Comparison of DOTA and NODAGA as chelates for ^{68}Ga-labelled CDP1 as novel infection PET imaging agents

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Abstract

The cathelicidin derived peptide (CDP1) is a human antimicrobial peptide that preferentially targets bacterial membranes in response to infection. CDP1 was functionalised with NODAGA and DOTA for complexation with gallium-68 to evaluate its potential as an infection imaging tracer. The synthesis of [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1 were optimised for pH, molarity, incubation time and temperature, and product purification. The integrity and protein binding were investigated employing [^{68}Ga]$GaCl_3$ and [^{68}Ga]Ga-DOTA-TATE as internal references. [^{68}Ga]Ga-NODAGA-CDP1 displayed good labelling properties with higher product yield compared to [^{68}Ga]Ga-DOTA-CDP1. In contrast, [^{68}Ga]Ga-DOTA-CDP1 showed better stability and is the preferred candidate for an *in vivo* investigation.

Keywords

CDP1, NODAGA, DOTA, ^{68}Ga, antimicrobial peptides, infection imaging, PET

Introduction

According to the World Health Organisation 2016 Global Health Estimates, more than half of all deaths in developing countries are caused by infectious diseases, such as lower respiratory infections, diarrhoeal diseases, human immune-deficiency virus/acquired immune-deficiency syndrome (HIV/AIDS), tuberculosis (TB) and malaria (WHO, 2018). In addition, the challenge of global antimicrobial resistance (AMR) has resulted in the increase of multi drug-resistant and extensively drug-resistant TB (World Health Organization., 2017). It has been reported that the overuse of antibiotics is the major cause of AMR due to inappropriate initiation or excessive duration of the therapy (Boyles and Wasserman, 2015). It is therefore crucial to accurately diagnose bacterial infections and subsequently prescribe the appropriate therapy. Biomarkers such as pro-calcitonin (PCT), give false positive results in chronic kidney diseases with no bacterial infection while C-reactive protein (CRP) is negligible in fulminant hepatic failure and both PCT and CRP are ineffective in liver and renal dysfunction and corticosteroid therapy. PCR methods and culture-based tests give poor prognostic accuracy and have relatively low sensitivity. An ideal biomarker for infectious diseases must detect the causative agent with high sensitivity and specificity. It must possess prognostic capacity, provide therapeutic monitoring, determine the duration of the treatment and provide for quick turnaround time (Hwang et al., 2018, Albrich and Harbarth, 2015).

The radiolabelling of biomarkers and the use of positron emission tomography (PET) for imaging could increase diagnostic accuracy because of the higher sensitivity and quantification accuracy provided by PET. Although 2-Deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG) is largely nonspecific to bacterial infection, it was successfully used to monitor the response to therapy in pulmonary and disseminated TB with HIV co-morbidity (Sathekge et al., 2018). 6”-[^{18}F]fluoromaltotriose appears to be specific for bacterial infections

due to uptake of the maltodextrin transporter in gram-negative and –positive bacteria (Gowrishankar et al., 2017) while sodium [^{18}F]fluoride has been used to image chronic TB in mice models (Ordonez et al., 2015). However, [^{18}F]fluoride is cyclotron produced, a limitation in developing countries which are most affected by infective diseases.

Positron-emitting radionuclides, such as the generator-produced gallium-68 (^{68}Ga) ($t_{1/2}$ = 68 min, Emax β+ = 1.899 MeV, 89% β+ yield), have been widely used clinically, mainly in the diagnosis of neuroendocrine tumours ([^{68}Ga]Ga-DOTA-TATE) (Haug et al., 2012, EDQM, 2014) and prostate cancer ([^{68}Ga]Ga-PSMA-11) (Uprimny, 2019, Giovacchini et al., 2018, Counago et al., 2018). Infection imaging studies showed that [^{68}Ga]Ga-citrate and [^{68}Ga]Ga-DOTA-TBIA101 are non-specific, accumulating in metabolically active tissue and as such, are unable to distinguish TB infection from inflammation (Mokaleng et al., 2015, Ebenhan et al., 2017). However, a *Staphylococcus aureus* infection was detected using [^{68}Ga]Ga-NOTA-UBI29-41 (Ebenhan et al., 2014b, Vilche et al., 2016) and recently a "first in-human" evaluation of [^{68}Ga]Ga-NOTA-UBI was published (Ebenhan et al., 2018). This indicates a strong interest in gallium-68 and possibly a PET solution for low-income countries.

The oligomeric peptide H_2N-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-OH (CDP1) is a fragment of a human cationic antimicrobial peptide (hCAP18) that exhibits bacterial cell membrane selectivity (Larrick et al., 1995). Strong electrostatic forces govern the attraction and attachment of CDP1 to the anionic lipopolysaccharide bacterial outer membrane and teichoic acids (Brogden, 2005). CDP1 demonstrated antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* (Gordon et al., 2005, Dorschner et al., 2001).

