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Electroless semi-automatic synthesis of ^{225}Ac-labeled PSMA-derivatives for clinical application and evaluation of rechelation capability☆

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ABSTRACT

Introduction: Sustainable green radiochemistry is the result of current research, in which radiolabeling strategies are increasingly being developed in the direction of kit labeling. This includes the use of sunlight for reactions, but also tailor-made chelators allowing a to quantitative chelation of the corresponding radionuclide even at temperatures around 20 °C. The ultimate goal is to use these reaction conditions for labeling kit development in which only the radionuclide needs to be added and the reaction then occurs quantitatively in a sterile vial. Chelators such as HBED are already included in approved kits for the production of [^{68}Ga]Ga-PSMA-11 (Locametz®, IsoPROtrace11®, and Illuccix®) and allow radiolabeling at 20 °C. This paper reports on the semi-automatic labeling method of macropa(mcp)-based PSMA derivatives with ^{225}Ac, which was performed at 20 °C without heating and, according to subsequent quality control, is technically ready-to-use for patient applications. To further strengthen the green aspect, critical chemicals like environmental persistent trifluoroacetic acid (TFA) were considered to be replace against more eco-friendly chemicals. Furthermore, additives like ascorbic acid or DTPA, which are usually used for protection against radiolysis or scavenge free progenies were considered to be eliminated from the process due to the reason, that the mcp-based precursor might be able to rechelate free progenies, as soon as they are generated from their parent nuclide ^{225}Ac.

Methods: An iQS module was equipped with sterile single-use components and an already automated radiolabeling process was adopted to the semi-automatic system. Three validation batches for two different mcp-based PSMA derivatives were produced including quality control with criteria fulfilling clinical standards.

Results: Three consecutive validation batches were successfully produced for each mcp-M-PSMA and mcp-D-PSMA derivatives to evaluate the safety and robustness of the labeling method. Overall, the six batches reached radiochemical yields >80% and hit the quality parameters such as radiochemical purity of >95% for clinical application.

Additionally, the replacement of environmental persistent TFA of the HPLC eluents with phosphoric acid (H_3PO_4) led to similar retention times combined with a higher resolution in the radio-HPLC chromatograms.

The effect of rechelation was carefully evaluated using an excess amount of precursor. It was found that, with increasing concentration, a rechelation of free progenies occurred from 22 to 144 h. Either the reaction solution or the diluted product solution was analyzed by TLC and HPLC.

Conclusions: The electroless synthesis of ^{225}Ac-mcp-PSMA derivatives was successfully implemented to the iQS module, and the products fulfilled all criteria for patient application. H_3PO_4 has successfully replaced environmental persistent TFA within the HPLC eluents. With the use of mcp-based precursors, the addition of radiolysis protection agents was no longer mandatory. Rechelation of free progenies was observed at higher precursor concentration from 22 up to 144 h in the diluted product solutions. In future, this labeling method could be used for safe production of ^{225}Ac-labeled macropa-containing peptides without electricity.

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Advances in knowledge: Mcp-based precursors enable for electroless reactions at 20 °C yielding ^{225}Ac-radiopharmaceuticals with 10× increased molar activity compared to DOTA- or DOTAGA-based precursors and diluted product solution leads to higher stability and lesser radiolysis due to the rechelation effect compared to the reaction solutions.

1. Introduction

Targeted alpha therapy (TAT) is an upcoming approach for the treatment of cancer, especially but not limited to patients who developed resistance to beta-minus emitter therapy [1]. Application of ^{225}Ac ($T_{1/2}$ = 9.92 d) dramatically reduces the activity used for peptide receptor radionuclide therapy by a factor of 1000 in comparison to ^{90}Y, ^{177}Lu or ^{188}Re while maintaining the therapeutic outcome. TAT is meaningful for out treated patients with palliative status, who have no other option available. Patients who are treated with ^{225}Ac are consistently monitored for their blood and kidney values as those are the critical parameters to decide for targeted alpha therapy. This monitoring is of great importance in light of the challenges associated with image-based dosimetry, which will persist until the widespread availability of next-generation SPECT-CT systems. The use of ^{225}Ac for cancer therapy has three distinct advantages over conventional therapies with beta-minus emitters: The short range of alpha radiation in human tissue (less than 0.1 mm), corresponding to only a few cell diameters, allowing selective killing of targeted cancer cells while sparing surrounding healthy tissue. At the same time, the high energy (several MeV) of alpha particles and its associated high linear energy transfer leads to high rate of cell deaths [2]. Consequently, alpha radiation can destroy cells, which otherwise exhibit resistance to treatment with beta or gamma irradiation or chemotherapeutic drugs, and thus can offer a therapeutic option for tumors resistant to conventional therapies. Thirdly, the radiation safety for personnel is higher. ^{225}Ac-therapeutic doses are in the MBq range (~100 kBq/kg) compared to several GBq used commonly for ^{90}Y- and ^{177}Lu-therapy. To date, the chelator DOTA is commonly used for ^{225}Ac-labeling of peptides, antibodies and small molecules [3–5] together with the well-known diagnostic partner nuclide ^{68}Ga ($T_{1/2}$ = 68 min). Therefore, common diagnostic radiotracers such as TATE, PSMA or RGD are available for ^{225}Ac-labeling and therapy [5–7]. Recent results demonstrate the remarkable therapeutic efficacy of alpha emitters to treat various cancers underlining the clinical potential of TAT although direct comparison between beta-minus- and alpha-therapy had similar outcome [8]. However, DOTA is not the ideal chelator for ^{225}Ac because of the long labeling time, harsh radiolabeling conditions, and the need for high precursor amounts (typically 10–20 μg/MBq). As a consequence, improved chelators are currently under development [9–14]. For example, the chelator macropa (mcp) forms stable ^{225}Ac-complexes within 5–15 min at room temperature and at lower precursor concentrations (1 μg/MBq). The positron-emitting radionuclides ^{132}La ($T_{1/2}$ = 4.6 h) and ^{133}La ($T_{1/2}$ = 3.9 h) form stable complexes with mcp as well and are being discussed as the diagnostic partner nuclide [9,15].

Meanwhile, the supply with ^{225}Ac is rising and despite sources only available for research (e.g. PRISMAP consortium) also commercial sources are entering the market by companies like ITM (Munich, Germany), E&Z (Berlin, Germany), and NorthStar (Beloit, WI, USA), additionally to already established suppliers (Rosatom, RIAR). Automated synthesis systems are ideal for GMP-compliant production in controlled and closed environments for safe and reliable synthesis methods to minimize waste production and protect environment and operator against contamination. Several automated systems for example from E&Z [16,17], Elysia-Raytest [18], IBA molecular [19], iPHASE [20], Scintomics [21,22], and Trasis [23] are currently available for diverse GMP-compliant diagnostic and therapeutic radiotracer production.

