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A rigidified AAZTA-like ligand as efficient chelator for 68Ga radiopharmaceuticals

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Author Comments:	Dr. Neville Compton Editor, Chemistry A European Journal Wiley WCH Germany

	December 18, 2015 Dear Dr Compton, We are submitting a manuscript entitled "A rigidified AAZTA-like ligand as efficient chelator for ^{68}Ga radiopharmaceuticals" that reports on the synthesis of a new rigidified AAZTA-like ligand and on a detailed study on thermodynamic stability, transmetallation and ligand exchange kinetics of the Ga(III) complex. The optimization of the ^{68}Ga radiolabelling in different experimental conditions (pH, temperature, buffer of reaction) is also reported. The novelty and significance of our work can be summarised as follows: a) the synthesis of a new cyclohexane fused polyaminocarboxylic chelator is reported; b) the thermodynamic stability of the Ga(III) complex was tested via pH-potentiometry and ^{71}Ga NMR; c) the kinetic inertness was investigated via transmetallation and ligand exchange reactions with Cu(II) and human serum transferrin, respectively. This procedure aims to be the most reliable to predict the behaviour of Ga(III) complexes in body fluids with respect to those reported in the literature so far; d) whereas the ^{68}Ga labelling was successful at room temperature and can be of interest for temperature sensitive molecules, the labelling at higher temperatures for lower ligand concentrations may be advantageous for reaching higher specific activity for peptide radiopharmaceuticals; e) the stability tests of the ^{68}Ga-labelled AAZTA-like ligand in human serum confirmed the high stability in body fluids. In our opinion, the results of this work present an additional tool useful for the future development of Ga-based radiopharmaceuticals based on a wide spectrum of possible bifunctional chelators that can be prepared starting from this ligand. As such, we believe this contribution may be of interest to a broad readership. Yours sincerely, Lorenzo Tei (on behalf of all the authors)
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A rigidified AAZTA-like ligand as efficient chelator for ^{68}Ga radiopharmaceuticals

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Abstract: The new cyclohexane fused CyAAZTA ligand was synthesized to increase the structural rigidity of the heptadentate chelator AAZTA with the aim to improve the overall stability of its Ga^{III} complex. The stability constant of Ga(CyAAZTA)$^-$, determined both by pH-potentiometry (logK_{GaL} = 21.39) and by ^{71}Ga NMR (logK_{GaL} = 21.92), was found similar to that of GaAAZTA (logK_{GaL} = 22.18). The kinetic inertness of Ga(CyAAZTA)$^-$ was investigated by following its transmetallation and ligand exchange reactions with Cu^{2+} and human serum transferrin, respectively. The formation of a hydroxo-complex near pH 7 decreases the half-life ($t_{1/2}$) of the dissociation reactions for Ga(CyAAZTA)$^-$ with respect to Ga(AAZTA)$^-$ (8.5 h vs 21 h, pH 7.4). However, at pH < 7 the $t_{1/2}$ of Ga(CyAAZTA)$^-$ is much longer (234h at pH 6). Finally, CyAAZTA was successfully radiolabelled with ^{68}Ga in acetate buffer at pH 3.8, 15 min reaction at room temperature and [CyAAZTA]=10 μM, with a labelling yield higher than 80%. 1 μM solution of CyAAZTA was successfully labelled (L.Y.: 97.4%) in 5 min at 90°C. Stability tests in human serum and in the presence of 50 mM DTPA showed that ^{68}GaCyAAZTA was highly stable over 90 min.

Introduction

The widespread clinical application of ^{68}Ge-based radioisotope generators ($t_{½}$ (^{68}Ge)=270.8 days) for the production of the PET isotope ^{68}Ga ($t_{½}$ = 67.71 min, $E_{β+,max}$=1.89 MeV, 89% decays through positron emission), together with the favorable properties of such isotope, *i.e.* half-life sufficient for production and application of tracers by minimizing the radiation dose to the patient, has boosted the research activity with the aim of designing effective, specific and safe ^{68}Ga-based radiopharmaceuticals.[1,2] The established diagnostic power of the PET technique coupled to the increasing availability of new receptor specific peptides and high affinity proteins have led to the design and testing of a large number of ^{68}Ga-based radiopharmaceuticals in oncology, cardiology, neurology, and infection diseases.[3] These tracers allow a personalized medical approach with accurate quantitative diagnosis and staging for subsequent selection and planning of therapeutic methods.[4] In this context, a prostate-specific membrane antigen (PSMA) inhibitor for imaging and therapy for ^{68}Ga-based PET and ^{177}Lu-based endoradiotherapeutic treatment in patients with metastatic and castration-resistant disease is currently under intense scrutiny.[5]

A ^{68}Ga-based radiopharmaceutical consists of a thermodynamically stable and kinetically inert Ga^{III} complex linked to a specific vector, most often represented by peptides or pseudo-peptides. Ga^{3+} ions should be complexed, typically at acidic pH to avoid the formation of $Ga(OH)_3$, by a chelator having at least 6 donor atoms (N- or O-donors) that is able to wrap around the metal ion and prevent the transmetallation reaction with endogeneous metal ions (Cu^{2+}, Zn^{2+}, Ca^{2+}) or ligand exchange reactions with proteins such as transferrin. It is well established that macrocyclic chelators such as 1,4,7,10-tetraazacyclododecane tetraacetic acid (DOTA) and 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), either free or conjugated to peptides, form very stable complexes with ^{68}Ga.[6] Recently, it has been reported that a NOTA-phosphinate analog (TRAP, Tri-Azacyclononane-tri-Phosphinic acid) and its conjugated multimeric systems show an enhanced efficacy in forming stable ^{68}Ga-complexes at ambient temperature, very low pH (<1) and low chelator concentrations.[7] It is straightforward to note that an efficient ^{68}Ga-labeling reaction, without the need to work at high temperatures (*i.e.* 95°C as for DOTA-based systems) represents a very attractive opportunity when producing fragile temperature sensitive macromolecular tracers.[8] In this context, a polyaminopolycarboxylate heptadentate ligand based on a 1,4-diazepine scaffold (AAZTA = 6-amino-6-methylperhydro-1,4-diazepinetetraacetic acid, Scheme 1) has been thoroughly studied as chelator for Gd^{3+} ions for Magnetic Resonance Imaging applications.[9] This ligand has already been proved to form thermodynamically stable and kinetically inert Ga^{III} complexes[10] and an AAZTA-RGD peptide conjugate have been recently shown to complex ^{68}Ga at room temperature, in acetate buffer at pH 3.8.[11] Moreover, Parker and co-workers carried out the ^{68}Ga-labelling with a series of hexadentate diazepine-based chelators similar to AAZTA synthesized by removing one carboxylate arm on the hexocyclic amine (AAZ3A, Scheme 1).[12] They reported that, whereas ^{68}GaAAZTA was efficiently formed as a mixture of three isomers, the AAZ3A-

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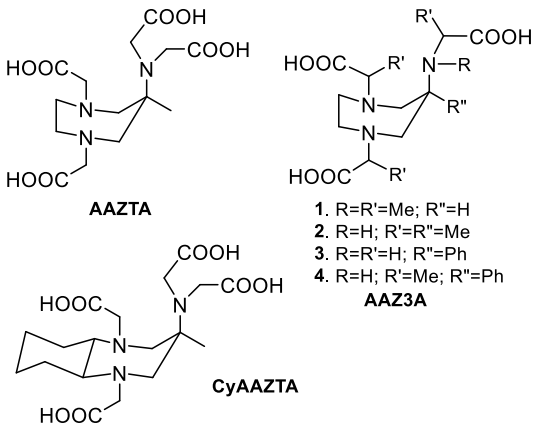
based ligands can bind ^{68}Ga rapidly and selectively in acetate buffer at pH 4 to 7, forming kinetically stable complexes.

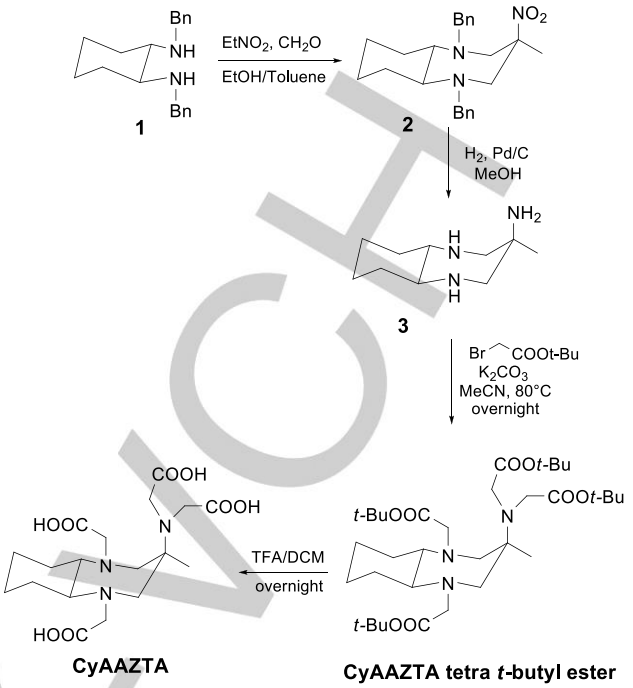


Scheme 1. 1,4-diazepine-based polyaminopolycarboxylate ligands for Ga(III) complexation

With the aim to improve further the performance of the AAZTA chelator, we designed a new ligand having a cyclohexane ring fused on the seven-membered diazepine ring to enhance its structural rigidity. It is known that the structural stiffening of the ethylenediaminetetraacetic acid (EDTA) backbone induced by the substitution of the 1,2-diaminoethane moiety by the *trans*-1,2-diaminocyclohexyl group (Cyclohexyl-DTA - CDTA) results in the increase of the thermodynamic stability and kinetic inertness of the metal complexes formed by these ligands.[13] Therefore, to establish the effect of the presence of *trans*-1,2-diaminocyclohexyl moiety on the coordination ability of the AAZTA skeleton, herein we report the synthesis of *trans*-3-amino-3-methyldecahydro-1H-1,5-benzodiazepine-N,N',N'',N''' tetraacetic acid (CyAAZTA, Scheme 1) and a full account of the solution thermodynamics of its Ga^{III} complex, along with a detailed kinetic study of the principal dissociation pathways. The ^{68}Ga labelling of CyAAZTA was also carefully tested in order to find the best conditions for an efficient labelling.