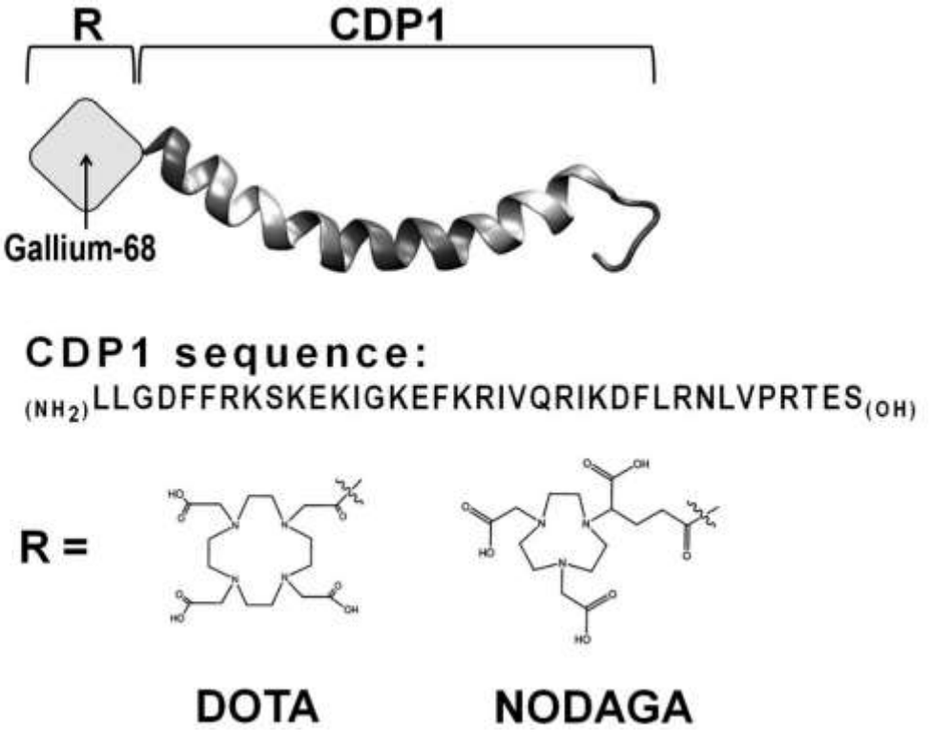

Fig. 1 Structural representation of ^{68}Ga-complexation with DOTA- or NODAGA-conjugated CDP1

Structurally, in order to carry out nuclear medical imaging, a radioisotope must be incorporated or attached to a biomolecule responsible for targeting. A convenient and readily established approach would be the complexation of a radioisotope with a chelating component, preferably without exerting any effect to the function of the tracer nor compromise the integrity of the radiotracer. As a potential PET biomarker for infection, CDP1 was functionalized with the aza-macrocyclic chelates, 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NODAGA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) to subsequently allow for complexation with the [^{68}Ga]Gallium ion (Fig. 1).

Preliminary radiolabelling of [^{68}Ga]Ga-NODAGA-CDP1 was first described in 2015 (Mdlophane et al., 2015). Later, Dutta et al. (2017) emphasised the synthesis and evaluation of [^{nat}Ga]Ga-

NODAGA-CDP1 *in vitro* and exemplified labelling NODAGA-CDP1 with gallium-68 (^{68}Ga) using a non-customised in-house method (adopted from [^{nat}Ga]Ga-NODAGA-CDP1 complexation). This study aimed to *optimise* the radiolabelling protocol for NODAGA functionalised CDP1 with gallium-68 following the *preliminary* radiosynthesis reported in 2015. Additionally, we radiolabelled DOTA-CDP1 and subsequently evaluated the integrity of each tracer to determine which will be suitable for further *in vivo* investigation. However, due to observed NODAGA-CDP1 degradation, efforts were made to re-synthesise it, but it was costly and unsuccessful. Thus, serum stability and protein binding tests were demonstrated only for DOTA-CDP1.

Experimental

Material and Reagents

The SnO_2 matrix-based germanium-68 (^{68}Ge)/^{68}Ga-generator (1.85 GBq) was obtained from iThemba LABS (Somerset West, South Africa) and utilised for experiments between 11 and 24 months after the manufacturing date. For radiosynthesis, supra-pure HCl, sodium acetate tri-hydrate (NaOAc), pH strips, reaction vessels and Millex GV 0.22 μm membrane sterile filters were procured from Sigma-Aldrich, Germany. High-performance liquid chromatography (HPLC) grade water (deionised; 18.2 MΩcm) was produced in-house by a Simplicity 185 Millipore system (Cambridge, USA). CDP1 (4493.03 Da) and NODAGA-CDP1 (4849.03 Da) were obtained from the Catalysis and Peptide Research Unit (UKZN Durban, South Africa) (Dutta et al., 2017). DOTA-CDP1 (4879.26 Da) was purchased from GL Biochem Ltd. (Shanghai, China). The internal control DOTA-(Tyr3)-Octreotate (DOTA-TATE) was purchased from Auspep Clinical Peptides

(Tullamarine, Australia). Gatifloxacin and human serum were bought from Sigma-Aldrich, Germany.

Quality control analyses

Radio-HPLC: Two analytical HPLC systems were used. System 1: An Agilent 1200 series HPLC instrument was coupled to a 6100 Quadruple MS detector (Agilent Technologies Inc., Wilmington, USA) and diode array detector. System 2: A Varian ProStar 230 HPLC system coupled to a 325 UV-Vis detector (SMM Instruments, Midrand, South Africa). Both systems were coupled to a radioactive detector (Gina Star, Raytest, Straubenhardt, Germany). A reversed-phase HPLC column (Zorbax StableBond C18, 4.6 × 250 mm, 5 µm; Agilent Technologies, USA) was used. The mobile phase in both HPLC systems consisted of solvent A (H_2O/0.1 % TFA) and solvent B (CH_3CN/0.1% TFA). Routinely, System 1 operated a linear A-B gradient (0% B to 100% B in 6 min, flow rate 1 mL/min) while System 2 was operated on a linear A-B gradient (5% to 95% B over 20 min, flow rate 1 mL/min) providing a better compound discrimination.

Radio-iTLC: Incorporation of $[^{68}Ga]Ga^{3+}$ was either analysed using silica-gel impregnated instant thin-layer chromatography paper (iTLC-SG) developed in 0.1 M sodium citrate or 50:50 ammonium acetate/ methanol (v/v) (Ebenhan et al., 2014a, de Blois et al., 2011a) followed by evaluation on a multi-channel chromatogram scanner (Veenstra VCS-201, Veenstra Instruments, Joure, Netherlands) and further characterization by radio-HPLC (Dutta et al., 2017). Quantitative analysis included calculation of the labelling efficiency (%LE) or radiochemical purity (%RCP).

Evaluation of radiolabelling parameters

Following generator elution by eluate fractionation (EF) as described (Ebenhan *et al*., 2014a), compound molarity, eluate acidity, incubation duration and temperature were evaluated to optimise the radiosynthesis of [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1.

Compound molarity: Lyophilised NODAGA-CDP1 and DOTA-CDP1 was divided in ready-to-use aliquots of 0.1-0.25 mg which were freshly reconstituted in deionised water yielding 1 µg/µL stock solutions based on published methods (Ebenhan et al., 2014a). Samples of 0.15 mL buffered [^{68}Ga]Ga^{3+} (50-55 MBq, % ^{68}Ga/buffer ratio: 23/87) were added to 0.5-12 nmol of each bio conjugate and incubated for 20 min at 21 ± 1 °C (NODAGA-CDP1) or 15 min at 95 ± 2 °C (DOTA-CDP1).

