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A methodical ^{68}Ga-labelling study of DO2A-(butyl-L-tyrosine)$_2$ with cation-exchanger post-processed ^{68}Ga: practical aspects of radiolabelling

Patrick J. Riss[a,b], Carsten Burchardt[a] and Frank Roesch[a]*

Positron emission tomography (PET) with ^{68}Ga is a fast-growing field in molecular imaging, both in research and in clinical routine. The availability of ^{68}Ga via the ^{68}Ge/^{68}Ga radionuclide generator facilitates the development and production of radiopharmaceuticals independent of a cyclotron. The presented work shows a complete ^{68}Ga labelling study exemplified on [^{68}Ga]DO2A-(butyl-L-tyrosine)$_2$, a potential tumour tracer for PET. A methodical sequence is followed to optimize the ^{68}Ga-labelling reaction. Practical aspects are described and the different parameters contributing to the labelling yield are demonstrated. The influence of temperature, time, amount of labelling precursor and pH value on the radiochemical yields is demonstrated. A conventional heating method is compared with microwave irradiation as an alternative labelling method. Finally, purification of ^{68}Ga-labelled compounds via solid-phase extraction and quality control is shown. The procedure described in this manuscript may serve as a guideline for optimizing ^{68}Ga labelling reactions. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: ^{68}Ge/^{68}Ga radionuclide generator; post-processing; radiolabelling; amino acids; DO2A

1. INTRODUCTION

Preclinical and clinical positron emission tomography (PET) with positron emitter ^{68}Ga-based imaging probes is a prospering field in molecular imaging (1,2). The radionuclide ^{68}Ga is instantaneously available via the ^{68}Ge/^{68}Ga radionuclide generator. The high cost of setting up and maintaining a cyclotron facility for cyclotron-produced positron emitters like ^{11}C or ^{18}F creates a significant financial burden for PET centres, while the use of generator-based ^{68}Ga appears to be more cost-effective.

With a half-life of 67.7 min and a positron abundance of 89% ^{68}Ga provides extraordinarily suitable characteristics for PET imaging. However, owing to the chemical characteristics of Ga^{3+}, it is hardly possible to attach gallium to bioactive molecules via covalent binding. In comparison to the cyclotron-produced 'organic' radionuclides ^{11}C and ^{18}F, entirely different labelling (co-ordination) chemistry has to be employed. To label a biomolecule with ^{68}Ga, the radiometal most often has to be complexed in multidentate, bifunctional chelators (1,2). In some special cases, for example ^{68}Ga-labelled transferrin or porphyrins, the biomolecule itself is capable of forming stable complexes with ^{68}Ga (3,4). In other cases, some simple chelators like citrate or diethylenetriaminepentaacetic acid (DTPA) labelled with ^{68}Ga were used as radiopharmaceuticals (5–8). However, in most cases a bifunctional chelator (BFC) has to be attached to a biomolecule to label it with ^{68}Ga. On the one hand the BFC has to form ^{68}Ga complexes with sufficient thermodynamic and kinetic stability *in vivo*. On the other hand it has to offer a functionality to attach a biomolecule finally functioning as physiological or molecular targeting vector.

Most of the suitable BFCs for ^{68}Ga today are based on macrocyclic ligands like 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (9) or 1,4,7,-triazacyclononane-1,4,7-triacetic acid (NOTA) (10,11) combining high thermodynamic stability with kinetic inertness. The potential of ^{68}Ga-labelled radiopharmaceuticals was demonstrated on the example of ^{68}Ga-labelled DOTA-conjugated somatostatin agonists (12–14). These prototypes are frequently used in clinical routine to image over-expression of G-protein coupled receptors in neuroendocrine tumours by means of PET/CT. Other peptides, like RGD or bombesin (15–17), but also small molecules like amino acids (18), are currently under investigation for their applicability as PET radiopharmaceuticals. Increasing need and prevalent clinical application of ^{68}Ga-labelled tracers in turn raise the demand for simple but efficient radiochemical labelling pathways.

The radiochemical yields of a ^{68}Ga-labelling process are influenced by various different parameters, like temperature, reaction time, stoichiometry and pH value. Hence the labelling procedure has to be optimized for each tracer. In this context, the rather 'classical' approaches towards synthesizing Me(III) imaging agents as used for example with Gd(III) for contrast imaging agents (MRI) definitely fail if adopted directly to the 'no-carrier-added' (n.c.a.) radionuclide ^{68}Ga(III).

