



DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

“Novel Approaches for Improved PET-Radiotracer
Synthesis and Quality Control”

verfasst von / submitted by

Sarah Pfaff, BSc MSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree
of

Doktorin der Naturwissenschaft (Dr. rer. nat)

Wien, 2019 / Vienna 2019

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on the student
record sheet:

A 796 605 419

Dissertationsgebiet lt. Studienblatt /
field of study as it appears on the student record sheet:

Doktoratsstudium Naturwissenschaften: Chemie

Betreut von / Supervisor:

Assoc. Prof. Mag. Dr. Wolfgang Wadsak

„It always seems impossible until it's done“

-Nelson Mandela

ACKNOWLEDGEMENTS

First of all, I want to thank my supervisor Prof. Dr. Wolfgang Wadsak who guided me during my thesis and always provided enough leeway for realization of my own ideas while giving good advice in key situations.

I want to thank Prof. Marcus Hacker for giving me the opportunity to work in his department.

Furthermore, I want to thank Verena Pichler for supervising, advising, discussing and introducing me into new scientific fields.

Especially, I want to express immense gratitude to Chrysoula Vraka, for inspiration and any scientific discussions.

Special thanks go to Erwin Schaier and Tina Nehring who assisted me by performing experiments.

Furthermore, I want to thank the whole working groups of Prof. Wolfgang Wadsak and Prof. Markus Mitterhauser for working out experimental designs and performing them in a good atmosphere.

Moreover, the LBI working group is thanked for any scientific advice.

Finally, thanks to my family, my friends and Karl Bugkel for supporting me during the time of my doctoral thesis.

Abstract

Radiotracers for positron emission tomography (PET) are a powerful imaging tool with ever increasing clinical relevance for diagnosis in versatile applications comprising tumor staging, cardiac perfusion, neuropsychiatric disorders among others. Most relevant PET-nuclides for radiolabeling of those imaging vectors are carbon-11, fluorine-18 and gallium-68. Since radiochemical procedures are constrained by radioactive decay, this small niche of chemistry requires special prerequisites like a short reaction time as well as an optimized radiopharmaceutical quality control and precaution regarding radiation protection during all operative steps. In this light, many chemical synthesis procedures and respective quality control assays of radiopharmaceuticals emerge as rather sophisticated, cumbersome or even unsuitable. Thus, an improvement of those processes is beneficial. Consequently, research is requested to put emphasis on the optimization of radiochemical syntheses and quality control procedures for a suitable application within a standardized clinical setting.

This doctoral thesis comprises of the improvement of radiosynthesis and quality control using a couple of model systems and radiotracers out of clinical trials and routine. In particular, ^{68}Ga -labeling of [^{68}Ga]Ga-DOTA-NOC, [^{68}Ga]Ga-PSMA-11 and [^{68}Ga]Ga-NODAGA-RGDyk using a HEPES buffered system was performed by means of continuous flow microfluidic- and microwave assisted synthesis.

Microfluidic ^{68}Ga -labeling was performed successfully in high radiochemical yields. Likewise, microwave assisted synthesis showed excellent radiochemical conversion rates, especially for the radiotracers, [^{68}Ga]Ga-PSMA-11 and [^{68}Ga]Ga-NODAGA-RGDyk. Hence, this study revealed high efficiency of unconventional microfluidic and microwave assisted ^{68}Ga -labeling, showing that those devices are perfectly feasible for challenging ^{68}Ga -labeling in particular situations as well as routine production.

Quality control procedures *via* TLC according to the European Pharmacopoeia (Ph. Eur.) and HPLC for prescribed HEPES quantification were evaluated and a novel, improved TLC assay was developed by changing (1) the spotting method, (2) mobile phase, (3) reference solution, (4) staining method and (5) using a free software for quantitative analysis.

Visual quantification of HEPES by TLC according to the Ph. Eur. was neither conclusive nor reproducible. An HPLC assay according to Antunes *et al.* achieved a good linearity of the created calibration curve. Furthermore, a novel TLC method was developed which is tremendously outperforming the TLC assay currently published in the Ph. Eur. in terms of limit of linearity,

reliability and conclusiveness. The optimized procedure, developed within this PhD thesis, is considered for publication Ph. Eur. monograph for [^{68}Ga]Ga-PSMA-11, highlighting its reproducibility, feasibility and scientific quality.

Additionally, the influence of HEPES on cell viability in three different PC3 cell lines was assessed by means of an MTT assay. Synergistic effects of HEPES and [^{68}Ga]Ga-PSMA-11 were investigated by incubation of the radiotracer in presence of different HEPES concentrations in PSMA expressing PC3 B9 cells. The *in vitro* evaluation of HEPES' influence on cell viability resulted in a non-toxic behavior up to a concentration that exceeds the currently prescribed limit by more than a factor of 10^3 . Preliminary results showed similar [^{68}Ga]Ga-PSMA-11 uptake in PC 3 B9 cells in the presence of different HEPES concentrations and no influence on the uptake at higher HEPES amounts present.

Another challenging experimental setting is radiolabeling using carbon-11 due to its short half-life of 20 min. In this context, sophisticated ^{11}C -labeling of (+)-HNO was significantly improved by omitting heating and cooling procedures during radiosynthesis and therefore, accelerating the whole process.

Skipping heating and cooling during (+)-[^{11}C]PHNO synthesis resulted in a remarkable reduction of synthesis duration of about 5 min counteracting radioactive decay of about 13%. Moreover, byproducts and intermediates of (+)-[^{11}C]PHNO synthesis were synthesized on purpose and chromatograms thereof were compared for facilitated failure analysis after unsuccessful syntheses. The investigation of the influence of reagents on byproduct formation enabled the creation of a decision tree facilitating troubleshooting in a generalized way.

In summary, within this thesis the following achievements were made:

- (1) Microfluidic and microwave assisted ^{68}Ga -labeling was proven to be feasible in the preparation of several radiopharmaceuticals.
- (2) A novel TLC assay for HEPES quantification was developed helping operators for a reliable and good applicable procedure.
- (3) *In vitro* investigations showed that the HEPES limit is dramatically low and should be reconsidered, as HEPES is probably not toxic in the applied concentrations and is assumed to have no impact on [^{68}Ga]Ga-PSMA-11 uptake.
- (4) (+)-[^{11}C]PHNO activity yield was dramatically improved due to a reduction of the synthesis procedure.
- (5) A decision tree for troubleshooting in the radiosynthesis of (+)-[^{11}C]PHNO was developed that will tremendously facilitate a demanding error search in case of failed syntheses.

Ultimately, this successful work optimized particular procedures within PET-tracer synthesis and quality control. It evoked several questions that need further attention, especially about the reasonability of the currently prescribed HEPES limit and HEPES quantification.

Zusammenfassung

Radiotracer für die Positronen-Emissions-Tomographie (PET) sind ein effizientes Werkzeug in der Bildgebung mit weiterhin steigender klinischer Relevanz für Diagnosestellungen in vielen Anwendungsgebieten, wie dem „*Staging*“ von Tumoren, bei der Ermittlung der kardialen Perfusion, bei neuropsychiatrischen Störungen, und vielem mehr. Die wichtigsten Nuklide für Radiomarkierungen solcher Bildgebungsvektoren sind Kohlenstoff-11, Fluor-18 und Gallium-68. Durch die Einschränkung des Arbeitens mit radiochemischen Substanzen aufgrund ihrer Halbwertszeit, setzt dieses spezialisierte Gebiet der Chemie bestimmte Bedingungen voraus: kurze Reaktionszeiten, standardisierte und rasche Qualitätskontrolle, sowie Anwendung von Strahlenschutzmaßnahmen während des gesamten Arbeitsvorgangs. In dieser Hinsicht sind viele radiochemische Synthesen und die daran anschließende Qualitätskontrolle anspruchsvoll und würden von einer Verbesserung profitieren. Deswegen stellt die Optimierung von radiochemischen Synthesen und der Qualitätskontrolle immer noch einen wichtigen Forschungsschwerpunkt dar, um eine angenehme klinische Anwendbarkeit zu gewährleisten.

Diese Doktorarbeit umfasst Studien zur Verbesserung von Radiosynthesen und der anschließenden Qualitätskontrolle. Dazu wurden einige Modellsysteme und Radiotracer aus klinischen Studien und klinischer Routine untersucht. Im Speziellen wurden ^{68}Ga -Markierungen von DOTA-NOC, PSMA-11 und NODAGA-RGDyk in HEPES Puffer unter der Verwendung von einem „*continuous flow*“ mikrofluiden System und einer speziellen PET-Labormikrowelle vorgenommen. Es konnte gezeigt werden, dass mikrofluide ^{68}Ga -Markierungen erfolgreich mit hohen radiochemischen Ausbeuten durchgeführt werden können. Genauso erzielten Synthesen mittels Mikrowelle exzellente radiochemische Umsetzungsraten; insbesondere im Fall von $[\text{}^{68}\text{Ga}]\text{Ga-PSMA-11}$ and $[\text{}^{68}\text{Ga}]\text{Ga-NODAGA-RGDyk}$. Damit zeigt diese Arbeit die hohe Effizienz von unkonventionellen mikrofluiden und Mikrowellen gestützten ^{68}Ga -Markierungen auf. Als Folge dieser experimentellen Untersuchungen konnte festgestellt werden, dass diese Methoden für die Behandlung spezieller Problemstellungen als auch für Routineproduktionen anwendbar sind.

Methoden für die Qualitätskontrolle mittels DC laut Europäischem Arzneibuch (Ph. Eur.) und HPLC für die vorgeschriebene HEPES Quantifizierung wurden evaluiert und optimiert. Eine neue DC Methode wurde entwickelt, welche durch Veränderung (1) der Auftragungsmethode, (2) mobilen Phase, (3) Referenzlösung, (4) der Anfärbemethode und (5) der Verwendung eines frei-

erhältlichem, Software-basiertem Auswertungsprogramms für eine quantitative Bestimmung optimiert wurde. Eine visuelle Quantifizierung von HEPES mittels DC laut aktueller Ph. Eur. war weder konklusiv noch reproduzierbar. Die HPLC Methode nach Antunes et al. zeigte eine gute Linearität der erhaltenen Kalibrationskurve. Zusätzlich wurde eine neue DC Methode entwickelt, welche die bisherige Methode nach der Ph. Eur. hinsichtlich der Linearität, Verlässlichkeit und Schlüssigkeit in hohem Maße übertrifft. Die Brauchbarkeit und wissenschaftliche Qualität dieser verbesserten Analysemethode kann daran abgelesen werden, dass diese Methode für eine Publikation in der Ph. Eur. in der Monographie für [^{68}Ga]Ga-PSMA-11 vorgeschlagen wurde.

Des Weiteren wurde der Einfluss von HEPES auf die Zellviabilität in drei verschiedenen PC3 Zelllinien mittels MTT-Assay getestet. Synergistische Effekte von HEPES und [^{68}Ga]Ga-PSMA-11 wurden durch Inkubation des Radiotracers in Gegenwart von verschiedenen HEPES-Konzentrationen in PSMA-exprimierenden PC3 B9 Zellen untersucht. Diese *in vitro* Evaluierung ergab, dass HEPES bis zu Konzentrationen, die das vorgeschriebene Limit um mehr als einen Faktor von 10^3 übertreffen, keinen Einfluss zeigte. Erste Ergebnisse zeigten eine ähnliche Aufnahme von [^{68}Ga]Ga-PSMA-11 in PC3 B9 Zellen in Gegenwart von verschiedenen HEPES Konzentrationen.

Ein weiteres herausforderndes experimentelles Setting stellen Radiomarkierungen mit Kohlenstoff-11 dar, da dieses Nuklid eine kurze Halbwertszeit von 20 min aufweist. Deswegen wurde die ^{11}C -Markierung von (+)-HNO durch Überspringen von Heiz- und Kühschritten während der Radiosynthese verbessert. Eine (+)-[^{11}C]PHNO Synthese ohne Heizen und Kühlen resultierte in einer beachtlichen Reduktion der Synthesezeit von rund 5 min, wodurch ein Produktverlust aufgrund des radioaktiven Zerfalls von ungefähr 13% verhindert werden kann. Zusätzlich wurden in dieser Studie Neben- und Zwischenprodukte der (+)-[^{11}C]PHNO Synthese gezielt hergestellt und die zugehörigen Chromatogramme verglichen, um eine umfangreiche und detaillierte Analyse nach Fehlsynthesen durchführen zu können. Dies ermöglicht die Untersuchung des Einfluss der Reagenzien auf die Entstehung von Nebenprodukten und diene zur Generierung eines generalisierbaren Entscheidungsbaums, der die Fehlersuche vereinfacht.

Insgesamt umfasst diese Dissertation folgende Punkte:

- (1) Der Einsatz von mikrofluiden und Mikrowellen unterstützten Systemen in der Präparation von ^{68}Ga -Radiopharmaka konnte erfolgreich demonstriert werden.
- (2) Die Entwicklung einer neuen DC Methode für HEPES Quantifizierung schafft eine Erleichterung für die Anwender durch einen verlässlichen und gut anwendbaren Ablauf.
- (3) *In vitro* Untersuchungen zeigten auf, dass das derzeitige HEPES Limit immens niedrig ist und überdacht werden sollte, weil HEPES offenkundig in den applizierten Konzentrationen nicht toxisch ist und vermutlich keinen Einfluss auf die [^{68}Ga]Ga-PSMA-11 Aufnahme hat.
- (4) Es konnte eine erhebliche Verbesserung der (+)-[^{11}C]PHNO Aktivitätsausbeute durch Reduktion der Syntheszeit erreicht werden.
- (5) Es wurde ein Entscheidungsbaum, der eine aufwendige Fehleranalyse nach einer Fehlsynthese vereinfacht, entwickelt.

Insgesamt konnten in dieser Arbeit spezielle Abläufe der PET-Tracer Synthesen mit Berücksichtigung der Qualitätskontrolle optimiert werden, welche aber auch noch weitere Fragen aufwerfen (insbesondere über die Rationale des vorgeschriebenen HEPES Limits), welche in zukünftigen Studien zu bearbeiten sein werden.

Table of Content

Abstract	i
Zusammenfassung.....	v
I. Introduction.....	1
Molecular Imaging and Positron Emission Tomography (PET).....	1
General Requirements for PET-Tracer Production.....	3
Quality Control (QC) for Radiopharmaceuticals.....	4
Gallium-68	7
⁶⁸ Ge/ ⁶⁸ Ga-Generator	8
Cyclotron Produced Gallium-68	10
Elution Protocols and Post Processing	10
Chelators for ⁶⁸ Ga-Complexation	11
⁶⁸ Ga-Radiotracers	14
Labeling Methods.....	17
Fixed Tubing Based Systems.....	18
Cassette Based Systems	19
KIT Based Approaches	20
Microfluidic Synthesis.....	21
Microwave Assisted Synthesis.....	23
HEPES as Buffer for ⁶⁸ Ga-Labeling	25
Carbon-11	28
(+)-[¹¹ C]PHNO for Imaging the Dopaminergic System.....	31
(+)-[¹¹ C]PHNO Synthesis	32
II. Aim.....	35
III. Publications	37
Peer Reviewed Manuscripts.....	39
Manuscript I.....	41
Manuscript II.....	51
Manuscript III.....	63
Unpublished Data: Manuscripts in Preparation	89
Manuscript IV: Microwave Assisted ⁶⁸ Ga-Labeling.....	91
Manuscript V: HEPES: Impact on Cell Viability and [⁶⁸ Ga]Ga-PSMA-11 Accumulation	99
IV. Discussion	103
Improvement of Radiotracer Synthesis.....	103

Microfluidics vs. Microwave Assisted Synthesis	104
Improved Troubleshooting after (+)-[¹¹ C]PHNO Synthesis	105
Reliable and Quantitative HEPES Determination.....	105
Biological Data of HEPES in Three PC3 Cell Lines.....	106
V. Summary and Outlook	107
VI. Abbreviations	108
VII. Figure Caption.....	110
VIII. References	112

I. Introduction

Molecular Imaging and Positron Emission Tomography (PET)

Medical imaging for a better understanding and treatment of any kind of diseases is based on the discovery of x-rays by Wilhem Conrad Röntgen at the end of the 19th century.¹ As a consequence, an prodigious progress in developing and establishing imaging techniques to gain structural information of living organisms, like ultrasound imaging, computed tomography and magnetic resonance, was witnessed by the following century. In the 1950s the technique of positron emission tomography started to enable insights to physiological processes and molecular information.^{2,3} Nowadays, PET has become a standard technique to diagnose metastases of tumors and contributes to the so called 'molecular imaging' modalities. Molecular imaging describes the representation characterization and quantification of biological processes at the cellular, subcellular and molecular level *in vivo*.⁴⁻⁸ Therefore, the molecular design of a radiopharmaceutical has to be well conceived and incorporates a physiologically relevant moiety that is labeled with a radionuclide in order to accumulate in a particular target region. Nowadays, a huge variety of radiotracers has been developed including small molecules, peptides or antibodies. In nuclear medicine, the workhorse is the sugar based compound [¹⁸F]FDG that exploits the high glycolytic rate of tumors and predominantly targets neoplastic tissue.⁹

PET-radiotracers are usually injected intravenously and circulate in the blood vessels until they have reached their target region. After they have arrived there, they are detected due to their characteristic property of emitting antimatter, namely a positron: Once the positron is emitted, it travels a short distance depending on its energy until it crashes into its matter-counterpart, an electron. As a result of this annihilation event, two photons (gamma quants) with an energy of 511 keV are emitted in the opposite direction.¹⁰ These photons are recognized by detectors that spatially arranged in a ring shape which are able to reconstruct the origin of the annihilation radiation along the line of response (LOR). Consequently, an image of biological processes can be created (Fig. 1).

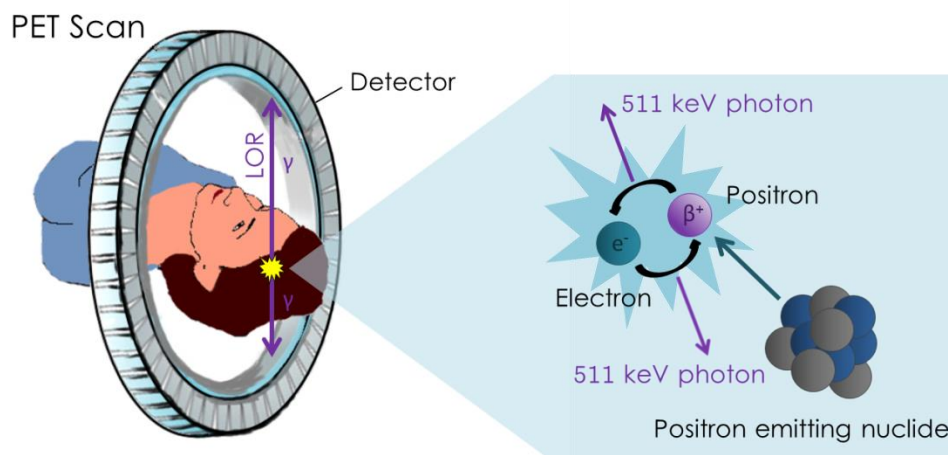


Figure 1: Scheme of a PET-scanner and the principle behind PET.¹¹

The drawback of a PET-image is its poor structural information resulting in demanding localization of tumors and metastases.¹² As a consequence, the hybrid imaging techniques of PET with computed tomography (PET/CT) as well as magnetic resonance imaging (PET/MR) were established in the recent years.^{13,14}

Application of PET-Nuclides

PET-labeling of biomolecules allows isotopic substitution by replacing naturally occurring nuclides with their radioactive counterparts like carbon-11, nitrogen-13, or oxygen-15. Therefore, the labeled compound is physico-chemically indistinguishable from the original, non-labeled compound (not considering the isotopic effect). In contrast, for non-isotopic substitution nuclides are not the same element, but are intended to not alter the respective compound dramatically. Therefore, the most common application is the substitution of a hydrogen atom by fluorine-18 due to their similar van der Waals radii. Furthermore, molecules can be labeled with a radiometal by linkage with a suitable chelator, which is associated with a relatively high modification of the biochemical behavior. Radiometal-labeled imaging vectors offer the possibility to exchange the PET-nuclide with an α - or β^- -emitter that shows high linear energy transfer with matter and can be used for therapy. Those approaches are so called “theranostic” approaches and are highly beneficial for therapy imaging or in terms of personalized medicine. A

typical theranostic nuclide couple is gallium-68, a PET-nuclide for diagnosis, and lutetium-177, a β^- -emitter for therapy.¹⁵ However, the choice of a nuclide strongly depends on the concrete research question. Hence, the biological half-life of the biomolecule should be feasible with the physiological half-life of the nuclide. Small molecules as peptides show usually a fast renal clearance. In contrast, high molecular antibodies are circulating in the blood system for a long period up to a few days. Hence, a nuclide with an appropriate half-life has to be chosen for reasonable imaging.¹⁶

General Requirements for PET-Tracer Production

In theory, radiochemistry does not differ from conventional chemistry except the low amounts of substances that are used in this small niche of chemistry and radioactive decay. Thus, this field is subjected to special conditions. The amounts of molecules (precursors) that are intended to be labeled differ from the low microgram-to the low milligram-range. However, the mass of applied radionuclides is dramatically lower by a factor of 10^3 - 10^4 resulting in pseudo-first-order reaction kinetics governed by the radionuclide.¹⁷ Considering the practical aspects of handling such small amounts, adequate vessels and tubes must be chosen to constraint the loss caused by unspecific binding. Therefore, all connections have to be as short as possible. Furthermore, radiochemical reactions have to be performed in a lead shielded area (hot cells) to keep the radiation exposure of the operator at a minimum in accordance with the ALARA (as low as reasonably achievable) principle. As a result, radiosyntheses are performed in fully automated synthesizers that are controlled by outside positioned unit. The most important factors for successful radiotracer production are time and the related decay. Hence, the common benchmark for a production duration including the quality control is two to three times the half-life of the respective radionuclide.¹⁷ As a result, fluorine-18 labeling comprises a vast diversity of reported synthesis approaches due to its relatively long half-life of 110 min. In contrast, carbon-11 has a half-life of 20 min, which makes the design of a rational synthesis period challenging as 16% of the activity is decayed after 5 min (Fig. 2).

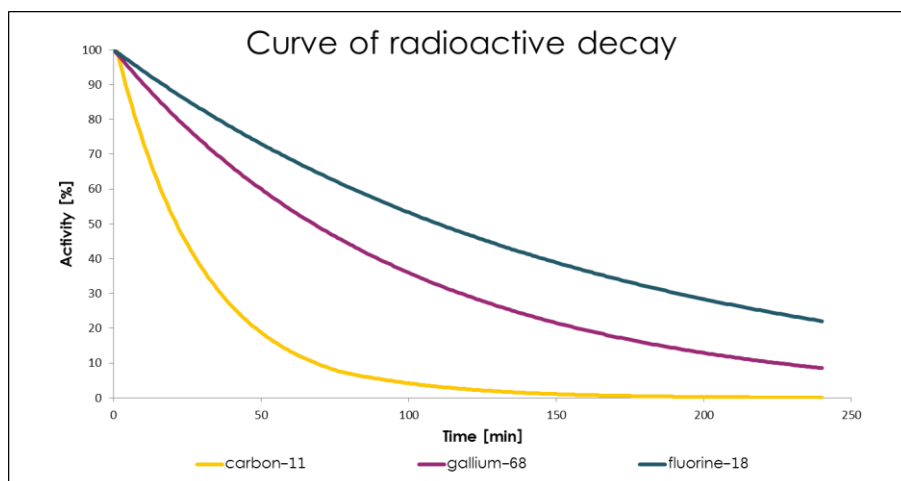


Figure 2: Radioactive decay of carbon-11, gallium-68 and fluorine-18.

The production of radionuclides does not necessarily deliver the desired isotope in its active form. Therefore, nuclides have to be transformed into a state that is amenable for labeling procedures. An example is the reduction of [^{11}C] CO_2 and subsequent iodination to finally obtain [^{11}C] CH_3I for methylation reactions.¹⁸ In case of generator produced radiometals a post processing of the eluate may play a pivotal role, which will be described within the next chapters.

Quality Control (QC) for Radiopharmaceuticals

Radiopharmaceuticals are drugs and have to meet certain quality criteria which are stipulated in the European Pharmacopeia (Ph. Eur.).¹⁹ As radiopharmaceuticals are freshly produced prior administration and decaying rapidly, a fast performance of the quality control process is pivotal. During this procedure following parameters are assessed: pH, osmolality, radionuclide purity, molar activity, radiochemical purity and chemical purity. pH and osmolality are highly relevant parameters as radiopharmaceuticals are usually injected intravenously and the injection is accompanied with pain and osmotic shock, if those parameters are out of range. The molar activity is defined as the ratio of activity and the total amount of the substance, which includes the amount of the non-radioactive, cold compound.²⁰ Radionuclide purity is understood as occurrence of unwanted radionuclides that may origin from cross contamination, byproducts in radionuclide production or generator breakthrough.²¹ Those nuclides are potentially harmful as

they accumulate in the body and ionize biological material apart the target region (e.g. germanium-68 in bones).²² Radiochemical purity describes the presence of radioactive compounds other than the product and is usually determined by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) using a radiodetector.^{21,23} The amount of those impurities is relatively low with respect to the used amount of reagents. Hence, radioactive impurities are probably not causing toxicity, but give rise to an additional signal and lead to false diagnosis. In contrast, evaluation of chemical purity involves occurring byproducts, solvents and detergents that are potentially harmful or impairing pharmacokinetics of the imaging agent e.g. acetonitrile from HPLC purification or HEPES as buffer substance. Additionally, sterility and endotoxins have to be assessed, which is performed after the release of the radiotracer.²⁴

Parameter	Technical equipment	Parameter range for gallium-68	Parameter range for carbon-11
pH	pH meter, pH paper	3.5-8.5	3.5-8.5
Osmolality	Osmometer	Depends on the application	Depends on the application (e.g. 250-300 mOsmol/kg for [¹¹ C]methionine)
Molar activity	HPLC, calculation	Depends on the application	Depends on the application (e.g. ≥ 7.07 GBq/ μ mol for [¹¹ C]PIB)
Radionuclide identity	1) half-life 2) γ -spectrometer	1) $t_{1/2} \pm 10\%$ 2) 511 keV	1) $t_{1/2} \pm 10\%$ 2) 511 keV
Radionuclide purity	γ -counter	⁶⁸ Ge $\leq 0.001\%$	
Radiochemical purity	HPLC, TLC	Typically 95%	Typically 95%
Chemical purity	HPLC, TLC, GC	Typically $\geq 90\%$, HEPES ≤ 200 μ g/V	Typically $\geq 90\%$ (5000 ppm for EtOH, 410 ppm for ACN)

Table 1: A selection of typically quality criteria for ⁶⁸Ga- and ¹¹C-radiopharmaceuticals.^{25,26} It should be considered that the guidelines differ within radiotracers and countries.

Quality criteria of radiopharmaceuticals are often individually adapted for a particular radiotracer. For instance, ^{68}Ga -radiotracers have to be tested for ^{68}Ge -breakthrough of the generator as depicted in table 1.

Gallium-68

Gallium is a group III element that was discovered in 1875.²⁷ In nuclear medicine, gallium-72 was applied for diagnostic and therapeutic approaches in osteosarcoma in the 1940's. Later, investigations focused on its congener gallium-67. It was found that gallium also accumulates in soft tissue lesions, which broadened the field of possible applications. Although gallium-68 was available since 1961 and it was one of the first PET-nuclides that was applied for tumor imaging, availability of this nuclide was not satisfying enough for further intensive investigations.^{28–30} Consequently, a landmark event in the history of gallium-68 was the development of generators that offer gallium-68 in chemically reactive form that is feasible for labeling reaction.³¹ Hence, so far ¹¹¹In-labeled octreotides for SPECT scans were labeled with gallium-68 to exploit PET for obtaining improved quality images (Fig. 3).³²

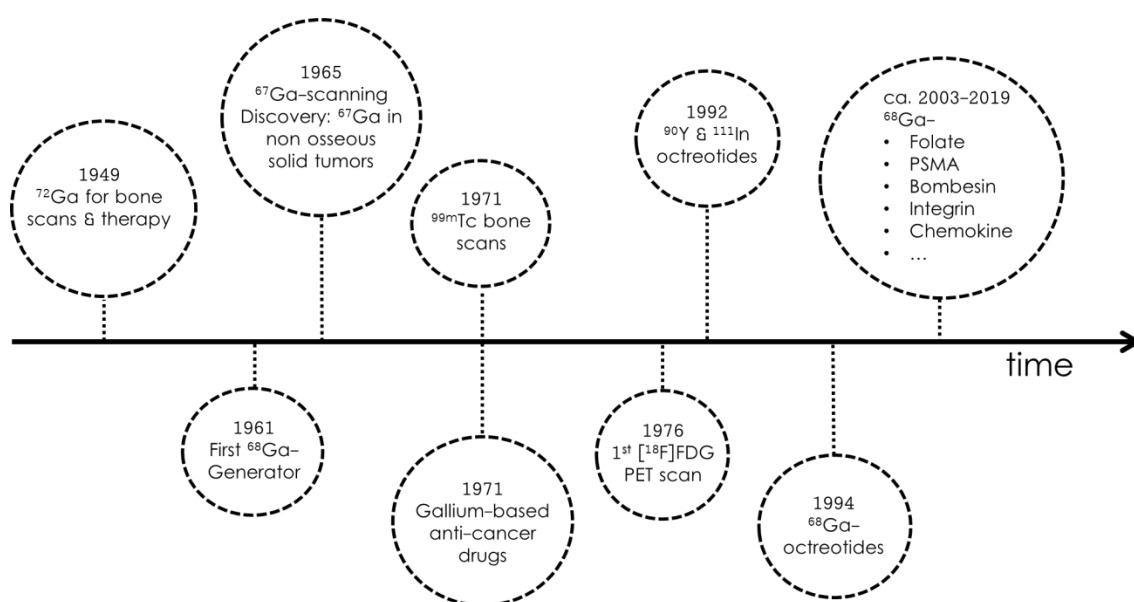


Figure 3: Timeline of ⁶⁸Ga-relevant events in history.^{28,33–39}

Gallium-68, built from germanium-68, decays with a convenient half-life of 68 min and emits positrons with an energy of 0.89 MeV to stable zinc-68 (Fig. 4). This energy is inferior in comparison to fluorine-18 with

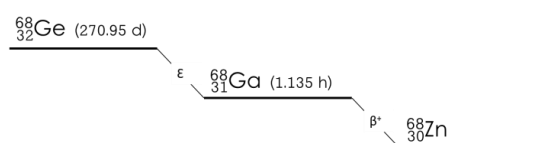


Figure 4: Scheme of decay from germanium-68 to gallium-68 and finally to zinc-68.

positrons of 0.63 MeV by means of image quality. However, the good availability of gallium-68 by a generator and not needing a costly cyclotron allows every radiochemistry laboratory the possibility of ^{68}Ga -radiotracer production.

From the chemical point of view, gallium is usually present in the oxidation state +III, but an abundant chloride concentration enables a presence of the +II state.²⁷ Gallium forms hydroxides with a poor solubility at a pH of 5.1 that start to precipitate even at lower pH, which is detrimental for ^{68}Ga -radiolabeling. Due to gallium's amphoteric nature, hydroxides dissolve at higher pH of 9.6.^{40,41} Due to its high charge and small radius, this metal is defined as hard Lewis acid that forms complexes with oxygen and nitrogen donor atoms like carboxylates, phosphonates and amines.⁴² Complexes are formed with coordination number of six mostly resulting in a distorted octahedral geometry. Gallium complexes are highly stable due to their d^{10} electron configuration.⁴³ At a low pH < 3.5, the formation of ^{68}Ga -complexes can be hindered by inactivation of donor atoms by protonation. However, ^{68}Ga -labeling can be performed at a small pH range of 3.5-5.1. Gallium(III) is chemically very similar to high spin iron(III) as they have comparable ionic radii (0.62 Å for Ga(III) and 0.65 Å for high spin Fe(III)).^{44,45} Consequently, gallium-68 is subject to exchange with the iron chelating blood serum protein transferrin, which results in an enhanced accumulation in lung and liver.³¹ Hence, ^{68}Ga -labeling strictly requires the absence of iron ions for a successful complexation.

$^{68}\text{Ge}/^{68}\text{Ga}$ -Generator

$^{68}\text{Ge}/^{68}\text{Ga}$ -generators are based on the radiochemical equilibrium of a long lived mother nuclide germanium-68, ($t_{1/2}=271$ days) and a short lived daughter nuclide (gallium-68, $t_{1/2}=68$ min). The half-life of a nuclide pair that is feasible for generator production may differ in a factor up to 100.⁴⁶ During the decay of germanium-68, the content of formed gallium-

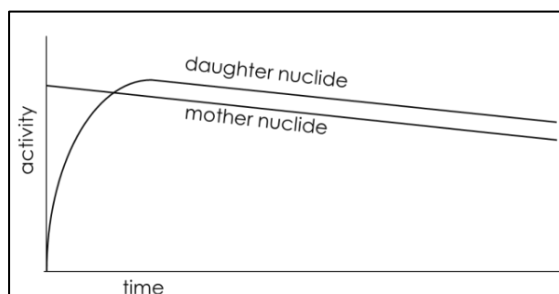


Figure 5: Equilibrium of long lived mother nuclide and its daughter nuclide.

68 increases until the nuclides have reached their equilibrium state (Fig. 5). At this point, the present amount of gallium-68 is limited by the decay of its mother nuclide and therefore, stays

almost constant until the generator is eluted. The technical design of a $^{68}\text{Ge}/^{68}\text{Ga}$ -generator is based on the chromatographic principle: Germanium-68 is loaded on a column and is intended to last there. In contrast, formed gallium-68 is accessible by elution with an appropriate solvent. The first generator system applying liquid-liquid extractions was reported in 1960 by Gleason *et al.* who used EDTA for separation of a $[^{68}\text{Ga}]\text{Ga-EDTA}$ complex.⁴⁷ During the following years, the innovation of an Al_2O_3 -column based “positron cow” set a landmark in $^{68}\text{Ge}/^{68}\text{Ga}$ -generator development.^{48,49} The eluate containing $[^{68}\text{Ga}]\text{Ga-EDTA}$ complexes could be directly used for administration, but this chemical form of gallium-68 was not feasible for performing further labeling procedures to obtain other ^{68}Ga -radiotracer. Accordingly, improvements of generator systems were implemented by applying diverse resin materials for ^{68}Ge -fixation and respective solvents for ^{68}Ga -elution in the following decades. Those approaches can be divided into two groups: 1) generators based on inorganic columns like TiO_2 , SnO_2 or Al_2O_3 with NaOH , EDTA or HCl as eluents and 2) organic resins that can be either eluted with hydrochloric acid, citric acid or phosphoric acid.^{50–53} However, during the last years, only one generator incorporating a TiO_2 column from Eckert&Ziegler (Galliapharm®, EZAG, Berlin, Germany), that is eluted with 5 mL of HCl (0.1 M), was approved for clinical use. Recently, a generator system, namely Galli Ad® (IRE-Elit, Fleurus, Belgium) requiring a remarkable small volume of 1.1 mL HCl (0.1 M), has been developed and accepted by authorities, for clinical application. As a result, the eluate can be used immediately for radiolabeling without any concentration steps. However, the drawback of this generator is its limitation of use by total volume of the intern hydrochloric acid due to the missing refill option.

General requirements on $^{68}\text{Ge}/^{68}\text{Ga}$ -generators are sterility and the absence of other metal ions. Metal ions like ubiquitous Fe^{3+} , Zn^{2+} derived from decay, $^{68}\text{Ge}^{4+}$ due to breakthrough and resin-desorbed Ti^{4+} or Sn^{4+} can govern or competitively hinder ^{68}Ga -complexation. Especially the presence of germanium-68 is a critical parameter due to its long half-life and poorly investigated pharmacological behavior and biodistribution. Consequently, authorities have to assume the worst case scenario in which this radioactive species accumulates in bone marrow and lasts there eternally.⁵⁴ To this end, the ^{68}Ge -content in the final radiopharmaceutical has to be below 0.001% of the starting activity.⁵⁵ As a result, several eluate-processing techniques are contemporary applied which are compiled within the next chapters.

Cyclotron Produced Gallium-68

An alternative to $^{68}\text{Ge}/^{68}\text{Ga}$ -generators is the production of gallium-68 *via* cyclotron. Besides solid targets that are subjected to a variety of hurdles described elsewhere⁵⁶, liquid targets have been developed for an improvement of this cyclotron production process within the last years.^{57,56,58} For the production of gallium-68 a zinc-68 enriched target is bombarded with protons resulting in gallium-68 and an ejected neutron ($^{68}\text{Zn}(p,n)^{68}\text{Ga}$). On the one hand, the advantage of cyclotron production is the increased activity of up to 6 GBq and the potential of an upscaling to 40 GBq by enhancing the content of the enriched nuclide.⁵⁶ On the other hand, the production procedure is prolonged due to the additional beaming duration and purification process. Finally, cyclotron produced gallium-68 may flourish in the future as the number of cyclotron equipped facilities is rising.⁵⁸

Elution Protocols and Post Processing

Most recently available $^{68}\text{Ge}/^{68}\text{Ga}$ -generators have to be eluted with hydrochloric acid in a particular concentration. Typical elution volumes are 5-10 mL except the elution volume of GalliEo and Galli Ad[®] that are eluted with 1.1 mL HCl. Volumes of 5-10 mL are relatively high considering the low amount of $[\text{}^{68}\text{Ga}]\text{Ga}^{3+}$ -ions that are dissolved ($4.69 \cdot 10^{12}$ atoms/800 MBq gallium-68).⁵⁹ Therefore, it is beneficial to reduce the volume of the eluate to obtain a higher activity concentration, which can

be managed by the application of two approaches e.g. fractionized elution and concentration using ion exchange cartridges.^{60,61}

A fractionized elution protocol exploits the variation of eluted activity within the elution volume.

An exemplary elution profile is

illustrated in figure 6 and shows that the first and the last few

milliliters contain a relatively low amount of activity and are discarded. Only the fraction that incorporates the highest amount of activity (main fraction) of about one to two milliliters is used

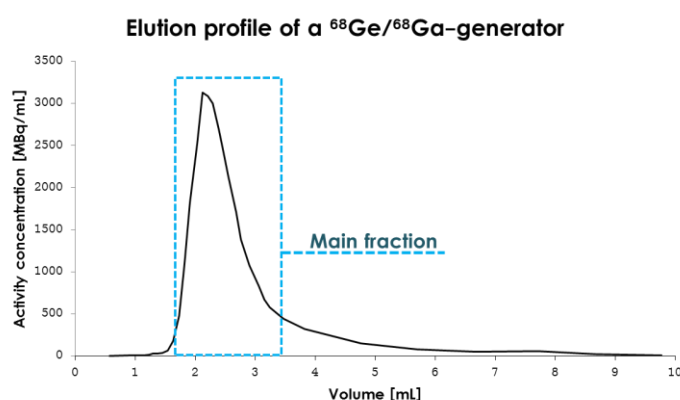


Figure 6: Representative elution profile of a $^{68}\text{Ge}/^{68}\text{Ga}$ -generator with highlighted main fraction.

for labeling. The drawback of this method is that about 80% of the eluate's total activity is employed for the labeling procedure.^{62,63} Furthermore, metal impurities like Zn(II), Ti(IV), Ge(IV) and Fe(III) are co-eluted and are able to disturb the complexation reaction, but their content in the main fraction is lower than in the total eluate.⁶³ The advantage of this method is its easy handling and direct application of the eluate for radiotracer synthesis without any time loss due to purification procedures.

For a minimization of the elution volume, but retaining more than 90% of its activity, the eluate can be concentrated by application of ion exchange columns. This strategy is implemented by using an anion exchange cartridge, a cation exchange cartridge or a combination thereof by exploiting the chemically different forms of gallium-68 (Fig. 7). The first one demands a high concentration of chloride to obtain the gallate-complex $[^{68}\text{Ga}][\text{GaCl}_4]^-$ which is usually realized by addition of HCl. $[^{68}\text{Ga}][\text{GaCl}_4]^-$ is retained at the cartridge and can be easily eluted with H_2O .⁶⁴ A cation exchange resin adsorbs the eluate directly and gallium-68 can be eluted with high chloride concentrations of HCl/Aceton or NaCl.^{59,65–67}

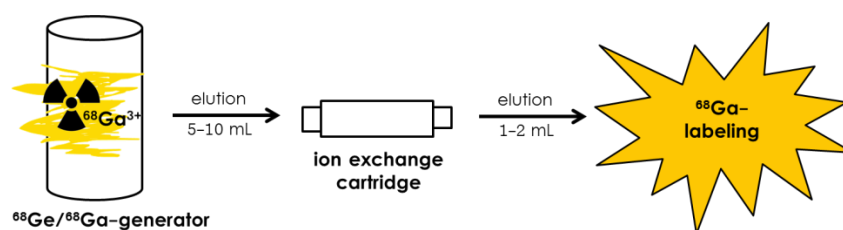


Figure 7: Scheme of elution of a $^{68}\text{Ge}/^{68}\text{Ga}$ -generator with subsequent concentration.

Chelators for ^{68}Ga -Complexation

Ideally, a chelator for radiometals should provide thermodynamic stability and kinetic inertness of the complex to ensure stability under physiological conditions. Besides, a chelator should preferentially form only one isomer due to a potential shift in pharmacokinetics of different isomers.⁶⁸ For isomeric forms that show the same behavior, this effect becomes negligible. Additionally, the metal complex must be able to stand radiolysis up to activities which are used in realistic settings. However, the choice of chelator has to be well considered in terms of the stability of the biomolecule during the labeling procedure: Usually, small molecules are able to withstand high temperatures during the radiolabeling process. In contrast, peptides and

especially antibodies are prone to fragmentation. By this, temperature sensitive biomolecules should be linked to a chelator that is capable of complexing at respective labeling parameters. Additionally, a metal exchange of the labeled biomolecule with transferrin should be avoided and therefore, complexes should reveal a high stability constant.⁴⁰ Nowadays, radiotracers are often designed to address theranostic approaches. In this way, the chelator moiety of biomolecules has to be amenable for complexing the diagnostic as well as the therapeutic nuclide. Consequently, the chelator may not be perfect for gallium-68 by means of e.g. the ionic radius, but has to be the best compromise for the intended radiometals. For the application of theranostic approaches, it should be considered that the radiotracer's pharmacological behavior may alter due to a different coordination geometry and charge. Finally, there is no "perfect" chelator for a certain nuclide; the properties have to be in accordance with the envisaged application.

