Validation of a radiosynthesis method and a novel quality control system for [68 Ga]Ga-MAA: is TLC enough to assess radiopharmaceutical quality?

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Abstract

Background: Technetium-99 m-labelled macroaggregated human serum albumin ([99mTc]Tc-MAA) is commonly used for lung perfusion scintigraphy. The European Pharmacopoeia (Eu.Ph.) specifies thin-layer chromatography (TLC) as the only method to assess its radiochemical purity (RCP). Similarly, TLC is the sole method reported in the literature to evaluate the RCP of Gallium-68-labelled MAA [68 Ga]Ga-MAA, recently introduced for lung perfusion PET/CT imaging. Since [68 Ga]Ga-MAA is prepared from commercial kits originally designed for the preparation of [99mTc]Tc-MAA, it is essential to optimize and validate the preparation methods for [68 Ga]Ga-MAA.

Results: We tested a novel, simplified method for the preparation of [68 Ga]Ga-MAA that does not require organic solvents, prewash or final purification steps to remove radioactive impurities. We assessed the final product using radio-TLC, radio-UV-HPLC, and radio SDS-PAGE. Overall, our quality control (QC) method successfully detected [68 Ga]Ga-MAA along with all potential impurities, including free Ga-68, [68 Ga]Ga-HSA, unlabeled HSA, which may occur during labelling process and HEPES residual, a non-toxic but non-human-approved contaminant, used as buffer solution. We then applied our QC system to [68 Ga]Ga-MAA prepared under different conditions (25°–40°–75°–95 °C), thus defining the optimal temperature for labelling. Scanning Electron Microscopy (SEM) analysis of the products obtained through our novel method confirmed that most [68 Ga]Ga-MAA particles preserved the morphological structure and size distribution of unlabeled MAA, with a particle diameter range of 25–50 μm, assuring diagnostic efficacy.

Conclusions: We optimized a novel method to prepare [68 Ga]Ga-MAA through a QC system capable of monitoring all impurities of the final products.

Keywords: [68 Ga]Ga-macroaggregated human serum albumin, Radiopharmaceuticals, High pressure liquid chromatography, SDS-PAGE, Morphological structure and size distribution, Lung perfusion imaging

Introduction

Radiometal-labelled macroaggregated human serum albumin (MAA) is widely used in perfusion imaging. Particularly, technetium-99 m-labelled ([^{99m}Tc]Tc-MAA) is commonly used in single photon emission computed tomography (SPECT) (Suga et al. 2004). Positron emission tomography/CT (PET/CT) can be also be performed with Gallium-68-labelled MAA ([^{68}Ga]Ga-MAA) and provides images with significantly higher resolution compared to SPECT. Ga-68 has a half-life of 68 min and the synthesis of 68 Ga-labelled radiopharmaceuticals is based on generator-produced Ga-68 that can be obtained on site and on demand, without the need for a medical cyclotron. [^{68}Ga]Ga-MAA for PET/CT perfusion imaging was first proposed in 2011 as an attractive alternative to [^{99m}Tc]Tc-MAA (Even and Green 1989a; Mathias and Green 2008). The first investigations on a method to directly label MAA with Ga-68 was performed in 1986 (Maziere et al. 1986). In 1989, a correlation between radiochemical yield (RY) and time and labelling temperature was reported (Even and Green 1989b). Consequently, a prewash step was introduced before adding the activity (Mathias and Green 2008). Different commercially available MAA kit systems have been tested, with similar results (Jain et al. 2016; Amor-Coarasa et al. 2014; Ament et al. 2013). To improve the Ga-68 labelling efficiency of MAA, several approaches have been pursued, including the removal of the reducing component stannous chloride (Shanehsazzadeh et al. 2017) and the addition of an excess of free albumin (HSA) (Amor-Coarasa et al. 2014; Hofman et al. 2011; Ament et al. 2013; Maus et al. 2011; Mueller et al. 2017), as well as the introduction of a final purification step (Shanehsazzadeh et al. 2017; Maus et al. 2011; Blanc-Béguin et al. 2022).