* *Correspondence to: F. Roesch, Institut fuer Kernchemie, Johannes Gutenberg-Universität Mainz, Fritz-Strassmann-Weg 2, D-55128 Mainz, Germany. E-mail: Frank.Roesch@uni-mainz.de*

a *P. J. Riss, C. Burchardt, F. Roesch*
Institut fuer Kernchemie, Johannes Gutenberg-Universität Mainz, Fritz-Strassmann-Weg 2, D-55128 Mainz, Germany

b *P. J. Riss*
The Wolfson Brain Imaging Centre, University of Cambridge, Robinson Way, Cambridge CB2 0QQ, UK

Herein, the contribution of different parameters onto labelling outcome is investigated. Particularly relevant aspects to address are: (a) The factors influencing labelling yields; (b) the effective combination of labelling chemistry and subsequent rapid purification using cGMP compliant materials and equipment; and (c) a reliable quality control methodology. Finally, (d) for new compounds, the stability of the formed ^{68}Ga-complexes has to be confirmed.

All procedures concerning these criteria are presented exemplified on [^{68}Ga]DO2A-(butyl-L-tyrosine)$_2$ (**1**) (Fig. 1) a promising new tracer for amino acid transporter over-expression (18). Practical aspects and pitfalls of ^{68}Ga-labelling are discussed and the relevant techniques and methods are displayed. Compound **1** further exemplifies a novel, bivalent combination of a radiometal complex and a targeting vector. Multivalent radiotracers combining a number of targeting moieties and a single metal complex have been shown to improve radiotracer–receptor binding interactions as elaborated in detail elsewhere (19–22). In the case of compound **1**, bivalency is readily achieved using two identic L-tyrosine moieties on the basis of a DO2A chelating moiety.

2. RESULTS AND DISCUSSION

2.1. No-carrier-added ^{68}Ga radiochemistry vs synthesis of metal containing based contrast agents

On the first view, ^{68}Ga labelled PET tracers and metal based MRI contrast agents appear to be quite similar. Both are based on metals with the oxidation state +III and both need a chelator to form a stable complex. To some extent similar chelators are suitable and, with DOTA as the best known example, are currently used. On closer inspection, however, significant differences in between both metal complexes can be identified.

A Gd-based contrast agent can be synthesized without time pressure. Reaction times of up to 24 h can be found in literature. In most cases, defined equimolar amounts of Gd(III) and multidentate chelators are reacted on a gram-scale at elevated pH values (23). Using starting materials and reagents of commercial quality (99.8% purity) and common purification methods (24–27), a high purity can be expected for the product as well. Apart from their contrast-enhancing, physical properties, high thermodynamic stability and sufficient kinetic inertness, the relatively high concentrations of the Gd complexes needed for NMR contrast are crucial, for example, to avoid nephrotoxic effects in living subjects.

In contrast, reaction times have to be very short in consequence of the half-life of ^{68}Ga (67.7 min). The chemical amount of Ga(III) involved under n.c.a. conditions is very low, let alone the amount of ^{68}Ga. For example 100 MBq of ^{68}Ga are equivalent to 0.68 ng (10 pgat). Reactions under stoichiometric conditions will hardly be possible and trace impurities in commercial reagents cannot be omitted. The concentration of common metallic impurities such as Fe(III) in laboratory reagents or ^{68}Zn(II) (as co-generated within the ^{68}Ge-^{68}Ga-^{68}Zn decay chain) has to be taken into account (26) to avoid confounding stoichiometric effects in reactions involving n.c.a. ^{68}Ga(III).

A sophisticated purification of ^{68}Ga(III) solutions is thus mandatory in order to maintain the typical amounts of labelling precursor per batch synthesis in the nanomolar range (1,2,26). Ga(III) is already hydrolysed at pH ~4 and even under such mildly acidic conditions, oxygenated species are formed. These are less suitable for the labelling process owing to the kinetically disfavoured exchange of hydroxyl ligands (1,2). As opposed to Gd(III) handling, the pH value has to be kept low in Ga(III) chemistry. Consequently, protonation of the donor functions in *α*-aminocarboxylate chelators, which increasingly impedes the complex formation, has to be considered at decreasing pH.

Furthermore, owing to the short physical half-life of ^{68}Ga, instant purification and quality control procedures for ^{68}Ga-pharmaceuticals are mandatory. When saturable biological systems such as ligand receptor binding or enzyme inhibition are imaged *in vivo* and ultra-low concentrations of the ^{68}Ga compounds are desired, the specific activity of a radiotracer formulation might become relevant. The specific activity (A_s) is calculated as the ratio of the radioactivity of a radiotracer formulation and the molar amount of the sum of all isotopic forms of the compound in this formulation ($[A_s] = \text{Bq}^*\ \text{mol}^{-1}$). Since the amount of substance for the sample includes radioactive, as well as nonradioactive nuclides or molecules of the same compound, the specific activity will decrease with radioactive decay over time. In the aforementioned cases, the specific activity might become a critical release criterion after production or define the expiration time and date of a radiotracer formulation. Table 1 summarizes some aspects of Gd(III) and Ga(III) coordination chemistry.