The first Ga-chelators used in nuclear medicine were linear chain chelators, like citrate and EDTA.⁶⁹ The latter one was used for the labeling of proteins and monoclonal antibodies. However, those chelators show an appreciable hydrophilic nature.⁷⁰ Therefore, more lipophilic chelators were investigated that should evoke a higher *in vivo* stability.^{70–73} Furthermore, a major aspiration was the crossing of the blood brain barrier, which could not be achieved so far. Nowadays, the mostly used chelators for ⁶⁸Ga-complexations are macrocyclic chelators like DOTA (1,4,7,10-tetraazacyclododecane-tetraacetic acid) or NOTA (1,4,7-triazacyclononanetriacetic acid) as they show kinetic inertness and are feasible for fast and relatively mild labeling (Fig. 8).⁷⁴ Especially the NOTA chelator proofed to be beneficial for ⁶⁸Ga-complexations in comparison to DOTA as the ionic radius of the trivalent ion fits in perfectly.^{75,76} DOTA suffers the disadvantage of requiring high labeling temperatures for achieving high conversions with gallium-68, but its benefit lies in the feasibility to complex a wealth of metal ions.⁷⁷ In recent years, research still puts effort in the development of new and improved chelators for ⁶⁸Ga-radiolabeling, which resulted in the development of second generation macrocyclic compounds like TRAP, NOPO and many more.^{78–80} Additionally, several derivatives of DOTA and NOTA (e.g. DO3A, DOTAGA or NODAGA) were investigated as they offer a further carboxylic entity for metal chelation and alter the overall charge and therefore, the pharmacokinetic behavior of a biomolecule. Besides, novel linear chain chelators were employed for ⁶⁸Ga-labeling as well like CP256, DFO, THP or HBED-CC.^{81–83} On the one hand, the advantage

of linear chain chelators lies in the fast labeling kinetic in contrast to macrocyclic chelators. On the other hand, macrocyclic chelators usually provide a higher stability.

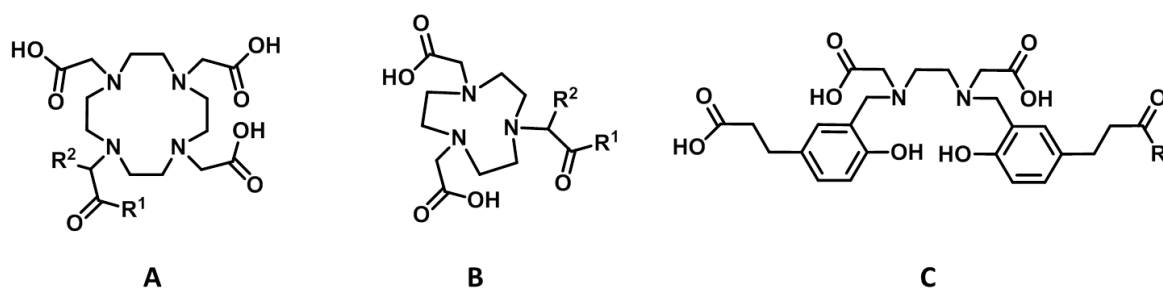


Figure 8: Chemical structure of A: DOTA (R^1 =linkage with biomolecule, R^2 =H) and DOTAGA (R^1 =OH, R^2 =Linkage with biomolecule); B: NOTA (R^1 =linkage with biomolecule, R^2 =H) and NODAGA (R^1 =OH, R^2 =Linkage with biomolecule) and C: HBED-CC (R =Linkage with biomolecule).

[⁶⁸Ga]Ga-DOTA-Octreotides

⁶⁸Ga-octreotides ([⁶⁸Ga]Ga-DOTA-TOC, [⁶⁸Ga]Ga-DOTA-TATE and [⁶⁸Ga]Ga-DOTA-NOC (see section: Abbreviations) widely displaced their SPECT-counterpart, [¹¹¹In]In-octreotide, due to their associated ameliorated spatial resolution of the scans.⁸⁴ Those compounds incorporate an octapeptide moiety that is linked to a DOTA chelator for radiometal-complexation. As somatostatin (SST) analogues, they are targeting the somatostatin receptor (SSTR) that is overexpressed in neuroendocrine tumors (NETs). Consequently, they visualize NETs and their metastases in an agonistic fashion. [⁶⁸Ga]Ga-DOTA-TOC, [⁶⁸Ga]Ga-DOTA-TATE as well as [⁶⁸Ga]Ga-DOTA-NOC are targeting the receptor subtype SSTR2, but as they show structural differences, their affinities to other SSTR subtypes differs (Fig. 9). In this way, [⁶⁸Ga]Ga-DOTA-TOC and [⁶⁸Ga]Ga-DOTA-TATE both incorporate tyrosine at position three, but differ in position eight they contain threonine and its alcohol, respectively, in their amino acid sequence. In [⁶⁸Ga]Ga-DOTA-NOC, tyrosine is substituted by naphthalene resulting in an altered aromatic entity. Position eight is Thr(ol) likewise [⁶⁸Ga]Ga-DOTA-TOC. As a consequence, those SST analogues reflect neoplastic tissue in a different manner.^{85,86} Furthermore, DOTA-octreotides are perfect candidates for theranostic approaches by complexing lutetium-177 instead of gallium-68.¹⁵

	SST-analogue	R ₁	R ₂	Targeted SSTR-subtype
	[⁶⁸ Ga]Ga-DOTA-TOC	Tyr	Thr(ol)	SSTR2, SSTR5
	[⁶⁸ Ga]Ga-DOTA-TATE	Tyr	Thr	SSTR2
	[⁶⁸ Ga]Ga-DOTA-NOC	Nal	Thr(ol)	SSTR2, SSTR3, SSTR5

Figure 9: Molecular structure of [⁶⁸Ga]Ga-DOTA-TOC, [⁶⁸Ga]Ga-DOTA-TATE and [⁶⁸Ga]Ga-DOTA-NOC, and the SSTR subtypes they are showing the highest affinity to.

$[^{68}\text{Ga}]\text{Ga-PSMA-11}$

$[^{68}\text{Ga}]\text{Ga-PSMA-11}$ was described by Eder *et al.* in 2012, which set a milestone in diagnosis of prostate cancer and its metastases.⁸² This relatively new radiotracer engenders better contrast images than the commonly used ^{18}F -choline derivatives and offers a good availability.⁸⁷ Hence, diagnostic tool attracted appreciably high attention from radiopharmacy departments and ranks among the most successful PET-tracers in clinical trials within the recent years.^{88,89} This molecular imaging vector targets the prostate specific membrane antigen (PSMA), which is a transmembrane enzyme and shows folate hydrolase I (FOLHI) as well as glutamate carboxypeptidase II (GCP II) activity.⁹⁰ This enzyme is highly overexpressed in most prostate cancer tissue and serves as eponym of the ^{68}Ga -labeled compound.^{91–93} The targeting entity contains an urea derivative, Glu-NH-CO-NH-Lys, that shows affinity of the carboxylic groups with Zn^{2+} -ions of the enzyme's active center (Fig. 10).^{94–96} This binding motif is linked with a HBED-CC chelating moiety. $[^{68}\text{Ga}]\text{Ga-PSMA-11}$ provides the special feature that the aromatic chelator is pivotal for the affinity as it interacts with a second lipophilic pocket of PSMA's active binding side.⁸² Besides, HBED-CC offers ^{68}Ga -labeling within a few minutes even at room temperature.

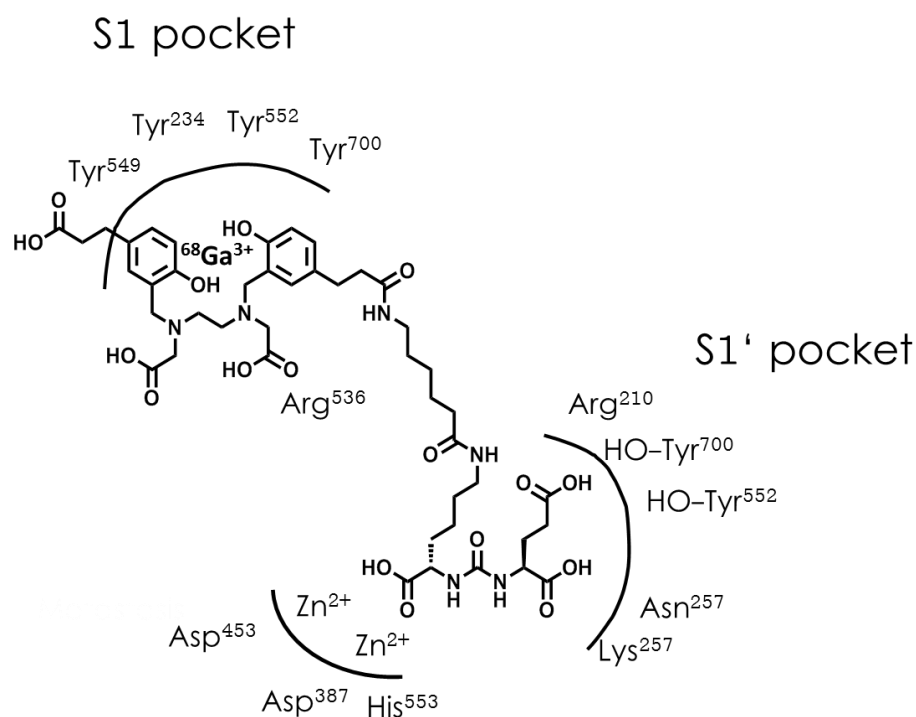


Figure 10: Assumption of potential binding of $[^{68}\text{Ga}]\text{Ga-PSMA-11}$ to PSMA adapted from Liu *et al.*⁹⁵

[⁶⁸Ga]Ga-NODAGA RGD

[⁶⁸Ga]Ga-NODAGA-RGD is a cyclic pentapeptide that is attached to the macrocyclic NODAGA chelator and is described in form of two different amino acid sequences: 1) NODAGA-RGDfK that incorporates a phenylalanine within the cyclic peptide and 2) NODAGA-RGDyK (Fig. 11) in which the phenylalanine is substituted by a tyrosine.^{97–99} The kinked tripeptidic vector arginine-glycine-aspartate (RGD) targets $\alpha_v\beta_3$ -integrin, which is a transmembrane glycoprotein.¹⁰⁰ This receptor is overexpressed in activated endothelial cells and therefore involved in angiogenesis and tumor progression.¹⁰¹ Hence, [⁶⁸Ga]Ga-NODAGA-RGD is a potential PET-tracer for gaining information about the localization, invasiveness as well as metastatic potential of a tumor. In contrast to the demanding production of ¹⁸F-labeled RGD derivatives, ⁶⁸Ga-labeling of NODAGA-RGD is quite simple and works quantitatively.¹⁰²

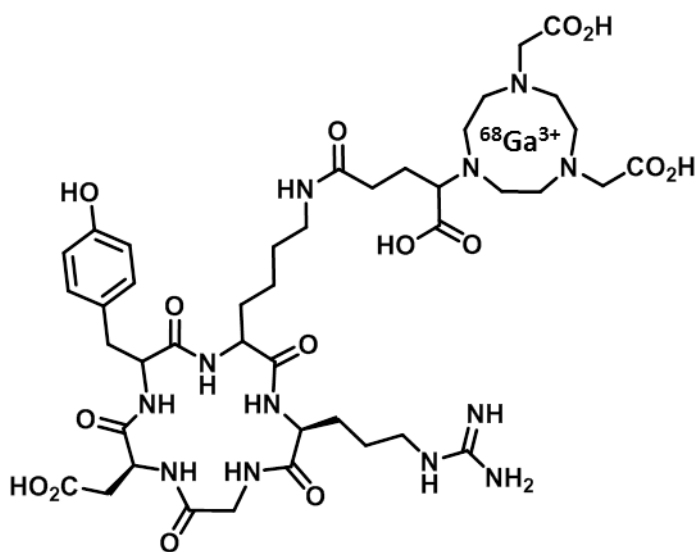


Figure 11: Simplified representation of the chemical structure of [⁶⁸Ga]Ga-NODAGA-RGDyK.

Labeling Methods

Basically, ^{68}Ga -labeling is commonly performed by adding the ^{68}Ga -eluate (main fraction or concentrated eluate) to the respective progenitor in buffer solution. The reaction mixture is heated for several minutes, cooled down and retained on a reversed phase (RP) C18 solid phase extraction (SPE) cartridge, while unbound $[\text{}^{68}\text{Ga}]\text{Ga}^{3+}$ is washed through the cartridge. Subsequently, the labeled compound is eluted with ethanol and colloidal ^{68}Ga -species remain on the cartridge. Finally, the radiotracer is formulated and sterile filtered (Fig. 12). Therefore, ^{68}Ga -labeling is an easy performable process requiring relatively cheap instrumentation compared to radiotracer production associated with a cyclotron. Accordingly, radiopharmaceutical institutions are delighted by theoretically unlimited access to ^{68}Ga -radiotracers. Those tangible benefits spur the flourishing appearance of ^{68}Ga -laboratories in nuclear medicine facilities as well as the interest of industry in producing fully automated ^{68}Ga -radiolabeling synthesizers, so called modules. Beside various commercially available devices like 'fixed tubing based'- and 'cassette based'-systems for clinical routine, unconventional approaches as microfluidic techniques and microwave assisted synthesis are investigated.^{103,104} All synthesizers for radiolabeling incorporate a reactor, a heating unit, storage containers for adding the reagents and a controlling unit that is usually a laptop with the respective software.

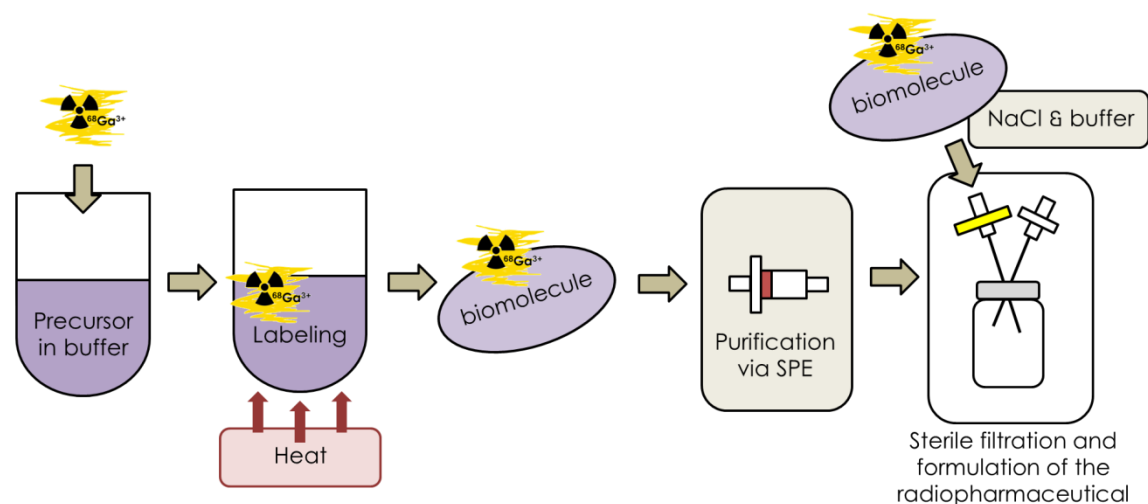


Figure 12: Scheme of commonly performed ^{68}Ga -labeling processes.

Fixed Tubing Based Systems

This kind of synthesizer comprises a reactor and storage vessels which are permanently attached (Fig. 13). Hence, all vessels and connections between them have to be washed properly prior synthesis. Nowadays, those modules are mostly used for ^{11}C -labeling and sparsely for routine ^{68}Ga -productions. The advantage of those easy accessible vials is the opportunity to perform versatile synthesis sequences. Resultantly, those synthesizers are often applied as research instruments, which also evolved from the fact that the focus of clinical routine production has shifted towards cassette based systems.¹⁰⁵

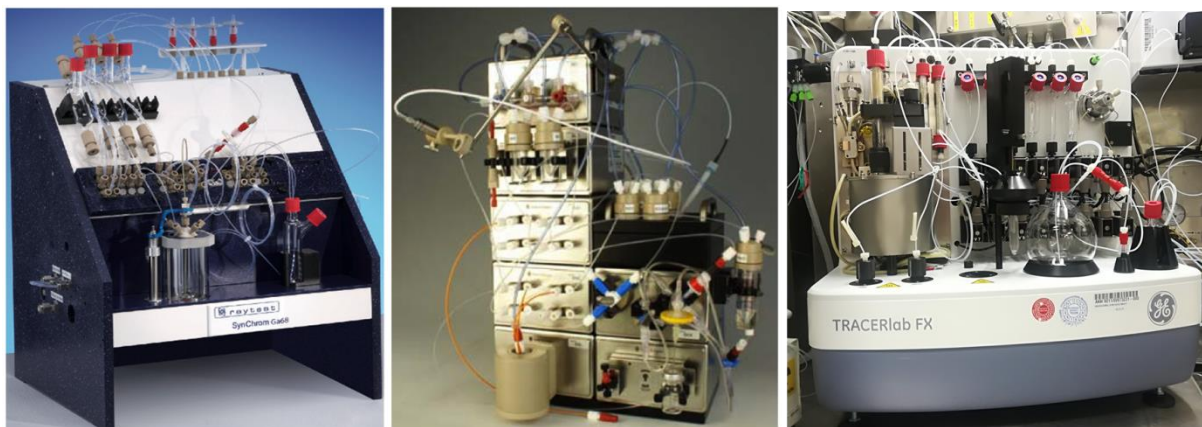


Figure 13: Synchrom Ga from raytest (left), Modular-Lab Standard from Eckert & Ziegler (middle) and TRACERlab FX from GE (right).¹⁰⁵

Cassette Based Systems

An innovative approach in ^{68}Ga -synthesizer development was the production of modules applying disposable cassettes. The cassettes and the reagent KITs are fabricated in accordance with GMP and sterilized with gamma irradiation, which is of utmost importance as most countries are restricted to a GMP compliant production of radiopharmaceuticals. Cassettes are intended for single use resulting in an obviation of any contaminations by chemicals, bacteria, *etc.* The manifolds of the cassettes are designed to be easily mounted on the synthesizer by any technical staff. However, the correct connection of the tubes have to be checked prior synthesis start as an intervention during the synthesis process is not possible.¹⁰⁵



Figure 14: Modular-Lab PharmTracer from Eckert & Ziegler (left) and FASTlab from GE (right).¹⁰⁵

KIT Based Approaches

Radiotracer production has to be uncomplicated and easily performable by any operator. A widespread implementation of this approach is given by the KIT based production of ^{99m}Tc -radiotracers. Ideally, freeze dried reagents are directly mixed with ^{68}Ga -eluate and after a few minutes a quantitative conversion is achieved. Resultantly, no expensive synthesizer is required for radiolabeling. The creation of such KITs is subjected to the condition that every compound of the lyophilisate is compatible in terms of a safe administration. Furthermore, the precursor has to be suitable for quantitative labeling due to the absence of a purification step. However, several KIT-based approaches have been reported in literature.¹⁰⁶ One of the authorized KITs is SomaKit® TOC for diagnosis of NETs and many novel Kits involve the 'blockbuster-compound' [^{68}Ga]Ga-PSMA-11 or other PSMA targeting tracers (e.g. [^{68}Ga]Ga-THP-PSMA, 'Galliprost®').^{26,107–}

111

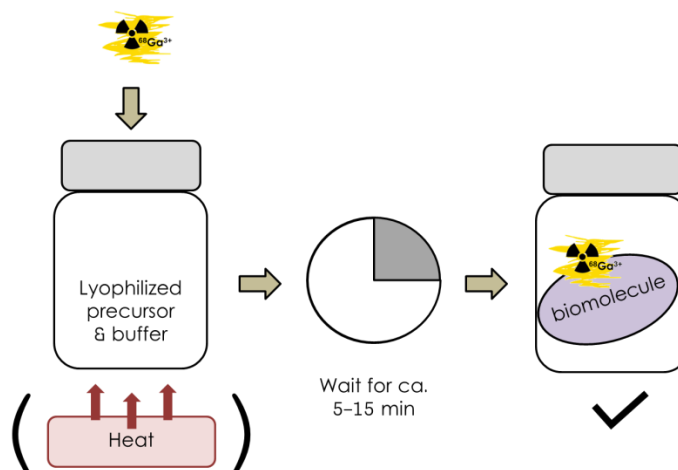


Figure 15: Scheme of ^{68}Ga -labeling via KITs.

Microfluidic Synthesis

Microfluidics developed from analytical systems like gas chromatography or HPLC, was harnessed for microanalysis like DNA sequencing among others. The establishment of this technique was even supported by the US Department of Defense within projects of the Advanced Research Projects Agency (DARPA) to detect chemical and biological weapons in the 1990s.¹¹² Beside analytical applications, microfluidics is contemporary applied in synthesis of versatile organic compounds.^{113–116} The appreciably high interest in microfluidic devices originates from their high efficiency and superiority to macroscale synthesis in terms of short synthesis times, tremendously low amounts of reagents and suppression of byproduct formation.^{117–119}

From the physical point of view, fluids have the same direction and flow speed in this miniaturized situation, which engenders a prevalent laminar flow.¹²⁰ Accordingly, the Reynolds number (Re), that describes the ratio between inertial forces and viscosity, is in a low range of $Re < 10^2$. In contrast, liquids in traditional macroscale reaction performed in round-bottomed flasks are churned resulting a turbulent flow and high Reynolds numbers of $Re > 10^3$.¹²¹ However, the fundament of every chemical reaction is the encounter of molecules, which is realized by mixing of the reactants. In a laminar flow the mixing of components is constrained by diffusion that is relatively slow in macroscale flow regiments due to large distances of mass transfer. In contrast, mixing within a laminar flow is extremely time efficient in typical microfluidic dimensions of 100 μm .¹¹⁸ Microfluidic tools are not restricted to a laminar flow: turbulences can be induced by an adequate design of the channel's geometry.¹¹⁷ Hence, this technique offers abroad range of geometrically different micro reactor set ups. Basically, microfluidic reactors are grouped in two main reactor categories, namely chip reactors and tubular continuous flow tools.¹²²

Since small amounts of starting materials and minimal synthesis durations are highly relevant in PET-tracer production, microfluidic-assisted syntheses have become quite attractive in the field of radiochemistry. As a result, several proof of principle studies of well-known radiotracer synthesis labeling have been accomplished like the synthesis of workhorse [^{18}F]FDG, $^{99\text{m}}\text{Tc}$ -labeling of KITs, radioiodinations and ^{11}C -.^{123–132} Furthermore, dose-on-demand approaches have been successfully performed by radiofluorination of different PET tracers.^{133,134} The benefits of microscale approaches are attributed to distinct physical features: The small size of the reactor causes a highly efficient heat transfer, which allows fast heating and cooling. Additionally, the

usage of high pressure enables the implementation of an unusually high temperature above the boiling point of the respective solvent. The application of elevated temperatures can initiate enhanced conversion rates.¹³⁵ As a consequence, microfluidic systems offer the application of reduced reagent amounts without a loss of yield, which is a weighty argument for radiochemical applications considering the high costs of GMP-certified precursors and low amounts of radionuclides.

Prerequisites for materials of microfluidic devices are solvent resistance and capability to withstand a high temperature, a dramatically elevated pressure and radiolysis. Hence, favorable materials for the production of these reactors are metals, glasses, silicones and polydimethylsiloxane (PDMS). The choice of the material strongly depends on the particular purpose.¹³⁶ An example of a commercially available system incorporating a continuous flow reactor is distributed by Advion (NanoTek) and is used for microfluidic synthesis within this thesis. For the sake of a better understanding, the scheme of a NanoTek synthesizer (Fig. 16) and a cursory explanation is given below.

The NanoTek device incorporates two storage loops in which the reactants are loaded. Two pumps simultaneously transfer the reagents from those loops into a preheated continuous flow reactor where the labeling is performed. Reaction parameters like flow rate and the temperature of the reactor have to be set prior the synthesis. The reactor is a two meter long silica capillary with a volume capacity of 15.6 μL . After the reaction mixture passes the reactor, it is collected in a dedicated vial.

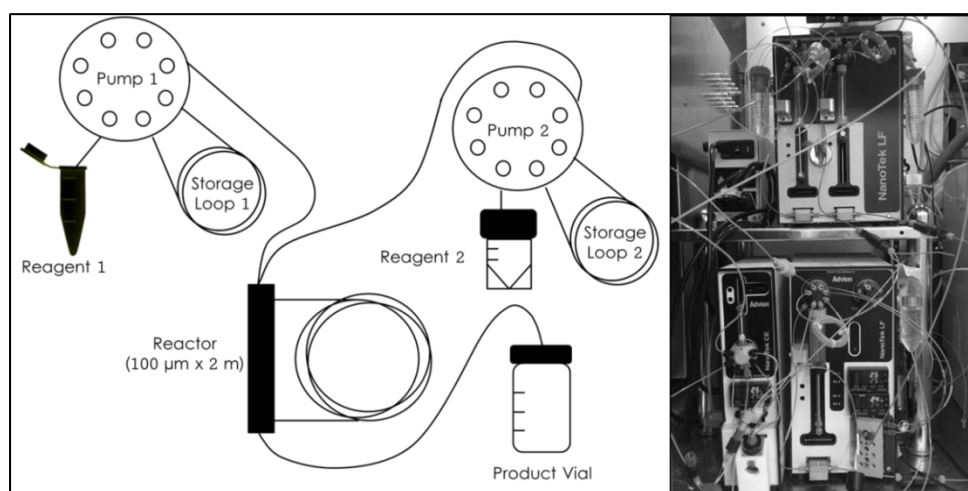


Figure 16: : Schematic arrangement of a NanoTek microfluidic synthesizer and image of the device (adapted from Pfaff et al.¹³⁷)

Microwave Assisted Synthesis

Microwaves are electromagnetic waves with a wavelength of 1 mm to 1 m with respective frequencies of 0.3 to 3 GHz. Commercially available microwave heaters irradiate with a frequency of 2.45 GHz. This kind of irradiation contains a magnetic and an electric component, but only the latter one is predominately involved in the heating process. For a successful heating process, the solvent has to be capable of absorbing the microwave energy and reveal a dipole moment. The electric field initiates dipolar polarization as well as ionic conduction. As the electric field is oscillating, the irradiated molecule changes its alignment within the corresponding frequency and is continuously reorienting with a small phase difference (Fig. 17). Consequently, molecular friction and collisions are caused, which results in dielectric heating.¹³⁸

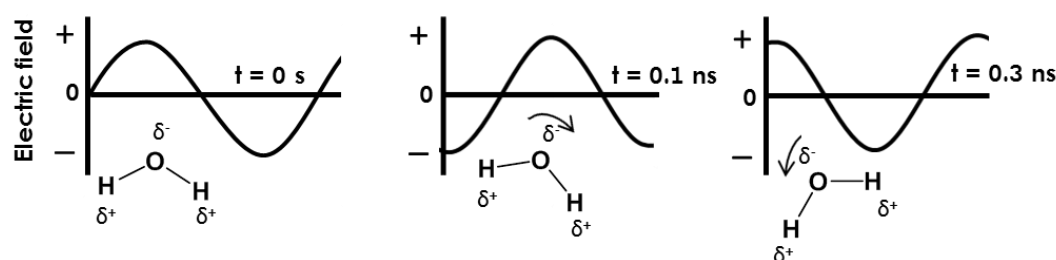


Figure 17: Alignment of a H_2O molecule in the electric field adapted from Kappe et al.¹³⁸

In synthesis chemistry, microwave heating provides a homogenous heating of a reaction mixture in contrast to the classical thermal heating of round bottom flasks (Fig. 18). In the latter one, a built up heat gradient starting from the flask's wall can lead to localized over-heating of the solvent and reagents resulting in a slow, insufficient energy transfer to the "inner" solution. Hence, microwave assisted synthesis is able to yield appreciably increased conversions in reduced reaction times.^{139,140} Due to the enhanced efficient energy transfer, microwave heating is also termed a "green" process.¹⁴¹

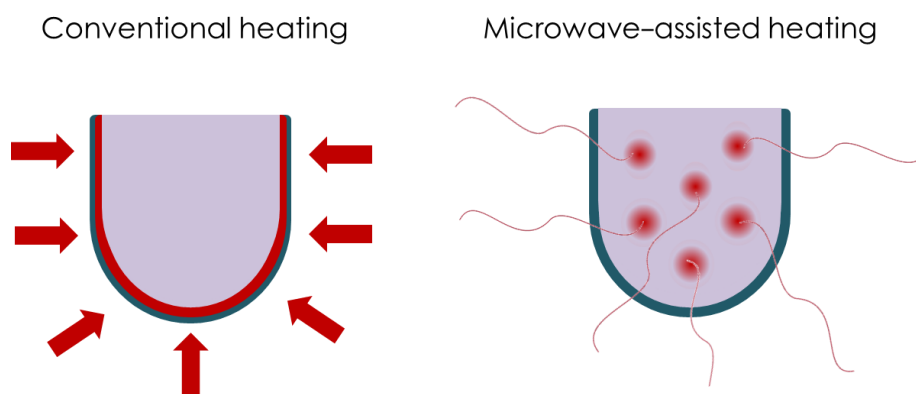


Figure 18: Conventional heating (left) showing a heterogeneous temperature distribution with a higher temperature at the reactor walls. Microwave heating (right) showing dielectric heating within the solution for an almost homogenous heating.

The advantages of microwave assisted synthesis were recognized by chemical and radiochemical institutions resulting in an increased interest in this technology. Therefore, various radiochemical labeling processes exploited this high yielding approach for radiofluorinations, ^{11}C -labeling as well as metal complexations.^{142–144} Hence, the radiolabeling peptides is an interesting perspective as they are prone to decomposition due to overheating. Accordingly, in several publications the advantage of ^{68}Ga -radiolabeling *via* microwave heating was highlighted and radiolabeling by applying this technique could be a flourishing approach in the future.^{145–149}

HEPES as Buffer for ^{68}Ga -Labeling

For successful ^{68}Ga -radiolabeling a particular pH range (about 3.5-5.1) is demanded to guarantee the presence of the reactants in their active form. Therefore, the reaction mixture for complexations has to be buffered. Accordingly, several buffer systems have been evaluated, which revealed acetate, succinic acid and -4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as the most suitable ones for ^{68}Ga -complexations (Fig. 19).¹⁵⁰ Moreover, Velikyan *et al.* showed that the latter one achieved the highest radiochemical yield at low precursor amounts of DOTATOC.¹⁴⁵

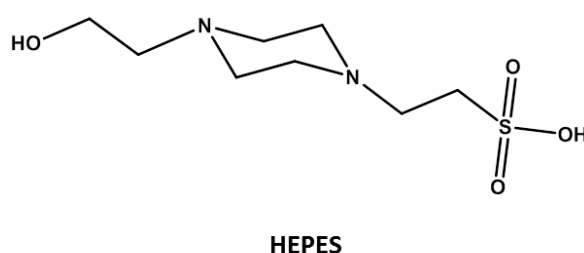


Figure 19: Chemical structure of HEPES.

HEPES is a zwitterionic taurine derivative that is able to form weak complexes with gallium-68 which may prevent the formation and precipitation of ^{68}Ga -hydroxy- and ^{68}Ga -oxygen-species for higher radiochemical yields.^{31,151} In contrast, a detrimental ^{68}Ga -complexation by NOTA was reported at low precursor amounts and high HEPES concentration. However, the formation constant of the Ga-HEPES complex is $\log K_f = 1.99 \pm 0.01 \text{ mol dm}^{-3}$ and the coordination sites are provided by the oxygen and one or two nitrogen atoms, respectively (Fig. 20).^{151,152}

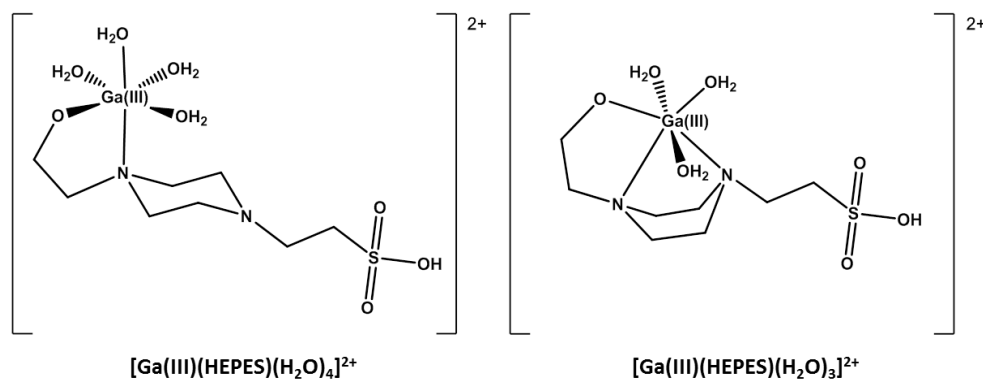


Figure 20: Possible chemical structure of Ga-complexes of HEPES based on Martin *et al.*¹⁵¹

Although HEPES should be the buffer of choice for ^{68}Ga -radiolabeling, this compound suffers from the drawback of an accompanied semi-quantitative, prescribed testing procedure by the Ph. Eur. as it is defined as an impurity. Theoretically, HEPES should be removed by a purification procedure using a C18 reversed phase cartridge, but a TLC assay has to ensure that the buffer substance is below a certain limit.⁶⁴ The stipulated limit of HEPES is 200 μg per recommended administration volume (V) and the formulated product has to undergo a visual comparison *via* iodine stained TLC plate with reference solution that contains the maximum concentration.⁵⁵ This analysis is highly dependent on the operator and not quantitative nor reliable.¹⁵³ As a consequence, novel analysis assays by means of HPLC were investigated by several working groups.^{153–155}

The obstacle in HEPES quantification is that this buffer belongs to the group of “Good’s buffers” and is designed for not disturbing any analytical method by its own signals. Hence, HEPES should be undetectable by using conventional parameters and shows a UV signal at 195 nm. Ultimately, most commercially available methods for ^{68}Ga -radiolabeling in clinical routine are performed by using sodium acetate as an alternative buffer to circumvent the onerous HEPES testing procedure, although HEPES may provide an enhanced radiochemical yield. In this light, the background and reasonability of the remarkably low limit of 200 $\mu\text{g}/\text{V}$ is questionable as this substance precludes toxicity issues.^{106,156} Furthermore, commercial suppliers report a relatively high oral LD_{50} of 2 g/kg HEPES in rats (comparison: NaCl has an oral LD_{50} of 3 g/kg in rats). The data safety sheet describes no reported toxicity symptoms, no germ cell mutagenicity in *escherichia coli* and *salmonella typhimurium*, no chromosome aberrations in human lymphocytes and no toxicity in a target organ.¹⁵⁷ Moreover, HEPES is used for a plethora of biological experiments, which indicates, that it does not negatively affect living cells.^{158–161}

Additionally, this buffer has been used for several years for *in vitro* fertilization (IVF). Although the influence of HEPES on IVF is controversial, studies that report a negative impact of HEPES, provide a vast leeway for data interpretation with respect to the experimental set up and reproducibility.^{162,163} However, HEPES is still used in artificial reproductive technologies *e.g.* in an oocyte *in vitro* maturation phase 3 study that started in December 2018 (gov Identifier: NCT03773263).¹⁶⁴ Besides, HEPES demonstrated anti-inflammatory effects *in vitro* and *in vivo*.^{165,166} In another study by Theodore *et al.* the toxicity of HEPES in dogs was assessed by administering 88 times a remarkably high dose up to 400 mg/kg intravenously. The study

revealed that the highest dose could start to cause hematopoietic and reticuloendothelial changes.

Theodore *et al.* found indications that HEPES could positively influence the immune system.¹⁶⁶ A case report by this working group showed that HEPES is able to act as painkiller in three cancer patients with bone metastases. In this study, HEPES was repeatedly administered in 1000 mg or 5000 mg doses intravenously and no adverse events were reported. Furthermore, administration of HEPES reduced tumor markers in other studies.¹⁶⁷ A patent about HEPES as analgesic agent, anti-tumor drug and for stabilization of cells recommends the administration of 70-75 mg/kg orally or parentally.¹⁶⁸ Finally, all the mentioned studies indicate that the limit of 200 µg/V HEPES is probably too low and is worth a reconsideration.

Carbon-11

Carbon-11 is a PET-nuclide with a relatively short half-life of 20.4 min that appeared in 1939 for the first time.¹⁶⁹ This nuclide is predominantly produced by bombardment of $^{14}\text{N}_2$ with accelerated protons ($^{14}\text{N}_2(\text{p},\alpha)^{11}\text{C}$) by usage of cyclotron.¹⁷⁰ The most frequently used primordial synthons for ^{11}C -labeling strategies are $[^{11}\text{C}]\text{CH}_4$ and $[^{11}\text{C}]\text{CO}_2$ whose formation is governed by adding sparse contents of either oxygen (20-20000 ppm) or nitrogen (5-10%) to the target gas, respectively.¹⁷ Due to the ubiquitous presence of natural carbon that prevalently consists of carbon-12, ^{11}C -labeling reactions are prone to be contaminated with this perturbing nuclide. Both nuclides show the same reactivity resulting in a certain ratio of the radiolabeled biomolecule and the non-radioactive content of the compound (^{11}C - and $^{\text{nat}}\text{C}$ -labeled) which is described as molar activity A_m (measured activity per mole of compound in GBq/ μmol).²⁰ As both compounds behave identically regarding pharmacology (neglecting the isotopic effect), a low molar activity promotes enhanced target occupancy by the cold compound. By this, the risk of pharmacological effects and a poor image quality is increased.¹⁷¹ In contrast, tremendous advantages of carbon-12's pervasive nature are versatile opportunities for isotopic substitution without changing the pharmacological properties of a compound. In the last decades, several ^{11}C -labeling pathways have been established.¹⁷² Some of them are comprised in figure 21. In theory, the whole spectrum of organic chemistry can be employed for ^{11}C -radiotracer production subject to the condition of a rational synthesis duration considering the half-life. Resultantly, radiolabeling a plethora biomolecules and derivatives thereof have been reported like endogenous $[^{11}\text{C}]\text{methionine}$, $[^{11}\text{C}]\text{choline}$ or $[^{11}\text{C}]\text{acetate}$ for oncological applications or ligands to target neurological disorders like $[^{11}\text{C}]\text{PIB}$, $[^{11}\text{C}]\text{harmine}$ or (+)- $[^{11}\text{C}]\text{PHNO}$ ((+)-4-Propyl-3,4,4a,5,6,10b-hexahydro-2H-naphtho[1,2-b][1,4]oxazin-9-ol).¹⁷³⁻¹⁷⁹

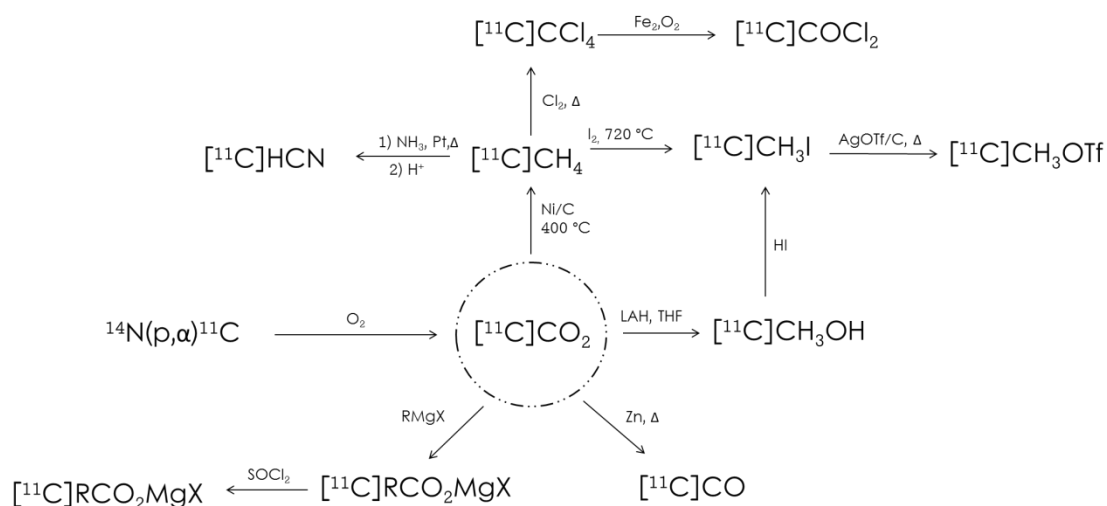


Figure 21: A selection of ^{11}C -labeling reactions starting with $[\text{}^{11}\text{C}]\text{CO}_2$.

