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Radiochemistry of cyclen-derived chelators comprising five-membered azaheterocyclic arms with $^{212}Pb^{2+}$, $^{213}Bi^{3+}$, and $^{225}Ac^{3+}$☆,☆☆

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ABSTRACT

In this work, we introduce the cyclen-based metal chelator DOTThia comprising four methylthiazole arms for metal complexation. Together with the recently described congener DOTI-Me bearing four methylimidazole arms, the radiochemistry of these two compounds with ^{212}Pb, ^{213}Bi, and ^{225}Ac was investigated thoroughly. Radiolabeling experiments were performed at various pH values and temperatures to determine the optimal conditions for quantitative radiochemical conversions. Experiments revealed excellent complexation properties of DOTThia for ^{212}Pb at room temperature, comparable to those of the current gold standard for Pb complexation, TCMC, while it was not well suited for ^{213}Bi. Contrarily, DOTI-Me exhibited quantitative radiochemical conversions for ^{213}Bi at pH 5.5 and room temperature, outperforming the metal chelator macropa, but was not able to quantitatively complex ^{212}Pb under any conditions investigated. Of note, both novel chelators were not able to bind ^{225}Ac. In preliminary experiments, we could also show that a functionalized DOTI-Me derivative is capable of complexing ^{213}Bi selectively from ^{225}Ac solutions. This feature may allow the preparation of ^{213}Bi-labeled radiotracers directly from ^{225}Ac solutions without the need for an $^{225}Ac/^{213}Bi$ generator. However, more detailed studies are needed to fully explore this potential application. Altogether, our results support the future development of ^{212}Pb-labeled radiopharmaceuticals using bifunctional derivatives of DOTThia as well as of ^{213}Bi-labeled radiotracers based on the DOTI-Me scaffold.

1. Introduction

Alpha emitters, such as Actinium-225 (^{225}Ac), Bismuth-213 (^{213}Bi), and Lead-212 (^{212}Pb), offer significant advantages in the treatment of tumors, especially in the context of targeted alpha therapy (TAT). TAT offers an improved therapeutic option for patients who are resistant to conventional therapies or targeted radioligand therapy using beta-emitters such as ^{177}Lu. In contrast to beta-emitters, alpha particles are highly effective due to their high linear energy transfer (LET) delivering a high energy over a very short range, typically around 50–100 μm, allowing for precise destruction of cancer cells with minimal damage to surrounding healthy tissue. With typical particle energies of 5–9 MeV, the resulting LET is approximately 80–100 keV/μm, which is orders of magnitude higher compared to beta or gamma radiation. This highly localized treatment results in the destruction of tumor cells while sparing normal tissues, which is a significant advantage over traditional therapies like external beam radiation or chemotherapy that often also affect healthy cells. The alpha-emitters ^{225}Ac, ^{213}Bi, and ^{212}Pb each have unique decay characteristics that make them particularly useful in cancer treatment by TAT. The radionuclide ^{225}Ac, for example, decays with a relatively long half-life of $t_{1/2}$ = 9.92 d through a series of daughter products (Scheme 1A), including ^{213}Bi, delivering altogether 4 alpha and 2 beta particles with a high LET (150 keV/μm), allowing for an extended therapeutic effect. The radionuclides ^{213}Bi and ^{212}Pb with their shorter half-lives of $t_{1/2}$ = 45.6 min and 10.6 h, respectively, provide a rapid and intense radiation dose, which is highly effective for

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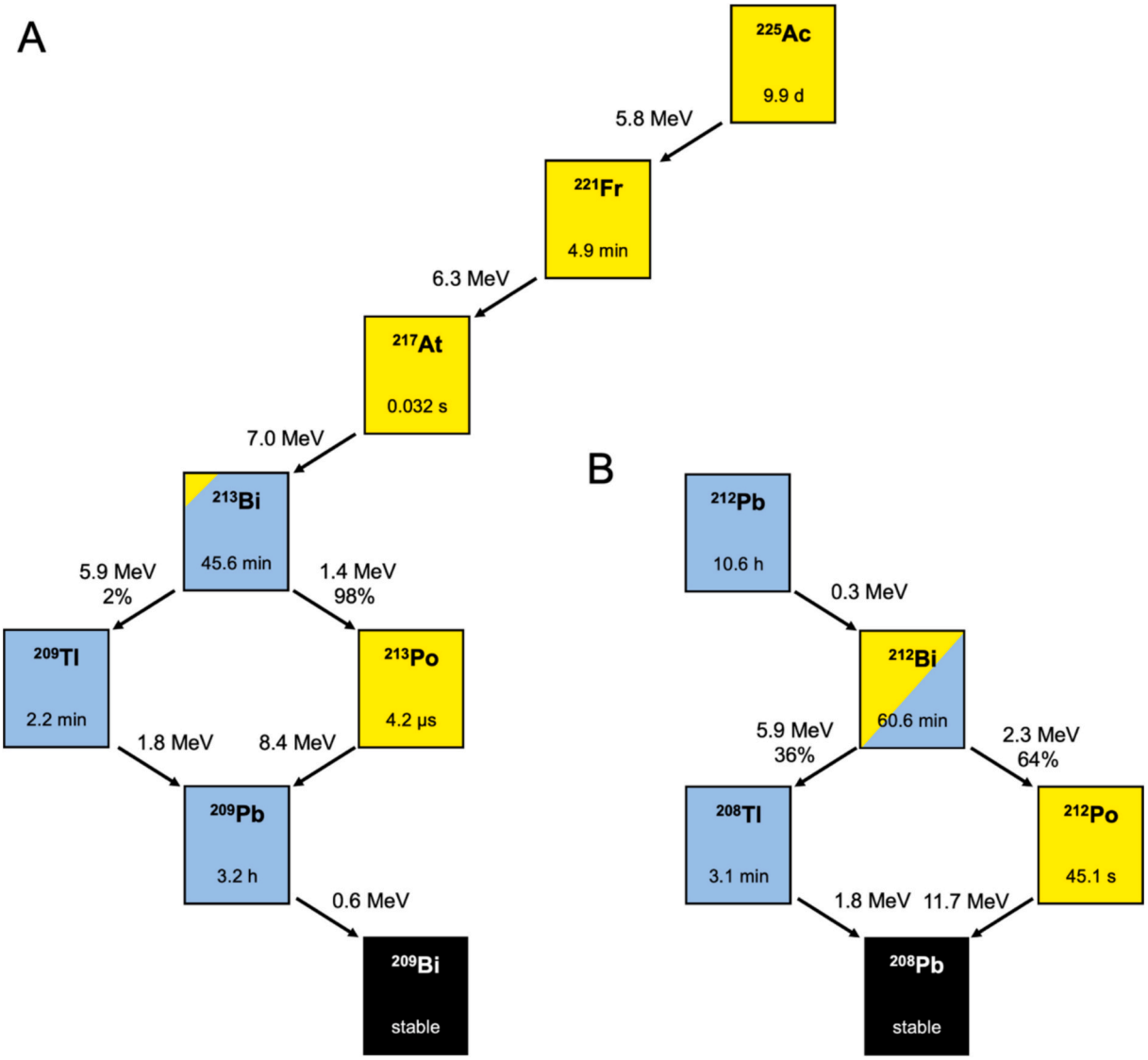
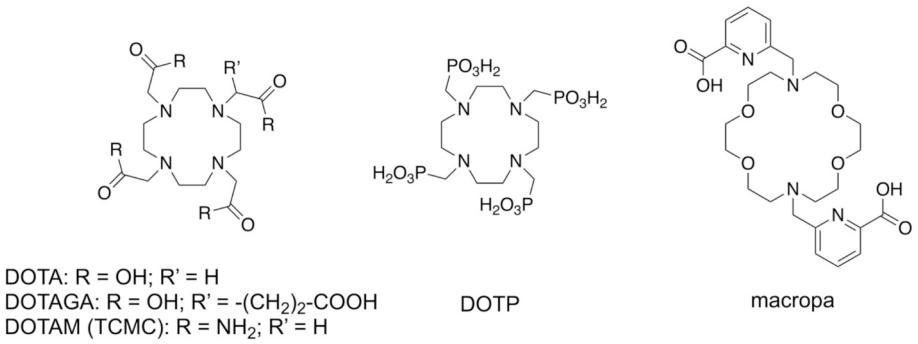
treating smaller tumors or micrometastases. The radionuclide ^{213}Bi emits only one alpha particle per decay and is available by ^{225}Ac/^{213}Bi generator systems [1–3]. The beta-emitter ^{212}Pb is the parent of the alpha-emitter ^{212}Bi ($t_{1/2}$ = 60.6 min) and is also available from generator systems, including ^{224}Ra/^{212}Pb and ^{228}Th/^{212}Pb generators [4–6]. Their physical decay properties and ready availability by generator systems make ^{212}Pb and ^{213}Bi highly attractive for TAT applications (Scheme 1). Furthermore, their relatively short half-lives match perfectly with targeting vectors exhibiting rapid pharmacokinetics such as small molecules and peptides.