The aim of this work was to evaluate the efficiency and reliability of the radiosynthesis of ^{225}Ac-labeled PSMA-ligands containing mcp as chelator (Fig. 1) [14] and to establish the electroless translation of the synthesis to the synthesis platform iQS Fluidic Labeling Module (ITM) for clinical routine production [24]. The iQS is a cassette-based module operated by manual pressurizing through sterile filters and back-pressure valves using sterile syringes. The cassette can be self-assembled from sterile single components. Additionally, the reaction vial can be heated. The ML EAZY process from a former work [25] was adopted using SPE purification method by CM-cartridge. The method was further validated and optimized allowing a convenient ^{225}Ac-radiolabeling in high radiochemical yields.

Additionally, since mcp is able to chelate ^{225}Ac at 20 °C a rechelation phenomenon might occur within the final product solution. To evaluate the capacity of rechelation after radiosynthesis, the amount of mcp-precursor was increased up to 20fold from 1 μg/MBq to 20 μg/MBq. The reaction solution and a diluted fraction (v:v = 1:9) were evaluated for free ^{213}Bi as marker for consumption of uncomplexed mcp-precursor excess from the solution up to 144 h after synthesis.

2. Material and methods

The precursors mcp-M-PSMA (M = 1325 g/mol) and mcp-D-PSMA (M = 2118 g/mol) were synthesized as previously described [14] and obtained from HZDR (Helmholtz-Zentrum Dresden-Rossendorf, Germany). All other reagents and solvents were purchased in the highest available purity from Merck (Darmstadt, Germany). SepPak CM light (WAT023531) cartridges were purchased from Waters (Milford, MA, USA). The synthesis module (iQS) was obtained from ITM (Medical Isotopes GmbH, Garching, Germany). Solvents for quality control were stored at 4 °C. Buffer and precursor were stored at −20 °C; other chemicals were stored at room temperature. The dose calibrator (calibrated by a Cs-137 source AN-1426) and the CoMo-170 for separate α-detection and β/γ-detection were obtained from NUVIA Instruments (Dresden, Germany). The pH was acquired by a QuantoFix Relax reflection photometer (91346) with the corresponding pH test strips 5.5 × 85 mm pH-Fix 2.0–9.0 (92118) (Macherey-Nagel, Düren, Germany). ITLC-SG strips (SGI0001) were obtained from Agilent and silica gel on aluminum strips from Merck. Measurement of the radionuclide purity (RNP) was performed with the TLC scanner MiniScanPRO+ provided by E&Z and a HPGe detector GC2018 provided by Canberra (Rüsselsheim, Germany) The endotoxin test device, EndoSafe, was obtained from

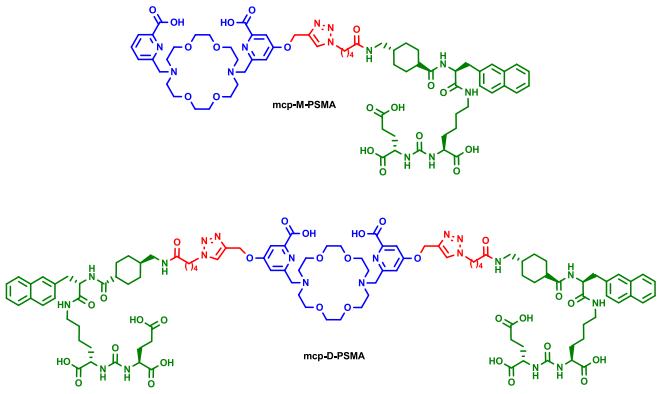

Fig. 1. Mcp-based PSMA-617 derivatives with one (mcp-M-PSMA) and two (mcp-D-PSMA) PSMA-binding motifs (green) and macropa chelator (blue) used in this work [14]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Charles River (Sulzfeld, Germany). Measurement of the radiochemical purity (RCY) by radio-TLC analyses were performed with a proportional gas-counter (AR-2000, Eckert & Ziegler Eurotope GmbH, Berlin, Germany) equipped with an ionization gas (90% Ar/10% CH_4 or 90% Ar/10% CO_2). CORGON-10 was purchased from Linde Gas (Dresden, Germany). The operator has to choose between Ar/CO_2 (90/10), a commonly used welding gas, or Ar/CH_4 (90/10), widely known as P10 gas. The gas flow during the operation was about 2–3 L/min. Radio-HPLC was performed on a LogiChromOne HPLC system (LabLogic Systems GmbH, Koblenz am Rhein, Germany), equipped with a reverse phase column (Merck HRChromolith RP-18e; 100 × 4.6 mm) plus a guard column 5 × 4.6 mm and a UV-diode array detector (220 nm). The solvent system used was a gradient of acetonitrile:water (containing 0.1% TFA or 0,05% H_3PO_4) (0–13 min: 0–60% MeCN) at a flow rate of 2 mL/min unless otherwise stated.

2.1. Radiochemistry – semi-automated synthesis

2 μg/MBq (1.5 nmol/MBq) of mcp-based precursor in 1 mL of 0.3 M sodium acetate/acetate buffer (pH = 5.0) was added to the corresponding ^{225}Ac activity in 0.1 M HCl (0.5–1.2 MBq). The reaction was maintained at 20 °C for 15 min in the reaction vial of the iQS module. Subsequently, 1 mL of 0.9% NaCl was added for dilution of the reaction. The whole reaction solution was passed through a CM cartridge and further through a vented sterile filter directly into the product vial. The reaction vial was rinsed with 1 mL of 0.9% NaCl solution and was again passed through the CM cartridge and the sterile filter into the product vial. Subsequently, quality control was performed addressing pH, endotoxin level, RNP, and RCP by TLC and HPLC.

2.2. Radiochemistry – rechelation experiments

10–20 μg/MBq of mcp-based precursor in 1 mL of 0.3 M sodium acetate/acetate buffer (pH = 5.0) was added to the corresponding ^{225}Ac activity in 0.1 M HCl (2.1–5.0 MBq). The reaction was maintained at 20 °C for 15 min. Subsequently, the reaction solution (1 mL) was divided in two parts. Part one (500 μL) maintained as reaction solution (2.1–5.0 MBq/mL) and part two (500 μL) was diluted 1:9 with 4.5 mL of 0.9% NaCl (0.1–0.25 MBq/mL). The ingrowth of free ^{213}Bi as marker for consumed excess of mcp-precursor was evaluated by TLC. The stability of ^{225}Ac-labeled product in reaction solution and diluted solution was evaluated by HPLC.

