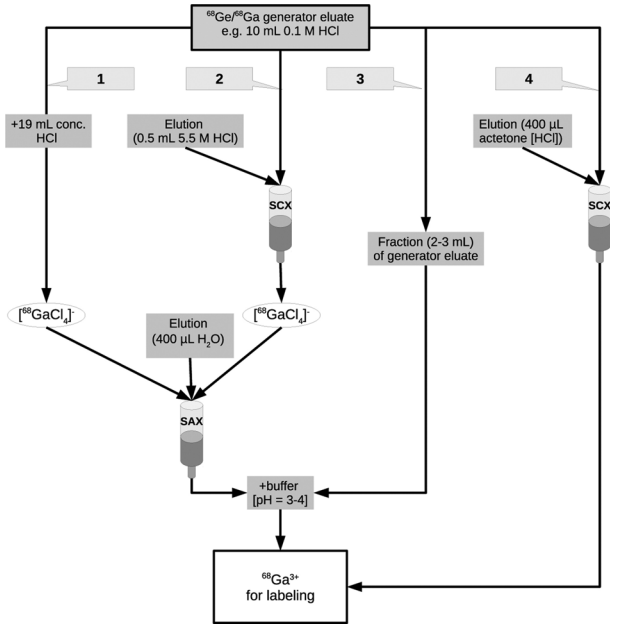

Figure 1. Schematic drawing of the widely applied ^{68}Ge/^{68}Ga generator elution and concentration method prior to ^{68}Ga labeling reactions (SCX, strong cation exchanger; SAX, strong anion exchanger; 1, anionic eluate concentration; 2, combined cationic/anionic eluate concentration; 3, fractioned method; 4, cationic eluate concentration). Reprinted by permission of the American Chemical Society from: Mueller, D., et al., Bioconjugate Chemistry, 2012. 23(8); p. 1712-1717.

1,4,7-triazacyclononane/multivalent effect

Due to its commercial availability and formation of an extremely stable six-coordinate Ga complex with high thermodynamic stability (45), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA, Figure 2) is recommended for the preparation of ^{68}Ga-labeled radiopharmaceuticals. However, the application of NOTA for a targeted PET probe design is restricted due to its limited bifunctionality. The coordinating ability of NOTA with ^{68}Ga is compromised by the loss of a coordinating pendant carboxylate group after its conjugation with a targeting vector. Therefore, NOTA derivatives containing a linker, such as 2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7 triacetic acid (p-SCN-Bn-NOTA), 2-[4,7-bis-(carboxymethyl)-1,4,7-triazonan-1-yl]-pentanedioic acid (NODAGA), and 1,4,7-triazacyclononane-1,4-bis[methylene-(hydroxymethyl)-phosphinic acid]-7-[methylene-(2-carboxyethyl)-phosphinic acid] (NOPO), have been developed to overcome this technical limitation (Figure 2) (9,46-50). Recent effort in ligand design has focused on studying and optimizing chelators N-functionalized with three pendant arms containing sites for both coordination and conjugation, such as 1,4,7-triazacyclononane-1,4,7-triglutaric acid (NOTGA) and 1,4,7-triazacyclononane-1,4,7-tris-[methyl-(2-carboxyethyl)-phosphinic acid] (TRAP), for multivalent conjugation of ligands and ^{68}Ga labeling (Figure 2) (51-53). An angiogenesis imaging marker (TRAP-RGD3) has been synthesized conjugating three RGD motives with TRAP,

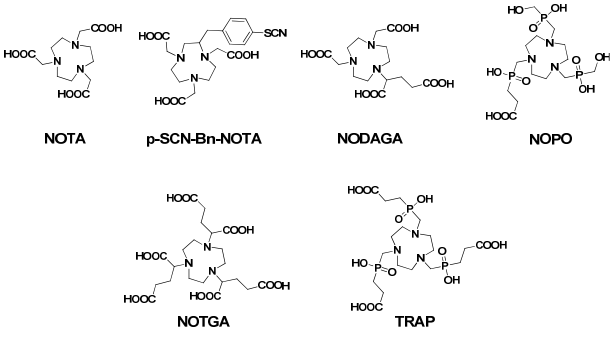

Figure 2. 1,4,7-triazacyclononane (TACN)-based bifunctional chelator for ^{68}Ga radiolabeling.

and its affinity compared with monovalent derivatives like NOPO-RGD. *In vivo* experiments with TRAP-RGD3 (5.33 ± 1.69) showed higher affinity than NOPO-RGD (2.02 ± 0.34) (50), ^{68}Ga-NODAGA-RGD (1.45 ± 0.11) (52) and ^{18}F-galacto-RGD (1.35 ± 0.53) (52) in $\alpha_v\beta_3$-positive M21 human melanoma xenografts (Figure 3). Another advantage of phosphonate-based chelators is that phosphonate-donating groups are less susceptible to transmetalation compared with acetic acid donors. Recently, ^{68}Ga-labeled multivalent TRAP-nitroimidazole (NI) derivatives for imaging hypoxia have been reported (54). The trivalent derivative (^{68}Ga-TRAP: 0.17 ± 0.04; ^{68}Ga-TRAP-NI1: 0.33 ± 0.04; ^{68}Ga-TRAP-NI2: 0.45 ± 0.09; ^{68}Ga-TRAP-NI3: 0.47 ± 0.05% ID/g) showed the highest uptake by tumor cells in biodistribution studies in CT-26 xenograft mice 1 h after injection. The trivalent derivative (^{68}Ga-TRAP: 0.10 ± 0.06; ^{68}Ga-TRAP-NI1: 0.20 ± 0.06; ^{68}Ga-TRAP-NI2: 0.33 ± 0.08; ^{68}Ga-TRAP-NI3: 0.59 ± 0.09) also showed the highest standard uptake value for tumor cells 1 h after injection in animal PET studies using CT-26 xenograft mice (54). Similarly, mono-, bis- and trimeric RGD conjugates have been prepared by conjugation of RGD peptides to 1,4,7-triazacyclononane-1,4,7-triglutaric acid (NOTGA) (55). The trimeric derivative showed high signal amplification leading to a 54% increase in tumor uptake in comparison with its monomeric version (55). The effect of different isomers (NOTGA, RRR/SSS and RRS/SSR) on radiosynthesis was also explored,

