SUPPLEMENTARY INFORMATION

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Gallium-68-NODASA-Functionalized D-Lysine Radiosynthesis and first-line *in vitro* characterization – an initial report on a prospective PET imaging agent for infection

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General Information Material and Equipment

All chemicals and reagents were purchased from Sigma Aldrich/Merck (Germany), DLD Scientific (South Africa) and Hangzhou Dayang Chem Co., Ltd (China). All solvents were dried by means of standard procedures. All the synthetic steps were characterized using a photodiode array (PDA) detector coupled ESI-LCMS (Shimadzu, Japan) with an YMC-Triart C18 (5 µm, 4.6 × 150 mm) column (YMC, Japan). Synthesized compounds were purified using a semi-preparative HPLC system (Shimadzu Prominence Preparative System, Japan) coupled to an ACE C18 preparative (150 x 21.2 mm I.D.) column (Avantor, USA). High resolution mass spectrometry (HRMS) was performed using a Bruker micro-TOF-Q II (Bruker, Germany) that operated at ambient temperatures. HPLC instrumentation used for radioanalysis include an Agilent 1200-series (System 1), or Agilent 1260 Infinity II (system 2), both coupled to a PDA detector and radioactive detector (Raytest Sockel 2 GABI Nova, Germany).

Note for the NMR spectra of the final compounds

The NMR spectra of the final compounds exhibited considerable overlap, resembling the pattern observed previously with NOTA derivatives.[1-3] This overlap was ascribed to the presence of rotamers and/or the chelator moieties adopting a bent conformation due to the 3D structure of the lactam, with a four-membered ring adjacent to a five/six-membered ring.[4] The inherent flexibility of the molecule contributed to signal broadening, leading to a poor resolution of the multiplets. The situation was further complicated by overlapping signals from multiple protons in similar environments. Although the integration aligned with the number of protons in the products, the spectra appeared 'messy' due to this overlap. To address this issue, consultations with NMR experts (collaborators in Sweden) involved subjecting the samples to temperature variation and complexing the chelators with either Zn or Cu.[5] Unfortunately, no significant changes in the spectra were observed. Consequently, NMR spectra of all starting materials were recorded and confirmed. The individual NMR spectra of the chelators also displayed substantial overlap. Due to these challenges, the final compounds underwent further

treatment and characterization following a methodology as per peptides in organic synthesis, where NMR spectra are not recorded. Instead, characterization relied on alternative means such as LC-MS traces and HRMS spectra.[6]

NDL-1/NLL-1 Synthesis

General synthesis: A procedure developed by Dutta *et al.*[7]for synthesising 1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid (NODASA)-functionalized peptides on resin was modified to functionalise D-lysine and L-lysine, respectively, with NODASA. All intermediates and products were characterized using a PDA-coupled LCMS to determine purity and to identify the molecular ions.

OFF-resin synthesis of NODASA precursor (4): A Michael addition reaction between 1,4,7-triazacyclononane (**1**) and monomethyl fumarate (**2**) was carried out in solution to produce stereomeric 4-methoxy-4-oxo-3-(1,4,7-triazonan-1-yl)butanoic acid (**3**). The reaction was performed at 3.84 mmol scale by dissolving 1,4,7-triazacyclononane (1.5 equiv.) with DIEA (1.6 equiv.) in DCM (30 ml/mmol scale) at 0°C, followed by drop-wise addition of monomethyl-fumarate (1 equiv. pre-dissolved together with another 1.6 equiv. of DIEA, in 5 ml/mmol DCM) over 20 minutes while stirring. The reaction was stirred for 4 hours until completion (TLC monitoring). The DCM was then removed *in vacuo* and the product was re-dissolved in minimal amount of DMF (~ 1.0 ml) and left to re-crystalise at -20°C overnight. The resulting solid product was isolated by centrifugation and decanting the DMF layer, followed by 3x washes with 5.0 ml toluene. The product was then dried *in vacuo* and weighed to determine yield, and characterized using LCMS. Almost quantitative yield of **3** was achieved (>98%) and it was used in the next step without further purification. LCMS analysis of the product molecular weight revealed desired product m/z of 260 $(M+H)^+$.

In order to prevent self-polymerization of **3** during the amide coupling reaction on resin, protection of the free secondary amines with Fmoc-OSu was carried out. Thus, 3-(4,7-bis(((9*H*-fluoren-9-yl)methoxy)carbonyl)-1,4,7-triazonan-1-yl)-4-methoxy-4-oxobutanoic acid (**4**) was synthesised from **3** by adding Fmoc-OSu (2.2 equiv.) slowly to **3** (1.0 equiv.) and sodium bicarbonate (5.0 equiv.) pre-dissolved in 1:1 water and acetone (150 ml/mmol). The reaction was stirred on ice overnight or until completed, as indicated by HPLC and LCMS analysis. Once completed, the acetone was removed *in vacuo* and residual Fmoc-OSu was removed from the remaining aqueous reaction mixture using liquid-liquid extraction with diethyl ether (25 ml/mmol, repeated 3 times). The aqueous layer was then acidified with 1 M HCl (pH 1) and **4** was extracted with diethyl ether (25 ml/mmol, repeated 3 times). Any residual water was removed from the extraction mixture with anhydrous magnesium sulphate followed by filtration. The diethyl ether was removed *in vacuo*, yielding a white powder product (78% yield). PDA-coupled LCMS analysis revealed a single peak with the molecular ion corresponding to the *m/z* of the desired product, (ESI-MS) *m/z* 704 ($[M+H]^+$) (R_T 18.2 min) with purity >98% (**Figure S1**).