3. Results

The concentration of mcp for quantitative complexation of ^{225}Ac was previously evaluated to be 1 μM (1 nmol/mL) [26]. Therefore, 2 μg/MBq (1.5 nmol/MBq) of mcp-based precursor were used for evaluation of the semi-automatic process, which is 10 times lower the amount of DOTA- or DOTAGA-based precursors used in earlier works [25].

3.1. Evaluation of the semi-automated radiolabeling with ^{225}Ac

In a previous work [25], the best reaction buffer was prepared from an EZ-102 reagent set (Eckert&Ziegler, Berlin, Germany) and the concentration was set to 0.1 M. Since then, new sources of ^{225}Ac (RIAR, Rosatom, NorthStar) were introduced to the market. To fit every molarity of HCl from the different suppliers, the buffer molarity was increased to 0.3 M. This concentration now is able to handle every ^{225}Ac-source which is currently on the market. Additionally, one batch of buffer can be stored at −20 °C for more than 6 months.

The iQS module (Fig. 2) was equipped with single sterile cassette components in order to produce radiopharmaceuticals in accordance to quality criteria for patient application with high radiochemical yield (RCY) and radiochemical purity (RCP). The following acceptance criteria were used to decide for a successful automated production:

- RCP >80% prospective, >95% retrospective (if activity is >80% prospective, a retrospective measurement will be >99% for silica gel on aluminum with citrate)
- Endotoxin level < 175 EU per injection, pH. Eur.
- RCY 80%–90%
- Product pH 4.0–8.0

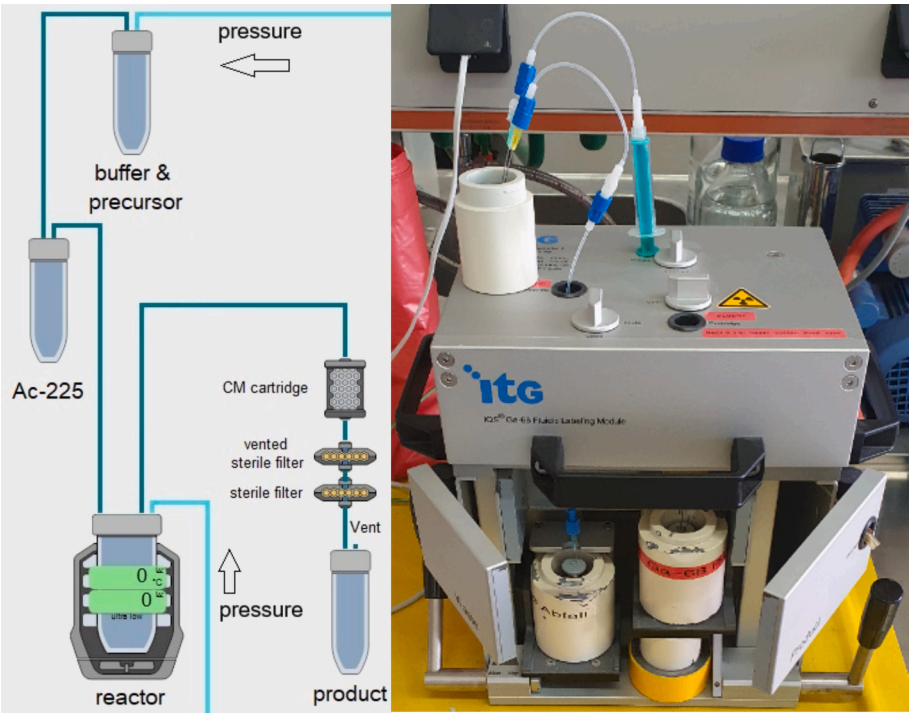

Fig. 2. A schematic and a real depiction of the iQS module. The pressure to transport liquids is added to the system via back-pressure valves and sterile syringes.

The reaction was started by rinsing the ^{225}Ac vial (0.5–1.2 MBq in 10–50 µL of 0.1 M HCl) with precursor-buffer solution (1 mL of 0.3 M sodium acetate/ acetic acid, pH 5.5–5.6, 2 µg of precursor) into the reaction vial. The reaction was executed at 20 °C for 15 min followed by dilution with 1 mL of 0.9% NaCl solution for injection. The diluted reaction solution was then transferred through a CM cartridge in order to adsorb unreacted ^{225}Ac and ^{213}Bi further through a sterile filter into the product vial. Finally, 3 mL of 0.9% NaCl solution was passed through the reaction vial to rinse residual product into the product vial. The process was completely performed without using electricity. For purification with the CM cartridge, mainly free ^{213}Bi and ^{225}Ac are trapped, while ^{225}Ac- and ^{213}Bi-labeled peptides and free ^{221}Fr are transferred into the product vial resulting in disruption of the equilibrium and challenges of the activity determination.

Subsequently, quality control was performed in accordance to the aforementioned quality criteria. Three validation batches were performed for each [^{225}Ac]Ac-mcp-M-PSMA-617 and [^{225}Ac]Ac-mcp-D-PSMA-617 derivatives to prove and ensure the reproducibility of the semi-automated syntheses. Additionally, technical personnel were trained to perform some of those validation batches in order to prove the robustness of this method in clinical routine. The results are summarized in Table 1.

3.2. Replacement of environmental persistent trifluoro acetate in HPLC eluent

TFA is accumulating worldwide in the environment including drinking water [27], and risks to human health might arise of the rising concentration. For further enhancing the green radiochemistry, the TFA in the HPLC eluent was replaced by different additives, namely formic acid (FA), methane sulfonic acid (MSA) ± 6 µM ethylene diamine tetra acetate (EDTA), 10–100 mM ammonium acetate (NH_4Ac) and 0.05% phosphoric acid (H_3PO_4) [28]. Experiments containing EDTA or MSA were performed to collect more data for comparison, even if those additives are not environmentally friendly alternatives at all. Subsequently, the chromatograms of three different radiopharmaceuticals [^{177}Lu]Lu-PSMA-I&T (Fig. 3 top row), [^{177}Lu]Lu-DOTA-TATE (Fig. 3 middle row) and [^{225}Ac]Ac-PSMA-I&T (Fig. 3 bottom row) were compared using the different eluents. It was found, that the addition of 0.05% H_3PO_4 not only led to the same retention time compared to 0.05–0.1% TFA, but also to a better resolution resulting in sharper peaks of the analytes (Fig. 3 B). Additionally, 0.03% MSA + 6 µM EDTA resulted in the same retention time and resolution of the peaks compared to 0.1% TFA. The additive NH_4Ac led to an inverted retention of the peaks of [^{177}Lu]Lu-PSMA-I&T and [^{177}Lu]Lu-DOTA-TATE but with the same resolution comparable to 0.1% TFA. However, the additives 0.01% MSA and 0.2% FA resulted in a broad smearing of the peaks probably due to metallic adsorption of the Glu-urea-Lys binding motif.

