Peptide synthesis, characterization and ^{68}Ga-radiolabeling of NOTA-conjugated ubiquicidin fragments for prospective infection imaging with PET/CT

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Short title:

^{68}Ga-labeling of NOTA ubiquicidin fragments

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This study was supported by grants from the National Research Foundation (NRF), the University of KwaZulu Natal (UKZN) and The Nuclear Technologies in Medicine and the Biosciences Initiative (NTeMBI)

Abstract

Introduction: Human antimicrobial peptides are of interest for the development of positron emission tomography (PET) tracers as they exhibit desirable characteristics that make them good candidates for targeting vectors. Due to their natural role in the innate immune system

they selectively bind to pathogenic bacteria and yeast, whilst remaining minimally immunogenic and cytotoxic to humans. Research into ubiquicidin (UBI)-based tracers has focused on ^{99m}Tc as a radionuclide, however, the use of bi-functional chelators such as 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), in combination with ^{68}Ga as a radionuclide, allows for a simple radiolabeling procedure which is preferable in a clinical setting using PET/CT. **Methods:** The peptides fragments UBI29-41, UBI30-41 were synthesized by standard microwave Fmoc/tert-butyl (tBu)-solid phase synthetic protocols. Characterizations were performed using analytical HPLC and LC/MS. Both NOTA-conjugated peptides were exposed to $^{nat}Ga^{3+}$; their complexed form was quantified by direct LC/MS injection. This complexation was utilized to testify bacterial and mammalian cell binding potential of fluorophore-linked NOTA-UBI29-41/30-41. ^{68}Ga labeled NOTA-UBI fragments were also tested for competitive interaction to Staphylococcus aureus to proof the binding target. ^{68}Ga was eluted from SnO_2- and TiO_2-based ^{68}Ge/^{68}Ga generators using fractionated elution and anion exchanged-based post-procession. NOTA-peptide radiolabeling was carried out including optimization of buffer molarity, NOTA-peptide concentration(s), incubation temperature and –duration as well as considering various SPE purification cartridges. **Results:** Pure UBI29-41, UBI30-41 and NOTA-UBI30-41 were successfully characterized. Both, NOTA-UBI fragments exhibited complexation rates to $^{nat}Ga^{3+} \geq 99\%$. The percentage binding was significantly higher to Staphylococcus aureus bacilli over Mt4 human leucocytes ($P > 0.05$) for NOTA-UBI29-41[Lys(Abz)] < NOTA-UBI30-41[Lys(Abz)]. Significant lower binding was observed for both ^{68}Ga-labeled NOTA-UBI fragments ($P > 0.03$) after pre-incubation with excess unlabeled NOTA-UBI. Reproducible ^{68}Ga radiolabeling ranged for 51-85% and 46-78% for NOTA-UBI29-41 and NOTA-UBI30-41, respectively. **Conclusion**: Aside from successful peptide syntheses the first ever ^{68}Ga-radiolabeling method is reported for NOTA-UBI fragments. The NOTA-conjugation didn't compromise the selective and specific interaction with bacterial cells *in vitro*. Both tracers are warranting prospective imaging of infection with PET/CT.

Keywords: *Ubiquicidin, NOTA, ^{68}Gallium, infection imaging, PET/CT, ^{68}Ga-NOTA-UBI*

Introduction

As positron emission tomography (PET) becomes more popular as a tool for the diagnosis and imaging of a wide range of medical conditions, it becomes necessary to develop and improve

tracers that allow for the rendering of better quality images without compromising the patients' health. It is important that the tracer does not expose the patient to excessive amounts of radiation from the radioisotope, and that potential cytotoxic or immunogenic effects of the targeting vector are minimal [1, 2]. In many cases a good tracer should show high specificity for certain physiological features or processes that are associated with a medical condition or pathogenesis, and exhibit good clearance from the background tissue or blood [3]. Biomolecules, such as peptides, that naturally occur in the human body are logical choices for further investigation as potential targeting vectors for PET tracers as they are unlikely to be cytotoxic or immunogenic to patients. Their biological role may also mean that they exhibit high selectivity for certain biological cell types or processes which may be of interest in the diagnosis of diseased individuals [4]. Such a biomolecule is ubiquicidin (UBI), a 6.6 kDa linear peptide (of 59 amino acids) that is found in humans and other eukaryotes [5, 6]. This peptide exhibits broad spectrum antimicrobial activity against pathogenic bacteria and *Candida* spp. yeasts, and is also believed to be part of the innate immune system of organisms in which it naturally occurs. Computer models suggest that this peptide contains *alpha* helices and *beta* sheets which allow it to bind to, and compromise the integrity of, prokaryote cell walls. Due to the difficulties in synthesizing the full-length peptide, studies were carried out with fragments of the peptide that are believed to be important in the preferential binding of the peptide to bacterial cells [6]. It was reported that certain fragments of UBI, including the 29-41 fragment exhibit features that make them good candidates for targeting vectors in the development of tracers for PET [5, 7]. Although most research into the development of radioactive tracers for detection and imaging of infection, including studies investigating UBI fragments, has been performed using ^{99m}Tc as a radionuclide [8], a variety of other radionuclides are available for use in tracers; problems associated with some radionuclides are poor image quality, excessive radiation dose to the patient, undesirably long or short radioactive half-life, or the requirement of an in-house cyclotron [4, 9]. Although ^{99m}Tc is still used in 90% of Nuclear Medicine

procedures today, a growing number of diagnostic radiotracers are based on molecules labeled with PET radionuclides such as ^{18}F, ^{11}C and more recently ^{68}Ga and ^{64}Cu. The ^{68}Ga radionuclide is well suited for use as a radiolabel for PET as it has a short half-life of 67.6 min, and emits two divergent gamma rays per decay, allowing the construction of three-dimensional images. It can also be obtained independently on-site, without the use of a cyclotron. Incorporation of the ^{68}Ga into the tracer is best achieved through the use of bi-functional chelators which can strongly chelate the gallium ion and are covalently bound to targeting vectors. The relatively straight-forward radiolabeling of most bi-functional chelators makes these kinds of tracers suitable for mass production in kit form that can be distributed to, and used by staff at hospitals and clinics where an on-site radionuclide generator is present [9, 10]. The most widely used bi-functional chelator for ^{68}Ga radionuclide labeling is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). However, more recently, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) has been investigated as a potential bi-functional chelator for use in tracers, as, unlike DOTA, efficient radiolabeling of NOTA tracers can occur without heating to temperatures which risk damaging targeting vectors such as peptides [9-11]. Due to the smaller dimensions of the macrocyclic cavity of NOTA, as compared to DOTA, the stability of a NOTA chelate containing a small cation such as ^{68}Ga is greater [10]. This leads to greater thermodynamic and kinetic stability [12, 13], which is advantageous as it makes the NOTA-peptide conjugate both easier to label and more resistant to trans-metallation in the body [9, 13]. If, as in most reported cases (for instance DOTATATE), the NOTA or DOTA chelator is linked to the peptide via one of the acid groups (as is also reported herein) the conjugation ability of the chelator is reduced as compared to linking to the peptide via the carbon backbone. However studies have shown that the chelation is sufficient for *in vivo* use and stable for 24 h even if challenged with a 10^4 fold molar excess of DTPA [10, 12]. When conjugating bi-functional chelators to a chemically synthesized peptide fragment such as UBI29-41 (TGRAKRRMQYNRR), different approaches are

possible. The conjugation can take place after the peptide has been cleaved from the resin and purified, however this method is inefficient, requiring a large excess of NOTA and attachment of the bi-functional chelator at specific sites is more difficult. Recently, Guérin and co-workers [14] described the solid phase synthesis of NOTA functionalized peptides. Conjugation of the NOTA moiety onto the peptide occurs prior to deprotection and cleavage of the peptide from the resin. This approach is more cost effective than post-purification conjugation methods which require a five times molar excess of NOTA [14]. Additionally, the amino terminus is conveniently available for covalent binding with the NOTA chelator prior to cleavage and deprotection in the most commonly used chemical peptide synthesis method. Herein, we describe an efficient method for the Fmoc solid phase peptide synthesis of UBI29-41 and UBI30-41 (GRAKRRMQYNRR). We also demonstrate the complexing of the NOTA-conjugated peptides with 'cold' ^{nat}Ga, their preferred interaction with bacterial cells as well as an optimized radiolabeling procedure with generator based ^{68}Ga. The study validates an efficient radiolabeling of ^{68}Ga-NOTA-UBI29-41 and ^{68}Ga-NOTA-UBI30-41 in comparison to ^{68}Ga-DOTA-TATE (DOTA-D-Phe, Tyr^3-octrotate, acetate salt) to warrant further preclinical application and potential use as diagnostic infection imaging agents.