Eluate acidity (pH): The influence of pH changes in the reaction mixture on ^{68}Ga-complexation was evaluated. Therefore, the ^{68}Ga-activity (0.5 mL) was mixed with different amounts of buffer solution to yield various concentrations (0.26 M - 0.72 M), thereby giving a pH range of 1.0 to 6.5. The buffered ^{68}Ga-activity was added to 20 µg NODAGA-CDP1 (total volume 0.20 mL) or 50 µg DOTA-CDP1 (total volume 0.25 mL) and immediately vortexed and incubated for 15-20 min as described earlier.

Incubation time and temperature: The buffered ^{68}Ga-activity was added to 20 µg NODAGA-CDP1 (total volume 0.20 mL) or 50 µg DOTA-CDP1 (total volume 0.25 mL) and incubated over time. Samples were taken at 5, 10 and 20 min for [^{68}Ga]Ga-NODAGA-CDP1 and at 10 and 20 min for [^{68}Ga]Ga-DOTA-CDP1. %LE was determined with radio-iTLC. Temperature measurements were performed with varying concentrations of the agents at 21 ± 1 °C and 95 ± 2 °C. Samples were taken at 20 min.

Product purification

Following radiolabelling, purification of [^{68}Ga]Ga-NODAGA-CDP1, [^{68}Ga]Ga-DOTA-CDP1 and [^{68}Ga]Ga-DOTA-TATE was conducted using a Sep-Pak light C8 or C18 cartridge units (Waters Corporation, Milford, USA). [^{68}Ga]Ga-DOTA-TATE, an established nuclear medicine tracer was labelled as a reference as previously described (Ebenhan et al., 2014a).

The cartridges were conditioned with absolute ethanol (4 mL) and equilibrated with 4 mL Millipore deionised water (2 mL for [^{68}Ga]Ga-DOTA-TATE) followed by loading of the crude peptide samples and subsequent rinsing with water or a saline solution. Increasing percentages of ethanol ranging from 10 to 100% (0.4 – 1 mL) were used to recover the products. [^{68}Ga]Ga-DOTA-TATE was purified using a C18 cartridge and recovered from the matrix using an ethanol/saline (1:1 v/v) solution as eluent. Quantitative analysis was done as previously described (Rossouw and Breeman, 2012). Briefly, the radioactive yield was calculated from the decay corrected product activity in relation to the sum of significant activities contained in the residual reaction mixture and in the C8/C18-cartridge, cartridge purging liquids and reaction vessels (micro centrifuge tubes were used to minimise adsorption of the product).

Integrity, protein binding and serum stability

Bench stability: The finished products, [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1 (isotonic formulation at physiological pH) and [^{68}Ga]Ga-DOTA-TATE (used as reference) remained at room temperature for 3 h. Several samples were taken over time and subsequently analysed on radio-HPLC. Unconjugated CDP1 (dissolved in water) and gatifloxacin (used as reference) was dissolved in 50% methanol/water and tested at 21± 1 °C for 3 h. Sample

identification and characterization of both agents was achieved by Maxis LC-MS (Shimadzu, Kyoto, Japan) as previously reported (Dutta et al., 2015).

Serum stability and protein binding: The serum stability and protein binding for [^{68}Ga]Ga-DOTA-CDP1 were evaluated as previously described (Beaino and Anderson, 2014). Briefly, *in vitro* stability of [^{68}Ga]Ga-DOTA-CDP1 was incubated with serum and PBS at 37 °C for 2 h. Samples were drawn at 0, 1 and 2 h; proteins were precipitated with ice-cold acetonitrile followed by centrifugation at 5000 rpm for 10 min at 25 °C (Digicen 21 R, Orto Alresa, Madrid, Spain). The supernatant was collected and diluted with deionised water and analysed on radio-HPLC (System 2). The characteristic low serum protein binding exhibited by [^{68}Ga]Ga-DOTA-TATE allowed for its application as a positive control, while neutralised [^{68}Ga]$GaCl_3$ was employed as a negative control due its high interaction with serum proteins. The ^{68}Ga-compounds were incubated in serum at 37 °C. Samples were drawn at 0, 1, and 2 h. The proteins were precipitated with ice-cold acetonitrile followed by centrifugation at 5000 rpm for 10 min at 25 °C. The acetonitrile washed protein precipitates (pellets) and supernatants were collected, and the radioactivity measured in a calibrated γ-counter (Hidex, Finland).

Statistical analysis

Analytical data was reported as average ± standard deviation (SD) or ± standard error of mean (sem) calculated using MS Excel Software (Microsoft, Albuquerque, USA). Variance was calculated by using a Student's *t*-test with differences at the 95% confidence interval ($p < 0.05$) considered to be statistically significant.

Results

Evaluation of labelling parameters

Compound molarity: Radiolabelling demonstrated significant differences between [^{68}Ga]Ga-DOTA-CDP1 and [^{68}Ga]Ga-NODAGA-CDP1 (Fig. 2). Poor labelling occurred at low molarity (≤6 µM). However, [^{68}Ga]Ga-NODAGA-CDP1 (21 ± 6%) had twice the %LE compared to [^{68}Ga]Ga-DOTA-CDP1 (11 ± 1%; $p < 0.03$). With increasing precursor molarity a faster increase in [^{68}Ga]Ga-NODAGA-CDP1 labelling occurred (between ~10 and 25 µM, always 2-fold higher than [^{68}Ga]Ga-DOTA-CDP1). At precursor concentration 33 µM, the %LE was similar, namely 99 ± 2% and 95 ± 2% for [^{68}Ga-]Ga-DOTA-CDP1 and [^{68}Ga]Ga-NODAGA-CDP1, respectively.