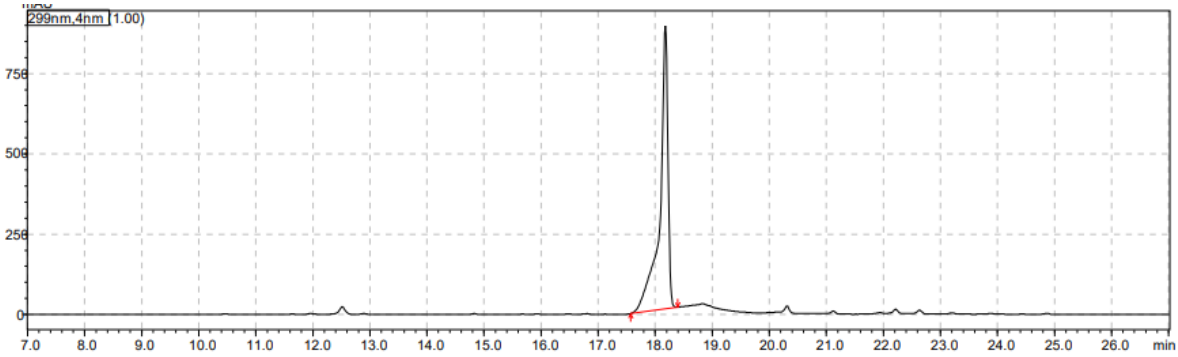


Figure S1: UV-LCMS results for compound 4. HPLC chromatogram with detection at 300 nm, indicating a single peak acquired for compound **4**, indicating >98% chemical purity, and mass spectra (positive mode) indicating desired (ESI-MS) *m/z* of 704 ($[M+H]^+$).

CTC-resin loading with Boc-Lysine(Fmoc)-OH: Enantiomerically-pure Boc-D-Lys(Fmoc)-OH and Boc-L-Lys(Fmoc)-OH was loaded respectively onto CTC-resin as follows: CTC-resin (0.25 mmol scale) was first activated by suspension in 10% (v/v) thionyl chloride in dry DCM for 2 hours; Activated CTC resin was washed with dry DCM, followed by Boc-Lys(Fmoc)-OH coupling (1.5 equiv.) with DIEA (10.0 equiv.) in dry DCM. After stirring for 1.5 hours, the resin was capped by addition of 100 µl methanol for 5 min and washed with DCM (2x 5.0 ml) and DMF (2x 5.0 ml). Coulometric ninhydrin test indicated the resin was fully loaded or capped. Fmoc deprotection was carried with 20% (v/v) piperidine in DMF for 5 min and washed again with DMF (2x 5.0 ml) and DCM (2x 5.0 ml).

Coupling (4) to Lysine on-resin to produce (7): Product **4** was respectively coupled to D-Lysine and L-lysine on-resin with a reaction mixture containing a 1:1:1 ratio of **4** (3.0 equiv.): diisopropylcarbodiimide (DIC) (3.0 equiv.): OxymaPure (3.0 equiv.) was dissolved in DMF (1.0 ml). After a reaction time of 1 hour, the resin was washed 3 times with DCM (5.0 ml) and DMF (5.0 ml), respectively. Ninhydrin test confirmed that the coupling reaction was complete. Before Fmoc-deprotection was carried out to yield **7**, an aliquot of the resin was cleaved from the resin under mild conditions (2% TFA, to prevent cleavage of Boc-protection) for 1 hour and monitored by PDA-coupled LCMS (**Figure S2**). The LCMS (ESI, positive mode) chromatogram (300 nm) indicated two product peaks. Both peaks correlated with molecular ions of the desired Fmoc-protected product, 7, (ESI-MS) *m/z* 832 ($[M-2Fmoc+H]^+$) (R_T 13.6 min), and the second peak (RT 18.9 min) corresponding to *m/z* 932 ($[M-2Fmoc-Boc+H]^+$). Both peaks combined indicated >98% conversion to desired product **7** for both D-Lysine and L-Lysine, respectively.

On-resin Fmoc deprotection of secondary amines was carried out with 20% (v/v) piperidine in DMF for 5 min and washed again with DMF (3x 5.0 ml) and DCM (3x 5.0 ml) to yield 2-((*tert*-butoxycarbonyl)amino)-6-(4-methoxy-4-oxo-3-(1,4,7-triazonan-1-yl)butanamido)hexanoic acid (**7**) on-resin.