While many efforts have been put in to develop more efficient radiosynthesis methods to label MAA with Ga-68, there is still the need to strengthen the current quality control (QC) system to accurately define the radiochemical purity (RCP%) of [^{68}Ga]Ga-MAA and quantitatively identify free Ga-68, and other impurities (colloidal Ga-68, [^{68}Ga]Ga-HSA and unlabeled HSA that may be produced during the labelling procedure (Nelson et al. 2022; Velikyan 2015). The presence of radiochemical impurities increases the background radiation, reduces the target/nontarget ratio, and contributes to an unnecessary radiation burden for the patients without adding to the diagnostic information or improving treatment (Maisonial-Besset et al. 2014; Molavipordanjani et al. 2019). In the case of diagnostic radiopharmaceuticals, radiochemical impurities can impact the interpretation of scans and imaging accuracy (Molavipordanjani et al. 2019; Perrin et al. 2018).

A validated and standardized separation method is a key factor for discriminating between different (radio)chemical forms. To date, similarly to [^{99m}Tc]Tc-MAA, the only reported method described in the European Pharmacopoeia (Ph. Eur. 11th Ed.) to determine the RCP% of [^{68}Ga]Ga-MAA is TLC (Monograph on Technetium xxxx). TLC is indeed the only quality control method reported in literature for [^{68}Ga]Ga-MAA (Mathias and Green 2008; Maziere et al. 1986; Even and Green 1989b; Jain et al. 2016; Amor-Coarasa et al. 2014; Ament et al. 2013; Shanehsazzadeh et al. 2017; Hofman et al. 2011; Maus et al. 2011; Mueller et al. 2017; Blanc-Béguin et al. 2022). Although TLC is a very convenient technique, it has low sensitivity for some compounds, it cannot distinguish enantiomers and some isomers, and it is more a qualitative than a qualitative

method. Particularly, in the case of [^{68}Ga]Ga-MAA, TLC can only distinguish between unreacted Ga-68 and radio-labelled proteins, regardless of their size and aggregation form.

The lack of specific and different methods to assess the radiopharmaceutical quality of [^{68}Ga]Ga-MAA has led us to develop a reliable QC procedure that includes a new radio-UV-HPLC method and radio-SDS-PAGE. This QC system allowed us to monitor [^{68}Ga]Ga-MAA preparation under different conditions and—particularly—at different labelling temperatures, starting from a commercially available MAA labelling kit.

Results

Radiosynthesis conditions investigation and quality controls

Labelling was carried out at 4 different temperatures, i.e. 25, 45, 75 and 95 °C, to investigate the effect of temperature on labelling efficiency. We did not perform any purification steps at the end of MAA synthesis to improve the RCP of the final product, but we assessed a purification step after labelling. Radiosynthesis was performed 3 times at each temperature. Labelling efficiency and the optimal labelling temperature were evaluated by QC of [^{68}Ga]Ga-MAA by Radio-TLC, Radio-UV-HPLC and Radio-SDS-PAGE, as reported below.
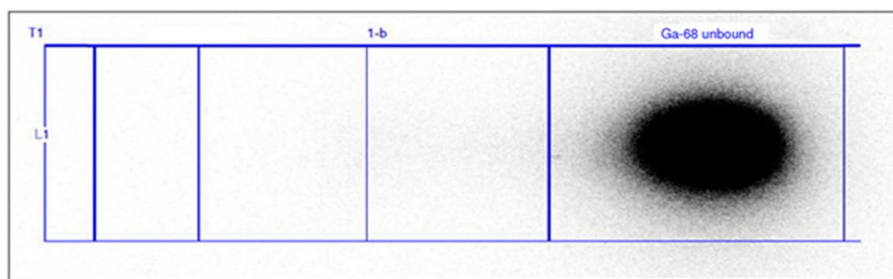
Radio-TLC