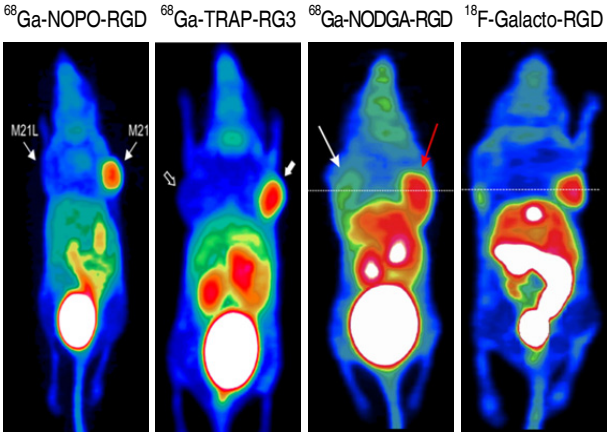

Figure 3. Micro PET images obtained 75 min after injection of ^{68}Ga-labeled RGD-derivatives in mice bearing $\alpha_v\beta_3$-negative M21L tumors on the left shoulder and $\alpha_v\beta_3$-positive M21 tumors on the right shoulder. Reprinted with permission of the American Chemical Society from: Simecek, J.; et al. Mol. Pharm. 2014, 11(5), 1687 and Notni, J., et al. Chemistry-A European Journal. 2011, 17(52), 14718.

showing that a diastereomeric mixture has no negative effect on the biological activity of RGD conjugates (56). The studies mentioned have been aimed to form trimeric bioconjugate species while keeping the coordinating atoms for ^{68}Ga radiolabeling.

Cyclen

The most commonly used cyclen-based BFCs for ^{68}Ga are DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and *p*-SCN-Bn-DOTA (2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, Figure 4), but their radiochemistry typically requires conventional or microwave heating to rapidly obtain high radiochemical yields and specific activities (57-61). ^{68}Ga is predisposed to form six-coordinate complexes, and although DOTA, which has eight potential coordinating groups, has extra carboxylic groups available for conjugation, NOTA, which only has six coordinating groups, compromises its stability when carboxylic groups are used for conjugation (62). Dicarboxymethyl pendant-armed cross-bridged cyclen [2,2'-(1,4,7,10-tetraazabicyclo[5.5.2]tetradecane-4,10-diyl) diacetic acid, CB-DO2A, Figure 4] gallium complexes have been synthesized and structurally characterized (63). *p*-NO_2-Bn-Oxo (1-oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid)

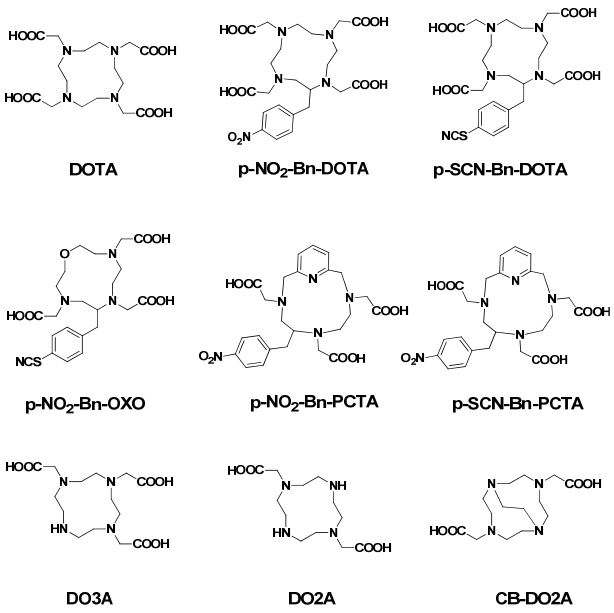

Figure 4. 1,4,7,10-tetraazacyclododecane (cyclen)-based bifunctional chelators for ^{68}Ga radiolabeling.