The use of ^{212}Pb, ^{213}Bi, and ^{225}Ac for TAT requires the utilization of bifunctional chelators (BFCs) that (1) tightly bind the radiometal and (2) possess at least one chemical functionality allowing covalent conjugation of disease-specific targeting vectors, such as antibodies, peptides, *etc.* The most prominent BFC is the ligand DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), shown in Scheme 2, and corresponding derivatives such as DOTAGA (1,4,7,10-tetraazacyclododecane-1-(glutaric acid)-4,7,10-triacetic acid) are commonly used for ^{225}Ac chelation, and to a lesser extent for ^{213}Bi chelation [7–9]. Phosphorus-containing ligands based on the macrocycle cyclen (1,4,7,10-tetraazacyclododecane), such as DOTP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylene phosphonic acid)) have shown superior labelling efficiencies for ^{213}Bi compared to DOTA [10]. In recent years, the ligand macropa (Scheme 2) and macropa-NCS have emerged as promising alternatives for ^{225}Ac and ^{213}Bi with minimal off-target organ accumulation, rapid complex formation kinetics at ambient temperature with high stability *in vivo* over an extended period of time [11,12]. The ligand DOTA and its derivative DOTAM (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamide), also known as TCMC (Scheme 2), where the carboxylate arms are modified to carbamoylmethyl groups, are the two most commonly used chelators for ^{212}Pb [13,14]. A direct comparison of DOTA–mAb and TCMC–mAb conjugates has shown that the latter binds Pb^{2+} cations more effectively [15].

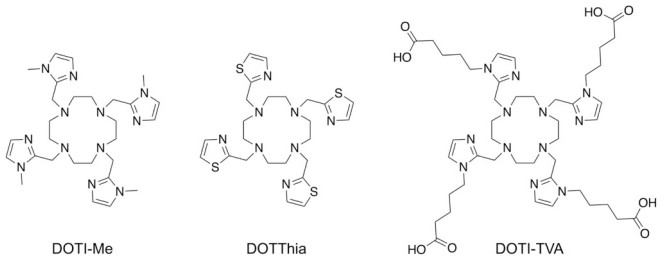
In our efforts to design novel chelators for radiopharmaceutical applications, we recently reported on the radiochemistry of the ligand DOTI-Me (1,4,7,10-tetrakis((1-methyl-1*H*-imidazol-2-yl)methyl)-1,4,7,10-tetraazacyclododecane), shown in Scheme 3, based on the cyclen macrocycle with four additional methylimidazole arms [16]. This ligand provides a $N_4N'_4$ donor set that exhibited distinct differences from the well-known chelator DOTA with a N_4O_4 donor set. DOTI-Me has shown excellent complexation properties for the radiometals $^{52g}Mn^{2+}$, $^{64}Cu^{2+}$, $^{111}In^{3+}$, $^{161}Tb^{3+}$, and $^{177}Lu^{3+}$, covering diagnostic as well as therapeutic radionuclides suggesting its applicability in radiopharmaceutical development [16]. In the present report, we also



Scheme 1. Decay schemes of (A) ^{225}Ac and ^{213}Bi, and (B) ^{212}Pb. Color coding: alpha decay (yellow), beta minus decay (blue).



Scheme 2. Commonly used metal chelators for ^{212}Pb, ^{213}Bi, and ^{225}Ac.



Scheme 3. Metal chelators presented in this work.

introduce its congener DOTThia (1,4,7,10-tetrakis(thiazol-2-ylmethyl)-1,4,7,10-tetraazacyclododecane) bearing four additional thiazole arms (Scheme 3) together with the results of the radiochemical evaluation of both ligands with the radiometals ^{225}Ac, ^{213}Bi, and ^{212}Pb. Additionally, we prepared the functionalized derivative DOTI-TVA (TVA = tetravaleric acid) comprising four carboxylic acid groups allowing covalent attachment of targeting vectors through peptide bond formation (Scheme 3).