The RCP of the reactants and the products of [^{68}Ga]Ga-MAA preparation were first assayed by Radio-TLC. [^{68}Ga]Ga^{3+} contained in the radioactive reagent migrated to a high Rf (Rf:1) (Fig. 1a), whereas [^{68}Ga]-HSA remained at the origin (Rf=0) (Fig. 1b). Following MAA labelling at 95 °C, only the spot at Rf=0 was observed, with no traces of residual [^{68}Ga]Ga^{3+} (Fig. 1c). Unbound [^{68}Ga]Ga^{3+} was found as a contaminant in preparations obtained at 25, 45 and 75 °C (Fig. S1).
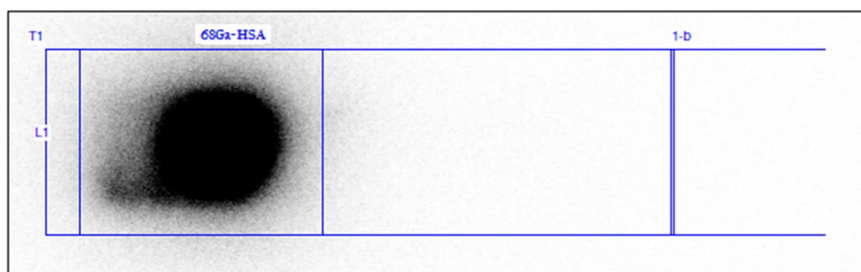
Radio-UV-HPLC

The reactants and the products of [^{68}Ga]Ga-MAA preparation were then analyzed by HPLC and Radio-HPLC. Preliminarily, we analysed the reactants and their known excipients, i.e. albumin (Proalbumin®), N-Acetyl-L-tryptophan, sodium octanoate, MAA (Pulmocis®) and [^{68}Ga]GaCl$_3$ (Fig. S2). The peaks corresponding to all species were well resolved and allowed to identify of all components in the reagents (Figs. S3 and S4), with retention times (Rt) of 17.18 min for HSA, 13.07 min for N-Acetyl-L-tryptophan and 21.9 min for sodium octanoate, respectively. The chromatogram of MAA (Pulmocis®) showed the presence of free HSA and sodium octanoate (Fig. S2b).

Following the optimization of the HPLC method, we analyzed [^{68}Ga]Ga-MAA obtained at different temperatures. The radio-UV-HPLC chromatograms of [^{68}Ga]Ga-MAA produced at 25 and 45 °C are reported in Fig. 2a and b, and exhibited a peak corresponding to free [^{68}Ga]Ga-HSA. Unlike radio-TLC, which could only detect 68Ga^{3+} as a contaminant, radio-UV-HPLC was capable of identifying free [^{68}Ga]Ga-HSA. In TLC, free [^{68}Ga]Ga-HSA migrated with the same Rf as [^{68}Ga]Ga-MAA (Fig. 1b), confirming that only radio-UV-HPLC can differentiate between the two. Notably, this impurity was absent in the radio-UV-HPLC chromatogram of the product obtained at 75 °C, which showed only the peak for free [^{68}Ga]Ga^{3+} (Fig. 2c). At 95 °C, both unbound [^{68}Ga]Ga^{3+}

(a)

(b)
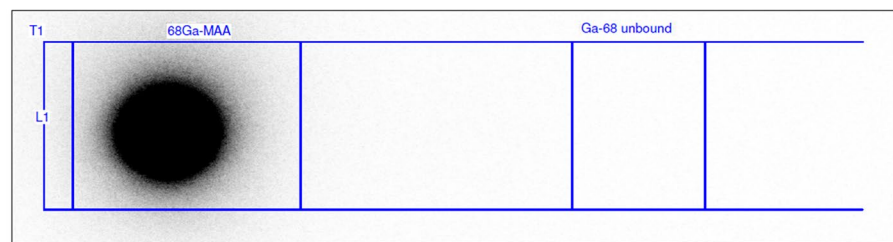
(c)