Material and methods

Peptide synthesis and NOTA conjugation

Rink amide resin and all 9-fluorenylmethoxycarbonyl (Fmoc) protected L-amino acids and coupling reagents were purchased from GLS Biochem Systems, Inc (Shanghai, China). All solvents for synthesis and purification of the peptides were of high performance liquid chromatography (HPLC) grade and purchased from Sigma-Aldrich. Peptides were synthesized on a 0.1 mmol scale using a CEM microwave peptide synthesizer by standard Fmoc/*tert*-butyl (tBu)-solid phase synthetic protocols. The peptides were synthesized on a Rink amide resin

with a loading value of 0.68 mmol/g. Approximately 0.167 g was required, in its dry state, for each peptide. Solutions of commercially available protected L-amino acids were prepared by dissolving the required mass of each amino acid in an appropriate volume of dimethylformamide (DMF) (Table 1). The peptide chain was elongated by sequential coupling and Fmoc deprotection of Fmoc-Arg(Pbf/Mis)-OH, Fmoc-Arg(Pbf/Mis)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Met-OH, Fmoc-Arg(Pbf/Mis)-OH, FmocArg(Pbf/Mis)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf/Mis)-OH, Fmoc-Gly-OH, Fmoc-Thr(OtBu/Bz)-OH. All couplings were performed once using the standard microwave conditions (Table 2), except for arginine which was double coupled and also coupled for an extended time. An excess of 4 equivalents (eq.) for each amino acid derivative was activated *in situ* by the standard HBTU/DIPEA procedure and twice deprotected with 20% piperidine in DMF. The NOTA conjugation was performed according to an adapted version of Guerin et al. [14]. The amino terminus of the peptide on resin was functionalized with bromoacetic acid and diisopropylcarbodiimide in dichloromethane. Excess 1,4,7-triazacyclononane (10 eq.) and triethylamine (50 eq.) in acetonitrile was used to displace the bromine and the reaction mixture was collected through filtration and reused several times. The remaining secondary amines on the NOTA ring were substituted with *tert*-butyl 2-bromoacetate (3 eq.) in the presence of triethylamine. Peptide cleavage from the solid support and the simultaneous removal of all protecting groups was carried out by treating the resin-bound peptide with TFA/thioanisole/H_2O/EDT/TIS (92.5:2.5:2.5:2.5) for a minimum of three hours followed by filtration.

Peptide purification and characterization

Analytical reverse phase HPLC was carried out on an Agilent Series 1100 apparatus using a Waters X-Bridge C18 column, 4.6 x 250 mm x 5 µm with a flow rate of 1.0 ml/min. Preparative reverse phase HPLC was performed on a Shimadzu 8A apparatus equipped with an

UV Shimadzu detector using an ACE C18 column, 22 x 250 mm x 5 µm with a flow rate of 20 ml/min. All HPLC procedures made use of H_2O containing 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), and a linear gradient from 0 to 25% of B in 30 min. Mass spectral analyses were carried out on a Bruker MicroTOF QII electrospray ionization mass spectrometer coupled with an Agilent 1100 HPLC using a Waters X-Bridge C18 column, 3.0 x 250 mm x 2.1 µm with a flow rate of 0.3 ml/min. UBI29-41 and UBI30-41 respectively were purified from access amino acids and solvents; NOTA-UBI30-41 was purified from access NOTA which came out much earlier in the HPLC trace. The retention of the desired products during semi-preparative HPLC occurred at the following times, 7.42-7.96 min for UBI29-41; 5.97-7.52 min for UBI30-41; and 6.53-7.62 min for the NOTA-UBI30-41. The desired products were collected in eluted volumes of 5, 10 and 12 ml respectively. The eluent obtained from all the preparative runs performed on each peptide was pooled and the sample lyophilized for storage and later use. NOTA-UBI 29-41 was sourced from GLS Biochem Systems, Inc (Shanghai, China) for further investigations.

^{nat}Gallium(III)chloride labeling of NOTA-peptide fragments

Gallium(III)chloride (^{nat}Ga) was purchased from Sigma-Aldrich. Upon arrival, it was immediately diluted to a 0.5 M stock solution with double-distilled water and stored in 200 µM aliquots at -80 °C until further use. To allow for complexation of the stable ^{nat}Ga, the NOTA-peptide-conjugates (20 nM) were incubated 10 min at room temperature challenged by two molar eq. of gallium(III)chloride (40 nM) in 100 µl double distilled water. Following this incubation period, the sample was directly injected into a q-TOF mass spectrometer. Quantification of labeling was determined by calculating the relative peak height of the molecular masses corresponding to the complexed and uncomplexed NOTA-peptide-conjugates.

Selective cell association of NOTA-UBI fragments

A fluorometric method was employed as this technology is sufficiently sensitive to allow for the detection of low concentrations of peptides in a laboratory that is not certified to work with radioactive materials. All the fluorescently labeled peptides used for this assays were purchased from GL Biochem Systems, Inc (Shanghai, China). The fluorophore, 2-aminobenzyl (Abz), was introduced to the NOTA-peptides by a lysine linker [Lys(Abz)]. Along with NOTA-UBI29-41[Lys(Abz)] and NOTA-30-41[Lys(Abz)], the following control peptide was used in this assays: NOTA-GDAKDDMQYNDD[Lys(Abz)], a peptide with an equal amino acid sequence to NOTA-UBI30-41, however, the 5 Arginine (letter -R -cationic residue) were replaced by aspartic acid (letter-D- anionic residue) to reverse its electrostatic binding potential. Verification of peptide mass of the purchased fluorescent peptides by LC-MS showed that the peptides corresponded to the calculated mass and analytical HPLC showed that they had purity greater than 98%. *Staphylococcus aureus* ATCC 25923 was cultured for 12 h in lysogeny broth and then washed three times in 0.1 M phosphate buffered saline (PBS) before being diluted into aliquots of 2 x 10^7 colony forming units (CFU)/ml immediately before the cell binding assay. The Mt4 cell line was cultured at low passage number in RPMI 1640 medium supplemented with 10% fetal calf serum. Immediately prior to testing, the cells were washed three times with 0.1 M PBS, pooled and then diluted to a concentration of approximately 2 x 10^7 CFU/ ml. The peptides were allowed to chelate gallium by incubating them with a two-fold excess of ^{nat}Ga as described earlier. Thereafter, peptide-gallium complexes were purified using SepPak RP-C18, 500 mg cartridges and assembled in 3 ml of acetonitrile/water solution (1:4, v/v). The fluorescent ^{nat}Ga-NOTA-peptide derivatives were then evaporated to a desired concentration in a vacuum concentrator before being lyophilized. The binding of peptide-gallium complexes to cells were assessed at 37 °C as published for ^{99m}Tc-radiolabeled UBI binding assays [15, 16]. Briefly, 0.1 ml of a 30 μM solution of the

fluorescent ^{nat}Ga-NOTA-peptide derivative was transferred to an amber micro-centrifuge tube. Thereafter, 0.8 ml of incubation buffer [50% (v/v) of 0.01 M acetic acid in PBS containing 0.01% (v/v) Tween-80, pH 5] and 0.1 ml of PBS containing approximately 2 x 10^6 viable bacteria or mammalian cells were added. The mixtures were incubated for 1 h at 37 °C. Thereafter the suspensions were centrifuged in a pre-cooled micro-centrifuge tube at 2000 *g* for 5 minutes at 4 °C. The supernatant was collected and 200 µl aliquots were dispensed into a 96 well plate. Fluorescence was determined using a Biotek Synergy Mx fluorescent plate reader ($\lambda_{excitation}$ 320 nm and $\lambda_{emission}$ 420 nm). Fluorescence from cells incubated without fluorescent peptide derivatives were determined and employed as a blank. Results were expressed as percentage reduction and corrected for unspecific peptide interaction with laboratory material.

Radiopharmaceuticals and material

All reagents were purchased from commercial sources and used as received. All chemicals for radiolabeling purposes were pharmaceutical grade. The SPE columns and cartridges are commercially available (Waters and Phenomenex). The anionic exchange column was assembled in-house and was kindly provided by University Hospital, Leuven, Belgium. Suprapure hydrochloric acid (HCl) was purchased from Merck Sharp & Dohme (Readington, New Jersey, USA). Instant thin-layer chromatography-silica gel paper (ITLC-SG) was purchased from PALL Life Science (New York, USA). Sterile filters MILLEX GV 0.22 µm were obtained from Millipore (Millipore, New York, USA). High-performance liquid chromatography (HPLC) grade water (resistivity = 18.2 MΩcm) was produced in-house by a Simplicity 185 Millipore system (Cambridge, Massachusetts, USA).