and *p*-NO_2-Bn-PCTA (3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid, Figure 4), have been developed with higher (98%) radiochemical yields at room temperature after 5 min of reaction (64). *p*-NO_2-Bn-DOTA [2-(4-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra acetic acid] requires longer reaction time, higher concentrations of ligand, and heating than *p*-NO_2-Bn-PCTA and *p*-NO_2-Bn-Oxo to obtain equivalent radiochemical yields. The oxo derivative [^{68}Ga]oxo-DO3A-RGD has inferior characteristics (unstable *in vivo*) compared with the already existing ^{68}Ga-labeled RGD (65). ^{68}Ga radiolabeled PCTA-RGD shows similar *in vivo* behavior compared with ^{68}Ga-NOTA-RGD, but a lower kidney uptake, which can be advantageous for some imaging applications (62). ^{68}Ga-labeled amino acid derivatives, such as β-aminoalanine, γ-aminohomoalanine, lysine conjugates of DOTA, alanine derivatives of 1,4,7,10-tetraazacyclododecane-1, 7-diacetic acid (DO2A) and 1,4,7,10-tetraazacyclododecane-1, 4,7,-triacetic acid (DO3A) have been prepared (12, 66). The cyclen-based tetraphosphinate chelator DOTPI (1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetrakis[methylene(2-carboxyethyl)ph osphinic acid], Figure 4) comprises four additional carboxylic acid groups for bioconjugation (67). DOTPI was functionalized with four cyclo(Arg-Gly-Asp-D-Phe-Lys) RGD peptides through polyethylene glycol (PEG_4) linkers. Integrin $\alpha_v\beta_3$ affinities of non-radioactive Lu^{3}+- and Cu^{2+}-DOTPI$(RGD)_4$ complexes were 18 times higher (both IC_{50} values of approximately 70 picomol) than those of an c(RGDfK) peptide (IC_{50} = 1.3 nanomol). DOTPI can be used for ^{68}Ga radiolabeling of tetrameric bioconjugates.

Other macrocycles

1. Diaminosarcophagine

Diaminosarcophagine [1,8-diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane, $(NH_2)_2$sar] is a macrocyclic ligand structure alternative to the triaza and tetraazamacrocycles for ^{68}Ga (Figure 5) (68). Primarily, this type of ligands is used for ^{64}Cu-based radiopharmaceutical preparations (69-71). $(NH_2)_2$sar does not dissociate in the presence of transferrin, due to the formation of a highly stable six coordinate complex with ^{68}Ga characterized by distorted octahedral geometry, as determined by X-ray crystallography studies. Metal complexes with $(NH2)_2$sar show high stability and are kinetically inert, due to the encapsulating nature of the cage-like ligand. $(cRGDfk)_2(NH)_2$sar was prepared conjugating two RGD peptides with this ligand and showed promising *in vivo* characteristics in 66c14β3 mouse xenografts. However, due to its harsh radiolabeling conditions (85°C, 30 min), it is not recommended for proteins and antibodies (68). Dicarboxymethyl pendant-armed cross-bridged cyclam, [4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane, CB-TE2A, Figure 5] gallium complexes has been synthesized and structurally characterized (72). ^{68}Ga-CB-TE2A showed impressive acid inertness than ^{68}Ga-CB-DO2A. A sample of ^{68}Ga-CB-TE2A in 5 M DCl at 90°C was less than 20% demetalated even after 6 months (73).

2. DATA

Hexadentate tribasic ligands based on 1,4-diacepine-6-amino-triacetate (DATA), 6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid (AAZTA) and its derivatives (Figure 5) bind ^{68}Ga rapidly in the pH range of 4-7, forming radiolabeled complexes suitable for PET studies (14,74). These derivatives form ^{68}Ga complexes in an out of case manner. The chelators showed excellent radiochemical characteristics (≥96% RCY, 3 min reaction time) in a pH range of 4.0-6.8. These derivatives also showed no demetalation in the presence of transferrin or Fe^{3+} for 2 h (14,75). PET showed high rates of kidney and bladder uptake for DATA, with no evidence for retention in any other organ 25 min after injection in BALB/c mice (14).

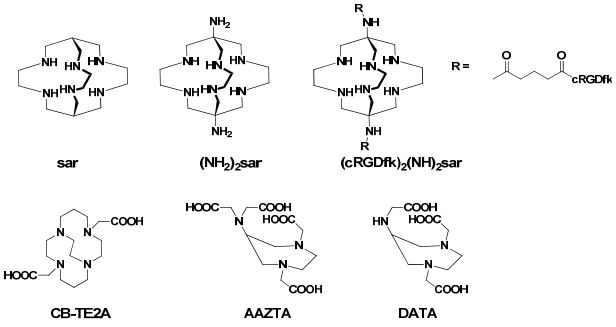

Figure 5. 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane, (diaminosarcophagine, $(NH_2)_2$sar)), 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TE2A), 1,4-diacepine-6-amino-triacetate (DATA) and 6-amino-6-methylperhydro-1,4-diazepinetetra-acetic acid (AAZTA) chelators for ^{68}Ga radiolabeling.

A ^{68}Ga-AAZTA-RGD (ST1646) derivative has been developed and used for PET imaging in U87MG tumor-bearing mice. ^{68}Ga-AAZTA-RGD showed slow kinetics and accumulation in the tumor. Forty min after injection, the tumor was more readily detectable compared with imaging data obtained using ^{68}Ga-AAZTA, due to improved contrast between the tumor mass and the surrounding tissues (74). The signal remained appreciable in the tumor until 70 min after injection. An AAZTA platform bearing arylamino and isothiocyanate groups for conjugation to biomolecules has been synthesized and used for MRI contrast imaging (76).

3. FSC

Siderophore fusarinine-C (FSC) is a deacetylated form of triacetylfusarinine-C (TAFC, Figure 6). The three primary amines of FSC can be used for conjugation to biomolecules such as RGD peptides. A ^{68}Ga-radiolabeled trimeric FSC-RGD conjugate, ^{68}Ga-FSC-(RGD)3, targeting $\alpha_v\beta_3$ integrin has been developed (77). Radiolabeling with ^{68}Ga was conducted at pH 4.5~5 at room temperature for 15 min. *In vitro* and biodistribution studies confirmed an improved tumor targeting for ^{68}Ga-FSC-(RGD)3 (4.25 ± 0.64 % ID/g) in comparison with ^{68}Ga-NODAGA-RGD (1.45 ±0.11 % ID/g) in an $\alpha_v\beta_3$-positive M21 tumor model, supporting the strategy of using FSC as a basis for the development of novel trimeric targeting bioconjugates for PET applications (77).