3.3. Evaluation of the rechelation capability of mcp-based precursors

It was hypothesized that, given mcp's ability to chelate ^{225}Ac and ^{213}Bi at 20 °C, a rechelation process could occur in the reaction solution as well as the diluted product solution after radiosynthesis. To evaluate the ability of rechelation, the precursor concentration was increased 10–20 times, reaching a concentration of 10–20 µg/MBq. The rechelation was evaluated in reaction solution (2.1–5.0 MBq/mL) and diluted solution (0.1–0.25 MBq/mL).

For 20 µg/MBq, a nearly quantitative rechelation was observed up to 144 h using radio-TLC in 0.1 M citrate buffer (pH = 5.0) or 1 M NH_4OAc in 50% methanol as control (Fig. 4, Table 2). As a result, no higher activity amount of free ^{213}Bi was observed in comparison to the radio- TLC developed 5 min after the radiolabeling. Significant radiolysis (in form of >10% radioactive peptide fragments, t_R ~ 5–9 min) was detected with radio-HPLC after 29 h in the reaction solution. Conversely, radiolysis was not significant for the diluted solution up to 144 h (Fig. 5).

For 10 µg/MBq, a nearly quantitative rechelation was observed up to 22 h and confirmed by radio-TLC. After that time, free ^{213}Bi accumulated in the reaction solutions as well as in the diluted solutions. Significant radiolysis was observed with HPLC already after 22 h in the reaction solution, whereas in the diluted solution the radiolysis was not significant up to 45 h (Fig. 6). Here it must be stated, that the same batch of precursor was used as for the 20 µg/MBq concentration. The stock solution of the precursor was stored in 50% EtOH for 7 days at −20 °C between the two experiments.

Radio-HPLC was used for identification of the ^{225}Ac-mcp-labeled peptides and detection of radiolysis, which was not detectable by radio-TLC. Even right after reaction, a significant portion of free progenies (^{221}Fr and ^{217}At) was always found in the dead volume fraction of the HPLC run (Fig. 7). Therefore, radio-HPLC was considered as identity control and radiolysis control. After 24 h, an increasing portion of radiolysed peptide fractions is found at earlier retention times compared to the main fraction of ^{225}Ac-mcp-labeled peptides at precursor concentrations of 10 µg/MBq (Fig. 6). No radiolysis protection agents (e.g. ascorbic acid, gentisic acid) or other chelators like DTPA were used, since the higher amount of precursor was considered to rechelate free ^{213}Bi and forming new ^{213}Bi-mcp-labeled peptides, which are considered to be a potent therapeutic agent as well. No significant amounts of colloidal ^{213}Bi-species were found even after 101 h. To ascertain the absence of adsorbed particles on the glass wall of the reaction vial, the reaction solution was removed from the glass vial followed by measuring of the residual activity.

4. Discussion

The development of new chelators able to form stable complexes at 20 °C is crucial for the application in green radiochemistry, since no electricity is needed for the radiolabeling process. Furthermore, heat can influence at least the PSMA-motive to some extend [29], which is prevented at 20 °C when mcp-based ligands are used. For ^{68}Ga-labeling, HBED is already used in approved PSMA-11-kits, while NOTA and NODAGA are reported to form stable complexes with ^{68}Ga at 20 °C as well [30]. Furthermore, newer chelators like THP show successful labeling with ^{68}Ga at 20 °C even when they are attached to affibodies [31].

Moreover, the application of DOTA- or DOTAGA-based ligands requires a high amount of precursor (20 µg/MBq) for the radiolabeling, which commonly results in a maximum dose of 200 µg/10 MBq ^{225}Ac-labeled peptide, which is equal to the amount used for routine ^{177}Lu-preparations with 8 GBq [33]. Typically, a single dose of 6–12 MBq is administered per patient containing 120–160 µg of peptide. With the

Table 1
Reaction outcome of six independent validation experiments with the iQS module.

precursor	amount	activity	RCY	RCP	particle	endotoxin level	pH value
mcp-M-PSMA	2 µg	0.5 MBq	0.4 MBq	97.6%	1.9%	<6.56 EU/mL	5.6
mcp-M-PSMA	2 µg	1.0 MBq	0.8 MBq	99.1%	1.1%	<5.0 EU/mL	5.7
mcp-M-PSMA	2 µg	1.1 MBq	0.9 MBq	98.5%	3.5%	<5.0 EU/mL	5.5
mcp-D-PSMA	2 µg	0.6 MBq	0.5 MBq	96.2%	1.3%	<5.51 EU/mL	5.6
mcp-D-PSMA	2 µg	1.1 MBq	0.9 MBq	98.2%	3.1%	<6.15 EU/mL	5.7
mcp-D-PSMA	2 µg	0.4 MBq	0.3 MBq	98.1%	3.0%	<6.39 EU/mL	5.5