^{68}Ge/^{68}Ga-generator elution and post-processing

^{68}Gallium(III)chloride (^{68}Ga) was routinely eluted from a SnO_2-based ^{68}Ge/^{68}Ga generator (1.85 GBq , iThemba LABS, South Africa) using moderate acidic conditions (10 ml of 0.6 N HCl) by fractionated elution performed as following: First 1.0 ml was discarded into a waste

vial, subsequent 2.0-3.0 ml eluate contained the ^{68}Ga-activity for subsequent radiolabeling and the remaining 6.0-7.0 ml were discarded into the waste vial (further referred as Method 1). Occasionally, ^{68}Ga was eluted from a TiO_2-based ^{68}Ge/^{68}Ga generator (1.1 GBq, Eckert & Ziegler, ca 100 days in use) using low acidic conditions (fractionated elution with 10 ml 0.1 N HCl); harvested in 7.5 ml eluate. This was dispensed in equal amounts of 30% HCl supradure to yield a molarity >5.5 M and subsequently transferred over an anionic Dowex 100-200 mesh resin (Fluka Analytics, Germany) for preliminary purification from potentially co-eluted metals and ^{68}Ge. The purified ^{68}Ga was desorbed with ultrapure water (Fluka Analytics, Germany) and collected in 0.84 ml acidic solution (Method 2A). Alternatively (Method 2B), a fractionated elution was performed on the TiO_2-based ^{68}Ge/^{68}Ga generator as described in Method 1.

Radiolabeling procedure

The peptide radiolabeling was performed by a procedure which implemented previously published knowledge by Rossouw et al. [17]. NOTA-UBI30-41 and NOTA-UBI29-41 were diluted to a stock solution of 10 mg/ml and stored below -20 ˚C in 50 µl aliquots. DOTA-TATE was purchased from ABX (Heidelberg, Germany) in 100 µg aliquots. Due to the amended protocol in this study, DOTA-TATE was used occasionally as reference compound to compare the labeling of the NOTA-conjugated peptides. Small scaled labeling in volumes ≤ 1.2 ml was performed in 1.8 ml polypropylene cryogenic vials (Nunc®, Sigma Aldrich, Munich Germany). For preclinical use, labeling was performed in certified sterile pyrogen-free sealed borosilicate glass vials provided by NTP Radioisotopes (Pelindaba, South Africa). In order to allow non-metallic transfer of the ^{68}Ge/^{68}Ga generator eluate into the reaction vial, the top of its septum was punctured by a Jelco 22G x 1'' polymer catheter (Smiths Medical, Croydon, South Africa). 2.5 M sodium acetate buffer (or equivalent milligram of sodium acetate salt) was used to adjust the pH. The pH value was assessed using a narrow range pH

paper method. Therefore the pH strip (pH Fix 0-6, Macherey-Nagel, Düren, Germany) was spiked with 5-8 µl of the product solution, allowed to dry, compared with the color scale and read off the corresponding pH-value in 0.5 increments. Following the addition of NOTA-UBI29-41, NOTA-UBI30-41 or DOTA-TATE, vortex stirring action was performed for 20 seconds, the reaction vial was allowed to incubate in a temperature controlled water bath followed by at least 2 min cooling. An aliquot was retained for quality control purposes.

Evaluation of labeling parameter

In order to achieve the highest labeling efficiency in optimal time, the following labeling parameters were studied for ^{68}Ga labeling of NOTA-UBI29-41 and NOTA-UBI30-41: (A) sodium acetate/$^{68}GaCl_3$ ratio (i.e. pH correlation), (B) incubation temperature, (C) incubation duration and (D) compound molarity. The labeling efficiency for the crude samples was determined by ITLC as described and the percentage labeling fraction of free ^{68}Ga, ^{68}Ga-labeled peptide(s) and ^{68}Ga-peptide-colloids were calculated. Elution Method 1 was applied. Therefore, (A) 1 ml of the ^{68}Ga eluate was mixed with different amounts of sodium acetate salt (range 0-600 mg), and subsequently divided in two equal aliquots of 0.5 ml. A total of 20 µg of either NOTA-UBI29-41 or NOTA-UBI30-41 was added, vortexed and allowed to incubate at 80-85 ˚C for 20 min. The optimal pH/salt concentration was applied, subsequently to B-D. For further evaluation (B/C), samples (0.5 ml ^{68}Ga eluate, sodium acetate salt and 20 µg NOTA-peptide) were incubated at different temperatures (range 30-100 ˚C) for up to 45 min and the optimal conditions thereof were applied to D. Therefore, total peptide amounts of 0.0016-50 µg were created by stock dilution and were subsequently mixed with 0.5 ml of ^{68}Ga eluate/respective amount of sodium acetate buffer and incubated 15 min at 80 ˚C.

Determination of labeling efficiency and colloid forming by instant thin layer chromatography (ITLC)

Labeling efficiency was determined as described elsewhere [18]. Briefly, ITLC impregnated silica gel paper (ITLC-SG) paper strips were spiked at the bottom with 3-6 µL of the crude or pure ^{68}Ga-peptide solution, using an insulin needle (Hamilton, Milano, Italy) following 4-5 min exposure to the mobile phase (0.1 M citrate, pH 4.5-5). For testing colloid forming a mixture of 1 M ammonium acetate/methanol 1:1 (v/v) was used. Strips were immediately dried and analyzed on an ITLC scanner (VSC-201, Veenstra Ind., Oldenzaal, Netherlands) using a gamma radiation detector (Scionix 25B25/1.5-E2, Bunnik, Netherlands) by obtaining chromatograms. If peak identification was possible, an "area under the curve" analysis allowed subsequent calculation of percentage peak recovery (Genie2000 software, Veenstra Ind., Oldenzaal, Netherlands).

Purification (solid phase extraction)

A panel of 3 SPE columns and 1 online cartridge were used (I) Strata™-X polymeric, 100 mg, (II) C18-E reversed phase 100 mg (both Phenomenex), (III) Sep-Pak online, light C18 reversed phase 100 mg and (IV) Sep-Pak C18 reversed phase 500 mg 3cc (both Waters,). I-IV were preconditioned with absolute ethanol followed by Millipore deionized water. Except for III, the matrix was not run dry during all steps of the purification. I-III were evaluated for use of small sample scale purification, whereas IV was used as standard for full scale production of labeled peptide purification. All crude peptide samples were loaded with a flow rate of 2.5 ml/min followed by 0.5-1 ml saline solution (0.9%) which was used to rinse the reaction vial to harvest residual ^{68}Ga-labeled peptide. I-III were subsequently rinsed with 3 ml saline solution, IV was rinsed with 8 ml, accordingly. All units were measured for their loaded radioactivity and the sample/saline fraction was tested on ITLC to calculate $^{68}Ga^{3+}$ and ^{68}Ga-peptide percentage recovery. If not stated otherwise, the units of I-III were treated with 0.15–0.3 ml of a

saline/ethanol mixture (1:1, v/v). Depending on the onwards use of the sample, units of IV were treated with mixtures of acetonitrile/water (1:4, v/v for bacterial cell binding), water/ethanol (3:1, v/v) or saline/ethanol (2:1, v/v). All units were additionally rinsed with ≥1 ml saline solution, drained from residual liquid and re-measured for retained radioactivity. If required, the ^{68}Ga-peptide solutions were sterile-filtered through a 0.22 µm membrane using a Millex, low protein-binding filter (Millipore).

Determination of labeling efficiency and radiochemical purity (RCP) by HPLC

Agilent 1200 series HPLC instrument coupled to 6100 Quadruple MS detector, (Agilent Technologies Inc., Wilmington DE, USA), diode array detector (DAD) and radioactive detector (Gina Star, Raytest, Straubenhardt, Germany) was used for analyses. Two different columns, (I) an Agilent SB C18 column, 4.6 x 250 mm x 5 µm and (II) Xterra (Waters) C18 column 4.6 x 250 mm x 5 µm were used. The mobile phase in both HPLC procedures consists of 0.1% TFA (trifluoroacetic acid) in H_2O (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). Gradient elution was carried out using 0-2 min 5%B, 2-32 min 65%B and 32-35 min 5%B. Column temperature and flow rate were set on 40 ˚C and 1.0 ml/min respectively. Under both methods free ^{68}Ga was eluted at 2.3-4 min for all tracers, ^{68}Ga-DOTA-TATE was eluted (I) 17.4-17.5 min; ^{68}Ga-NOTA-UBI29-41 was eluted (I) 11.1-12.2 min, (II) 10.5-11.2 min; ^{68}Ga-NOTA-UBI30-41 was eluted (I) 10.5 -12.6 min, (II) 11.0-11.2 min, respectively (Fig. 1). The retention times of the crude and SPE-purified compounds were corresponding to each other.

Radioactive compound integrity

For determination of tracer integrity the radiolabeled ^{68}Ga-peptides were incubated at room temperature post tracer production and analyzed at 30, 60, 120, 180 and 240 min by ITLC. The percentage recurrence of ^{68}Ga was calculated as described.