Acyclic chelators

1. Bis(thiosemicarbazones)

Optical and radionuclear imaging methods provide high detection sensitivity and specificity, and for this reason are ideal for molecular imaging. New fluorescent and biocompatible aromatic bis(thiosemicarbazones) labeled with ^{68}Ga or ^{111}In complexes that can be used for dual optical mode and PET or SPECT molecular imaging have been synthesized (Figure 6) (78). Bis(thiosemicarbazones) were rapidly radiolabeled with ^{68}Ga under mild conditions and high yields were obtained. Briefly, ^{68}Ga (in 98% acetone/0.02 M HCl) was added to a glass vial and evaporated to dryness under nitrogen stream at 90°C for 20 min in a heating block. Subsequently, Zn-allyl precursors (1 mg/mL) and 0.5 M HEPES buffer (pH ~3.9) were added to the vial and heated for 30 min at 90°C (78). ^{68}Ga was bound using a synthetic method based on transmetalation reactions from Zn^{2+} precursors (78). This method had been previously used for ^{64}Cu peptide labeling (79, 80). The skeleton of the ligand is modified to add aromatic groups, for the ligand to be used as a multimodal PET/optical agent with ^{68}Ga and other functional groups to increase aqueous solubility (78, 81).

2. ProtoporphyrinIX

ProtoporphyrinIX (PPIX) is another chelator that can be used for both *in vitro* fluorescence imaging and *in vivo* PET or SPECT imaging (Figure 6) (82). PPIX was conjugated to an

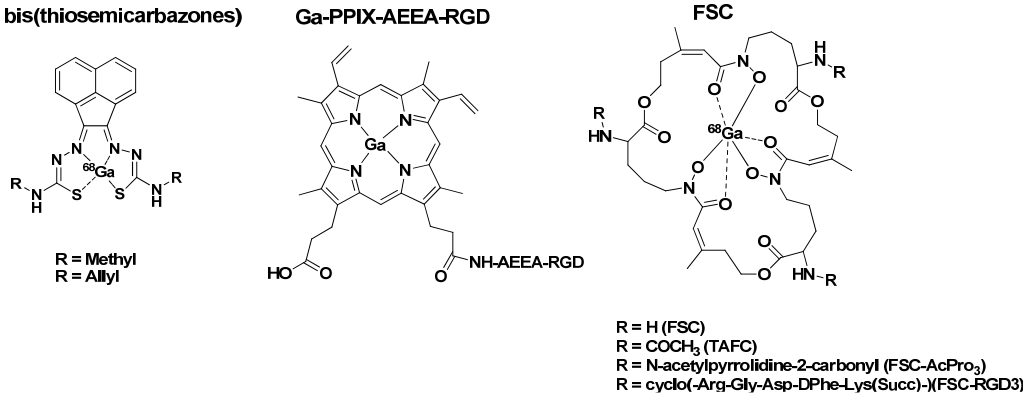

Figure 6. Bis(thiosemicarbazones)-, ProtoporphyrinIX (PPIX)- and siderophore fusarinine-C (FSC)-based bifunctional chelators for ^{68}Ga radiolabeling.

RGD peptide and labeled with ^{68}Ga under microwave heating. The resulting probe was evaluated in an MDA-MB-435 cancer cell line expressing integrin receptors, and demonstrated binding specificity. Porphyrins specifically accumulate in tumor tissues and five ^{68}Ga-labeled analogues were suggested as imaging agents (83). Preliminary results indicate accumulation in DS sarcoma tumors of rat models, however their uptake mechanism requires further investigation (83).

3. Tris(hydroxypyridinone) ligands

An acyclic chelator, the tripodal tris-(hydroxypyridinone) bifunctional chelator, 4-acetamido-N1,N7-bis-[(3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methyl]-4-(3-[(3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methylamino]-3-oxopropyl)heptanediamide (CP256) and its bifunctional maleimide derivative 4-(3-[3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido]propanamido)-N1,N7-bis[(3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methyl]-4-(3-[(3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methylamino]-3-oxopropyl)heptanediamide (YM-103) have been developed for ^{68}Ga (Figure 7) (84, 85). CP256 and YM103 show RCYs of 98-100% after 5 min of reaction at room temperature. ^{67}Ga stability studies proved that no transchelation occurred after 4 h of incubation with a 130-fold excess of Fe^{2+}, demonstrating that the complex is highly stable albeit including an acyclic chelator (85). It is likely to become the BFC of choice for the labeling of sensitive proteins with ^{68}Ga. Recently, two new tris(hydroxypyridinone) chelators based on 1,6-dimethyl-3-hydroxypyridin-4-one groups that contain pendant isothiocyanates [(N1,N7-bis((3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methyl)-4-(3-((3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methylamino)-3-oxopropyl)-4-(3-thiocyanatopropanamido)heptanediamide, H_3-THP-NCS, Figure 7) and (N1,N7-bis((3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methyl)-4-(3-((3-hydroxy-1,6-dimethyl-4-methylene-1,4-dihydropyridin-2-yl)methylamino)-3-oxopropyl)-4-(3-(3-(4-thiocyanatophenyl)thioureido)propanamido)heptanediamide, H_3-THP-Ph-NCS, Figure 7], for conjugation to primary amines have been reported (86). H_3-THP-NCS and H_3-THP-Ph-NCS were conjugated with cRGDfk peptides, labeled with ^{68}Ga and used for angiogenesis PET imaging (86).