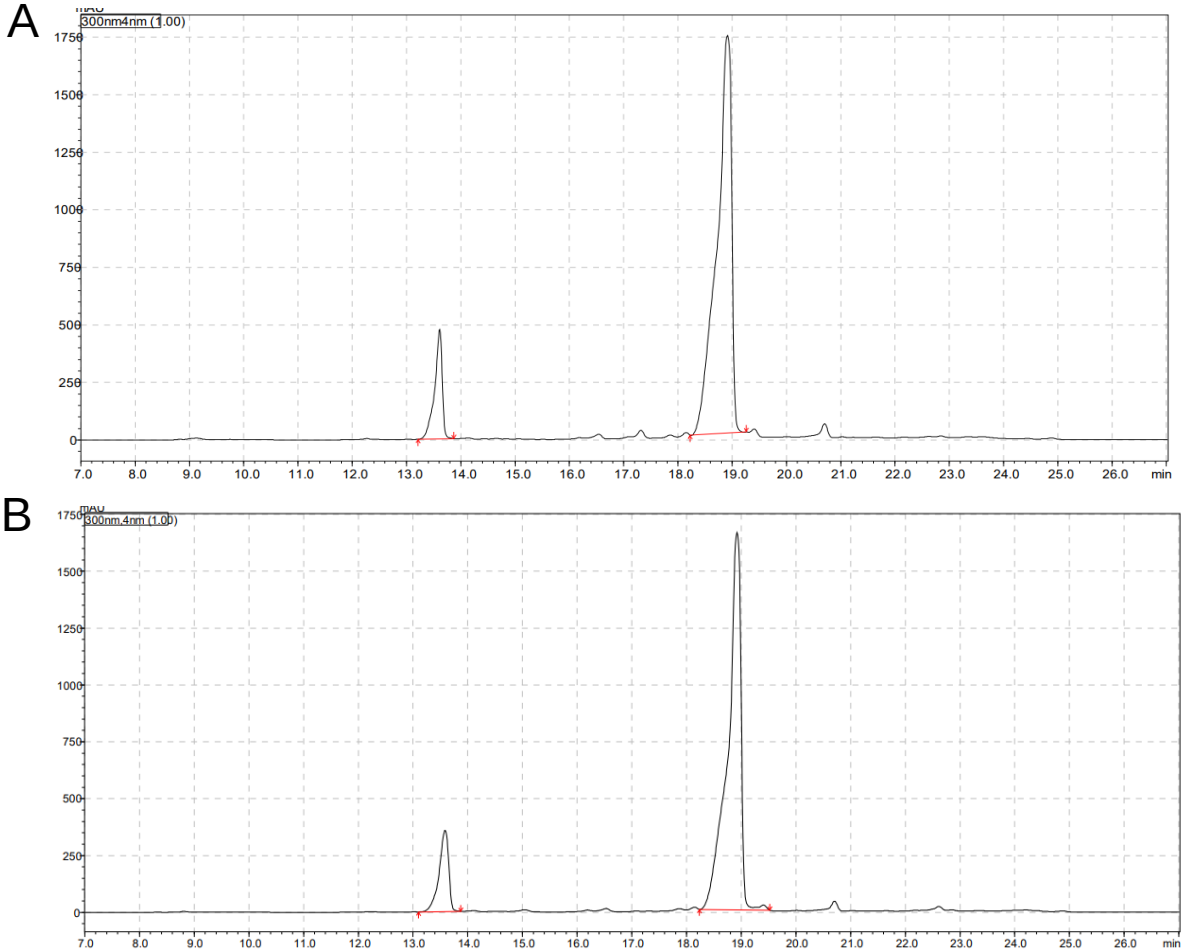


Figure S2: UV-LCMS results of coupling compound 4 to Boc-D-Lys (A) and Boc-L-Lys (B) on-resin (6), respectively. HPLC chromatogram with detection at 300 nm, indicating two product peaks. Mass spectra of both peaks showed the desired product, with the first peak corresponding to (ESI-MS) *m/z* 832 ($[M\text{-}2Fmoc+H]^+$) (R_T 13.6 min), and the second peak (RT 18.9 min) corresponding to *m/z* 932 ($[M\text{-}2Fmoc\text{-}Boc+H]^+$). Both peaks combined indicated >98% conversion to desired product for both D-Lysine (A) and L-Lysine (B), respectively.

Alkylation of (7) to produce (8): On-resin alkylation of the amines on **7** was performed to produce 6-(3-(4,7-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7-triazonan-1-yl)-4-methoxy-4-oxobutanamido)-2-((*tert*-butoxycarbonyl)amino)hexanoic acid on resin (**8**). This was achieved by addition of a reaction mixture consisting of *tert*-butyl bromoacetate (6.0 equiv.) and DIEA (6.0 equiv.) in *N*-methyl-2-pyrrolidone (NMP, 3.0 ml/0.1 mmol scale) to **7**, allowing it to react for 1 hour under sealed conditions. This was to prevent evaporation of the highly volatile *tert*-butyl bromoacetate. The resin was then washed with DMF (3x 5.0 ml) and DCM (3x 5.0 ml). A ninhydrin test confirmed that the alkylation reaction was completed. An aliquot of the resin was cleaved from the resin under mild conditions (2% TFA in water, to prevent cleavage of Boc- and Trt protecting groups) for 1 hour and analysed by UV-LCMS (**Figure S3**).

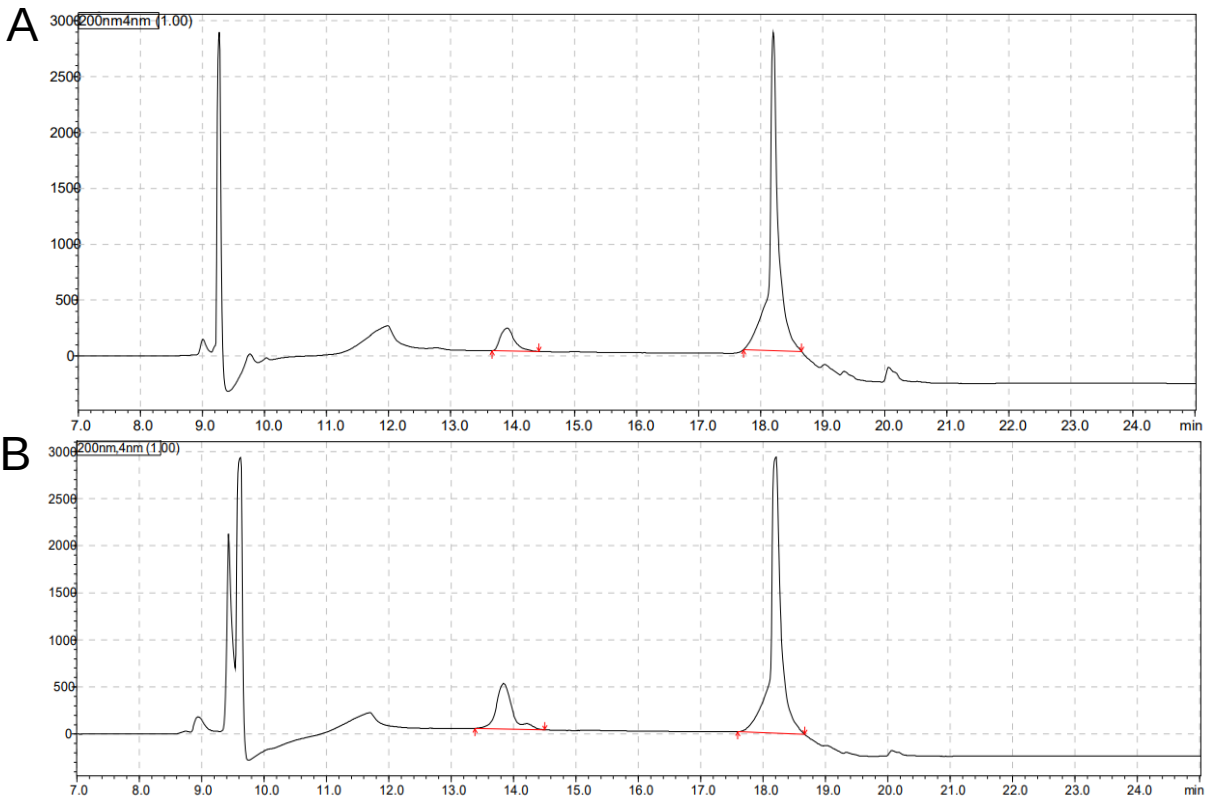


Figure S3: UV-LCMS analysis of on-resin alkylation of 7 to produce compound 8 in the process of synthesizing NDL-1 (A) and NLL-1 (B). PDA-coupled ESI-LCMS analysis of an aliquot of the resin cleaved under mild conditions revealed two HPLC peaks (retention times, RT, 13.8 min and 18.2 min) correlating with the protonated molecular ion and daughter ions of the desired product, 8, (ESI-MS) m/z 504 ([M-Boc-2tBu+H]+) (R_T 13.8 min), and the second peak (R_T 18.2 min) corresponding to m/z 716 ([M+H]+, 616 ([M-Boc+H]+), 560 ([M-Boc-tBu+H]+). Both peaks combined indicate >98% conversion to desired product for D-lysine and L-lysine, respectively.