Interaction of ^{68}Ga-labeled UBI fragments with Staphylococcus aureus

Staphylococcus aureus ATCC 25923 was cultured and prepared as mentioned earlier to yield a 1 x 10^8 CFU/ml stock solution. NOTA-UBI29-41 and NOTA-UBI30-41 were radiolabeled with ^{68}Ga and purified as explained earlier to yield a 370-450 MBq/ml stock concentration. The assay was carried out adapting published procedures [15, 16] and as elaborated earlier. 37-45 MBq (0.1ml) of the ^{68}Ga-NOTA-peptides were used in the mixtures and incubated with 2 x 10^7 CFU for 1 h at 37°C. For competition experiments, bacteria were pre-incubated for 30 min with 50-fold unlabeled NOTA-peptides. The radioactivity in the supernatant and the pellet containing bacteria was determined using a dose calibrator (Capintec, U.S.A). Mixtures containing radiolabeled peptides but lacking bacterial cells served as the control group to reveal unspecific peptide binding to surfaces. The bacteria-associated radioactivity was decay corrected and phrased as percentage ^{68}Ga-activity/2 x 10^7 CFU.

Statistical analysis

If systematic errors were non-existent, outliers were determined by the *Grubbs* Test. If not stated otherwise, analytical data were expressed as mean and standard deviation (±SD) as calculated using Microsoft Excel or Origin 8.1 software. In this study, one-way analysis of variance (ANOVA) was used to determine whether there were statistically significant differences in the levels of binding to bacterial cells and the mammalian cell line amongst the fluorescently-labeled NOTA-peptides. Significance of two mean values was calculated by *Student-t-test* (paired and unpaired comparison). The level of significance was set at $p < 0.05$.

Results

Peptide synthesis and NOTA conjugation

UBI29-41 and UBI30-41 were successfully synthesized using the Fmoc-polyamide synthetic strategy. The NOTA bi-functional chelator was successfully conjugated to the UBI30-41 peptide at the N-terminus (NOTA-UBI30-41). After purification, the molecular weights of the peptides were verified with q-TOF LC-MS which correlated to the expected molecular weight of the desired peptides or conjugate (Table 1).

^{nat}Ga labeling of the NOTA-UBI fragments

After incubating of the NOTA-conjugated peptides at room temperature in a two molar equivalent solution of ^{nat}Ga the solution was analyzed by LC-MS direct to determine the ratio of complexed and uncomplexed peptide by relative peak height of the peptides. It was found that greater than 99% of the peptides existed in the complexed state following the incubation. It should be noted that typical radiolabeling methods usually result in a nano-molar concentration of the expensive radionuclide being chelated with a large excess, often 100 fold, of the targeting vector-chelator conjugate to speed up complexation kinetics.

Selective cell association of fluorescent tagged NOTA-UBI fragments

The latter complexation method was used to evaluate the selectivity of the NOTA-conjugated peptides in a fluorometric *in vitro* interaction assay. A linear relationship existed between peptide concentration and fluorescence detected at concentrations ranging between 0.5 and 14 μM ($r^2 \geq 0.996$). This result meant that the Beer-Lambert law applied at these concentrations and that the concentration of peptides in the incubation buffer could be interpolated from detected fluorescence. Based on the results of this experiment it was decided that peptide binding could be assessed from an initial concentration of 3 μM, pH 5 was used to mimic

condition in the infection site. The average percentage association of the peptides to the Mt4 leukocyte cells was 9.4 ± 4.8%, 13.8 ± 7.4% and 9.3 ± 3.4% for the control peptide, NOTA-UBI29-41[Lys(Abz)] and NOTA-UBI30-41-[Lys(Abz)], respectively (Fig. 2A). The control peptide binding amounted to 29.1 ± 4.8%, NOTA-UBI29-41[Lys(Abz)] showed 45.4 ± 7.4% and NOTA-UBI30-41[Lys(Abz)] exhibited 97.9 ± 1.9% binding to the *Staphylococcus aureus*.

Specific interaction of ^{68}Ga-labeled UBI fragments with Staphylococcus aureus

To validate the targeted binding to *S. aureus*, competitive interaction studies (n=4) were performed using ^{68}Ga-NOTA-UBI29-41 and ^{68}Ga-NOTA-UBI30-41 amounting to 67.7 ± 4.3% and 81.2 ± 3.7% of the added radioactivity, respectively. Unspecific peptide interaction with material surfaces was 4.1± 1.8%. Significant decrease occurred for ^{68}Ga-NOTA-UBI29-41 ($P < 0.03$) and ^{68}Ga-NOTA-UBI30-41 ($P < 0.02$) in bacterial interaction following pre-incubation of *Staphylococcus aureus* with 50-fold unlabeled NOTA-peptides (target blockade) prior to the assay (Fig. 2B).

Generator elution and post processing

The fractionated elution of the SnO_2-based ^{68}Ge/^{68}Ga generator with method 1 assembled 95-98% of the ^{68}Ga-activity in 2-3 ml 0.6 N HCl with a new instrument and approximately 83-87% after 276 days (half-life span of ^{68}Ge), respectively. Elution method 2A using a TiO_2-based ^{68}Ge/^{68}Ga generator was able to yield highly consistent 98 ± 0.2% in 7.5 ml, which was predominately combined with a post-processing procedure in order to reduce the volume and enhance purity of the crude eluate, which can change in quality over time. Method 2B achieved 89 ± 1.3%; given the age of the TiO_2-based ^{68}Ge/^{68}Ga generator (100-130 days at the time of the study), it was similar to the performance of a SnO_2-based ^{68}Ge/^{68}Ga generator of the same age. The crude ^{68}Ga eluate was post-processed with an anionic exchange material yielded 81 ± 4.3% of purified $^{68}GaCl_3$ in a small volume aqueous solution (pH ≤ 1.5-2) within 5-8 min. The radioactivity collected in the waste was 9 ± 2.9%, the retained radioactivity on the Dowex

resin amounted to 9 ± 4.0%, which could be reconstituted with 3 ml 6 M HCl, subsequently or before use.

^{68}Ga radiolabeling procedure

The NOTA-conjugated peptides were evaluated towards various parameter' changes; the percentage of peptide-bound ^{68}Ga-activity can be strongly influenced by change of pH, hereby we used 2.5 M sodium acetate solution to adjust pH 2.5-3 (NOTA-UBI30-41) and 3.5 (NOTA-UBI29-41) for optimal labeling (Fig. 3A). Optimal labeling efficiencies were achieved at 80 ˚C (Fig. 3B). A minimum of 10-15 min incubation duration yielded a maximum percentage peptide labeling without being compromised by substantial ^{68}Ga-colloid forming (Fig. 3C). The labeling efficiency was strongly prone to changes in NOTA-peptide molarity given all other parameters were ideal. Noticeable ^{68}Ga-labeled NOTA-UBI29-41 was seen at 0.1-1 nM concentration and NOTA-UBI30-41 at 0.05-0.1 nM. Maximum percentage labeling occurs at a NOTA-peptide concentration of 5-10 nM (Fig. 3D). It should be noted that neutral pH, high temperatures, longer incubation durations and excess NOTA-peptides will lead to more than 15% ^{68}Ga-colloid forming. Based on the latter evaluation, the NOTA-conjugated peptides were routinely labeled in 20 independent radiosyntheses (Table 4) and compared to standard ^{68}Ga-DOTA-TATE labeling (n = 40). The complete ^{68}Ga-labeling including purification was performed in approximately 40 min, yielded in percentage labeling efficiency (%LE) of 62 and 67% for ^{68}Ga-NOTA-UBI29-41 and NOTA-UBI30-41, respectively, with no significant difference to the %LE of ^{68}Ga-DOTA-TATE (71%). It should be noted that more reproducible results were achieved with the NOTA-conjugated peptides compared to DOTA-TATE. No %LE ≤ 33 or ≥ 90% occurred for both NOTA-UBI29-41 and NOTA-UBI30-41, which is not apparent for DOTA-TATE. The procedure achieved a radiochemical purity ≥99% for all three tracers, detected by ITLC/HPLC analysis. The average end product activities for NOTA-UBI29-41 (279 ± 67 MBq) or NOTA-UBI30-41 (232 ± 80 MBq) yielded in similar amounts

compared to ^{68}Ga-DOTA-TATE (271 ± 47 MBq). The peptides were labeled at a specific activity of 9- 24 GBq/µmol. No unexpected losses to instruments, material or to the formation of ^{68}Ga-colloids were observed (9-16%).