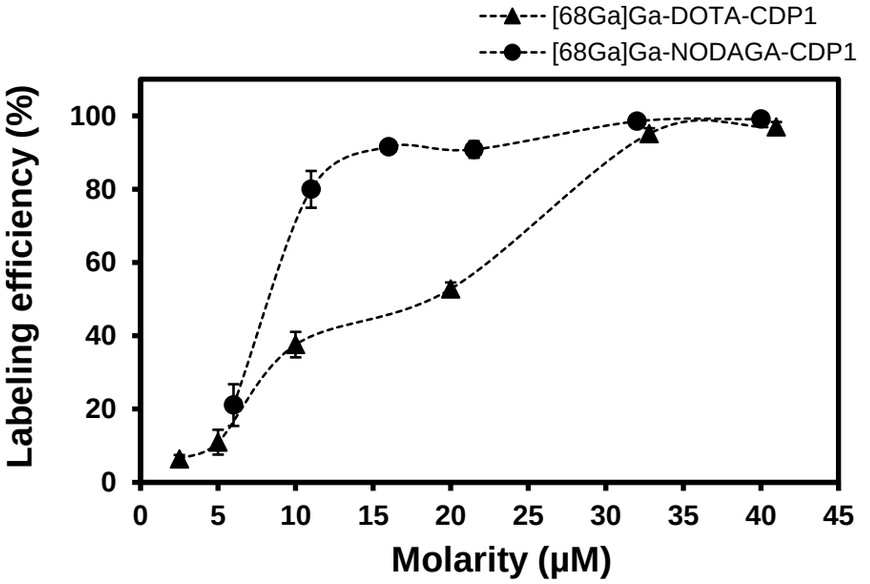

Fig. 2 The effect of compound molarity on the radiolabelling efficiency after reacting with buffered ^{68}Ga-activity for 15 - 20 min at 21 ± 1 °C for [^{68}Ga]Ga-NODAGA-CDP1 and 95 ± 2 °C for [^{68}Ga]Ga-DOTA-CDP1 (n = 3)

Eluate acidity (pH): **The** radiolabelling yield was determined over a range of pH values (pH 1 – 7) using the fractionated labelling method. The degree of complexation of gallium-68 with NODAGA-CDP1 and DOTA-CDP1 was pH dependent.

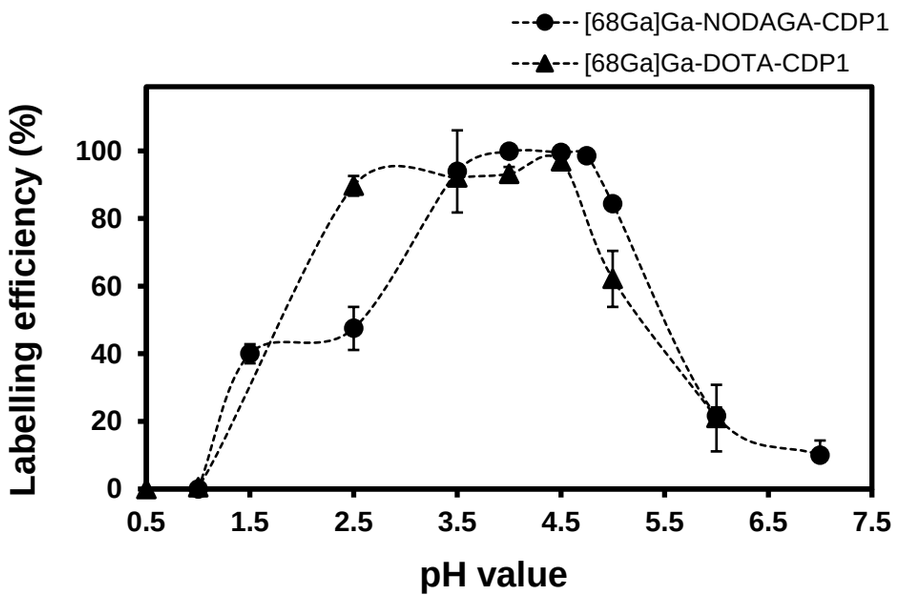

Fig. 3 The effect of pH on the labelling efficiencies of [^{68}Ga]Ga-NODAGA-CDP1 (n = 4) and [^{68}Ga]Ga-DOTA-CDP1 (n = 3)

The acidity of the eluate has an influence on the success of radiolabelling (Fig. 3). At pH 2.5, the [^{68}Ga]Ga-DOTA-CDP1 labelling was better (90 ± 3%; $p < 0.01$) than the labelling of [^{68}Ga]Ga-NODAGA-CDP1 (48 ± 6%). A steady increase in LE occurred for both compounds to a maximum of 100 ± 0% at pH 4.0 and 99 ± 1% at pH 4.5 for [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1, respectively. Both tracer radiolabelling performances were compromised towards physiological pH levels as a significant decline to minimal [^{68}Ga]Ga-NODAGA-CDP1 ($p < 0.001$) and [^{68}Ga]Ga-DOTA-CDP1 ($p < 0.002$) radiolabelling at pH >6 occurred.

Incubation time: [^{68}Ga]Ga-NODAGA-CDP1 displayed a 75 ± 17% LE within 5 min. There was no difference at 10 min (93 ± 2%) and 20 min (95 ± 2%) with [^{68}Ga]Ga-DOTA-CDP1 whereas [^{68}Ga]Ga-NODAGA-CDP1 labelling improved significantly from 10 to 20 min (84 ± 4% and 92 ± 1% ; $p < 0.03$). This indicates that 10 min is sufficient to achieve maximum radiolabelling with [^{68}Ga]Ga-DOTA-CDP1 (95 ± 2°C, 33 µM, pH 4.5) and 20 min for [^{68}Ga]Ga-NODAGA-CDP1 (21 ± 1 °C, 16 µM, pH 4.5).

Incubation temperature: The effect of time, molarity and temperature on the incorporation of [68Ga]$^{3+}$ NODAGA- and DOTA-CDP1 is summarized (Table 2) with emphasis on the optimal incubation duration. At 21 ± 1 °C, [^{68}Ga]Ga-NODAGA-CDP1 showed poor labelling with molarities ≤2 µM (5 ± 1%) but improved labelling with increasing molarity occurred to a quantitative radiolabelling of 99 ± 2% at 40 µM (Table 2). Increasing the temperature to 95 ± 2 °C was beneficial for both the 2 µM (13 ± 4%; $p < 0.05$) and 10 µM (100 ± 0%; $p < 0.002$) samples. Moreover, [^{68}Ga]Ga-NODAGA-CDP1 exhibited significantly higher %LE compared to [^{68}Ga]Ga-DOTA-CDP1 at 95 °C with 10 µM ($p < 0.001$).