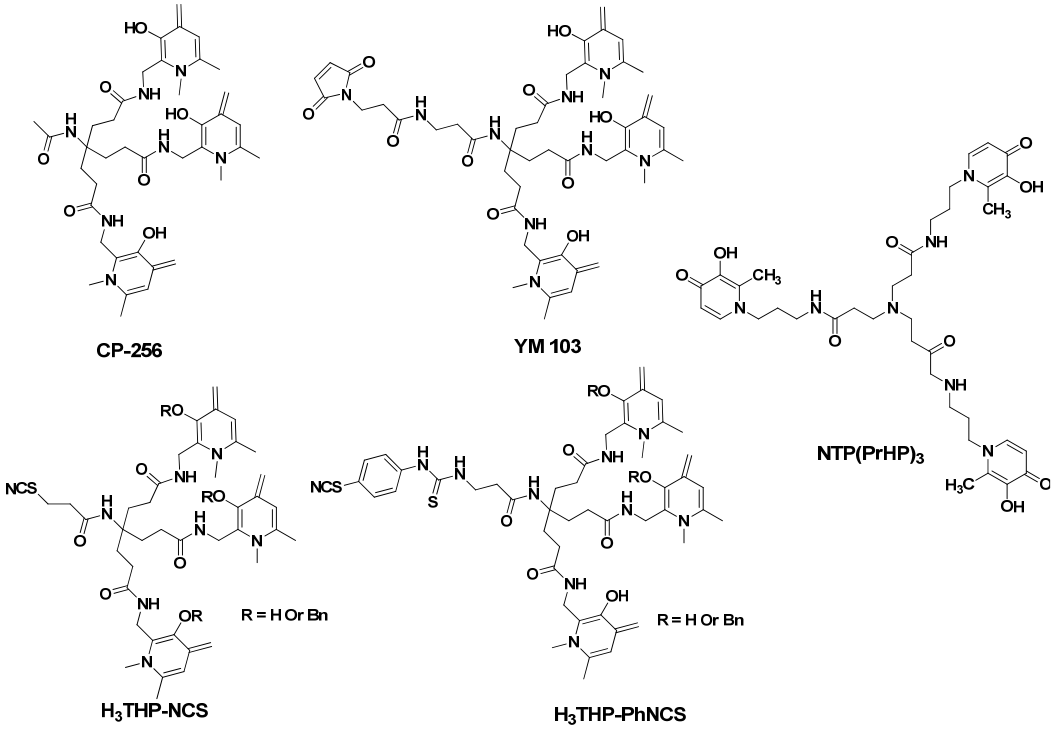

Figure 7. Tris(hydroxypyridinone) based chelators for ^{68}Ga radiolabeling.

4. HBED

Another promising chelator for ^{68}Ga is HBED (3,3'-(3,3'-(ethane-1,2-diylbis((carboxymethyl)azanediyl))bis(methylene)bis(4-hydroxy-3,1-phenylene))dipropanoic acid (Figure 8), which forms a Ga complex with a very high log K (38.5) (87) but shows low radiolabeling yield and slow blood clearance (88). Other derivatives, 3,3'-(3,3'-(ethane-1,2-diylbis((carboxymethyl)azanediyl))bis(methylene)bis(4-hydroxy-3,1-phenylene))dipropanoic acid (HBED-CC, Figure 8) and 3-(3-(((carboxymethyl)(2-((carboxymethyl)(2-hydroxy-5-(3-oxo-3-(2,3,5,6-tetrafluorophenoxy)propyl)benzyl)amino)ethyl)amino)methyl)-4-hydroxyphenyl)propanoic acid (HBED-CC-TFP, Figure 8) have been developed. HBED-CC-TFP has been used for protein labeling obtaining a yield of 80% upon incubation at pH 4.1 for 5 min.

5. DEDPA

Five versions of the DEDPA chelator were synthesized: a version with no reactive groups, 6,6′-((ethane-1,2-diylbis(azanediyl))bis(methylene))dipicolinic acid (DEDPA) (89), a bis-N-functionalized derivative, 3-(4-isothiocyanatophenyl)-propane-1,2-diamino-N,N'-bis[6-(carboxylato)-pyridin-2-yl]methylamine (H_2dp-bb-NCS), a mono-N-functionalized derivative, (1,2-[N,N'-(p-benzyl-isothiocyanato)-methyl]-N,N'-[6-(carboxylato)-pyridin-2-yl] methylamino)ethane (H_2dp-N-NCS), a bis-C-functionalized derivative, (1,2-[N,N'-(p-benzylamino)methyl]-N,N'-[6-(carboxylato)-pyridin-2-yl]methylamino)ethane (H_2dp-bb-NH_2), and a mono-C-functionalized derivative 3-(4-aminophenyl)-1,2-diamino-N,N'-bis[6-(carboxylato)-pyridin-2-yl]methylamino-propane (H_2dp-N-NH_2) (Figure 9) (90). All of the three types of ligands show quantitative radiolabeling after 10 min of incubation at room temperature and 97% of the complexes remained intact after transferrin challenge experiments. The bis-N-functionalized (^{68}Ga-H_2dp-bb-NH_2-RGD) and C-functionalized (^{68}Ga-H2dp-N-NCS-RGD) derivatives were tested as BFCs by conjugation with RGD (90). It was observed that the ^{68}Ga-H2dp-N-NCS-RGD conjugate shows high stability (92% after 2 h), whereas the ^{68}Ga-H_2dp-bb-NH_2-RGD conjugate shows a significantly lower stability (73% after 2 h). Quantitative PET analysis showed higher tumor uptake (^{68}Ga-H_2dp-bb-NH_2-RGD: 2.83 ± 0.63% ID/g; ^{68}Ga-H2dp-N-NCS-RGD: 2.02 ± 0.56% ID/g) after 2 h compared with biodistribution data (^{68}Ga-H_2dp-bb-NH_2-RGD: 1.13 ± 0.42% ID/g; ^{68}Ga-H2dp-N-NCS-RGD: 1.44 ± 0.59% ID/g) in U87MG tumor-bearing RAG2M mice. However, the identified slow clearance from blood requires further improvement of pharmacokinetic pro-