The different toxicities of the metal ions require purification of the final pharmaceutical product from free metal ions. While Gd^{3+} is known to be highly toxic for the living organism ($LD_{50} = 0.0004$ gat/kg), generator-derived ^{68}Ga is considered to be nontoxic. Furthermore, the injected amounts of ^{68}Ga-labelled radiotracer are so small (low picomolar range) that no pharmacological

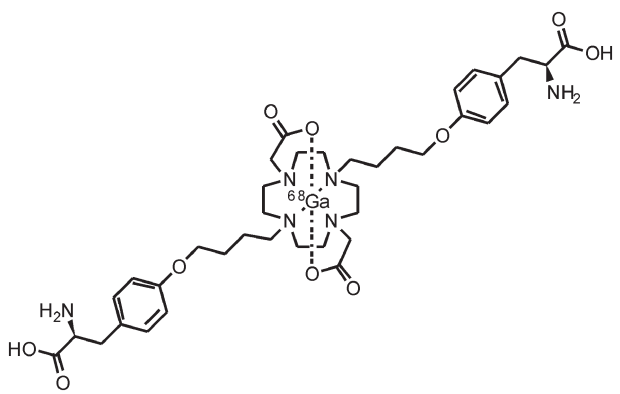

Figure 1. Structural formula of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ (**1**).

Table 1. Selected specifications of Gd(III) and ^{68}Ga(III) coordination chemistry

Parameters	Gd(III)	^{68}Ga(III)
Co-ordination number	8	6
Half-life	Stable	67.7 min
Synthesis period	~1 day	~10 min
Ligand concentration	Millimolar	Nanomolar
Typical stoichiometry Me(III):ligand	1:1	1:1000–10 000
pH limitations owing to Me(III) hydrolysis	pH < 7	pH < 4.5

effects are imminent. The reason for purifying the ^{68}Ga-radiopharmaceutical from free ^{68}Ga is thus to address a chemical and pharmacological problem: noncomplexed Ga^{3+} preferentially binds to transferrin and leads to a higher background. Further issues of ^{68}Ga release include undesired distribution patterns in the subject under investigation that do not mirror the distribution of the injected tracer but that of ^{68}Ga-transferrin, thereby disturbing the image acquisition.

2.2. Post-processing of ^{68}Ge/^{68}Ga radionuclide generators

In this study a 350 MBq and a 180 MBq ^{68}Ga/^{68}Ge-generator, based on titanium dioxide and produced by Cyclotron Co., Obninsk, Russian Federation, were used. This type of generator is eluted with 8 ml of 0.1 M hydrochloric acid. The volume of this generator eluate is too large and it is too acidic to perform a direct labelling. It also contains a significant amount of metal impurities, like ^{68}Ge(IV) breakthrough, Fe(III) and Ti(IV) as released from the inorganic generator matrix. An increasing amount of ^{68}Zn(II) will build up in between the elution cycles via the decay of ^{68}Ga. Furthermore, even hydrochloric acid of high purity is contaminated with considerable amounts of metal ions dramatically exceeding the number of ^{68}Ga atoms (1,26). The presence of long-lived ^{68}Ge activity in the final product, as well as in any waste streams during tracer production, is highly undesirable.

Complexes formed from Fe(III) and Zn(II) and the labelling precursor will indeed form additional chemical entities of unknown effect to the binding of the tracer. From a kinetic modelling or quantitative imaging point of view, the effect of non-^{68}Ga species in the final tracer formulation should be minimized in order to maintain a reasonably specificity to the biological target, in particular when studying ligand–receptor interactions where binding is saturable and binding affinity of molecular ligands is sensitive to structural modification (19). Notwithstanding that this effect might be of negligible influence in nonsaturable processes, such as uptake and conversion of 'metabolic' tracers, a post-processing of the generator eluate has to be performed to concentrate and purify the eluate.

There are different post-processing methods described in the literature, the fractionated elution method, a method using an anion-exchange resin and a method using a cation-exchange resin (24–27). All these procedures lead to volume reduction and lowering the content of ^{68}Ge, applicable for the labelling process in this form. However, we preferred to use a cation-exchange method (26). With this method the volume is readily reduced from any volume of 0.1 M HCl eluate to 400 µl. Nonradioactive metal impurities as well as ^{68}Ge are removed too. This post-processing was carried out prior to every labelling process and took less than 2 min.