Results and Discussion

Synthesis. The synthesis of CyAAZTA is analogous to that reported for the parent AAZTA ligand.[9a] (±) *trans*-1,2-dibenzylaminocyclohexane (**1**), prepared by reductive amination of (±) *trans*-1,2-diaminocyclohexane with benzaldehyde, was used in place of *N*,*N*'-dibenzylethylenediamine in the key cyclization step. The formation of the seven-membered ring was achieved through the high-performing double nitro-*Mannich* reaction with **1**, nitroethane and paraformaldehyde (Scheme 2). Reduction of the nitro group and combined hydrogenolysis of the *N*-benzyl groups was achieved by Pd/C catalysed hydrogenation. The triamine was then alkylated by reaction with *tert*-butyl bromoacetate in acetonitrile in the presence of K_2CO_3 as base. Finally, the *tert*-butyl esters were removed in a 1:1 mixture of TFA and dichloromethane to obtain the desired ligand.



Scheme 2. Synthesis of CyAAZTA.

Solution equilibrium studies.

Protonation equilibria: The protonation constants of CyAAZTA (logK_i^H, i = 1, 2…5), defined by Equation (1), were determined by pH-potentiometry (Figure S1) and are listed in Table 1 where are compared with those of AAZTA, EDTA and CDTA.

$$K_i^H = \frac{[H_iL]}{[H_{i-1}L][H^+]} \quad (1)$$

Table 1. Protonation constants of H_4CyAAZTA, H_4AAZTA, H_4CDTA and H_4EDTA (0.1 M KCl, 25°C)

	H_4CyAAZTA	**H_4AAZTA[a,b]**	**H_4CDTA[c]**	**H_4EDTA[c]**
logK_1^H	10.48 (2)	11.23	11.70	10.14
logK_2^H	6.43 (2)	6.52	6.12	6.02
logK_3^H	4.23 (2)	3.78	3.52	2.53
logK_4^H	2.76 (2)	2.24	2.43	1.99
logK_5^H	1.68 (2)	1.56	–	–
ΣlogK_i^H	25.58	25.33	23.77	20.69

[a] Ref. [14], [b] Ref. [10], [c] Ref. [15]

The comparison of protonation constants between CyAAZTA and AAZTA (Table 1) indicates that logK_1^H value for CyAAZTA is significantly lower, whereas logK_3^H for CyAAZTA is nearly half order of magnitude higher. The lower logK_1^H value of CyAAZTA might be explained by the presence of the *trans*-1,2-diaminocyclohexyl moiety which can distort the 7-membered ring resulting in the lower cooperation between the exocyclic- and

ring-N donor atoms during the first protonation process as shown to happen in case of AAZTA.[10] This view is supported by the similarity of the logK_1^H values for CyAAZTA (10.48) and for 1,4-diazacycloheptane-*N,N*-diacetic acid (9.83).[16] The difference in the logK_3^H values might be accounted for the higher basicity of one carboxylate O-atom due to the electron donation of the cyclohexyl ring. An analogous explanation was forwarded to explain the logK_3^H value for CDTA in comparison with EDTA. Noteworthy, the total basicity ($\Sigma\log K_i^H$) of CyAAZTA is comparable with that of AAZTA (Table 1).

Complexation properties: The stability and protonation constants of Ga(CyAAZTA)$^-$ and Cu(CyAAZTA)$^{2-}$ are defined by Eqs. (2) and (3):

$$K_{ML} = \frac{[ML]}{[M][L]} \quad (2)$$

$$K_{MH_iL} = \frac{[MH_iL]}{[MH_{i-1}L][H^+]} \quad (3)$$

The stability constants of Ga(CyAAZTA)$^-$ (Table 2) were determined by pH-potentiometry (Figure S1) following the competition between CyAAZTA and OH$^-$ ions for Ga^{3+} (Eq. (4)) at high pH values (pH>8) using the hydrolysis constants of the free Ga^{3+} ion (log$K_{[Ga(OH)]2+}$ = -2.97, log$K_{[Ga(OH)2]+}$ = -5.92, log$K_{[Ga(OH)3]}$ = -8.2 and log$K_{[Ga(OH)4]}$- = -16.81).[17-19].

$$Ga(CyAAZTA) + nOH^- \rightleftharpoons [Ga(OH)_n]^{3-n} + CyAAZTA \quad n=3,4 \quad (4)$$

The protonation constants of Ga(CyAAZTA)$^-$ were calculated from the titration curves obtained at 1:1 metal to ligand concentration ratios by titrating the complex with HCl and NaOH solutions, respectively. The titration data of Ga(CyAAZTA)$^-$ obtained at pH>6 indicate the occurrence of a base consuming process. This process can be interpreted by assuming the coordination of a OH$^-$ ion and the formation of [Ga(CyAAZTA)OH]$^{2-}$ according to Eqs. (5) and (6):

$$M + L + OH^- \rightleftharpoons ML(OH)^- \quad (5)$$

$$\beta_{MLH_{-1}} = \frac{[ML(OH)]}{[M][L][OH^-]}$$

$$ML(OH^-) + H^+ \rightleftharpoons ML + H_2O \quad (6)$$

$$K_{MLH_{-1}} = \frac{[ML]}{[ML(OH^-)][H^+]}$$

The stability constants of Ga(CyAAZTA)$^-$ is slightly lower than that of Ga(AAZTA)$^-$ (Table 2), probably as a result of the higher rigidity of the CyAAZTA chelator. Moreover, the formation of [Ga(CyAAZTA)OH]$^-$ (characterized by the logK_{GaLH-1} protonation constant) starts at pH>6.0, 3 pH units higher that of Ga(AAZTA)$^-$ takes place. The stability constant of Cu(CyAAZTA)$^{2-}$ (logK_{CuL} = 20.11 (2)) was determined by spectrophotometry in the [H$^+$] range of 0.01 – 1.0 M ([CyAAZTA]=[Cu^{2+}]= 2×10^{-3} M) and the data are reported in ESI.

The Ga^{3+}- CyAAZTA equilibrium system was also investigated by means of ^{1}H- and ^{71}Ga-NMR spectroscopy. ^{71}Ga- and ^{1}H-NMR spectra obtained at 1:1 Ga^{3+} to CyAAZTA ratio in the pH range 0.7-12.7 are presented in Figures 1 and S4, respectively. ^{71}Ga-NMR data were also used for the calculation of the stability constants of [Ga(CyAAZTA)OH]$^{2-}$. The integrals of the ^{71}Ga-NMR signals ([Ga(OH)$_4$]$^-$ at 223 ppm, Fig. 1B) were used for the calculation of the logβ_{GaLH-1} value of [Ga(CyAAZTA)OH]$^{2-}$ (Table 2).

Table 2. Stability and protonation constants of the Ga^{III}-complexes of CyAAZTA and AAZTA (0.1 M KCl, 25°C)

	Ga(CyAAZTA)		Ga(AAZTA)[a]	
	pH-pot.	**^{1}H- and ^{71}Ga-NMR**	**pH-pot.**	**^{1}H- and ^{71}Ga-NMR**
logK_{GaL}	21.39 (3)	21.92 (8)	22.18	22.36
logK_{GaHL}	4.09 (3)		3.05	
logK_{GaH2L}	2.32 (3)	+ logK_{GaLH-1}	0.82	+ logK_{GaLH-1}
logK_{GaLH-1}	7.31 (3)		4.49	
logβ_{GaLH-1}	14.08 (3)	14.61 (8)	17.69	17.87
pGa[b]	22.0	22.4	pGa[b]	22.0

[a] Ref. [10], [b] pGa=-log[Ga^{3+}]$_{free}$, [Ga^{3+}]$_{tot}$=1×10^{-8} M, [L]$_{tot}$=1×10^{-7} M, pH=7.4.