Fig. 1 Radio-TLC of **a** [^{68}Ga]GaCl$_3$, **b** [^{68}Ga]-HSA, and **c** [^{68}Ga]Ga-MAA crude product, obtained with a radiolabelling temperature of 95 °C
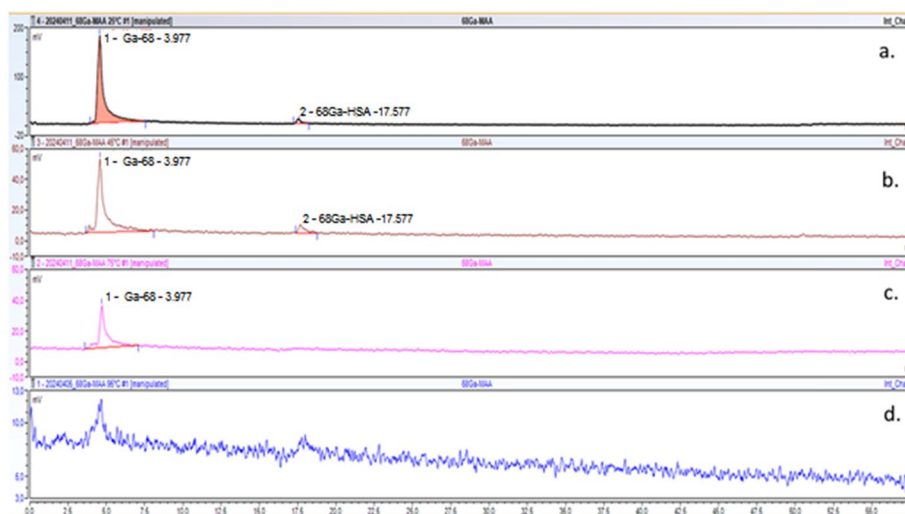

Fig. 2 Radio-UV-HPLC chromatograms of [^{68}Ga]Ga-MAA obtained at different temperatures: **a** 25 °C, **b** 45 °C, **c** 75 °C, **d** 95 °C

and [^{68}Ga]Ga-HSA were absent in the Radio-UV-HPLC of the radiopharmaceutical product (Fig. 2d), confirming that the only spot showed in the Radio-TLC (Fig. 1c) was referring to [^{68}Ga]Ga-MAA.

We then applied our TLC and UV-HPLC methods to assess the separation of [^{68}Ga]Ga-MAA from HEPES—an additive of the kit—y 0.22 µM filtration. HEPES is biocompatible and does not pose any toxicity risk but is not approved for use in humans. HPLC performed on the filtered and resuspended [^{68}Ga]Ga-MAA showed that the residual content of HEPES in the final preparation was lower in the HEPES test solution (12.5 µg/mL) (Fig. S5) confirming its almost complete removal. The large amount of residual HEPES in the solution coming from the filtration from the bottom was also evidenced (Fig. S6).

Validation of the radio-UV-HPLC analytical method to determine chemical purity

Under the chromatographic conditions described above, the HSA peaks were well-resolved. In (Fig. S7), a typical chromatogram of blank eluent is shown in comparison to the spiked samples of HSA analyzed for the radio-UV-HPLC method validation. The average retention time of HSA was 17.3 min ± 0.016. The calibration curve was linear over the concentration range 1–20 mg/mL of HSA. From the evaluated chromatograms, we determined the peak area and graphically plotted these values against the concentrations, thus obtaining the calibration curve (Fig. 3a). All data are provided in Supplemental Table S1. From the regression analysis, a linear equation was obtained ($y = 152.93x + 158.14$). The estimated correlation coefficient (R^2) of 0.9998 was in accordance with the acceptance criteria (Table S2), indicating a linear relationship between the concentration of the analyte and the area under the peak.

The relative standard deviation (RSD) value of the slope was 0.59%. For each point of calibration standard, the concentrations of HSA were recalculated from the equation of the linear regression curve. The average coefficient of variation (CV%) was less than 2%, which is in accordance with the acceptance criteria (Table S2). The bias% value was 98.14%. The limit of quantification (LOQ) and the limit of detection (LOD) for HSA, calculated according to ICH Q2 (R1) recommendations (ICH Guideline 1995), were 0.71 mg/mL and 0.23 mg/mL, respectively.

Validation of Radio-UV-HPLC analytical method to determine radiochemical purity

Under the same gradient chromatographic conditions described above, the [^{68}Ga]Ga-HSA peaks were well resolved. Figure S8 shows the typical chromatogram of [^{68}Ga]Ga-HSA analyzed for the analytical Radio-UV-HPLC method validation. The average retention time of the [^{68}Ga]Ga-HSA was 17.68 min. The calibration curve for the determination of [^{68}Ga]Ga-HSA was linear, and the linearity of this method was therefore statistically confirmed (Fig. 3b). The R^2 for calibration curve was equal to 0.9986 and the average CV% was less than 2% which is in accordance to acceptance criteria (Table S3).