2.3. pH value

The pH value plays a key role in ^{68}Ga-labelling, since changes in the pH value influence the labelling behaviour dramatically (1,2). Owing to the aqueous chemistry of gallium, the pH value has to be kept low to avoid the formation of Ga oxide and hydroxide species (28). On the other hand the pH value has to be high enough to deprotonate a sufficient number of donor functions of the BFC. In order to optimize the pH value for labelling, the dependency of the radiochemical yield (RCY) from the pH value of the labelling mixture was investigated thoroughly. A suitable range for ^{68}Ga-labelling of DO2A-(butyl-L-tyrosine)$_2$ was found being in between pH = 2 and pH = 4, although optimal results are achieved in a much more narrow range in between pH 3.2 and pH 3.8 (Fig. 2).

Based on these findings, a setup using 5 ml of Millipore water containing 2.6 nmol precursor per ml and 50 µl of the post-processed generator eluate was introduced (26). Besides the pH value, this setup has the advantage that eight labelling experiments can be performed under identical conditions with one elution of the generator. This allows a fast screening of different labelling parameters.

2.4. TFA salt vs desalted precursor

Initial labelling experiments were performed using the (TFA) trifluoro acetic acid salt of the precursor DO2A-(butyl-L-tyrosine)$_2$ (**2a**) as obtained from deprotection and subsequent HPLC purification. As described above, the labelling precursor (13 nmol) was dissolved in Millipore water (5 ml) and heated to the desired temperature. An aliquot of 50 µl of the post-processed generator eluate was added and samples were withdrawn from the resultant mixture after 1, 3, 5, 10 and 15 min. Monitoring of the reaction outcome was terminated after 15 min at 90 °C. Following this procedure, the resulting pH value of the labelling mixture was 3.2.

In order to compare the labelling yields with those obtained with a desalted labelling precursor **2a**, the TFA salts were removed using an anion-exchange resin prior to labelling. The labelling yields of the TFA salt **2a** and an equimolar amount of desalted precursor (**2b**) are shown in Fig. 3. It is clearly demonstrated that the desalted labelling precursor leads to higher radiochemical labelling yields and faster labelling. Consequently, the desalted precursor **2b** was used in all following labelling processes.

2.5. Amounts of labelling precursor

In the next step the dependency of the (RCY) radiochemical yield of the amount of precursor was investigated. Under the conditions mentioned above, the mass of labelling precursor was varied from 1 µg (1.3 nmol, 0.26 nmol/ml) to 25 µg (32.5 nmol, 6.5 nmol/ml). The RCY remained almost constant in the range of 10–25 µg (6.5–32.5 nmol). Conversely, the use of less than 5 µg precursor resulted in very inconsistent yields (Fig. 4). This might indicate a competing effect of metallic impurities present in the solutions. For further studies 10 µg labelling precursor was used.

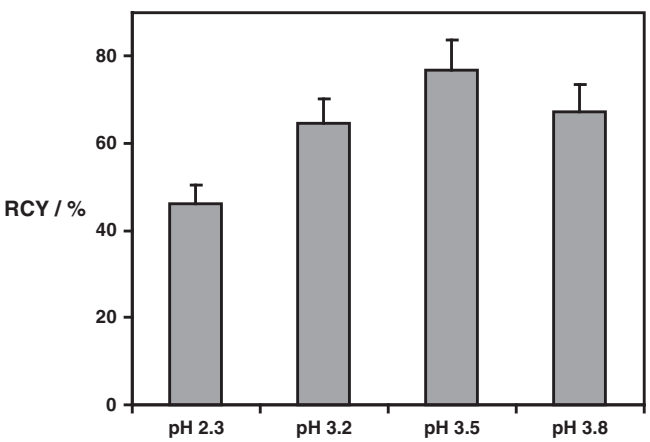

Figure 2. Comparison of the radiochemical yield of **1** using 10 µg (13 nmol) of labelling precursor and 50 µl processed ^{68}Ga generator eluate at 90 °C in 5 ml aqueous media at various pH values after 10 min.

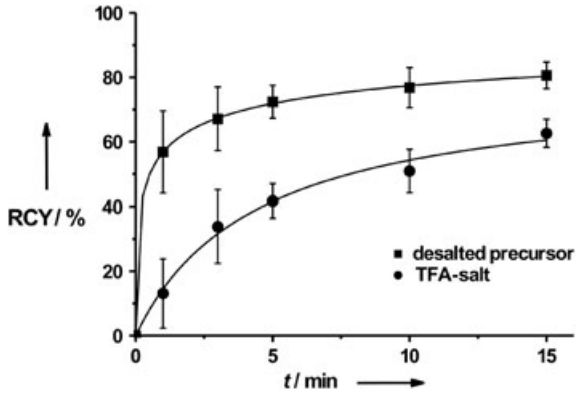

Figure 3. Comparison of the radiochemical yield of **1** obtained from desalted precursor **2b** and its TFA salt **2a**. Labeling conditions: 5 ml Millipore water, 90 °C, 13 nmol precursor, 50 µl processed ^{68}Ga generator eluate.