Influence of post-processing and SPE purification

Two processes, such as the generator eluate post-processing and a potent SPE purification procedure have an influence on the success of the labeling protocol. Therefore ^{68}Ga radiolabeling of both, NOTA-UBI29-41 and NOTA-UBI30-41 was performed with (Method 2A) and without (Method 2B) using the post-processing method of the crude ^{68}Ga eluate obtained from the SnO_2-based generator (Table 5). The main results indicate that a higher % LE and better reproducibility was achieved using elution Method 2A than by Method 1 (using a TiO_2-based generator) and Method 2B for both NOTA-peptide conjugates. Less colloids formed with NOTA-UBI29-41 labeling compared with NOTA-UBI30-41 labeling and better reproducibility by means of lower variability between syntheses was noted for NOTA-UBI29-41. There were no particular differences observed between method 1 and 2B, except a slight increase in % LE when using method 2B.

For an optimal purification of the crude radiopharmaceuticals, different SPE cartridges were compared with regards to the ideal type for maximum product desorption (Table 6) and its respective reproducibility (Fig. 4). All cartridges exhibited a high overall percentage desorption (82.7-98.0%) with ^{68}Ga-DOTA-TATE < ^{68}Ga-NOTA-UBI29-41 < ^{68}Ga-NOTA-UBI30-41. There was minimum loss of ^{68}Ga-labeled peptides whilst loading the SPE cartridges (2.1-5.5%). Consistent performance was noted for both SepPak cartridges (variability 0.7-3.4%). The performance of Strata™X 100 mg and C18-E light 100 mg was only highly reproducible for NOTA-UBI30-41 (variability 1.3-1.8%). Otherwise the variability was ≥ 4.8%. Overall the best results were achieved with Strata™X 100 mg and SepPak C18 500 mg 3 cc for all three ^{68}Ga-peptide conjugates tested.

Determination of radioactive compound integrity

The purified ^{68}Ga-labeled peptides were further incubated at room temperature and demonstrated an overall integrity range of 95-97% after 240 min. The percentage of free ^{68}Ga recurrence was calculated at 30, 60, 120, 180 and 240 min for ^{68}Ga-NOTA-UBI30-41 (n = 3): 0.33 ± 0.05% , 0.60 ± 0.66%, 1.0 ± 0.78%, 1.8 ± 0.82% and 3.4 ± 0.75%; for ^{68}Ga-NOTA-UBI29-41 (n = 3): 0.33 ± 0.23% , 0.82 ± 0.28%, 0.90 ± 0.69%, 2.3 ± 0.57% and 4.2 ± 0.80% and for ^{68}Ga-DOTA-TATE (n = 5): 0.32 ± 0.33%, 1.2 ± 1.2% , 1.8 ± 1.1%, 3.7 ± 3.1% and 4.5 ± 2.3%; respectively.

Discussion

Antimicrobial peptide fragments of ubiquicidin has been evaluated as potential infection imaging agents and clinical studies were successfully performed using SPECT imaging with ^{99m}Tc-UBI29-41[7, 19]. To date, there is no reported evidence that shows successful radiolabeling of UBI fragments with ^{68}Ga, which would allow image acquisition with PET. Although numerous examples of peptide fragments conjugated to chelator molecules are evident in literature [13, 18, 20, 21], chemically it remains a challenge to couple them with quantitative yields. The UBI29-41 peptide fragment proved to be more difficult to synthesize than the UBI30-41 peptide, requiring multiple attempts to produce satisfactory yields of the desired peptide. This was unexpected as previously published reports do not mention difficulties in the synthesis of UBI29-41 [5, 6, 22]. Since the syntheses were initiated at the C-terminus, it could be suggested that the problem may have occurred on addition of the last amino acid, threonine. This is supported by the observation that no such difficulties were encountered in the synthesis of either UBI30-41 or its NOTA-conjugate, both lacking the threonine residue at the amino terminal. The most common reason for persistent difficulty in synthesizing a peptide using microwave-assisted solid phase synthesis are unfavorable

intramolecular and/or intermolecular interactions of the nascent peptides, which may result in secondary structure conformations which inhibit coupling of additional amino acids [23-25]. Steric hindrance due to the presence of bulky protecting groups on amino acids may also be a factor that inhibits extension of the peptide [26, 27]. Peptides with sequences that give rise to *beta* sheet secondary structures, and those with very hydrophobic regions are most prone to aggregation and are generally the most problematic to synthesize [23, 28-32]. Sequence analysis of UBI29-41, using the Garnier-Robson and Chou-Fasman algorithms, predicted an *alpha* helical secondary structure for this peptide rather than a *beta* sheet structure [6, 33, 34]. Amphipathic *alpha* helices, such as UBI29-41, are generally considered less challenging to chemically synthesize than *beta* sheet or disulfide rich peptides [35]. A thorough search of the literature did not yield any conclusive reasons as to why the N-terminal-threonine should be an especially problematic residue to couple under the conditions used in this study [6, 24, 28, 36, 37], although it is possible that steric hindrance effects, due to its bulky side chain, may have prevented its coupling onto the nascent peptide [38].

An important proof of principle within this study was the general ability of the NOTA-conjugated peptides to chelate excess Ga^{3+} ions in solution at nano-molar concentrations as determined by mass spectrometry. The findings can be used to determine how the labeling efficiency with radiogallium will correlate to the given parameters. A similar study [39] investigated ^{68}Ga complexation with peptides functionalized with DOTA or NOTA. At equimolar concentrations it was found that under ideal conditions, more than 90% of the ^{68}Ga was complexed by the peptide-conjugate. Another study found that the bi-functional chelator NOTA gave complexation yields of 99 ± 1% when chelated at room temperature with ^{68}Ga [40]. These two studies support the findings of the study presented here with regards to complexation of $^{nat}Ga^{3+}$ ions by NOTA chelators in which the complexation method with $^{nat}Ga^{3+}$ was used to evaluate the selectivity of the NOTA-conjugated peptides in a fluorometric

cell association assay. Under the present study conditions the levels of binding to mammalian cells (Mt4 leukocyte cells) were higher than the < 4% found in a study by Ferro-Flores and colleagues [22], however the herein applied method of determining the selective cellular interaction differs with regards to the detection method of the assay, the choice of cell lines and the modifications of the peptide fragment with a fluorescent marker. The fluorescent assay showed great potential I) to reveal the selective potential of the NOTA-conjugated UBI fragments towards bacterial cells and II) to compare the change of interaction when the electrostatic potential of the amino acid sequence is significantly.

Whilst no statistically significant differences in cellular association to the Mt4 cell line amongst the peptides were observed ($p > 0.05$), there were clear and significant differences in the peptides' abilities to interact with the bacterial cells ($p < 0.05$). The low cellular association of the control peptide to the bacterial cell line was not unexpected, since this peptide has an overall anionic charge, and it is believed that electrostatic attraction between the peptide and bacterial cells is the main cause of such cell binding in these peptides [41]. Welling *et al.* [15] studied a ^{99m}Tc-UBI29-41 fragment with a scrambled amino acid sequence and reported significantly decreased bacterial binding, thus supporting our *in vitro* findings with *in vivo* results. The > 45% binding of (^{nat}Ga)NOTA-UBI29-41[Lys(Abz)] to the *S. aureus* cells was similar to the 34.6 ± 3.0% [22] and 40 - 50% [42] binding found for ^{99m}Tc-UBI29-41. NOTA-UBI30-41[Lys(Abz)] showed much higher levels of binding (almost 98%) than was found for ^{99m}Tc-UBI29-41. The large differences in bacterial cell binding between NOTA-UBI29-41[Lys(Abz)] and NOTA-UBI30-41[Lys(Abz)] were unexpected since these peptides differ by only one threonine residue. The R-group of threonine should not be ionized at pH 5 and would therefore not contribute to the overall charge of the peptide. The pH conditions applied in this experiment should cause minimal differences in electrostatic potential between NOTA-UBI29-41[Lys(Abz)] and NOTA-UBI30-41[Lys(Abz)]. A remarkable finding is that these compounds

show selective association bacterial cells despite the conjugation with NOTA, and could potentially be used to discriminate between sterile inflammation and bacterial infection *in vivo* as we discovered by a recent proof of concept study in rabbits [43]. Furthermore, we have successfully proven specific tracer interaction with bacteria cells for both NOTA-UBI fragments that were radiolabeled with ^{68}Ga. The radioactive assay revealed high compound binding/interaction targeting Staphylococcus aureus. The compound binding reduction by competition/blockade was 51% and 55% for ^{68}Ga NOTA-UBI29-41 and ^{68}Ga NOTA-UBI30-41, respectively when S.aureus was prior incubated with 50-fold of the un-radiolabeled NOTA-UBI fragments. Welling et al. [15] and also Nibbering et al. [16] observed in a similar assay a significant decrease in ^{99m}Tc-UBI29-41 binding to bacteria cells whilst pre-incubated with excesses unlabeled UBI [15, 16]. Our result indicates that NOTA would not affect the ability of the UBI-peptide fragments to specifically and selectively interact with the bacteria cell wall. The latter findings are of very high clinical relevance because ^{68}Ga is a radioisotope that is conveniently available on a daily basis from ^{68}Ge/^{68}Ga generators as numerous studies have pointed out [9, 18, 40, 44]. The concentration effect (achieved by full-scale elution of $^{68}GaCl_3$ reduced from 0.84 ml instead of 7.5 ml) was comparable to similar procedures published by de Blois *et al.* [44]. During optimization of the ^{68}Ga labeling for NOTA-conjugated peptides, both NOTA-conjugated peptides showed similar labeling efficiencies using 2.5 M sodium acetate as buffer as has been previously reported for DOTA conjugated peptides [17, 18]. Using sodium acetate (2.5 M) as buffer, both NOTA-conjugated peptides were labeled at 50 ˚C to yield between 40 and 50% labeled product, however, optimal labeling efficiencies were achieved at 85 ˚C.