Table 2 Summary of the radiolabelling efficiencies of various concentrations of [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1 labelled at 21 ± 1 °C and 95 ± 2 °C

Molarity (µM)	**Labelling efficiency (%)**		
	[^{68}Ga]Ga-NODAGA-CDP1		**[^{68}Ga]Ga-DOTA-CDP1**
	21± 1 °C [a]	**95 ± 2 °C** [b]	**95 ± 2 °C** [c]
2	5 ± 1	13 ± 4*	6 ± 1
10	80 ± 5	100± 0*	38 ± 4
16	91 ± 1	-	-
33	-	-	95 ± 2
40	99 ± 1	100 ± 0	97 ± 1

Incubation time for a) 20 min, b) 20 min, and c) 20 min
n = 3 for all results, *) $p < 0.05$ comparing NODAGA-CDP1 incubated at different temperatures. (e.g. significant benefit of heating step)

Purification

SPE using Sep-Pak cartridges has been suggested for purification of [^{68}Ga]-peptides from unbound [^{68}Ga] $(H_2O)_n$ and ^{68}Ga-colloids (Ebenhan et al., 2014a). [^{68}Ga]Ga-NODAGA-CDP1 recovery from the C18-matrix was not reproducible (Fig. 5a) and overall significantly lower than [^{68}Ga]Ga-DOTA-TATE ($p < 0.01$). Only 90 - 95% ethanol/saline (v/v) was capable to recover on average 45-66% of compound. Therefore, a cartridge with a C8-matrix was employed to purify [^{68}Ga]Ga-DOTA-/NODAGA-CDP1, reaching a higher compound recovery of 84 ± 2% ($p < 0.001$) [^{68}Ga]Ga-NODAGA-CDP1 with a 90% ethanoic water solution compared to [^{68}Ga]Ga-DOTA-CDP1 (23 ± 0.3%) (Fig. 5b). A single desorption with only 60% ethanoic water (v/v) yielded 67 ± 5% of [^{68}Ga]Ga-NODAGA-CDP1 while 95% ethanoic water (v/v) recovered only 65 ± 10% of [^{68}Ga]Ga-DOTA-CDP1 (not shown). The radioactive product purity after Sep-Pak-C8 treatment was determined as ≥ 99% (EOS ~40 min). Since a high concentration of ethanol was required to recover the compound from the C8 matrix, some post purification measures, such as heat evaporation or dilution, had to be employed to reduce the ethanol content to acceptable levels (<10 % for future *in vivo* testing).

Moreover, the required radiochemical purity for radiopharmaceuticals is >95% (EDQM, 2014). ^{68}Ga-DOTA-CDP1 had a 97% RCP, eliminating the need for SPE purification. The purification was performed to compare with SPE-C8 purification of ^{68}Ga-NODAGA-CDP1. Further investigation was beyond the scope of the paper.

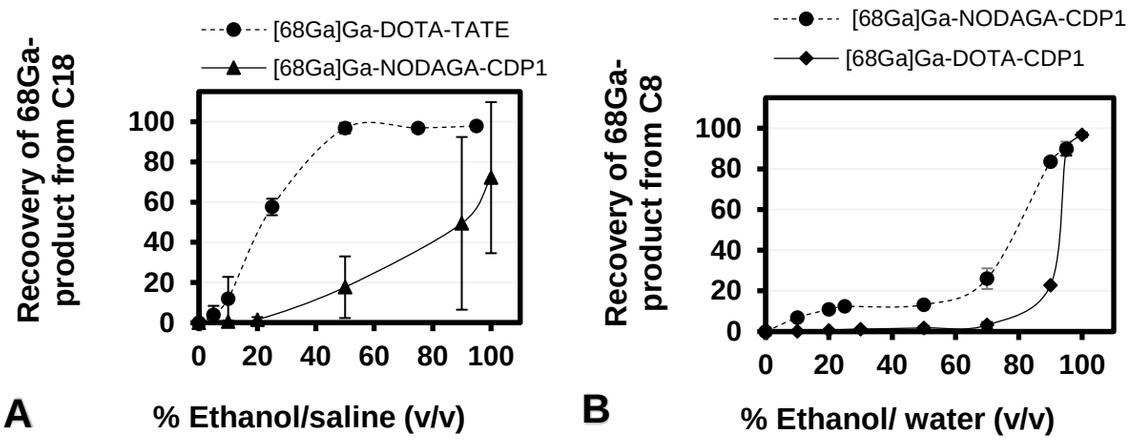

Fig. 5 Conditions for the purification of [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1 (n = 3). *[^{68}Ga]Ga-DOTA-TATE (n = 3) serves as a comparison with known C18 recovery

Integrity and serum stability

Bench stability: CDP1 quickly degraded by ~50% every hour until it reached a plateau after the third hour (Fig. 6a), exhibiting significantly lower bench stability than gatifloxacin ($p < 0.01$). Unconjugated CDP1 was dissolved in water (500ng/ml = 0.1025 µM) for the duration of the experiment. Peptides containing Asp, Glu, Lys, Arg (such as CDP1) are prone to moisture absorption from the air (lyophilised) and may be susceptible to bacterial degradation in solution. Bacterial degradation may occur because the peptide has a disordered structure that has poor antibacterial activity in $<10^{-6}$ M concentrations (Johansson et al., 1998).

On the contrary, the DOTA-/ NODAGA-conjugated CDP1 was lyophilised and dissolved in buffered ^{68}Ga-activity (2.5 M NaOAc, pH 4.5), after incubation, purified and diluted in PBS, pH 7. It is likely that the presence of acetate ions stabilised peptide molecules by 1) decreasing the electrostatic energy between the peptide molecules maintaining the peptide structure and preventing aggregation; and 2) promoting the α-helical structure formation which is associated

with increased antimicrobial activity – which could counteract bacterial degradation by killing contaminant bacteria. In addition, at the pH >5, α-helicity starts to form contributing to antibacterial activity (Johansson et al., 1998). Moreover, DOTA-/NODAGA-conjugation at the N-terminus may have contributed in the stability (see also discussion).