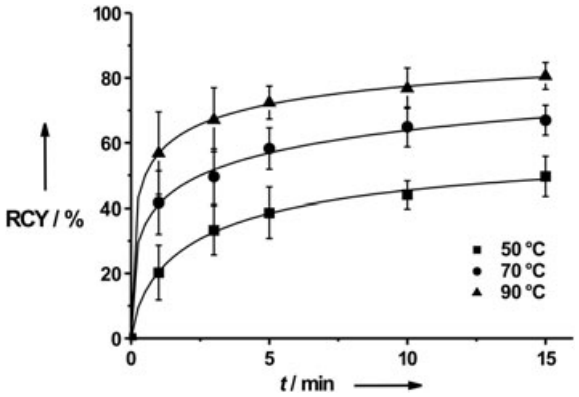

Figure 5. Radiochemical yield of **1** using 13 nmol desalted labelling precursor in 5 ml Millipore water and 50 µl processed ^{68}Ga generator eluate at 50, 70 and 90 °C.

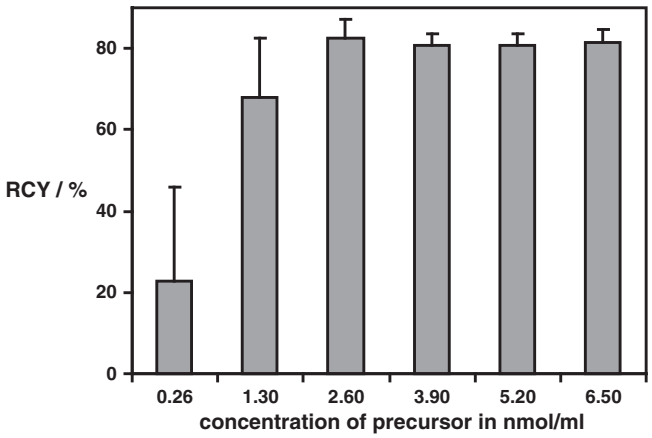

Figure 4. Comparison of the radiochemical yield **1** of using 1–25 µg (1.3–32.5 nmol) desalted labelling precursor at 90 °C in 5 ml Millipore water and 50 µl processed ^{68}Ga generator eluate after 15 min.

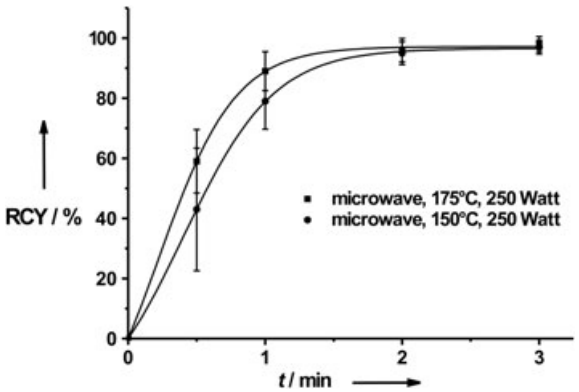

Figure 6. Microwave-supported synthesis of **1** of 13 nmol desalted labelling precursor in 5 ml Millipore water with the total volume of 400 µl processed ^{68}Ga generator eluate at 175 °C (16.5 bar) and 150 °C (12.2 bar).

2.6. Effects of reaction temperature

Radiochemical yields were determined for three different temperatures (50, 70 and 90 °C) over 15 min. The results are shown in Fig. 5. As expected, the highest yields and the fastest labelling kinetics are reached at 90 °C.

2.7. Microwave-supported method vs conventional heating method

The use of the entire volume of purified generator eluate (400 µl) leads to a lower pH in the reaction mixture, down to a value of pH 2.3, with RCY reduced to about 40%. To keep the pH value in an acceptable range, base solution (i.e. NaOH) or buffer (i.e. HEPES) can be used.

However, an alternative way to increase the labelling yields at more acidic pH, is a higher reaction temperature to overcome the impeding energetic barrier. Increased reaction temperatures are furthermore known to increase the reaction rate. Thus, in parallel, a microwave-supported labelling reaction was carried out (29). The time dependency of the labelling yield is exemplified at 150 and 175 °C and a pressure of 12.2 and 16.5 bar, respectively, using a focussed microwave reactor. An almost quantitative RCY of more than 98% was achieved within 3 min (Fig. 6). Further heating resulted in the thermal decomposition of the compound. Using microwave irradiation, the labelling reaction can be conducted in sterile-filtered water with 400 µl purified generator eluate. Interestingly, the absolute RCY increased significantly. This could be due to the higher reaction temperatures, which would certainly speed up complexation of less reactive Ga(III) species, i.e. partly oxygenated species. The formation of Ga(III) oxide/hydroxide species might impede higher labelling yields using the conventional method. Alternatively, microwave dipole interactions might affect direct transfer of activation energy to the components bearing the highest dipole moment, i.e. the Ga^{3+} or the chelator in the reaction mixture, thereby improving the reaction outcome.