Although a vast range of ^{11}C -labeling reactions is accessible, the widest applied one is $\text{S}_{\text{N}}2$ methylation of heteroatoms *via* the $[\text{}^{11}\text{C}]\text{CH}_3\text{I}$ -route, which was described in 1973 and is nowadays called the “wet” method. Thereby, $[\text{}^{11}\text{C}]\text{CO}_2$ is reduced to methanol and converted with HI to $[\text{}^{11}\text{C}]\text{CH}_3\text{I}$.^{17,180} This approach was superseded by the “gas phase” method by Larson *et al.* which starts with a catalytic reduction to $[\text{}^{11}\text{C}]\text{CH}_4$ with hydrogen and continues with free radical iodination at 720°C . The iodination is a circular process for a better conversion and ends up with an improved molar activity of the radiopharmaceutical (Fig. 21).¹⁸

A further intriguing approach for ^{11}C -radiolabeling is depicted by application of a Grignard reagent for the introduction of versatile organic groups. Grignard reactions are moisture and oxygen sensitive and performed by using aprotic ethers like THF for stabilization of the Grignard reagent. In detail, the organo-magnesium halide performs a nucleophilic attack to carbonyl group under formation of a new covalent C-C bond. Thereby, a six-membered transition state is formed (Fig. 22).¹⁸¹

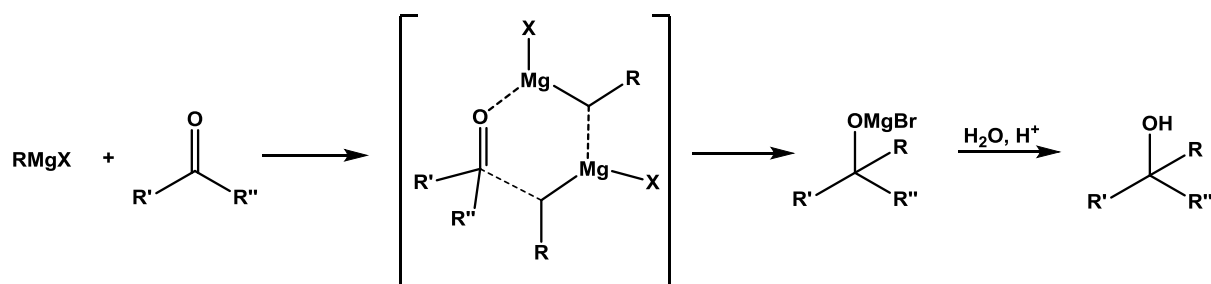


Figure 22: A cursory view of Grignard reaction mechanism.

In case of ^{11}C -labeling, $[^{11}\text{C}]\text{CO}_2$ is released to the Grignard reagent solution with a flow of <10 mL/min. Subsequently, the obtained carboxymagnesium halide can be hydrolyzed with water to obtain the respective carboxylic acid or transformed to an acid chloride for further reactions with amines.^{182–184} From the technical point of view, the Grignard reaction can be performed in a vial (“wet” method) or in a PE loop. For latter one, the Grignard reagent is pushed through a loop of a distinct length in an inert gas stream. Consecutively, $[^{11}\text{C}]\text{CO}_2$ is passed through the impregnated loop and forms the carboxymagnesium halide.^{185–188} This method is used for the synthesis of $[^{11}\text{C}]\text{acetate}$, $[^{11}\text{C}]\text{way-100635}$ and (+)- $[^{11}\text{C}]\text{PHNO}$.^{176,179}

(+)-[¹¹C]PHNO for Imaging the Dopaminergic System

The dopamine receptor subtypes D₂ and D₃ are believed to play a crucial role in several neurological disorders like Parkinson disease, schizophrenia and pathological gambling.^{189–191} Accordingly, development of several antipsychotic drugs focuses on targeting this receptor. The D₂ receptor is a G-protein coupled receptor (GPCR) that is present in two interconvertible states: A G-protein coupled state (D₂^{high}) which is functional and binds its endogenous ligand dopamine, and the uncoupled low affinity state (D₂^{low}).¹⁹² In contrast, a high and low affinity state of the D₃-subtype could not be proven so far, but it is known that the D₃-subtype has a 20 fold higher affinity for dopamine than D₂-receptor.^{193–196}

To assess the physiological processes in psychological disorders, several radiotracers have been developed. Those imaging agents can be subdivided in antagonists, that do not differentiate between high- and low affinity states and agonist, that selectively bind to the high affinity state of the D₂-receptor.¹⁹⁷ [¹¹C]raclopride belongs to the first class and was widely used for imaging of the dopaminergic system.¹⁹⁸ A rational design of the molecular structure could elicit agonist behavior of radiotracers like [¹¹C]PHNO that accumulates in D_{2/3}-receptor rich brain regions like *caudate putamen* and *globus pallidus* (Fig. 23).¹⁹⁹ As a consequence, the application of (+)-[¹¹C]PHNO provided improved insights into the dopaminergic system.²⁰⁰ The emphasis of investigations by using (+)-[¹¹C]PHNO lies in the exploration of physiological processes in patients suffering from schizophrenia.

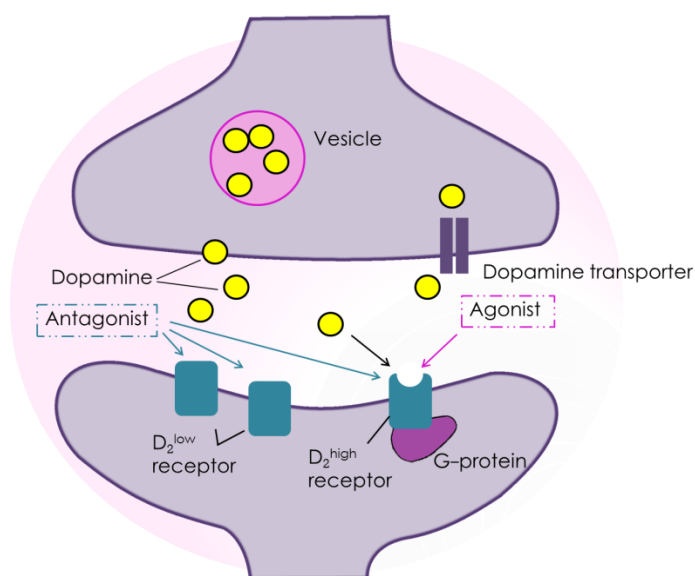


Figure 23: Simplified scheme of synaptic D₂-receptors showing high/low affinity states and the behavior of agonists.

(+)-[¹¹C]PHNO Synthesis

¹¹C-labeling of the progenitor (+)HNO to obtain (+)-[¹¹C]PHNO was firstly described by Wilson *et al.*²⁰¹ This method starts with a Grignard reaction of [¹¹C]CO₂ and EtMgBr as described in chapter ‘Carbon-11’. The addition of phthaloyl dichloride fuels the formation of propionic acid chloride that is distilled in a vial containing the precursor. Accordingly, the intermediate amide is formed and subsequently reduced to (+)-[¹¹C]PHNO at temperatures less than -30°C. Finally, the product is purified by reversed-phase HPLC. This synthesis was fully automated on a commercially available synthesizer by Plisson *et al.* in 2012.²⁰² In 2013, Rami-Mark *et al.* reported a further automated approach applying an ‘in-loop’ approach.¹⁷⁹ This work skips the distillation process and introduces the acid chloride directly into the reactor. Resultantly, the application of chloride donating agent with a high boiling point is redundant and thionyl chloride is used instead of phthaloyl dichloride (Table 2, Fig. 24).

	Wilson <i>et al.</i> 2005	Plisson <i>et al.</i> 2012	Rami-Mark <i>et al.</i> 2013
Synthesis	Robotic arm, automated	Fully automated	Automated
Gignard reaction	In-vial	In-vial	In-loop
Acid chloride formation	Phtaloyl dichloride	Phtaloyl dichloride	SOCl ₂
distillation	Yes	Yes	No
Acylation reactio	60°C	80°C	80°C
Lithium aluminum hydride (LAH) addition	<-30	-15°C	-15°C
Synthesis duration	40 min	35 min	37 min

Table 2: Comparison of (+)-[¹¹C]PHNO synthesis methods of Wilson *et al.*, Plisson *et al.* and Rami-Mark *et al.*^{179,201,202}

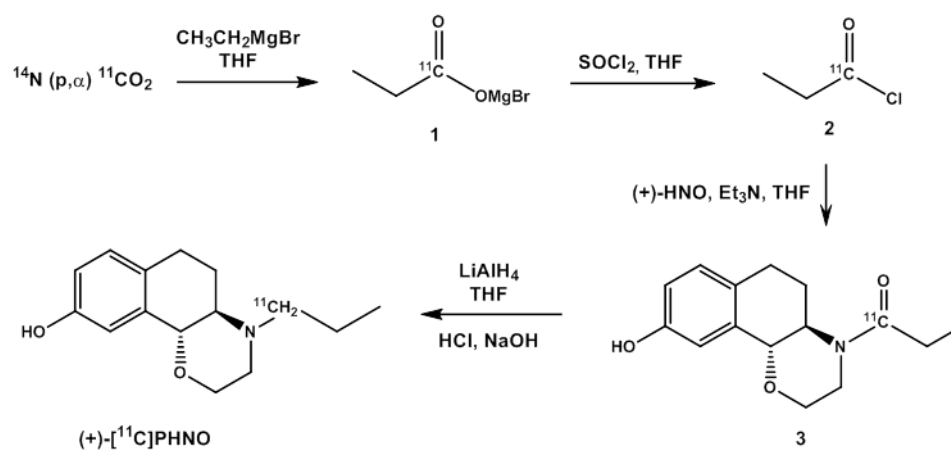


Figure 24: Synthesis scheme of (+)-[^{11}C]PHNO according to Rami-Mark et al.¹⁷⁹

In contrast to other ^{11}C -labeling methods, the synthesis of (+)-[^{11}C]PHNO is highly sophisticated four step procedure. The production prerequisites harsh reaction conditions and inertness as it is sensitive to humidity resulting in a high technical effort. As a consequence, (+)-[^{11}C]PHNO synthesis is prone to errors and demands a time intensive trouble shooting after a failed synthesis. In this light, the production procedure is worth an optimization and exploration of error sources.

II. Aim

This doctoral thesis deals with the improvement of PET-tracer synthesis and the associated quality control by means of HEPES quantification. In particular, [^{68}Ga]Ga-DOTA-NOC, [^{68}Ga]Ga-PSMA-11 and [^{68}Ga]Ga-NODAGA-RGDyk should be synthesized by applying a continuous flow microfluidic device. In this proof of principle study, temperature and precursor amount should be evaluated.

Additionally, ^{68}Ga -complexations of DOTA-NOC, PSMA-11 and -NODAGA-RGDyk should be performed by microwave assisted synthesis to achieve an enhanced conversion due to homogeneous, dielectric heating. Furthermore, a comparison of NaOAc and HEPES as buffer substances should proof the superiority of HEPES for ^{68}Ga -labeling. As HEPES quantification prior radiopharmaceutical release is prescribed by the Ph. Eur., existing analytical methods *via* TLC and HPLC should be evaluated and a novel and robust TLC method should be developed. Moreover, the relatively low limit of HEPES in radiopharmaceuticals was questioned resulting in the task of *in vitro* assessment of this substance. Hence, MTT assays using PC3 cells, PSMA-expressing PC3 B9 cells and PC3 B3 vector control cells should be performed to investigate the impact of HEPES on cell viability. The influence of HEPES on [^{68}Ga]Ga-PSMA-11 uptake should be clarified by uptake experiments in PC3 B9 and PC3 B3 cells.

A further aim of this thesis was the improvement of (+)-[^{11}C]PHNO synthesis for reduction of failed synthesis and not possible scans in clinical studies due to an insufficient activity yield. Therefore, the four step synthesis should be performed without heating and cooling steps after [^{11}C]CO₂ release to reduce synthesis duration and radioactive decay. Besides, the role of all involved reagents and their influence on byproducts as well as intermediate formation should be assessed for an ameliorated troubleshooting protocol.

In summary, this study should provide novel approaches for PET-tracer synthesis and quality control in order to facilitate the performance of clinical routine procedures and studies. Moreover, *in vitro* investigations on HEPES should evoke a reconsideration of the low HEPES limit resulting in a convenient use of this substance for clinical applications.

III. Publications

Peer Reviewed Manuscripts

Manuscript I

S. Pfaff, C. Philippe, V. Pichler, M. Hacker, M. Mitterhauser, W. Wadsak; Microfluidic ^{68}Ga -labeling: A proof of principle study. *Dalton Trans.*, 2018, 47 pp 5997-6004

Contribution: Experimental design, synthesis, analysis, evaluation of data, preparation of the manuscript

Manuscript II

S. Pfaff, T. Nehring, V. Pichler, J. Cardinale, M. Hacker, M. Mitterhauser, W. Wadsak; Development and evaluation of a rapid analysis for HEPES determination in ^{68}Ga -radiotracers. *EJNMMI Research*, 2018, 8 (95), in press (<https://doi.org/10.1186/s13550-018-0449-6>)

Contribution: Experimental design, synthesis, analysis, evaluation of data, preparation of the manuscript

Manuscript III

S. Pfaff, C. Philippe, L. Nics, N. Berroterán-Infante, K. Pallitsch, C. Rami-Mark, A. Weidenauer, U. Sauerzopf, M. Willeit, M. Mitterhauser, M. Hacker, W. Wadsak, V. Pichler; Toward the optimization of (+)-[^{11}C]PHNO synthesis: time reduction and process validation. Submitted in *Contrast Media Mol. Imaging* on 20.01.2019

Contribution: Experimental design, synthesis, HPLC measurements, evaluation of data, preparation of the manuscript.

Manuscripts in Preparation:

Manuscript IV: HEPES: Microwave Assisted ^{68}Ga -labeling

Manuscript V: HEPES: Impact on Cell Viability and [^{68}Ga]Ga-PSMA-11 Accumulation

Peer Reviewed Manuscripts

Manuscript I

Microfluidic ^{68}Ga -labeling: A proof of principle study

Sarah Pfaff^{1,2}, Cecile Philippe¹, Verena Pichler¹, Marcus Hacker¹, Markus Mitterhauser^{1,3},
Wolfgang Wadsak^{1,2,4,#}

1 Division of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria

2 Department of Inorganic Chemistry, University of Vienna, Vienna, Austria

3 Ludwig Boltzmann Institute Applied Diagnostics, Vienna, Austria

4 CBmed – Center for Biomarker Research in Medicine, Graz, Austria

#corresponding author

PAPER



Cite this: *Dalton Trans.*, 2018, **47**, 5997

Microfluidic ^{68}Ga -labeling: a proof of principle study

Sarah Pfaff,^{a,b} Cecile Philippe,^a Verena Pichler,^a Marcus Hacker,^a Markus Mitterhauser^{a,c} and Wolfgang Wadsak ^{*a,b,d}

Positron emission tomography (PET) as a tool for molecular imaging of cancer has gained huge interest in the last few years. Gallium-68 is a popular PET nuclide due to its favorable characteristics, like advantageous half-life (68 min) and independency of a cyclotron on-site for its production. Accordingly, several ^{68}Ga -complexes for cancer imaging via PET have been made available during the last few years. In this work, ^{68}Ga -labeled compounds were synthesized applying a commercially available microfluidic device for the first time. Therefore, a proof of principle study using three important radiotracers, [^{68}Ga]Ga-PSMA-11, [^{68}Ga]Ga-NODAGA-RGDyk and [^{68}Ga]Ga-DOTA-NOC, was designed. For all three radioligands, various synthesis parameters were evaluated and the feasibility of using a continuous flow reactor was assessed. All of the precursors were successfully radiolabeled with a radiochemical yield higher than 80%, proving the principle that a microfluidic set-up is a suitable approach for the production of ^{68}Ga -labeled tracers.

Received 12th January 2018,
Accepted 26th March 2018

DOI: 10.1039/c8dt00158h

rsc.li/dalton

Introduction

Treatment of cancer and its detection and evaluation using molecular diagnostics have become a hot topic in modern precision medicine. The application of radiotracers dramatically facilitated molecular tumor imaging and, even more important, tumor staging (e.g. detecting lymph node involvement and/or distant metastases). Additionally, this noninvasive technique is much sought-after in the field of personalized medicine. Thus, diversity and availability of radiotracers and especially radioligands for positron emission tomography (PET) in medical research are expanding fast. One favorable PET nuclide is gallium-68 as it offers production using a radio-nuclide generator and it has a suitable half-life (*i.e.* 68 min). Its breakthrough is attributed to ^{68}Ga -labeling of DOTA-octreotides used in the assessment of neuroendocrine tumors (NETs).¹ Since then, investigations in developing novel ^{68}Ga -radiotracers have become a new and relevant focus in nuclear medicine and the number of ^{68}Ga -related publications has been increasing dramatically.² Another reason for this nuclide's popularity is given by the fast and easy preparation

of ^{68}Ga -complexes, provided that the design of the precursor is feasible and an accurate system for labeling is present. In clinical routine, several devices for labeling with radionuclides including gallium-68 have been developed and commercialized in order to meet the demands of radiation protection and high quality standards of radiopharmaceuticals. Commonly used synthesizers are fully automated and either set up as fixed vessel based systems or single use cassette based systems. Moreover, several techniques like microfluidic syntheses have already been tested for production of PET tracers in general.³ These approaches should broaden the range of versatility of synthesis devices for individual synthesis optimization as well as facilitate the handling and enhance the radiochemical conversion. Especially for ^{18}F -labeling, microfluidic systems achieved higher conversions than conventional synthesizers or even enabled syntheses that failed using other modalities.^{4,5} Additionally, Selivanova *et al.* reported a microfluidic approach viable even for radiolabeling of peptides.⁶ These results were obtained by applying a flow through reactor featuring the technical characteristics of an extremely high surface to volume ratio that leads to an enhanced mass transfer.⁷ Furthermore, the small diameters of the reactor capillaries result in a high pressure offering the opportunity to heat solvents to a temperature above their boiling point making a higher chemical conversion rate possible. Besides, microfluidic techniques are highly suitable for optimizing synthesis parameters as they enable a high throughput evaluation using small volumes and therefore only require a minimum amount of precursor.^{8,9} Additionally, microfluidic synthesizers

^aDivision of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria.

E-mail: wolfgang.wadsak@meduniwien.ac.at; <http://www.meduniwien.ac.at/nuklear-medicin>; Tel: +43 (1) 40400-55330

^bDepartment of Inorganic Chemistry, University of Vienna, Vienna, Austria

^cLudwig Boltzmann Institute Applied Diagnostics, Vienna, Austria

^dCBMed – Center for Biomarker Research in Medicine, Graz, Austria

enable the preparation of only small product fractions in dose on demand mode.¹⁰ For example, Pascali *et al.* described the successful set-up of a dose on demand study labeling different choline derivatives with fluorine-18 to produce [¹⁸F]fluoromethylcholine, [¹⁸F]fluoroethylcholine and [¹⁸F]fluoropropylcholine, respectively, with remarkable yields.^{11,12} Additionally, Liang *et al.* demonstrated that radiopharmaceuticals prepared within a continuous flow microreactor can be applied in a clinical setting.¹³

In the literature, there are only a very limited number of publications reporting ⁶⁸Ga-labeling in a microfluidic synthesizer so far.^{8,14,15} As a consequence, this work aims at designing an experimental set up using a commercially available, microfluidic, flow-through system and demonstrating its principal feasibility for ⁶⁸Ga-complexation reactions. Furthermore, pre-clinical investigations of ⁶⁸Ga-radiotracers do not require high amounts of radioactivity. Thus, this study additionally aims at the production of ⁶⁸Ga-radiotracers following a dose on demand concept forthcoming.

For evaluation of radiolabeling applying a microfluidic flow-through reactor [⁶⁸Ga]Ga-PSMA-11 (Glu-CO-Lys(Ahx)-[[⁶⁸Ga]Ga(HBED-CC)], [⁶⁸Ga]Ga-NODAGA-RGDyK (cyclo[Arg-Gly-Asp-D-Tyr-Lys[NODAGA([⁶⁸Ga]Ga)]] and [⁶⁸Ga]Ga-DOTA-NOC (DOTA([⁶⁸Ga]Ga)-D-Phe-Cys-1-Nal-D-Trp-Lys-Thr-Cys-Thr(ol), cyclic disulfide) were chosen as representative ⁶⁸Ga-radiotracers as they provide three different chelating systems (*i.e.*, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), NOTA (2,2',2''-(1,4,7-triazacyclononane-1,4,7-triyl)triacetic acid), and HBED (*N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid))^{16–18} (Fig. 1). These chelators are widely used in radiotracer development as well as in established imaging agents. In this study, the reaction parameters, *e.g.*, reaction temperature and precursor concentration, for ⁶⁸Ga-labeling of the respective precursors were optimized.

In parallel to our investigations, Máté *et al.* recently reported ⁶⁸Ga-complexation in a self-made microfluidic system comprising a PEEK loop as the reaction vessel and focusing on two different RGD-compounds and their chelating moieties.⁸ In contrast, the aim of this study was the first use of a commercially available device incorporating a silica capillary with a volumetric capacity of 15.6 µL as the reaction tube that is applied to three different classes of compounds, including PSMA-11 (targeting the prostate specific membrane antigen), NODAGA-RGDyK (targeting α_vβ₃ integrin receptor) and DOTA-NOC (targeting somatostatin receptors).

Experimental

Materials

Gallium-68 was obtained from a ⁶⁸Ge/⁶⁸Ga-generator (1.85GBq, Galliapharm, Eckert & Ziegler, Berlin, Germany) that was eluted with sterile, GMP certificated 0.1 M HCl purchased from ROTEM (Mishor Yamin D.N Arava, Israel). The GMP-grade precursors (PSMA-11, DOTA-NOC and NODAGA-RGDyK) as well as HEPES buffer were obtained from ABX (Radeberg, Germany). TraceSELECT® water from Fluka (Neu-Ulm, Germany) was used for washing the microfluidic synthesizer. All other chemicals were of analytical grade, purchased from Sigma Aldrich, Merck (Vienna, Austria) or Fluka and used without further purification. High pressure liquid chromatography (HPLC) was performed using an Agilent Technologies HPLC system (Series 1200) with a variable wavelength detector, a scintillation detector from Elysia Raytest (Gabi Star 2 × 2") and a Chromolith® Performance, RP-18e, 100-4.6 mm from Merck (Germany). Radio thin layer chromatography (TLC) was performed using silica gel impregnated paperstrips purchased from Agilent Technologies (Santa Clara, USA). ITLC (instant

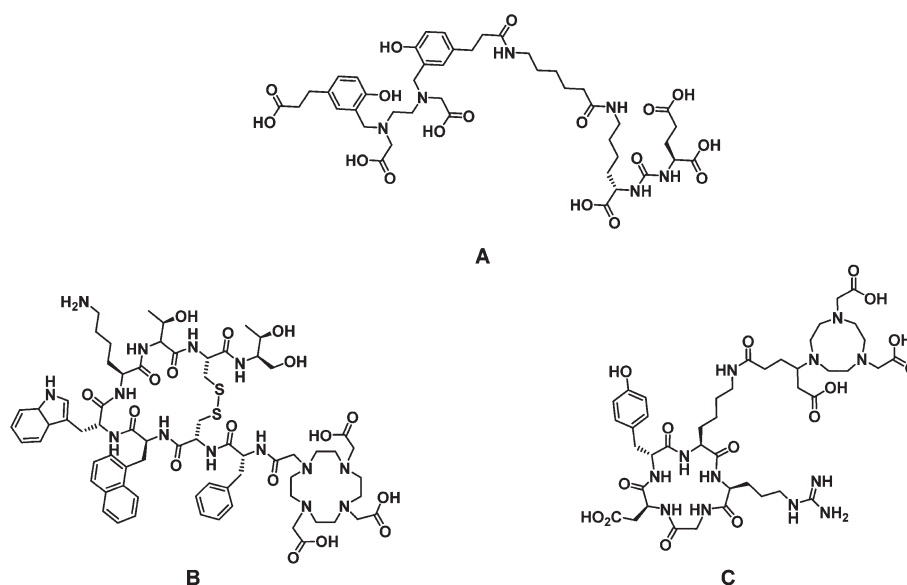


Fig. 1 Chemical structures of the precursors for ⁶⁸Ga-labeling: PSMA-11 (A), DOTA-NOC (B), NODAGA-RGDyK (C).

TLC) strips were analyzed with a Canberra-Packard Instant Imager (PerkinElmer, Watford, UK). All syntheses were conducted in a lead-shielded hot cell (Comecer, Castel Bolognese, Italy). The labelling reactions were performed using an Advion NanoTek microfluidic synthesizer (Ithaca, NY, USA) containing a concentrator unit (CE) and a liquid flow reaction unit (LF) operated by a dedicated control software (Advion, version 1.3.2). The microreactor is made of a fused silica tube ($\varnothing = 100\ \mu\text{m}$, length 2 m, volume 15.6 μL) embedded in a brass ring for optimized heat transfer.

General procedure

Gallium-68 was obtained by eluting a $^{68}\text{Ge}/^{68}\text{Ga}$ -generator with 0.1 M HCl according to a fractionized protocol and the fraction containing the highest amount of activity ("main fraction" *ca.* 2 mL) was used for radiolabeling.¹⁹ For all one-step complexation reactions, the ^{68}Ga -eluate was placed in the concentrator unit and the HEPES-buffered (5.5 M) precursor solution was prepared in an Eppendorf vial next to pump 1 that is responsible for transferring the solution (see Fig. 2). The two reactants, *i.e.*, the precursor and ^{68}Ga -eluate, were loaded into the intended respective storage loops *via* pump 1 and pump 2. Preset volumes (up to the whole volume of the loop; 20–370 μL) were used for the complexation reactions. Accordingly, the chosen volumes of the reaction solutions were simultaneously pumped through the pre-heated flow through reactor at a flow rate of 50 $\mu\text{L}\ \text{min}^{-1}$ for complexation reactions at 25 $^{\circ}\text{C}$ and 80 $\mu\text{L}\ \text{min}^{-1}$ at 100 and 150 $^{\circ}\text{C}$, respectively. After finishing the reactants' transfer, the system was swept with highest purity water (TraceSELECT®). No washing steps were performed between the reactions. The synthesizer was washed before loading of the loop and after the performed experiments with TraceSELECT® water. The crude product was collected and TLC- and HPLC-measurements were performed for determining conversion rates.

Recovery experiments of $[^{68}\text{Ga}]\text{Ga}^{3+}$ ions were performed by applying the same set up, but without a precursor.

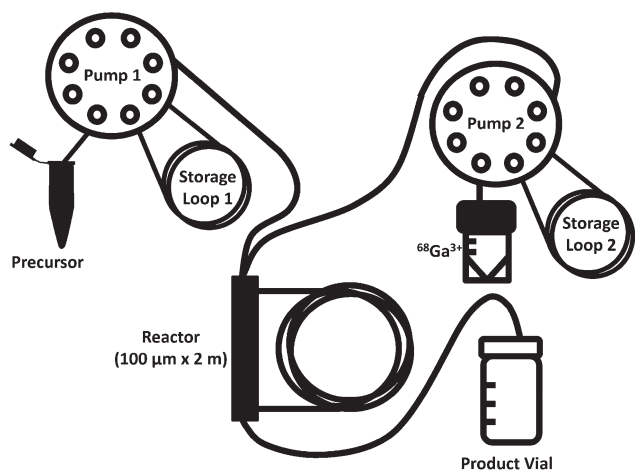


Fig. 2 Scheme of the Advion Nanotek LF synthesizer used for radio-labeling of DOTA-NOC, NODAGA-RGDyk and PSMA-11 with gallium-68.

Calculation of the radiochemical yield (RCY)

The starting activity of the ^{68}Ga -eluate varied for each experiment from 9–250 MBq, depending on the chosen volume of the bolus and the time of elution. The crude product's activity yield was determined by calculating the starting activity per unit volume and, further on, the corresponding activity of the initial volume of the eluate. Accordingly, the radiochemical yield was determined as follows:

- The activity of the main fraction of the eluate (A_e) with a specific volume (V_e [μL]) was measured with a dose calibrator.
- The theoretically used activity per experiment (A_1) was calculated using the respective volume (V_1) in μL .

$$\frac{A_e}{V_e} \cdot V_1 = A_1$$

- The activity of the crude mixture (A_c) in the product vial was measured.
- The radiochemical conversion rate (RCR [%]) of the crude mixture was determined *via* HPLC and TLC.
- The product activity (A_p) was then calculated by multiplying the total radioactivity measured in the crude product (A_c) with the RCR and corrected for decay. (Half-life $^{68}\text{Ga} = 67.8\ \text{min}$; Δt is the time between measurement of A_c and A_1 .)

$$A_p = \frac{A_c \times \frac{\text{RCR}}{100}}{e^{-\frac{\ln 2 \times \Delta t}{67.8}}}$$

- Finally, the RCY [%] was calculated by dividing the product activity (A_p) by the initial starting activity (A_1) and is in accordance with nomenclature guidelines.²⁰

$$\text{RCY} = \frac{A_p}{A_1} \times 100[\%]$$

For comparison reasons, also the radiochemical conversion rate (RCR [%]) which is often given in published work was determined. RCR directly reflects the RCP and is determined by analyzing all impurities *via* HPLC and TLC.

Reaction conditions

For evaluation of ^{68}Ga -labeling using a microfluidic device, gallium-68 was complexed by PSMA-11, DOTA-NOC and NODAGA-RGDyk applying the parameters shown in Fig. 3.

Chromatographic analyses

Radiochemical purity was determined by HPLC and TLC. For HPLC measurements, the mobile phase was a mixture of aqua bidest (A) and acetonitrile (B), both containing 0.1% TFA with a flow rate of 2 $\text{mL}\ \text{min}^{-1}$ (gradient: 0.0–0.6 min 95% A, linear reduction to 50% A from 0.6 to 6.0 min, isocratic flow with 50% A from 6.0 to 7.0 min). Radio TLC was performed by spotting the product on silica gel impregnated paper and using two solvent mixtures: (1) 0.1 M citrate solution (pH 5); (2) 1 M $\text{NH}_4\text{OAc}/\text{MeOH}$ (1 : 1).

Temperature [°C]	Flow [mL/min]	[⁶⁸ Ga]Ga-DOTA-NOC	[⁶⁸ Ga]Ga-NODAGA-RGDyk	[⁶⁸ Ga]Ga-PSMA-11
Concentration [μg/mL]				
25	50	• 30 • 40	• 5 • 10 • 15 • 20	• 1 • 3 • 5
100	80			
150	80			

Fig. 3 Overview on reaction parameters tested for the complexation reaction of gallium-68 with DOTA-NOC, NODAGA-RGDyk and PSMA-11.

Statistical analyses

If not stated otherwise, all data are given as arithmetic means $\pm$ standard deviation. Statistical analysis of the mean values of experimental data ($n \geq 3$) was performed using a one-way or two-way ANOVA multiple comparison test according to Tukey (or Sidak's analytical comparison of two concentrations) within GraphPad Prism (Graphpad software, California, USA). Differences of data with $P \leq 0.05$ were considered as statistically significant. All figures display only highly significant ($P \leq 0.01$) differences.

Results & discussion

The here presented study shows the successful synthesis of three different gallium-68 labeled compounds by means of a commercially available microfluidic continuous flow system for the first time. Three precursors, namely PSMA-11, DOTA-NOC and NODAGA-RGDyk, were chosen due to their clinical relevance. In this study, temperatures of 25, 100, and 150 °C as well as different precursor concentrations were evaluated. All tested compounds were successfully labeled with gallium-68 when adequate reaction conditions were applied.

Flow rate

In general, the flow rate was set as high as possible in order to minimize the reaction time to counteract decay. A flow rate of 80 $\mu\text{L min}^{-1}$ was chosen according to the pressure limit of the synthesizer. Furthermore, preliminary experiments showed that a reduced flow rate to 60 $\mu\text{L min}^{-1}$ did not influence the results of the experiments. Nevertheless, this flow rate was too high for labeling at 25 °C and caused overpressures due to the higher viscosity of liquids at lower temperature. Therefore, all further experiments at 25 °C were conducted with a reduced flow of 50 $\mu\text{L min}^{-1}$.

Compound concentration & temperature

There was a trend of enhanced RCY at higher temperatures observable within the tested radiotracers. However, results showed distinct discrepancies between the radiochemical yield (RCY) and the radiochemical conversion rate (RCR). Therefore, it is of high interest to have a closer look on these differences. In the following section, RCY, RCR, and the reproducibility of the syntheses of [⁶⁸Ga]Ga-PSMA-11, [⁶⁸Ga]Ga-DOTA-NOC, and [⁶⁸Ga]Ga-NODAGA-RGDyk are described in detail.

[⁶⁸Ga]Ga-DOTA-NOC

⁶⁸Ga-labeling of DOTA-NOC was investigated in two concentrations 30 and 40 $\mu\text{g mL}^{-1}$ at 25, 100 and 150 °C (Fig. 4). The

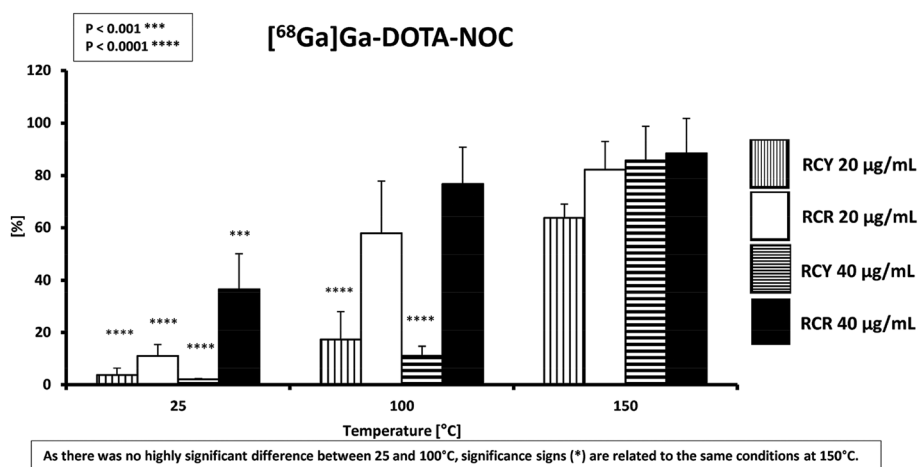


Fig. 4 Comparison of RCY and RCR (conversion) of the radiolabeling of DOTA-NOC with gallium-68 conducted using different concentrations as well as temperatures.

highest turnover in radiolabeling was achieved at 150 °C in terms of RCR and RCY. Labeling-temperatures below 100 °C resulted in poor conversion rates for DOTA-NOC, as already published elsewhere for DOTA peptides.²¹ A higher precursor concentration caused a trend of enhanced RCR at all temperatures. An increased temperature from 25 to 150 °C showed a highly significant ($P \leq 0.001$) enhancement of the RCR as well as RCY for both concentrations. Accordingly, higher RCYs were obtained by increasing the temperature from 100 to 150 °C ($P \leq 0.0001$).

Besides, reproducibility increased at higher temperatures and an enhanced precursor amount. Furthermore, a higher precursor concentration shows a trend of higher reproducibility at 25° and 100 °C although the used precursor concentrations showed no significant difference in terms of RCY and RCR. Furthermore, enormous discrepancies occurred between the RCR and RCY for the reaction conducted at 25 and 100 °C. These results implicate a high unspecific binding to the synthesizer material of either [⁶⁸Ga]Ga-DOTA-NOC or gallium-68 species at 25 and 100 °C, which will be discussed later in this section. However, by increasing the temperature to 150 °C, the results for the RCR and RCY could be successfully approximated. This advantageous application of temperatures above the boiling point of the aqueous reaction mixture can be performed easily in a microfluidic device with respect to applied high pressures (at 150 °C the water's vapor pressure is ≈ 470 kPa). As a result, ⁶⁸Ga-labeling of DOTA-NOC might be favorably performed exploiting microfluidic devices, leading to almost quantitative conversion.

[⁶⁸Ga]Ga-NODAGA-RGDyk

The reaction of gallium-68 with NODAGA-RGDyk was performed at lower concentrations (5, 10, 15 and 20 $\mu\text{g mL}^{-1}$), but at the same temperatures as for [⁶⁸Ga]Ga-DOTA-NOC (Fig. 3 and 5). The complexation did not work at the lower precursor concentrations of 5 and 10 $\mu\text{g mL}^{-1}$. A concentration of at

least 15 $\mu\text{g mL}^{-1}$ was necessary to obtain the ⁶⁸Ga-labeled product. The RCR of NODAGA-RGDyk was comparable to the one observed for DOTANOC at different temperatures.

The highest RCY of $127.2 \pm 65.0\%$ was found at 150 °C and a precursor concentration of 15 $\mu\text{g mL}^{-1}$ (Fig. 5). An RCY exceeding 100% can be explained by two possible reasons: [⁶⁸Ga]Ga³⁺ ions are not distributed equally within the eluate that is pumped into the storage loop, which is contrary to our basic assumption for calculating the RCY (described above). Hence, one bolus can contain more [⁶⁸Ga]Ga³⁺ ions than calculated and results in an RCY surpassing 100%. The second reason could be sticking of uncomplexed or complexed gallium-68 species in the microreactor which are released at the following higher temperature reaction. However, an enhancement of the concentration from 15 $\mu\text{g mL}^{-1}$ to 20 $\mu\text{g mL}^{-1}$ did neither improve RCY nor reproducibility. Ultimately, both concentrations demonstrated a trend of a higher RCY at labeling temperatures of 100 and 150 °C. RCY and RCR are comparable for both concentrations, which is in contrast to results observed for [⁶⁸Ga]Ga-DOTA-NOC. More than 90% of gallium-68 could be incorporated at 100 and 150 °C and both precursor amounts.

[⁶⁸Ga]Ga-PSMA-11

⁶⁸Ga-complexation with PSMA-11 was performed with precursor concentrations ranging from 1 to 5 $\mu\text{g mL}^{-1}$ (Fig. 6). PSMA-11 showed excellent RCRs and RCYs even at low precursor concentrations and temperatures, which is in good accordance with the literature.^{22,23} Interestingly, a precursor concentration of 1 $\mu\text{g mL}^{-1}$ showed the poorest RCR at the highest temperature unlike other radiotracers. These results could be attributable to a possible decomposition of the precursor at high temperatures. However, concentrations of 3 and 5 $\mu\text{g mL}^{-1}$ enabled conversion rates of more than 95% at all tested temperatures. In contrast, RCYs are following the scheme of higher RCY at higher temperatures. The best RCY ($114.4 \pm 4.0\%$) for

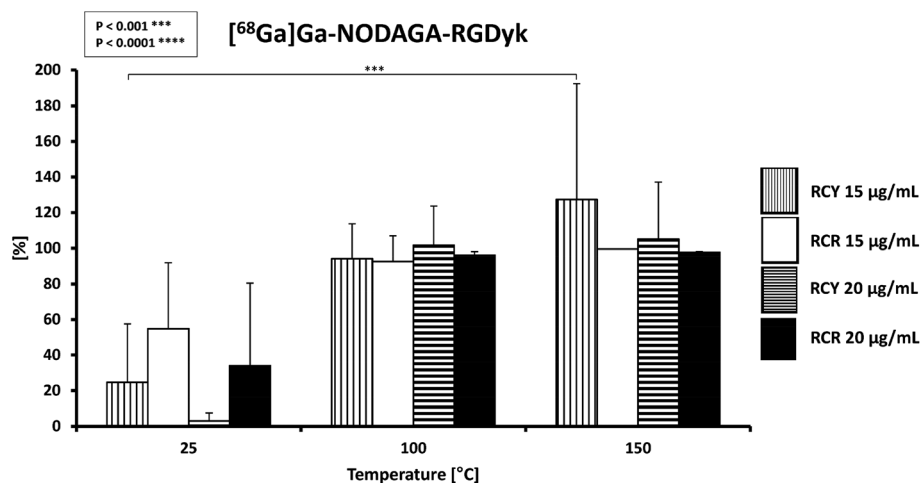


Fig. 5 Comparison of RCY and RCR (conversion) of the radiolabeling of NODAGA-RGDyk with gallium-68 conducted using different concentrations as well as temperatures.

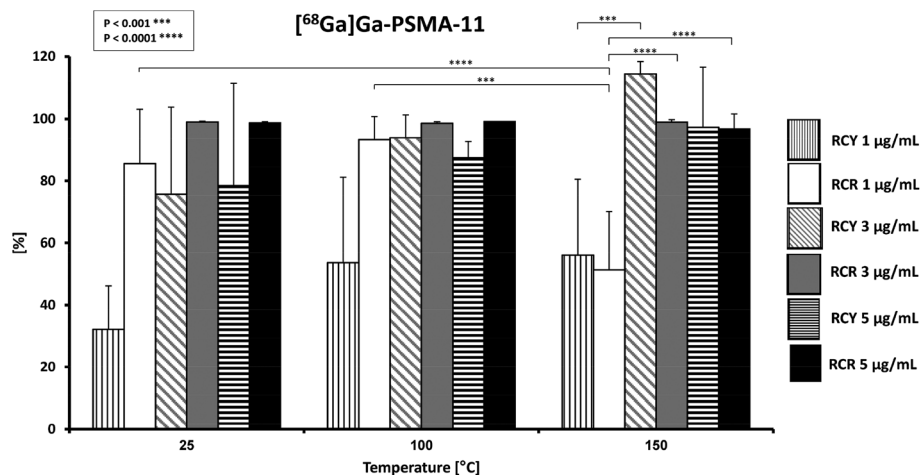


Fig. 6 Comparison of RCY and RCR (conversion) of the radiolabeling of PSMA-11 with gallium-68 conducted using different concentrations as well as temperatures.

$[^{68}\text{Ga}]\text{Ga-PSMA-11}$ was found for a concentration of $3\text{ }\mu\text{g mL}^{-1}$ at $150\text{ }^{\circ}\text{C}$. Additionally, results showed that concentrations of 3 and $5\text{ }\mu\text{g mL}^{-1}$ are superior to $1\text{ }\mu\text{g mL}^{-1}$ in terms of reproducibility (*i.e.*, lower standard deviations).

In general, ^{68}Ga -complexation using a microfluidic workflow showed best results for PSMA-11 at all temperatures, followed by NODAGA-RGDyk at $150\text{ }^{\circ}\text{C}$ and with the lowest turnover for DOTA-NOC. This is probably attributable to the different chelating units. In contrast to DOTA and NODAGA, PSMA-11's chelator, HBED-CC, is an acyclic chelating unit. Hence, formation of the ^{68}Ga -complex is much faster in

comparison with the cyclic DOTA and NODAGA ligands. ^{68}Ga -complexation of NODAGA is superior to DOTA as the ion radius and coordination number of gallium are more suitable for incorporation into NODAGA.