Base-hydrolysis to produce (9): Base-hydrolysis of methyl ester **8** was carried out to produce 6-(3-(4,7-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7-triazonan-1-yl)-3-carboxypropanamido)-2-((*tert*-butoxycarbonyl)-amino)-hexanoic acid (**9**) on-resin. This was done by addition of a 1:1 mixture of THF/MeOH saturated with LiOH for 30 minutes. The resin was then washed with THF (2x 5.0 ml), DMF (3x 5.0 ml) and lastly DCM (3x 5.0 ml) and left to dry under vacuum.

Compound **9** was fully deprotected and cleaved from resin using a 95:5 mixture (v/v) TFA/ H_2O (2.0 ml/0.1 mmol scale) at 50ºC for 4 hours. The cleavage reaction solution containing the desired product was then filtered from the resin into a falcon tube, with an additional 1.0 ml TFA wash. Residual TFA was evaporated with the aid of N_2-gas bubbling through the mixture until roughly 0.5 – 1.0 ml remained. The product (NDL-**1** or NLL-**1**, respectively) was precipitated in diethyl ether (5.0 ml), followed by centrifugation at 10000 rpm for 5 min, after which the diethyl ether was decanted. This was repeated twice, after which the resulting solid product was dried under vacuum to remove residual diethyl ether. UV-LCMS analysis was performed to characterize and determine the purity of crude products (**Figure S4**).

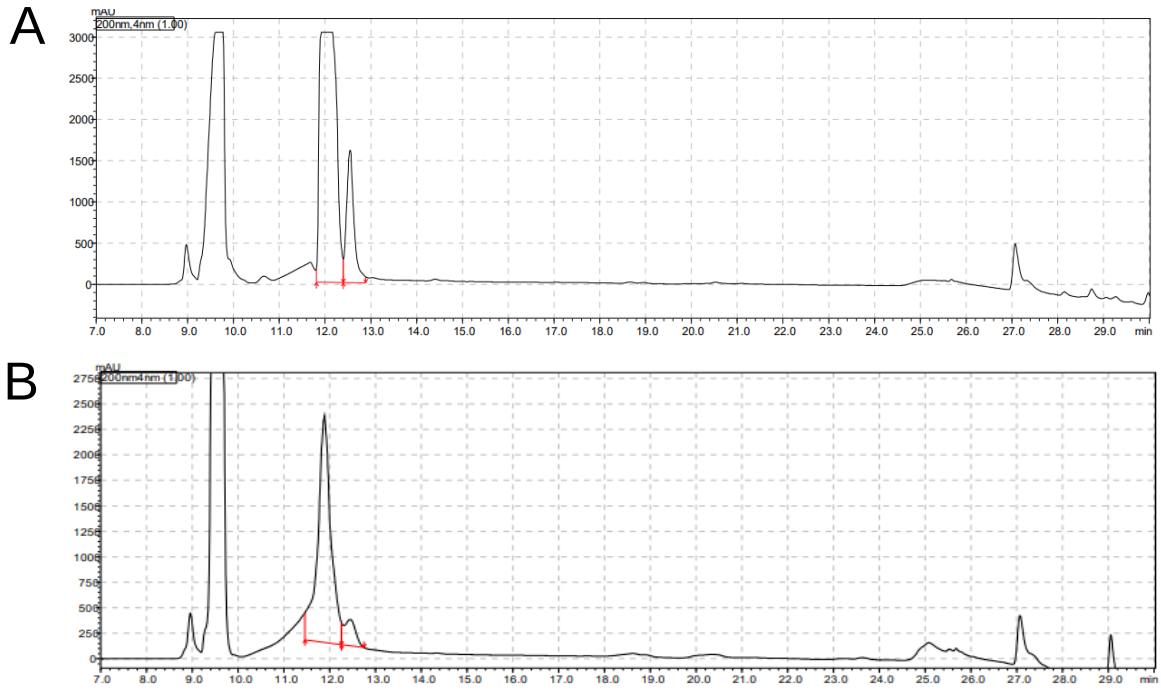


Figure S4: UV-LCMS chromatograms (200 nm) acquired from the analysis of NDL-**1** (A) and NLL-**1** (B). The protonated molecular ions of NDL-**1** and NLL-**1** with (ESI-MS) *m/z* of 490.2 ($[M+H]^+$) were observed by LCMS.