2. Methods

2.1. General

Chemicals were of reagent grade and were purchased from Sigma Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), and Tokyo Chemical Industry (Eschborn, Germany). [^{212}Pb]$PbCl_2$ was eluted from a ^{228}Th generator (TRIUMF, Vancouver, BC, Canada) according to previously published procedures [17]. A ^{225}Ac/^{213}Bi generator system was used as a source of [^{213}Bi]Bi^{3+}. The preparation of the generator and isolation of $^{213}Bi^{3+}$ from $^{225}Ac^{3+}$ was performed using previously reported methods (TRIUMF, Vancouver, BC, Canada) [18,19]. The radionuclide [^{225}Ac]$Ac(NO_3)_3$ was obtained from Van Overeem Nuclear BV (Breda, The Netherlands). Water (>18.2 MΩ·cm at 25 °C) was used without further purification. Solvents for reactions were of reagent grade. Evaporation of the solvents was performed under reduced pressure by using a rotary evaporator (Rotavapor R-300, Büchi Labortechnik AG, Flawil, Switzerland). ^{1}H and ^{13}C NMR spectra were measured in deuterated solvents on a Bruker AV-400 (^{1}H: 400 MHz, ^{13}C: 100.6 MHz) or a Bruker AV-500 (^{1}H: 500 MHz, ^{13}C: 125.8 MHz) spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) relative to the resonance of the residual solvent peaks. Coupling constants (J) are reported in Hz. Peak multiplicities are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad signal). Analytical high-performance liquid chromatography (HPLC) experiments were performed by using an Agilent 1260 Infinity II System (Waldbronn, Germany) with a Phenomenex Jupiter Proteo (250 × 4.60 mm, 5 µm) column equipped with an Agilent 1260 VWD detector and a FlowStar [2] LB 514 (Berthold Technologies, Bad Wildbad, Germany) in series and a 1260 quat pump VL. Semi preparative HPLC was performed on a Rigol L-3000 equipped with a L-3500 UV-VIS detector and a L-3245 quaternary pump (Techlab, Germany). The solvent system for both was A = H_2O (0.1 % TFA = trifluoroacetic acid) and B = acetonitrile (0.1 % TFA). The gradient for ligand purification was 0–1 min 5 % B, 1–10 min 5–45 % B, 10–13 min 45–95 %, 13–15 min 95 % B, 15–19 min 95–5 % B, 19–20 min 5 % B with a flow rate of 12 mL min^{-1}, the gradient for complex purification was 0–1 min 0 % B, 1–16 min 0–50 % B, 16–18 min 50–95 % B, 18–20 min 95 % B, 20–21 min 95–5 % B, 21–22 min 5 % B with a flow rate of 7 mL min^{-1}. The gradient for analytical purposes was 0–1 min 0 % B, 1–16 min 0–50 % B, 16–18 min 50–95 % B, 18–20 min 95 % B, 20–22 min 95–0 % B at a flow rate of 1 mL min^{-1}. Low resolution mass spectrometry ((+)-LR-ESI-MS) was performed on an Advion expressionCMS (Ithaca, NY, USA). High resolution mass spectrometry ((+)-HR-ESI-MS) was performed on an AB Sciex API 5500 QTRAP spectrometer (Framingham, MA, USA). Samples were lyophilized using a Christ Alpha 1–2 LD plus lyophilizer (Osterode am Harz, Germany). Radioactive reactions were monitored by using instant thin-layer chromatography (radio-iTLC). Glass-fiber iTLC plates impregnated with silica-gel or salicylic acid (iTLC-SG or iTLC-SA, Agilent Technologies) were developed by using an aqueous mobile phase containing EDTA (ethylenediamine tetraacetic acid) (50 mM, pH 5.0) for ^{212}Pb and ^{213}Bi analysis and DTPA (diethylenetriamine pentaacetic acid) (50 mM, pH 7.4) for ^{225}Ac analysis, and were analyzed on a radio-TLC detector (MiniGita, Elysia Raytest, Straubenhardt, Germany), an AR-2000 TLC Scanner (Eckert and Ziegler, Berlin, Germany) or Typhoon TRIO imager (GE Healthcare, Munich, Germany). When using aqueous mobile phases containing EDTA (50 mM, pH 5.0), radiochemical conversion (RCC) was determined by integrating the data obtained by the radio-TLC plate reader and determining both the percentage of radiolabelled product (R_f = 0.0) and 'free' activity (R_f = 1.0; present in the analyses as $[M(EDTA)]^{x-}$). Integration and data analysis were performed by using MiniGita software (Gina Star TLC™, Raytest), AR-2000 TLC Scanner software (Eckert and Ziegler, Berlin, Germany) or FIJI (on GitHub) using the WinScan V3 software (Academic Software Inc., Lexington, USA). Appropriate background and decay corrections were applied as necessary. All instruments measuring radioactivity were calibrated and maintained in accordance with previously reported routine quality-control procedures [20]. Radioactivity was measured using an Activimeter ISOMED 2010 (Nuklear-Medizintechnik, Dresden, Germany).

2.2. Ligand synthesis

1,4,7,10-tetrakis((1-methyl-1*H*-imidazol-2-yl)methyl)-1,4,7,10-tetraazacyclododecane (DOTI-Me) (1).

The ligand DOTI-Me was prepared as previously described and characterization data matched with the literature [16]. Fractions containing the product were combined and lyophilized to give DOTI-Me as yellowish resin. Yield: 794 mg (90 %). ^{1}H NMR (400 MHz, 298 K, D_2O) δ

(ppm): 7.44 (m, 8H), 4.14 (s, 8H), 3.82 (s, 12H), 3.04 (s, 16H). ^{13}C NMR (101 MHz, 298 K, D_2O) δ (ppm): 144.28, 127.22, 122.73, 53.46, 49.75, 37.11 ppm. LR-ESI-MS: *m/z* calc. for $C_{28}H_{45}N_{12}$ $[M + H]^+$: 549.4, found 549.4. HR-ESI-MS: *m/z* calc. for $[M + H]^+$: 549.38901, found 549.38767. RP-HPLC (semipreparative): t_R = 10 min. RP-HPLC (analytical): t_R = 8.6 min, purity 99 %.

1,4,7,10-tetrakis(thiazol-2-ylmethyl)-1,4,7,10-tetraazacyclododecane (DOTThia) (2).

Cyclen (345 mg, 2 mmol, 1 eq.) was suspended in THF (tetrahydrofurane) and 2-formylthiazole (1.360 mg, 1.05 mL, 12 mmol, 6 eq.) was added. The mixture was stirred for 120 min at r.t. (room temperature). Then, sodium triacetoxyborohydride (2.540 mg, 12 mmol, 6 eq.) was added portion-wise. The reaction was stirred for 18 h at r.t. The solvent was removed under reduced pressure and the crude was purified by column chromatography silica gel with n-hexane/ethyl acetate 1:2, then pure ethyl acetate. To rinse the column, an increasing amount of methanol was added to ethyl acetate. Fractions containing the product were combined and the solvent was removed under reduced pressure. Yield: 537 mg (0.96 mmol, 48 %). ^{1}H NMR (400 MHz, 298 K, $CDCl_3$) δ (ppm): 7.86 (d, J = 3.3 Hz, 4H), 7.49 (d, J = 3.4 Hz, 4H), 4.66 (s, 8H), 3.45 (s, 16H). ^{13}C NMR (101 MHz, 298 K, $CDCl_3$) δ (ppm): 143.19, 121.75, 116.19, 114.03, 52.44, 49.01, 30.94, 29.71. LR-ESI-MS: *m/z* calc. for $C_{24}H_{33}N_8S_4$ $[M + H]^+$: 561.2, found 561.4 calc. for $[M + Na]^+$ 583.2, found 583.4. HR-ESI-MS: *m/z* calc. for $C_{24}H_{33}N_8S_4$ $[M + H]^+$: 561.16328, found 561.16935. RP-HPLC (analytical): t_R = 13.0 min, purity 95 %.

5,5′,5″,5‴-(((1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetrakis(methylene))tetrakis(1*H*-imidazole-2,1-diyl))tetrapentanoic acid DOTI-TVA (3).

Methyl 5-(2 formyl-1H-imidazol-1-yl)pentanoate was prepared as recently described [21]. The macrocycle cyclen (0.34 g, 2.00 mmol) was dissolved in THF (30 mL) and mixed with methyl 5-(2 formyl-1H-imidazol-1-yl)pentanoate (1.26 g, 6.00 mmol, 3 eq.). The reaction was stirred at 70 °C for 19 h. After cooling to r.t., sodium triacetoxyborohydride (3.82 g, 18.0 mmol, 18 eq.) was added portion-wise and the reaction stirred at r.t. for another 17 h. The suspension was centrifuged and MeOH was added to the supernatant. After the gas evolution ceased, solvents were removed under reduced pressure and the residue was redissolved in THF (30 mL). Then, a second reduction amination analogous to the first reaction was performed using methyl 5-(2 formyl-1H-imidazol-1-yl)pentanoate (1.24 g, 5.90 mmol, 2.9 eq.) and sodium triacetoxyborohydride (3.82 g, 18.0 mmol, 18.0 eq.). After workup of the reaction mixture similar to the previous step, the crude product was obtained an orange oil. This was dissolved in water and the solution acidified with TFA to pH 2. The crude was then purified by reverse phase flash chromatography. Yield: 268 mg, 0.28 mmol, 14 %. ^{1}H NMR (400 MHz, 298 K, MeOD-d_4) δ (ppm): 7.51 (d,$^3J_{HH}$ = 2.0 Hz, 4H), 7.42 (d,$^3J_{HH}$ = 2.1 Hz, 4H), 4.16 (s, 1H), 4.11 (m, 8H), 3.63 (s, 12H), 3.03 (s, 16H), 2.39 (t,$^3J_{HH}$ = 7.2 Hz, 8H), 1.89–1.76 (m, 8H), 1.63–1.49 (m, 8H). ^{13}C NMR (101 MHz, 298 K, D_2O) δ (ppm): 176.30, 141.20, 122.96, 52.08, 50.62, 47.33, 32.67, 28.57. LR-ESI-MS: *m/z* calc. for $C_{48}H_{78}N_{12}O_8$ $[M + 2H]^{2+}$ 475.0, found 475.3 RP-HPLC (analytical): t_R = 14.1 min.