Recovery

Having a closer look on the results regarding unspecific binding to materials of the synthesizer, the obtained data indicate a correlation between the temperature and overall recovered radioactivity. The quantification of recovery is depicted in Fig. 7; subsequently, the decay-corrected radioactivity of the

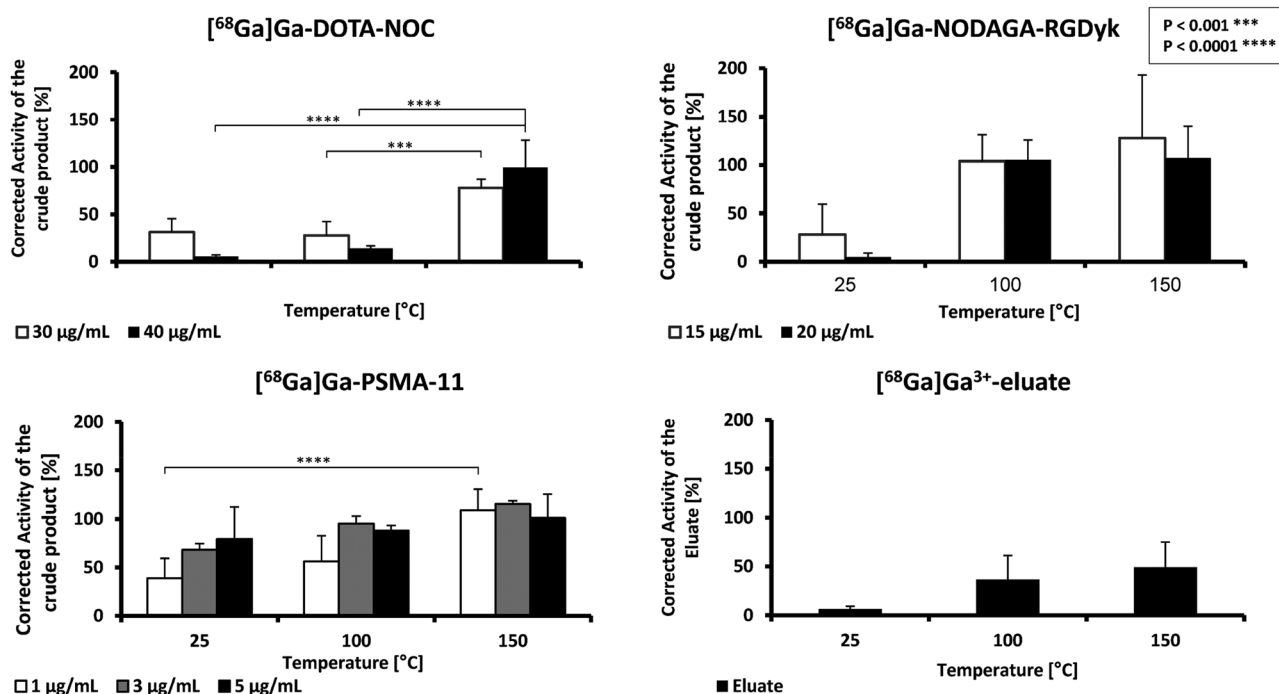


Fig. 7 Overview on the recovery observed for the reaction of gallium-68 with DOTA-NOC, NODAGA-RGDyk, PSMA-11, respectively, as well as for $[^{68}\text{Ga}]\text{Ga}^{3+}$ without complexation agents.

crude product (sum of all ^{68}Ga -species present; as percentage of the starting activity) was plotted against the temperature for each tracer.

All compounds showed a clear trend of increased recovery at higher temperatures. Differences between the ligands and chemical species are evident. Furthermore, values higher than 100% were observed supporting the assumption that the respective compound, which was adsorbed in previous runs, could be partially recovered in later experiments.

In this context, also the elucidation of the adsorption of unbound $^{68}\text{GaGa}^{3+}$ ions in the microfluidic device was assessed. For this purpose, the reaction conditions were mimicked using ^{68}Ga -eluate, HEPES buffer in the absence of the precursor. Then, these two solutions were pumped at different temperatures through the reactor and collected in a product vial. The adsorption of the unbound $^{68}\text{GaGa}^{3+}$ ions proved to be dependent on the temperature and showed a trend of lower activity loss at higher temperatures. A possible explanation for this phenomenon is an enhanced vibration and rotation of molecular bonds in the reaction solution (or even in the synthesizer materials), which result in lower capability of unspecific binding. Consequently, these results suggest to apply higher reaction temperatures for successful increase of the recovered activity.

Having demonstrated that a microfluidic set-up can be successfully used for ^{68}Ga -radiosyntheses, its advantages regarding radiation protection for the operators during optimization of the reaction conditions and its usefulness in dose-on-demand preparation of different radiotracers from one single batch of radionuclide can now be fully exploited. As research in radiochemistry is mainly based on manual radiolabeling experiments resulting in a high radiation exposure to the operators, the results of this study additionally lay the foundation for performing high throughput screening of synthesis parameters applying the proposed microfluidic set-up in the future. Consequently, a drastic reduction of the radiation dose could be achieved by the researchers. Besides, it is now possible to design a dose-on-demand production also for ^{68}Ga -radiotracers according to recently published work using other radionuclides.¹⁰ Especially, in view of upcoming production options for gallium-68 in high amounts and radioactivity concentrations using cyclotrons,²⁴ the possibility to produce two or more radiotracers back to back from one single batch of the radionuclide is tempting and could reduce radiolysis as already shown for fluorine-18.²⁵ These opportunities could provide a boost to the field of microfluidics in radio-pharmaceutical preparations.

Conclusions

^{68}Ga -incorporation for radiotracer production was successfully performed for the first time by means of a commercially available microfluidic continuous flow synthesizer using gallium-68 and several clinically relevant compounds. This study clearly demonstrated the suitability of a microfluidic device for

^{68}Ga -incorporation for radiotracer production. Optimized labeling conditions for three representative compounds, *i.e.*, $^{68}\text{GaGa}$ -PSMA-11, $^{68}\text{GaGa}$ -DOTA-NOC, and $^{68}\text{GaGa}$ -NODAGA-RGDyk, were determined. However, discrepancies between RCR and RCY were observed that were effectively reduced by applying higher temperatures, which lead to a substantial increase in recovery of radiolabeled species. Summarizing, all tested precursors were successfully radiolabeled with high radiochemical yields, proving the principal suitability of this approach. Hence, setting up a dose on demand approach with its already described advantages is possible for ^{68}Ga -radiopharmaceuticals in the future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Ingrid Leitinger and Friedrich Girschele are acknowledged for their help in standard ^{68}Ga -radiosyntheses and QC. Andreas Krcal and Thomas Zenz are thanked for their technical support.

References

- 1 M. Henze, *et al.*, PET imaging of somatostatin receptors using $^{68}\text{GaGa}$ -DOTA-D-Phe1-Tyr3-octreotide: first results in patients with meningiomas, *J. Nucl. Med.*, 2001, **42**, 1053–1056.
- 2 I. Velikyan, Prospective of ^{68}Ga -Radiopharmaceutical Development, *Theranostics*, 2014, **4**, 47–80.
- 3 C.-C. Lee, Multistep Synthesis of a Radiolabeled Imaging Probe Using Integrated Microfluidics, *Science*, 2005, **310**, 1793–1796.
- 4 C. Philippe, *et al.*, ^{18}F -FE@SNAP—A new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1): Microfluidic and vessel-based approaches, *Bioorg. Med. Chem.*, 2012, **20**, 5936–5940.
- 5 J. Ungersboeck, *et al.*, Microfluidic preparation of ^{18}F -FE@SUPPLY and ^{18}F -FE@SUPPLY:2 — comparison with conventional radiosyntheses, *Nucl. Med. Biol.*, 2011, **38**, 427–434.
- 6 S. Selivanova, *et al.*, Single-step radiofluorination of peptides using continuous flow microreactor, *Org. Biomol. Chem.*, 2012, **10**, 3871–3874.
- 7 P. Miller, Radiolabelling with short-lived PET (positron emission tomography) isotopes using microfluidic reactors, *J. Chem. Technol. Biotechnol.*, 2009, **84**, 309–315.
- 8 G. Máté, *et al.*, Multiparametric labeling optimization and synthesis of ^{68}Ga -labeled compounds applying a continuous-flow microfluidic methodology, *J. Flow Chem.*, 2016, **6**, 86–93.

- 9 H. Audrain, Positron Emission Tomography (PET) and Microfluidic Devices: A Breakthrough on the Microscale?, *Angew. Chem., Int. Ed.*, 2007, **46**, 1772–1775.
- 10 L. Matesic, A. Kallinen, I. Greguric and G. Pascali, Dose-on-demand production of diverse 18 F-radiotracers for pre-clinical applications using a continuous flow microfluidic system, *Nucl. Med. Biol.*, 2017, **52**, 24–31.
- 11 G. Pascali, G. Mazzone, G. Saccomanni, C. Manera and P. A. Salvadori, Microfluidic approach for fast labeling optimization and dose-on-demand implementation, *Nucl. Med. Biol.*, 2010, **37**, 547–555.
- 12 G. Pascali, G. Nannavecchia, S. Pitzianti and P. A. Salvadori, Dose-on-demand of diverse 18F-fluorocholine derivatives through a two-step microfluidic approach, *Nucl. Med. Biol.*, 2011, **38**, 637–644.
- 13 S. H. Liang, *et al.*, First Human Use of a Radiopharmaceutical Prepared by Continuous-Flow Microfluidic Radiofluorination: Proof of Concept with the Tau Imaging Agent [18F]T807, *Mol. Imaging*, 2014, **13**, 7290201400025.
- 14 D. Zeng, *et al.*, Microfluidic radiolabeling of biomolecules with PET radiometals, *Nucl. Med. Biol.*, 2013, **40**, 42–51.
- 15 Y. Liu, M. Tian and H. Zhang, Microfluidics for Synthesis of Peptide-Based PET Tracers, *BioMed Res. Int.*, 2013, **2013**, 1–8.
- 16 F. L. Eplattenier, I. Murase and A. E. Martell, New Multidentate Ligands. VI. Chelating Tendencies of N,N'-Di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic Acid, *J. Am. Chem. Soc.*, 1967, **89**, 837–843.
- 17 H. Stetter and W. Frank, Complex Formation with Tetraazacycloalkane-N,N',N'',N'''-tetraacetic Acids as a Function of Ring Size, *Angew. Chem., Int. Ed. Engl.*, 1976, **15**, 686–686.
- 18 M. Fani, J. P. André and H. R. Maecke, 68Ga-PET: a powerful generator-based alternative to cyclotron-based PET radiopharmaceuticals, *Contrast Media Mol. Imaging*, 2008, **3**, 53–63.
- 19 H. Eidherr, F. Girschele, M. Mitterhauser and W. Wadsak, Synthesis of [68Ga]gallium DOTA-(Tyr3)-octreotide acetata (68 Ga-DOTATOC), in *Radiochemical Syntheses*, Wiley, 2012, vol. 1, pp. 321–334.
- 20 H. H. Coenen, *et al.*, Consensus nomenclature rules for radiopharmaceutical chemistry—Setting the record straight, *Nucl. Med. Biol.*, 2017, **55**, v–xi.
- 21 G.-J. Meyer, H. Mäcke, J. Schuhmacher, W. H. Knapp and M. Hofmann, 68Ga-labelled DOTA-derivatised peptide ligands, *Eur. J. Nucl. Med. Mol. Imaging*, 2004, **31**, 1097–1104.
- 22 M. Eder, *et al.*, ⁶⁸Ga-Complex Lipophilicity and the Targeting Property of a Urea-Based PSMA Inhibitor for PET Imaging, *Bioconjugate Chem.*, 2012, **23**, 688–697.
- 23 H. Eidherr, *et al.*, Fully automated preparation of 68Ga PSMA-HBEC-CC using fractionated elution, *Eur. J. Nucl. Med. Mol. Imaging*, 2014, **41**, 428.
- 24 M. Lin, U. Mukhopadhyay, W. Waligorski, J. Balatoni and C. Gonzales-Lepera, Production of Curie quantities of 68Ga with a medical cyclotron via the 68Zn(p,n)68Ga reaction, *J. Nucl. Med.*, 2017, **58**, 336.
- 25 C. Rensch, *et al.*, Microfluidic reactor geometries for radiolysis reduction in radiopharmaceuticals, *Appl. Radiat. Isot.*, 2012, **70**, 1691–1697.

Manuscript II

Development and evaluation of a rapid analysis for HEPES determination in ^{68}Ga - radiotracers

Sarah Pfaff^{1,2}, Tina Nehring^{1,3,4}, Verena Pichler¹, Jens Cardinale^{1,3}, Markus Mitterhauser^{1,3}, Marcus Hacker¹, Wolfgang Wadsak^{1,2,4#}

¹ *Division of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria*

² *Department of Inorganic Chemistry, University of Vienna, Vienna, Austria*

³ *Ludwig Boltzmann Institute Applied Diagnostics, Vienna, Austria*

⁴ *CBmed – Center for Biomarker Research in Medicine, Graz, Austria*

#corresponding author

ORIGINAL RESEARCH

Open Access



Development and evaluation of a rapid analysis for HEPES determination in ^{68}Ga -radiotracers

Sarah Pfaff^{1,2}, Tina Nehring^{1,3,4}, Verena Pichler¹, Jens Cardinale^{1,3}, Markus Mitterhauser^{1,3}, Marcus Hacker¹ and Wolfgang Wadsak^{1,2,4*} 

Abstract

Background: HEPES is a favorable buffer for ^{68}Ga -complexations in radiochemical laboratories. The drawback of this buffer is its prescribed limit of 200 μg per recommended application volume in the final formulation. Currently, a TLC test according to the European Pharmacopoeia (Ph. Eur.) has to be performed for quantification, but this analysis suffers from low reliability and reproducibility and is based on a subjective, semi-quantitative visual evaluation. In this study, the TLC method according to the Ph. Eur. and two literature-known HPLC assays for HEPES quantification were evaluated. Additionally, the development of an improved TLC method was performed.

Results: The assay according to Antunes et al. provided a reasonable quantification of HEPES using HPLC. Additionally, a reliable and conclusive TLC method was developed, which facilitates quantitative analysis by means of a pixel-based evaluation. A comparison of those two methods with the Ph. Eur. TLC assay pinpoints the superiority of the HPLC as well as the new TLC assay. Furthermore, evaluation of HEPES contents using both TLC assays by 28 subjects supported the conclusion that the newly developed TLC method is clearly favorable.

Conclusion: The TLC method according to the Ph. Eur. provides unsatisfactory results in terms of conclusiveness and reproducibility. In contrast, a reported HPLC assay showed valid results, with the drawback of high technical effort. An optimized alternative is provided by the improved TLC method described in this work that results in reliable outcomes and additionally offers quantitative analysis.

Keywords: HEPES, Gallium-68, ^{68}Ga -radiotracer, TLC, HPLC, Quality control, PET

Background

Radiopharmaceutical chemistry deals with short-lived nuclides, especially PET nuclides. Therefore, syntheses of PET radiotracers in this small niche of chemistry have to fulfill special prerequisites like short synthesis duration and minimal radiation exposure of the operator. In the first line, the duration of the semi- to fully automated production should be kept as short as possible to reduce the decay of the nuclide during the synthesis to a minimum. As a result, reaction conditions have to be

optimized to meet the demands of rational PET tracer production.

In this article, the emphasis is put on one important parameter, the pH value that has to be taken into account, especially for complexations with radiometals. At a low pH, the coordinating moieties of the chelator can be protonated and therefore inactivated [1]. Focusing on gallium-68, the pH must be in a range of 3.5 to 5.5 due to the fact that gallium starts to form insoluble colloids and hydroxides at a higher pH that are not available for complexation [2, 3]. Correspondingly, pH buffering systems are necessary for a successful product formation. Appropriate buffers for ^{68}Ga -labeling are supposed to be weak gallium-complexing agents that hinder hydrolysis to hydroxides and keep the metal in its active ionic state [4, 5]. Sodium acetate (NaOAc), sodium

* Correspondence: wolfgang.wadsak@meduniwien.ac.at; <https://radnuk.meduniwien.ac.at/nuklearmedizin/>

¹Division of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

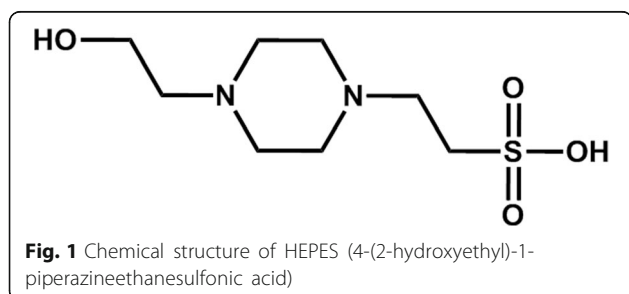
²Department of Inorganic Chemistry, University of Vienna, Vienna, Austria
Full list of author information is available at the end of the article

succinate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were reported as the most favorable buffers for ^{68}Ga -labeling so far [6]. Nevertheless, the latter one was reported to result in better radiochemical yields for ^{68}Ga [Ga]-DOTATOC than the first ones at low precursor amounts [7]. Hence, HEPES should be the buffer of choice for clinical routine productions and especially scientific labeling approaches. However, the drawback of using HEPES is that the European Pharmacopoeia (Ph. Eur.) defines HEPES as an impurity that has to be below a specified limit [8].

One of the standard purification techniques prior to the final formulation of ^{68}Ga -radiopharmaceuticals is the solid-phase extraction using reversed phase cartridges (e.g., SepPak C18 RP), followed by washing steps and finally the elution of the product. While in theory, the buffer should be removed completely during this procedure, in reality, those separations are rarely quantitative, and thus, the remaining content of the buffer has to be quantified prior to administration to patients [9]. For the development of a feasible analytical method, the chemical and physical properties of HEPES should be considered.

HEPES (Fig. 1) is one of the so-called Good's buffers that is designed to meet several demands for biological experiments, for instance, a high water solubility being helpful for ^{68}Ga -labeling in aqueous media [10]. Furthermore, this buffer should not interfere with analytical determination, e.g., UV/vis absorption by its intrinsic absorbance below 200 nm, which impedes the quantification at a low concentration range.

The Ph. Eur. sets a limit of 200 μg HEPES per maximum administration volume (V) (maximum recommended dose in milliliters). V depends on the total activity of the formulated product (A_p) (see model calculation in Fig. 2). The higher the product activity is, the lower is the recommended administered volume (V) that contains the activity which is preferably administered to a patient (A_A) and vice versa. Therefore, V depends on the starting activity (A_0) which is limited by the capacity of the used $^{68}\text{Ge}/^{68}\text{Ga}$ -generator. As a result, V cannot be determined until the activity of the product is known.



The Ph. Eur. stipulates a certain chromatographic testing method to determine the HEPES concentration of the final formulated product in comparison with a reference solution that requires V for its calculation. The prescribed thin-layer chromatography (TLC) analysis is performed by spotting the product solution next to a reference solution. The latter contains the limiting HEPES concentration (which depends on the product activity as explained above) in water. The spotted volume of each solution has to be the recommended administration volume V (in mL) divided by 2000 and spotted in 1 μL steps. After development of the TLC plate, HEPES is visualized by iodine staining and any spot must not be more intense than the reference solution, consequently containing only an equal or lower amount of HEPES.

This TLC assay was reported as neither reliable nor suitable and an improvement of this method would be highly advantageous [11]. As a result, a lot of effort has been made in the optimization and validation of an improved method for quantification of HEPES in radiopharmaceuticals by means of HPLC chromatography according to Kvaternik et al. and Antunes et al. [11–13].

The aim of this study was the comparison and evaluation of the TLC method, proposed by the European Directorate for the Quality of Medicine and Healthcare (EDQM), and established methods described in literature for quantification of HEPES in a complex matrix such as a formulated radiopharmaceutical. Finally, we aimed towards the development of a new, reliable, and reproducible method for quantitative HEPES analysis dealing with identified drawbacks as well as keeping the technical demands as low as possible.

Methods

Materials

All chemicals were used without further purification. The precursors, Pentixafor and PSMA-11 (both GMP grade), for ^{68}Ga -labeling were provided from ABX (Radeberg, Germany). All other chemicals were obtained from Sigma Aldrich, Merck, Fluka, or Honeywell. Syntheses were performed using a synthesizer (GRP®) from Scintomics (Fürstfeldbruck, Germany) with disposable single-use cassettes. TLC for radiochemical purity assessment was done by applying chromatography paper impregnated with silica gel (iTLC-SG plates) purchased from Agilent Technologies (Santa Clara, USA). HEPES testing was performed using silica gel 60 F245 20 $\times$ 20 cm aluminum plates and aluminum oxide plates 60 F254 20 $\times$ 20 cm obtained from Merck. HPLC analysis was performed using a VWR Hitachi Chromaster system (VWR International, Radnor, USA; pump 5160, column oven 5310, UV detector 5410) and separate scintillation detectors Gabi Star (2 $\times$ 2 mm) and

Model Calculation of Maximal HEPES Concentration & Spotting Volume**Assumption:**

starting activity (A_0) = 800 MBq
product activity (A_p) = 400 MBq
preferred administered activity (A_A) = 200 MBq
product volume (V_p) = 16 mL

Calculation:

recommended volume of application (V) = $V_p \times (A_A/A_p)$ = 8 mL
maximal HEPES concentration ($c_{\max}$): $200 \mu\text{g}/8 \text{ mL} = 25 \mu\text{g/mL}$
spotting according to Eur. Ph.: $V/2000 \text{ mL}$ in 1 μL steps
4 μL in 1 μL steps

Fig. 2 Model calculation of the product volume that has to be spotted on the TLC plate according to the Ph. Eur.

Ramona from Elysia-Raytest (Straubenhardt, Germany) controlled by a clarity software from Data Apex (Prague, Czech Republic).

Radiochemical synthesis of ^{68}Ga -labeled PET tracers

Radiolabeling was performed according to a cationic purification protocol described elsewhere [14]. Briefly, the $[^{68}\text{Ga}]\text{Ga}^{3+}$ -containing eluate was concentrated using a P-SH+ cartridge. Gallium-68 was eluted with NaCl solution (5 M) and transferred to a reactor containing either 40 μg Pentixafor or 2.5–10 μg PSMA-11 in 1.5 mL HEPES buffer (1 M). The reaction mixture was heated to 126 °C for 6 min and subsequently purified applying a SPE cartridge (C-18, Sep-Pak Light). The purified product was diluted with PBS buffer and transferred to a sterile vial. The product was tested for its HEPES content using HPLC and/or TLC aside from all other routine quality control tests.

HPLC methods for HEPES quantification

The injection volume was 20 μL of reference solution and the product sample, respectively. All solutions were injected undiluted.

HPLC method according to Kvaternik et al. [12]

An isocratic flow of 0.5 and 0.8 mL/min was set using an Obelisc N column (5 μm , $4.6 \times 150 \text{ mm}$, Sielc, USA). The mobile phase consisted of 80/20 $\text{H}_2\text{O}/\text{ACN}$ (% v/v) under addition of 0.05% H_3PO_4 . Absorbance was measured at 195 nm.

HPLC method according to Antunes et al. [11]

An isocratic flow of 0.7 mL/min was used. As mobile phase, ammonia formate (pH 9.5) and, as stationary phase, an XBridge C18 column (5 μm , $4.6 \times 150 \text{ mm}$, Waters, Austria) were used. The UV channel was recorded at 195 nm.

Thin-layer chromatography methods for HEPES quantification

TLC analysis was performed according to the Ph. Eur. for comparison reasons, and the following parameters were evaluated for improving the assay: different spotting methods, stationary phases, mobile

phase compositions (Table 2), various forms of iodine staining, and quantification instruments.

TLC assay according to Ph. Eur. [8]

The Ph. Eur. stipulates for product solutions with a certain maximum volume of administration V [mL] a spotting method of “ $V/2000$ ”-times 1- μL droplets of product solution next to a reference solution containing the maximum allowed concentration (see the “Background” section for a more detailed explanation and a model calculation in Fig. 2). The TLC plate was developed in $\text{ACN}/\text{H}_2\text{O}$ (3/1 v/v) as mobile phase. After drying, the TLC plate rested in an iodine chamber for 4 min. The two resulting HEPES spots ($R_f = 0.3$) were compared visually. For release, the product solution spot has to be less or equally intense than the one of the reference solution.

Schedule for creating a new and improved TLC assay

In order to develop a new TLC assay, the reference solution was prepared in the same composition as the product solution. Afterwards, the spotting volume and spotting method were optimized for a focused HEPES spot that was comparable or even more focused than spotting according to the Ph. Eur. assay. Different mobile phases were tested to obtain an even more focused spot compared to the diffuse spot of the product solution according to the Ph. Eur. All evaluations were performed using a warmed iodine chamber.

Reference solution

A reference solution was freshly prepared for every product activity concentration containing the maximum allowed HEPES concentration (200 μg /recommended administration volume). The reference solution was further adjusted to the product solution of ^{68}Ga -radiopharmaceuticals produced with a Scintomics GRP synthesizer. Therefore, the matrix consisted of $\text{PBS}/\text{EtOH}/\text{H}_2\text{O}$ (16/1/1, $v/v/v$).

Sample spotting

For TLC spotting, five different patterns were tested to enhance interpretability of results: 8 μL in 1 μL steps, 8 μL in 2 μL steps, 16 μL in 2 μL steps, 25 μL in 5 μL steps, and 40 μL in 5 μL steps.

Mobile phase

Ten mobile phases were tested that are listed in Table 1.

Optical determination of the HEPES content

Initially, iodine-dyed TLC plates had to be observed visually and the HEPES content was subjectively determined. Judgment whether HEPES was below the allowed limit or not and if the used method was able to give useful information had to be done in a non-standardized manner only based on visual inspection. To improve this procedure, a photo of the TLC plate was analyzed by ImageJ free software to evaluate the contents of HEPES [15, 16]. Therefore, the photo was set to 32 bit. Regions of interest (ROIs) were drawn manually, and the same frame was used across the row. Afterwards, the spots were plotted and the area was measured. Comparison of the ROIs of the HEPES sample and a set of reference solutions enabled standardized relative quantification.

Evaluation of different methods by determining the HEPES content of a product sample

For the comparison of the tested method, a HEPES-containing product sample synthesized using a Scintomics GRP module was measured using the different methods, i.e., TLC (Ph. Eur.), TLC (improved), and HPLC (Antunes et al.). The TLCs were additionally quantified using ImageJ.

Evaluation of different TLC methods by using a questionnaire

For evaluation whether the new TLC conditions were more reliable or not, 28 volunteers completed a questionnaire consisting of photographed TLC plates which were performed according to the Ph. Eur. method and the new assay, respectively. Accordingly, a sample with a HEPES concentration unknown to the subjects had to be compared with a spot of a reference solution and determined if the sample contained more or less HEPES than the reference. In this case, a recommended volume of $V = 8$ mL was arbitrarily chosen. Consecutively, the

maximum allowed HEPES concentration was calculated as 25 $\mu\text{g/mL}$.

Results

In order to implement an HPLC-based quantification method for HEPES in our facility, two literature-known assays were tested for suitability: the methods according to Kvaternik et al. and Antunes et al., respectively [11, 12]. In parallel, the TLC method according to the Ph. Eur. was refined in order to obtain reliable and reproducible results, even when conducted by different operators.

Comparison of different HPLC assays

The HPLC assay according to Kvaternik et al. [11, 12] was not possible to establish on our VWR Hitachi HPLC system. The VWR Hitachi HPLC device was not capable of separating the HEPES peak from the NaCl peak even if the flow rate was reduced from 0.8 to 0.5 mL/min (Fig. 3).

The method developed by Antunes et al. could be successfully adapted on the VWR Hitachi HPLC system. A quantifiable HEPES peak was found applying a flow rate of 0.7 mL/min and a mobile phase of 15 mM ammonium formate at pH 9.5. The HEPES peak was baseline separated from all other components of the formulation and quantifiable at a retention time of 3.64 min as depicted in Fig. 4. The respective calibration curve shows a high linearity with an $R^2 = 0.9905$ (Fig. 5).

Comparison of different TLC methods

The TLC assay according to the protocol of the Ph. Eur. gave different shapes of iodine spots of product samples (same formulation as the synthesized radiopharmaceutical) and the reference solution (in water). Figure 6 shows that the spot of the reference solution was more focused, whereas the product spot appeared more diffuse. Furthermore, iodine staining of the TLC plate was not reproducible.

Improvement of the TLC method involved the evaluation of different spotting patterns, using the same formulation of the reference standard and sample solution, testing different mobile phases, pre-heating of the iodine chamber, and developing a method as well as image analysis.

Several spotting methods were tested (i.e., 8×1 μL , 4×2 μL , 8×2 μL , 5×5 μL , 8×5 μL), and results showed that spotting four times 2 μL gave intense spots after staining. For preparation of the reference solution, dissolving HEPES in a solution of the same composition as a clinically used product formulation resulted in a similar shape of the stained spots, but the spot shape was still quite diffuse (see Fig. 6). Accordingly, a new mobile phase consisting of ACN/H₂O/MeOH (15/3/2) (mobile phase B) was used that resulted in more focused

Table 1 Composition of the tested mobile phases

Mobile phase	Composition (v/v)
A (Ph. Eur.)	ACN/H ₂ O (3/1)
B	ACN/H ₂ O/MeOH (15/3/2)
C	ACN/0.25 M NaOAc (3/1)
D	ACN/H ₂ O/MeOH (15/5/1)
E	H ₂ O/MeOH (1/1)
F	ACN/H ₂ O/MeOH (13/3/4)
G	ACN/NaOAc/MeOH (15/3/2)
H	ACN/MeOH (4/1)
I	ACN/H ₂ O/MeOH (11/4/3)
J	ACN/MeOH (1/1)

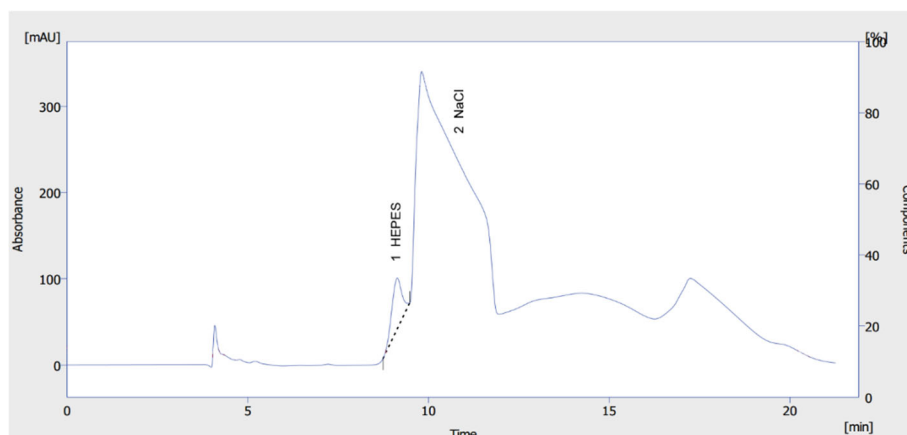


Fig. 3 Representative HPLC chromatogram of HEPES (1 mg/mL) obtained by the method according to Kvaternik et al.

spots and showed a short time of development of less than 10 min. The retention factor (R_f) was about 0.35 using this mobile phase. Additionally, warming the iodine chamber to 30–40 °C achieved uniform staining [17].

The evaluation of the validity of both methods was executed by 28 subjects who filled out a questionnaire that contained photos of a set of TLC strips. These fictive quality controllers had to determine whether or not samples contained more HEPES than a corresponding reference solution. This examination leads to the following results:

- The concentration of HEPES in the solution was underestimated for the method according to Ph. Eur. This resulted in a high rate of false assignments at concentrations of 20 and 30 µg/mL which were above the allowed maximum concentration assumed for a maximum amount of 200 µg in 8 mL (Fig. 7).

- Evaluation of the stained TLC plates was additionally interpreted by means of a pixel-based analysis (using ImageJ software). Applying this quantitative analysis, only at two concentrations (30 and 35 µg/mL, respectively), one false assignment was observed out of two measurements.
- Using the newly established method, the subjects overestimated the HEPES content in the sample at lower concentrations (Fig. 7).
- Pixel-based evaluation of those TLC plates also led to two false results at concentrations 15 and 30 µg/mL for one out of two measurements.

For both TLC methods, a calibration curve was drawn for the pixel-based analysis. The method for the Ph. Eur. method showed a limit of linearity (LOL) at about 35 µg/mL (Fig. 8). Additionally, values at 20 and 30 µg/mL showed a high standard deviation of

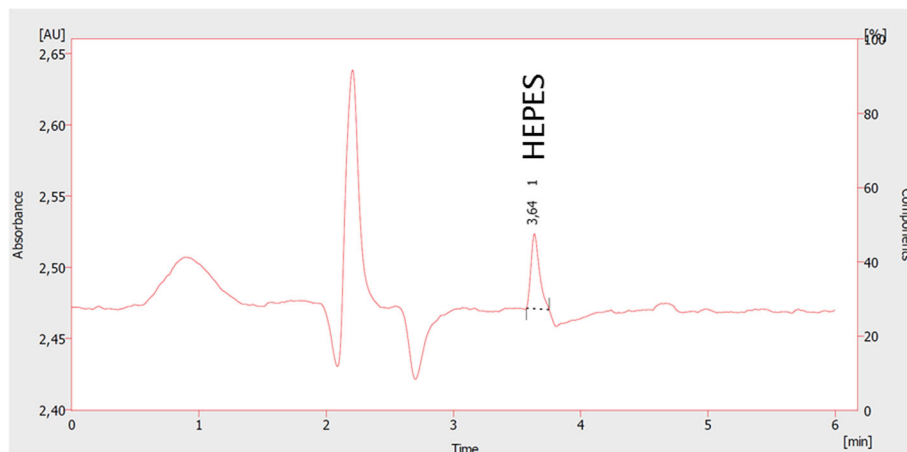


Fig. 4 Representative HPLC chromatogram of HEPES (35 µg/mL) obtained by the method according to Antunes et al.

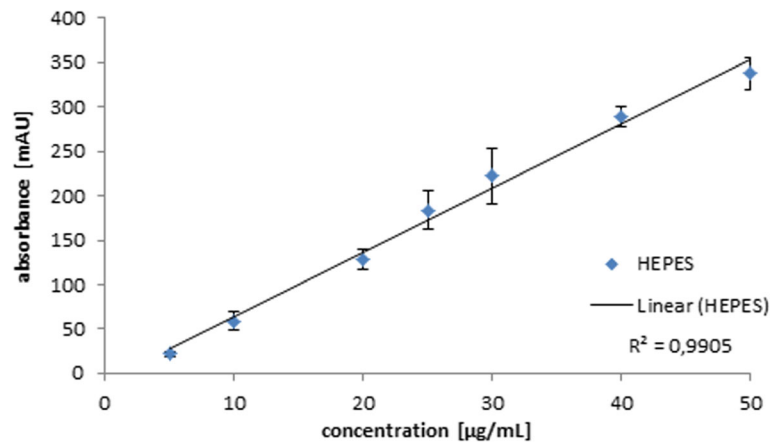


Fig. 5 Calibration curve of HEPES applying the HPLC assay according to Antunes et al. ($n = 3$)

21,210 ± 5875 (28% CoV) and 27,041 ± 6702 (25% CoV) area under the curve, respectively.

TLC analysis using the improved method and ImageJ for evaluation gave a calibration curve with an excellent $R^2 = 0.9956$ throughout a linear range of 10 to 70 µg.

Determining the HEPES content of three $[^{68}\text{Ga}]\text{Ga-Pentixafor}$ product samples

The HEPES concentrations determined by HPLC and ImageJ analysis of TLC (improved) gave comparable results for all three product samples (Table 2). In contrast, the ImageJ analysis of TLC (Ph. Eur.) gave only comparable

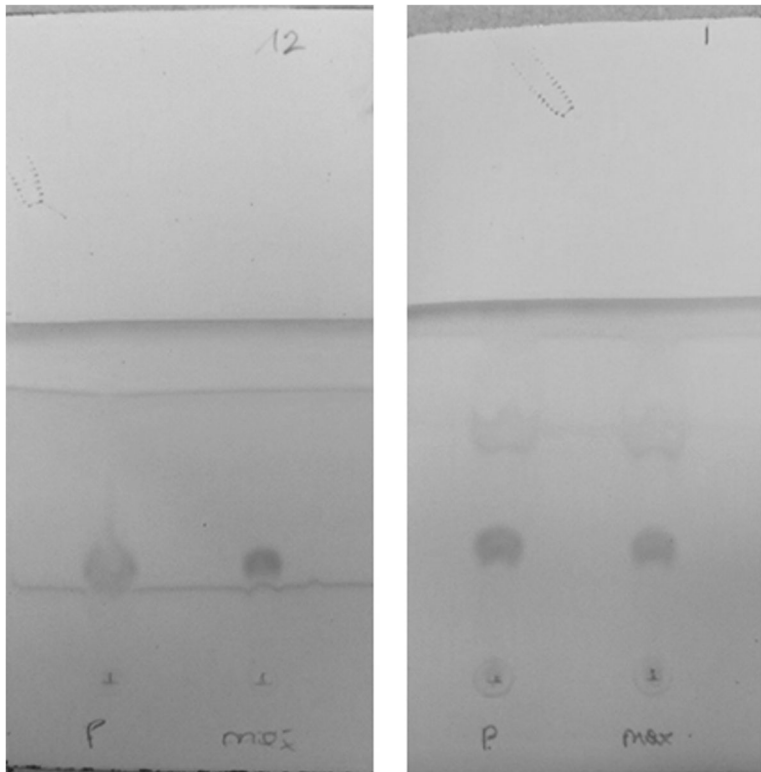


Fig. 6 Representative images of iodine-stained TLC plates (35 µg/mL HEPES) with the sample (“P”) spotted in the left column and the maximum allowed concentration (“max,” 25 µg/mL in this experimental setup) in the right column of each plate. The plates were processed according to the Ph. Eur. (left image) and the improved method (right image) and were used for the operator questionnaire

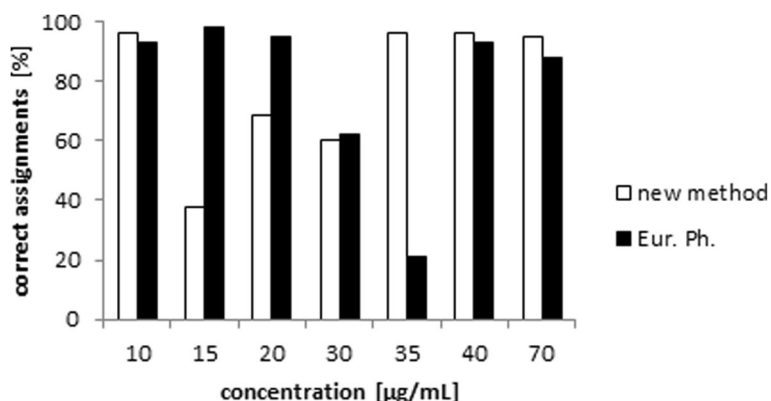


Fig. 7 Evaluation of the questionnaires. Correct assignments of HEPES concentration in comparison to the reference solution using visual inspection

results in accordance with the other methods for product sample 2. The other two measurements using this assay resulted in a concentration deviation of more than 100% without a trend to higher or lower values.

For a model calculation of a potential applicable patient dose of 150 MBq and an achieved product activity of 900 MBq in 16 mL, the maximum concentration of HEPES according to Ph. Eur. is 75 $\mu\text{g/mL}$. Therefore, the standard TLC (Ph. Eur.) resulted in one false negative result, whereas our improved TLC method as well as the HPLC method by Antunes et al. led to release of all three product batches.

Discussion

Advantages and limitations of the HPLC method

The HPLC assay according to Kvaternik et al. could not be set up or adapted with a reduced flow of 0.5 mL/min using our HPLC device. Kvaternik et al. described this HPLC assay by applying an Agilent system. In contrast,

we used a VWR Hitachi system that was not feasible for a baseline separation of the HEPES peak.

The HPLC method according to Antunes et al. was valid in our laboratory setup and achieved an excellent linearity in a concentration range of 5–50 $\mu\text{g/mL}$ HEPES (Fig. 5) for a reliable HEPES quantification. However, a second HPLC run using the same HPLC system as for routine quality control requires proper pre-conditioning of the column after switching methods. This process including column changing, conditioning, and the HPLC run takes a rather long period of about 30 min that is not feasible for the quality control procedure of short-lived radiopharmaceuticals due to substantial radioactive decay. As an alternative, a second HPLC system for performing two simultaneous runs could be an adequate option. Basically, most radiochemical laboratories are equipped with only one HPLC system for the quality control of radiopharmaceuticals. Therefore, this approach is not implementable in all radiochemical laboratories due to the high effort in equipment. In contrast, every radiochemical

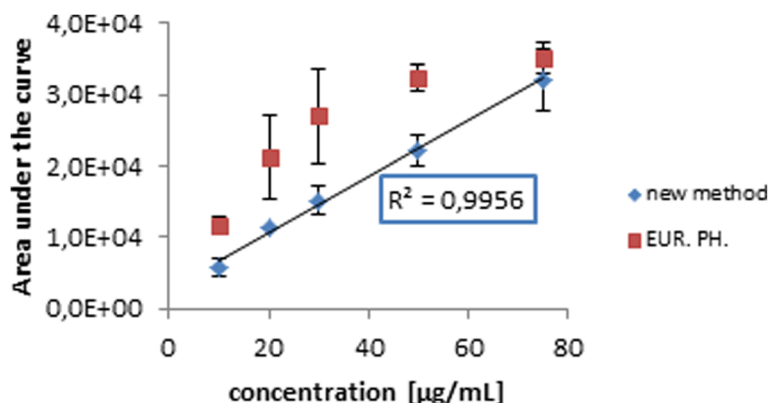


Fig. 8 Calibration curves of HEPES concentration obtained from a pixel-based analysis method by means of ImageJ applied to the Ph. Eur. and the improved assay

Table 2 Model calculation for maximum allowed HEPES concentration and determination of HEPES content in exemplary [^{68}Ga]Ga-Pentixafor product samples measured with HPLC and TLC (ImageJ)

Model calculation			
Product activity	Product volume	Activity for administration	Calculated max. HEPES concentration
900 MBq	16 mL	150 MBq (in 2.67 mL)	200 $\mu\text{g}/2.67\text{ mL} = 75\text{ }\mu\text{g/mL}$
Determination of HEPES concentration in a radiopharmaceutical			
Method	[^{68}Ga]Ga-Pentixafor 1 [$\mu\text{g/mL}$]	[^{68}Ga]Ga-Pentixafor 2 [$\mu\text{g/mL}$]	[^{68}Ga]Ga-Pentixafor 3 [$\mu\text{g/mL}$]
TLC (Ph. Eur.)	169.4 (no release)	23.7	2.6
TLC (improved)	71.6	23.9	6.6
HPLC (Antunes et al.)	65.97	19.17	8.83

laboratory is provided with TLC equipment. Thus, a reliable TLC analysis is definitely preferred to meet the demands of a feasible quality control in terms of a manageable instrumental setup and time efficiency.