Both ^{68}Ga-compounds demonstrated a high degree of stability (>95%) within 3 h at 21 ± 1°C (Fig. 6b). The [^{68}Ga]Ga-DOTA-TATE stability was better than the stability of [^{68}Ga]Ga-DOTA-CDP1 and [^{68}Ga]Ga-NODAGA-CDP1 ($p < 0.001$). The NODAGA-peptide displayed significantly lower stability compared to [^{68}Ga]Ga-DOTA-CDP1 ($p < 0.01$).

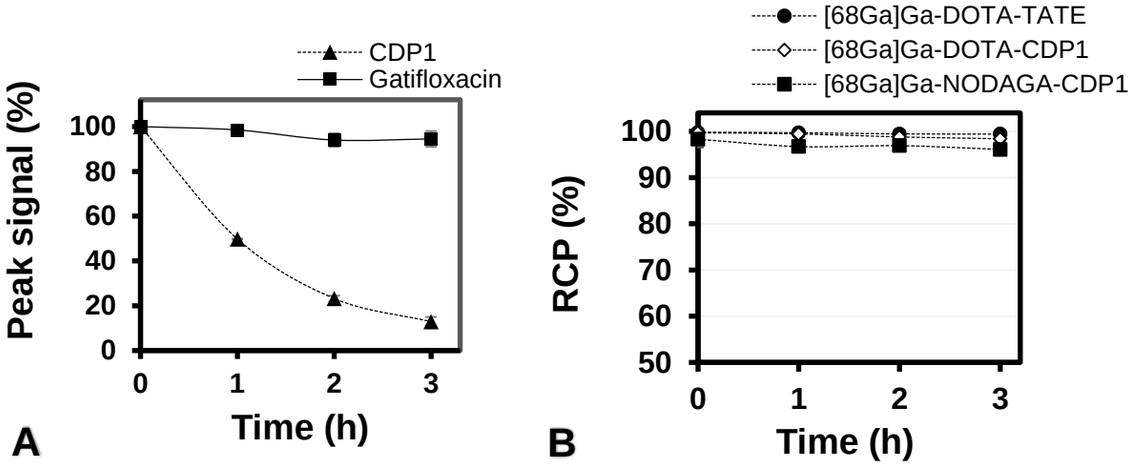

Fig. 6 (A) Bench stability of unconjugated CDP1 compared with gatifloxacin (internal reference) (n = 3). (B) CDP1 after conjugation with NODAGA or DOTA and radiolabeled with [^{68}Ga]$^{3+}$ (n = 3) and [^{68}Ga]Ga-DOTA-TATE (n = 3); all agents were tested at neutral pH up to 3 h

Serum stability and protein binding: The results for [^{68}Ga]Ga-DOTA-CDP1 protein binding were significantly higher than the results for [^{68}Ga]Ga-DOTA-TATE ($p < 0.001$)(positive control) and similar to the results for [^{68}Ga]$GaCl_3$ (negative control). The uptake of [^{68}Ga]Ga-DOTA-CDP1

into serum protein at 37 °C was cumulative with a resultant 3 ± 1% remaining unbound after 2 h (Fig. 7a). The unbound fraction was further analysed by radio-HPLC displaying high compound integrity after 2 h in serum (98 ± 2% RCP), indicating that no quantifiable metabolic decay occurred (Fig. 7b).

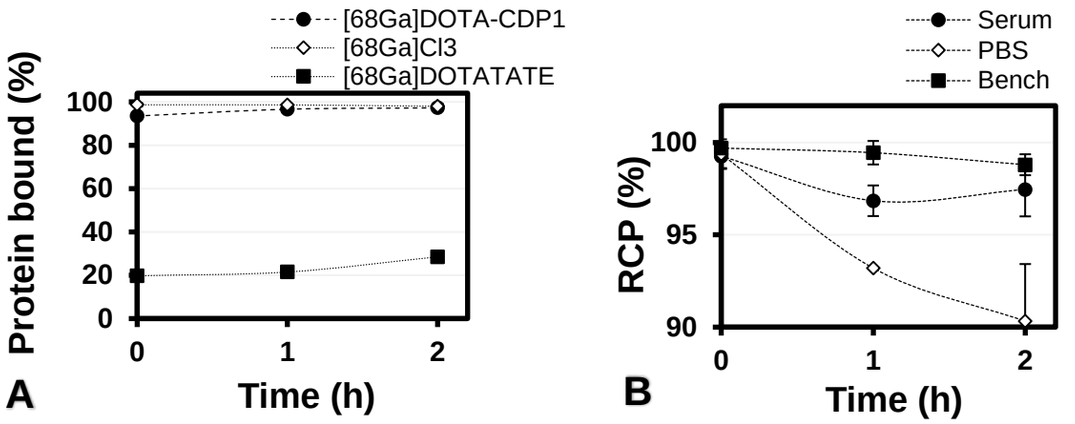

Fig. 7 (A) Serum protein binding of [^{68}Ga]Ga-DOTA-CDP1, [^{68}Ga]$GaCl_3$ and [^{68}Ga]Ga-DOTA-TATE at 37°C. (B) [^{68}Ga]Ga-DOTA-CDP1 serum and PBS integrity at 37 °C and 21 ± 1 °C (bench); n = 3

Although product degradation was relatively faster at 37 °C in PBS (90 ± 3%) after 2 h, there was no significant difference in serum and PBS at 37 °C and at 21 ± 1 °C (bench: 99 ± 1%).