The DOTI-TVA methylester (76.8 mg, 0.086 mmol) was dissolved in hydrochloric acid (30 %, trace metal basis, 10 mL) and refluxed for 36 h. The solvent was removed under reduced pressure to give the final product as colorless resin. Yield: 36 mg, 0.040 mmol, 47 %. ^{1}H NMR (400 MHz, 298 K, D_2O) δ (ppm): 7.52 (d,$^3J_{HH}$ = 2.0, 4H), 7.44 (d,$^3J_{HH}$ = 2.0, 4H), 4.22 (s, 8H), 4.15 (t,$^3J_{HH}$ = 7.3 Hz, 8H), 3.07 (s, 16H), 2.40 (t,$^3J_{HH}$ = 7.2 Hz, 8H), 1.90–1.77 (m, 8H), 1.62–1.49 (m, 8H). ^{13}C NMR (101 MHz, 298 K, D_2O) δ (ppm): 177.76, 141.04, 123.03, 120.24, 50.78, 47.46, 47.36, 32.48, 28.56, 20.79. HR-ESI-MS: *m/z* calc. for $C_{44}H_{67}N_{12}O_8$ $[M-H]^-$: 891.52103, found 891.51932. RP-HPLC (analytical): t_R = 10.8 min, purity 93 %.

2.3. Preparation of non-radioactive metal complexes

For the preparation of the non-radioactive metal complexes, a stock solution of DOTI-Me (free base) in EtOH was prepared (0.03 M). Metal salts (trace metal basis, >99.999 %) were dissolved in H_2O to give 0.03 M solutions. The solutions were mixed in 1:1 or 1:1.2 M ratio. To prepare the non-radioactive complexes of the chelator DOTThia, an aqueous solution (1.0 M) of the chelator was used and mixed with aqueous solutions (1.0 M) of the corresponding metal salts. The solutions were mixed in a 1:1 ratio and heated at 95 °C for 15 min.

2.4. [Pb(DOTI-Me)]$(NO_3)_2$

A volume of each 30 μL of the stock solutions of DOTI-Me and Pb $(NO_3)_2$ were mixed in 0.1 M NH_4Ac (ammonium acetate) pH 5.4 (84 μL) and heated for 30 min at 95 °C. After purification by semi-preparative HPLC, the complex was isolated. Yield: 9.0 mg (10 μmol, 34 %). ^{1}H NMR (400 MHz, 298 K, D_2O) δ (ppm): 7.44–7.40 (m, 4H), 7.30 (s, 3H), 7.03 (s, 1H), 4.42 (s, 4H), 4.19 (s, 4H), 3.81 (s, 7H) 3.76 (s, 9H), 3.05 (s, 12H). ^{13}C NMR (101 MHz, 298 K, D_2O) δ (ppm): 163.06, 162,70, 141.00, 124.54, 124.33, 50.76, 46.89, 34.43 ppm. LR-ESI-MS: *m/z* calc. for $C_{28}H_{44}N_{12}Pb$ $[M + Pb]^{2+}$ 378.2, found 377.7. HR-ESI-MS: *m/z* calc. for $C_{28}H_{44}N_{12}Pb$ $[M + Pb]^{2+}$ 378.17853, found 378.17715. RP-HPLC (analytical): t_R = 10.6 min.

2.5. [Bi(DOTI-Me)]Cl_3

A volume of 30 μL of the DOTI-Me stock solution was mixed with 36 μL of $BiCl_3$ stock solution in 0.1 M NH_4Ac pH 5.4 (84 μL) and heated for 30 min at 95 °C. After purification by semi-preparative HPLC, the complex was isolated. Yield: 7.4 mg, 7.8 μmol, 26 %. LR-ESI-MS: *m/z* calc. for $[Bi(DOTI\text{-}Me)]Cl^{2+}$ $C_{28}H_{44}BiClN_{12}$ 396.2, found 396.5. HR-ESI-MS: *m/z* calc. for $[Bi(DOTI\text{-}Me)]^{3+}$ 252.45387, found 252.45326. RP-HPLC (analytical): t_R = 10.0 min.

2.6. [Pb(DOTThia)]$(NO_3)_2$

A volume of 36 μL of the 1 M $Pb(NO_3)_2$ stock solution was added to 30 μL of the chelator stock solution and incubated at r.t. for 30 min. The complex formation was monitored by LR-ESI-MS. Once the reaction was complete, the solvent was evaporated, and the compound was obtained as white powder. Yield: 15.41 mg (0.17 mmol, 57 %). ^{1}H NMR (400 MHz, 298 K, D_2O) δ (ppm): 7.50 (d, J = 3.3 Hz, 4H), 7.12–7.05 (m, 4H), 4.36 (s, 8H), 3.57 (s, 4H), 3.27 (s, 4H), 2.89 (s, 8H). ^{13}C NMR (101 MHz, 298 K, D_2O) δ (ppm): 169.10, 140.98, 121.54, 55.27, 50.32. LR-ESI-MS: *m/z* calc. for $C_{24}H_{32}N_8PbS_4$ $[M + Pb]^{2+}$ 384.1, found 384.3. HR-ESI-MS: *m/z* calc. for $C_{24}H_{32}N_8PbS_4$ $[M + Pb]^{2+}$ 384.06924, found 384.06939. RP-HPLC (analytical): t_R = 14.1 min.

2.7. ^{212}Pb labeling experiments

A volume of 94 μL of the corresponding buffer (0.1 M) was mixed with 1 μL of the corresponding chelator stock solution (10^{-3} M) in ultrapure water. The buffers used were NaAc (sodium acetate) pH 4.0, NaAc pH 4.5, NH_4Ac pH 4.5, NaAc pH 5.5, MES pH 5.5, NH_4Ac pH 7.0, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4, PBS (phosphate buffered saline) pH 7.4, HEPES pH 8.2, and NH_4Ac pH 8.2. A volume of 5 μL of activity (150 kBq/5 μL) in NH_4Ac pH 7.0 were added and the reaction was incubated for 15 min at r.t. or 80 °C. Samples were analyzed by iTLC. For the dilution experiments, 85 μL buffer were mixed with 10 μL of the chelator-dilution in the corresponding buffer before adding 5 μL of activity. The chelator dilutions were prepared in the corresponding buffer in the concentration range of 10^{-3} to 10^{-7} M.

2.8. Serum stability measurements of ^{212}Pb-labeled compounds

100 μL of human serum (Human male AB plasma, Sigma Aldrich) were mixed with 20 μL of the ^{212}Pb-labelling solution and the samples were incubated at 37 °C for 24 h. Samples were analyzed by iTLC.