TLC limitations and comparison

The TLC method according to the Ph. Eur. was found to be neither reliable nor fully conclusive. The spots of reference and product solution displayed diverging spot shapes, which made them incomparable. This work showed that this problem originates from different formulations of the HEPES solutions (Fig. 6). The matrix of the reference solution was water while the product solution contained PBS and ethanol. Hence, we refined this method using the same composition of the matrix for both, the reference and the product solution, which resulted in spots with a similar shape. However, the shape was still quite diffuse, which indicated the necessity of a different mobile phase. Among the tested mobile phases (Table 1), the composition of ACN/ H_2O /MeOH (15/3/2) attained highly focused spots after iodine staining (Fig. 6).

Another error source was the iodine chamber that was assumed to contain an inhomogeneous distribution of iodine, which resulted in unreliability and irreproducibility. Thus, the iodine chamber was pre-heated for an even distribution of the gas, which achieved reproducible iodine staining of the HEPES spot. Further changes in the spotting method could facilitate the TLC assay. A spotting method of spotting four times 1 μL gave a relatively weak staining according to the low amount of HEPES in 4 μL and was prone to pipetting errors. If the recommended application volume is more than 8 mL with regard to the activity of the generator and, accordingly, the product, the spotting (V/2000 mL in 1 μL steps, see Fig. 2) is time consuming, especially considering that a maximally focused spot had to be achieved by letting the spotted microliters dry before the next one was added. Therefore, using this method for the radioactive probe is not ideal in terms of radiation protection and decay of the product.

On the basis of the high number of error sources, a low validity and reproducibility of the assay according to Ph. Eur. was not surprising. However, this TLC method and subsequent evaluation by comparative, visual quantification is still a semi-quantitative method, which is worthwhile to be substituted by quantitative analysis. Eventually, this could be managed by application of a pixel-based analysis by means of ImageJ. As a result, errors caused by subjective evaluation and inter-observer variability are potentially erased. For evaluations of the TLC methods using ImageJ analysis, a calibration curve with an impressive R^2 of 0.9956 was created for the newly developed TLC assay, in contrast to the calibration curve of the TLC method according to Ph. Eur. in which it was not possible to assign a reasonable linearity. Those results confirm that a refinement of the TLC assay according to Ph. Eur. is of imperative urgency.

For evaluation, all three methods were compared in a realistic setting: Three samples of [^{68}Ga]Ga-Pentixafor obtained from routine production were analyzed by HPLC and both TLC methods. The HPLC assay by Antunes et al. and the improved TLC method resulted in comparable HEPES concentrations, whereas the TLC method according to Ph. Eur. was only for one sample in a comparable range with the other methods.

A correct assignment of the HEPES content determined by HPLC and the new TLC method seems more reliable and reproducible than the method proposed in Ph. Eur. However, in clinical routine, it is not necessary to determine the exact HEPES content; a limit test to determine whether or not the HEPES amount in the sample is below the maximum limit is sufficient. Therefore, a direct comparison with the maximum concentration provides sufficient information. For evaluation, a questionnaire involving 28 subjects was set up providing information about the feasibility of the assay according to Ph. Eur. and the improved method when the sample solution is spotted next to the reference solution with the maximum concentration. The HEPES content according to the Ph. Eur. method was basically underestimated, which facilitated the release of radiopharmaceuticals that

might fail the required quality criteria. In contrast, many subjects overestimated the HEPES concentration using the new TLC method, which provided an improvement in terms of inherent safety. On the other hand, a radiotracer that is in accordance with the guidelines may not be released in this case. To overcome this inter-observer variability, a pixel-based evaluation of the TLC plates using ImageJ software was introduced and proved to be reliable and time effective.

Therefore, the optimum setup for quick results consists of a TLC analysis applying the improved method and performance of a visual evaluation. The product sample can directly be spotted on a TLC strip next to the reference solution. If the HEPES spot is not clearly distinguishable from the reference spot of 200 µg/V, a subsequent pixel-based analysis is recommended, which takes about 3 min. Overall, the novel procedure offers a significant improvement to the available methods for HEPES testing.

Conclusion

In the present work, different available methods for the determination of HEPES content in radiopharmaceutical formulations were evaluated. It was shown that the TLC assay according to Ph. Eur. was neither reliable nor reproducible to determine the HEPES content in radiopharmaceutical product formulations in our laboratory setting. Since a previously presented HPLC method gave satisfactory results, but required a high additional technical effort, we developed an improved, simple, and valid TLC assay that proved its feasibility for visual evaluation and additional software-based, quantitative analysis. In summary, the newly developed TLC method facilitates HEPES determination substantially and is worth to replace the TLC assay proposed in Ph. Eur. for a straightforward evaluation of the HEPES content.

Abbreviations

ACN: Acetonitrile; CoV: Coefficient of variance; HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC: High-performance liquid chromatography; NaOAc: Sodium acetate; PET: Positron emission tomography; Ph. Eur.: European Pharmacopoeia; TLC: Thin-layer chromatography

Acknowledgements

Ingrid Leitinger and Friedrich Girschele are thanked for the routine ^{68}Ga -radiopharmaceutical production and quality control. Andreas Krcal and Thomas Zenz are acknowledged for the technical support. Thomas Mindt is thanked for the helpful discussions and advice. Especially, Chrysoula Vraka is thanked for her continuing support.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Authors' contributions

SP and WW designed the experiments. SP and TN performed the experiments. JC optimized the iodine staining. VP established the pixel-based analysis. SP, TN, VP, JC, and WW analyzed and interpreted the data. MM and MH enabled the research and discussed outcomes. SP, VP, and WW wrote the paper. All authors read, revised, and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Division of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. ²Department of Inorganic Chemistry, University of Vienna, Vienna, Austria. ³Ludwig Boltzmann Institute Applied Diagnostics, Vienna, Austria. ⁴CBmed – Center for Biomarker Research in Medicine, Graz, Austria.

Received: 5 September 2018 Accepted: 10 October 2018

Published online: 23 October 2018

References

1. Roesch F, Riss PJ. The renaissance of the $^{68}\text{Ge}/^{68}\text{Ga}$ radionuclide generator initiates new developments in ^{68}Ga radiopharmaceutical chemistry. *Curr Top Med Chem*. 2010;10(16):1633–68.
2. Moerlein SM, Welch MJ. The chemistry of gallium and indium as related to radiopharmaceutical production. *Int J Nucl Med Biol*. 1981;8(4):277–87.
3. Weiner RE, Thakur ML. Chemistry of gallium and indium radiopharmaceuticals. In: Welch MJ, Redvanly CS, editors. *Handbook of radiopharmaceuticals: radiochemistry and applications*. New York: J. Wiley; 2003. p. 848.
4. Martins AF, Prata MIM, Rodrigues SPJ, Gerales CFGC, Riss PJ, Amor-Coarasa A, et al. Spectroscopic, radiochemical, and theoretical studies of the Ga^{3+} -N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES buffer) system: evidence for the formation of Ga^{3+} - HEPES complexes in (^{68}Ga) labeling reactions. *Contrast Media Mol Imaging*. 2013;8(3):265–73.
5. Velikyan I. ^{68}Ga -based radiopharmaceuticals: production and application relationship. *Mol*. 2015;20(7):12913–43.
6. Bauwens M, Chekol R, Vanbilloen H, Bormans G, Verbruggen A. Optimal buffer choice of the radiosynthesis of ^{68}Ga -Dotatoc for clinical application. *Nucl Med Commun*. 2010;31(8):753–8.
7. Velikyan I, Beyer GJ, Långström B. Microwave-supported preparation of ^{68}Ga bioconjugates with high specific radioactivity. *Bioconjug Chem*. 2004;15(3):554–60.
8. European Directorate for the Quality of Medicines (EDQM). European Pharmacopoeia 7.7 (01/2013:2482 gallium (^{68}Ga) edotreotide injection). *Eur Pharm*. 2011;23:310–3.
9. Meyer GJ, Mäcke H, Schuhmacher J, Knapp WH, Hofmann M. ^{68}Ga -labelled DOTA-derivatised peptide ligands. *Eur J Nucl Med Mol Imaging*. 2004;31(8):1097–104.
10. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RMM. Hydrogen ion buffers for biological research. *Biochemistry*. 1966;5(2):467–77.
11. Antunes I, Zijlma R, van der Woude G, Luurtsema G, Boersma H, Elsinga PH. A new fast and reliable HPLC method to detect HEPES in [^{68}Ga]-radiopharmaceuticals. (meeting abstract) *J Lab Comp Radiopharm*. 2017;60(Suppl.1):293.
12. Kvaternik H, Barowitsch C, Hausberger D, Müller R, Aigner RM. Bestimmung von HEPES in ^{68}Ga -markierten Peptiden mittels HPLC. (poster) Annual Meeting of the Austrian Society of Nuclear Medicine and Molecular Imaging, Zell am See; 2015.
13. Sasson R, Vaknin D, Bross A, Lavie E. Determination of HEPES in ^{68}Ga -labeled peptide solutions. *J Radioanal Nucl Chem*. 2010;283(3):753–6.
14. Martin R, Jüttler S, Müller M, Wester HJ. Cationic eluate pretreatment for automated synthesis of [^{68}Ga]CPCRA.2. *Nucl Med Biol*. 2014;41(1):84–9.

15. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9(7):671–5.
16. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol Reprod Dev*. 2015;82(7–8): 518–29.
17. Merck AG. Anfärbereagenzien für Dünnschicht- und Papier-Chromatographie. http://phoenix.tuwien.ac.at/pdf/DC/DC_Farbreagent.pdf. Accessed 26 June 2018.

Submit your manuscript to a SpringerOpen[®] journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com

Manuscript III

Toward the optimization of (+)-[¹¹C]PHNO synthesis: time reduction and process validation

Sarah Pfaff,¹ Cécile Philippe,¹ Lukas Nics,^{1,#} Neydher Berroterán-Infante,¹ Katharina Pallitsch,² Christina Rami-Mark,¹ Ana Weidenauer,³ Ulrich Sauerzopf,³ Matthäus Willeit,³ Markus Mitterhauser,^{1,4} Marcus Hacker,¹ Wolfgang Wadsak,^{1,5} Verena Pichler¹

¹ *Department of Biomedical Imaging and Image-guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, Vienna, Austria*

² *Institute of Organic Chemistry, University of Vienna, Vienna, Austria*

³ *Department of Psychiatry and Psychotherapy, Division of General Psychiatry, Medical University of Vienna, Vienna, Austria*

⁴ *Ludwig-Boltzmann-Institute Applied Diagnostics, Vienna, Austria*

⁵ *CBmed GmbH - Center for Biomarker Research in Medicine, Graz, Austria*

#corresponding author

Submitted in *Contrast Media Mol. Imaging* on 20.01.2019

Toward the optimization of (+)-[¹¹C]PHNO synthesis: time reduction and process validation

Sarah Pfaff,¹ Cécile Philippe,¹ Lukas Nics,^{1,#} Neydher Berroterán-Infante,¹ Katharina Pallitsch,² Christina Rami-Mark,¹ Ana Weidenauer,³ Ulrich Sauerzopf,³ Matthäus Willeit,³ Markus Mitterhauser,^{1,4} Marcus Hacker,¹ Wolfgang Wadsak,^{1,5} Verena Pichler¹

¹ *Department of Biomedical Imaging and Image-guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, Vienna, Austria*

² *Institute of Organic Chemistry, University of Vienna, Vienna, Austria*

³ *Department of Psychiatry and Psychotherapy, Division of General Psychiatry, Medical University of Vienna, Vienna, Austria*

⁴ *Ludwig-Boltzmann-Institute Applied Diagnostics, Vienna, Austria*

⁵ *CBmed GmbH - Center for Biomarker Research in Medicine, Graz, Austria*

Corresponding author:

Lukas Nics

Department of Biomedical Imaging and Image-guided Therapy,

Division of Nuclear Medicine,

Medical University of Vienna,

Waehringer Guertel 18-20,

A-1090 Vienna, Austria

Tel.: +43 1 40400 45350.

E-mail address: lukas.nics@meduniwien.ac.at (L. Nics).

Abstract

(+)-[¹¹C]PHNO, a dopamine D_{2/3} receptor agonistic radiotracer, is applied for investigating the dopaminergic system *via* positron emission tomography (PET). An improved understanding of neuropsychiatric disorders associated with dysfunctions in the dopamine system and the underlying mechanism is a necessity in order to promote the development of new potential therapeutic drugs. In contrast to other broadly applied ¹¹C-radiopharmaceuticals, the production of this radiotracer requires a challenging four-step radiosynthesis involving harsh reaction conditions and reactants as well as inertness. Consequently, the production is prone to errors and troubleshooting after failed radiosyntheses remains time consuming. Hence, we aimed to optimize the radiosynthesis of (+)-[¹¹C]PHNO for achieving better activity yields without loss of product quality. Therefore, we synthesized (+)-[¹¹C]PHNO and omitted all heating and cooling steps leading to higher activity yields. As a result, radiosynthesis at room temperature led to a highly time-reduced production procedure that saves about 5 min, which is an appreciable decay-prevention of around 15% of the activity yield. Additionally, we established a troubleshooting protocol by investigating reaction intermediates, byproducts and impurities. Indeed, partial runs enabled the assignment of byproducts to their associated error source. Finally, we were able to generate a decision tree facilitating error detection in (+)-[¹¹C]PHNO radiosynthesis.

Introduction

The dopamine system is a key player in many neuropsychiatric disorders like schizophrenia, Parkinson's disease or attention deficit hyperactivity disorder.¹⁻³ (+)-PHNO ((+)-4-propyl-3,4,4a,5,6,10b-hexahydro-2H-naphtho[1,2-b][1,4]oxazin-9-ol hydrochloride) is a well-described agonist for the dopamine D_{2/3} receptor subtypes.⁴⁻⁶ This compound binds to dopamine D₂ receptors in their high affinity states and has high affinity for dopamine D₃ receptor subtypes, making it a virtual D₃ selective probe in ventrobasal striatum and brainstem dopaminergic nuclei.⁴ It is the current gold standard for measuring changes of extracellular dopamine, as it can be easily displaced by endogenous dopamine in so-called competition experiments, where dopamine-releasing agents such as amphetamine are administered.^{6,7} This compound was radiolabeled using the positron emission tomography (PET) radionuclide carbon-11 to image the involvement of the dopaminergic system in *in vivo* mechanisms of schizophrenia. (+)-[¹¹C]PHNO shows high potential to bring new insights into poorly understood physiological processes of a variety of neuropsychiatric disorders. Recently, the application of this tracer in healthy subjects and patients suffering from schizophrenia showed an enhanced dopamine release in patients with schizophrenia.^{8,9} Basically, the synthesis of ¹¹C-radiopharmaceuticals is highly challenging due to the relatively short half-life of carbon-11 (20.4 min). Consequently, a prerequisite for achieving a reasonable activity yield is a reliable production within two to three half-lives.¹⁰ Furthermore, a fundamental requirement for this particular radiotracer is high molar activity as pharmacological doses cause side effects, like e.g. nausea.¹¹ (+)-[¹¹C]PHNO radiosynthesis has been published by several working groups describing synthetic routes *via* carbon-11 radiolabeling either on the 1-position or the 3-position of the propyl moiety (see Fig. 1).¹²⁻¹⁵ Radiolabeling of the carbonyl position is possible by using [¹¹C]propionic acid chloride, which can be obtained by a Grignard reaction from [¹¹C]CO₂ and ethylmagnesium bromide and subsequent conversion with thionyl chloride (SOCl₂) (Fig. 1, compound 1 and 2).¹⁶⁻¹⁸ This highly reactive acyl chloride readily reacts with the precursor ((+)-HNO) to give an intermediate amide (Fig. 1, compound 3). Finally, the amide is reduced and the product is purified by semi-preparative high performance liquid chromatography (HPLC). However, this radiolabeling procedure is very demanding as inert conditions and extremely harsh reagents are required. Therefore, the automated synthesis needs high technical effort. As an alternative to the conventional vessel-based approach our working group reported an in-loop method for (+)-[¹¹C]PHNO synthesis. This method facilitates the establishment of (+)-[¹¹C]PHNO in other PET facilities.¹⁹

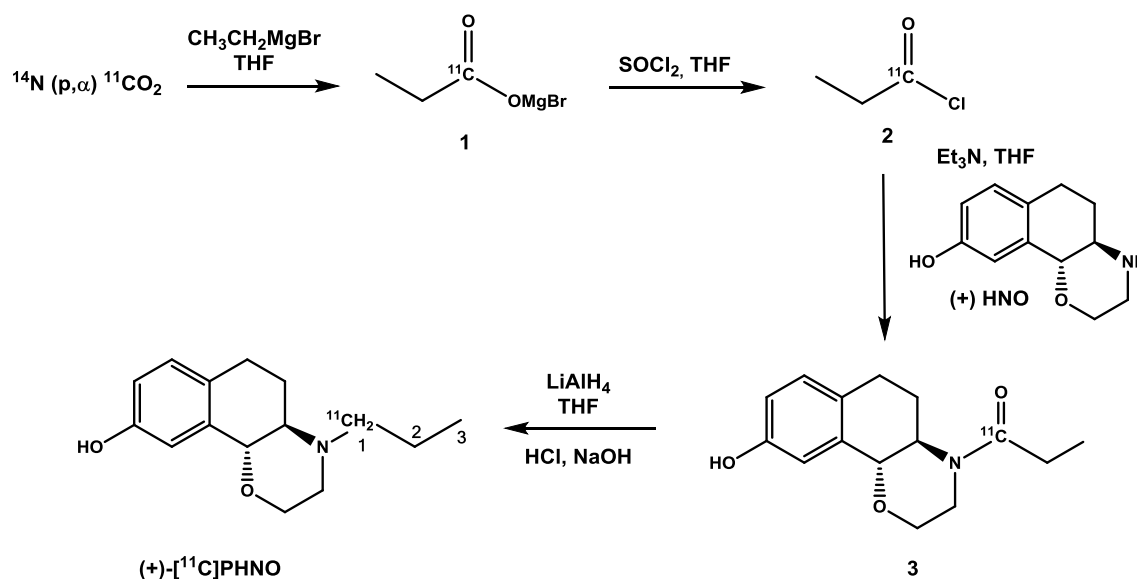


Figure 1: Synthesis scheme for the four-step radiosynthesis of (+)-[^{11}C]PHNO starting from cyclotron-produced [^{11}C]CO₂ and ethylmagnesium bromide.

Although this method improved the production of (+)-[^{11}C]PHNO, the susceptibility to humidity as well as oxygen contamination remains an unsolved problem. As a consequence, (+)-[^{11}C]PHNO synthesis has the highest failure rate within our facility. After a failed production run, an extensive validation process starts for investigating which of the numerous steps led to the radiosynthetic failure. During troubleshooting, the impact of the reaction temperature needs special considerations. Wilson et al. reported that the applied temperature regulated the formation of byproducts: the reduction of the carboxyl-group at temperatures below -30°C results in a lower number of side products compared to higher temperatures.¹²

By this, the major goal of this study was to reduce the duration of the radiosynthesis by shortening heating and cooling times and to validate the impact of these omitted temperature regulating procedures. Furthermore, we aimed to evaluate each reaction step in order to understand the influence of the individual reagents on the production process. The assignment of reaction intermediates (Fig. 2) and their chromatographic patterns directs to a better understanding of the error-prone reaction steps and consequently, to stable radiotracer production.

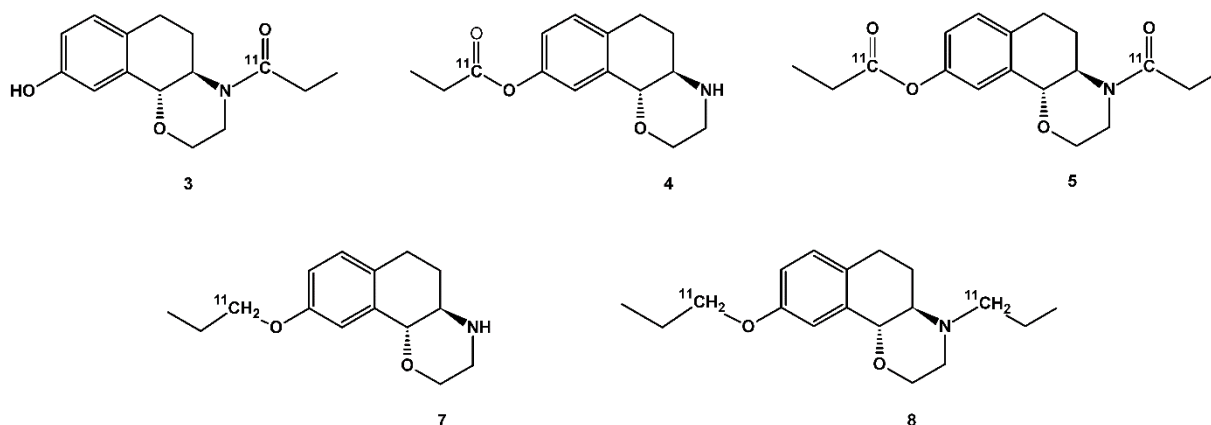


Figure 2: Chemical structure of the desired intermediate amide (compound 3) as well as non-reduced form of the side products (compound 4 and 5) and the reduced side products (compound 6 and 7).

Materials and methods

Ethylmagnesium bromide (EtMgBr, 3.0 M in diethylether, in Sure-Seal™), thionyl chloride (SOCl_2) (99%), triethylamine (TEA; 99.5%) and tetrahydrofuran (THF, anhydrous, $\geq 99.9\%$, inhibitor-free) were purchased from Sigma Aldrich (St. Louis, USA). Lithium aluminum hydride (LAH) (1.0 M in THF), (+)-PHNO standard and the precursor (+)-HNO hydrochloride (GMP and non GMP) were obtained from ABX (Advanced Biochemical Compounds, Radeberg, Germany). All reagents were used without further purification. Sterile water and 0.9% saline solution were purchased from B. Braun (Melsungen, Germany). Sterile phosphate-buffered saline solution (0.021 M phosphate buffer, 0.188 M NaCl, pH 7.7) was obtained from the Vienna General Hospital's Pharmacy (Vienna, Austria). The loop for the Grignard reactions is made of a 90 cm polyethylene (PE) tubing (fine bore polythene tubing REF 800/100/280; ID: 0.86 mm OD: 1.52 mm, Smiths Medical International Ltd.; Kent, UK). Ascarite II (20–30 mesh) was purchased from Thomas Scientific (Swedesboro, USA). Removal of acetonitrile was performed by using solid phase extraction (SPE) cartridges (SepPak C18-plus) from Waters (Waters Cooperation; Milford, MA, USA). $[^{11}\text{C}]\text{CO}_2$ was produced by a GE PETtrace cyclotron 860 (General Electric Medical Systems; Uppsala, Sweden) via the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction by irradiation of a gas target (aluminum, irradiation parameters: 16 MeV protons, 10–25 mA) filled with high purity N_2 (+1% O_2) (Air Liquide Austria GmbH, Schwechat, Austria). Automated syntheses were performed on a TRACERlab™ FX C Pro synthesizer (GE Healthcare, Uppsala, Sweden) including a semi-preparative HPLC system featuring a Linear Instruments Model 200 UV/Vis detector and a LaPrep HPLC pump (VWR International GmbH; Vienna, Austria). For analytical HPLC an Agilent 1260 infinity system

(Agilent Technologies GmbH; Santa Clara, USA) incorporating a quaternary pump (G1311B), a column oven (G1316A), a manual injector (G1328C), a multi wavelength UV-detector (G1365D) and a NaI (TI) detector from Berthold Technologies (Bad Wildbad, Germany) was used. The HPLC device is controlled by GINA Star v5.9 SP17 controlling software (Elysia-Raytest; Straubenhardt, Germany).

Time optimization on the automated synthesizer

A reduced reaction time was achieved by omitting all heating and cooling steps with exception of CO₂-trap heating. In particular, the target chamber was flushed twice with target gas prior to [¹¹C]CO₂ production and molecular sieves were preheated to 400°C for at least 15 min to minimize the content of non-radioactive [^{nat}C]CO₂ within the target chamber and synthesizer. Afterwards, respective lines and tubings of the synthesizer were flushed with helium. After the production of the required amount of radioactivity, [¹¹C]CO₂ was released to the synthesizer and trapped on molecular sieve. Subsequently, the trapped [¹¹C]CO₂ was released by heating to 400°C under a He stream (5 mL/min) to a PE tube, which was beforehand impregnated with a solution of ethylmagnesium bromide (EtMgBr) in THF. The impregnation was performed by diluting 500 µL EtMgBr with 1000 µL THF and pushing the solution through the column, then the loop was flushed with helium for around 5 sec to remove the excess of the impregnation solution. After the [¹¹C]CO₂ reaction with EtMgBr that last for around 5 min, SOCl₂ was pushed through the tube to obtain the acid chloride and the solution was simultaneously transferred to a reactor containing a solution of (+)-HNO (1.9-2.4 mg) in TEA (50 µL) and THF (400 µL). The intermediate amide species was obtained after 5-6 min stirring at room temperature. Afterwards a solution of 120 µL lithium aluminum hydride (LAH) in THF (400 µL) was added to reduce the intermediate amide. The reaction was quenched by addition of aqueous HCl (1 M, 900 µL) and neutralized with aqueous NaOH (1 M, 900 µL). The resulting suspension was filtered over cotton wool and purified by semi-preparative HPLC. The collected product peak was diluted in 80 mL water and trapped on a C18-SPE cartridge. Afterwards, the product was eluted with 1.5 mL ethanol, diluted with phosphate buffered saline and sterile filtered.

Evaluation of temperatures for the reduction step with lithium aluminium hydride (LAH)

In order to evaluate the influence of the reaction temperature on the efficacy of the reduction of the intermediate amide with lithium aluminum hydride (LAH) to (+)-[¹¹C]PHNO, the synthesis was carried out using the following conditions:

- -40°C (experimental set up as for -15°C)

- -15°C (as previously published by our group¹⁹)
- 22°C (shortened synthesis time, no heating and cooling)

Partial runs for the investigation of failed syntheses

Small scale reactions and partial runs were conducted in borosilicate glass vials sealed with plastic septa as reaction vessel at room temperature. For all syntheses including the formation of the intermediate amide, a precursor solution of (+)-HNO (1-2 mg) in THF (400 μL) and TEA (50 μL) was used. The reaction was quenched by addition of H_2O (1 mL). For the identification of byproducts and investigation of the impact of individual reagents leading to failed synthesis, partial runs were performed as depicted in Fig. 3.

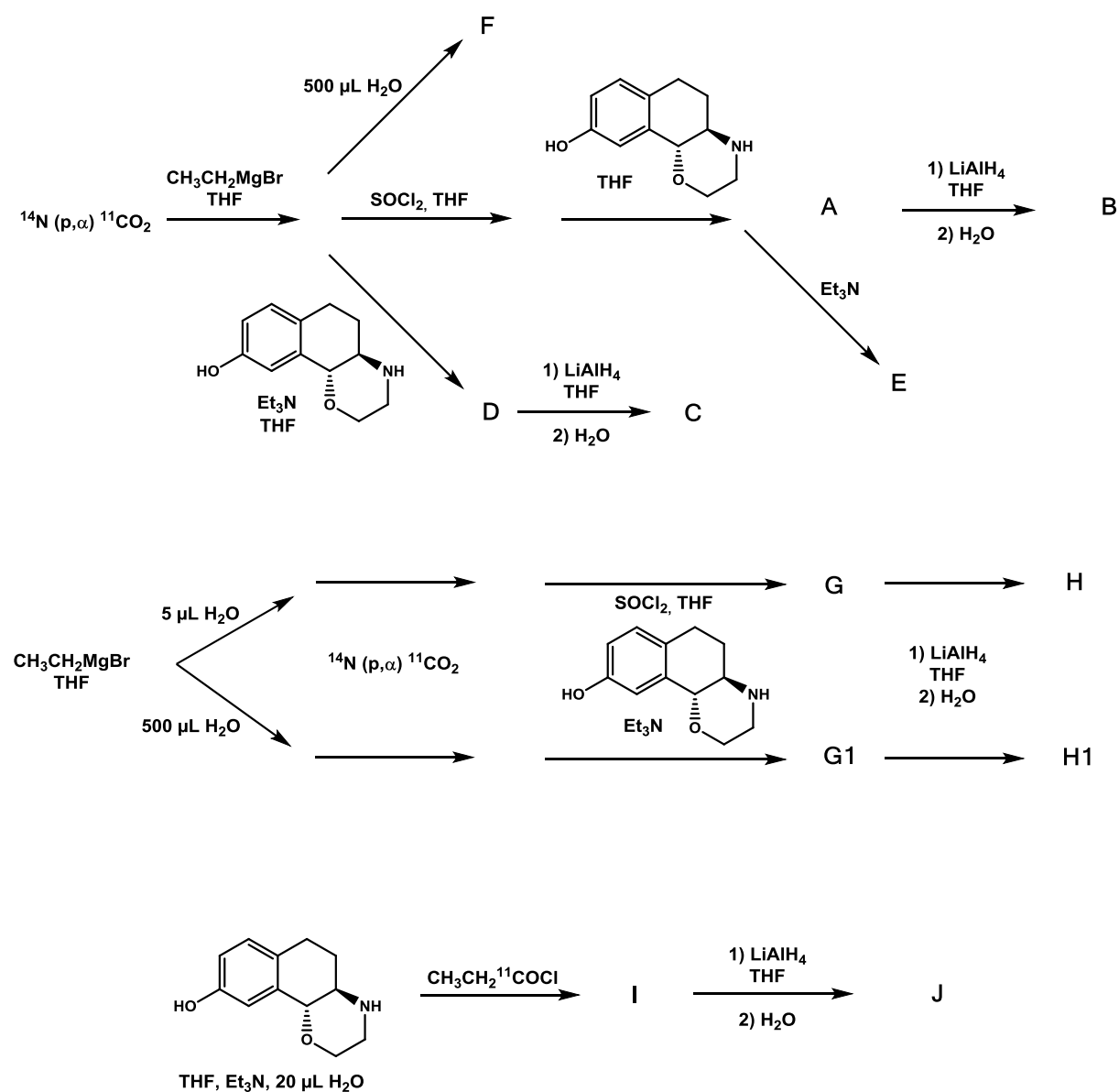


Figure 3: Reaction scheme of partial runs for investigation of reaction intermediates formed during (+)-[¹¹C]PHNO radiosynthesis.

- 1) Absence of TEA: The synthesis was conducted as described above, but without the use of triethylamine. The reaction was stopped either after the amide formation (A) or at the end of the whole production scheme (B).
- 2) Absence of SOCl₂: The synthesis procedure was performed either without the addition of SOCl₂ (C) or in absence of both, SOCl₂ and LAH (D).
- 3) Absence of LAH: The synthesis was stopped before LAH addition to simulate a failed reduction of the amide (E).

Small scale reactions and partial runs for the analysis of insufficient inertness

As Grignard reactions are especially sensitive to moisture, the impact of insufficient inertness and therefore contamination of the reagents with traces of water was investigated.

a) Influence of moisture on the Grignard reaction

The effect of moisture before and after trapping of [¹¹C]CO₂ in EtMgBr was investigated as follows:

- The PE tube was impregnated with a mixture of 0.5 mL Grignard solution (3 M in Et₂O) in 1 mL THF and then the activity was released through the loop. To quench the reaction and hydrolyse the radioactive intermediate, 0.5 mL of water were pushed through the impregnated loop and the reaction mixture was analyzed (F).
- 5 µL of water were added to the Grignard reagent solution and the reaction was stopped after amide formation (G) or after the reduction step (H).
- The PE tube was impregnated with the Grignard reagent solution and the loop was flushed with 0.5 mL of water. The reaction was stopped after amide formation (G1) or after the reduction step (H1).

b) Influence of moisture on acylation

The impact of moisture on the amide formation was investigated by adding H₂O (20 µL) to a solution of (+)-HNO (1-2 mg) in THF (400 µL) and TEA (50 µL) prior to the addition of [¹¹C]propionic acid chloride (I). Additionally, the same synthesis was performed and the resulting reaction mixture further treated with LAH (J).

Analytical and semi-preparative HPLC measurements

All crude, small scale reaction mixtures were analyzed by analytical HPLC measurements. The stationary phase was an X-Bridge BEH Shield RP-18, 4.6 x 50 mm, 2.5 μ m, 130 Å column (Waters Cooperation; Milford, MA, USA). The mobile phase consisted of solvent A (ammonia phosphate buffer (100 mM), sodium-1-octasulfonate (5 mM), pH 2.1 adjusted with H₃PO₄); solvent B (90% acetonitrile (ACN) / 10% water); solvent C (water); and solvent D (ammonium phosphate buffer (50 mM), pH 9.3 adjusted with NaOH). The gradient started with a composition of 33% A, 17% B, 17% C, and 33% D. Subsequently, B is increased over 2 min from 17% to 34% whereas C is reduced from 17% to 0%. The UV/Vis signal was detected at a wavelength of 280 nm with a reference wavelength of 450 nm. The initial flow rate (1.5 mL/min) was decreased after 25 s to 1.0 mL/min. The retention time was compared to the reference standard of (+)-PHNO.

A part of the partial runs were additionally investigated on a semi-preparative HPLC system with a Phenomenex Luna C18 column (250 x 10 mm, 10 μ m; Phenomenex Ltd., Aschaffenburg, Germany) as stationary phase. The mobile phase consisted of 25 mM phosphate buffered saline (PBS) (pH 7.0)/ACN (60/40 v/v) with a flow rate of 5.8 mL/min. The UV/Vis signal was measured at 280 nm. The peaks were collected and correlated with the respective peaks of the analytical HPLC.

Assignment of the chromatographic peaks of [¹¹C]CO₂ and [^{nat}C]propionic acid

[¹¹C]CO₂ was trapped in THF and the solution was analyzed by analytical HPLC. The intermediate product propionic acid was injected to the analytical HPLC and its retention time was compared to the radioactive impurity peaks of the crude mixture.

Synthesis of **3 using [^{nat}C]CO₂**

The intermediate amide **3** was synthesized by using [^{nat}C]CO₂ gas that was bubbled through an EtMgBr solution (15 μ L, 3.0 M in Et₂O) in THF (200 μ L) for 3 min under He atmosphere. Afterwards, a solution of SOCl₂ (5 μ L) in THF (400 μ L) was added to generate the acyl chloride. After 4 min, the precursor solution of (+)-HNO (5 mg, non GMP) in TEA (50 μ L) and THF (200 μ L) was added to the reaction mixture. The resulting suspension was extracted three times with CH₂Cl₂ (about 500 μ L). The combined organic phases were injected into the semi-preparative HPLC system. The collected peaks were analyzed by analytical HPLC.

Characterization of intermediate compounds and side products

Characterization and identification of the compounds separated by HPLC was performed by high resolution mass spectrometry (HRMS) on a Bruker maXis UHR-TOF device (electrospray

ionization (ESI); qQ-TOF, mass accuracy < 5 ppm) in either positive or negative mode depending on the respective molecular structure.

Definition of yield determination

According to the consensus of nomenclature, the activity yield is defined as the amount of product that is obtained after a synthesis in MBq or GBq and is not corrected for decay. This term can be expressed in [%] as radiochemical yield not corrected for decay.²⁰

Statistical analysis

Study's data were obtained from all syntheses performed in our department between 01/2017 and 08/2017 with starting activity of 100-150 GBq at the end of bombardment (EOB) of $[^{11}\text{C}]\text{CO}_2$. Values are given as mean values $\pm$ SD as calculated with Microsoft Excel 2010 if not stated otherwise. Statistical analysis was performed by using a t-test as implemented in Graph Pad Prism 6 (GraphPad Software, La Jolla, USA).

Results and Discussion

Optimization of radiosynthesis duration

The previously described radiosynthetic process for (+)- $[^{11}\text{C}]\text{PHNO}$ preparation by Rami-Mark et al. involves a heating step to 80°C for the acylation reaction, followed by the addition of LAH at –15°C.¹⁹ Afterwards, the residual THF is removed *via* distillation (Fig. 4).

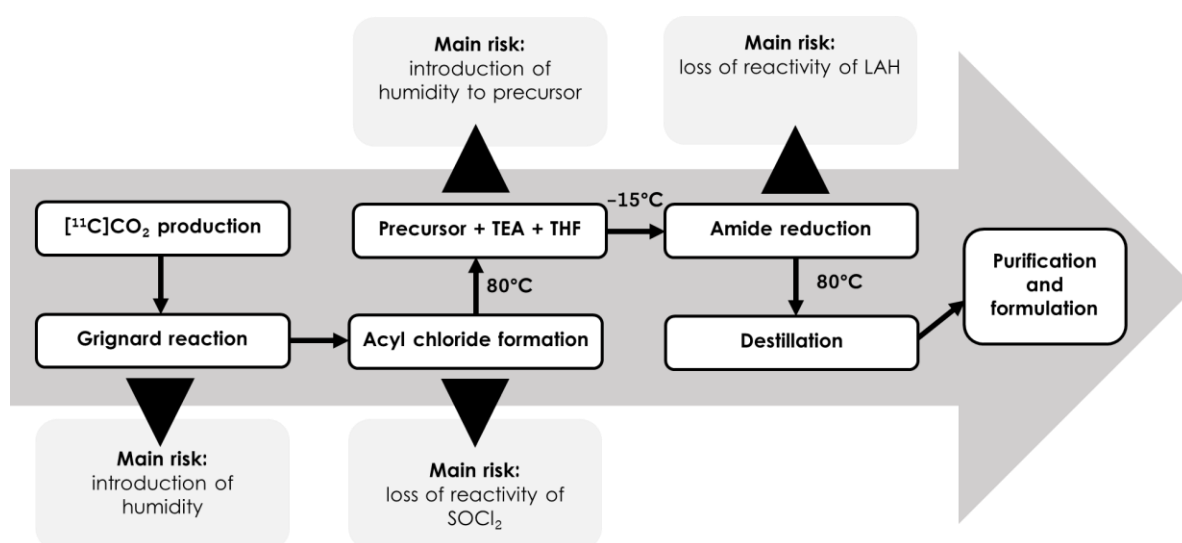


Figure 4: Reaction steps of (+)- $[^{11}\text{C}]\text{PHNO}$ production according to Rami-Mark et al.¹⁹ with the corresponding main risk for each reaction step.

In the improved method all reaction steps of (+)-[^{11}C]PHNO synthesis were performed at room temperature. In detail, the acylation with [^{11}C]propionic acid chloride was performed without heating and cooling as well as the addition of LAH. Additionally, (+)-[^{11}C]PHNO synthesis was conducted as previously described by our group (Fig. 4).¹⁹ Furthermore, LAH was added at -40°C as described by Wilson et al. for comparison. Performing all reaction steps at room temperature realized a tremendous reduction of the overall synthesis time by 5 min (13% in comparison to the previously published synthesis¹⁹). This time reduction is especially beneficial for the production of a ^{11}C -radiotracer as it impedes loss of radiolabeled product by decay of around 15% (Fig. 5). As a result, the activity yield was dramatically increased in comparison to previously published synthetic procedures. Synthesis at room temperature led to a significantly increased isolated radiochemical yield not corrected for decay of $1.4 \pm 0.8\%$. Indeed, the experiments in which the reduction was carried out at -15 or -40°C resulted in $0.53 \pm 0.17\%$ and $0.50 \pm 0.11\%$ isolated radiochemical yield without decay correction, respectively (Fig. 5). Thus, this study clearly showed that (+)-[^{11}C]PHNO synthesis can be successfully performed at room temperature without a forfeit of activity yield.

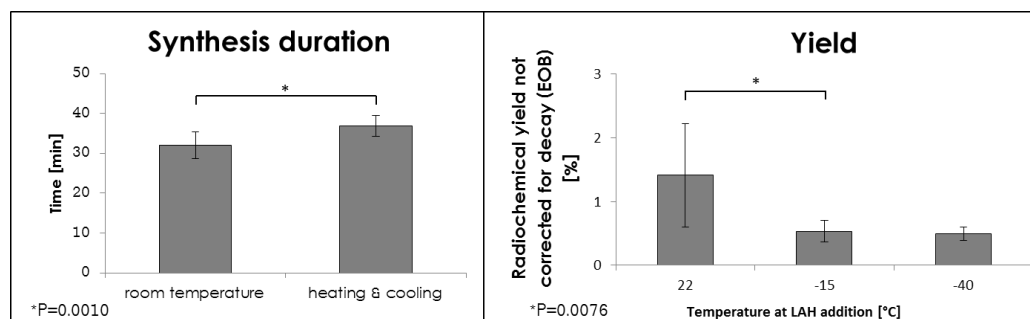


Figure 5: Left: Comparison of the synthesis duration of (+)-[^{11}C]PHNO synthesis at room temperature (n=16) and according to our previously published method including heating and cooling (n=9). Right:

Influence of the temperature at LAH addition on the isolated radiochemical yield not corrected for decay (22°C: n=16, -15°C : n=9, -40°C : n=3)

The limiting parameter of the reaction at room temperature is the reactor volume as the solvent is not evaporated and an overflow of the reaction mixture must be avoided. If an adequate reactor is available, this facilitated four-step synthesis procedure can be performed in every radiochemistry laboratory that is equipped with a cyclotron.