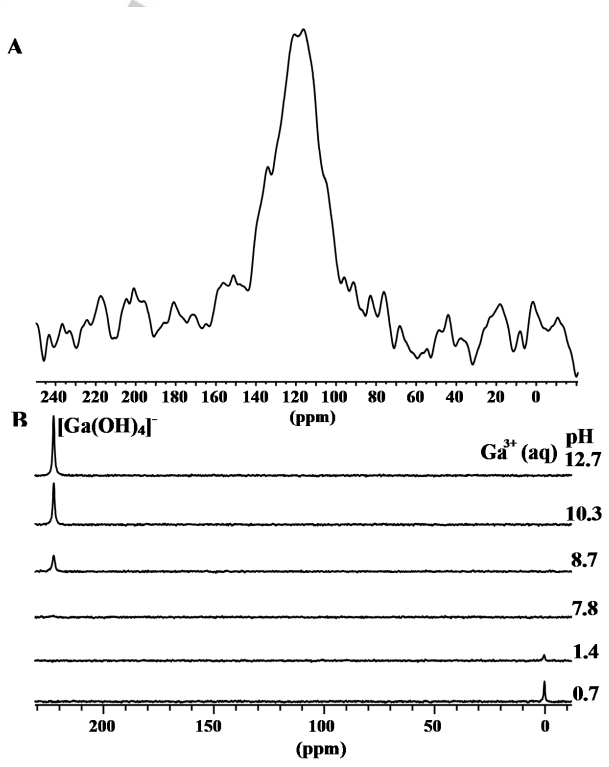


Figure 1. ^{71}Ga-NMR spectra (122 MHz) of Ga(CyAAZTA) ([GaL]=0.02 M, 75°C, pH=5.0, D_2O) (**A**) and Ga^{3+}-CyAAZTA systems ([Ga^{3+}]= [CyAAZTA]= 0.015 M, 0.1 M KCl, 25°C (**B**).

The logβ_{GaLH-1} values of [Ga(CyAAZTA)OH]$^{2-}$ obtained by pH-potentiometry (logβ_{GaLH-1} = 14.08 (3)) and NMR spectroscopy (logβ_{GaLH-1}=14.61 (8)) are in good agreement (Table 2). The equilibrium data obtained by the pH-potentiometric titration combined with the results of NMR studies, allowed to calculate the species distribution diagram for the Ga^{3+}-CyAAZTA system (Figure 2). The species distribution diagram and ^{71}Ga-NMR spectra indicate that the complex formation is complete at

pH>1.5. The gallium resonance is relatively sharp for the highly symmetric $[Ga(H_2O)_6]^{3+}$ species (Fig. 1B, $\nu_{½}$ = 51 Hz) at pH~0.7. The intensity of the ^{71}Ga-NMR signal of the aquaion decreases by increasing the pH due to the formation of protonated $Ga(H_2CyAAZTA)^+$ and Ga(HCyAAZTA) complexes in the pH range 0.7 - 1.4. In $Ga(AAZTA)^-$, the Ga^{3+} ion is coordinated by 3 amino-N and 3 carboxylate-O donor atoms with a distorted octahedral geometry (one of the carboxylate-O donors does not coordinate),[10] and presumably the coordination geometry of $Ga(CyAAZTA)^-$ is similar. In the pH range 1.4 - 5.0, the deprotonation of the $Ga(H_2CyAAZTA)^+$ and Ga(HCyAAZTA) results in a slight upfield shift of all signals in the ^{1}H NMR spectrum (Fig. S4) which indicates that these processes take place at the weakly- or non-coordinated carboxylate groups of the ligand. At pH>6.0 the formation of the $[Ga(CyAAZTA)OH]^{2-}$ species become evident by the extra equivalent base consumption during the pH-potentiometric titration and the new set of signals in the ^{1}H-NMR spectra (Fig. S4). At pH=8, the $[Ga(CyAAZTA)OH]^{2-}$ is the predominant species (Fig. 2). The OH^- ion of $[Ga(CyAAZTA)OH]^-$ is coordinated directly to the metal ion replacing one carboxylate O-atom. At pH > 8.0, the appearance of the ^{71}Ga-NMR signal of the $[Ga(OH)_4]^-$ (Fig. 1B, δ_{Ga}=223 ppm) and the ^{1}H-NMR signals of the free CyAAZTA indicate that CyAAZTA is getting replaced by the OH^- ions in the competition reaction for Ga^{3+} ion with the formation of $Ga(OH)_3$ and $[Ga(OH)_4]^-$ species. The ^{1}H-NMR signal of the methyl protons of the free and the complexed CyAAZTA are well separated (δ_H=0.92 and 1.12 ppm, respectively). The intensity and the chemical shifts of the methyl protons of complexed and free CyAAZTA indicates the dissociation of $[Ga(CyAAZTA)OH]^{2-}$ and the deprotonation of free CyAAZTA in the 8.2 – 12.2 pH range.

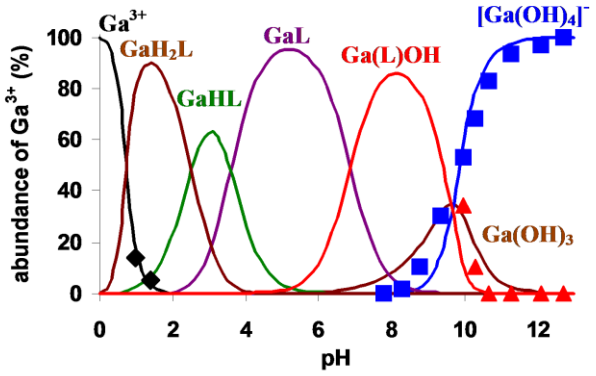


Figure 2. Species distribution of Ga^{3+} - CyAAZTA system calculated from the pH-potentiometric titration data. The percentage of Ga^{3+}_{aq} (◆), $[Ga(CyAAZTA)OH]^{2-}$ (▲) and $[Ga(OH)_4]^-$ (■) calculated from the NMR data. ($[Ga^{3+}]$ = [CyAAZTA] = 15 mM, 0.1 M KCl, 25°C).

At pH=5.0 and 298 K, the ^{71}Ga-NMR signal of $Ga(CyAAZTA)^-$ is very broad and almost not observable, even broader than that of $Ga(AAZTA)^-$ (δ_{Ga}=118 ppm, $\nu_{½}$ = 2200 Hz).[10] This is clearly the result of the asymmetric coordination environment of the Ga^{3+} ion provided by the rigid CyAAZTA. Generally, the broadening or sharpening of the ^{71}Ga-NMR signal could be interpreted either by an exchange processes or by the interaction of the nuclear quadrupole moment with the electric-field gradient at the ^{71}Ga nucleus.[20] In particular, the VT - ^{71}Ga-NMR spectra of Ga(CyAAZTA) (Fig. S5-B) show a sharpening of the linewidth with increasing temperature from $\nu_{½}$= 4700 Hz (308 K, Fig. S5-B) to $\nu_{½}$= 2400 Hz (348 K, Figure 1A).

Dissociation kinetics

Nowadays, as far as the *in vivo* use of metal complexes concerns, there is a large consensus in saying that the kinetic inertness is as important as the thermodynamic stability, since the products of the dissociation reactions (both the free metal ions and ligands) could be toxic for the living systems. The dissociation rate of the metal ion from a Ga^{III} complex is typically measured in strong acidic ($[H^+]$>1.0 M) and basic conditions ($[OH^-]$>0.1 M).[7a,20,21] However, the kinetic data obtained in such conditions, which are considerably different from the physiological ones, are not fully reliable to predict the behaviour of Ga^{III} complexes in body fluids. In fact, after intravenous administration, the Ga^{III}-complex may also interact with the endogenous ions (Cu^{2+}, Zn^{2+} and Ca^{2+}) that can exchange and release toxic Ga^{3+} ion forming the corresponding metal complexes.

Transmetallation reactions: To obtain information about the kinetic inertness of $Ga(CyAAZTA)^-$ near physiological conditions, we studied the kinetics of the transmetallation reactions occurring between the Ga^{III} chelate and Cu^{2+} ions in the presence of citrate excess to prevent the hydrolysis of Ga^{3+} and Cu^{2+} ions at pH 6.0 – 9.0. Under such conditions Cu^{2+} is predominantly present as $Cu(Cit)H_{-1}$ species, whereas the Ga^{3+} ion forms $Ga(Cit)H_{-1}$ and $Gd(Cit)_2$ complexes.[10,22,23] The absorption spectra and the species distribution of the $Ga(CyAAZTA)^-$ and Cu^{2+}-citrate reacting system are presented in Figures 3 and S3.

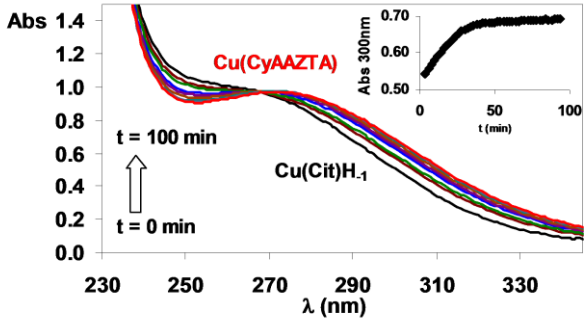


Figure 3. Absorption spectra of $Cu(CyAAZTA)^{2-}$ and $Ga(CyAAZTA)^-$ - Cu(Citrate) reacting system as a function of time at pH=7.50. Inserted figure shows the absorbance values of the reacting system at 300 nm as a function of time ($[Ga(CyAAZTA)\text{-}]$ = 1.0 mM, $[Cu(CyAAZTA)^{2-}]$ = 0.3 mM, $[Cu^{2+}]_{tot}$=0.3 mM, $[Cit^{3-}]_{tot}$ = 2.0 mM, [HEPES] = 0.01 M, l=1 cm, 0.1 M KCl, 25°C).