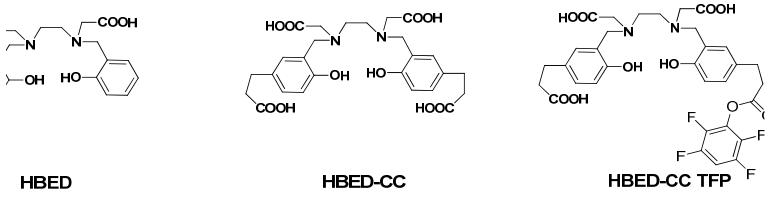

Figure 8. 3,3'-(3,3'-(ethane-1,2-diylbis((carboxymethyl)azanediyl))bis(methylene)bis(4-hydroxy-3,1-phenylene))dipropanoic acid) (HBED) and its derivatives.

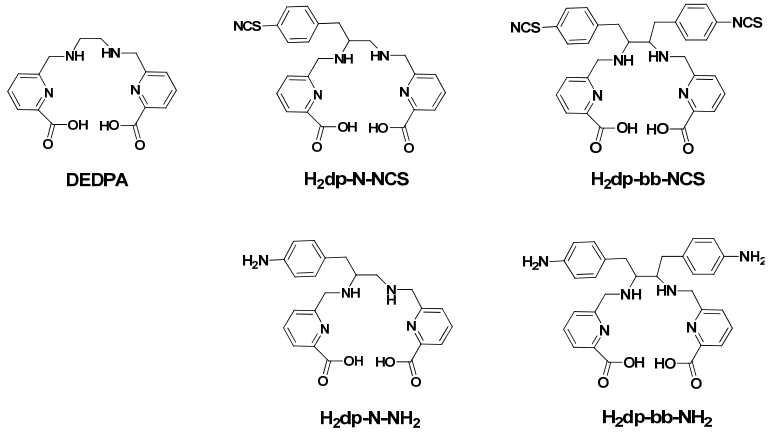

Figure 9. 6,6′-((ethane-1,2-diylbis(azanediyl))bis(methylene))dipicolinic acid (DEDPA)-based bifunctional chelators for ^{68}Ga radiolabeling.

perties. Boros, E., et al. subsequently synthesized a diazide derivative that can be used for Cu^{1+}-catalyzed azide alkyne cycloaddition reactions for bioconjugation and showed versatility in forming complexes with a range of radiometals (91).

6. TAME-Hex

1,1,1-tris(aminomethyl)ethane (TAME) (Figure 10) is a tridentate ligand which is used as a starting material to synthesize the tris(aminomethyl)-ethane-*N,N,N′,N′,N″,N″*-hexaacetic acid (TAME-Hex, Figure 10) (92). A potentially sexadentate ligand H_3[(5-MeO-sal)-TAME] was synthesized by reaction of TAME with 5-methoxy- salicylaldehyde in hot ethanol (93). Two versions of TAME-Hex chelator were synthesized by Arslantas et al.,(94); a version with isothiocyanate group, 2,2',2'',2'''-(2-((bis(carboxymethyl)amino)methyl)-2-((4-thiocyanatobenzyl oxy)methyl)propane-1,3 diyl)bis(azanetriyl)tetraacetic acid (TAME-Hex-A, Figure 10), and a 2,2',2'',2'''-(2-((bis(carboxymethyl)amino)methyl)-2-((dibenzylamino)methyl)propane-1,3diyl)bis(azanetriyl)tetraacetic acid (TAME-Hex-B, Figure 10). 2,2',2'',2'''-(2-((bis(carboxymethyl)amino)methyl)-2-((4-nitrobenzyloxy)methyl)propane-1,3-diyl)bis(azanetriyl)tetraacetic acid, (p-NO_2-Bn-TAME-Hex, Figure 10), is a intermediate compound to synthesize the TAME-Hex-A. The stabilities of the ^{67}Ga-p-NO_2-Bn-TAME-Hex and ^{67}Ga-TAME-Hex-B were tested by performing *trans*-chelation experiments using DTPA as the competing ligand (94). ^{67}Ga-p-NO_2-Bn-TAME-Hex was very stable with 94% remaining intact after 10 days, and ^{67}Ga-TAME-Hex-B was even more stable with around 99% still intact after 10 days. Both the gallium chelates were stable against *trans*-chelation by a 1000-fold excess of DTPA and thus are potentially highly effective candidates for use in radioimaging (94). TAME-Hex-B was useful for coupling to peptides and proteins.