In terms of reaction intermediates, all experiments showed a similar pattern in semi-preparative as well as analytical HPLC chromatograms, but a different intensity of the peaks (Fig. 6). However, after purification by semi-preparative HPLC, (+)-[^{11}C]PHNO could be obtained in an excellent purity of >95% for all applied methods.

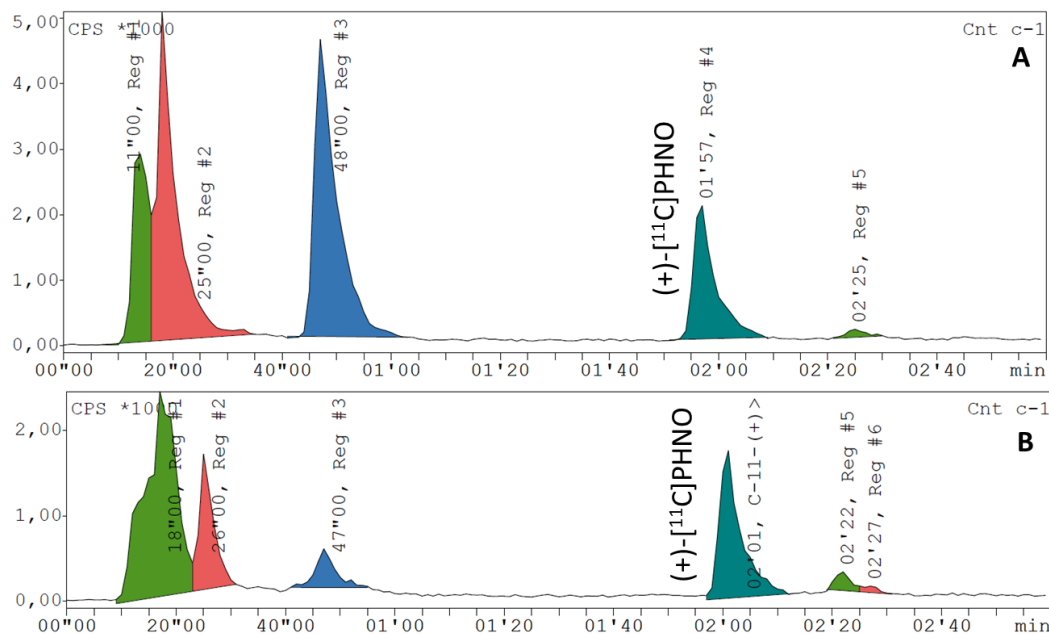


Figure 6: Analytical HPLC chromatograms of the crude reaction mixtures obtained by addition of LAH at room temperature (A) or at -40°C (B).

Investigations of failed synthesis and associated byproducts

Partial runs of the synthesis were performed to assign characteristic patterns of the chromatograms to the respective failure in the synthesis procedure. Within (+)-[^{11}C]PHNO synthesis, radioactive reaction intermediates and byproducts were shown at retention times of 2-3 min, 3-4 min, 4.8 min, 6 min and 8 min within semi-preparative HPLC, as well as of 20 s, 45 s and 2.5 min in analytical HPLC, respectively (see Fig. 6 A).

Partial runs for the investigation on failed synthesis

Absence of triethylamine

Simulation of failed (+)-HNO deprotonation was realized by performing a small scale preparation of the intermediate amide (A) or until the end of synthesis (B). In route A, semi preparative HPLC showed the main species at a retention time of 2.5-3 min (Fig. 7). Further peaks with smaller intensities were observed at higher retention times (4.5 min and 6 min). Therefore, those minor impurities are assumable potential formed carbonyl byproducts (compounds **3**, **4** or **5**) resulting from an insufficient deprotonation of the precursor, which impede the acylation process. In

analytical HPLC the main peak was observed at 15-20 s and comprised about $66.8 \pm 0.2\%$, usually representing small hydrophilic compounds (Fig. 8). Hence, this early eluting peak presumably incorporates hydrolyzed acid chloride, namely $[^{11}\text{C}]\text{propionic acid}$. In contrast, reaction scheme B includes a reduction step that enhanced the content of species that elute at 15-20 s to $73.6 \pm 0.7\%$. The pattern of the other main impurities was similar in analytical HPLC. Furthermore, no product formation was observed. Hence, deprotonation of (+)-HNO is pivotal for a successful (+)- $[^{11}\text{C}]\text{PHNO}$ synthesis.

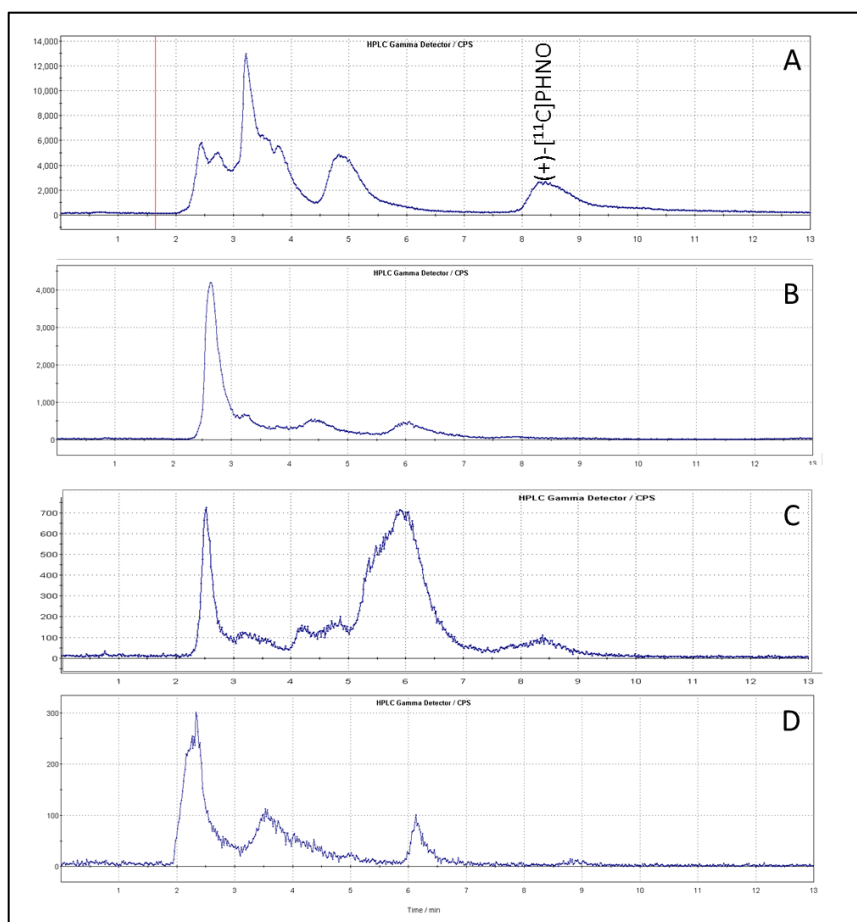


Figure 7: Chromatogram of semi-preparative HPLC of full synthesis at room temperature (A), route A synthesis without TEA and LAH (B), route E without LAH (C) and route F in which the reaction is quenched after Grignard reaction (D).

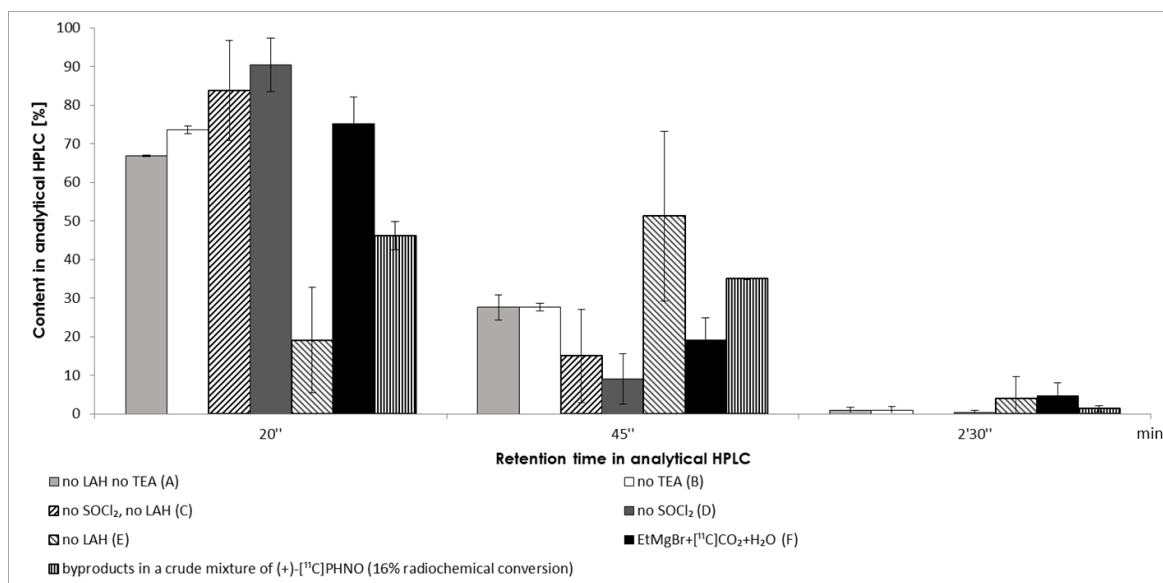


Figure 8: Content of impurities in analytical HPLC for reaction route A, B, C, D, E, F and byproducts of a crude (+)-[¹¹C]PHNO mixture with 16% radiochemical conversion (n=2).

Absence of SOCl₂

All experiments which were performed without SOCl₂, either stopped after theoretical amide formation (C) or after the reduction step (D), showed no conversion to (+)-[¹¹C]PHNO. Semi-preparative HPLC showed only peaks with retention time <3.5 min in experiments without SOCl₂. A similar picture was observed for analytical HPLC, where the main radioactive peak was at a retention time of 15-20 s of $83.8 \pm 13.0\%$ and $90.5 \pm 6.9\%$ for routes C and D, respectively (Fig. 8). Thus, absence of high retention-time peaks indicates inactivation of SOCl₂ presumably by hydrolysis.

Absence of LAH

Simulation of a failed LAH reduction step was done by stopping prior the reduction step (E). Here, a radioactive peak at a retention time of 6 min could be observed in semi-preparative HPLC. This peak also occurred in other experiments without LAH (see route B). Hence, this peak potentially represents a carbonyl species, namely **3**, **4** or **5**. This peak could further be assigned to the 45 s peak in analytical HPLC. In accordance, the crude reaction mixture showed the most prominent signal at 45 s of $51.2 \pm 22.1\%$. (Fig. 8) To that end, a highly dominant peak in the semi-preparative HPLC chromatogram at 6 min without product formation points to problems regarding the LAH reduction. As a consequence, a new bottle of LAH should be used for following syntheses and special attention should be paid to inertness.

Small scale reactions and partial runs for the analysis of insufficient inertness

Grignard reactions are known to be extremely moisture sensitive. Therefore, the radioactive impurities were analyzed that are formed by adding water to the Grignard reagent before addition of [^{11}C]CO₂. Additionally, the influence of the hydrolysis of the Grignard reagent on the following acylation was investigated.

Moisture-sensitivity of the Grignard reagent

For evaluation of impurities that are formed in the presence of water, [^{11}C]CO₂ was released to EtMgBr and water (500 μL) was added (F). The quenched species were loaded onto the semi-preparative HPLC column, which showed signals at retention times of 2-3 min, 3-5 min and 6.0-6.5 min (Fig. 8). In this respect, analytical HPLC displayed main signals at a retention time of 15-20 s ($75.3 \pm 6.9\%$). Surprisingly we also observed peaks at 45 s and 2.5 min: Addition of water after release of radioactivity is expected to lead to the formation of radioactive propionic acid and [^{11}C]CO₂, which are expected to be eluted relatively early on a C18-column. However, signals at similar retention times were observed in other experiments. Accordingly, Fig. 6B shows two not baseline separated peaks at a retention time of 2 min 20 s and 2 min 30 s.

Continuing synthesis until amide formation after Grignard quenching

Simulation of a Grignard reaction with insufficient inertness on the acylation was performed by directly adding 5 μL water to the Grignard reagent (G). The reaction process was stopped after amide formation. Semi-preparative HPLC measurements showed four main peaks, including a relatively intensive peak at 6 min, which suggests a successful amide formation (Fig. 9). Hence, the occurrence of this peak shows that 5 μL water are not quenching the Grignard reaction completely due to stoichiometric reasons: 15 mmol of EtMgBr are used for the reaction and 0.28 mmol H₂O were added. Besides, the peaks of the semi-preparative HPLC and analytical HPLC were correlated to each other as follows: below 3 min (semi-preparative) equals peak at 20 s (analytical), the peaks between 3 min and 5 min 30 s are similar to the one at 2 min 30 s and the 6 min peak to the one at 45 s. Accordingly, the two peaks of 3-5 min 30 s and 6 min in semi-preparative HPLC switched their retention order in the analytical run. A similar reaction was performed by applying 500 μL water (G1). Only one radioactive product occurred at 15-20 s in the analytical method, therefore, 500 μL water quenched the Grignard reagent completely. (Fig. 10)

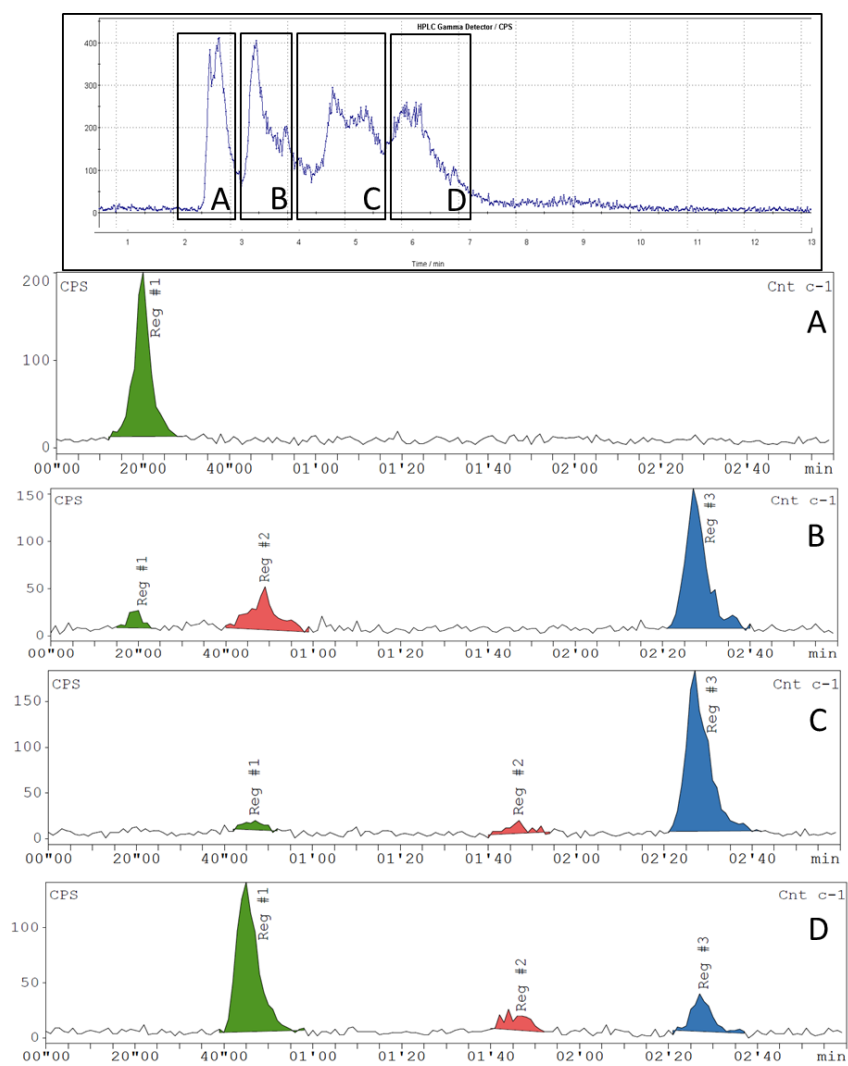


Figure 9: Semi-preparative HPLC chromatogram of partial run G and the corresponding analytical chromatograms of the respective peak.

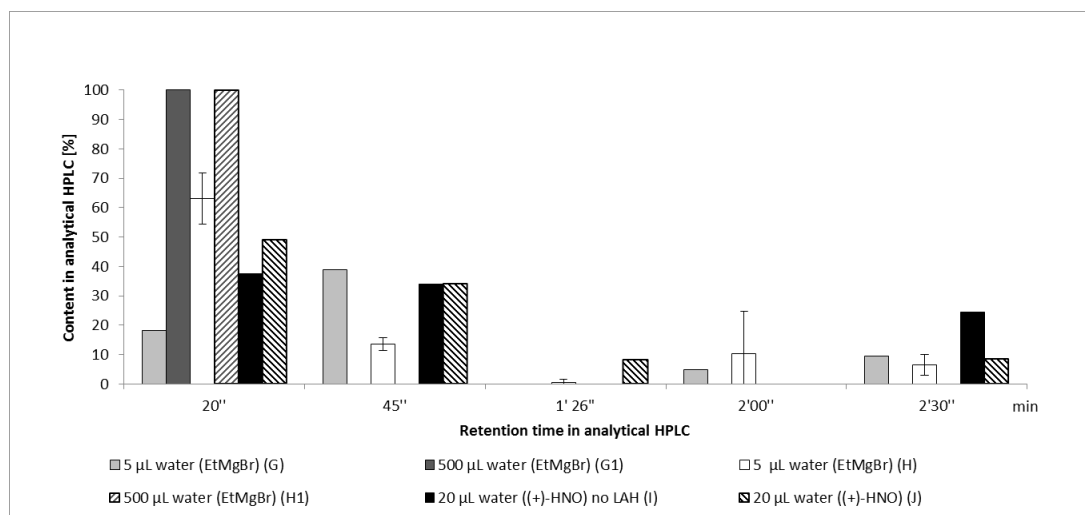


Figure 10: Radioactive peaks within the analytical HPLC chromatogram obtained by the reaction routes G, G1, H1, I, J (all $n=1$) and H ($n=2$).

Continuing the whole production procedure after Grignard quenching

Addition of 5 μL water to the Grignard solution and performing the whole synthesis scheme (H) lead to minor amounts of (+)- ^{11}C PHNO. Further investigation of quenching the Grignard reagents by addition of 500 μL of water (H1) showed one peak at 15-20 s in analytical HPLC (Fig. 10). Both results agree with the data obtained from G and G1, respectively.

Influence of moisture on acylation

To get a better understanding of the influence of water on the acylation process, 20 μL water were added to the precursor solution (400 μL THF, 50 μL TEA, 0.9-1.4 mg (+)-HNO) prior to addition of intact propionic acid chloride. The synthesis was stopped after formation of the amide (I) or after the reduction (J). For partial run I the ratio of the reaction intermediates was as follows: 15-20 s and 45 s with 37.5% and 34.0%, respectively. Due to the high intensity of the peak at 45 s, it can be assumed that the reaction was not completely quenched and one of the carbonyl species were partly formed (Fig. 10). Performing the entire synthetic route for (+)- ^{11}C PHNO production and adding 20 μL water to the precursor solution (J) lead to a peak at 1 min 26 s with 8.3% conversion, but no signal at 2 min for the product.

Assignment of the chromatographic peaks of ^{11}C propionic acid and ^{11}C CO₂

One intermediate of (+)- ^{11}C PHNO synthesis is ^{11}C propionic acid, which were identified by the non-radioactive reference compound ^{11}C propionic acid. The chromatogram showed a signal at

15-40 s at 280 nm wavelength (Fig. 11). Furthermore, $[^{11}\text{C}]\text{CO}_2$ was trapped in THF to assign the respective peak as well. The signal was found at an early retention time of 20-40 s similar to $[\text{nat}\text{C}]\text{propionic acid}$ (Fig. 11). Hence, both compounds, dissolved $[^{11}\text{C}]\text{CO}_2$ as well as $[^{11}\text{C}]\text{propionic acid}$, were present in nearly every analytical chromatogram.

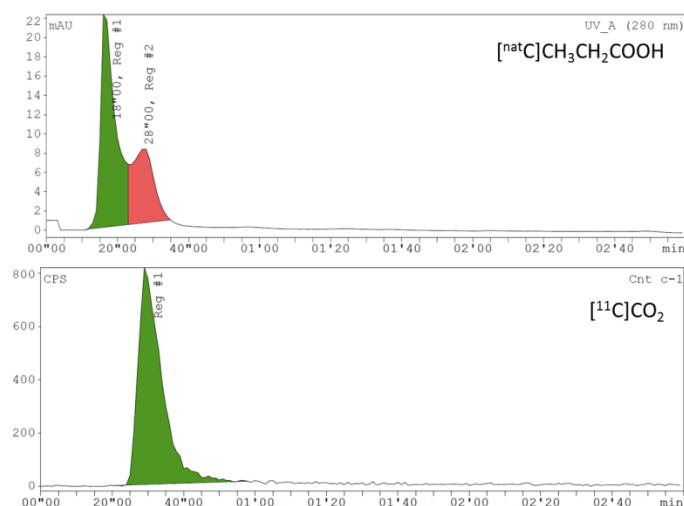


Figure 11: Top: UV/Vis-chromatogram of $[\text{nat}\text{C}]\text{propionic acid}$. Bottom: Radio-channel chromatogram of $[^{11}\text{C}]\text{CO}_2$

Synthesis of **3** using $[\text{nat}\text{C}]\text{CO}_2$ for assignment of HPLC peaks

The cold synthesis of the intermediate amide resulted in a crude reaction mixture that was transferred onto semi-preparative HPLC. The fractions were collected and measured by HRMS (high resolution mass spectrometry). The respective signal of the precursor, as well as of compounds **3**, **4** and **5** could be identified (see materials). As **3** and **4** have the same molecular weight, those two molecules cannot be differentiated by mass spectrometry.

Establishment of a troubleshooting protocol

As shown in this study, failure of (+)- $[^{11}\text{C}]\text{PHNO}$ radiosynthesis can be attributed to various reasons, but the chromatogram of the semi-preparative HPLC provide information on error sources. Based on our results, we created a decision scheme that helps troubleshooting in case of a failed (+)- $[^{11}\text{C}]\text{PHNO}$ production (Fig. 12). The presence of intensive and broad peaks in the region of 5-7 min shows a successful carbonylation, but a failed reduction to (+)- $[^{11}\text{C}]\text{PHNO}$. Consequently, the LAH should be renewed. If there is at least one broad peak with low intensity in this range, the amide formation was incomplete, which is mainly attributed to moisture. In addition, a single, sharp peak between 5-7 min suggests the presence of water after the

Grignard reaction. A chromatogram that is missing those signals indicates a failure in the coupling and can be assigned to poor inertness or decomposed SOCl_2 . Hence, the SOCl_2 should be changed. This scheme will facilitate troubleshooting and lead to an improved rate of successful (+)- ^{11}C PHNO.

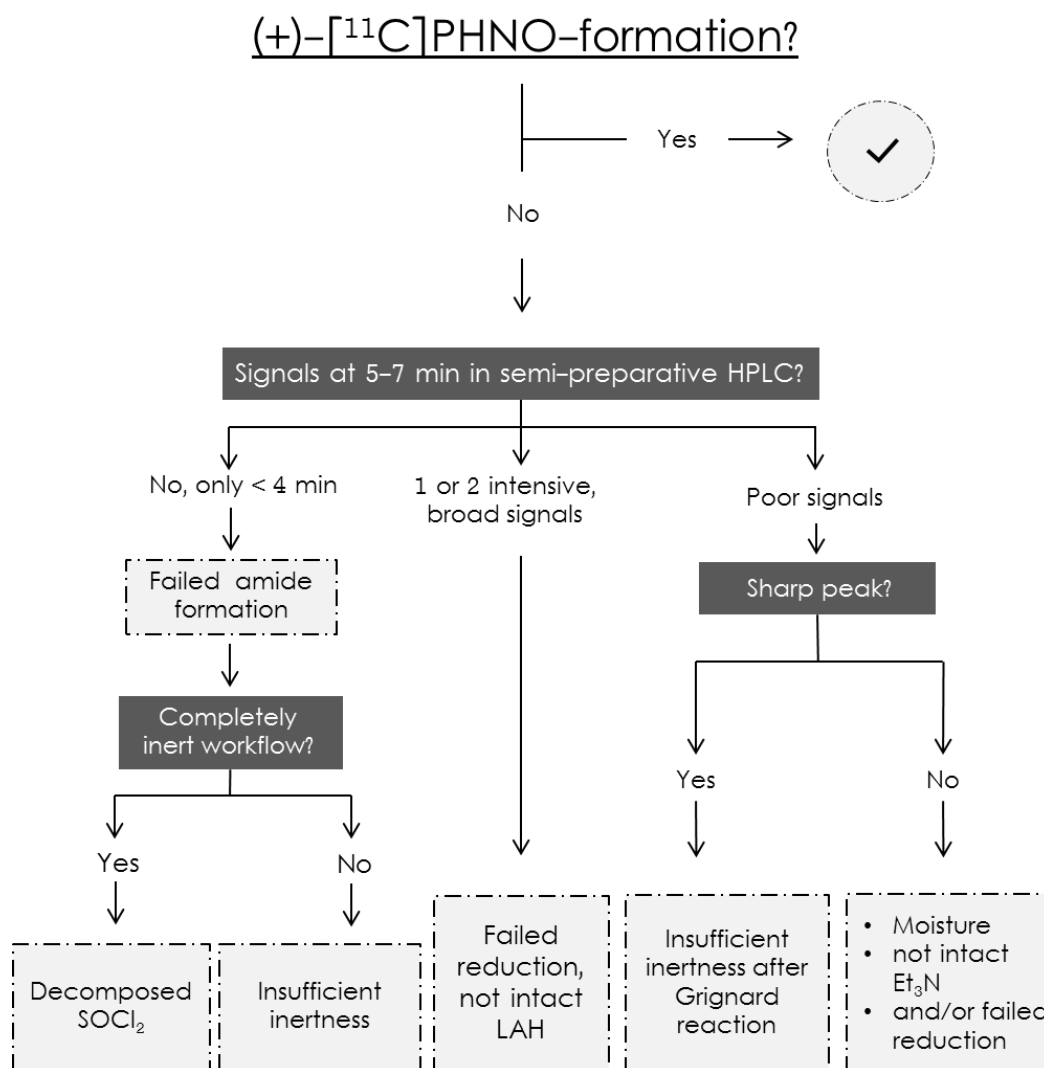


Figure 12: Schematic decision tree aiding troubleshooting after a failed (+)- ^{11}C PHNO formation.

Conclusion

The complex synthesis of (+)- ^{11}C PHNO is a challenge for every radiochemist in terms of time efficiency, reduction of failed syntheses and error evaluation. Here, we present a new method allowing a tremendous reduction of the radiosynthetic duration of approx. 5 min by omitting heating and cooling steps, which enhances the activity yield significantly. Moreover, the investigation of side products and intermediate species facilitates the error evaluation after a

failed synthesis. Accordingly, we propose a decision tree to support troubleshooting and facilitating a stable and continuous radiotracer production that is a necessity for clinical studies.

Data availability

All data used to support the findings of this study are included within the article.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

We thank Thomas Zenz and Andreas Krcal for technical support and their “around the clock” availability. Moreover, Thomas Kalina, Chrysoula Vraka, Jens Cardinale, Marie Brandt and Marius Ozenil are thanked for scientific discussions and support.

1. Seeman, P., Ulpian, C., Larsen, R. D. & Anderson, P. S. Dopamine receptors labelled by PHNO. *Synapse* **14**, 254–262 (1993).
2. Breier, A. *et al.* Schizophrenia is associated with elevated amphetamine-induced synaptic dopamine concentrations: Evidence from a novel positron emission tomography method. *Proceedings of the National Academy of Sciences* **94**, 2569–2574 (1997).
3. Hisahara, S. & Shimohama, S. Dopamine Receptors and Parkinson's Disease. *International Journal of Medicinal Chemistry* **2011**, 1–16 (2011).
4. Graff-Guerrero, A. *et al.* Brain region binding of the D2/3 agonist [11C]-(+)-PHNO and the D2/3 antagonist [11C]raclopride in healthy humans. *Human Brain Mapping* **29**, 400–410 (2008).
5. Gallezot, J.-D. *et al.* Affinity and selectivity of [11C]-(+)-PHNO for the D3 and D2 receptors in the rhesus monkey brain in vivo. *Synapse* **66**, 489–500 (2012).
6. Willeit, M. *et al.* First Human Evidence of d-Amphetamine Induced Displacement of a D2/3 Agonist Radioligand: A [11C]-(+)-PHNO Positron Emission Tomography Study. *Neuropsychopharmacology* **33**, 279–289 (2008).
7. Ginovart, N. *et al.* Binding characteristics and sensitivity to endogenous dopamine of [11C]-(+)-PHNO, a new agonist radiotracer for imaging the high-affinity state of D2 receptors in vivo using positron emission tomography. *Journal of Neurochemistry* **97**, 1089–1103 (2006).
8. Mizrahi, R. *et al.* Increased Stress-Induced Dopamine Release in Psychosis. *Biological Psychiatry* **71**, 561–567 (2012).
9. Tseng, H.-H. *et al.* Nigral Stress-Induced Dopamine Release in Clinical High Risk and Antipsychotic-Naïve Schizophrenia. *Schizophrenia Bulletin* **44**, 542–551 (2018).
10. Ametamey, S. M., Honer, M. & Schubiger, P. A. Molecular Imaging with PET. *Chemical Reviews* **108**, 1501–1516 (2008).
11. Willeit, M. *et al.* High-Affinity States of Human Brain Dopamine D2/3 Receptors Imaged by the Agonist [11C]-(+)-PHNO. *Biological Psychiatry* **59**, 389–394 (2006).

12. Wilson, A. A. *et al.* Radiosynthesis and Evaluation of [^{11}C]-(+)-4-Propyl-3,4,4a,5,6,10b-hexahydro-2 H -naphtho[1,2- b][1,4]oxazin-9-ol as a Potential Radiotracer for in Vivo Imaging of the Dopamine D2 High-Affinity State with Positron Emission Tomography. *Journal of Medicinal Chemistry* **48**, 4153–4160 (2005).
13. Plisson, C. *et al.* Automated preparation of the dopamine D2/3 receptor agonist ligand [11C]-(+)-PHNO for human PET imaging studies. *Applied Radiation and Isotopes* **70**, 380–387 (2012).
14. Oya, S., Joseph, S. K., Carberry, P., Divgi, C. R. & Koren, A. O. Preparation of [11C]-(+)-PHNO using an automated synthesizer coupled with a stand-alone system for making [11C]propionyl chloride. *Journal of Labelled Compounds and Radiopharmaceuticals*. 466 (2013).
15. Shoup, T. M. *et al.* Synthesis of the dopamine D2/D3 receptor agonist (+)-PHNO via supercritical fluid chromatography: preliminary PET imaging study with [3-11C]-(+)-PHNO. *Tetrahedron Letters* **55**, 682–685 (2014).
16. McPherson, D. W. *et al.* A simple one-pot synthesis of cyclopropane [11C]carbonyl chloride. Synthesis and biodistribution of [11C]cyclorphan. *Journal of Labelled Compounds and Radiopharmaceuticals* **23**, 505–514 (1986).
17. Luthra, S. K., Le Bars, D., Pike, V. W. & Brady, F. Preparation of NCA [1-11C]acid chlorides as labelling agents for amides. *Journal of Labelled Compounds and Radiopharmaceuticals* **26**, 66–68 (1989).
18. Luthra, S. K., Pike, V. W. & Brady, F. Preparation of some NCA [1-11C]acid chlorides as labelling agents. *International Journal of Radiation Applications and Instrumentation. Part A. Applied Radiation and Isotopes* **41**, 471–476 (1990).
19. Rami-Mark, C. *et al.* Reliable set-up for in-loop 11C-carboxylations using Grignard reactions for the preparation of [carbonyl-11C]WAY-100635 and [11C]-(+)-PHNO. *Applied Radiation and Isotopes* **82**, 75–80 (2013).

20. Coenen, H. H. *et al.* Consensus nomenclature rules for radiopharmaceutical chemistry — Setting the record straight. *Nuclear Medicine and Biology* **55**, v–xi (2017).

Unpublished Data: Manuscripts in Preparation

Manuscript IV: Microwave Assisted ^{68}Ga -Labeling

Materials and Methods

DOTA-NOC (GMP), NODAGA-RGDyk and PSMA-11 (GMP) for ^{68}Ga -labeling were provided by ABX (Radeberg, Germany). A $^{68}\text{Ge}/^{68}\text{Ga}$ -generator was purchased from Obninsk (Cyclotron Co., Ltd, Obninsk, Russia). GMP certificated 0.1 M HCl was provided by ROTEM (Mishor Yamin D.N Arava, Israel). All other chemicals were obtained from Sigma Aldrich or Merck (Vienna, Austria). All chemicals were used without further purification. High performance liquid chromatography (HPLC) was conducted using a HPLC system from Agilent Technologies (Series 1200) including a variable wavelength detector, a scintillation detector obtained from Elysia Raytest (Gabi Star 2x2'') and a Chromolith® Performance column, RP-18e, 100-4.6 mm from Merck (Germany). Radio thin layer chromatography (ITLC, instant TLC) was carried out using silica gel impregnated paperstrips obtained from Agilent Technologies (Santa Clara, USA). A Canberra-Packard Instant Imager (Perkin Elmer, Watford, UK) was used for ITLC measurements. Syntheses were performed by using a microwave emitting Discover PETwave from CEM Corporation (Matthews, United States) incorporating a controlling unit and a synthesis unit.

^{68}Ga -complexations of DOTA-NOC, PSMA-11 and NODAGA-RGDyk were performed using 660 μL of the respective precursor solution in HEPES (1.5 M, pH=5.5). $[^{68}\text{Ga}]\text{Ga}^{3+}$ in HCl (0.1 M) was obtained by elution according to a fractionized elution protocol. 900 μL of the ^{68}Ga -eluate (100-250 MBq/mL) were added to the precursor solution to obtain a pH of 4 - 4.5. Respective reaction conditions (comprised in Fig. 25) were set using the microwave controlling unit. Syntheses were performed by applying a full power mode by which the respective power is used to heat the reaction solution to the set temperature. Furthermore, each radiotracer was synthesized in a NaOAc buffered solution for comparison.

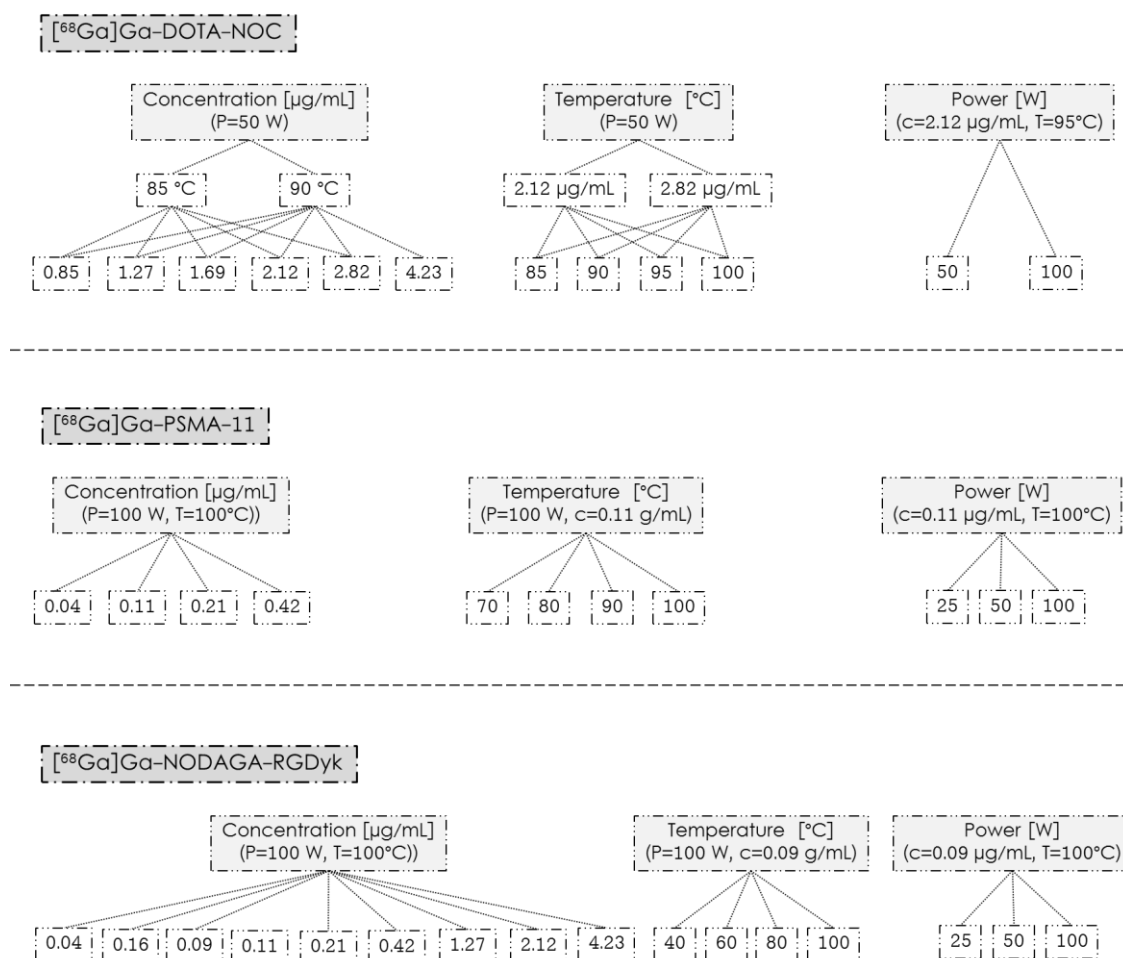


Figure 25: Scheme of tested parameters for microwave assisted synthesis of [⁶⁸Ga]Ga-DOTA-NOC, [⁶⁸Ga]Ga-PSMA-11 and [⁶⁸Ga]Ga-NODAG-RGDyk.

Radiochemical conversion rate (RCR) was assessed by HPLC and TLC. For HPLC measurements a mobile phase consisting of aqua bidest. (A) and ACN (B), both containing 0.1% TFA, was used. For [⁶⁸Ga]Ga-NODAG-RGDyk the following assay was used at a flow rate of 2 mL/min: 0.0 - 2.0 min 97% A. From 2.0 min to 8.0 min the content of A was reduced to 40%. From 8.0 - 9.0 min A was further reduced to 95% and subsequently enhanced to 97% within 0.5 min for equilibration.

[⁶⁸Ga]Ga-PMSA-11 and [⁶⁸Ga]Ga-DOTA-NOC were measured at a flow rate of 2 mL/min (gradient: 0.0-0.6 min 95% A, linear reduction to 50% A from 0.6 to 6.0 min, isocratic flow with 50% A from 6.0 to 7.0 min). Radio TLC was performed by spotting the product on silica gel impregnated paper and using two solvent mixtures: (1) 0.1 M citrate solution (pH 5); (2) 1 M NH₄OAc/MeOH (1:1).

Data are given as mean values $\pm$ SD as calculated with Microsoft Excel 2010 or GraphPad 6 Prism (GraphPad Software, La Jolla, USA), if not stated otherwise. Statistical analyses were carried out by applying a t-test *via* GraphPad Prism 6 (GraphPad Software, La Jolla, USA).

Results

All compounds were successfully radiolabeled with gallium-68 *via* microwave assisted synthesis. The reaction time was dependent on the time of microwave heating, but all complexations could be performed in less than 1 min. Detailed results are comprised below.

[⁶⁸Ga]Ga-DOTA-NOC

⁶⁸Ga-labeling of DOTA-NOC using different precursor concentrations at a temperature of 85°C or 90°C and a power of 50 W showed a trend of enhanced RCR within increasing concentrations and temperatures (not involving the concentration of 2.12 µg/mL) (Fig. 26 and 27).

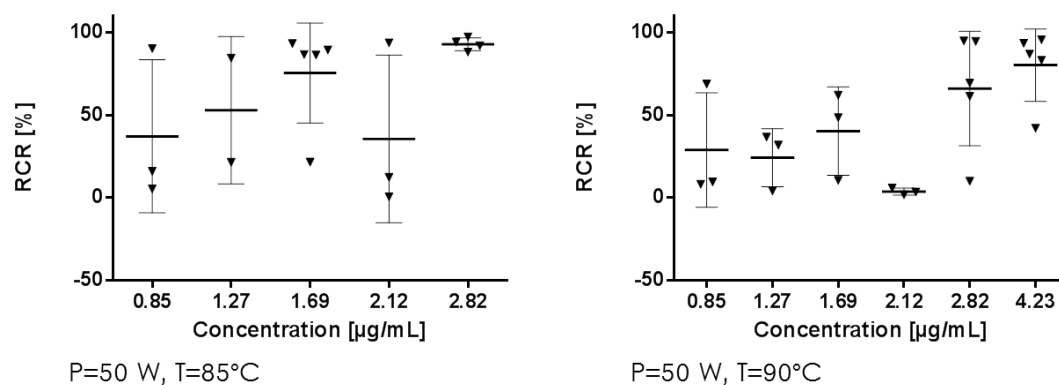


Figure 26: Left: Conversion of [⁶⁸Ga]Ga-DOTA-NOC at a temperature of (left) 85°C or (right) 90°C and a power of 50 W applying different precursor concentrations.