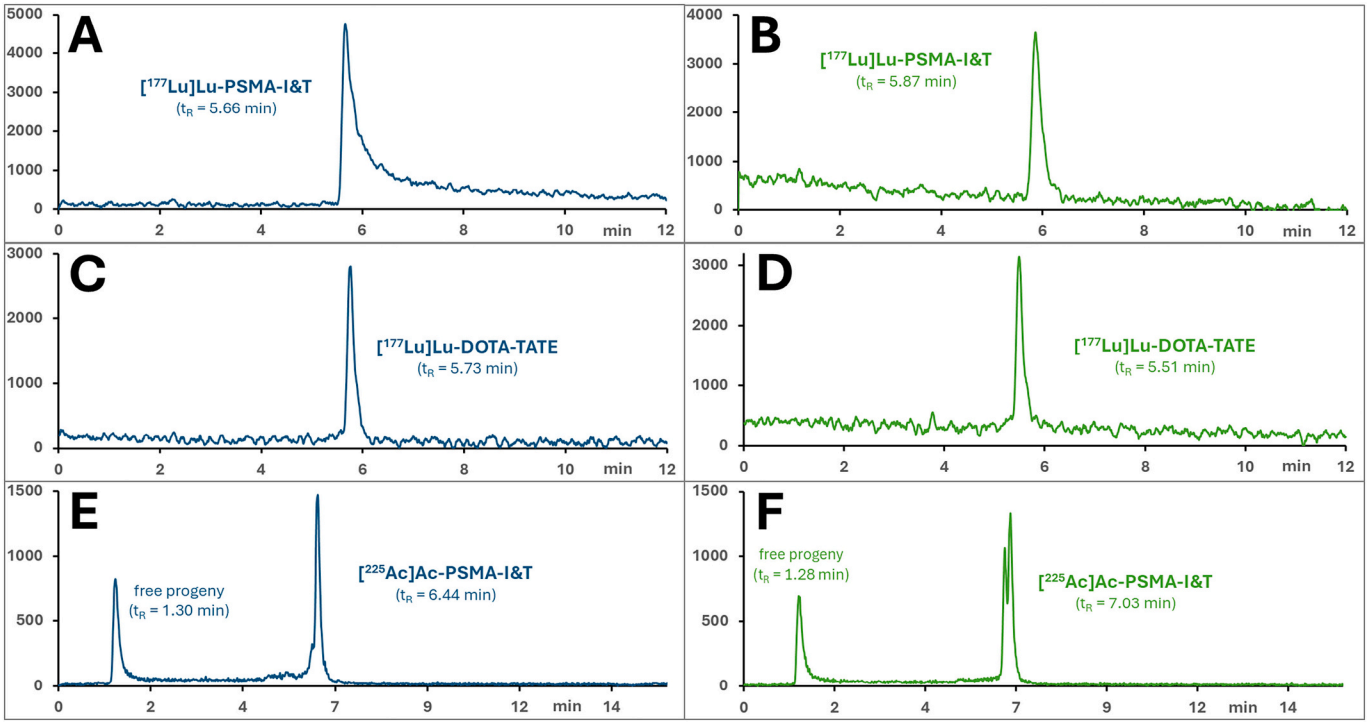

Fig. 3. Representative radio-HPLC chromatograms of [^{177}Lu]Lu-PSMA-I&T (**A**, **B**), [^{177}Lu]Lu-DOTA-TATE (**C**, **D**) and [^{225}Ac]Ac-PSMA-I&T (**E**, **F**) either with 0.1% TFA (blue colored chromatograms) and 0.05% H3PO4 (green colored chromatograms) as additive in the H_2O and acetonitrile eluents. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

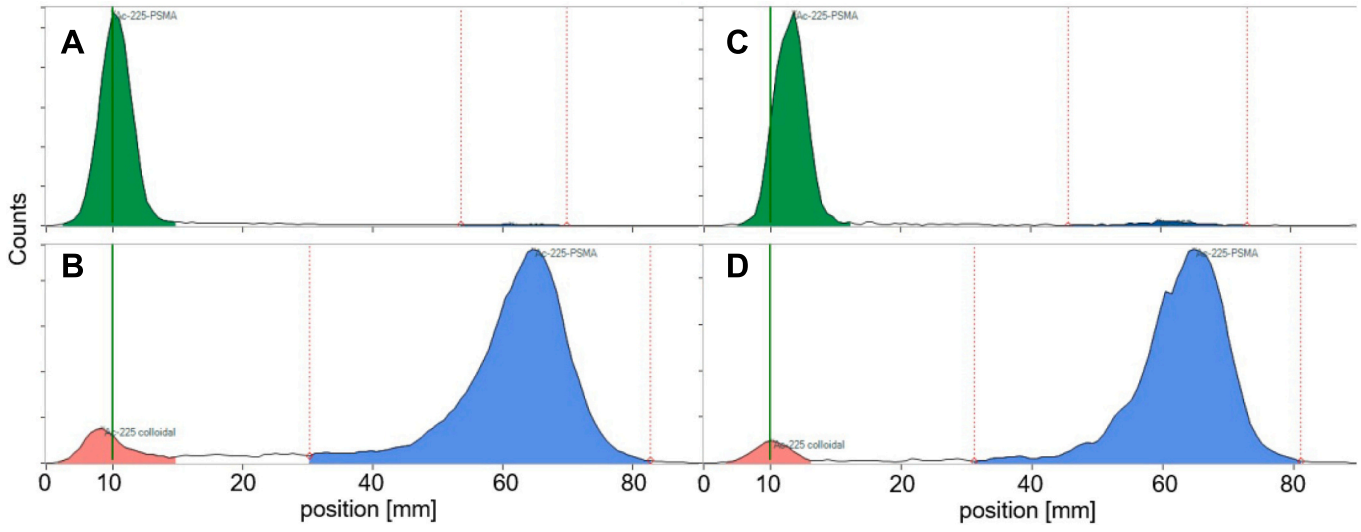

Fig. 4. Representative radio-TLC chromatograms of reaction solution with 99% ^{225}Ac-mcp-M-PSMA (**A** - citrate) and 8% colloidal ^{225}Ac-species (**B** - NH_4Ac) and diluted product solution with 96% ^{225}Ac-mcp-M-PSMA (**C** - citrate) and 5% colloidal ^{225}Ac-species (**D** - NH_4Ac) with 2.1 MBq starting activity of ^{225}Ac and precursor concentration of 20 μg/MBq after 144 h.

introduction of mcp-based ligands, the amount of precursor was drastically reduced by a factor of 10 to 2 μg/MBq [26].

To shed light on rechelation, one can assume that ^{213}Bi and ^{225}Ac are in equilibrium at the beginning of the radiolabeling reaction. DOTA or DOTAGA can chelate ^{225}Ac and ^{213}Bi at high temperature with >95% efficiency. The product is a mixture of e.g. 5 MBq ^{225}Ac-DOTA and 5 MBq ^{213}Bi-DOTA, which can be determined via radio-HPLC but not via radio-TLC. From the time point after the CM purification, new free ^{213}Bi is formed from the ^{225}Ac-decay, which can no longer be chelated by DOTA or DOTAGA, because the product solution is now at room temperature and formulated for injection. Further, 5–30 min after CM purification the radio-TLCs start to show a rising signal of free activity, which is mainly the newly formed ^{213}Bi, since the spots on the radio-TLC with the free activity have a half-life of 46 min. In the case of using macropa as chelator, there would also be a mixture of e.g. 5 MBq ^{225}Ac-macropa and 5 MBq ^{213}Bi-macropa. But now, no free ^{213}Bi can be detected on the radio-TLCs 5–30 min after purification. If higher amounts of macropa-precursor were used the effect persists for even longer periods as stated in this work. The now formed ^{213}Bi is chelated by macropa at 20 °C. Therefore, this is an indirect proof for the rechelation hypothesis. Any free activity, which is detected via radio-HPLC, is related to ^{221}Fr, which cannot be chelated either by DOTA and DOTAGA

Table 2
Comparison of TLC measurements for determining the rechelation capacity (%).