2.9. Stability experiments with ^{212}Pb-labeled compounds

For stability testing in a physiological cocktail of metals, 440 μL PBS were mixed with 1.35 μL 1 M $Ca(NO_3)_2$, 3.5 μL 10 mM $Fe(SO_4)_2$, 0.5 μL 10 mM $Cu(SO_4)_2$, and 5 μL 10 mM $Zn(NO_3)_2$ solution before addition of 50 μL of the ^{212}Pb-labelling solution with final concentrations of 2.1 mM–2.7 mM for Ca^{2+}, 54 mM – 72 mM for Fe^{2+}, 10^{-18} M – 10^{-13} M for Cu^{2+}, and 10–100 μM for Zn^{2+}. To test the stability of the compound in presence of $^{nat}Pb^{2+}$, 440 μL PBS were mixed with 10 μL of a 10 mM Pb $(Ac)_2$ solution followed by addition of 50 μL of the ^{212}Pb-labelling solution resulting in an ~15,000-fold molar excess of $^{nat}Pb^{2+}$. The stability in an excess of EDTA was tested by mixing 50 μL of the ^{212}Pb-labelling solution with 450 μL of a 50 mM EDTA solution providing a final concentration of EDTA in the challenge experiment of 5 mM. As the control, 50 μL of the ^{212}Pb-labelling solution were mixed with 450 μL PBS. All samples were prepared in triplicates and incubated at 37 °C for the duration of the experiment. Samples were analyzed by iTLC.

2.10. ^{213}Bi labelling experiments

A volume of 40 μL of the corresponding buffer (0.5 M) were mixed with 5 μL chelator solution and 5 μL ^{213}Bi-solution (~10–15 kBq) and incubated for 7 min at r.t. The buffers used were NaAc pH 4.0, MES pH 5.5, and NH_4Ac pH 7.0. The chelator dilutions were prepared in the corresponding buffer in the concentration range of 10^{-3} to 10^{-7} M. Samples were analyzed by iTLC.

2.11. ^{225}Ac labelling experiments

A volume of 10 μL of the chelator stock solution (1 mM) was mixed with 89 μL of the corresponding buffer (0.1 M) before addition of 1 μL of the ^{225}Ac solution (3 kBq μL^{-1}). The buffers used were NaAc pH 4.0 and MES pH 5.5. The reactions were incubated at r.t. or at 80 °C for 60 min. For analysis, 5 μL of the labeling solution were quenched by addition of 5 μL of a 50 mM DTPA solution (pH 7.4). Samples were analyzed by iTLC.

2.12. iTLC analysis

iTLC analyses of ^{212}Pb- and ^{213}Bi-labeled compounds were carried out with a 50 mM EDTA solution (pH 5) as mobile phase using TLC-SA plates. For ^{225}Ac, 50 mM DTPA (pH 7.4) was used as the eluent. A volume of 5–10 μL of each sample was spotted onto the TLC plates and developed after drying. For ^{212}Pb, samples were measured after 5 h, which correlated to 5 half-lives of the daughter ^{212}Bi.

To account for non-soluble forms of radioactivity in the TLC experiments, blank runs using only the radiometals were performed and the reported RCCs have been corrected accordingly.

For TLC analyses, plates were counted using an AR-2000 TLC Scanner (Eckert and Ziegler, Berlin, Germany) with a counting time of 1 min per sample. The readout for ^{225}Ac was performed with a Typhoon TRIO imager (GE Healthcare, Munich, Germany). The TLC plates were placed on photosensitive plates (Storage Phosphor Screen, GE Healthcare, Munich, Germany) for 15 s before the readout.

3. Results

To evaluate the applicability of DOTI-Me and DOTThia for radiopharmaceutical development, their radiochemistry was evaluated with ^{212}Pb, ^{213}Bi, and ^{225}Ac at different ligand concentrations over the pH range of 4.0 to 8.2 using different buffer systems. Reactions were performed at r.t. and 80 °C. For comparison, the current gold standards for complexing radionuclides of Pb^{2+} and Bi^{3+} the ligands macropa and TCMC were tested under identical conditions, respectively. All experiments were performed in triplicate. Determination of radiochemical conversion (RCC) was performed by radio thin layer chromatography (radio-TLC) using silica plates and 50 mM EDTA solution (pH 5.0) as the eluent. Under these conditions the radiolabeled complexes remained at the origin ($R_f = 0$), whereas unbound radiometals migrated with the solvent front ($R_f = 1$).

3.1. Radiolabeling and stability experiments with ^{212}Pb

Radiolabeling of DOTI-Me and DOTThia with ^{212}Pb was first evaluated in different buffer systems (all 0.1 M) in the pH range of 4.0 to 8.2 at r.t. as well as 80 °C using ligand concentrations of 10^{-5} M. Samples were incubated for 15 min. A graphical representation of the results is given in Fig. 1. For DOTI-Me, RCCs ranging from ~43–75 % were noted at r.t. depending on the pH, which slightly improved to ~73–93 % at 80 °C. Quantitative RCCs (> 95 %) could however not be achieved for DOTI-Me. In contrast, the ligand DOTThia gave quantitative RCCs with ^{212}Pb over the investigated pH range already at ambient temperature as well as at 80 °C. Only for PBS at r.t., the RCC decreased slightly to 87 ± 5 %. The ligand TCMC was also tested in 0.1 M NH_4Ac pH 7.0 as positive control providing RCCs of >95 % at pH = 7 after 15 min incubation at r.t.

Next, the maximum apparent molar activity for [^{212}Pb]Pb-DOTThia and [^{212}Pb]Pb-TCMC was determined by ligand dilution experiments at selected pHs and buffers. For this, radiolabeling experiments were performed for ligands at pH 4.0 (0.1 M NaAc), 5.5 (0.1 M MES), and 7.0 (0.1 M NH_4Ac) in the ligand concentration range from 10 to 0.01 μM. Samples were incubated for 30 min at r.t. As can be seen from Fig. 2, quantitative RCCs were achieved for DOTThia at all pHs at ligand concentrations as low as 10 μM resulting in molar activities of ~1.5 MBq nmol^{-1} being comparable to those determined for TCMC. Differences were noted in the nature of the buffer. While quantitative RCCs were still

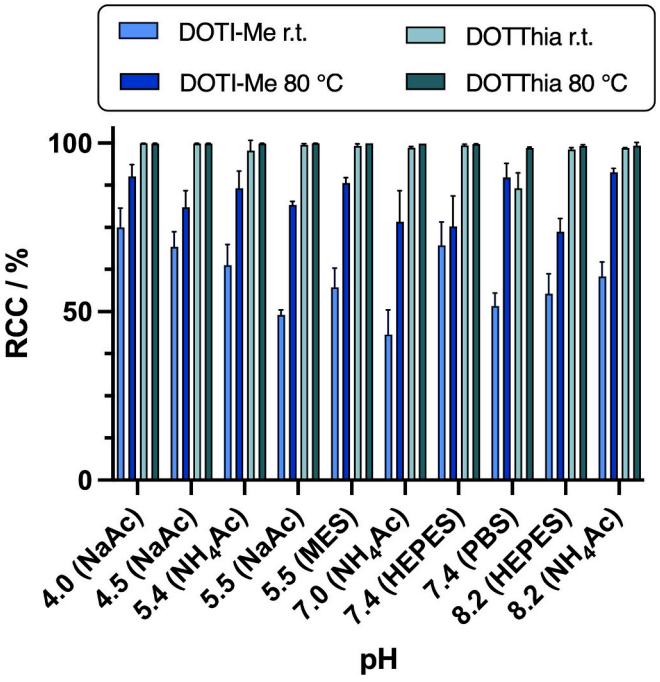


Fig. 1. Radiolabeling experiments of the ligands DOTI-Me and DOTThia with ^{212}Pb at various pH values and different temperatures using ligand concentrations of 0.01 mM. All buffers used were 0.1 M. Results are presented as RCCs as mean ± SD ($n \geq 3$).