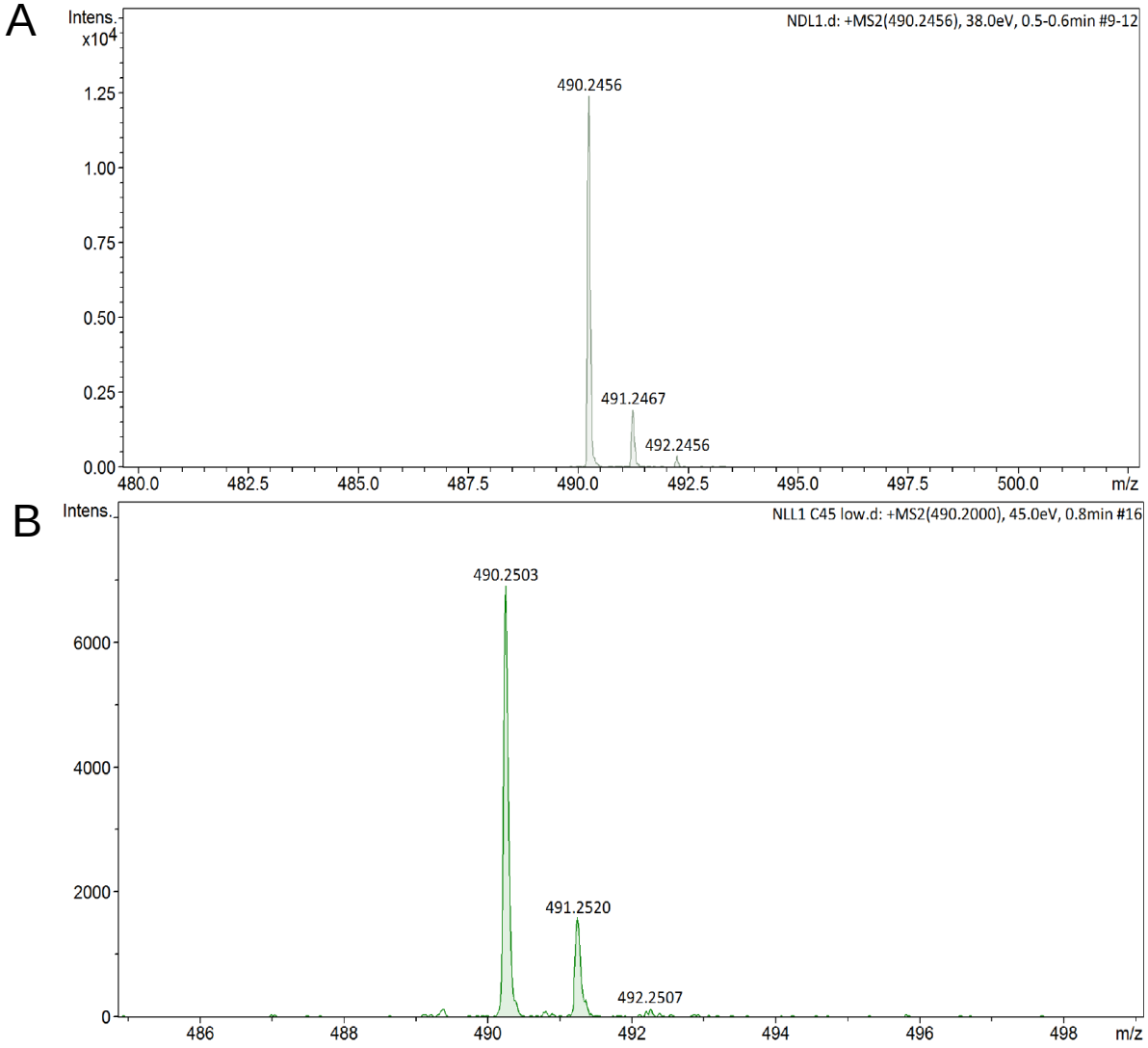
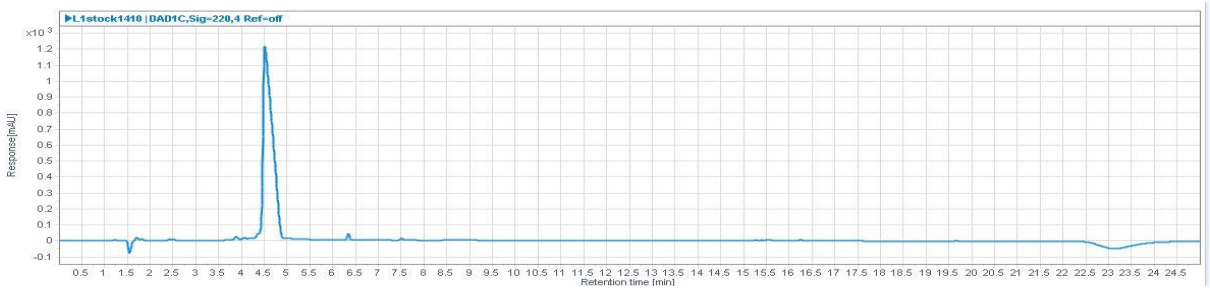


Figure S5: HRMS (ESI+) for NDL-**1** (A) and NLL-**1** (B). Calculated for $C_{20}H_{35}N_5O_9$ $[M+H]^+$ 490.2508, found 490.2456 and 490.2503 for NDL-**1** and NLL-**1**, respectively.

A



B

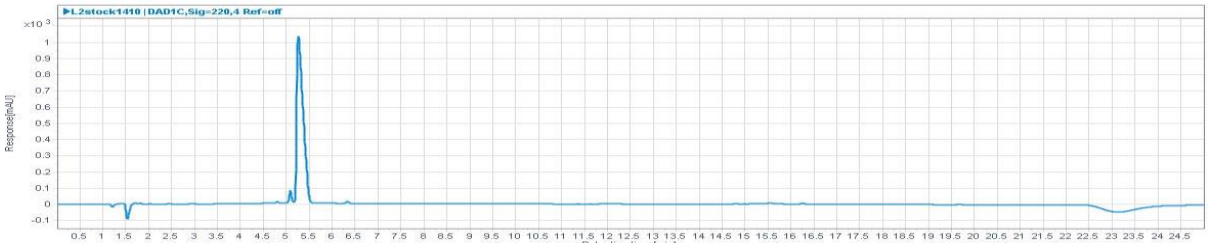
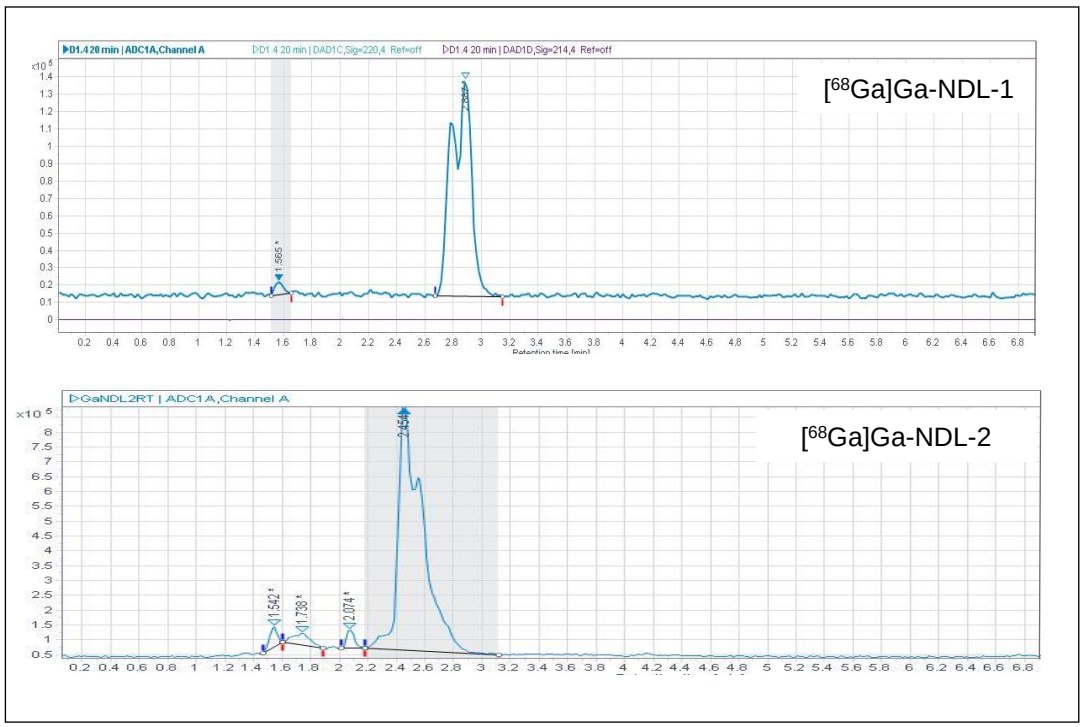


Figure S6: HPLC chromatograms of purified NDL-**1** (A) and NLL-**1** (B) with detection at 220 nm.