Sampling time	^{225}Ac-PSMA-I&T 20 µg/MBq		^{225}Ac-TATE 20 µg/MBq		^{225}Ac-mcp-M-PSMA 10 µg/MBq		Diluted ^{225}Ac-mcp-M-PSMA 10 µg/MBq		^{225}Ac-mcp-M-PSMA 20 µg/MBq		Diluted ^{225}Ac-mcp-M-PSMA 20 µg/MBq	
	Citrate	NH_4Ac	Citrate	NH_4Ac	Citrate	NH_4Ac	Citrate	NH_4Ac	Citrate	NH_4Ac	Citrate	NH_4Ac
5 min after synthesis	98	96	99	99	99	98	n.d.	n.d.	99	98	n.d.	n.d.
1 h after synthesis	n.d.	n.d.	n.d.	n.d.	99	99	98	98	99	99	99	97
3 h after synthesis	n.d.	n.d.	n.d.	n.d.	99	98	99	96	99	99	99	94
5 h after synthesis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99	98	99	95
24 h after synthesis	83*	85*	86*	78*	99	99	99	98	99	98	96	96
47 h after synthesis	n.d.	n.d.	n.d.	n.d.	99	96	99	98	99	98	98	95
73 h after synthesis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99	97	99	97
101 h after synthesis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99	97	99	94
120 h after synthesis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99	97	99	98
144 h after synthesis	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	99	92	96	95

* values >95% after 4 h of TLC development due to the decay of ^{213}Bi.

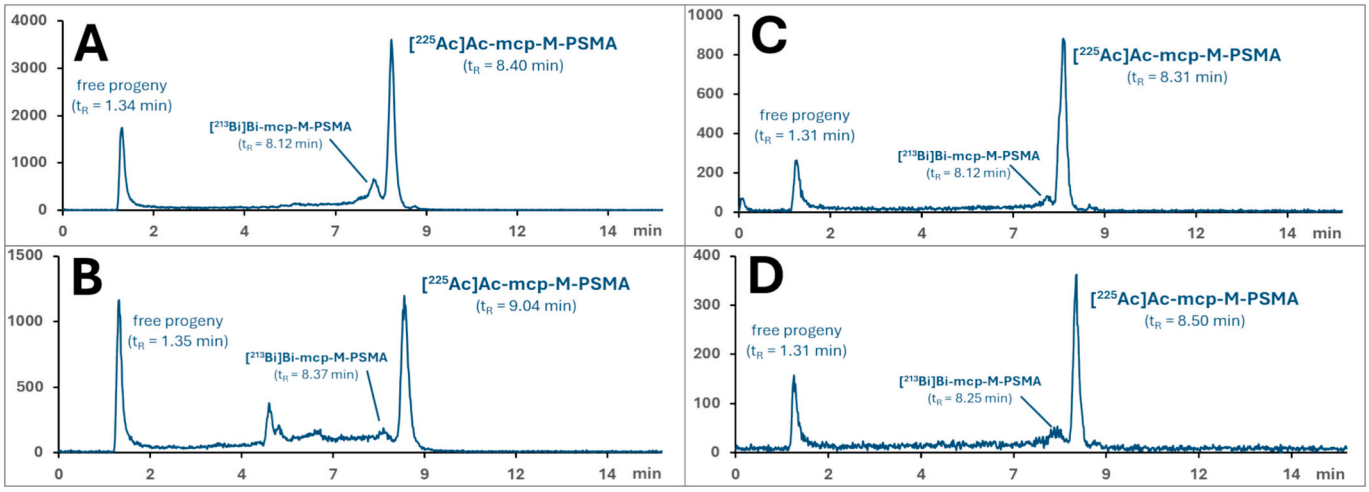

Fig. 5. Representative radio-HPLC chromatograms of [^{225}Ac]Ac-mcp-M-PSMA reaction solution (**A**, **B**) and diluted product solution (**C**, **D**) with 2.1 MBq starting activity of ^{225}Ac and precursor concentration of 20 µg/MBq after 29 (top) and 144 h (bottom).

nor by macropa.

After performing different rechelation experiments, it was found that higher amounts of mcp-ligands (5–10 µg/MBq) led to nearly quantitative rechelation of the progeny ^{213}Bi in a time span of 24–47 h in the reaction solution and 48–144 h in the diluted solution, respectively. Any ^{213}Bi generated due to the decay process released by recoil from the mcp-chelator must have been rebound by free uncomplexed mcp-ligands in the reaction and diluted solutions, since no further waiting time after the development of radio-TLCs was necessary due to the rechelation of ^{213}Bi already right after the synthesis (Table 2). The radio-TLC to determine free ^{225}Ac in citrate buffer must be performed on SiO_2-coated aluminum sheets. Otherwise, both [^{225}Ac]Ac-mcp-M-PSMA and [^{225}Ac]Ac-mcp-D-PSMA migrate together with free ^{225}Ac with the front when using ITLC-SG. The radio-TLC for colloidal ^{225}Ac-hydroxide in NH_4Ac:MeOH can be performed on ITLC-SG for both.