Radio-SDS-PAGE

To further investigate the binding of [^{68}Ga]Ga^{3+} to MAA and devise a strategy to distinguish [^{68}Ga]Ga-labelled MAA from [^{68}Ga]Ga-labelled free HSA, we preliminarily evaluated the separation of non-labelled MAA by SDS-PAGE. As-is resuspended Pulmocis$^{®}$,
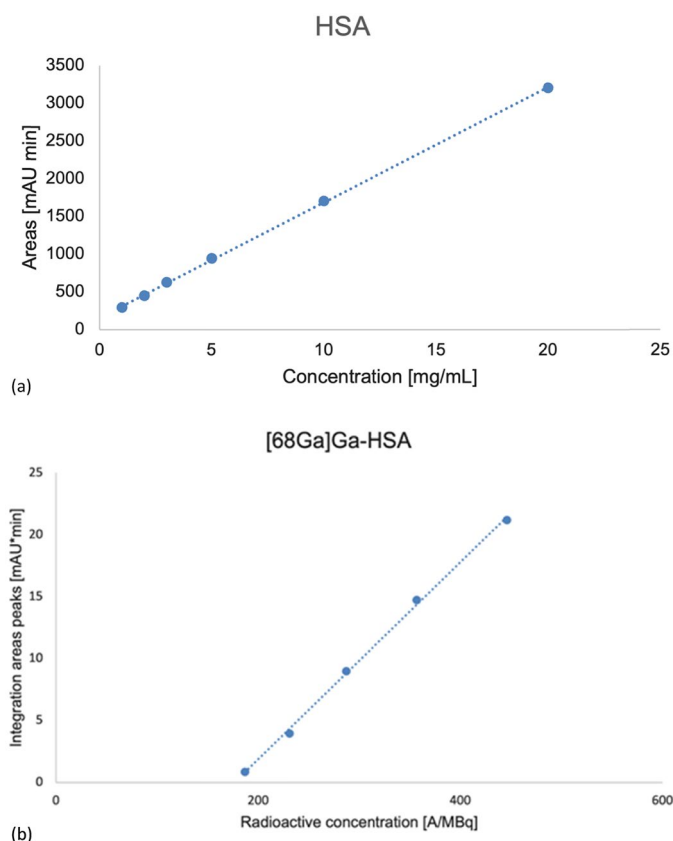
Fig. 3 Calibration curve obtained with **a** the values of peak areas of five different concentrations (20, 10, 5, 3, 2, 1 mg/mL) of HSA; **b** the average values of peak areas of [68 Ga]Ga-HSA

as well as the permeate and the retentate upon filtration with a 0.22 μm filter, were denatured under different conditions (a, b and c in Fig. 5a) and loaded onto a 12% SDS-PAGE gel. All samples displayed a single band at around 65 kDa, consistent with HSA (Mw 66.5 kDa) (Fig. 4a). The denaturing conditions did not affect the intensity of the bands (conditions a and b in Fig. 4). In the absence of 2-mercaptoethanol in the sample buffer, a higher electrophoretic mobility was observed, indicative of one or more intramolecular disulfide bridges. Under no conditions were high molecular weight aggregates observed, confirming that filtration followed by SDS-PAGE effectively separates HSA derived from MAA and free HSA. Densitometric analysis of the bands, adjusted for dilution coefficients, indicates that 68.8% of resuspended Pulmocis® consists of free HSA (Fig. S9).

The same separation protocol was applied to [68 Ga]Ga-MAA obtained at 95 °C. We then measured the radioactivity of the gel with a dose calibrator (CAPINTEC 25R®). A band consistent with HSA could be observed only in the lane containing the retentate, whereas no radioactivity was observed in the permeate, indicating that Ga-68 was bound to MAA (Fig. 4b).