The transmetallation reaction between $Ga(CyAAZTA)^-$ and Cu^{2+} is expressed by the following equation:

$$Ga(CyAAZTA)^- + Cu^{2+} \rightleftharpoons Cu(CyAAZTA)^{2-} + Ga^{3+} \qquad (7)$$

The rates of the transmetallation reactions were studied in the presence of excess of Ga(CyAAZTA) ($[GaL]_{tot}/[Cu^{2+}]_{tot}$ = 5) when a pseudo-first order kinetic model can be applied and the rates of transmetallation can be expressed by Eq. (8):

$$-\frac{d[GaL]_t}{dt} = \frac{d[CuL]_t}{dt} = k_d[GaL]_t \qquad (8)$$

where k_d is a pseudo-first-order rate constant, $[GaL]_t$ and $[CuL]_t$ are the concentration of complexes (e.g. GaL, $Ga(L)H_{-1}$, CuL, $Cu(L)H_{-1}$), at the time t, respectively. During the progress of the transmetallation reactions the concentration of Cu(CyAAZTA) and $Cu(CyAAZTA)H_{-1}$ complexes increases (the deprotonation of the Cu(CyAAZTA) complex takes place in the pH range 8 – 11), while that of $Cu(Cit)H_{-1}$ decreases. By the use of 1.0 cm cells, the first-order rate constant, k_d can be expressed by Eq. (9):

$$k_d = \frac{\Delta Abs}{\Delta t} \times \frac{1}{\varepsilon_{CuL} - \varepsilon_{Cu(Cit)H_{-1}}} \times \frac{1}{[GaL]_t} \qquad (9)$$

In the Eq. (9) $\Delta Abs/\Delta t$ values (the increase of the absorbance during the time Δt) are calculated from the slope of the kinetic curves. $\varepsilon_{Cu(Cit)H-1}$ and ε_{CuL} are the molar absorptivities of the $Cu(Cit)H_{-1}$ (921 $M^{-1}cm^{-1}$), Cu(CyAAZTA) (2435 $M^{-1}cm^{-1}$) and $Cu(CyAAZTA)H_{-1}$ (2811 $M^{-1}cm^{-1}$) complexes at 300 nm (the absorption of the Ga^{3+} containing species at 300 nm can be neglected). The ε_{CuL} of Cu(CyAAZTA) species at given pH can be expressed as a weighted average of the molar absorptivities of the Cu(CyAAZTA) and $Cu(CyAAZTA)H_{-1}$ complexes by taking into account the protonation constant of $Cu(CyAAZTA)H_{-1}$ ($\log K_{CuLH-1}$=9.62 (2)) determined by pH-potentiometric titration of the Cu(CyAAZTA) at 25°C in 0.1 M KCl). The obtained pseudo-first-order rate constants for the reaction of $Ga(CyAAZTA)^-$ with Cu^{2+} at different pH and $[OH^-]$ values in the presence of citrate are shown in Figure 4.

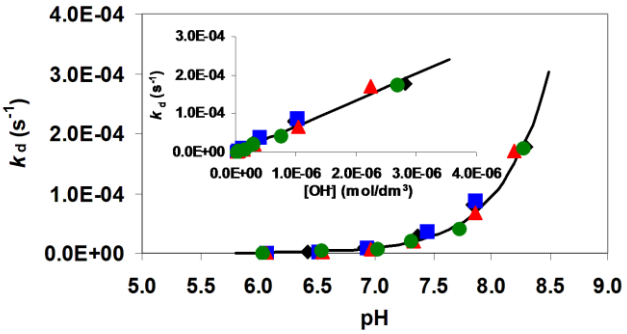


Figure 4. *k*d values vs. pH for the reaction between $Ga(CyAAZTA)^-$ and Cu^{2+}. ($[Ga(CyAAZTA)^-]$ = 1.0 mM, $[Cu^{2+}]$=0.2 mM [Cit]=1.0 mM (♦), 2.0 mM (■), 3.0 mM (▲), 4.0 mM (●), [MES]=[HEPES] = 0.01 M, 0.1 M KCl, 25°C).

Since the reaction rate is independent from the concentration of citrate and $Cu(Cit)H_{-1}$ (Figure 4), it can be assumed that the rate determining step is the dissociation of $Ga(CyAAZTA)^-$, followed by the fast metal exchange reaction between $Cu(Cit)H_{-1}$ species and the free CyAAZTA ligand. The increase of k_d values with the increase of $[OH^-]$ (Figure 4) can be explained by the equilibrium formation of $[Ga(CyAAZTA)OH]^{2-}$ (Eq. (6)), which slowly dissociates in spontaneous (Eq. (10)) and OH^- assisted (Eq. (11)) reactions.

$$[Ga(CyAAZTA)OH]^{2-} \xrightarrow{k_{OH}} Ga^{3+} + H_xCyAAZTA + OH^- \qquad (10)$$

$$[Ga(CyAAZTA)OH]^{2-} + OH^- \xrightarrow{k_{OH^2}} Ga^{3+} + H_xCyAAZTA + 2OH^- \qquad (11)$$

By taking into account these two pathways, the rate of the transmetallation of $Ga(CyAAZTA)^-$ can be expressed by Eq. (12).

$$-\frac{d[GaL]_t}{dt} = k_{OH}[Ga(L)OH] + k_{OH^2}[Ga(L)OH][OH^-] \qquad (12)$$

Considering the total concentration of the complex ($[GaL]_{tot}$ = [GaL]+[Ga(L)OH]), the pseudo-first-order rate constant (k_d) can be expressed as follows:

$$k_d = \frac{k_{OH}K_{Ga(L)OH}[OH^-] + k_{OH^2}K_{Ga(L)OH}[OH^-]^2}{1 + K_{Ga(L)OH}[OH^-]} \qquad (13)$$

where $K_{Ga(L)OH}$ equilibrium constant, k_{OH} and k_{OH}^2 rate constants characterizing the spontaneous and OH^- assisted dissociation of $[Ga(CyAAZTA)OH]^{2-}$, respectively. The rate and equilibrium constants characterising the transmetallation reaction of $Ga(CyAAZTA)^-$ in the presence of citrate were calculated by fitting the k_d values presented in Figure 4 to the Eq. (13) and the values obtained are shown and compared with those of $Ga(AAZTA)^-$ in Table 3. By taking into the ionic product of water for the related condition (pK_w=13.87, 0.1 M KCl, 25°C), the $\log K_{GaLH-1}$ of $Ga(CyAAZTA)^-$ can be calculated from the $K_{Ga(L)OH}$ equilibrium constant obtained from the kinetic data ($K_{GaLH-1}=1/K_w \times K_{Ga(L)OH}$). The "deprotonation" constant ($\log K_{GaLH-1}$) obtained by pH-potentiometry ($\log K_{GaLH-1}$=7.31 (3)) and from the kinetic data ($\log K_{GaLH-1}$=7.34 (8)) are in very good agreement.

Table 3. Rate and equilibrium constants and half-lives ($t_{1/2}=\ln 2/k_d$) for the transmetallation reactions of $Ga(CyAAZTA)^-$ and $Ga(AAZTA)^-$ complexes (0.1 M KCl, 25°C)

	Ga(CyAAZTA)⁻	Ga(AAZTA)[a]
k_{OH} (s^{-1})	(1.7 ± 0.1)×10⁻⁵	3.0×10⁻⁶
k_{OH}^2 ($M^{-1}s^{-1}$)	68 ± 5	10
$K_{Ga(L)OH}$ (M^{-1})	(3.4 ± 0.1) ×10⁶	1.4 ×10⁹
pK_w[b]	13.87	
$\log K_{GaLH-1}$	7.34 (1)	4.72
k_d (s^{-1}) at pH=7.4	2.3×10⁻⁵	9.2×10⁻⁶
$t_{1/2}$ (h) at pH=7.4	8.5	21

[a] Ref. [5], [b] determined by pH-potentiometry (0.1 M KCl, 25°C)

The k_{OH} and k_{OH}^2 rate constants for $[Ga(CyAAZTA)OH]^{2-}$ are 6 - 7 times higher than the corresponding rate constants of $[Ga(AAZTA)OH]^{2-}$. The spontaneous dissociation of $[Ga(CyAAZTA)OH]^{2-}$ and $[Ga(AAZTA)OH]^{2-}$ likely proceeds through the intramolecular rearrangement of the Ga^{III} complexes that results in the simultaneous de-coordination of all donor atoms with the consequent release of Ga^{3+} ion. The faster spontaneous dissociation of $[Ga(CyAAZTA)OH]^{2-}$ might be interpreted by the coordination of OH^- to the Ga^{3+}-ion which cause the weakening of the interaction between the Ga^{3+}-ion

and both ring N donor atoms due to the rigidity of the CyAAZTA skeleton.