Following the literature, DTPA is frequently added to the product for the complexation of free progenies to facilitate faster renal excretion [7], as well as ascorbic acid to prevent radiolysis [7]. One can assume, the only reason for adding DTPA to the product solution is to chelate free ^{213}Bi in the patient solution. DTPA works at room temperature. This is done in advance, since the product solution is waiting for injection due to quality control at least 1 h after synthesis, in which a substantial amount of free ^{213}Bi is generated. Free ^{213}Bi is mainly accumulated to the kidneys, which could be harmful to the patient, whereas, DTPA-chelated ^{213}Bi forces the elimination from the kidneys in a higher and faster extend. Nonetheless, free ^{213}Bi would be removed relatively fast by renal elimination [32,33]. Currently, the amount of DTPA is recommended to be 0.1 mg/mL. For a 5 mL product solution, 500 µg of additional DTPA must be added. An elevated rechelation amount of macropa-precursor is 10 µg/MBq, resulting in 50 µg overall for 5 MBq ^{225}Ac. It would be very unlikely, that one tenth amount of macropa precursor should be more harmful in comparison to DTPA. Advantageously, the rechelation effect lasted for at least 24 h or longer. The stability of the macropa compound was proven via radio-HPLC. Therefore, despite the literature, it is possible to exclude DTPA from the final formulation, even if a purification method with CM cartridges is used to remove free, unbound ^{225}Ac. In this case, the remaining mcp-based precursors are able to rechelate free ^{213}Bi coming from the ^{225}Ac-decay directly in the reaction and diluted solution even at lowest precursor concentration of 2 µg/MBq for about 5 h. However, a higher amount of precursor peptide (10–20 µg/MBq) resulted in the rechelation of ^{213}Bi from 22 up to 144 h. Therefore, DTPA is considered to be no longer necessary in the final formulation and could therefore be excluded from the product formulation for the sake of viability. It is known that macropa is highly specific for large trivalent metal ions such as La^{3+} and Ac^{3+} as well as large divalent cations such as Ba^{2+} and Ra^{2+} [26]. It therefore forms only weak or no complexes with other, smaller cations such as Fe^{3+}, Cu^{2+} or Zn^{2+}. Additionally, the chelation with DOTA requires high temperatures. Since the amount of macropa precursor is not higher than 50 µg in total for a dose of 5 MBq ^{225}Ac, the concept of microdosing and tracer principle come into effect. To our knowledge, no adhere pharmacologic, allergic or even toxic effects are considered at such low doses. However, the higher amount (50 µg) of precursor accounts only for the rechelation setting. For patient dose

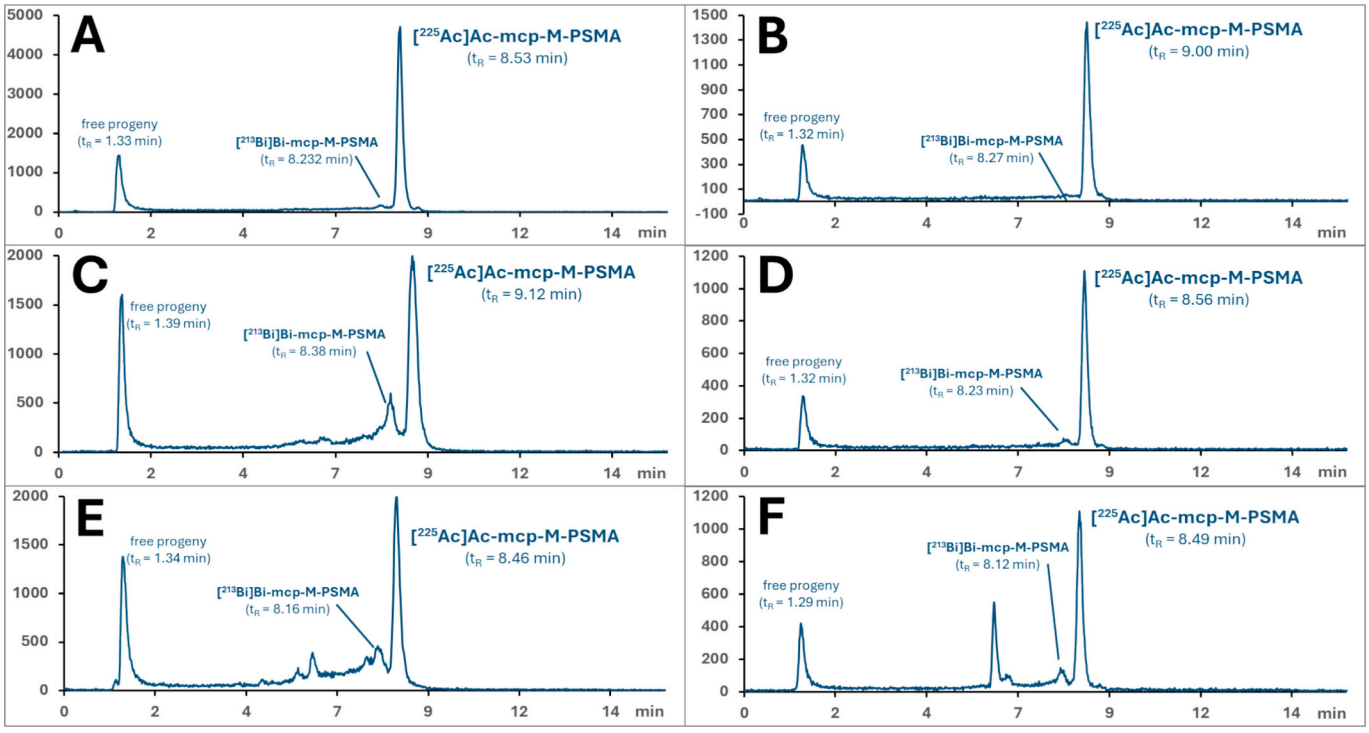

Fig. 6. Representative radio-HPLC chromatograms of [^{225}Ac]Ac-mcp-M-PSMA reaction solution (**A**, **C**, **E**) and diluted product solution (**B**, **D**, **F**) with 5.0 MBq starting activity of ^{225}Ac and precursor concentration of 10 μg/MBq after 3 h (**A**, **B**), 22 h (**C**, **D**) and 45 h (**E**, **F**).

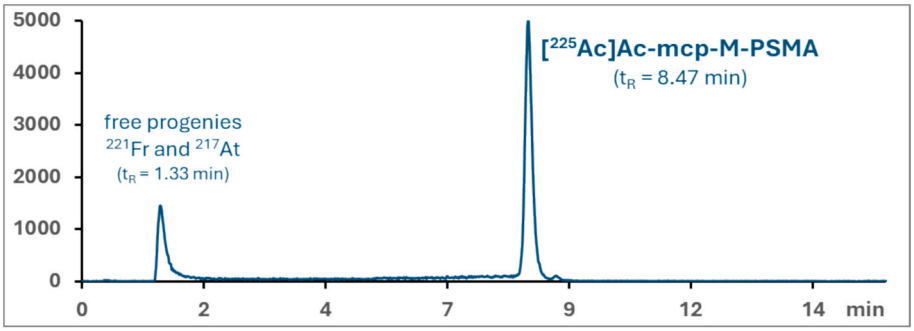

Fig. 7. Representative radio-HPLC chromatogram of [^{225}Ac]Ac-mcp-M-PSMA reaction solution with 5.0 MBq starting activity of ^{225}Ac and precursor concentration of 10 μg/MBq. The short-living free progenies ^{221}Fr and ^{217}At are found in the death volume peak (t_R = 1.33 min) even right after the reaction.

application, 20 μg or less is needed to achieve nearly quantitative chelation of 10 MBq of ^{225}Ac. In contrast, 200 μg of the rather unspecific DOTAGA precursor is routinely used to reach nearly quantitative chelation 10 MBq of ^{225}Ac, without any adhere side effects observed in our or other clinics [34].