Although a higher RCY was achieved compared with the conventional heating method, the amount of labelling precursor could not be reduced for microwave labelling. Different amounts of labelling precursor from 1 to 20 µg (1.3–26 nmol) were tested at 175 °C and 3 min. The results are shown in Fig. 7.

2.8. Product purification

Two alternative purification methods were used for the isolation and purification of n.c.a. radiotracers. Semi-preparative HPLC

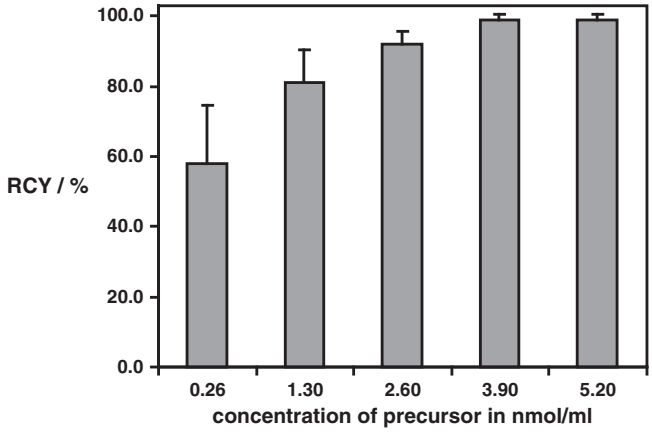

Figure 7. Microwave-supported synthesis of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ using 1–20 µg (1.3–26 nmol) desalted labelling precursor in 5 ml Millipore water and 400 µl of processed ^{68}Ga generator eluate at 175 °C after 3 min of reaction time.

purification was compared with solid-phase extraction (SPE). HPLC provides a much higher resolution, capable of separating even complex reaction mixtures. Compared with the HPLC-purification, SPE is less time-consuming, which is beneficial for handling short-lived positron emitters. SPE cartridges are single-use devices that can be produced and maintained under (cGMP) current Good Manufacturing Practice conditions. cGMP-compliant equipment, procedures and documentation are a prerequisite for the production of radiotracers for human application.

Fortunately, the separation of the product from multiple by-products is not a common case in n.c.a. complex chemistry. Therefore, SPE cartridges are sufficient for isolation and purification. Waters C_{18} light, Waters Silica light and Merck Lichrolute SCX cartridges were investigated. The amount of radioactivity extracted from the reaction mixture, the percentage of activity remaining on the cartridge and the purity of the labelled compound after the SPE-purification are shown in Table 2. For these experiments batches with labelling yields of about 70%, containing up to 30% nonreacted $^{68}Ga^{3+}$, were used. In this case, the SCX cartridge was very suitable for the purpose of purification. About 99% of the overall ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was adsorbed on the cartridge and the radiopharmaceutical could readily be eluted with (PBS) Phosphate Buffered Saline buffer pH 7.4, with the nonreacted $^{68}Ga^{3+}$ being retained on the cartridge.

2.9. *In vitro* stability of the labelled product

For an application in PET imaging, it is crucial for the ^{68}Ga radiotracer to remain intact over the period required for specific biodistribution *in vivo*. Different from therapeutic doses of pharmaceuticals or contrast agents, PET examinations are ideally conducted with nanomolar radiotracer quantities. With progressing distribution throughout a living system, a ^{68}Ga radiotracer will be challenged with a multifacetted variety of chelating biomolecules and metal ions at significantly higher concentrations. It is therefore recommended to investigate the release and transchelation of the radiometal under physiological conditions (1). *In vitro* competition experiments against physiologically relevant species can be used as models with satisfying validity (1). Release and transchelation of $^{68}Ga^{3+}$ can be assessed by adding, for example, a chelator to the formulated radiotracer and incubating the obtained mixture at 37 °C for a few hours. DTPA was used since this chelator readily forms a complex with Ga(III) at moderate temperature. Formation of a new ^{68}Ga complex will then indicate dissociation of the parent complex. More elegantly, the iron carrier *apo*-transferrin can be used as a species to simulate chelating ^{68}Ga to plasma proteins (1). Aliquots of the labelled product were incubated with a 750-fold excess of DTPA or an about 750-fold excess of *apo*-transferrin at 37 °C for 2 h, revealing that this ^{68}Ga complex was completely stable over this period of time (Fig. 8).