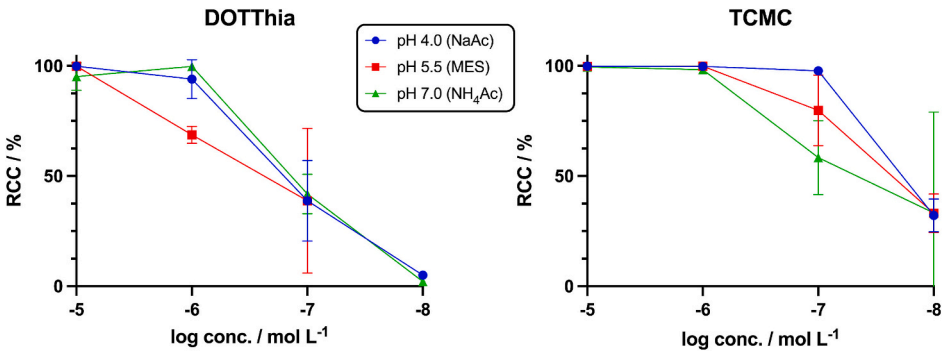


Fig. 2. RCCs of DOTThia (left) and TCMC (right) with ^{212}Pb at r.t. and selected pH values in dependence of ligand concentrations. Results are presented as mean ± SD ($n \geq 3$). All buffers were 0.1 M.

achieved with NaAc and NH_4Ac, RCC decreased to ~65 % for the non-coordinating MES buffer at 1 μM concentrations. For TCMC, the nature of the buffer did not as strongly influence the RCC as quantitative RCCs occurred at concentrations as low as 1 μM, independently of the three buffers and the pHs. At concentrations ≤0.1 μM, differences in RCCs became noticeable.

The kinetic inertness of the ^{212}Pb-labeled complexes of DOTThia and TCMC was assessed *in vitro* by three competition assays (Fig. 3). Both radiolabeled complexes were challenged by incubation in (1) a cocktail of physiological metal cations, (2) in the presence of an excess of $^{nat}Pb^{2+}$, (3) in a 50 mM EDTA solution, and (4) in PBS for up to 48 h. Both complexes remained intact over 95 % for up to 24 h in three assays. At 48 h, the [^{212}Pb]Pb-DOTThia complex was intact to 88 ± 2 % in the EDTA challenge experiment and to 90 ± 3 % in the cocktail mixture. The intact [^{212}Pb]Pb-TCMC complex at 48 h was 92 ± 1 % in the EDTA challenge experiment and 94 ± 2 % in the cocktail mixture. The kinetic inertness was further tested by incubating the radiolabeled complexes in human serum at 37 °C for up to 18 h. Both ^{212}Pb-labeled complexes revealed high stability in the serum with 98 ± 1 % of the complex intact after 18 h.

3.2. Radiolabeling experiments with ^{213}Bi

Similar to the labeling experiments for ^{212}Pb, radiolabeling of DOTI-Me and DOTThia with ^{213}Bi was first evaluated in different buffer systems (all 0.5 M) in the pH range of 4.0 to 7.0 at r.t. using ligand concentrations of 0.1–1.0 mM. A graphical summary is given in Fig. 4.

In this experiment, both chelators DOTI-Me and DOTThia were able to bind ^{213}Bi with the best RCCs being achieved in MES buffer at pH 5.5. However, quantitative RCCs could not be achieved for both ligands under any of the conditions investigated, which could be attributed to impurities in the generator eluate. In general, DOTI-Me gave higher RCCs than DOTThia suggesting its superiority over DOTThia for complexing ^{213}Bi.

In the next step, a dilution experiment was performed in MES buffer pH 5.5 as these conditions gave the best RCCs for both chelators. As can be seen in Fig. 4, the ligand DOTI-Me was able to complex ^{213}Bi to >90 %, even at very low concentrations (0.1 μM), whereas higher concentrations were necessary for DOTThia (1 mM) and macropa (1 μM), respectively.

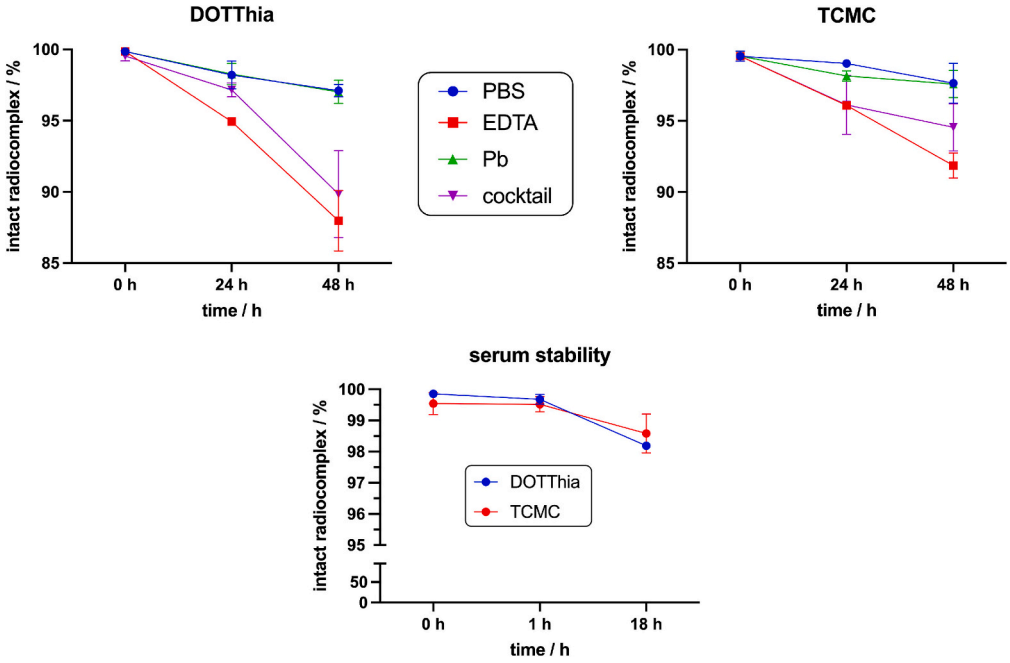


Fig. 3. Comparative *in vitro* stability studies of the [^{212}Pb]Pb-DOTThia (top left) and [^{212}Pb]Pb-TCMC (top right) at 37 °C for up to 48 h in PBS as well as under different challenging conditions using EDTA (final concentration 5 mM), $^{nat}Pb^{2+}$ (~15,000-fold molar excess), or a cocktail of physiological metal cations with final concentrations of 2.1 mM–2.7 mM for Ca^{2+}, 54 mM – 72 mM for Fe^{2+}, 10^{-18} M – 10^{-13} M for Cu^{2+}, and 10–100 μM for Zn^{2+}. Bottom: Serum stability of both radiocomplexes in human serum at 37 °C for up to 18 h. Results are presented as a percentage of the intact radiolabeled complex with mean ± SD ($n \geq 3$).