A



B

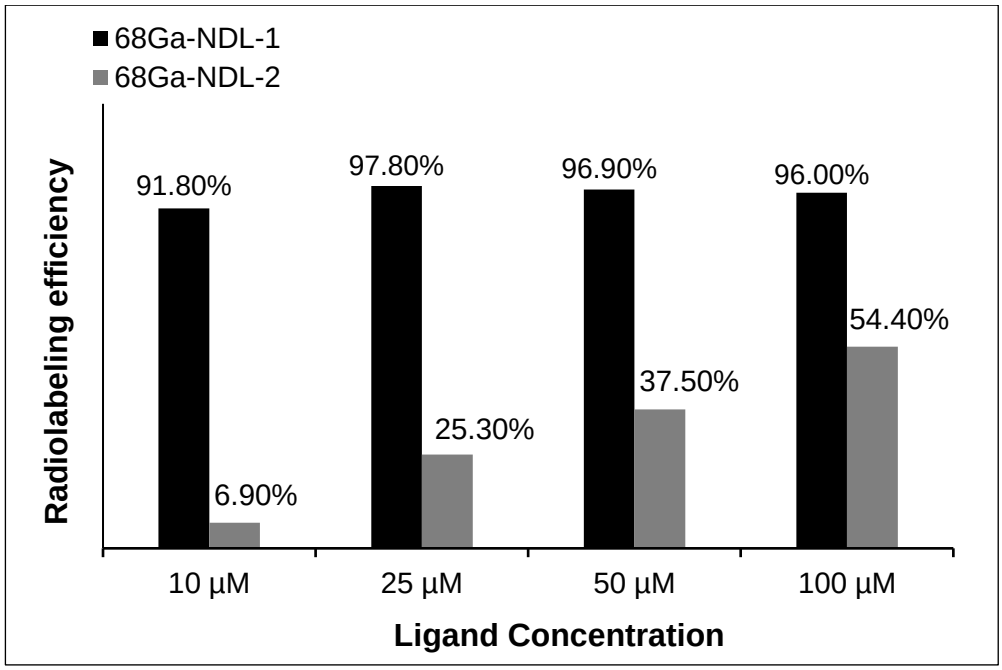
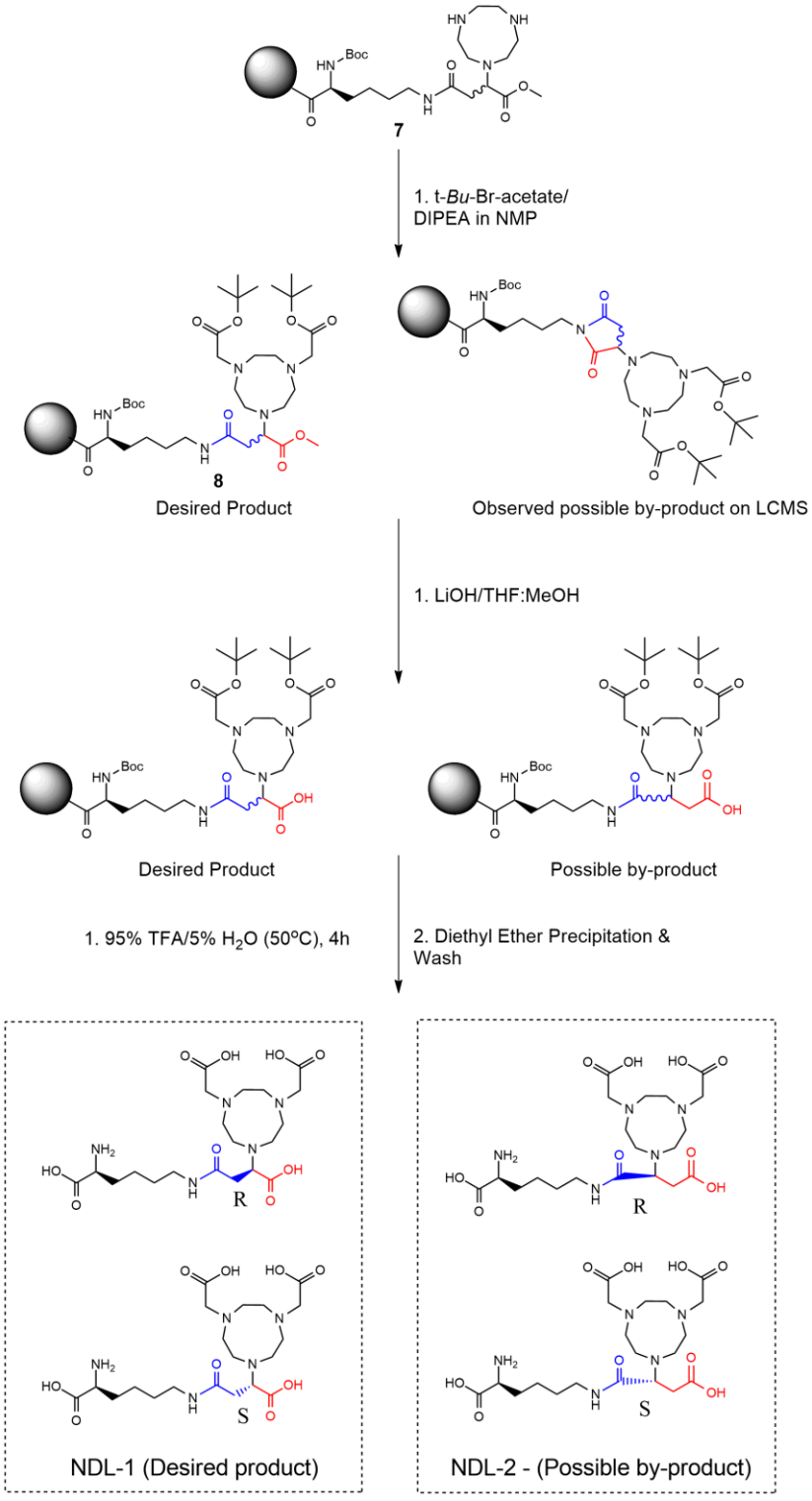