2.10. Quality control

The purified compound ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was analysed with radioTLC and radioHPLC. For radioTLC, silica-gel plates were used. It is recommended to use two different radioTLC-methods to avoid misinterpretation of two or more overlapping spots. When citrate buffer pH 4 is used as a mobile phase, a ^{68}Ga–citrate complex is formed *in situ*, which progresses with an R_f of 0.9, reflecting the percentages of unlabelled ^{68}Ga, while ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ remains on the starting line. In parallel, a mixture of 25% ethanol in 5% NaCl solution was used. Under these conditions, residual noncomplexed ^{68}Ga remains at the starting line and ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ is identified at an R_f of 0.4. A Phenomenex Synergy Max RP 5 × 250 mm column was used as stationary phase for quality control by radioHPLC. The mobile phase was a mixture of Millipore water–methanol (90:10, 0.1% TFA). The labelling yields of all reactions were cross-checked under these two TLC conditions and compared with the results of the analytical HPLC. All quality control methods led to equivalent results.

Table 2. Comparison of three SPE-methods for purification of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$

SPE cartridge	Adsorption[a] (%)	Eluent[b]	Purity[c]
Waters SepPak® light silica	82 ± 5% (at 4 °C)	0.9% NaCl solution	90 ± 3%
Waters SepPak® light C-18	63 ± 7%	Ethanol	84 ± 5%
Merck Lichrolut® SCX	97 ± 2%	PBS buffer pH 7.4	99 ± 1%

[a]Adsorption of activity from the reaction mixture.
[b]Eluent used for recovery of the trapped product.
[c]Purity of the formulated product after SPE.

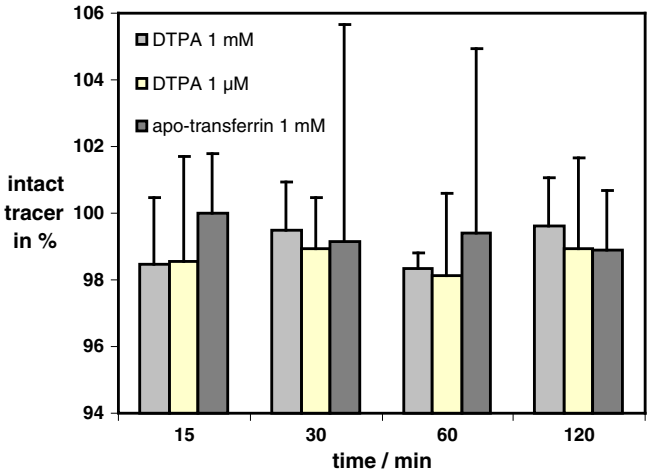

Figure 8. Challenge experiment of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ (2.6 nmol/ml) with 10 ml of DTPA solution in two concentrations (1 mM, 750-fold excess; 1 µM, 0.75 equiv.) and 5 ml *apo*-transferrin solution (1 mM, 750-fold excess) at 37 °C.

3. CONCLUSIONS

In this study the RCY of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was optimized for different volumes of the processed ^{68}Ga generator eluate. Cation-exchange post-processed ^{68}Ga allows all procedures to be reliably performed in pure water; no buffer is needed. Under these conditions, desalted labelling precursors were found to be superior to TFA salts. Microwave-supported radiosynthesis results in remarkably high, almost quantitative, yields and is very time-effective. SPE purification with SCX cartridges can be used as a time-effective alternative to HPLC purification. The optimized labelling procedure including generator elution, post-processing, microwave-supported labelling and SPE purification takes only 13 min. Representative challenge experiments indicate high *in vitro* stability of the ^{68}Ga labelled compound.

The labelled ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was formulated in PBS buffer pH 7.4 ready for injection. The same formulation can be readily employed in animal PET studies. Specific activities of up to 100 GBq/µmol were obtained under relatively mild conditions. A further improvement should be possible using a ^{68}Ge/^{68}Ga generator initially delivering higher activities.

The investigated methods and procedures were exemplified by the labelling precursor DO2A-(butyl-L-tyrosine)$_2$. The procedure can easily be transferred to any other new derivative. Each novel labelling precursor, nevertheless, will show particular behaviour, and a systematic labelling study will be necessary to achieve high yields.