With the use of the rate and equilibrium constants presented in Table 3, the half-lives ($t_{1/2}$=ln2/k_d) of the dissociation reactions of Ga(CyAAZTA)$^-$ and Ga(AAZTA)$^-$ can be calculated for any pH by Eq. (13). The $t_{1/2}$ values of Ga(CyAAZTA)$^-$ and Ga(AAZTA)$^-$ calculated for pH=7.4 are 8.5 and 21 hours, which indicate a lower kinetic inertness of Ga(CyAAZTA)$^-$ due to the faster spontaneous dissociation of [Ga(CyAAZTA)OH]$^{2-}$. However, by calculating the dissociation half-lives at pH=6.0, $t_{1/2}$ values of 234 and 64 hours can be found for Ga(CyAAZTA)$^-$ and [Ga(AAZTA)OH]$^{2-}$, respectively. These data highlight a higher kinetic inertness of Ga(CyAAZTA)$^-$ at lower pH due to its significantly higher conformational rigidity than that of [Ga(AAZTA)OH]$^{2-}$ (Ga(CyAAZTA)$^-$ is the predominant species at pH=6.0).

Transferrin challenge reactions. Another mechanism that could be responsible for the dissociation of Ga(CyAAZTA)$^-$ in body fluids is related to the strong affinity of Ga^{3+} for transferrin (Ga^{3+}-transferrin: logK_{GaTf}=18.88, logK_{Ga2Tf}=17.65).[24] Because of the relatively high concentration of the transferrin in human plasma,[25] this protein could compete with CyAAZTA for the Ga^{3+} ion leading to the significant dissociation of Ga(CyAAZTA)$^-$. In order to determine the extent of the competition between CyAAZTA and transferrin, the ligand exchange reaction between Ga(CyAAZTA)$^-$ and human serum transferrin (sTr) was followed by spectrophotometry at the absorption band of the Ga^{3+}-sTf complex in the 240 - 250 nm range (Figure 5). The absorbance values of Ga(CyAAZTA)$^-$ - sTf reacting systems obtained at [sTf]=10 and 15 μM are shown in Figure S6. The ligand exchange reaction between Ga(CyAAZTA)$^-$ and human sTf is expressed by the following equation:

$$Ga(CyAAZTA)^- + sTf \rightleftharpoons Ga(sTf) + CyAAZTA \quad (14)$$

The rates of the ligand exchange reaction were studied at different concentrations of the exchanging human transferrin ([sTf]=10 and 15 μM) in the presence of high excess of Ga(CyAAZTA)$^-$ ([Ga(CyAAZTA)]=0.1 mM). At such condition, the ligand exchange reaction can be treated as a pseudo-first-order process and the rate of the reactions can be expressed with the Eq. (8). By taking into account the molar absorptivity of Ga(sTf) (ε_{246}=13800 cm^{-1}M^{-1}),[10] the rate constants (k_d) were calculated from the slope of the kinetic curves (ΔAbs/Δt, Figure S6) with Eq. (9) (the middle term of Eq. (9) has been replaced by 1/$\varepsilon_{Ga(sTf)}$). The slope of the kinetic curves are considered up to 40% conversion in order to be sure of the Ga(sTf) complex formation. The rate constants (k_d) obtained at 10 and 15 μM human sTf concentration were 2.8×10^{-5} and 3.0×10^{-5} s^{-1}, respectively (average value 2.9 ×10^{-5} s^{-1}).

The k_d rate constants characterizing the ligand exchange reaction of Ga(CyAAZTA)$^-$ with sTf (k_d=2.9×10^{-5} s^{-1}) and the metal exchange reaction between Ga(CyAAZTA)$^-$ and Cu^{2+} in the presence of citrate (k_d=2.3×10^{-5} s^{-1}) at pH=7.4 and 25°C in 0.1 M KCl are essentially equal. These findings suggest that human transferrin has no effect on the rate of dissociation, which practically takes place through the spontaneous and hydroxide assisted dissociation of [Ga(CyAAZTA)OH]$^{2-}$ followed by the fast reaction between the released Ga^{3+} ion and human sTf at physiological condition.

In conclusions, the dissociation half-life values of Ga(CyAAZTA)$^-$ calculated from the transmetallation studies ($t_{1/2}$=8.5 h) and from the ligand exchange reactions ($t_{1/2}$=6.6 h) indicates that the kinetic inertness of Ga(CyAAZTA)$^-$ at physiological conditions is 7 times higher than the half-life of the radioactive decay of ^{68}Ga isotope ($t_{1/2}$=67.71 min). On the basis of these results the Ga(CyAAZTA) could represent a good candidate for the development of the Ga^{3+}-based radiodiagnostics.

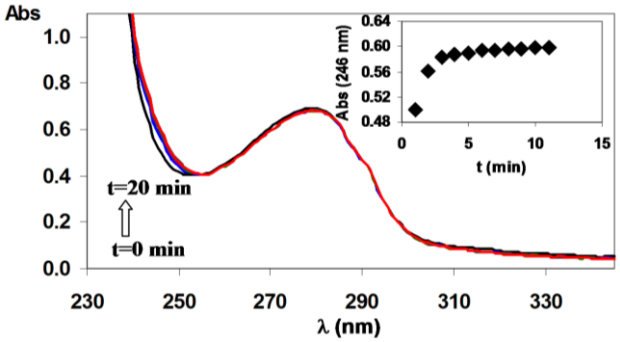


Figure 5. Absorption spectra of the Ga(CyAAZTA)$^-$ - transferrin system. Inserted figure shows the absorbance values of the reacting system at 246 nm as a function of time ([GaL] = 0.1 mM, [Trf] = 10 μM, [$NaHCO_3$]=25 mM, pH = 7.4, 0.15 M NaCl, 25°C).

^{68}Ga labelling. AAZTA is a pseudo-macrocyclic chelator which has the advantages of either fast complexation kinetics typical of the open chain ligands and of forming stable chelates typical of macrocyclic ones.[10] AAZTA was shown to form the ^{68}Ga-labelled complex at room temperature and pH 3.8 (acetate buffer).[11] These experimental conditions were used for the ^{68}Ga-labelling of a 10 μM solution of CyAAZTA (25 °C, 0.25 M acetate buffer pH 3.8, 15 min, 15-20 MBq of ^{68}Ga). A labelling yield of 80.7 ± 0.8 % was measured by radio-TLC, demonstrating the excellent efficiency of the novel chelate for ^{68}Ga incorporation at room temperature. HPLC analysis of the radiolabelled complex showed the formation of a single species (Figure 6a), differently from what observed earlier in case of ^{68}GaAAZTA.[12a] Since the use of an appropriate buffer during the radiolabelling step is essential to maximize the labelling yield and the specific activity,[26] 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer was also selected, due to its widely usage for ^{68}Ga labelling, as it has a weak metal complexing capacity to avoid the formation of colloidal gallium. In spite of the favourable expectations, the labelling using HEPES at pH 3.8 (0.2 M) and 25°C for 15 min gave unsatisfactorily results (L.Y.: 53 ± 3%, 10 μM) with respect to acetate buffer. The ^{68}Ga labelling of CyAAZTA at different temperatures (25, 37 and 90 °C) and at different ligand concentrations (0.5-100 μM) was tested (Figure 6b). A labelling yield > 80% was found at room temperature for concentrations higher than 10 μM whereas at lower concentrations the labelling efficiency decreased. The labelling at 37°C only slightly increased the yield for each concentration; in particular, 85.8 ± 0.9 % yield could be obtained for 10 μM ligand concentration. For 1 μM CyAAZTA concentration (2.4 nmol), the labelling efficiency could be improved by increasing the temperature to 90°C. In fact, a radiochemical incorporation of 96.9% was accomplished after 5 min heating. Similar results were obtained for higher concentrations of the chelator whereas the further decrease of the ligand concentration to 0.5 μM did not yield a successful incorporation of the radiometal. The fast

and nearly quantitative labelling at 90°C may result in higher specific activity of CyAAZTA-conjugated peptide or peptide-mimetics that is essential for an accurate quantification of PET examinations. The kinetics of ^{68}Ga incorporation for 1 μM CyAAZTA concentration in acetate buffer (pH 3.8) at 25 and 37°C was also studied (Figure 6c). The data show a slightly faster complexation kinetic at higher temperatures and confirm that the ^{68}Ga labelling of CyAAZTA can be efficiently carried out either at room or physiological temperature. Therefore, this chelator is suitable for conjugation to temperature labile vectors such as antibody derived fragments (Fab, diabodies, nanobodies, affibodies)[27,28] or antibody-like molecules (anticalins).[27] This claim prompts by considering that AAZTA was modified in several ways in order to conjugate it to biomolecules, *i.e.* bifunctional AAZTA chelators bearing amino,[9c] isothiocyanate,[9c] carboxylate[29] or hydroxyl[9b] groups have been reported in the literature. Thus, also CyAAZTA can be functionalized with the same reactive groups using analogous procedures.