Figure S7: NDL-1 and NDL-2 radio-HPLC analysis and radiolabeling efficiency. (A): the radio-HPLC analysis shown for ^{68}Ga-NDL-**1** (top, R_T = 2.79/2.88 min) and ^{68}Ga-NDL-**2** (bottom, R_T = 2.46/2.55 min). Observed split peaks are proposed to be slight separation of diastereoisomeric mixtures. Free (unchelated) ^{68}Ga-species can be observed at R_T = 1.4 - 2.2 min. Identical results were obtained for ^{68}Ga-NLL-**1** and ^{68}Ga-NLL-**2** (data not shown). **(B):** the compared %RCY for NDL-**1** and NDL-**2**. Radiolabeling reactions were performed using 0.5 ml buffered Gallium-68 activity (2.5M NaOAc, pH 4.5) at room temperature.



Scheme S1: NDL-**1** and NLL-**1** synthesis with a proposed aspartimide-forming side reaction and rearrangement to NDL-**2** and NLL-**2**.

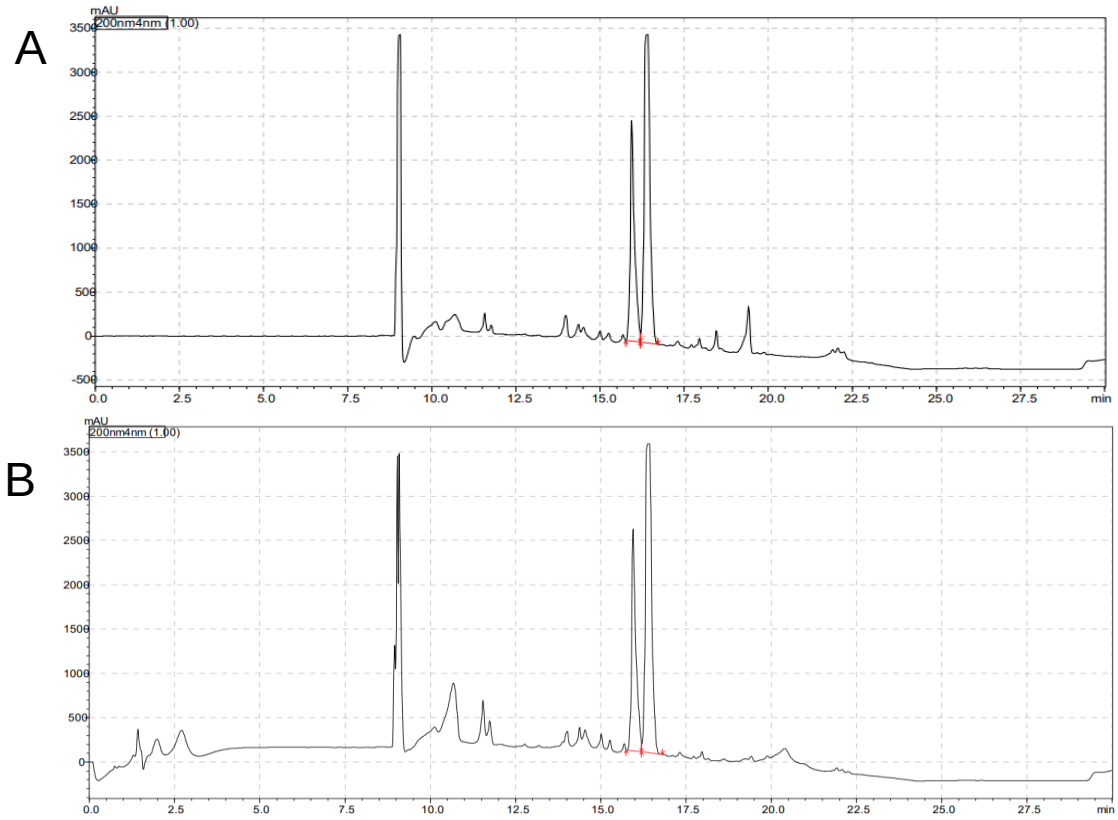
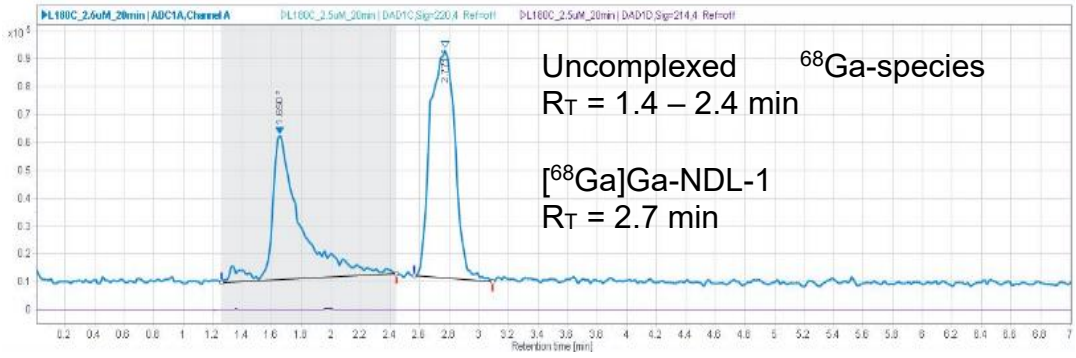


Figure S8: Alkylation reaction product when using 3.0 equiv. *tert*-Bu-Br-Acetate/DIEA. Peak 1 is the desired product (*m/z*: 716.42, $[M+H]^+$), peak 2 is the aspartimide by-product (*m/z*: 684.42, $[M+H]^+$).