Table 3 Summary of the optimum labelling parameters and compound integrity for [^{68}Ga]Ga-NODAGA-CDP1 and [^{68}Ga]Ga-DOTA-CDP1

	DOTA-CDP1	**NODAGA-CDP1**	**DOTA-TATE**
Number of radiosynthesis (n)	11	16	10
Precursor (nmol)	20	8	35.0
Molarity (μM)	40	16.0	24.6
Incubation time (min)	10	15-20	10
Temperature (°C)	95 ± 2	25 ± 2	95 ± 2
Optimal pH	4.5	4.0	3.5
E/H(%) of SPE C8*	95	60	50
Radiochemical yield (%, d.c.)[#]	65 ± 10	67 ± 5	> 90
Radiochemical purity (%)	> 99	> 99	> 95
Specific activity (MBq/nmol)	2 ± 1	6 ± 2	

Bench stability (2 h, 25±2°C) (%)	99 ± 1	96 ± 1	99 ± 1
Integrity (PBS, pH 7, 2 h, 37°C) (%)	90 ± 3	-	> 95 [1)]
Serum integrity (2 h, 37°C) (%)	98 ± 2	-	> 95 [1)]
Protein binding (2 h, 37°C) (%)	98 ± 1	-	29 ± 1

*) E/H (%)of SPE C8= percentage of an ethanol/water solution required to recover the maximal compound from a solid-phase extraction C8 matrix; [#]) decay corrected; [1]) Previously published (Ebenhan et al., 2014a)

The optimised labelling summary (Table 3) tabulated only the optimum labelling conditions after the optimisation of all the labelling parameters was performed. The runs were carried out for multiple experiments, some of which are beyond the scope of this manuscript.

Discussion

Technologies such as PET-CT can aid in localising deep seeded infection of unknown origin by providing a holistic, non-invasive insight in the whole body, hence detect the site of infection without relying on blood cultures or collection of other bodily fluids. One of the current roadblocks to more targeted imaging of infection is the lack of radiotracers that are selective for the microorganism or endotoxins released by the microorganism. Currently, [^{18}F]FDG PET-CT and radiolabelled white blood cell imaging are the most commonly utilized procedures to diagnose infectious diseases, both procedures with major disadvantages. This study introduces the optimisation of a recently developed [^{68}Ga]Ga-NODAGA-CDP1 (Mdlophane et al., 2015, Dutta et al., 2017) and provides radiolabelling comparison with a newly synthesised [^{68}Ga]Ga-DOTA-CDP1. Moreover, we report a radio-TLC analysis and stepwise optimisation of peptide molarity, pH, reaction time, temperature, SPE-C8 and C18 product purification and integrity at 21 ± 1 °C with [^{68}Ga]Ga-NODAGA-CDP1, and serum stability and protein binding data with [^{68}Ga]Ga-DOTA-CDP1 whereas Dutta et al (Dutta et al., 2017) differed by demonstrating that NODAGA-

CDP1 can be identified by radio-HPLC and UV analysis as a radiolabelled ^{68}Ga-product with sufficient retention time to decipher it from free ^{68}Ga-species. The reported 'radiochemical purity' at 5 min was actually tested with a very high peptide concentration. Most importantly, the main emphasis was on peptide synthesis and the evaluation of [^{nat}Ga]Ga-NODAGA-CDP1 *in vitro*.

A large proportion of literature on ^{68}Ga-peptide radiolabelling methods were developed mainly for short peptides (<20 amino acids), while proteins were rarely labelled with ^{68}Ga-activity. Short ^{68}Ga-peptides for infection imaging described [^{68}Ga]Ga-DOTA-TATE as reference (Mokaleng et al., 2015, Ebenhan et al., 2014a). To the best of our knowledge, clinically proven examples of ^{68}Ga-oligopeptides are currently not available to use as references for the ^{68}Ga-labelling of our oligomeric peptide (CDP1), therefore some challenges with the radiosynthesis were anticipated. Hence, [^{68}Ga]Ga-DOTA-TATE, a short peptide, was used as a guide and a reference due to its extensively reported radiochemistry and established clinical application. Furthermore, the DOTA-TATE labelling approach translated to NODAGA-CDP1 radiolabelling was advantageous but had its limitations. Thus, employing translatable radiolabelling approaches used for short peptides in oligopeptides should be accepted with its merits and the limitations thereof.

For this study, understanding the peptide characteristics and the influence of the chelate was essential to develop a robust radiolabelling solution. The aim of the study was to develop a fast, if not instant, radiolabelling procedure by optimizing the radiolabelling parameters. We compared the two chelates to determine which one meets the criteria for a radiopharmaceutical imaging agent for infections to be tested in a preclinical trial. DOTA and NODAGA each have their advantages and challenges. DOTA is considered a more universal chelate accepting not only gallium-68 but

also other radionuclides including copper (II), indium (III), yttrium (III), zirconium (IV) and scandium (III) (Wadas et al., 2010). DOTA shows stable and robust metal complexation. However, it may be incompatible with thermo-labile peptides or proteins due to the required heating step for metal complexation. In addition, metal complexation with DOTA is sometimes compromised by conjugation with the peptide, resulting in lower specific activity compared to NODAGA, TRAP or NOPO (Notni et al., 2012).

Due to its smaller size, NODAGA forms stable complexes with fewer radiometals, but can complex $[^{68}Ga]Ga^{3+}$ and $[^{64}Cu]Cu^{2+}$ ions. Unlike DOTA, its chelating properties may allow efficient radiolabelling at 21 ± 1 °C (Kubíček et al., 2010, de Sá et al., 2010, Fani et al., 2011), kit formulation is allowed. However, there is no consensus on the superiority of NODAGA over DOTA. Poor *in vivo* stability of NODAGA-peptides compared to NOTA and DOTA has been reported, as it defluorinated *in vivo* from the $Al^{18}F$-complex (Blasi et al., 2014). Increased stability due to favourable cyclo-protective encapsulation of the $[^{68}Ga]Ga^{3+}$ molecule, minimising demetalation and thus *in vivo* trans-chelation to transferrin was also reported (Dumont et al., 2011, Clarke and Martell, 1991).