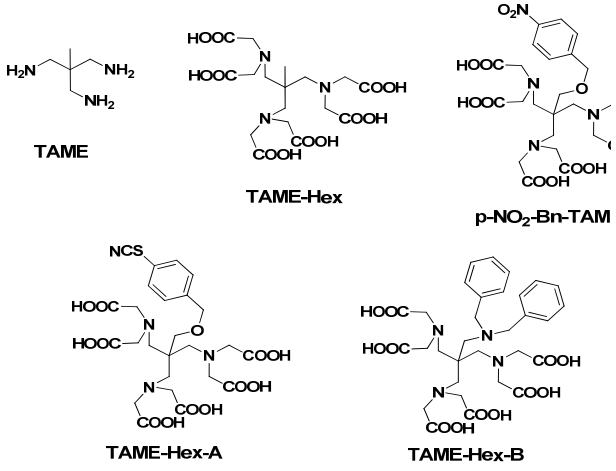

Figure 10. Tris(aminomethyl)-ethane-*N,N,N′,N′,N″,N″*-hexaacetic acid based bifunctional chelators for 68Ga radiolabeling.

7. Mercapto amino chelators

Various mercapto amino chelators, 1,1'-(ethane-1,2-diylbis (azanediyl))bis(2-methylpropane-2-thiol) (4SS), 2,3-bis(2-mercapto-2-methylpropylamino)propanoic acid (5SS), 2,2'-(ethane-1,2-diylbis((2-mercapto-2-methylpropyl)azanediyl))diacetic acid (6SS), ethane-1,2-diylbis(2-mercaptoethylcarbamic acid) (EC), 2,2'-(ethane-1,2-diylbis((2-mercaptoethyl)azanediyl))diacetic acid (EDAA-SS), and 2,2',2' '-nitrilotris(ethanethiol) (NS3), its carboxylic acid derivative, 2-(bis(2-mercaptoethyl)amino)-3-mercaptopropanoic acid (NS3-COOH) and tris(2-mercaptobenzyl)amine (NS3-Bn) have been developed (Figure 11) (95,96).

Among them hexadentate ligands, EDDA-SS and 6SS ligands are the best candidates for forming bifunctional chelators through covalent linkage to biomolecules (95). The ^{68}Ga-NS3 complex was easily formed with a radiochemical purity of 95%, due to its lipophilic nature it easily crosses the blood-brain-barrier, and accumulates in the heart and exhibits a high heart-to-blood ratio (65). However, in this form the chelator is not suitable for conjugation to targeting vectors like peptides and proteins. Its carboxylic acid derivative, NS3-COOH, is a bifunctional chelator, which allows easy conjugation of targeting domains such as peptides or can serve as anchor for pharmacologically modifying groups (65,97). ^{68}Ga-labeling of NS3-COOH can be done at room temperature in high radio-

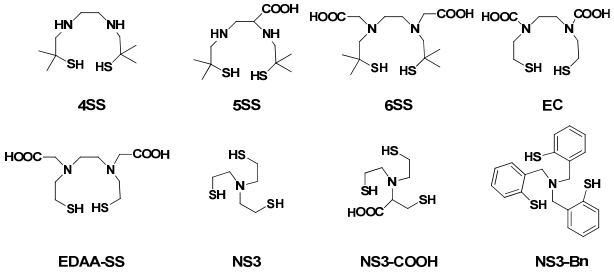

Figure 11. Mercapto amino based bifunctional chelators for ^{68}Ga radiolabeling.

chemical yields. An angiogenesis imaging agent [^{68}Ga]NS3-RGD has been developed and found to be unstable in serum, after 30 min (50% intact), and 60min (0%) than ^{68}Ga-DOTA-RGD (99%) and ^{68}Ga-NODAGA-RGD (96%) derivatives (65).

Microfluidic radiolabeling

Automated synthesis reduces radiation exposure to operator, improves robustness of production and provides on-line documentation of manufacturing processes, thus improving GMP compliance (98). Recently, fully automated and programmable synthesis modules for ^{68}Ga radiopharmaceutical production with high radiochemical yields and shorter synthesis time (Figure 12) have been reported (99). ^{68}Ga-NOTA-RGD was synthesized using an auto synthesizer and its feasibility for routine synthesis was evaluated. The radiolabeling yield of NOTA-RGD was 65.4 ± 2.42%, and radiochemical purity was greater than 97% with a total synthesis time of 20 min (99). c(Arg-Gly-Asp-DPhe-Lys) peptide-conjugated 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) were labeled with ^{68}Ga using the microfluidic method. The microreactor radiolabeling conditions were optimized varying temperature, concentration and residence time (100). Radiolabeling yields of the NOTA-RGD and DOTA-RGD derivatives were greater than 80% after 10 min at 37°C using the microfluidic method (100). Autoclabeling is a method that combines ^{68}Ga-labeling and steam sterilization. Somatostatin receptor-DOTA-