The use of the adequate purification method can be paramount for the overall yield, radiochemical purity, specific activity and reproducibility. Even though we did not find any correlation between the ^{68}Ga-NOTA-peptide labeling efficiency and the age of the ^{68}Ge/^{68}Ga

generator(s) (data not shown), we have implemented a SAX-based method to process the crude eluate. Blois *et al.* [44] recently compared different anionic exchange purification (SAX) methods with a maximum of 83% desorption of purified ^{68}Ga and similar amounts of retained ^{68}Ga-activity. Interestingly, the authors also tested cationic exchange purification (SCX) method, with no particular difference compared to the SAX based method. The fractionated elution method yielded a very high ^{68}Ga-activity, but it has been reported that SCX/SAX post-processing is more efficient than fractionated elution of the generator [44-48], mainly reducing co-eluted metal impurities and ^{68}Ge-breakthrough levels. We focused on the impact that a simple post-processing procedure has on the crude %LE of the NOTA-peptide conjugates. We could not find any significant differences in the %LE with or without the SAX-based method applied. The tendency observed was; a 10% higher efficiency in labeling and partial reduction in the percentage colloid forming was achieved using fractionated elution combined with the SAX-based method. This trend might become significant with prolonged use of a generator or with using older generators. The choice of an optimal SPE performance has been emphasized previously [44] .

The challenge within this study was to find a compromise of the SPE material towards a full scale production and an alternative production with smaller sample volumes or concentration. The SPE units tested were all suitable to purify the ^{68}Ga-NOTA-peptides and ^{68}Ga-DOTA-TATE (sample desorption $\geq$ 83%) from free ^{68}Ga and ^{68}Ga-colloids, but also from ^{68}Ge and other cationic metal impurities (data not shown). Radiochemical purities of >99% were achieved after SPE purification. SPE eluent solvents such as ethanol and acetonitrile could be minimized by standard procedures. The integrity of the purified labeled compounds was positively tested up to four hours to warrant the prospective use in preclinical studies. The obtained tracer activity was high enough to inject two larger animals from one production. The Preliminarily studies in healthy vervet monkeys and *Staphylococcus aureus* bearing rabbits

resulted in favorable *in vivo* biodistribution and stability [49] and specific imaging of infection [43].

Conclusion

We have synthesized and characterized two ubiquicidin fragments and reported successful NOTA-conjugation for UBI30-41. The successful NOTA-peptide complexation with "cold" gallium facilitated the subsequent testing in a fluorescent bacterial binding assay of both NOTA-UBI29-41 and NOTA-UBI30-41 *in vitro* where we could demonstrate that the NOTA-conjugation did not compromise the selective binding to bacterial cells. To our knowledge, we are reporting the first ^{68}Ga radiolabeling procedure for UBI peptide fragment derivatives with a similar performance compared to ^{68}Ga-DOTA-TATE labeling. This method can potentially be optimized using a (semi)automated procedure conforming to current good manufacturing practices making use of kits. Following preclinical evaluation a first in human trial for diagnosis of infection of unknown origin is envisaged.

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Captions to Figures

Fig. 1. Representative radio- HPLC chromatogram of **A**) ^{68}Ga-NOTA-UBI29-41 **B**) ^{68}Ga-NOTA-UBI30-41and **C**) ^{68}Ga-DOTA-TATE. Retention times are referred in material and methods.

Fig. 2. **A**) Percentage association of fluorescent-marked NOTA-UBI-fragment-gallium(III) complexes to *Staphylococcus aureus* 29523 and Mt4 leukocyte cell line. # = ^{nat}Ga-NOTA-peptide complexes were structurally N-terminal prolonged by a lysine linker with 2-aminobenzoic acid (fluorescent marker). Results are mean ± SEM of N =5 individual experiments, expressed as percentage association per 2 x 10^6 CFU. **B**) Selective interaction of ^{68}Ga-labeled NOTA-UBI fragments to *Staphylococcus aureus* 29523 with (open columns) and without (filled columns) competition. Blockade experiments used 50-fold of unlabeled NOTA-UBI fragments as competitor. Results are mean ± SEM of N=4 individual experiments, expressed as percentage of ^{68}Ga-activity added per 2 x 10^7 CFU. *****= statistically significant difference was achieved ($p < 0.05$) via Student t-test.

Fig. 3. Effect of various factors on ^{68}Ga-labeling for NOTA-conjugated peptides. Percentage ^{68}Ga-peptide labeling related to (**A**) sodium acetate buffer molarity, (**B**) incubation temperature, (**C**) incubation duration and (**D**) NOTA-peptide concentration ($N \geq 3$).

Fig. 4. Reproducibility of solid phase extraction performance by means of percentage ^{68}Ga-activity desorption of (**A**) ^{68}Ga DOTA-TATE, (**B**) ^{68}Ga-NOTA-UBI30-41 and (**C**) ^{68}Ga-NOTA-UBI29-41(N = 4-10). *****= statistically significant difference was achieved ($p < 0.05$) via Student t-test.

Tables

Table 1

Preparation of the protected L-amino acid solutions for peptide synthesis

Protected L-amino acid	Mass of protected L-amino acid (g)	
	UBI29-41	UBI30-41
Ala	0.31	0.31
Arg	3.50	3.50
Asn	0.60	0.60
Gln	0.61	0.61
Gly	0.30	0.30
Lys	0.47	0.47
Met	0.37	0.37
Thr	0.40	-
Tyr	0.46	0.46

Table 2

Microwave conditions for coupling and deprotection

	Microwave power (Watts)	Temperature (°C)	Time (sec)
Standard coupling (30 min)			
	0	25	900
	35	73	900
Arginine coupling (60 min)			
	0	25	2700
	35	73	900
Deprotection			
	40	73	180

Table 3

Yield and purity of peptides

Peptide	Yield (mg)	Purity (%)	calculated molecular mass (g/mol)	observed molecular mass (g/mol)
UBI29-41*	4.1	> 97	1693	1693
NOTA-UBI29-41	5.2	≥ 97	1978	1978
UBI30-41*	39.8	> 97	1592	1592
NOTA-UBI29-41*	35.3	≥ 96	1877	1877
DOTA-TATE	1	≥ 97	1434.6	1434.8

*= Peptides and NOTA-conjugated peptides were produced by the herein described method

Table 4

Summary of routine labeling after routine generator elution (Method 1)

^{68}Ga-peptide conjugate	**NOTA-UBI29-41**	**NOTA-UBI30-41**	**DOTA-TATE**
Number of syntheses (N)	20	20	40
Starting ^{68}Ga-activity			
Half scale (GBq)*	0.75 ± 0.15	0.52 ± 0.14	0.65 ± 0.16
Full scale (GBq)*	1.10 ± 0.11	-	1.35 ± 0.38
Ratio of 2.5 M Sodium acetate/ $^{68}GaCl_3$	12/88	16/84	18/82
Peptide concentration (nmol/ml)	25	26	17
Optimal pH value	3.2 ± 0.2	3.4 ± 0.4	3.7 ± 0.4
Temperature (˚C) / duration (min)	80 / 15	80 /15	90 /10
SPE C18-cartridge type			
Small sample volume <0.5 ml (A-D)	A,C	A,C	C
Large sample volume >0.5 ml (A-D)	D	D	D
SPE C18-cartridge elution mixture			
Default (v/v)	EtOH/Saline (1:3)	EtOH/Saline (1:3)	EtOH/Saline (1:1)
Alternative (v/v)	CH_3CN/H_2O (1:4)	CH_3CN/H_2O (1:4)	EtOH/Saline (1:4)
Specific activity (GBq/µmol)	13.0 ± 0.8	8.9 ± 0.7	24.3 ± 1.9
Time EOL to purified product (min)	39.9 ± 11.3	38.5 ± 6.6	40.1 ± 7.0
Recovery of radioactivity (%)	98.3 ± 2.1	98.4 ± 2.4	100.6 ± 5.1
Radiochemical purity			
Crude product ITLC / HPLC (%)	64.3 / 73.6	62.1 / 77.9	70.7 / 88.5
Final product ITLC / HPLC (%)	99.4 / 99.7	99.0 / 99.4	99.1 / 99.4
Loss to apparatus and colloids (%)	11.9 ± 6.2	8.8 ± 6.4	15.8 ± 12.1
Reproducibility			
Average %LE (Range min-max)	62.2 (51-85)	66.6 (46-78)	65.5 (15-99)
LE < 33%: N, (%)	-, (0)	-, (0)	3, (7.5)
LE > 66%: N, (%)	7, (35)	11,(55)	22, (55)
LE > 90%: N, (%)	-, (0)	-, (0)	5, (12.5)
Average end product activity			
half scale (MBq)*	279 ± 67	232 ± 80	271 ± 47
full scale (MBq)*	381 ± 87	-	545 ± 208

%LE = percentage labeling efficiency, A-D = names and specification of C18 SPE cartridges are listed in Material and methods, *= full scale -a full generator elution was used for radiolabeling whereas half scale- the activity was divided in two equal aliquots post pH adjustment.