Finally, although kinetic studies and transferrin challenge reactions on GaCyAAZTA were discussed in detail previously, the relative stability of the radiolabelled complex was also assessed to confirm the data reported for the cold chelate. ^{68}GaCyAAZTA (10 μM, formed at 90 °C, 0.25 M acetate buffer pH 3.8, 5 min, 15-20 MBq of ^{68}Ga) was diluted in the presence of an excess DTPA and in human serum and its stability was checked by HPLC and radio-TLC as a function of time at 37°C (Figure S7). No transmetallation reaction over 90 min was measured. Noteworthy, the reported data on ^{68}Ga CyAAZTA labelling and stability compare favourably with the behaviour described for related acyclic and macrocyclic ligands.[1,2,6-8]

Conclusions

The presence of the *trans*-1,2-diaminocyclohexyl group in the newly synthesized ligand CyAAZTA led to the formation of a structurally rigid Ga^{III} complex having good thermodynamic stability. The kinetic inertness of $Ga(CyAAZTA)^-$ was determined via transmetallation and ligand exchange reactions with Cu^{2+} ions and transferrin, respectively, showing a slight tendency to decomplex the Ga^{3+} ion through the formation of the hydroxo-complex at physiological pH. Remarkably, the kinetic data obtained in such conditions appear more reliable to predict the behaviour of Ga^{III} complexes in body fluids than those typically used in the literature, *i.e.* dissociation rate of the metal ion measured in strong acidic and basic conditions. The thermodynamic stability and kinetic inertness of $Ga(CyAAZTA)^-$ is well suitable for ^{68}Ga PET studies as it is considerably longer than the radioactive half-life of the ^{68}Ga isotope. The complexation of ^{68}Ga with CyAAZTA was observed at RT and pH 3.8 within 15 min with the yield of >80% and at 90°C with almost quantitative yield (in 5 min). ^{68}GaCyAAZTA was confirmed to be stable in human plasma and in excess of DTPA at 37 °C. The potential of the proposed chelator is strenghtened by the wide spectrum of possible bifunctional chelators that can be prepared adapting to CyAAZTA the procedures used for the synthesis of functionalized AAZTA ligands. Therefore, whereas the labelling at room temperature can be of interest for temperature sensitive molecules, labelling at higher temperatures may be advantageous for reaching higher specific activity in peptide radiopharmaceuticals.

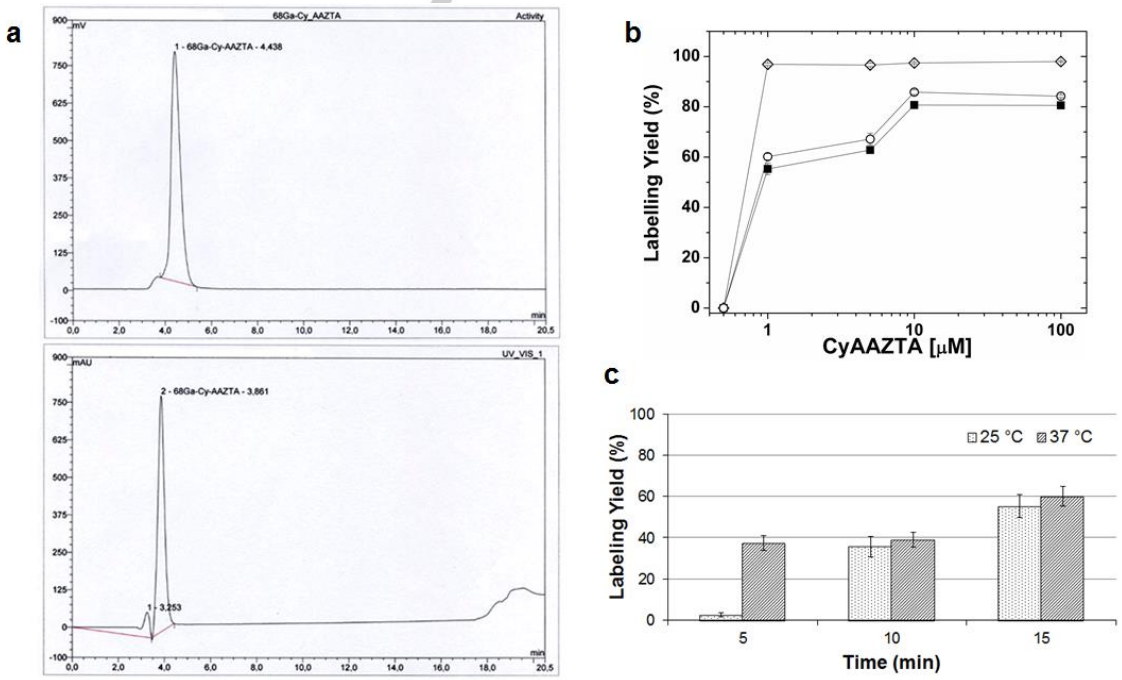


Figure 6. a) Radio-HPLC analysis of ^{68}GaCyAAZTA; b) Radiochemical yield (^{68}Ga incorporation) as a function of CyAAZTA concentration (0.5, 1, 5, 10 and 100 μM) at 25°C (■), 37°C (○) and 90°C (◊) (15-20 MBq in 500 μL, pH 3.8 acetate buffer); c) Time course of ^{68}Ga complexation reaction at 25°C and 37°C for 1 μM CyAAZTA concentration.

Experimental Section

General All reagents and solvents were obtained from commercial suppliers and directly used without further purification. The NMR spectra were recorded on Bruker Avance III (11.74) instruments. Chemical shifts are reported in δ relative to an internal standard of residual solvent. Electrospray ionization mass spectra (ESI MS) were recorded on a SQD 3100 Mass Detector (Waters), operating in positive or negative ion mode, with 1% v/v formic acid in methanol as the carrier solvent. 1.0 M HCl for elution of $^{68}Ge/^{68}Ga$ generator was prepared from HCl (Suprapur®) and water (Ultrapur®), both obtained from Merck. Ultrapur® water was also used for all radiochemical works, including the preparation of precursors and buffers solutions. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was obtained from Merck and sodium acetate from Sigma. Radio-TLC was performed on Varian glass microfiber chromatography papers impregnated with silicic acid (#A120B12). Readout of TLC chromatograms was done using a Bioscan TLC scanner, consisting of B-MS-1000 scanner, B-EC-1000 detector with a B-FC-3600 GM tube. Analytical HPLC was performed using a Shimazu HPLC System with low pressure gradient unit, equipped with a Nucleosil C18-RP column (250x4.6 mm, 5 µm particle size) from Machery-Nagel, at flow rate of 1 ml/min, and a SPD 20A Variable Wavelength Detector (UV/VIS) set at 254 nm. Eluent were water and acetonitrile, both containing 0.1 % trifluoroacetic acid (TFA).

Materials $Ga(NO_3)_3$ was prepared by dissolving Ga_2O_3 (99.9%, Fluka) in 6M HNO_3 and evaporating of the excess acid. The solid $Ga(NO_3)_3$ was dissolved in 0.1 M HNO_3 solution. The concentration of the $Ga(NO_3)_3$ solution was determined by using the standardized Na_2H_2EDTA in excess. The excess of the Na_2H_2EDTA was measured with standardized $ZnCl_2$ solution and xylenol orange as indicator. The concentration $ZnCl_2$ (Sigma) and $CuCl_2$ (Sigma) solutions were determined by complexometric titration with standardized Na_2H_2EDTA and xylenol orange ($ZnCl_2$) and murexide ($CuCl_2$) as indicator. The H^+ concentration of the $Ga(NO_3)_3$ solution was determined by pH potentiometric titration in the presence of excess Na_2H_2EDTA. The concentration of $H_4CyAAZTA$ was determined by pH-potentiometric titrations in the presence and absence of a 40-fold excess of Ca^{2+}. The citrate solution was prepared from $H_3Citrate$ (Sigma) and its concentration was determined by pH-potentiometry. The pH-potentiometric titrations were made with standardized 0.2 M KOH.

*Synthesis of (±) trans-1,2-dibenzylaminocyclohexane diacetate (**1**)* Benzaldehyde (2.26 mL, 22.4 mmol) was dropped to a suspension of (±) trans-1,2-diaminocyclohexane (1.16 g, 10.2 mmol) and a small amount of $MgSO_4$ (ca. 200 mg) in 10 mL of DCM. $NaBH_4$ (1.54 g, 40.7 mmol) was slowly added to the mixture which was then stirred vigorously at room temperature. After 18 h the mixture was washed with water (3 x 10 mL). The organic phase was then dried over Na_2SO_4, acidified with acetic acid (1.5 mL) and evaporated to obtain an oil that was further washed with hexane (3 x 10 mL) to remove unreacted benzaldehyde. (±) trans-1,2-dibenzylaminocyclohexane diacetate (**1**) was obtained as a yellow oil after drying under vacuum (3.15 g, 7.6 mmol, 74.5% yield). ^{1}H-NMR $CDCl_3$ 400 MHz: δ 1.75 (m, CH_2, 4H), 2.11 (m, CH_2, 4H), 3.60 (s, CH_2, 4H), 7.22-7.37 (m, CH_{Ph}, 10H); ^{13}C-NMR $CDCl_3$ 100 MHz: δ 1.75 23.1 (CH_2), 31.1 (CH_2), 56.4 (CH_2), 71.4 (CH_2), 128-130 (CH_{Ph}). ESI^+-MS: 295.45 $[M+H^+]$, MW calc. for $C_{20}H_{26}N_2$ = 294.43.