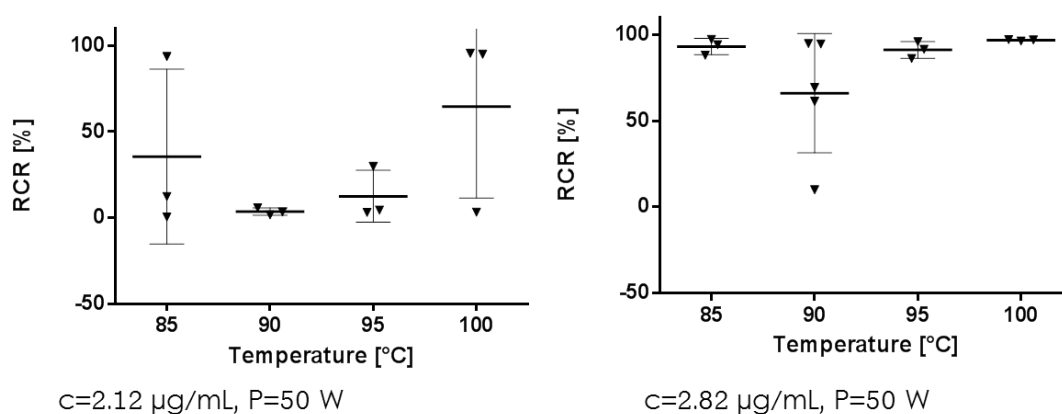


Figure 27: Conversion of $[^{68}\text{Ga}]\text{Ga-DOTA-NOC}$ with a precursor concentration of (left) $2.12 \mu\text{g/mL}$ or (right) $2.82 \mu\text{g/mL}$ and a power of 50 W applying different temperatures.

Statistical analysis of complexations at 85, 90, 95 and 100°C showed no significant difference in conversion at both precursor concentrations of $2.12 \mu\text{g/mL}$ and $2.82 \mu\text{g/mL}$. Furthermore, no difference was found in power settings of 50 W and 100 W for a precursor concentration of $2.12 \mu\text{g/mL}$ and a temperature of 95°C .

$[^{68}\text{Ga}]\text{Ga-PSMA-11}$

^{68}Ga -complexations of PSMA-11 were assessed at a concentration range of $0.04 - 0.42 \mu\text{g/mL}$ with an applied temperature of 100°C and 100 W. Results showed an enhanced RCR within increasing precursor concentrations (Fig. 28).

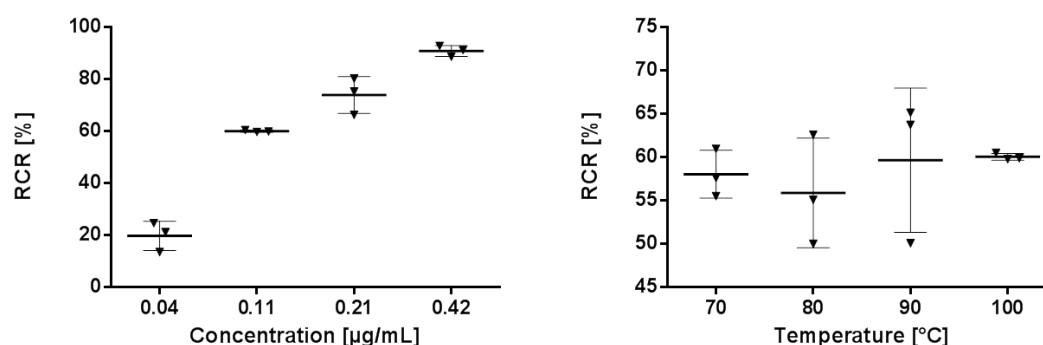


Figure 28: Left: Conversion of [^{68}Ga]Ga-PSMA-11 at a temperature of 100°C and a power of 100 W applying different precursor concentrations. Right: Influence of temperature on the conversion at a precursor concentration of 0.11 µg/mL and 100 W.

Power settings of 25, 50 and 100 W at a precursor concentration of 0.11 µg/mL and a temperature of 100°C showed conversions of 49.4±4.9%, 64.7±5.8% and 60.0±0.4%, respectively (Table 3). Statistical analysis using an unpaired t-test resulted in a significant difference in power settings of 25 W and 50 W ($P < 0.5$).

	Conversion at 100°C, 0.11 µg/mL PSMA-11		
Power	25 W (n=3)	50 W (n=2)	100 W (n=3)
RCR	49.4±4.9%	64.7±5.8%	60.0±0.4%

Table 3: Influence of power on the conversion at a precursor concentration of 0.11 µg/mL and 100°C.

[^{68}Ga]Ga-NODAGA-RGDyk

Microwave assisted synthesis of [^{68}Ga]Ga-NODAGA-RGDyk showed a clear trend of enhanced conversion within increasing precursor concentration until a concentration of 0.42 µg/mL at 100°C and 100 W (Fig. 29). At this concentration the curves starts to reach a plateau resulting in a nearly quantitative conversions of >95%. The influence of temperature was assessed by applying a precursor concentration of 0.09 µg/mL and 100 W. Results showed a trend towards increased yield at enhanced temperatures of 80 and 100°C. Different power settings of 50 W and

100 W did not influence the conversion at a similar concentration and 100°C and gave RCRs of $60.9 \pm 13.8\%$ and $59.2 \pm 6.9\%$, respectively.

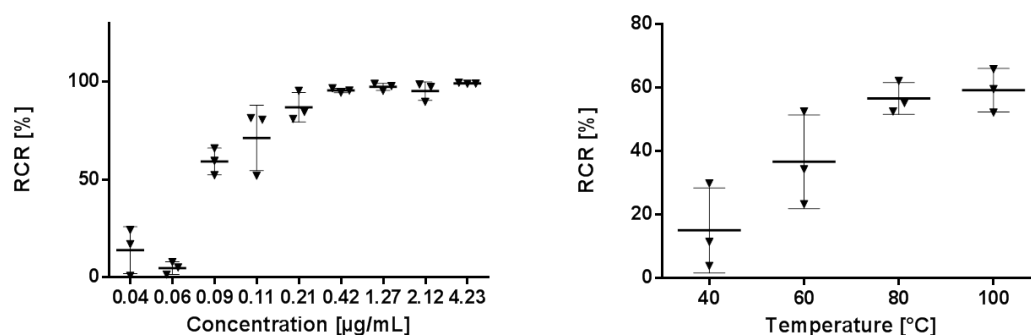


Figure 29: Conversion of $[^{68}\text{Ga}]\text{Ga-NODAGA-RGDyk}$ at a temperature of 100°C and a power of 100 W applying different precursor concentrations. Right: Influence of temperature on conversion at a precursor concentration of 0.09 µg/mL and 100 W.

Buffer System

^{68}Ga -complexation was performed in HEPES as well as sodium acetate. Results for $[^{68}\text{Ga}]\text{Ga-DOTA-NOC}$ in NaOAc buffer showed a higher RCR than for HEPES buffered solution, but statistical analysis revealed no significant difference (Fig. 30). In contrast, synthesis of $[^{68}\text{Ga}]\text{Ga-PSMA-11}$ and $[^{68}\text{Ga}]\text{Ga-NODAGA-RGDyk}$ revealed a highly significant enhancement of RCR by applying HEPES.

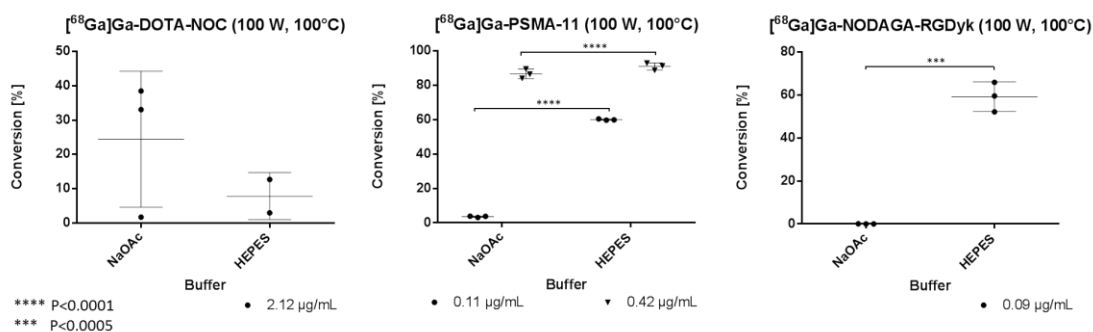


Figure 30: Comparison of RCR using HEPES or NaOAc for ^{68}Ga -complexation of DOTA-NOC, PSMA-11 and NODAGA-RGDyk.

Discussion

^{68}Ga -radiolabeling of DOTA-NOC, PSMA-11 and NODAGA-RGDyk was performed *via* microwave assisted synthesis in high radiochemical conversion rates.

$[^{68}\text{Ga}]\text{Ga-DOA-NOC}$

^{68}Ga -labeling of DOTA-NOC was carried out using different precursor concentrations at a temperature of 85°C and 90°C, respectively. A temperature of 85°C showed high standard deviations at concentrations below 2.12 µg/mL (1.40 µM) and no trend of RCR. Interestingly, standard deviation of radiolabeling at 90°C was higher applying this precursor concentration and RCR was lower at 90°C. Basically, there was a trend of enhanced RCR within increasing precursor concentrations, but due to the high standard deviations either the concentration range or the labeling temperature was probably not at optimum. Hence, ^{68}Ga -labeling applying different temperatures was assessed resulting in high standard deviations for a concentration of 2.12 µg/mL. A recent study by Yagi *et al.* proving the superiority of microwave heating over conventional heating showed that a RCR of over 95% could be achieved for $[^{68}\text{Ga}]\text{Ga-DOTA-TOC}$ in 2 min at a concentration of 3.5 µM.²⁰³ Therefore, ^{68}Ga -labeling of DOTA-NOC may be improved by expanding the reaction time to 2 min and increasing the precursor amount. However, Velikyan *et al.* reported quantitative RCYs by microwave heating of HEPES buffered DOTA-comprising precursor solutions in the nanomolar range within 1 min.¹⁴⁵ Discrepancies within those results may be attributed due to the application of different microwave devices. However, results indicate that the used experimental set up is immature and its improvement will be pursued to exploit reported advances of microwave assisted synthesis for radiolabeling of DOTA-NOC.

$[^{68}\text{Ga}]\text{Ga-PSMA-11}$

Microwave assisted ^{68}Ga -complexations of PSMA-11 showed a clear, linear trend of enhanced RCR within increasing precursor concentrations. The chosen precursor concentrations are in a relatively low sub-micromolar range and started to achieve quantitative, reproducible RCR at an applied concentration of 0.42 µg/mL (0.40 µM). For evaluation of the temperature, a precursor concentration that gave a moderate RCR was used to visualize the impact on RCR. Resultantly,

the optimum temperature for ^{68}Ga -labeling of PSMA-11 was found at 100°C as this setting displayed the lowest standard deviation. Comparison of applied power settings resulted in a significant difference of RCR between 25 W and 100 W. Although there was no significant difference for the conversion between 50 W and 100 W, higher power may be beneficial in terms of a faster synthesis.

[^{68}Ga]Ga-NODAGA-RGDyk

^{68}Ga -labeling of NODAGA-RGDyk using different concentrations showed likewise [^{68}Ga]Ga-PSMA-11 a linear trend of increased RCR at higher precursor concentrations. Furthermore, radiolabeling of NODAGA-RGDyk started to give quantitative RCRs at 0.42 $\mu\text{g/mL}$ (0.41 μM), similar to [^{68}Ga]Ga-PSMA-11.

Buffer System

A comparison of HEPES and NaOAc with respect to the RCR showed a superiority of HEPES for [^{68}Ga]Ga-PSMA-11 and [^{68}Ga]Ga-NODAGA-RGDyk. Radiolabeling of PSMA-11 showed a tremendous difference of the RCR at low precursor amounts that converges at a higher precursor amount. This phenomenon was already reported by Velykian *et al.* for microwave assisted ^{68}Ga -labeling of DOTA-TOC.¹⁴⁵ Interestingly, results for [^{68}Ga]Ga-DOTA-NOC showed a higher RCR in a NaOAc buffered system, although the reported results should be similar in theory as both compounds incorporate the same chelator. However, SD-values are quite high and, as already discussed above, it is assumed that the experimental set up for microwave assisted DOTA-NOC synthesis will be improved. Consequently, obtained results are presumably not representative.

Finally, parameters for microwave assisted synthesis has to be further evaluated for [^{68}Ga]Ga-DOTA-NOC. In contrast, ^{68}Ga -labeling of PSMA-11 and NODAGA-RGDyk resulted in striking RCRs at low precursor amounts. Hence this technique is definitely worthwhile a consideration for routine. Moreover, HEPES showed a superior performance for ^{68}Ga -complexation of PSMA-11 and NODAGA-RGDyk compared to NaOAc suggesting the application of this buffer for improved syntheses in clinical routine.

Manuscript V: HEPES: Impact on Cell Viability and [⁶⁸Ga]Ga-PSMA-11 Accumulation

Materials and Methods

PC3 cells were provided by ATCC®. PMSA transfected PC3 cells (PC3 B9) and PC3 vector cells (PC3 B3) were a generous gift from the working group of Denise O'Keeffe from the University of Texas Health Science Center, San Antonio (Department of Urology). RPMI medium 1640 (1X), 0.05% Trypsin-EDTA (1X), Accutase, FBS (qualified, HI), L-glutamine and DPBS buffer were obtained from Gibco® (Paisley, UK). For absorption measurements, a Synergy HTX Multi-Mode Reader controlled by a Gen5 software version 2.09 (BioTek® Instruments, Inc., Winooski, U.S.A.) was used. IC₅₀ values were also obtained from the calculation of Gen5 software. HEPES, DMSO, and Thiazolyl Blue Tetrazolium bromide (MTT) were provided by Sigma Aldrich (Sigma-Aldrich GmbH, Steinheim, Germany). PSMA-11 (GMP) for ⁶⁸Ga-labeling was obtained from ABX (Radeberg, Germany). Osmolality was determined using a Wesco Vapro® osmometer (Sanoya Medical Systems, Vienna, Austria). A wizard² 3'' 2480 automatic Gamma Counter controlled by a dedicated Wizard² software was obtained from PerkinElmer (Singapore).

MTT-Assay

The influence of HEPES on cell viability was assessed by means of an MTT-assay of PC3 cell lines. By this, PC3, PC3 B3 and PC3 B9 cells were cultivated in RPMI 1640 media containing 1% L-glutamine and 10% FBS. For each cell line, 9×10^3 cells per well in 100 µL were seeded in a 96-well plate (0.32 cm² growth area) and were allowed to settle for 24 h. Then, the cells were treated in triplicates with 100 µL of a solution of 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 mM HEPES in RPMI 1640 as well as untreated wells as control. After 48 h incubation at 37°C, the media was removed and the cells were treated with an MTT (5 mg/mL in DPBS)/RPMI solution (1:10). After 4 h of incubation, the MTT solution was removed and 100 µL DMSO were added to every well and shaken until all formazane crystals were dissolved. Subsequently, the 96-well plate was analyzed with a multi-mode reader at 490 nm and a reference wavelength of 650 nm. The inhibitory concentration at which 50% of cells lost viability (IC₅₀) was calculated by Gen5 software. All experiments were performed in at least three repetitions.

Influence of HEPES on [⁶⁸Ga]Ga-PSMA-11 Accumulation

For the evaluation of the impact on HEPES on [⁶⁸Ga]Ga-PSMA-11 accumulation on PC3-B9 cells, cells were cultivated as described above and 27×10^3 cells/well in 1.5 mL were seeded in 6-well plates (9.6 cm² growth area) 24 h prior experiments. The cells were treated with 1 mL of a [⁶⁸Ga]Ga-PSMA-11 solution in DPBS with HEPES concentrations of 20, 20×10^2 , 20×10^3 and 20×10^6 μ M to obtain a final volume of 2 mL with an activity concentration of 1 MBq/mL. One triplicate was performed in wells without cells as blank to assess the unspecific binding to the plate material and control wells were treated with [⁶⁸Ga]Ga-PSMA-11 not containing HEPES. After the cells were incubated for 1 h, the solution was removed and the cells were washed with 1 mL DPBS. Subsequently, the cells were digested with 200 μ L trypsin or accutase. After the trypsinization was complete (about 5 min), the enzyme was quenched with 800 μ L RPMI. 200 μ L cell suspension of each sample were transferred into a vial for measuring the activity in a gamma-counter device.

Results

MTT-Assay

An MTT assay for evaluation of the impact of HEPES on viability of different PC3 cell lines was performed and all IC₅₀ values were in the micromolar range. In particular, HEPES showed IC₅₀ at concentrations of 321 ± 32 mM, 36 ± 13 mM and 93 ± 20 mM for PC3, PC3 B3 and PC3 B9 cells, respectively (Fig. 31). A potential reason for the antiproliferative effect can be attributed to high osmolality. Osmolality of a 1 M HEPES solution was measured and resulted in 796 mmol/kg.

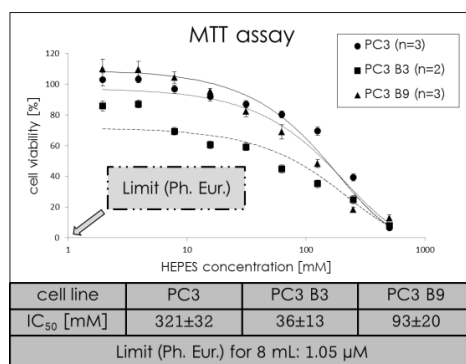


Figure 31: Cell viability of PC3, PC3 B3 and PC3 B9 cells after treatment with different concentrations of HEPES.

Influence of HEPES on [^{68}Ga]Ga-PSMA-11 Accumulation

[^{68}Ga]Ga-PSMA-11 accumulation experiments without HEPES showed an absolute uptake of $1.03 \pm 0.03\%$ after an incubation time of 1 h and an applied radiodose of 2 MBq in 2 mL in stable PSA transfected cells PC3 B9. In contrast, the control cell line PC3 B3 (PSMA non expressing) showed $0.18 \pm 0.02\%$ [^{68}Ga]Ga-PSMA-11 accumulation (Fig. 32).

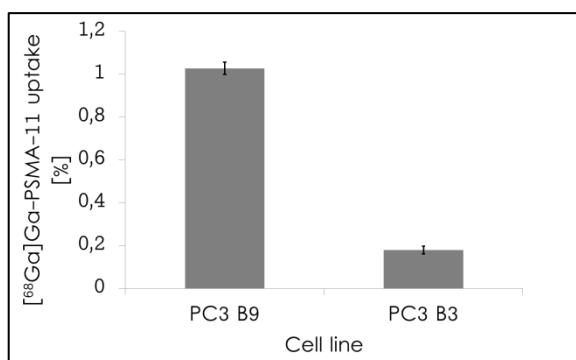


Figure 32: Uptake of [^{68}Ga]Ga-PSMA-11 in PC3 B9 and PC3 B3 cells

Normalized data in figure 33 show that HEPES had no or only minor impact on the accumulation of [^{68}Ga]Ga-PSMA-11 throughout the whole concentration range.

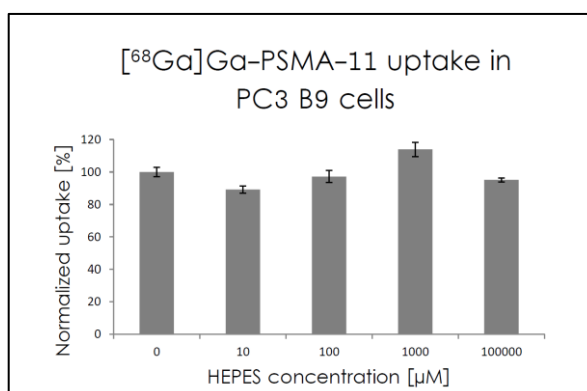


Figure 33: [^{68}Ga]Ga-PSMA-11 uptake of solutions with different HEPES concentrations relative to a solution without HEPES.

Discussion

The low limit of HEPES in radiopharmaceutical formulations of 200 µg/V raises the question if the compound is toxic in biological material or influences the behavior of pharmaceuticals. Consequently, the impact of HEPES on cell viability was assessed by means of an MTT assay using PC3 cells stable transfected with PSMA (PC3 B9) and the according control cell line (PC3 B3). Furthermore, the HEPES' influence on [⁶⁸Ga]Ga-PSMA-11 accumulation in PC3 B9 cells was investigated.

Results of the MTT assay showed that HEPES treatment of all three cell lines reveals IC₅₀ values in the millimolar range but differ from 36±13 mM for PC3 B3 to 321±32 mM for PC3 cells. HEPES concentrations until 3.91 mM did not evoke any impairment of cell viability. In comparison, the limit according to the Ph. Eur. for a recommended administration volume of 8 mL is 1.05 µM which is more than 10³ fold lower. Those results indicate that the prescribed limit can probably be increased. Furthermore, the highest HEPES concentration in medium for treating cells showed an tremendously high osmolality of 796 mmol/kg. Consequently, cells treated with high concentrations of HEPES presumably died due to hyperosmosis. Those assumptions are in accordance to literature stating that HEPES administration is in compliance with safety.^{106,156} Moreover, in a clinical case study were 5 g HEPES per day intravenously administered to humans resulting in a reduction of pain and no adverse events. Therefore, toxicity is probably not a reasonable cause for the low limit for HEPES administration subject to the condition of a physiological pH and osmolality.

A possible constraint on the administration of HEPES is a potential synergistic effect with the applied radiotracer. Resultantly, [⁶⁸Ga]Ga-PSMA-11 accumulation in PC3 B9 cells in the presence of different HEPES concentrations was investigated. Preliminary results showed a normalized uptake in a range of 89-114%. The relatively small differences of uptake within the HEPES concentrations will probably be relativized by future experiments. However, HEPES is an outstanding buffer for ⁶⁸Ga-radiolabeling as described in the section 'Microwave Assisted Synthesis'. Hence, further *in vitro* and *in vivo* data proving that HEPES is not actuating any detrimental effects are imperatively needed for a convenient and safe application of ⁶⁸Ga-radiopharmaceuticals obtained from a HEPES-buffered synthesis.

IV. Discussion

Improvement of Radiotracer Synthesis

One aim of this thesis was the improvement of radiotracer synthesis by implementation of different approaches. In particular, three different ^{68}Ga -radiotracers, namely [^{68}Ga]Ga-DOTA-NOC, [^{68}Ga]Ga-NODAGA-RGDyk and [^{68}Ga]Ga-PSMA-11, were synthesized by using a microfluidic continuous flow device and performing microwave assisted synthesis. Additionally, the highly error prone synthesis of (+)-[^{11}C]PHNO was significantly improved.

One of the most important factors in radiochemical optimization is to reduce the synthesis duration to a minimum due to the short half-life of PET-nuclides. Hence, this parameter was optimized for all investigated radiotracers and synthesis devices. Synthesis time of microfluidic ^{68}Ga -labeling depends on the set flow rate and the volume. In contrast microwave assisted synthesis and (+)-[^{11}C]PHNO synthesis are performed in a vial and therefore, have a fixed synthesis duration. For the proof of principle study using the microfluidic device, the flow rate was set as high as possible to keep the reaction time at a minimum subject to a feasible pressure range. Likewise, the synthesis duration of microwave assisted ^{68}Ga -labeling was performed in an appreciable short duration of one minute. However, synthesis of [^{68}Ga]Ga-DOTA-NOC is probably improvable by extending the synthesis time. The time limiting parameters in (+)-[^{11}C]PHNO synthesis are the heating and cooling procedures. Therefore, the synthesis was performed at room temperature skipping all heating and cooling steps. Consequently, the activity yield could be increased by constraining the time for decay. Those results show that (+)-[^{11}C]PHNO synthesis is relatively independent from the applied temperature. In contrast, successful microfluidic ^{68}Ga -labeling was strongly associated with the temperature setting as radioactive species showed a reduced sticking in the device at high temperatures. Experiments using a microwave showed an advantageous outcome at enhanced temperatures as well. As ^{68}Ga -complexations are performed in aqueous media, the feasibility of NaOAc and HEPES were tested to obtain an optimized set up for microwave assisted synthesis. In contrast, an inert atmosphere is pivotal for (+)-[^{11}C]PHNO synthesis.

Microfluidics vs. Microwave Assisted Synthesis

In order to compare RCR obtained by microfluidic and microwave assisted ^{68}Ga -labeling, similar concentrations for radiolabeling were consulted (table 4). ^{68}Ga -complexation of PSMA-11 showed a similar RCR for both devices. In contrast, RCR of $[^{68}\text{Ga}]\text{Ga}$ -DOTA-NOC and $[^{68}\text{Ga}]\text{Ga}$ -NODAGA-RGDyk obtained by microwave assisted synthesis dramatically surpassed RCR achieved by microfluidic syntheses. Therefore, microwave assisted synthesis of those radiotracers was proven superior in terms of RCR.

100 °C	Synthesis using a microfluidic device		Microwave assisted synthesis	
^{68}Ga -complex	Concentration [$\mu\text{g/mL}$]	RCR $\pm$ SD [%]	Concentration [$\mu\text{g/mL}$]	RCR $\pm$ SD [%]
$[^{68}\text{Ga}]\text{Ga}$ -DOTA-NOC	15.00	57.9 $\pm$ 20.0	4.23	80.3 $\pm$ 21.9
$[^{68}\text{Ga}]\text{Ga}$ -PSMA-11	0.50	93.3 $\pm$ 7.5	0.42	91.0 $\pm$ 2.1
$[^{68}\text{Ga}]\text{Ga}$ -NODAGA-RGDyk	7.50	92.5 $\pm$ 14.4	0.42	95.7 $\pm$ 1.1

Table 4: Comparison of RCR of $[^{68}\text{Ga}]\text{Ga}$ -DOTA-NOC, $[^{68}\text{Ga}]\text{Ga}$ -PSMA-11 and $[^{68}\text{Ga}]\text{Ga}$ -NODAGA-RGDyk *via* microfluidic and microwave assisted ^{68}Ga -labeling.

Microwave assisted synthesis achieved a reaction time of less than one minute and is more flexible as a feasible reaction vessel for the volume of the reaction mixture can be chosen (Fig. 34). In contrast, reaction time using the microfluidic device was more than one minute depending on the applied reaction volume and set flow rate. Moreover, the preparation procedure of the microfluidic synthesizer takes more time as the reactants have to be loaded into the dedicated loops. Hence, implementation of a microwave is highly time efficient. However, a microfluidic device is amenable for a dose on demand approach for future perspectives regarding high activities of gallium-68 *via* cyclotron production. Additionally, an enhanced radiolysis due to high activities could be alleviated. In contrast, an advantage of microwave assisted synthesis is its feasibility for a preparation according to KIT like approach that contains an appreciably low amount of precursor.

From the scientific point of view, a microfluidic device is capable of performing a huge number of batches consecutively. In this way, this technique is perfectly applicable for a fast screening of reaction parameters and synthesis optimization.

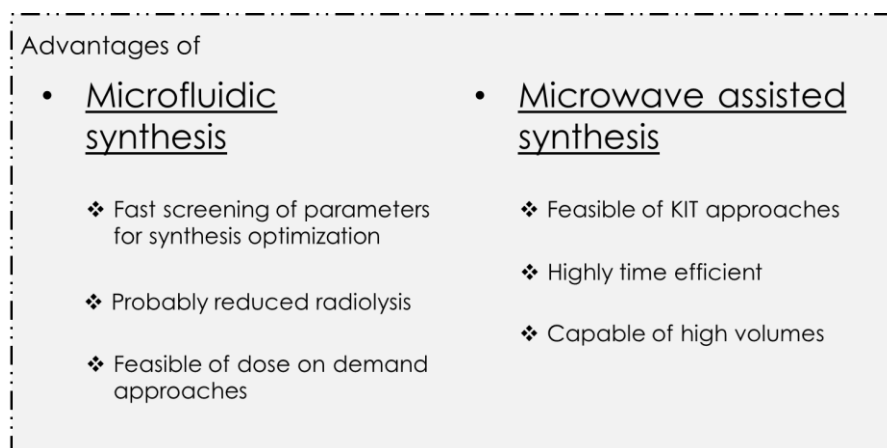


Figure 34: Advantages of microfluidic synthesis and microwave assisted synthesis

Finally, both synthesizers reveal excellent features for respective purposes. However, a prediction about a superior feasibility of a microfluidic device or a microwave for a particular synthesis cannot be made in advance. Hence, both approaches have to be evaluated for finding an optimal technique for radiolabeling.

Improved Troubleshooting after (+)-[¹¹C]PHNO Synthesis

Besides optimization of (+)-[¹¹C]PHNO synthesis, awareness of the error source after a failed synthesis is essential for a continuous workflow in clinical routine. Hence, possible byproducts and intermediates by omitting the addition of particular reagents were synthesized in order to assign chromatogram patterns to the origin of an error. As a consequence, a decision tree for finding the error source was created, which dramatically facilitated troubleshooting.

Reliable and Quantitative HEPES Determination

The superiority of HEPES compared to NaOAc was shown by means of microwave assisted ⁶⁸Ga-labeling within this thesis. However, the content of this substance in radiopharmaceutical formulations has to be tested prior release and the prescribed TLC method by the Ph. Eur. is described as not reliable. Evaluation of the TLC assay and alternative HPLC methods revealed the

impracticability of first one and good results for the latter one. As an HPLC assay demands high technical effort, a new TLC method was developed by stepwise analysis of the obstacles within the method according to the Ph. Eur. and adjusting all parameters (reference solution, mobile phase, spotting method, iodine staining and quantitative analysis) to obtain reproducible and reliable assay. Consequently, HEPES determination of radiopharmaceutical solutions within quality control is tremendously facilitated, which may enhance the number of facilities working with HEPES for clinical applications. Furthermore, the application of a software for quantitative analysis prevents subjective interpretations by individual operators resulting in a standardized method.

Biological Data of HEPES in Three PC3 Cell Lines

The reasonability of the appreciable low HEPES limit was questioned within this thesis. Therefore, the influence of HEPES on cell viability was investigated by means of an MTT assay that is broadly applied for gaining cytotoxicity data. Results showed no impact on cell viability up to a HEPES concentration that exceeds the prescribed limit more than a thousand fold. Additionally, the influence on radiotracer uptake was evaluated by treating PSMA-expressing cell lines with [^{68}Ga]Ga-PSMA-11 in presence of HEPES in different concentrations. The obtained data support the hypothesis that the HEPES limit should be reconsidered

V. Summary and Outlook

Radiochemistry is a small niche field in chemistry with special prerequisites which can cause challenging radiotracer synthesis. Hence, an important aspect is the development of improved synthesis procedures and associated quality control assays. Accordingly, this work provides studies of successfully performed ^{68}Ga -labeling *via* two unconventional approaches: microwave assisted synthesis and application of a microfluidic device. Both of them achieved high conversion in a HEPES buffered system, which is associated with cost effectiveness. Resultantly, those devices show a great potential for further routine and scientific applications. Furthermore, a novel TLC assay for determining the HEPES concentration in radiopharmaceutical formulations was developed. This method is conclusive and reliable in contrast to the prescribed method by the Ph. Eur. As a result, the new assay facilitates HEPES quantification in clinical routine dramatically. Hence, the developed assay is currently considered for publication in Ph. Eur. Additionally, the HEPES limit set by the Ph. Eur. was questioned and *in vitro* tests investigating the influence of HEPES on 1) cell viability and 2) [^{68}Ga]Ga-PSMA-11 accumulation were performed. Preliminary results show no influence on cell viability in concentrations that exceed the prescribed limit by a factor of 10^3 . The first experiment of the impact on radiotracer accumulation leads to the assumption that there is no difference caused by HEPES. Consequently, the HEPES limit should be reconsidered and further investigations regarding HEPES are needed. Besides, the challenging synthesis of (+)-[^{11}C]PHNO was optimized by skipping all heating and cooling steps after release of [^{11}C]CO₂ leading to a dramatic time reduction of 5 min. A consecutive study of byproducts and impurities formation led to a scheme of HPLC chromatogram patterns of failed (+)-[^{11}C]PHNO formation which are assigned to probable error sources. Therefore, time intensive troubleshooting after a failed synthesis will help tremendously.

Ultimately, this work describes the improvement of various radiotracer synthesis and HEPES testing during the quality control procedure. The prescribed limit of HEPES was reasonably questioned and reconsidered on base of *in vitro* assays. Resultantly, pursuing the implemented approaches within this thesis could appreciably facilitate radiosynthesis and quality control in clinical studies and routine.

VI. Abbreviations

ACN	acetonitrile
AUC	Area under the curve
CT	Computed tomography
DOTA-NOC	L-Cysteinamide, N-[[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododec-1-yl]acetyl]-D-phenylalanyl-L-cysteinyl-3-(1-naphthalenyl)-L-alanyl-D-tryptophyl-L-lysyl-L-threonyl-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)propyl]-, cyclic (2→7)-disulfide
DOTA-TATE	DOTA-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH, cyclic disulfide
DOTA-TOC	L-Cysteinamide, N-[[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododec-1-yl]acetyl]-D-phenylalanyl-L-cysteinyl-L-tyrosyl-D-tryptophyl-L-lysyl-L-threonyl-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)propyl]-, cyclic(2→7)-disulfide
EDTA	Ethylenediaminetetraacetic acid
EtMgBr	Ethylmagnesium bromide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
LAH	Lithium aluminum hydride
LOR	Line of response
MeOH	Methanol
MR	Magnetic resonance

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NaOAc	Sodium acetate
NODAGA-RGDyk	Cyclo[L-arginylglycyl-L- α -aspartyl-D-tyrosyl-N6-([4,7-bis(carboxymethyl)-1,4,7-triazonan-1-yl]acetyl)]-L-lysyl]
PET	Positron emission tomography
Ph. Eur.	European Pharmacopoeia
PSMA	Prostate specific membrane antigen
PSMA-11	Glu-NH-CO-NH-Lys(Ahx)-HBED-CC
RCR	Radiochemical conversion rate
RCY	Radiochemical yield
SD	Standard deviation
SST	Somatostatin
SSTR	Somatostatin rezeptor
TEA	Triethylamine
TLC	Thin layer chromatography
(+)-HNO	2H-Naphtho[1,2-b]-1,4-oxazin-9-ol, 3,4,4a,5,6,10b-hexahydro-hydrochloride
(+)-PHNO	(+)-4-Propyl-3,4,4a,5,6,10b-hexahydro-2H-naphtho[1,2-b][1,4]oxazin-9-ol

VII. Figure Caption

Figure 1: Scheme of a PET-scanner and the principle behind PET. ¹¹	2
Figure 2: Radioactive decay of carbon-11, gallium-68 and fluorine-18.	4
Figure 3: Timeline of ⁶⁸ Ga-relevant events in history. ^{28,33–39}	7
Figure 4: Scheme of decay from germanium-68 to gallium-68 and finally to zinc-68.	7
Figure 5: Equilibrium of long lived mother nuclide and its daughter nuclide.	8
Figure 6: Representative elution profile of a ⁶⁸ Ge/ ⁶⁸ Ga-generator with highlighted main fraction.	10
Figure 7: Scheme of elution of a ⁶⁸ Ge/ ⁶⁸ Ga-generator with subsequent concentration.	11
Figure 8: Chemical structure of A: DOTA (R ¹ =linkage with biomolecule, R ² =H) and DOTAGA (R ¹ =OH, R ² =Linkage with biomolecule); B: NOTA (R ¹ =linkage with biomolecule, R ² =H) and NODAGA (R ¹ =OH, R ² =Linkage with biomolecule) and C: HBED-CC (R=Linkage with biomolecule).	13
Figure 9: Molecular structure of [⁶⁸ Ga]Ga-DOTA-TOC, [⁶⁸ Ga]Ga-DOTA-TATE and [⁶⁸ Ga]Ga-DOTA-NOC, and the SSTR subtypes they are showing the highest affinity to.	14
Figure 10: Assumption of potential binding of [⁶⁸ Ga]Ga-PSMA-11 to PSMA adapted from Liu et al. ⁹⁵	15
Figure 11: Simplified representation of the chemical structure of [⁶⁸ Ga]Ga-NODAGA-RGDyK.	16
Figure 12: Scheme of commonly performed ⁶⁸ Ga-labeling processes.	17
Figure 13: Synchrom Ga from raytest (left), Modular-Lab Standard from Eckert & Ziegler (middle) and TRACERlab FX from GE (right). ¹⁰⁵	18
Figure 14: Modular-Lab PharmTracer from Eckert & Ziegler (left) and FASTlab from GE (right). ¹⁰⁵	19
Figure 15: Scheme of ⁶⁸ Ga-labeling <i>via</i> KITs.	20
Figure 16: : Schematic arrangement of a NanoTek microfluidic synthesizer and image of the device (adapted from Pfaff <i>et al.</i> ¹³⁷)	22
Figure 17: Alignment of a H ₂ O molecule in the electric field adapted from Kappe <i>et al.</i> ¹³⁸	23
Figure 18: Conventional heating (left) showing a heterogeneous temperature distribution with a higher temperature at the reactor walls. Microwave heating (right) showing dielectric heating within the solution for an almost homogenous heating.	24
Figure 19: Chemical structure of HEPES.	25
Figure 20: Possible chemical structure of Ga-complexes of HEPES based on Martin <i>et al.</i> ¹⁵¹	25
Figure 21: A selection of ¹¹ C-labeling reactions starting with [¹¹ C]CO ₂ .	29

Figure 22: A cursory view of Grignard reaction mechanism.	30
Figure 23: Simplified scheme of synaptic D ₂ -receptors showing high/low affinity states and the behavior of agonists.	31
Figure 24: Synthesis scheme of (+)-[¹¹ C]PHNO according to Rami-Mark et al. ¹⁷⁹	33
Figure 25: Scheme of tested parameters for microwave assisted synthesis of [⁶⁸ Ga]Ga-DOTA-NOC, [⁶⁸ Ga]Ga-PSMA-11 and [⁶⁸ Ga]Ga-NODAG-RGDyk.	92
Figure 26: Left: Conversion of [⁶⁸ Ga]Ga-DOTA-NOC at a temperature of (left) 85°C or (right) 90°C and a power of 50 W applying different precursor concentrations.....	93
Figure 27: Conversion of [⁶⁸ Ga]Ga-DOTA-NOC with a precursor concentration of (left) 2.12 µg/mL or (right) 2.82 µg/mL and a power of 50 W applying different temperatures.	94
Figure 28: Left: Conversion of [⁶⁸ Ga]Ga-PSMA-11 at a temperature of 100°C and a power of 100 W applying different precursor concentrations. Right: Influence of temperature on the conversion at a precursor concentration of 0.11 µg/mL and 100 W.	95
Figure 29: Conversion of [⁶⁸ Ga]Ga-NODAGA-RGDyk at a temperature of 100°C and a power of 100 W applying different precursor concentrations. Right: Influence of temperature on conversion at a precursor concentration of 0.09 µg/mL and 100 W.	96
Figure 30: Comparison of RCR using HEPES or NaOAc for ⁶⁸ Ga-complexation of DOTA-NOC, PSMA-11 and NODAGA-RGDyk.	96
Figure 31: Cell viability of PC3, PC3 B3 and PC3 B9 cells after treatment with different concentrations of HEPES.	100
Figure 32: Uptake of [⁶⁸ Ga]Ga-PSMA-11 in PC3 B9 and PC3 B3 cells.....	101
Figure 33: [⁶⁸ Ga]Ga-PSMA-11 uptake of solutions with different HEPES concentrations relative to a solution without HEPES.	101
Figure 34: Advantages of microfluidic synthesis and microwave assisted synthesis.....	105

VIII. References

1. Scatliff, J. H. & Morris, P. J. From Röntgen to Magnetic Resonance Imaging: The History of Medical Imaging. *N. C. Med. J.* **75**, 111–113 (2014).
2. Sweet, W. H. The Uses of Nuclear Disintegration in the Diagnosis and Treatment of Brain Tumor. *N. Engl. J. Med.* **245**, 875–878 (1951).
3. Brownell, G. L. & Sweet, W. H. Localization of brain tumors with positron emitters. *Nucleonics* **11**, 40–45 (1953).
4. Haberkorn, U. & Eisenhut, M. Molecular imaging and therapy—a programme based on the development of new biomolecules. *Eur. J. Nucl. Med. Mol. Imaging* **32**, 1354–1359 (2005).
5. Massoud, T. F. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* **17**, 545–580 (2003).
6. Weissleder, R. & Mahmood, U. Molecular Imaging. *Radiology* **219**, 316–333 (2001).
7. Doubrovin, M., Serganova, I., Mayer-Kuckuk, P., Ponomarev, V. & Blasberg, R. G. Multimodality in Vivo Molecular-Genetic Imaging. *Bioconjug. Chem.* **15**, 1376–1388 (2004).
8. Mankoff, D. A. A definition of molecular imaging. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **48**, 18N, 21N (2007).
9. Kubota, K. From tumor biology to clinical PET: A review of positron emission tomography (PET) in oncology. *Ann. Nucl. Med.* **15**, 471–486 (2001).
10. Herzog, H. Methods and applications of positron-based medical imaging. *Radiat. Phys. Chem.* **76**, 337–342 (2007).
11. Li, Z. & Conti, P. S. Radiopharmaceutical chemistry for positron emission tomography. *Adv. Drug Deliv. Rev.* **62**, 1031–1051 (2010).
12. Pichler, B. J. *et al.* Performance test of an LSO-APD detector in a 7-T MRI scanner for simultaneous PET/MRI. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **47**, 639–647 (2006).