A



B

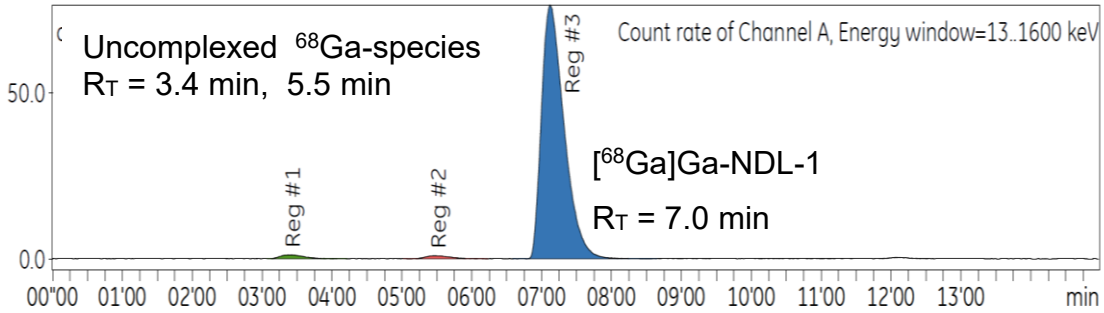


Figure S9: Example of radio-chromatograms showing baseline-separated free (ionic) ^{68}Ga-species obtained for low %RCY [^{68}Ga]Ga-NDL-**1** using radio-HPLC method 1 (top) and radio-HPLC method 2 (bottom, but RCY>97%).

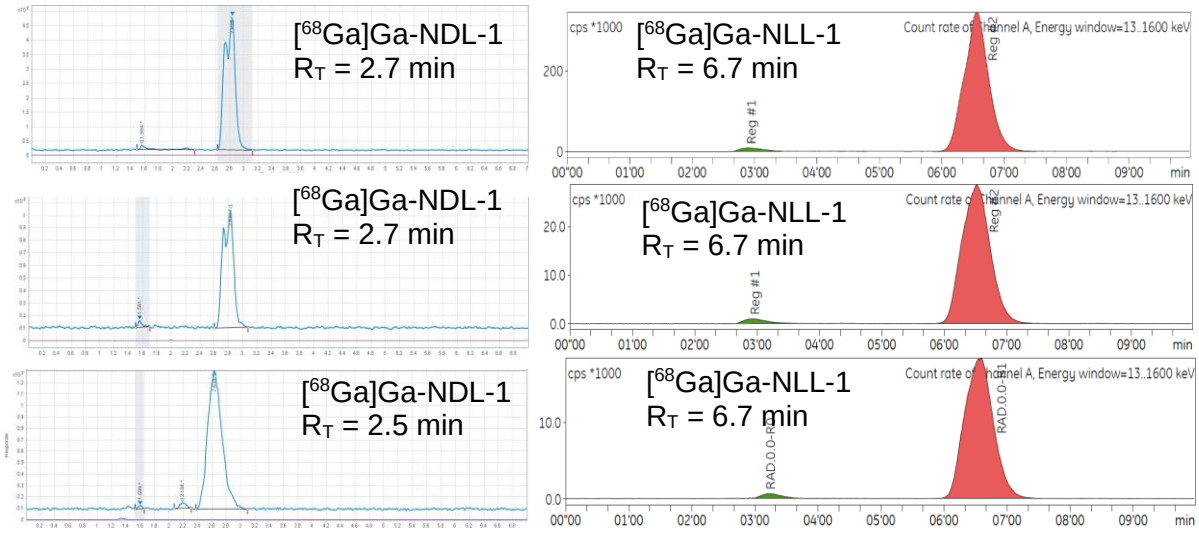


Figure S10: Example radio-chromatograms of [^{68}Ga]Ga-NDL-**1** (left, top) and [^{68}Ga]Ga-NLL-**1** (right, top) at the start of the experiment, after 180 minutes bench-top incubation (middle) and PBS (bottom) at 37°C.

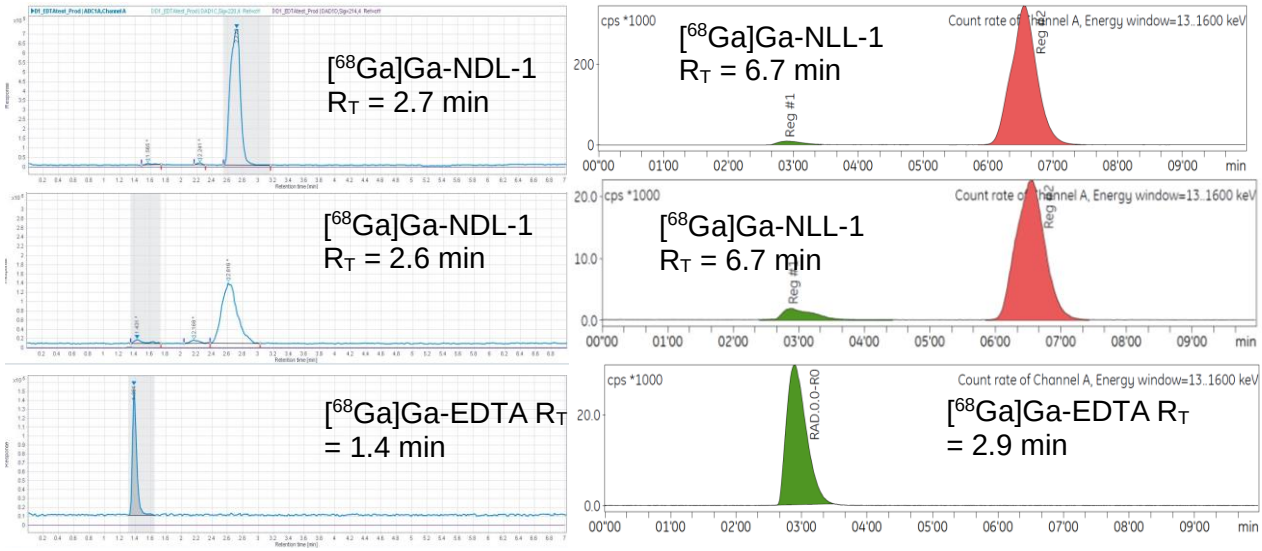


Figure S11: Examples of radio-chromatograms of [^{68}Ga]Ga-NDL-**1** (left, top) and [^{68}Ga]Ga-NLL-**1** (right, top) at the start of the experiment, after 180 minutes incubation in 1000-fold excess EDTA (middle) and [^{68}Ga]Ga-EDTA control (bottom).

References

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