4. EXPERIMENTAL

All chemicals were procured from commercial suppliers, in the highest available quality and purity. Deionized water was filtered through a Millex® Millipore filter membrane (0.54 µm) prior to use. For radio-TLC, Merck Silica F_{254} TLC-Plates were used. Flatbed detection was conducted on a Canberra Packard Instant Imager. Analytical HPLC was performed using a Phenomenex® Synergy 4 µ Max RP 80A 250 × 4.6 mm, 4 µm column, a Sykam UV-Detektor S3240, a Sykam S1122 pump and a radioactivity detector (ISOMED 110, Nuklear-Medizintechnik Dresden GmbH). Reactions under microwave irradiation were carried out in a CEM Discover focussed microwave reactor. Citrate buffer for TLC was obtained from VWR [Merck Buffer solution; citric acid–sodium hydroxide–hydrogen chloride; colour, red, traceable to SRM from NIST and PTB pH 4.00 (20 °C) CertiPUR®].

4.1. General

Generally all contaminations with other metals should be avoided. Therefore in all experiments plastic spatulas were used in place of metal ones. As labelling media, freshly prepared sterile-filtered water was utilized. ^{68}Ga has a considerably higher β^+-energy than, for example, ^{18}F. Adequate shielding is necessary, especially to avoid high radiation dose to the extremities.

4.2. Procedure for desalting

The synthesis of DO2A-(butyl-L-tyrosine)$_2$ was performed as described elsewhere (9). The TFA salt **2a** of the labelling precursor DO2A-(butyl-L-tyrosine)$_2$ (100–250 mg) was dissolved in 5 ml Millipore water and adsorbed on an anion-exchange resin (~5 g in a small column), then rinsed neutral with about 40 ml of water and eluted with an increasing gradient of methanol in water. The desalted product **2b** was collected in 10 ml fractions. The desalted precursor was obtained as a colourless powder after lyophilization.

4.3. Standard labelling conditions

The precursors (1 mg) were dissolved in 1 ml Millipore water to form a stock solution, which was stored at −20 °C. The reaction mixture with 5 ml Millipore water and the desired amount of labelling precursor from the stock solution was, in the case of conventional heating, preheated for 2 min to the desired labelling temperature. The desired volume of the post-processed ^{68}Ga generator eluate was added to form an acidic reaction medium. For microwave-supported synthesis, all components were mixed in a 10 ml microwave reaction vial, the vial was capped and the reaction started. After the desired labelling period the reaction vial was removed from the oil bath or the microwave heating was aborted.

4.4. RadioTLC

At specific time points (1, 3, 5, 10 and 15 min) 2 µl fractions were withdrawn from the reaction vial and spotted on silica-gel TLC plates. To avoid misinterpretation of overlapping TLC bands, TLC plates were developed in duplicate using citrate buffer (pH 4) and 25% EtOH in 5% NaCl solution as mobile phases. Using citrate buffer, a ^{68}Ga citrate complex was formed from nonchelated ^{68}Ga(III) *in situ* on the TLC plate. The complex progressed with an R_f of 0.9, the product complex remaining on the starting line. Using 25% EtOH in saline, ^{68}Ga remained on the starting line and the labelled product ^{68}Ga-**1** had an R_f of 0.4.

4.5. Radio HPLC

For HPLC, a Phenomenex® Synergy 4 µ Max RP 80A 250 × 4.6 mm column was used as the stationary phase. A mixture of Millipore water–methanol (90:10) containing 0.1% TFA was used as mobile phase. For each run 20 µl of the labelling mixtures was injected.

4.6. Purification

All SPE cartridges (Waters Silica, Waters C_{18} small, Merck Lichrolute SCX) were preconditioned following the instructions of the manufacturer. The mixture of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ and 'free' ^{68}Ga was transfered over the preconditioned cartridges. The

cartridges were rinsed with 10 ml Millipore water, then the product was eluted. In the case of the C_{18} cartridge, ethanol p.a. was used with the drawback that the ethanol had later on to be removed before the formulation of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ in sterile 0.9% NaCl solution. The reaction mixture had to be cooled to 0 °C in an ice bath before SPE using a silica cartridge. After the use of 5 ml Millipore water the product was received utilizing 3 ml of preheated 0.9% NaCl-solution. On the SCX cartridge the whole activity of ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was adsorbed. The cartridge was rinsed with 5 ml water and the pure ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was collected in 3 ml PBS-buffer pH 7.4, ready to inject after sterile filtration.

4.7. Stability

Three vials were charged with DTPA solution (10 ml, 0.1 M), and three vials were charged with *apo*-transferrin-solution (5 ml, 0.2 mM). The solutions were placed in an incubator set to 37 °C. The purified product was formulated in 3 ml DPBS-buffer pH 7.4. Aliquots of this solution containing 500 µl of the radiotracer formulation were added to each vial. At specific time-points (15, 30, 60 and 120 min), samples (100 µl) were withdrawn from the reaction mixture. These samples were analysed by radioTLC and the amount of intact parent compound ^{68}Ga-DO2A-(butyl-L-tyrosine)$_2$ was determined at each time point.

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