13. von Schulthess, G. K., Steinert, H. C. & Hany, T. F. Integrated PET/CT: Current Applications and Future Directions. *Radiology* **238**, 405–422 (2006).
14. Shao, Y. *et al.* Simultaneous PET and MR imaging. *Phys. Med. Biol.* **42**, 1965–1970 (1997).
15. Baum, R. P. & Kulkarni, H. R. THERANOSTICS: From Molecular Imaging Using Ga-68 Labeled Tracers and PET/CT to Personalized Radionuclide Therapy - The Bad Berka Experience. *Theranostics* **2**, 437–447 (2012).
16. Lundqvist, H. & Tolmachev, V. Targeting peptides and positron emission tomography. *Biopolymers* **66**, 381–392 (2002).
17. Ametamey, S. M., Honer, M. & Schubiger, P. A. Molecular Imaging with PET. *Chem. Rev.* **108**, 1501–1516 (2008).
18. Larsen, P., Ulin, J., Dahlstrøm, K. & Jensen, M. Synthesis of [11C]iodomethane by iodination of [11C]methane. *Appl. Radiat. Isot.* **48**, 153–157 (1997).
19. *Ph. Eur. 8th edition. General monographs: radiopharmaceutical preparations.* (2014).
20. Coenen, H. H. *et al.* Consensus nomenclature rules for radiopharmaceutical chemistry — Setting the record straight. *Nucl. Med. Biol.* **55**, v–xi (2017).
21. Schmidt, A., Schottelius, M., Herz, M. & Wester, H.-J. Production of clinical radiopharmaceuticals: general pharmaceutical and radioanalytical aspects. *J. Radioanal. Nucl. Chem.* **311**, 1551–1557 (2017).
22. Decristoforo, C., Pickett, R. D. & Verbruggen, A. Feasibility and availability of 68Ga-labelled peptides. *Eur. J. Nucl. Med. Mol. Imaging* **39**, 31–40 (2012).
23. Machulla, H. J., Laufer, P. & Stöcklin, G. Radioanalytical quality control of 11C, 18F and 123I-labelled compounds and radiopharmaceuticals. *J. Radioanal. Chem.* **32**, 381–400 (1976).
24. Meyer, G.-J. *et al.* Quality Assurance and Quality Control of Short-Lived Radiopharmaceuticals for PET. in *Radiopharmaceuticals for Positron Emission Tomography*

- (eds. Stöcklin, G. & Pike, V. W.) 91–150 (Springer Netherlands, 1993). doi:10.1007/978-94-015-8204-9_3
25. INTERNATIONAL ATOMIC ENERGY AGENCY. *Quality control in the production of radiopharmaceuticals*. (INTL ATOMIC ENERGY AGENCY, 2018).
 26. Notni, J. & Wester, H.-J. 68Ga-Radiopharmaka: Methode oder Episode? *Nukl.* **41**, 335–347 (2018).
 27. Boisbaudran, L. Ueber das Gallium. Mit dem Namen Gallium und dem Symbol Ga bezeichnet. *Zeitschr. f. Anal. Chem.* **16**, 239–242 (1877).
 28. Hayes, R. L. The medical use of gallium radionuclides: a brief history with some comments. *Semin. Nucl. Med.* **8**, 183–191 (1978).
 29. Anger, H. O. & Gottschalk, A. LOCALIZATION OF BRAIN TUMORS WITH THE POSITRON SCINTILLATION CAMERA. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **4**, 326–330 (1963).
 30. Banerjee, S. R. & Pomper, M. G. Clinical applications of Gallium-68. *Appl. Radiat. Isot.* **76**, 2–13 (2013).
 31. Fani, M., André, J. P. & Maecke, H. R. 68Ga-PET: a powerful generator-based alternative to cyclotron-based PET radiopharmaceuticals. *Contrast Media Mol. Imaging* **3**, 53–63 (2008).
 32. Decristoforo, C. Gallium-68 -- a new opportunity for PET available from a long shelf-life generator - automation and applications. *Curr. Radiopharm.* **5**, 212–220 (2012).
 33. Smith-Jones, P. M. *et al.* Gallium-67/gallium-68-[DFO]-octreotide--a potential radiopharmaceutical for PET imaging of somatostatin receptor-positive tumors: synthesis and radiolabeling in vitro and preliminary in vivo studies. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **35**, 317–325 (1994).
 34. McAfee, J. G., Subramanian, G., Gagne, G., Schneider, R. F. & Zapf-Longo, C. 99mTc-HM-PAO for leukocyte labeling--experimental comparison with 111In oxine in dogs. *Eur. J. Nucl. Med.* **13**, 353–357 (1987).

35. Banerjee, S., Ambikalmajan Pillai, M. R. & Ramamoorthy, N. Evolution of Tc-99m in diagnostic radiopharmaceuticals. *Semin. Nucl. Med.* **31**, 260–277 (2001).
36. Hart, M. M. & Adamson, R. H. Antitumor Activity and Toxicity of Salts of Inorganic Group IIIa Metals: Aluminum, Gallium, Indium, and Thallium. *Proc. Natl. Acad. Sci.* **68**, 1623–1626 (1971).
37. Krenning, E. P. *et al.* ¹¹¹In-octreotide scintigraphy in oncology. *Metabolism* **41**, 83–86 (1992).
38. Mathias, C. J. *et al.* Preparation of ⁶⁶Ga- and ⁶⁸Ga-labeled Ga(III)-deferoxamine-folate as potential folate-receptor-targeted PET radiopharmaceuticals. *Nucl. Med. Biol.* **30**, 725–731 (2003).
39. Schuhmacher, J. *et al.* GRP receptor-targeted PET of a rat pancreas carcinoma xenograft in nude mice with a ⁶⁸Ga-labeled bombesin(6-14) analog. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **46**, 691–699 (2005).
40. Moerlein, S. M. & Welch, M. J. The chemistry of gallium and indium as related to radiopharmaceutical production. *Int. J. Nucl. Med. Biol.* **8**, 277–287 (1981).
41. Moeller, T. & King, G. L. Some physicochemical studies on gallium (III) salt solutions. *J. Phys. Colloid Chem.* **54**, 999–1011 (1950).
42. Pearson, R. G. Hard and Soft Acids and Bases. *J. Am. Chem. Soc.* **85**, 3533–3539 (1963).
43. Bandoli, G., Dolmella, A., Tisato, F., Porchia, M. & Refosco, F. Mononuclear six-coordinated Ga(III) complexes: A comprehensive survey. *Coord. Chem. Rev.* **253**, 56–77 (2009).
44. Harris, W. R. & Pecoraro, V. L. Thermodynamic binding constants for gallium transferrin. *Biochemistry* **22**, 292–299 (1983).
45. Bartholomä, M. D. Recent developments in the design of bifunctional chelators for metal-based radiopharmaceuticals used in Positron Emission Tomography. *Inorganica Chim. Acta* **389**, 36–51 (2012).

46. Roesch, F. Maturation of a key resource - the germanium-68/gallium-68 generator: development and new insights. *Curr. Radiopharm.* **5**, 202–211 (2012).
47. Gleason, G. I. A positron cow. *Int. J. Appl. Radiat. Isot.* **8**, 90–94 (1960).
48. Greene, M. W. & Tucker, W. D. An improved gallium-68 cow. *Int. J. Appl. Radiat. Isot.* **12**, 62–63 (1961).
49. Yano, Y. & Anger, H. O. A GALLIUM-68 POSITRON COW FOR MEDICAL USE. UCRL-11387. *UCRL Rep. US At. Energy Comm.* **31**, 87–90 (1964).
50. Nakayama, M. *et al.* A new ⁶⁸Ge/⁶⁸Ga generator system using an organic polymer containing N-methylglucamine groups as adsorbent for ⁶⁸Ge. *Appl. Radiat. Isot.* **58**, 9–14 (2003).
51. Schuhmacher, J. & Maier-Borst, W. A new ⁶⁸Ge/⁶⁸Ga radioisotope generator system for production of ⁶⁸Ga in dilute HCl. *Int. J. Appl. Radiat. Isot.* **32**, 31–36 (1981).
52. Loc'h, C., Mazière, B. & Comar, D. A new generator for ionic gallium-68. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **21**, 171–173 (1980).
53. Romero, E. & Morcillo, M. A. Inorganic oxides with potential application in the preparation of a ⁶⁸Ge/ ⁶⁸Ga generator system. *Appl. Radiat. Isot.* **119**, 28–35 (2017).
54. Velikyan, I., Antoni, G., Sörensen, J. & Estrada, S. Organ biodistribution of Germanium-68 in rat in the presence and absence of [(⁶⁸Ga)Ga-DOTA-TOC for the extrapolation to the human organ and whole-body radiation dosimetry. *Am. J. Nucl. Med. Mol. Imaging* **3**, 154–165 (2013).
55. *Ph. Eur., 6. Nachtrag Monographie [68Ga]Galliumedotreotid Injektionslösung.*
56. Alves, F. *et al.* Production of copper-64 and gallium-68 with a medical cyclotron using liquid targets. *Mod. Phys. Lett. A* **32**, 1740013 (2017).
57. Engle, J. W. *et al.* Very high specific activity ⁶⁶/68Ga from zinc targets for PET. *Appl. Radiat. Isot.* **70**, 1792–1796 (2012).

58. Synowiecki, M. A., Perk, L. R. & Nijsen, J. F. W. Production of novel diagnostic radionuclides in small medical cyclotrons. *EJNMMI Radiopharm. Chem.* **3**, (2018).
59. Zhernosekov, K. P. *et al.* Processing of Generator-Produced ^{68}Ga for Medical Application. *J. Nucl. Med.* **48**, 1741–1748 (2007).
60. Eidherr, H., Girschele, F., Mitterhauser, M. & Wadsak, W. Synthesis of [^{68}Ga]gallium DOTA-(Tyr3)-octreotide acetata (^{68}Ga -DOTATOC). in *Radiochemical Syntheses* **1**, 321–334 (Wiley, 2012).
61. Eidherr, H. *et al.* Fully automated preparation of ^{68}Ga PSMA-HBEC-CC using fractionated elution. *Eur. J. Nucl. Med. Mol. Imaging* **41**, 428 (2014).
62. de Blois, E. *et al.* Characteristics of SnO_2 -based $^{68}\text{Ge}/^{68}\text{Ga}$ generator and aspects of radiolabelling DOTA-peptides. *Appl. Radiat. Isot.* **69**, 308–315 (2011).
63. Breeman, W. A. P. *et al.* Radiolabelling DOTA-peptides with ^{68}Ga . *Eur. J. Nucl. Med. Mol. Imaging* **32**, 478–485 (2005).
64. Meyer, G.-J., Mäcke, H., Schuhmacher, J., Knapp, W. H. & Hofmann, M. ^{68}Ga -labelled DOTA-derivatised peptide ligands. *Eur. J. Nucl. Med. Mol. Imaging* **31**, 1097–1104 (2004).
65. Mueller, D. *et al.* Simplified NaCl Based ^{68}Ga Concentration and Labeling Procedure for Rapid Synthesis of ^{68}Ga Radiopharmaceuticals in High Radiochemical Purity. *Bioconjug. Chem.* **23**, 1712–1717 (2012).
66. Martin, R., Jüttler, S., Müller, M. & Wester, H.-J. Cationic eluate pretreatment for automated synthesis of [^{68}Ga]CPCR4.2. *Nucl. Med. Biol.* **41**, 84–89 (2014).
67. Asti, M. *et al.* Validation of $^{68}\text{Ge}/^{68}\text{Ga}$ generator processing by chemical purification for routine clinical application of ^{68}Ga -DOTATOC. *Nucl. Med. Biol.* **35**, 721–724 (2008).
68. Liu, S. The role of coordination chemistry in the development of target-specific radiopharmaceuticals. *Chem. Soc. Rev.* **33**, 445–461 (2004).
69. Lavender, J. P., Lowe, J., Barker, J. R., Burn, J. I. & Chaudhri, M. A. Gallium 67 citrate scanning in neoplastic and inflammatory lesions. *Br. J. Radiol.* **44**, 361–366 (1971).

70. Mathias, C. J. *et al.* Targeting radiopharmaceuticals: Comparative biodistribution studies of gallium and indium complexes of multidentate ligands. *Int. J. Rad. Appl. Instrum. B* **15**, 69–81 (1988).
71. Moerlein, S. M., Welch, M. J., Raymond, K. N. & Weitzl, F. L. Trictecholamide analogs of enterobactin as gallium- and indium-binding radiopharmaceuticals. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **22**, 710–719 (1981).
72. Hunt, F. C. Phenolic Aminocarboxylic Acids as Gallium-Binding Radiopharmaceuticals. *Nuklearmedizin* **23**, 123–125 (1984).
73. Green, M. A., Welch, M. J., Mathias, C. J., Taylor, P. & Martell, A. E. Evaluation of PLED as a chelating ligand for the preparation of gallium and indium radiopharmaceuticals. *Int. J. Nucl. Med. Biol.* **12**, 381–386 (1985).
74. Stasiuk, G. J. & Long, N. J. The ubiquitous DOTA and its derivatives: the impact of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid on biomedical imaging. *Chem. Commun.* **49**, 2732–2746 (2013).
75. Notni, J., Pohle, K. & Wester, H.-J. Comparative gallium-68 labeling of TRAP-, NOTA-, and DOTA-peptides: practical consequences for the future of gallium-68-PET. *EJNMMI Res.* **2**, 28 (2012).
76. Zhang, Y. *et al.* Positron Emission Tomography Imaging of CD105 Expression with a ⁶⁴Cu-Labeled Monoclonal Antibody: NOTA Is Superior to DOTA. *PLoS ONE* **6**, e28005 (2011).
77. Fu, R., Carroll, L., Yahioglu, G., Aboagye, E. O. & Miller, P. W. Antibody Fragment and Affibody ImmunoPET Imaging Agents: Radiolabelling Strategies and Applications. *ChemMedChem* **13**, 2466–2478 (2018).
78. Notni, J., Šimeček, J., Hermann, P. & Wester, H.-J. TRAP, a Powerful and Versatile Framework for Gallium-68 Radiopharmaceuticals. *Chem. - Eur. J.* **17**, 14718–14722 (2011).

79. Šimeček, J., Zemek, O., Hermann, P., Wester, H.-J. & Notni, J. A Monoreactive Bifunctional Triazacyclononane Phosphinate Chelator with High Selectivity for Gallium-68. *ChemMedChem* **7**, 1375–1378 (2012).
80. Price, T. W., Greenman, J. & Stasiuk, G. J. Current advances in ligand design for inorganic positron emission tomography tracers ^{68}Ga , ^{64}Cu , ^{89}Zr and ^{44}Sc . *Dalton Trans.* **45**, 15702–15724 (2016).
81. Berry, D. J. *et al.* Efficient bifunctional gallium-68 chelators for positron emission tomography: tris(hydroxypyridinone) ligands. *Chem. Commun.* **47**, 7068 (2011).
82. Eder, M. *et al.* ^{68}Ga -Complex Lipophilicity and the Targeting Property of a Urea-Based PSMA Inhibitor for PET Imaging. *Bioconjug. Chem.* **23**, 688–697 (2012).
83. Tsionou, M. I. *et al.* Comparison of macrocyclic and acyclic chelators for gallium-68 radiolabelling. *RSC Adv* **7**, 49586–49599 (2017).
84. Virgolini, I. *et al.* Procedure guidelines for PET/CT tumour imaging with ^{68}Ga -DOTA-conjugated peptides: ^{68}Ga -DOTA-TOC, ^{68}Ga -DOTA-NOC, ^{68}Ga -DOTA-TATE. *Eur. J. Nucl. Med. Mol. Imaging* **37**, 2004–2010 (2010).
85. Kabasakal, L. *et al.* Comparison of ^{68}Ga -DOTATATE and ^{68}Ga -DOTANOC PET/CT imaging in the same patient group with neuroendocrine tumours. *Eur. J. Nucl. Med. Mol. Imaging* **39**, 1271–1277 (2012).
86. Antunes, P. *et al.* Are radiogallium-labelled DOTA-conjugated somatostatin analogues superior to those labelled with other radiometals? *Eur. J. Nucl. Med. Mol. Imaging* **34**, 982–993 (2007).
87. Eder, M. *et al.* Novel Preclinical and Radiopharmaceutical Aspects of [^{68}Ga]Ga-PSMA-HBED-CC: A New PET Tracer for Imaging of Prostate Cancer. *Pharmaceuticals* **7**, 779–796 (2014).

88. Afshar-Oromieh, A. *et al.* PET imaging with a [⁶⁸Ga]gallium-labelled PSMA ligand for the diagnosis of prostate cancer: biodistribution in humans and first evaluation of tumour lesions. *Eur. J. Nucl. Med. Mol. Imaging* **40**, 486–495 (2013).
89. Haberkorn, U., Eder, M., Kopka, K., Babich, J. W. & Eisenhut, M. New Strategies in Prostate Cancer: Prostate-Specific Membrane Antigen (PSMA) Ligands for Diagnosis and Therapy. *Clin. Cancer Res.* **22**, 9–15 (2016).
90. Anilkumar, G. *et al.* Prostate-specific membrane antigen association with filamin A modulates its internalization and NAALADase activity. *Cancer Res.* **63**, 2645–2648 (2003).
91. Ghosh, A. & Heston, W. D. W. Tumor target prostate specific membrane antigen (PSMA) and its regulation in prostate cancer. *J. Cell. Biochem.* **91**, 528–539 (2004).
92. Silver, D. A., Pellicer, I., Fair, W. R., Heston, W. D. & Cordon-Cardo, C. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **3**, 81–85 (1997).
93. Perner, S. *et al.* Prostate-specific membrane antigen expression as a predictor of prostate cancer progression. *Hum. Pathol.* **38**, 696–701 (2007).
94. Barinka, C. *et al.* Interactions between Human Glutamate Carboxypeptidase II and Urea-Based Inhibitors: Structural Characterization. *J. Med. Chem.* **51**, 7737–7743 (2008).
95. Liu, T., Toriyabe, Y., Kazak, M. & Berkman, C. E. Pseudoirreversible Inhibition of Prostate-Specific Membrane Antigen by Phosphoramidate Peptidomimetics. *Biochemistry* **47**, 12658–12660 (2008).
96. Hillier, S. M. *et al.* Preclinical Evaluation of Novel Glutamate-Urea-Lysine Analogues That Target Prostate-Specific Membrane Antigen as Molecular Imaging Pharmaceuticals for Prostate Cancer. *Cancer Res.* **69**, 6932–6940 (2009).
97. Knetsch, P. A. *et al.* [⁶⁸Ga]NODAGA-RGD for imaging $\alpha\text{v}\beta\text{3}$ integrin expression. *Eur. J. Nucl. Med. Mol. Imaging* **38**, 1303–1312 (2011).

98. Buchegger, F. *et al.* ^{68}Ga -NODAGA-RGDyK for $\alpha\text{v}\beta 3$ integrin PET imaging: Preclinical investigation and dosimetry. *Nuklearmedizin* **50**, 225–233 (2011).
99. Liu, S. Radiolabeled Cyclic RGD Peptide Bioconjugates as Radiotracers Targeting Multiple Integrins. *Bioconjug. Chem.* **26**, 1413–1438 (2015).
100. Heckmann, D. & Kessler, H. Design and Chemical Synthesis of Integrin Ligands. *Methods in Enzymol.* **426**, 463–503 (Elsevier, 2007).
101. Hood, J. D. & Cheresh, D. A. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2**, 91–100 (2002).
102. Dumont, R. A. *et al.* Novel ^{64}Cu - and ^{68}Ga -Labeled RGD Conjugates Show Improved PET Imaging of $\alpha\text{v}\beta 3$ Integrin Expression and Facile Radiosynthesis. *J. Nucl. Med.* **52**, 1276–1284 (2011).
103. Schultz, M. K., Mueller, D., Baum, R. P., Leonard Watkins, G. & Breeman, W. A. P. A new automated NaCl based robust method for routine production of gallium-68 labeled peptides. *Appl. Radiat. Isot.* **76**, 46–54 (2013).
104. Decristoforo, C. *et al.* A fully automated synthesis for the preparation of ^{68}Ga -labelled peptides: *Nucl. Med. Commun.* **28**, 870–875 (2007).
105. Boschi, S., Lodi, F., Malizia, C., Cicoria, G. & Marengo, M. Automation synthesis modules review. *Appl. Radiat. Isot.* **76**, 38–45 (2013).
106. Velikyan, I., Maecke, H. & Langstrom, B. Convenient Preparation of ^{68}Ga -Based PET-Radiopharmaceuticals at Room Temperature. *Bioconjug. Chem.* **19**, 569–573 (2008).
107. Young, J. D. *et al.* ^{68}Ga -THP-PSMA: A PET Imaging Agent for Prostate Cancer Offering Rapid, Room-Temperature, 1-Step Kit-Based Radiolabeling. *J. Nucl. Med.* **58**, 1270–1277 (2017).
108. Ebenhan, T. *et al.* Development of a Single Vial Kit Solution for Radiolabeling of ^{68}Ga -DKFZ-PSMA-11 and Its Performance in Prostate Cancer Patients. *Molecules* **20**, 14860–14878 (2015).

109. Maus, S. & Schreckenberger, M. GMP Ga-68-PSMA Cold Kit Cost effective Alternative to synthesizer based method. **supplement 2**, 1537 (2016).
110. Derlin, T. *et al.* Imaging Characteristics and First Experience of [68Ga]THP-PSMA, a Novel Probe for Rapid Kit-Based Ga-68 Labeling and PET Imaging: Comparative Analysis with [68Ga]PSMA I&T. *Mol. Imaging Biol.* **20**, 650–658 (2018).
111. Beheshti, M. *et al.* Optimal time-point for 68Ga-PSMA-11 PET/CT imaging in assessment of prostate cancer: feasibility of sterile cold-kit tracer preparation? *Eur. J. Nucl. Med. Mol. Imaging* **45**, 1188–1196 (2018).
112. Whitesides, G. M. The origins and the future of microfluidics. *Nature* **442**, 368–373 (2006).
113. Mitchell, M. C., Spikmans, V. & Mello, A. J. de. Microchip-based synthesis and analysis: Control of multicomponent reaction products and intermediates. *The Analyst* **126**, 24–27 (2001).
114. Kim, H., Lee, H.-J. & Kim, D.-P. Integrated One-Flow Synthesis of Heterocyclic Thioquinazolinones through Serial Microreactions with Two Organolithium Intermediates. *Angew. Chem. Int. Ed.* **54**, 1877–1880 (2015).
115. Kawaguchi, T., Miyata, H., Ataka, K., Mae, K. & Yoshida, J. Room-Temperature Swern Oxidations by Using a Microscale Flow System. *Angew. Chem. Int. Ed.* **44**, 2413–2416 (2005).
116. Shestopalov, I., Tice, J. D. & Ismagilov, R. F. Multi-step synthesis of nanoparticles performed on millisecond time scale in a microfluidic droplet-based system. *Lab. Chip* **4**, 316 (2004).
117. Rodrigues, T., Schneider, P. & Schneider, G. Accessing New Chemical Entities through Microfluidic Systems. *Angew. Chem. Int. Ed.* **53**, 5750–5758 (2014).
118. deMello, A. J. Control and detection of chemical reactions in microfluidic systems. *Nature* **442**, 394–402 (2006).

119. Watts, P., Pascali, G. & Salvadori, P. A. Positron Emission Tomography Radiosynthesis in Microreactors. *J. Flow Chem.* **2**, 37–42 (2012).
120. Reynolds, O. An Experimental Investigation of the Circumstances Which Determine Whether the Motion of Water Shall Be Direct or Sinuous, and of the Law of Resistance in Parallel Channels. *Philos. Trans. R. Soc. Lond.* **174**, 935–982 (1883).
121. Squires, T. M. & Quake, S. R. Microfluidics: Fluid physics at the nanoliter scale. *Rev. Mod. Phys.* **77**, 977–1026 (2005).
122. Harrison, D. J. *et al.* Micromachining a Miniaturized Capillary Electrophoresis-Based Chemical Analysis System on a Chip. *Science* **261**, 895–897 (1993).
123. Gillies, J. M. *et al.* Microfluidic reactor for the radiosynthesis of PET radiotracers. *Appl. Radiat. Isot.* **64**, 325–332 (2006).
124. Wester, H.-J., Schoultz, B. W., Hultsch, C. & Henriksen, G. Fast and repetitive in-capillary production of [18F]FDG. *Eur. J. Nucl. Med. Mol. Imaging* **36**, 653–658 (2009).
125. Lee, C.-C. Multistep Synthesis of a Radiolabeled Imaging Probe Using Integrated Microfluidics. *Science* **310**, 1793–1796 (2005).
126. Steel, C. J., O'Brien, A. T., Luthra, S. K. & Brady, F. Automated PET radiosyntheses using microfluidic devices. *J. Label. Compd. Radiopharm.* **50**, 308–311 (2007).
127. Haroun, S., Wang, L., Ruth, T. J. & Li, P. C. H. Computational fluid dynamics study of the synthesis process for a PET radiotracer compound, [11C]raclopride on a microfluidic chip. *Chem. Eng. Process. Process Intensif.* **70**, 140–147 (2013).
128. Ungersboeck, J. *et al.* Optimization of [11C]DASB-synthesis: Vessel-based and flow-through microreactor methods. *Appl. Radiat. Isot.* **70**, 2615–2620 (2012).
129. Selivanova, S. *et al.* Single-step radiofluorination of peptides using continuous flow microreactor. *Org. Biomol. Chem.* **10**, 3871–3874 (2012).
130. Ungersboeck, J. *et al.* Microfluidic preparation of [18F]FE@SUPPY and [18F]FE@SUPPY:2 — comparison with conventional radiosyntheses. *Nucl. Med. Biol.* **38**, 427–434 (2011).

131. Ungersboeck, J. *et al.* Radiolabeling of [18F]altanserine — a microfluidic approach. *Nucl. Med. Biol.* **39**, 1087–1092 (2012).
132. Philippe, C. *et al.* [18F]FE@SNAP—A new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1): Microfluidic and vessel-based approaches. *Bioorg. Med. Chem.* **20**, 5936–5940 (2012).
133. Pascali, G., Mazzone, G., Saccomanni, G., Manera, C. & Salvadori, P. A. Microfluidic approach for fast labeling optimization and dose-on-demand implementation. *Nucl. Med. Biol.* **37**, 547–555 (2010).
134. Pascali, G., Nannavecchia, G., Pitzianti, S. & Salvadori, P. A. Dose-on-demand of diverse 18F-fluorocholine derivatives through a two-step microfluidic approach. *Nucl. Med. Biol.* **38**, 637–644 (2011).
135. Bouvet, V., Wuest, M., Tam, P.-H., Wang, M. & Wuest, F. Microfluidic technology: An economical and versatile approach for the synthesis of O-(2-[18F]fluoroethyl)-L-tyrosine ([18F]FET). *Bioorg. Med. Chem. Lett.* **22**, 2291–2295 (2012).
136. Zacheo, A. *et al.* Radioactivity resistance evaluation of polymeric materials for application in radiopharmaceutical production at microscale. *Microfluid. Nanofluidics* **11**, 35–44 (2011).
137. Pfaff, S. *et al.* Microfluidic ⁶⁸Ga-labeling: a proof of principle study. *Dalton Trans.* **47**, 5997–6004 (2018).
138. Kappe, C. O., Dallinger, D. & Murphree, S. S. *Practical microwave synthesis for organic chemists: strategies, instruments, and protocols.* (Wiley-VCH, 2009).
139. Majetich, G. & Hicks, R. Applications of microwave-accelerated organic synthesis. *Radiat. Phys. Chem.* **45**, 567–579 (1995).
140. Dallinger, D. & Kappe, C. O. Microwave-Assisted Synthesis in Water as Solvent. *Chem. Rev.* **107**, 2563–2591 (2007).

141. Roberts, B. A. & Strauss, C. R. Toward Rapid, “Green”, Predictable Microwave-Assisted Synthesis. *Acc. Chem. Res.* **38**, 653–661 (2005).
142. Elander, N., Jones, J. R., Lu, S.-Y. & Stone-Elander, S. Microwave-enhanced radiochemistry. *Chem. Soc. Rev.* **29**, 239–249 (2000).
143. Bauman, A., Valverde, I. E., Fischer, C. A., Vomstein, S. & Mindt, T. L. Development of ⁶⁸Ga- and ⁸⁹Zr-Labeled Exendin-4 as Potential Radiotracers for the Imaging of Insulinomas by PET. *J. Nucl. Med.* **56**, 1569–1574 (2015).
144. Schieferstein, H., Betzel, T., Fischer, C. R. & Ross, T. L. 18 F-click labeling and preclinical evaluation of a new 18 F-folate for PET imaging. *EJNMMI Res.* **3**, 68 (2013).
145. Velikyan, I., Beyer, G. J. & Långström, B. Microwave-Supported Preparation of ⁶⁸Ga Bioconjugates with High Specific Radioactivity. *Bioconjug. Chem.* **15**, 554–560 (2004).
146. Maecke, H. R., Hofmann, M. & Haberkorn, U. (⁶⁸Ga)-labeled peptides in tumor imaging. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **46 Suppl 1**, 172S–8S (2005).
147. Cantorias, M. V. *et al.* Development of high-specific-activity ⁶⁸Ga-labeled DOTA-rhenium-cyclized α -MSH peptide analog to target MC1 receptors overexpressed by melanoma tumors. *Nucl. Med. Biol.* **36**, 505–513 (2009).
148. Behnam Azad, B., Cho, C.-F., Lewis, J. D. & Luyt, L. G. Synthesis, radiometal labeling and in vitro evaluation of a targeted PPIX derivative. *Appl. Radiat. Isot.* **70**, 505–511 (2012).
149. Pellico, J. *et al.* Fast synthesis and bioconjugation of ⁶⁸Ga core-doped extremely small iron oxide nanoparticles for PET/MR imaging: Chelator-free ⁶⁸Ga-iron oxide nanoparticles. *Contrast Media Mol. Imaging* **11**, 203–210 (2016).
150. Bauwens, M., Chekol, R., Vanbilloen, H., Bormans, G. & Verbruggen, A. Optimal buffer choice of the radiosynthesis of ⁶⁸Ga-Dotatoc for clinical application: *Nucl. Med. Commun.* **31**, 753–758 (2010).
151. Martins, A. F. *et al.* Spectroscopic, radiochemical, and theoretical studies of the Ga³⁺-N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES buffer) system: evidence for the

- formation of Ga³⁺ - HEPES complexes in (68) Ga labeling reactions. *Contrast Media Mol. Imaging* **8**, 265–273 (2013).
152. Azab, H. A., Abou El-Nour, K. M. & Sorrow, S. H. Metal Ion Complexes Containing Dipeptides, Tripeptides, and Biologically Important Zwitterionic Buffers. *J. Chem. Eng. Data* **52**, 381–390 (2007).
 153. Antunes, I. *et al.* A new fast and reliable HPLC method to detect HEPES in [68Ga]-radiopharmaceuticals. in *Journal of Labelled Compounds and Radiopharmaceuticals* **60**, 293 (wiley, 2017).
 154. Kvaternik, H., Barowitsch, C., Hausberger, D., Müller, R. & Aigner, R. M. Bestimmung von HEPES in Ga-68markierten Peptiden mittels HPLC. *Annual Meeting of the Austrian Society of Nuclear Medicine and Molecular Imaging, Zell am See* (2015).
 155. Sasson, R., Vaknin, D., Bross, A. & Lavie, E. Determination of HEPES in 68Ga-labeled peptide solutions. *J. Radioanal. Nucl. Chem.* **283**, 753–756 (2010).
 156. Velikyan, I. 68Ga-Based radiopharmaceuticals: production and application relationship. *Mol. Basel Switz.* **20**, 12913–12943 (2015).
 157. Merckmillipore. Safety Data Sheet HEPES, Free Acid, ULTROL® Grade-CAS 7365-45-9-Calbiochem 391338.
 158. McFadden, G. I. & Melkonian, M. Use of Hepes buffer for microalgal culture media and fixation for electron microscopy. *Phycologia* **25**, 551–557 (1986).
 159. Dainiak, M. B., Kumar, A., Plieva, F. M., Galaev, I. Y. & Mattiasson, B. Integrated isolation of antibody fragments from microbial cell culture fluids using supermacroporous cryogels. *J. Chromatogr. A* **1045**, 93–98 (2004).
 160. Takezawa, T., Mori, Y. & Yoshizato, K. Cell Culture on a Thermo-Responsive Polymer Surface. *Nat. Biotechnol.* **8**, 854–856 (1990).
 161. Lee, J., Ishihara, A., Oxford, G., Johnson, B. & Jacobson, K. Regulation of cell movement is mediated by stretch-activated calcium channels. *Nature* **400**, 382–386 (1999).

162. Will, M. A., Clark, N. A. & Swain, J. E. Biological pH buffers in IVF: help or hindrance to success. *J. Assist. Reprod. Genet.* **28**, 711–724 (2011).
163. Swain, J. E. & Pool, T. B. New pH-buffering system for media utilized during gamete and embryo manipulations for assisted reproduction. *Reprod. Biomed. Online* **18**, 799–810 (2009).
164. New Application of Sequential in Vitro Maturation System for Infertility Patients With Ovary Syndrome. Downloaded from <https://clinicaltrials.gov/ct2/show/NCT03773263?term=HEPES&cond=cancer&rank=1> (26.02.2019)
165. McLoughlin, D. M., Stapleton, P. P. & Bloomfield, F. J. Influence of taurine and a substituted taurine on the respiratory burst pathway in the inflammatory response. *Biochem. Soc. Trans.* **19**, 73–78 (1991).
166. Stapleton, P. P., Mahon, T. M., Nowlan, P. & Bloomfield, F. J. Effects of In-vivo Administration of Taurine and HEPES on the Inflammatory Response in Rats. *J. Pharm. Pharmacol.* **46**, 745–750 (1994).
167. Theodore, T. R., Van Zandt, R. L. & Carpenter, R. H. Preliminary Evaluation of a Fixed Dose of Zwitterionic Piperazine (TVZ-7) in Clinical Cancer. *Cancer Biother. Radiopharm.* **12**, 351–353 (1997).
168. Danhof, I. E. Role of N-2-hydroxy-ethy-piperazine-N'-2-ethane sulfonic acid (HEPES) in pain control and reversal of demyelination injury. Downloaded from <https://patentimages.storage.googleapis.com/22/37/e0/c363d2d331128f/US20110071157A1.pdf> (26.02.2018)
169. Wolf, A. P. & Redvanly, C. S. Carbon-11 and radiopharmaceuticals. *Int. J. Appl. Radiat. Isot.* **28**, 29–48 (1977).
170. Barkas, W. H. Some New Reactions in Light Nuclei with High Energy Protons. *Phys. Rev.* **56**, 287–287 (1939).

171. Pichler, V. *et al.* Molar activity – The keystone in ^{11}C -radiochemistry: An explorative study using the gas phase method. *Nucl. Med. Biol.* **67**, 21–26 (2018).
172. Scott, P. J. H. Methods for the Incorporation of Carbon-11 To Generate Radiopharmaceuticals for PET Imaging. *Angew. Chem. Int. Ed.* **48**, 6001–6004 (2009).
173. Pichler, V. *et al.* L-[S-methyl- ^{11}C]methionine – An example of radiosynthetic optimization. *Appl. Radiat. Isot.* **141**, 107–111 (2018).
174. Lodi, F. *et al.* Synthesis of oncological [^{11}C]radiopharmaceuticals for clinical PET. *Nucl. Med. Biol.* **39**, 447–460 (2012).
175. Hara, T., Kosaka, N., Shinoura, N. & Kondo, T. PET imaging of brain tumor with [methyl- ^{11}C]choline. *J. Nucl. Med. Off. Publ. Soc. Nucl. Med.* **38**, 842–847 (1997).
176. Mitterhauser, M. *et al.* New aspects on the preparation of [^{11}C]acetate—a simple and fast approach via distillation. *Appl. Radiat. Isot.* **61**, 1147–1150 (2004).
177. Mathis, C. A. *et al.* Synthesis and Evaluation of ^{11}C -Labeled 6-Substituted 2-Arylbenzothiazoles as Amyloid Imaging Agents. *J. Med. Chem.* **46**, 2740–2754 (2003).
178. Bergström, M., Westerberg, G., Kihlberg, T. & Långström, B. Synthesis of some ^{11}C -labelled MAO-A inhibitors and their in vivo uptake kinetics in rhesus monkey brain. *Nucl. Med. Biol.* **24**, 381–388 (1997).
179. Rami-Mark, C. *et al.* Reliable set-up for in-loop ^{11}C -carboxylations using Grignard reactions for the preparation of [carbonyl- ^{11}C]WAY-100635 and [^{11}C]-(+)-PHNO. *Appl. Radiat. Isot.* **82**, 75–80 (2013).
180. Antoni, G. Development of carbon-11 labelled PET tracers-radiochemical and technological challenges in a historic perspective: Development of carbon-11 labelled PET tracers. *J. Label. Compd. Radiopharm.* **58**, 65–72 (2015).
181. Maruyama, K. & Katagiri, T. Mechanism of the Grignard reaction. *J. Phys. Org. Chem.* **2**, 205–213 (1989).

182. Luthra, S. K., Le Bars, D., Pike, V. W. & Brady, F. Preparation of NCA [1-¹¹C]acid chlorides as labelling agents for amides. *J. Label. Compd. Radiopharm.* **26**, 66–68 (1989).
183. Luthra, S. K., Pike, V. W. & Brady, F. Preparation of some NCA [1-¹¹C]acid chlorides as labelling agents. *Int. J. Rad. Appl. Instrum. [A]* **41**, 471–476 (1990).
184. Henriksen, G. ¹¹C-Labelled Tracers for use in PET: Concepts and Selected Labelling Strategies for Preparative Radiosynthesis. in *Munich Molecular Imaging Handbook Series 1*, 75–102 (SCINTOMICS Print Media and Publishing, 2010).
185. Krasikova, R. N. *et al.* A fully automated one-pot synthesis of [carbonyl-¹¹C]WAY-100635 for clinical PET applications. *Appl. Radiat. Isot.* **67**, 73–78 (2009).
186. Hall, H. *et al.* Autoradiographic localization of 5-HT_{1A} receptors in the post-mortem human brain using [3H]WAY-100635 and [11C]WAY-100635. *Brain Res.* **745**, 96–108 (1997).
187. McCarron, J. A., Turton, D. R., Pike, V. W. & Poole, K. G. Remotely-controlled production of the 5-HT_{1A} receptor radioligand, [carbonyl-¹¹C]WAY-100635, via ¹¹C-carboxylation of an immobilized Grignard reagent. *J. Label. Compd. Radiopharm.* **38**, 941–953 (1996).
188. Wadsak, W. *et al.* Simple and fully automated preparation of [carbonyl-¹¹C]WAY-100635. *Radiochim. Acta* **95**, 417–422 (2007).
189. Seeman, P., Ulpian, C., Larsen, R. D. & Anderson, P. S. Dopamine receptors labelled by PHNO. *Synapse* **14**, 254–262 (1993).
190. Breier, A. *et al.* Schizophrenia is associated with elevated amphetamine-induced synaptic dopamine concentrations: Evidence from a novel positron emission tomography method. *Proc. Natl. Acad. Sci.* **94**, 2569–2574 (1997).
191. Boileau, I. *et al.* In vivo evidence for greater amphetamine-induced dopamine release in pathological gambling: a positron emission tomography study with [11C]-(+)-PHNO. *Mol. Psychiatry* **19**, 1305–1313 (2014).

192. Graff-Guerrero, A. *et al.* The Dopamine D2 Receptors in High-Affinity State and D3 Receptors in Schizophrenia: A Clinical [11C]-(+)-PHNO PET Study. *Neuropsychopharmacology* **34**, 1078–1086 (2009).
193. Gallezot, J.-D. *et al.* Affinity and selectivity of [11C]-(+)-PHNO for the D3 and D2 receptors in the rhesus monkey brain in vivo. *Synapse* **66**, 489–500 (2012).
194. Freedman, S. B. *et al.* Expression and pharmacological characterization of the human D3 dopamine receptor. *J. Pharmacol. Exp. Ther.* **268**, 417–426 (1994).
195. Seeman, P., McCormick, P. N. & Kapur, S. Increased dopamine D2High receptors in amphetamine-sensitized rats, measured by the agonist [3H](+)PHNO. *Synapse* **61**, 263–267 (2007).
196. Sokoloff, P. *et al.* Pharmacology of human dopamine D3 receptor expressed in a mammalian cell line: comparison with D2 receptor. *Eur. J. Pharmacol. Mol. Pharmacol.* **225**, 331–337 (1992).
197. George, S. R., Watanabe, M. & Seeman, P. Dopamine D₂ Receptors in the Anterior Pituitary: A Single Population Without Reciprocal Antagonist/Agonist States. *J. Neurochem.* **44**, 1168–1177 (1985).
198. Ehrin, E., Gawell, L., Högberg, T., de Paulis, T. & Ström, P. Synthesis of [methoxy-3H]- and [methoxy-11C]-labelled raclopride. Specific dopamine-D2 receptor ligands. *J. Label. Compd. Radiopharm.* **24**, 931–940 (1987).
199. Ginovart, N. *et al.* Binding characteristics and sensitivity to endogenous dopamine of [11C]-(+)-PHNO, a new agonist radiotracer for imaging the high-affinity state of D2 receptors in vivo using positron emission tomography. *J. Neurochem.* **97**, 1089–1103 (2006).
200. Willeit, M. *et al.* First Human Evidence of d-Amphetamine Induced Displacement of a D2/3 Agonist Radioligand: A [11C]-(+)-PHNO Positron Emission Tomography Study. *Neuropsychopharmacology* **33**, 279–289 (2008).

201. Wilson, A. A. *et al.* Radiosynthesis and Evaluation of [¹¹ C]-(+)-4-Propyl-3,4,4a,5,6,10b-hexahydro-2 H -naphtho[1,2- b][1,4]oxazin-9-ol as a Potential Radiotracer for in Vivo Imaging of the Dopamine D2 High-Affinity State with Positron Emission Tomography. *J. Med. Chem.* **48**, 4153–4160 (2005).
202. Plisson, C. *et al.* Automated preparation of the dopamine D2/3 receptor agonist ligand [11C]-(+)-PHNO for human PET imaging studies. *Appl. Radiat. Isot.* **70**, 380–387 (2012).
203. Yagi, Y. *et al.* Efficient gallium-68 radiolabeling reaction of DOTA derivatives using a resonant-type microwave reactor. *J. Label. Compd. Radiopharm.* (2019). doi:10.1002/jlcr.3704
204. Schindelin, J., Rueden, C. T., Hiner, M. C. & Eliceiri, K. W. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol. Reprod. Dev.* **82**, 518–529 (2015).
205. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).