*Synthesis of trans-1,5-dibenzyl-3-methyl-3-nitrodecahydro-1H-1,5-benzodiazepine (**2**)* Paraformaldehyde (250 mg, 8.3 mmol) and nitroethane (178.7 mg, 2.38 mmol) were added to a solution of (±) trans-1,2-dibenzylaminocyclohexane diacetate (987 mg, 2.38 mmol) in 100 mL of a mixture of EtOH and Toluene (1:1 v/v). The mixture was then heated to reflux and stirred overnight. The evaporation of the solvent gave 1.5 g of crude product which was purified by column chromatography (silica gel, eluents: 9:1 Petrol Ether/Ethyl acetate, R_f = 0.56) to obtain **2** as a pale yellow oil (0.55 g; 1,4 mmol, 59% yield). ^{1}H-NMR $CDCl_3$ 500 MHz: δ 1.03 (d q, CH_2, 2H), 1.28 (b , CH_2, 4H), 1.34 (s, CH_3, 3H), 1.76 (m, CH_2, 2H), 1.85 (b, CH_2, 1H), 2.11 (d, CH_2, 1H), 2.46 (t, CH, 1H), 2.69 (b, CH_2, 1H), 2.87 (d, CH_2, 1H), 3.35 (d, CH_2, 1H), 3.55 (d, CH_2, 1H), 3.64 (d, CH_2, 1H), 3.87 (d, CH_2, 1H), 3.98 (d, CH_2, 1H), 4.11 (d, CH_2, 1H), 4.17 (s, CH_2, 1H), 7.28-7.36 (m, CH_{Ar}, 10H); ^{13}C-NMR $CDCl_3$ 125 MHz: δ 24.1 (CH_3), 24.6 (CH_2), 25.1 (CH_2), 26.4 (CH_2), 31.9 (CH_2), 51.1 (CH_2), 56.5 (CH_2), 56.6 (CH_2), 58.9 (CH_2), 60.4(CH), 66.4 (CH), 93.2 (C), 127-129 (5 CH_{Ar}), 139.1 (C_{Ar}). ESI^+-MS: 394.36 $[M+H^+]$, MW calc. for $C_{24}H_{31}N_3O_2$ = 393.24.

*Synthesis of trans-3-amino-3-methyldecahydro-1H-1,5-benzodiazepine (**3**)* Compound **2** (0.55 g, 1.4 mmol) was dissolved in MeOH (10 mL) and acetic acid (160 uL, 2.8 mmol). After addition of Pd/C (26 mg, 10% w/w), the reaction was carried out under H_2 pressure (5 Bar) and vigorous stirring at room temperature overnight (18h). The catalyst was removed by filtration and the methanol was removed to obtain 410 mg of the amine **3** as diacetate salt (96% yield). ^{1}H-NMR MeOD 500 MHz: δ 1.19 (m, CH_2, 4H), 1.38 (s, CH_3, 3H), 1.79 (b. CH_2, 4H), 2.0 (s, NH, 2H), 2.64 (b, CH_2, 2H), 2.86 (b, CH_2, 2H), 3.11 (m, CH, 1H), 3.33 (m, CH, 1H); ^{13}C-NMR $CDCl_3$ 125 MHz: δ 22.3 (CH_3), 24.2 (CH_2), 31.5 (CH_2), 54.8 (C), 56.9 (CH_2), 66.9 (CH); ESI^+-MS: 184.18 $[M+H^+]$, MW calc. for $C_{10}H_{21}N_3$ = 183.29.

*Synthesis of trans-1,5-bis(t-butoxycarbonylmethyl)-3-[bis(t-butoxycarbonylmethyl)]amino-3-methyldecahydro-1H-1,5benzodiazepine (CyAAZTA tetra-t-butylester, **4**)* tert-butilbromoacetate (473 µL, 3.2 mmol) was added to a suspension of compound **3** (120 mg, 0.40 mmol) and K_2CO_3 (550 mg, 4 mmol) in 10 mL of acetonitrile. The mixture was stirred at 80°C overnight. After then it was filtered and the solvent was evaporated to obtain an orange oil. After purification by column chromatography (silica gel, eluents: 9:1 Petrol Ether/Ethyl acetate, R_f = 0.55), **4** was obtained as a pale yellow oil (140 mg, 0.22 mmol, 55% yield). ^{1}H-NMR $CDCl_3$ 500 MHz: δ 1.08-1.13 (b, CH_2, 4H), 1.37 (s, tBu CH_3, 36H), 1.42 (b, CH_3, 3H), 1.64 (b, CH_2, 4H), 2.39-2.47 (b, CH_2, 4H), 2.80-2.98 (m, CH, 2H), 3.4-3.5 (m, CH_2, 8H); ^{13}C-NMR $CDCl_3$ 125 MHz: δ 14.2 (CH_3), 22.6 (CH_2), 25.7 (CH_2), 28.2 (CH_3^{tBu}), 51.4 (CH_2), 60.3 (CH_2), 61.9 (CH), 69.4 (C), 80.5 (C_{tBu}), 172.7 (C=O); ESI^+-MS: 640.45 $[M+H^+]$, MW calc. for $C_{34}H_{61}N_3O_8$ = 639.86.

Synthesis of trans-3-amino-3-methyldecahydro-1H-1,5-benzodiazepine-N,N',N'',N'''tetraacetic acid (CyAAZTA) Compound **4** (140 mg, 0.22 mmol) was dissolved in 2 mL of DCM. Trifluoroacetic acid (2 mL) was then added and the solution was stirred overnight at room temperature. After evaporation of the solvent, the solid was washed three times with diethyl ether (5 mL) and then dried under vacuum to obtain the ligand **L** as the mono-trifluoroacetic salt (101 mg, 0.19 mmol, 87% yield). The solid was then dissolved in 2-3 mL of water and the pH was raised to 6.5 by addition of a few drops of a 1 M solution of NaOH and then lyophilized. ^{1}H-NMR D_2O 500 MHz: δ 1.23 (s, CH_3, 3H), 1.25 (m, CH_2, 2H), 1.38-1.44 (m, CH_2, 2H), 1.75 (m, CH_2, 2H), 1.98 (m, CH_2, 2H), 3.12-3.20 (m, CH, 2H), 3.36-3.62 (m, CH_2, 4H), 3.72-3.79 (m, CH_2, 8H); ^{13}C-NMR D_2O 125 MHz: δ 14.1 (CH_3), 24.2 (CH_2), 26.6 (CH_2), 48.0, 51.0, 53.6, 54.7 (CH_2), 64.2 (CH), 66.2 (C), 170.0, 173.5 (C=O); ESI^+-MS: 416.4 $[M+H^+]$, MW calc. for $C_{18}H_{30}N_3O_8$ = 416.44.

Equilibrium measurements The stability and protonation constants of $Ga(CyAAZTA)^-$ were determined by pH-potentiometric titration from acidic to basic pH range by studying the competition reaction between CyAAZTA and OH^- for Ga^{3+} ($[CyAAZTA]=[Ga^{3+}]= 3\times10^{-3}$ M). The protonation constants of $Cu(CyAAZTA)^{2-}$ were determined by pH-potentiometric titrations of CuL complex in the pH range of 1.7 – 11.7 ($[Cu(CyAAZTA)]= 3\times10^{-3}$ M). For pH measurements and pH-potentiometric titrations, a Metrohm 785 DMP Titrino titration workstation and a Metrohm-6.0233.100 combined electrode were used. Equilibrium measurements were carried out with 120 s waiting time between two pH-

KOH datapairs at a constant ionic strength (0.1 M KCl) in 6 ml samples at 25 °C. The solutions were stirred, and N_2 was bubbled through them. The titrations were made in the pH range of 1.7-11.7. KH-phthalate (pH=4.005) and borax (pH=9.177) buffers were used to calibrate the pH meter. For the calculation of $[H^+]$ from the measured pH values, the method proposed by Irving et al. was used. A 0.01M HCl solution was titrated with the standardized KOH solution in the presence of 0.1 M KCl ionic strength. The differences between the measured (pH_{read}) and calculated pH (-log$[H^+]$) values were used to obtain the equilibrium H^+ concentration from the pH values, measured in the titration experiments. The ionic product of water (pK_w) at 25°C in 0.1 M KCl was found to be 13.87.[30] The stability constant of $Cu(CyAAZTA)^{2-}$ was determined by spectrophotometry in the $[H^+]$ range of 0.01 – 1.0 M ($[CyAAZTA]=[Cu^{2+}]=2\times10^{-3}$ M). Seven samples were prepared for determination and the H^+ concentration ($[H^+]$=0.010, 0.025, 0.050, 0.10, 0.30, 0.50 and 1.0 M) in the samples was adjusted with the addition of calculated amounts of 2.0 M HCl. The samples were kept at 25 °C for 7 days in order to attain the equilibrium (the time needed to reach the equilibria was determined by spectrophotometry). The absorbance values of the samples were measured at 11 wavelengths (575, 595, 615, 635, 655, 675, 695, 715, 735, 755 and 775 nm). The ionic strength of samples with $[H^+]$=0.30, 0.50 and 1.0 M was not constant (the ionic strength of samples with $[H^+]+[K^+]$=0.1 M). For the equilibrium calculations, the molar absorptivities of the Cu^{2+}, Cu(CyAAZTA), $Cu(HCyAAZTA)^-$, $Cu(H_2CyAAZTA)$, $Cu(H_3CyAAZTA)^+$ and $Cu(CyAAZTA)H_{-1}$ were used. The molar absorptivities of Cu^{2+} and $Cu(CyAAZTA)^{2-}$ complexes were determined by recording the VIS spectra (λ=400 – 800 nm) of 1.0×10^{-4}, 2.0×10^{-4}, 3.0×10^{-4} and 4.0×10^{-4} M solutions in the pH range 1.7 – 11.7 (0.1 M KCl, 25°C). The pH was adjusted by stepwise addition of concentrated KOH or HCl. The spectrophotometric measurements were made with the use of a Cary 1E spectrophotometer at 25 °C, using 1.0 cm cells. The protonation and stability constants were calculated with the PSEQUAD program.[31]