Radiolabelling

The interdependence of compound concentration, time and temperature were emphasised in this study, where it was found that complex formation with gallium-68 increases with time and depending on the nature of the chelate, the presence of heat or the absence thereof was critical to the reaction. Effective bioconjugate molarity was largely affected by the chelate where DOTA-CDP1 required double the concentration of NODAGA-CDP1 to reach comparable LE (Fig. 2),

similar to findings by Notni et al. (Notni et al., 2012). Heating [^{68}Ga]Ga-NODAGA-CDP1 was not beneficial except at the lower precursor concentration, but the overall small radiochemical yields as well as poor reproducibility did not justify the use of low concentrations (Table 2). Although heat is essential to efficiently radiolabel DOTA-peptides, we found that prolonged heating (≥35 min at 95 °C) compromised the integrity of the peptide which presented recurring UV peaks on HPLC (Fig. S1 & Fig. S2). As such, practical incubation times (10-20 min) are preferred, particularly when developing an agent for routine clinical use. In this study, the [^{68}Ga]Ga-DOTA-CDP1 radiosynthesis demonstrated sufficiently-high LE and radiolabelling yield in as little as 10 min with optimal precursor amount.

CDP1 changes in structure from a 'circular dichroism spectrum consistent with a disordered structure' (Johansson et al., 1998, Oren et al., 1999) to an α-helical oligomeric structure as the pH rises (pH 2-13). Radiolabelling the bioconjugate (NODAGA-CDP1) is, however, highly affected by pH due to the complex ^{68}Ga-chemistry. As expected, the LE was highest between pH 4 and 4.75 and began to decline above pH 4.75. [^{68}Ga]Ga-DOTA-CDP1 had a notably higher %LE at pH 2.5, highlighting a distinct advantage of the DOTA over the NODAGA chelate. Similar findings were reported (de Blois et al., 2011, Breeman et al., 2005).

SPE is an established separation method used to remove trace impurities and colloids from radiopharmaceuticals with the added advantage of increasing the radioactivity concentration in the product. Purifying a large peptide using reversed-phase SPE is a challenge due to the likelihood of poor recovery of the product. The restrictive use of clinically acceptable organic solvents for product elution and accepted content of ethanol in the final product for intravenous administration (EDQM, 2014) are additional concerns. Alternatively, using a less retentive sorbent, such as the reversed-phase C8 was considered. However, [^{68}Ga]Ga-DOTA-CDP1 showed significantly lower

recovery than [^{68}Ga]Ga-NODAGA-CDP1 suggesting that molecular size was the limiting factor in this instance. Nevertheless, as low as 5 µCi (0.185 MBq) has been used for *in vitro* tests (Beaino and Anderson, 2014) to meet with the required injection volumes for rodent imaging studies, physiological pH, isotonicity and specific activity. On this basis, the labelled [^{68}Ga]Ga-DOTA-CDP1 thus acceptable.

Due to degradation of the NODAGA analogue that was identified, it was decided that DOTA-CDP1 is a valid alternative to compare to NODAGA-CDP1 for prospective application for the *in vitro* evaluation and in further studies.

Integrity and Serum Stability

CDP1 degraded significantly compared to gatifloxacin, a fourth generation fluoroquinolone with excellent stability which made it a desirable control compound to compare the stability of other compounds. Consequently, gatifloxacin was radiolabeled with Technetium-99m to evaluate it as a radiopharmaceutical to allow for its biological evaluation (Motaleb et al., 2011). It must be noted that CDP1 stability conditions were different from that of the conjugated peptide in that the former was dissolved in water while the latter was dissolved in acetate buffer, thus they are incomparable. However, the presence acetate ions in solution as well as acetylation of the N-terminus have been known to stabilise peptides (Johansson et al., 1998, John et al., 2008, Strömstedt et al., 2009). Thus, in our case, the conjugation with NODAGA or DOTA at the N-terminus yielded a labelled product of high integrity (>95%) that complies with the standard shelf-life requirements of a radiopharmaceutical (Saha, 2004, EDQM, 2014).

The bench stability of the tested bioconjugates can be summarised as follows: [^{68}Ga]Ga-DOTA-TATE > [^{68}Ga]Ga-DOTA-CDP1 > [^{68}Ga]Ga-NODAGA-CDP1. Contrary to the reported stability

of NOTA and its derivatives (Fani et al., 2011, Clarke and Martell, 1991), it can be deduced that DOTA had a significant advantage over NODAGA in this regard, in accordance with results reported by Domnanich et al. (Domnanich et al., 2016). High protein binding of [^{68}Ga]Ga-DOTA-CDP1 may be the effect of electrostatic forces of attraction between the cationic peptide and the net ionic negative charge of serum proteins at physiologic pH (Baler et al., 2014), which suggests that slow blood clearance may be anticipated *in vivo*. Furthermore, the integrity of the unbound fraction of [^{68}Ga]Ga-DOTA-CDP1 is an indication that no transchelation of the [^{68}Ga]Ga^{3+} occurred in serum. However, *in vivo* testing is required to validate the significance of these findings.

Study Scope and Limitations

Developing a radiolabelling procedure for an oligomeric peptide, such as CDP1, has certain limitations., Therefore, the scope of the study was set out to determine suitable parameters for optimal radiolabelling and comparing two known bi-functional aza-macrocycles as possible moieties to complex gallium-68 to CDP1; whilst most of the results were within the scope, some of our investigations were limited. These include i) low specific activity associated with large peptides, ii) low radiochemical yield due to poor recovery using RP-SPE, iii) high ethanol content in the product which can be evaporated, iv) potential product degradation as a result of heat and high levels of ethanol; or v) increased product volume resulting from dilution (further lowering the radioactivity concentration). As mentioned above, ^{68}Ga-peptide data suggested mainly C18 - and to a lesser extent, strata X and C8- SPE for product recovery of small peptides, which were subsequently tested in this study. Although it is beyond the scope of this study, the application of other SPE units, such as C4, C2 and size-exclusion units is warranted for preparations used in safety evaluation studies in larger mammals.

Conclusion

A novel AMP, CDP1 was successfully labelled with gallium-68, comparing DOTA and NODAGA chelates. [^{68}Ga]Ga-NODAGA-CDP1 exhibits better labelling properties with gallium-68 than [^{68}Ga]Ga-DOTA-CDP1. However, [^{68}Ga]Ga-DOTA-CDP1 showed better bench and serum stability, favouring it for *in vivo* investigations. Low product recovery post purification and low specific activity remains a challenge for CDP1. High serum protein binding may be indicative of slower blood clearance and may warrant further evaluation *in vivo*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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