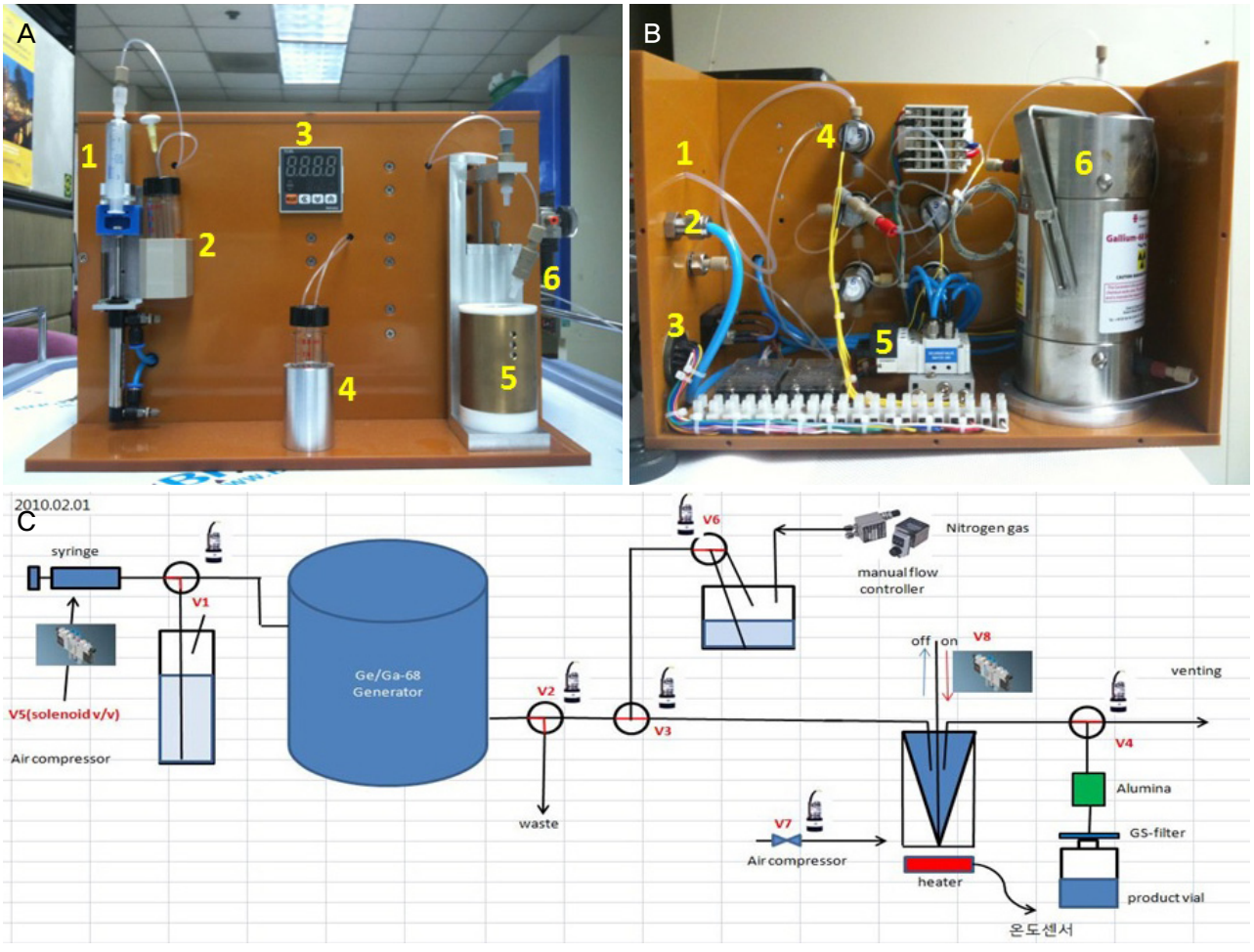

Figure 12. Fully automated and programmable synthesis module for ^{68}Ga radiopharmaceutical production. A) Auto module front view (1. eluent dispensing syringe, 2. 0.1 M HCl, 3. temperature controller, 4. saline, 5. heater, 6. needles connected to vial containing peptides). B) Auto module rare view (1. He gas supply line, 2. compressed air supply line, 3. PLC connection cable, 4. 2 or 4 ways valve, 5. pneumatic accumulator, 6. ^{68}Ge/^{68}Ga generator). C) Flow chart of synthesis procedure.

TATE and -NOC have been evaluated with this method and used for PET imaging of neuroendocrine tumors (101). The peptides DOTA-TATE and-NOC were labeled with ^{68}Ga at 121°C for 15 min in acetate buffer (pH ~ 4.3) and confirmed that the final product was sterile and did not undergo peptide degradation. However, this method is only selective for heat-sensitive biomolecules (101).

Automation provides the possibility for harmonized and standardized multicenter clinical studies that can accelerate the introduction of new radiopharmaceuticals as well as their regulatory approval. This in turn might motivate investments for research and development of novel ^{68}Ga-based radiopharmaceuticals (98).

Conclusion

^{68}Ga is an emerging radionuclide for positron emission tomography (PET). It is produced using a ^{68}Ge/^{68}Ga generator and has a half-life of 271 days. It provides a convenient way to produce an amount of ^{68}Ga sufficient for more than one year, and the cost of the generator is comparable with the ones of other radionuclides used for PET. However, open-chain chelators serve the aim of rapid chelation at room temperature despite the difficulty to compete with macrocyclic chelators based on TACN and cyclen that provide stability, selectivity, fast association kinetics and extremely slow dissociation kinetics, as well as high thermodynamic stability. However, TACN-based chelators offer an advantage over cyclen-based chelators with respect to fast complexation kinetics at room temperature and higher selectivity for ^{68}Ga.

The production of ^{68}Ga-based imaging agents can be accomplished either under GMP or under radiopharmaceutical practice, and it is a cost-effective complement to cyclotron-based tracers. Most importantly, it will enable PET-CT investigations globally and in structures without access to accelerators and radiopharmaceutical distribution centers, thus promoting PET worldwide for faster and/or better diagnostics and representing a step forward in individualized medicine.

Acknowledgements

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