The stability of the final ^{225}Ac-labeled peptides was also tested, since the radiolysis and alpha decay is often discussed to be fatal for the radioligands [35]. Radiolysis might be suppressed by dilution and long half-life of ^{225}Ac. One has to keep in mind, that not more than 1:1000 macropa molecules contain an ^{225}Ac or ^{213}Bi, more likely 1:10,000. At this point, alpha radiation completely destroys one molecule or at least the chelator due to the recoil effect. It is very unlikely, that the recoil effect impairs other intact non-chelated macropa precursor molecules due to their high dilution in the solution, the distance and the low range of alpha particles in solution. However, the intact precursor molecules are able to bind ^{213}Bi resulting from the ^{225}Ac- decay. However, it can be observed that, at a certain point in time after radiosynthesis, free ^{213}Bi becomes detectable via radio-TLC (Table 2). The formation of ^{213}Bi is proven by the half-life which relates to ^{213}Bi and not to ^{225}Ac. To prove this hypothesis, the ^{225}Ac-labeled peptides remained in the product vial for 24 h at room temperature after the synthesis without DTPA or ascorbic acid before sampling. Right after development, <5% of activity was found as unbound activity on the TLC chromatogram. Concurrently, the activity of the spot for unbound radionuclides decreased over time and > 2 h after development of the TLC, the RCP was even >98%, indicating no significant loss of ^{225}Ac from the radiolabeled peptide. Additionally, the main product peak was found to be prominent over radiolytic side products in radio-HPLC analyses after 24 h. However, when using 5–10 times excess of precursor the portion of stable product could be increased from 47 h (5 times precursor excess) up to 144 h (10 times precursor excess) in the diluted product formulation presumably due to the hypothesized rechelation effect. While rechelation can be observed up to 144 h with 20 μg/MBq of mcp-precursor in the reaction solution via radio-TLC (Fig. 4, Table 2), significant radiolysis was exclusively detectable by HPLC in the reaction solution already after 22 h with 2.1 MBq starting activity in 1 mL reaction volume. This radiolysis was not observable via HPLC in the diluted (0.5 MBq/mL) product solution up to 144 h. A combination of radio-HPLC and radio-TLC is

therefore recommended, since the radio-TLC can only detect the rechelation, but not the radiolytic side products. This important fact could lead to centralized production of diluted ^{225}Ac-labeled peptides in future. A product volume of 10–20 mL for 5–10 MBq would be recommended for this approach.

The GMP-compliant electric system (Ac-EAZY) [25] has some minor drawbacks like sporadic connection issues to the computer, blown fuses which can occur, impractical service of the canula for cassette attachment, and software issues. This comes always into play when using automated procedures and software. But the ^{225}Ac-cassette is commercially available and easy to install. All those points do not arise with the electroless system. The only drawback is, that liquids have to be transported by manual handling via syringes and that the "cassette" has to be assembled from sterile single-use components. Advantageously, no heating equipment is needed, which reduces the cost of the radiosynthesis. The use of lower precursor amounts is beneficial as well, as it reduces the costs of a GMP-compliant production. The environmentally persistent TFA is successfully substituted by eco-friendly, non-hazardous H_3PO_4. Overall, the synthesis contains no other hazardous chemicals. The main goal was to demonstrate the possibility of producing ^{225}Ac-labeled ligands for clinical applications, which was confirmed by experimental evidence. It must be stated that only radiolabeling and formulation were performed without electricity. Based on experience from numerous syntheses, it is known that the conversion of the labeling reaction is >90% when the CM cartridge has a residual activity of <0.2 MBq after purification. Even then an electric dose calibrator has to be used for measuring the developed TLCs, other non-electric quality control equipment might be pH strips.

Next steps are based on the development of kits for labeling, since the iQS is only needed for radiation safety purposes when handling with very high activities of ^{225}Ac. For single doses of 5–10 MBq per patient, the procedure could ultimately be performed manually using kits, without higher doses accumulating on the fingers or the body of the technical staff.

5. Conclusion

The electroless synthesis of ^{225}Ac-based mcp-PSMA derivatives was successfully implemented using the iQS module. The resulting ^{225}Ac-radioconjugates satisfied all the quality control criteria for a safe patient application. In conclusion, the system is technically ready for routine clinical application. Moreover, H_3PO_4, a sustainable alternative, has been employed to substitute for environmentally persistent TFA in radio-HPLC eluent, achieving successful results in the process. The employment of mcp-based precursors rendered the application of radiolysis protection agents in diluted product solutions redundant. The rechelation of free progenies (^{213}Bi) was observed for higher precursor concentrations, from 22 to 144 h, in the diluted product solutions. It is hypothesized that subsequent advancements may result in the creation of kits for convenient ^{225}Ac-labelling, as well as modifications to Ph. Eur. monographies analogous to [^{177}Lu]Lu-PSMA-I&T (pH. Eur. 3170) in order to facilitate the application of H_3PO_4 as an additive in the radio-HPLC eluent. The rechelation effect has the potential to result in the centralized production of mcp-based ligands. This would involve the use of slightly higher amounts of the relevant substances, as required for complete radiolabeling. The purpose of this process would be to ensure stability of the ^{225}Ac-radioconjugates for 24–48 h and this would facilitate delivery options over mid- and long-term transportation distances.

Abbreviations

DTPA diethylenetriaminepentaacetic acid
DOTA 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
HBED *N*,*N*′-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid
NODAGA 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid
NOTA 1,4,7-triazacyclononanetriacetic acid
THP tris(hydroxypyridinone)

CRediT authorship contribution statement

Marc Pretze: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Petra Gehlmann:** Validation, Methodology, Investigation. **Florian Brandt:** Writing – review & editing, Methodology, Investigation, Data curation. **Constantin Mamat:** Writing – review & editing, Visualization, Resources, Investigation, Conceptualization. **Ralph A. Bundschuh:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The are no conflict of interests to declare.

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