Table 5

Influence of post-processing on the ^{68}Ga-NOTA-peptide labeling efficiency (N ≥ 3)

Peptide conjugate	Elution method*	Percentage labeling efficiency (%LE)		Colloids (%)
		ITLC	HPLC	ITLC
^{68}Ga-NOTA-UBI30-41	2A	91 ± 4	85 ± 5	8 ± 3
	2B	81 ± 9	76 ± 7	6 ± 3
^{68}Ga-NOTA-UBI29-41	2A	86 ± 11	82 ± 4	9 ± 3
	2B	76 ± 6	72 ± 8	16 ± 4

*= Elution method 2A (with post-processing) and 2B (without post-processing) combined with standard labeling procedure were applied (see material and methods).

Table 6

Comparison of solid phase extraction performance (N ≥ 4)

Cartridge type	Mean percentage (%) ^{68}Ga-desorption (range: max-min)		
	^{68}Ga-NOTA-UBI29-41	^{68}Ga-NOTA-UBI30-41	^{68}Ga-DOTA-TATE
Strata ^{TM}-X 100 mg polym.	93.4 (97.9-83.9)	96.7 (98.3-96.0)	94.8 (99.0-88.8)
C18-E light 100 mg	86.2 (88.8-83.0)	90.7 (92.7-88.9)	82.7 (90.2-78.4)
SepPak C18 online light	95.0 (98.5-89.8)	94.7 (96.0-93.9)	93.4 (96.0-88.9)
SepPak C18* 500 mg 3cc	96.4 (98.8-92.0)	98.0 (98.8-96.8)	96.4 (98.3-93.5)

*= Results from cartridge elution with ethanol/saline solution (1:1, v/v) are displayed. The cartridge desorption performance shows no significant difference when alternative elution mixtures (described in material and methods) were used.

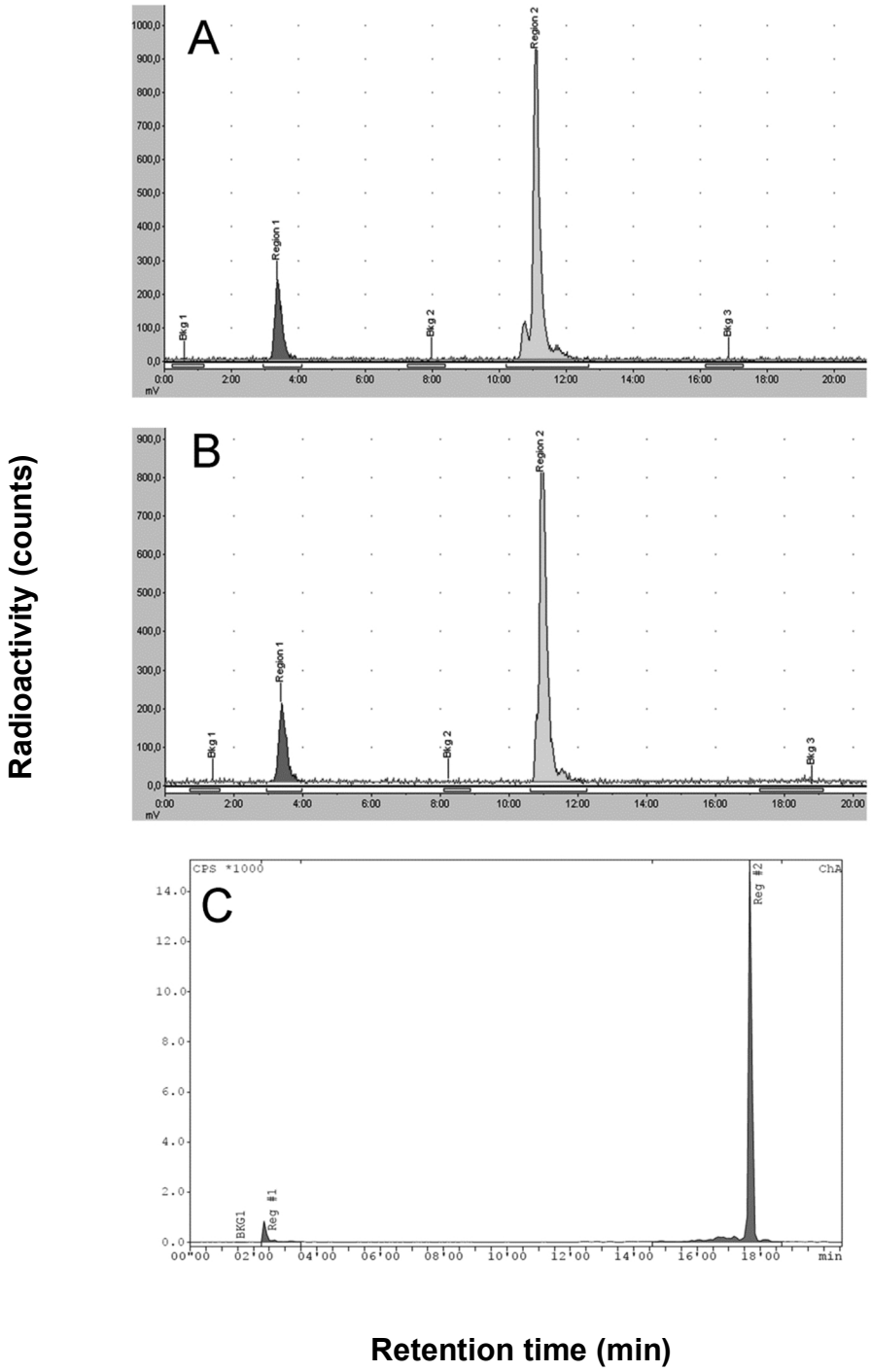
A
B
C
Region 1
Region 2
Bkg 1
Bkg 2
Bkg 3
mV
CPS *1000
ChA
Reg #1
Reg #2
BKG1
min
Radioactivity (counts)
Retention time (min)

Fig. 1

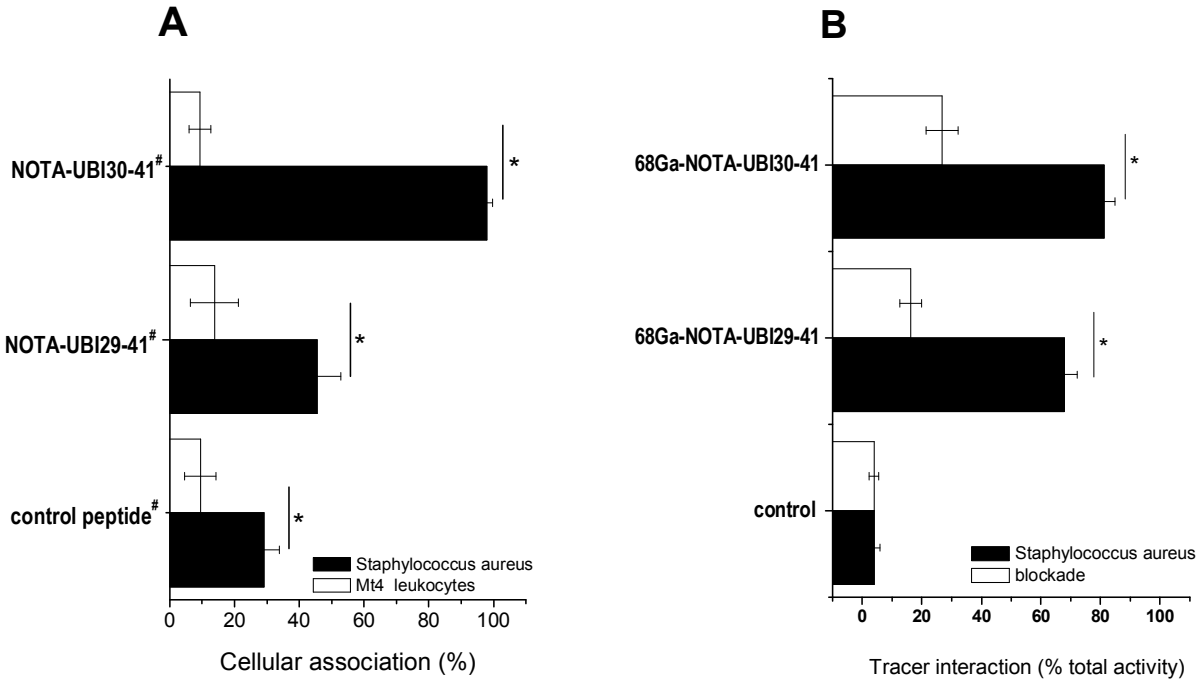
A
NOTA-UBI30-41#
NOTA-UBI29-41#
control peptide#
*
*
*
Staphylococcus aureus
Mt4 leukocytes
0
20
40
60
80
100
Cellular association (%)
B
68Ga-NOTA-UBI30-41
68Ga-NOTA-UBI29-41
control
*
*
Staphylococcus aureus
blockade
0
20
40
60
80
100
Tracer interaction (% total activity)