NMR experiments ^{1}H- and ^{71}Ga-NMR measurement were performed with a Bruker DRX 400 (9.4 T) equipped with a Bruker VT-1000 thermocontroller and a BB inverse z gradient probe (5 mm). The formation and protonation/deprotonation processes of the Ga(CyAAZTA) were followed from acidic to basic pH range at 298K in 0.1 M KCl. For these experiments, 0.015 M solution of the Ga(CyAAZTA) in H_2O was prepared (a capillary with D_2O was used for lock). The pH was adjusted with the addition of the concentrated solution of KOH and HCl. Because of the metal exchange between the Ga(CyAAZTA)OH and $[Ga(OH)_4]^-$ was in the "slow exchange regime" on the actual NMR timescale, the calculation of the $\log\beta_{GaLH-1}$ value of Ga(CyAAZTA)OH was performed by using the integrals of the ^{71}Ga-NMR signal of $[Ga(OH)_4]^-$ complex. The molar integral values of ^{71}Ga-NMR signal of $[Ga(OH)_4]^-$ complex were determined by recording the ^{71}Ga NMR spectra of 0.01, 0.015, 0.02 and 0.025 M solutions of $[Ga(OH)_4]^-$ complex (pH=12.5, 0.1 M KCl, 25°C).

Transmetallation kinetic The rates of the exchange reactions taking place between Ga(CyAAZTA) and Cu^{2+} in the presence of citrate were studied by spectrophotometry, following the formation of the Cu(CyAAZTA) or $Cu(CyAAZTA)H_{-1}$ complexes at 300 nm, with the use of 1.0 cm cells and a Cary 1E spectrophotometer. The concentration of Cu^{2+} was 0.2 mM, while that of Ga(CyAAZTA) was 5 times higher, in order to ensure pseudo-first-order conditions. In order to prevent the hydrolysis of Ga^{3+} and Cu^{2+} ions, the transmetallation reactions were studied in the presence of citrate excess ($[Cit]_t$=1.0 – 4.0 mM). The exchange rates were studied in the pH range about 6.0 - 9.0. For keeping the pH values constant, MES (pH range 6.0 - 7.0), HEPES (pH range 7.0 - 8.5) and piperazine (pH range 8.5 - 9.0) buffers (0.01 M) were used. The temperature was maintained at 25°C and the ionic strength of the solutions was kept constant (0.1 M KCl). The formation rates of Cu(CyAAZTA) or $Cu(CyAAZTA)H_{-1}$ were determined by the initial rate method. The pseudo-first-order rate constants (k_d) were calculated from the tangent to the absorbance vs. time curves ($\Delta Abs/\Delta t$) with Eq. (9). For the calculations, the molar absorptivities of $Cu(CyAAZTA)^{2-}$, $Cu(CyAAZTA)H_{-1}$ and $Cu(Cit)H_{-1}$ were used, which were determined at 300 nm by recording the spectra of 1.0×10^{-4}, 2.0×10^{-4}, 3.0×10^{-4} and 4.0×10^{-4} M solutions in the pH range 5 - 10 (0.1 M KCl, 25°C). The calculations were performed with the use of the computer program Micromath Scientist, version 2.0 (Salt Lake City, UT, USA).

Ligand-exchange kinetics with transferrin The ligand exchange reaction between $Ga(CyAAZTA)^-$ and human serum transferrin (Sigma, partially Fe^{3+} saturated) have been studied by spectrophotometry, following the formation of Ga(sTf) complex at 246 nm and pH=7.4 with the use of 1.0 cm cells and Cary 1E spectrophotometer. The concentration of the human serum transferrin solution was determined from the aborbance at 280 nm using the molar absorptivity ε_{280}=91200 $cm^{-1}M^{-1}$.[32] In order to ensure the pseudo-first-order condition, the rate of the ligand exchange reactions were studied in the presence of high excess of $Ga(CyAAZTA)^-$ ([Ga(CyAAZTA)]=0.1 mM, [sTf]=10 and 15 μM)). The temperature was maintained at 25°C, the ionic strength and the hydrogen-carbonate concentration of the samples were kept constant; 0.15 M for NaCl and 0.025 M for $NaHCO_3$, respectively.

^{68}Gallium labelling Manual synthesis was used for investigation of radiolabeling properties, namely buffer, concentration and time dependencies of radioactivity incorporation. A ^{68}Ge/^{68}Ga-generator with SnO2 matrix (obtained from ITHEMBA LABS, South Africa) was eluted with 1.0 M aq. HCl. To a fraction of 1.20 mL containing the highest activity (ca. 700 MBq). For buffer dependency ^{68}Ga incorporation study, a solution of HEPES (0.2 M, 800 μL) or sodium acetate (0.25 M, 500 μL plus 300 μL of 1.0 M aq. HCl) was added to the eluate and mixed by brief shaking, resulting in 2.0 mL solution at pH 3.8. Of this solution, 50 μL aliquots were transferred into eppendorf vials containing 450 μL volume of stock solutions of CyAAZTA ligand with appropriate concentrations (ranging from 0.1x10–3 to 0.5×10–6 M), resulting in 500 μL reaction solutions. Heating (when applied) was performed by placing the closed eppendorf vials into a thermostated water bath. After heating, labeling reactions were interrupted by placing the cups into a cold water bath. Radioactivity incorporation was determined by radio-TLC, while samples were withdrawn after completed cooling or, in case of reactions at 25 °C, during the reaction at several time points, for kinetic of incorporation studies.

Quality control Radio-TLC of the product was performed on VARIAN glass microfiber chromatography papers impregnated with silicic acid (cat. #A120B12), using two different solvents. TLC-A: 0.1 M aq. sodium citrate as mobile phase, where free $^{68}Ga^{3+}$ is eluted with the solvent front as citrate complex (R_F = 0.8–1) and the product stays at the origin (R_F = 0). TLC-B: 1.0 M NH_4OAc/MeOH (1:1) as mobile phase, where insoluble colloidal $^{68}Ga^{III}$ stays at the origin (R_F = 0) and the radiolabeled product is eluted with the solvent front (R_F = 0.5-0.6). Radio-TLCs were evaluated using a BIOSCAN TLC scanner consisting of B-MS-1000 scanner, B-EC-1000 detector with a B-FC-3600 GM tube. Radio-HPLC was performed on a Shimazu system using a Nucleosil C18-RP column (MAchery-Nagel, 250×4.6 mm) with radioactivity and UV detection (254 nm). Eluents were water (A) and acetonitrile (B), both containing 0.1 % TFA (flow rate 1 mL/min, isocratic elution with a gradient from 15 % B to 20 % B in 10 min and isocratic elution with 90 % B for 5 min). Retention time of CyAAZTA ligand 10 μM radiolabeled at 25 and 37 °C, and CyAAZTA ligand 1 μM radiolabeled at 90 °C was invariably 4.4 min (activity channel). Radiochemical purity was of ^{68}Ga-CyAAZTA was >98.0 % determined by radio-HPLC, and > 97.0 % determined by radio-TLC.

Stability tests Stability of the ^{68}Ga-CyAAZTA was tested by adding 100 μL of the product to 900 μL of human serum freshly prepared and 900 μL of 50 mM DTPA solution, followed by incubation at 37 °C for 90 min. Stability of ^{68}GaCyAAZTA complex was determined with radio-TLC, by withdrawing samples (2.5 μL) during the reaction at 30, 60 and 90 min and spotting onto the TLC strip, and with radio-HPLC by injecting 50 μL of the ^{68}Ga-CyAAZTA/50mM DTPA reaction solution at 30, 60 and 90 min.

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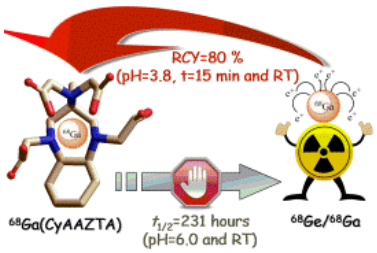
Entry for the Table of Contents

FULL PAPER

A new rigid chelator based on a cyclohexane ring fused into a AAZTA-like ligand was synthesized. The thermodynamic stability and kinetic inertness of the Ga^{III} complex were investigated by pH potentiometry, ^{71}Ga NMR and UV-spectrophotometry. The radiolabeling with ^{68}Ga was tested with different buffers, ligand concentrations and temperatures. Finally, the stability of $^{68}GaCyAAZTA$ was checked in human serum and with excess DTPA at 37°C.



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A rigidified AAZTA-like ligand as efficient chelator for ^{68}Ga radiopharmaceuticals