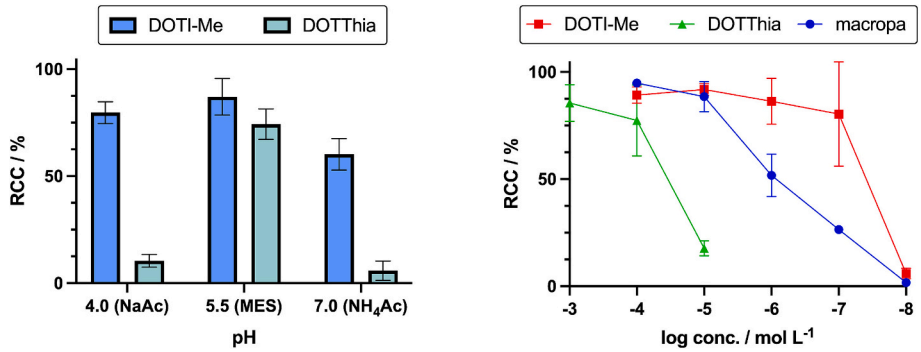


Fig. 4. Left: Radiolabeling experiments of the ligands DOTI-Me and DOTThia with ^{213}Bi at various pH values at r.t. (c = 1 mM). All buffers used were 0.1 M. RCCs are given as mean ± SD ($n \geq 3$). Right: RCCs of DOTI-Me, DOTThia, and macropa with ^{213}Bi at r.t. and pH 5.5 (MES 0.1 M) in dependence of ligand concentrations. Results are presented as mean ± SD ($n \geq 3$).

3.3. Radiolabeling experiments with ^{225}Ac

The aim of the ^{225}Ac labeling experiments was to determine conditions for the exclusive complexation of the daughter radionuclides (*vide infra*), we focused on conditions that were optimal for complexing ^{212}Pb and ^{213}Bi, namely pH 4.0 NaAc and pH 5.5 MES. At pH 4.0 and ambient temperature, DOTI-Me and DOTThia were able to bind ^{225}Ac to 7 ± 4 % and 9 ± 2 %, respectively. At 80 °C, the RCCs for both ligands did not significantly increase and gave similar results indicating that higher RCCs could not be achieved at elevated temperatures. At pH 5.5 at ambient temperature, the RCCs for DOTI-Me and DOTThia were comparable to those obtained at pH 4.0 with 9 ± 2 % and 7 ± 1 %, respectively. Contrarily, the RCCs significantly increased for DOTI-Me at 80 °C to 22 ± 10 %, whereas DOTThia did not bind ^{225}Ac with only 3 ± 2 % being in the complexed form.

3.4. Radiolabeling experiments of DOTI-TVA methyl ester with ^{225}Ac

The findings of the ^{213}Bi and ^{225}Ac labeling experiments with DOTI-Me prompted us to check if DOTI-Me could be used to selectively complex ^{213}Bi from ^{225}Ac solutions. This would allow the preparation of ^{213}Bi-labeled radiopharmaceuticals directly from ^{225}Ac solutions without the need of a ^{225}Ac/^{213}Bi generator system. In this respect, we performed preliminary labeling experiments using ^{225}Ac solutions with the DOTI-TVA methyl ester. This bifunctionalized derivative allowed the separation of the labeled compound from the ^{225}Ac solution using C_{18} Sep Pak cartridges. The ligand DOTI-Me was not suitable for these experiments because corresponding metal complexes were too hydrophilic to be retained on the C_{18} Sep Pak cartridge. Consequently, ^{225}Ac solutions in NaAc pH 4.0 buffer were treated with DOTI-TVA methyl ester for 15 min at r.t. The solution was then passed through a C_{18} Sep Pak cartridge, which should retain any labeled DOTI-TVA methyl ester. Measurements of cartridges in a gamma counter in the ^{213}Bi (350–500 keV) and ^{221}Fr (150–270 keV) windows after rinsing with water gave decay curves as shown in Fig. 5. Non-linear regression of the curves allowed the determination of a half-life of 35.0 ± 2.9 min with $R^2 = 0.99$ ($n = 3$) being in the range of the half-life of ^{213}Bi ($t_{1/2} = 45.6$ min), suggesting complexation of ^{213}Bi by DOTI-TVA Me ester from the ^{225}Ac solution.

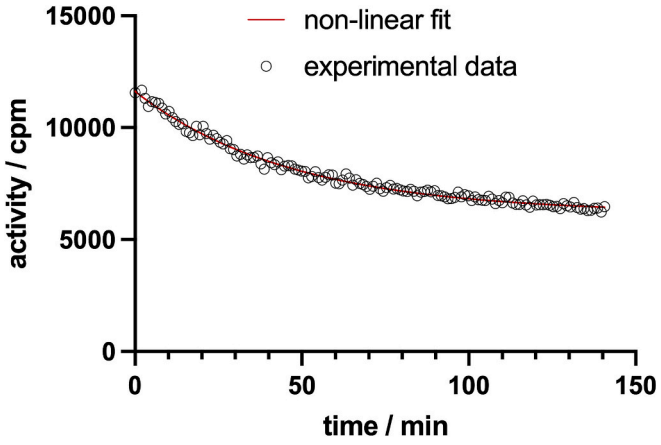


Fig. 5. Representative decay curve of a C_{18} Sep Pak cartridge measured in a gamma-counter after incubation of the DOTI-TVA methyl ester with ^{225}Ac in NaAc pH 4.0 buffer for 15 min at r.t. and passing the reaction mixture through the cartridge. Non-linear regression gave a half-life of 35.0 ± 2.9 min with $R^2 = 0.99$ ($n = 3$) being comparable to that of ^{213}Bi ($t_{1/2} = 45.6$ min), suggesting complexation of ^{213}Bi by DOTI-TVA Me ester from the ^{225}Ac solution.

4. Discussion

We recently developed metal chelators for radiopharmaceutical applications based on the macrocycle 1,4,7-triazacyclononane (tacn) comprising up to three additional five-membered azaheterocycles giving rise to the ligands NOTI (1,4,7-tris((1*H*-imidazol-2-yl)methyl)-1,4,7-triazacyclononane), NOTI-Me (1,4,7-tris((1-methyl-1*H*-imidazol-2-yl)methyl)-1,4,7-triazacyclononane), and NOTThia (1,4,7-tris(thiazol-2-ylmethyl)-1,4,7-triazacyclononane) [22]. These ligands are characterized by their excellent complexation properties for Cu^{2+}, in particular for the PET nuclide ^{64}Cu [22]. Moreover, the imidazole-type ligands are capable of stably complexing the PET isotope ^{68}Ga and the gamma emitter ^{111}In [23,24]. More recently, we expanded our approach to the macrocycle cyclen, preparing the chelator DOTI-Me with four methylimidazole arms and reported on its radiochemistry with ^{52g}Mn, ^{64}Cu, ^{68}Ga, ^{111}In, ^{161}Tb, and ^{177}Lu [16]. In the present work, we successfully prepared and characterized the thiazole congener DOTThia with four thiazole arms as well as the functionalized imidazole derivative DOTI-TVA allowing the future conjugation of up to four functionalities such as tumor-specific targeting vectors by peptide bond formation (Scheme 3). To explore the potential of these metal chelators for radiopharmaceutical applications, we investigated the radiochemistry of DOTI-Me and DOTThia with the radiometals ^{212}Pb, ^{213}Bi, and ^{225}Ac in the present work.