Fig. 2

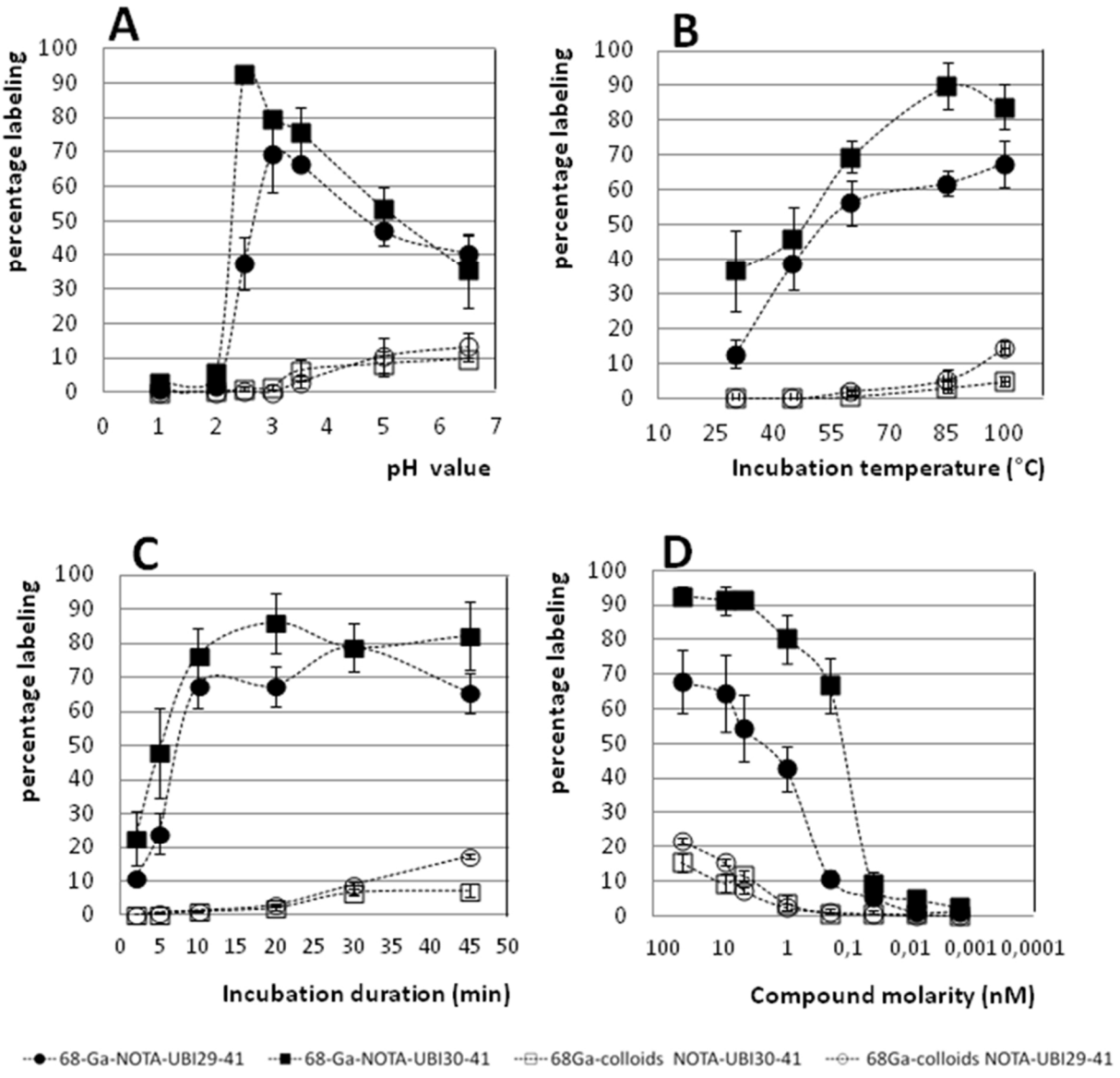
A
percentage labeling
pH value
B
percentage labeling
Incubation temperature (°C)
C
percentage labeling
Incubation duration (min)
D
percentage labeling
Compound molarity (nM)
68-Ga-NOTA-UBI29-41
68-Ga-NOTA-UBI30-41
68Ga-colloids NOTA-UBI30-41
68Ga-colloids NOTA-UBI29-41

Fig. 3

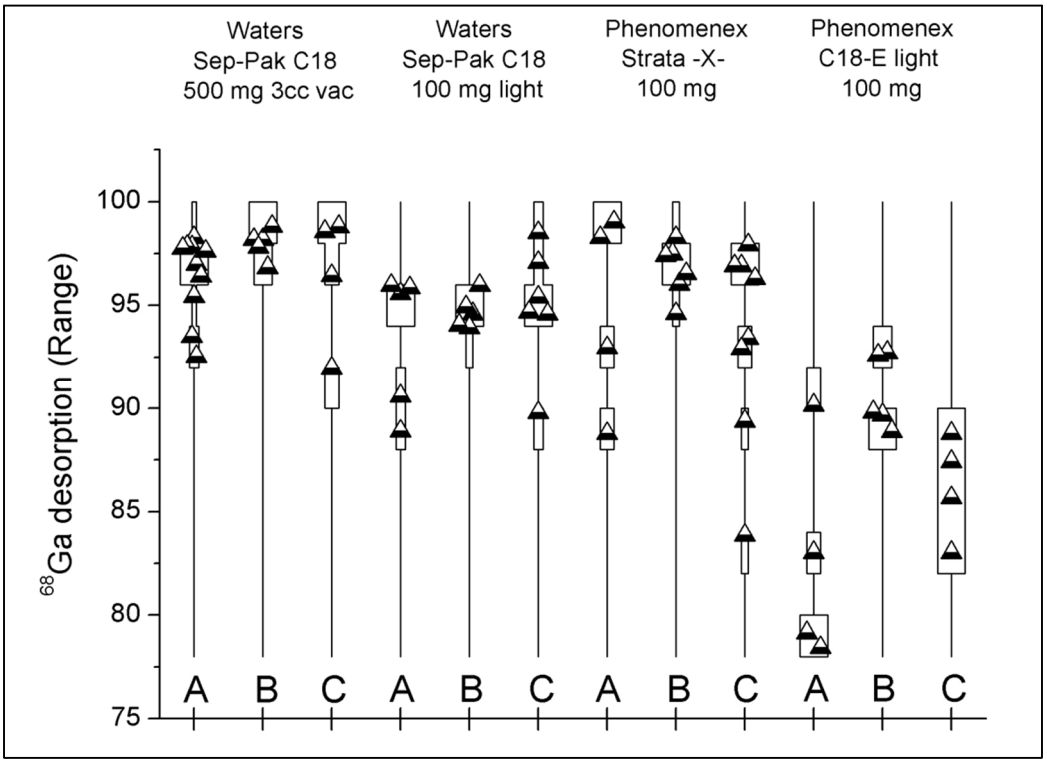
Waters
Sep-Pak C18
500 mg 3cc vac
Waters
Sep-Pak C18
100 mg light
Phenomenex
Strata -X-
100 mg
Phenomenex
C18-E light
100 mg
68Ga desorption (Range)
100
95
90
85
80
75
A
B
C
A
B
C
A
B
C
A
B
C

Fig. 4