The thiazole-type ligand DOTThia showed excellent chelating properties for ^{212}Pb, achieving quantitative RCCs at r.t. and concentrations of 10 μM, being comparable to the current gold standard TCMC. In contrast, the ligand DOTI-Me appeared to be less suited for ^{212}Pb. Quantitative RCCs could be obtained for DOTThia independently of the pH and the nature of the buffer. The only exemption was PBS, which gave only ~90 % conversion at ambient temperature, most likely due to the competitive nature of the phosphate anions. Heating to 80 °C, however, restored the quantitative RCCs for PBS. This feature may allow

the preparation of directly injectable formulations of ^{212}Pb-labeled radiopharmaceuticals in the future. In a dilution study in selected buffers, differences between DOTThia and TCMC were noted at a ligand concentration of 0.1 μM. At this concentration, quantitative RCCs were only achieved for TCMC in NaAc at pH 4.0. In general, at very low ligand concentrations, TCMC exhibited slightly higher RCCs than DOTThia. Moreover, ^{212}Pb complexes of DOTThia and TCMC also exhibited high stability in several stability experiments *in vitro,* suggesting a potential application of DOTThia in radiopharmaceutical development.

For ^{213}Bi, the ligand DOTI-Me showed excellent binding properties, while DOTThia appeared not to be able to complex the radiometal in a satisfactory amount. Interestingly, DOTI-Me outperformed the current gold standard macropa for ^{213}Bi in the dilution experiments at pH 5.5, in which RCCs of 80 ± 25 % could still be achieved for DOTI-Me at extremely low ligand concentrations of 0.1 μM, whereas only 26 ± 2 % RCCs were obtained for macropa. These findings suggest that DOTI-Me and corresponding derivatives may be an interesting alternative to existing metal chelators for ^{213}Bi chelation.

Since we recently found that DOTI-Me is capable of complexing the radiometals $^{111}In^{3+}$, $^{161}Tb^{3+}$, and $^{177}Lu^{3+}$ [16], we decided to study the capability of DOTI-Me and DOTThia for complexing $^{225}Ac^{3+}$ despite their differences in ionic radii for the coordination number 6 (Ac^{3+} 1.12, $^{111}In^{3+}$ 0.8, $^{161}Tb^{3+}$ 0.92, and $^{177}Lu^{3+}$ 0.86) [25] due to their shared oxidation state +3. Additionally, the metal chelator DOTA, which is also based on the macrocycle cyclen, is commonly used for $^{225}Ac^{3+}$ complexation [7–9]. Interestingly, neither DOTI-Me nor DOTThia proved to be suited for Ac^{3+} chelation with very low RCCs for both compounds. This feature, together with the excellent chelating properties of DOTI-Me for $^{213}Bi^{3+}$ prompted us to test if the ligand DOTI-Me could be used to directly generate ^{213}Bi-labeled compounds from ^{225}Ac solutions in preliminary experiments. Due to the hydrophilicity of the DOTI-Me metal complexes, experiments for the generation of ^{213}Bi-labeled compounds from ^{225}Ac solutions were performed using the model compound DOTI-TVA methyl ester, which allows the future covalent conjugation of up to four targeting vectors/functionalities. This way, the ^{213}Bi-labeled DOTI-TVA methyl ester could be separated from the ^{225}Ac solution by C_{18} Sep Pak cartridges. Indeed, we have been able to retain a radioactive species on the cartridge that exhibited a rapid decay indicating the presence of a ^{213}Bi-labeled compound. A non-linear fit of the decay curves allowed the calculation of a half-life of 35.0 ± 2.9 min, which corresponds well with the half-life of ^{213}Bi ($t_{1/2}$ = 45.6 min). The underestimation of the half-life is indicative for the absence of radiolabeled compounds with longer-lived radionuclides, in particular of ^{225}Ac ($t_{1/2}$ = 9.92 d). Of note, more detailed studies using an automated gamma-counter to also determine the ^{213}Bi and ^{221}Fr contents of the loading and elution solutions proved to be none trivial due to their short half-lives and the rapid reestablishment of the decay equilibria. More detailed studies are required to fully explore this application and are part of ongoing work.

Another possible application of the two chelators presented in this work might be the purification of ^{225}Ac by selective removal of the daughter radionuclides ^{213}Bi and ^{209}Pb. This could possibly be achieved by incorporating both chelators in ion exchange resins. Passing ^{225}Ac solutions through such DOTI-Me and DOTThia-functionalized resins, on which the daughter radionuclides are then selectively retained, may provide purified ^{225}Ac solutions free of the daughters ^{213}Bi and ^{209}Pb. Future works will also focus on this potential application.

5. Conclusions

In this work, we successfully studied the radiochemistry of the two cyclen-based chelators DOTI-Me and DOTThia with the radiometals ^{212}Pb, ^{213}Bi, and ^{225}Ac. Results suggest that DOTI-Me may be a promising alternative to existing chelators for ^{213}Bi chelation, while its thiazole congener exhibited excellent complexation properties for ^{212}Pb. Both chelators are not suited for ^{225}Ac. Interestingly, this feature allowed the preparation of the ^{213}Bi-labeled DOTI-TVA methyl ester directly from ^{225}Ac solutions. This may pave the way for the future development of ^{213}Bi-labeled radiotracers without the need of a ^{213}Bi/^{225}Ac generator. While the imidazole-type chelators can readily be functionalized with targeting vectors by the introduction of chemical handles at the non-coordinating NH groups of the imidazole residues as in the case of the DOTI-TVA scaffold, the development of bioconjugatable DOTThia derivatives is part of ongoing work. Altogether, our results support the future development of ^{212}Pb-labeled radiopharmaceuticals using bifunctional derivatives of DOTThia as well as of ^{213}Bi-labeled radiotracers based on the DOTI-Me scaffold.

Abbreviations

BFC	bifunctional chelator
cyclen	1,4,7,10-tetraazacyclododecane
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DOTAM (TCMC)	1,4,7,10-tetraazacyclodocecane-1,4,7,10-tetraacetamide
DOTAGA	1,4,7,10-tetraazacyclodocecane-1-(glutaric acid)-4,7,10-triacetic acid
DOTI-Me	1,4,7,10-tetrakis((1-methyl-1*H*-imidazol-2-yl)methyl)-1,4,7,10-tetraazacyclododecane
DTPA	diethylenetriamine penta-acetic acid
DOTP	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylene phosphonic acid)
EDTA	**e**thylendiaminetetraacetic acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
LET	linear energy transfer
MES	2-(N-morpholino)ethanesulfonic acid
NOTI	1,4,7-tris((1*H*-imidazol-2-yl)methyl)-1,4,7-triazacyclononane
NOTI-Me	1,4,7-tris((1-methyl-1*H*-imidazol-2-yl)methyl)-1,4,7-triazonane
PET	positron emission tomography
RCC	radiochemical conversion
SA	salicylic acid
tacn	1,4,7-triazacyclononane
TAT	targeted alpha therapy
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TVA	tetravaleric acid

CRediT authorship contribution statement

Ina Hierlmeier: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Parmissa Randhawa:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Brooke L. McNeil:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Elisa Oliveri:** Methodology, Investigation, Formal analysis, Data curation. **Stephan Maus:** Writing – original draft, Methodology, Formal analysis, Data curation. **Valery Radchenko:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Formal analysis. **Florian Rosar:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Samer Ezziddin:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition. **Mark D. Bartholomä:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nucmedbio.2025.109034.

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