Morphological structure and size distribution of the MAA and [68 Ga]Ga-MAA particles

After reconstitution, Pulmocis® was analysed with a polarized light optical microscope, highlighting the presence of aggregates (Figs. 5a and b).
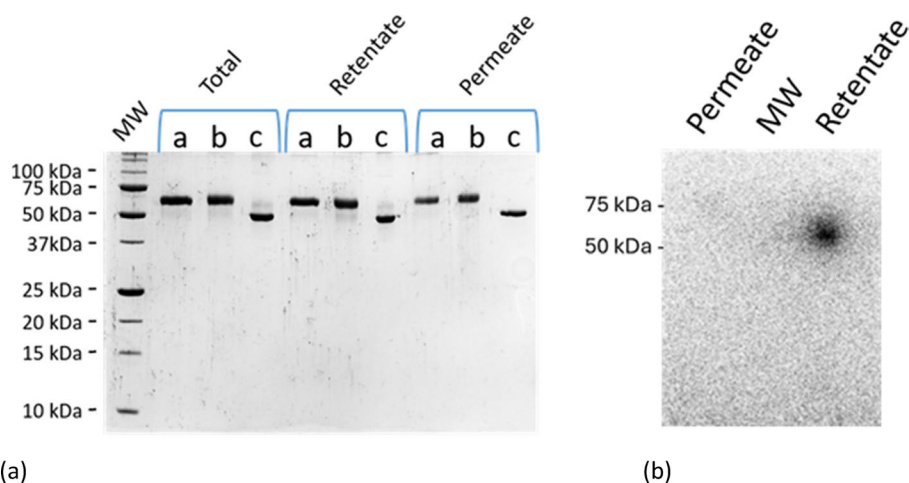
Fig. 4 **a** 12% SDS-PAGE of resuspended Pulmocis® (total), and the retentate and permeate upon filtration. SDS-PAGE samples were prepared at 100 °C for 20 min in the presence of 2 mM 2-mercaptoethanol (condition *a*), at 100 °C for 45 min in the presence of 2 mM 2-mercaptoethanol (condition *b*) and at 37 °C for 20 min with no 2-mercaptoethanol (condition *c*). The three original samples were diluted to achieve similar protein concentration for densitometric analysis; **b** radio SDS-PAGE of [^{68}Ga]Ga-MAA Pulmocis® separated by filtration
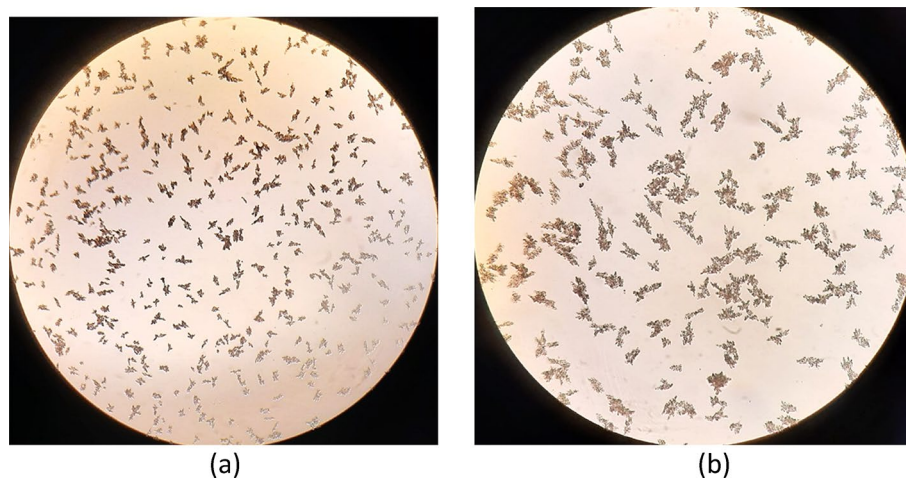

Fig. 5 Microscope images of Pulmocis® sample after reconstitution. **a** Magnification 100x **b** Magnification 200x

[^{68}Ga]Ga-MAA obtained at 95 °C was analysed with the microscope at 100x (Fig. 6a) and 200x (Fig. 6b), revealing the presence of macroaggregates and single particles deriving from the disaggregation of MAA, likely due to the high labelling temperature adopted.

The same Pulmocis® and [^{68}Ga]Ga-MAA samples were submitted to particle size distribution analysis by laser light diffraction. The data are reported in Fig. 7 for Pulmocis® and [^{68}Ga]Ga-MAA, respectively. Pulmocis® presented a bimodal distribution with a first more abundant population centred around 30 μm and a second around 180 μm (Fig. 7a). The volume diameter of the 10th percentile (Dv_{10}) of the particle population was 17 μm, that of the 90th percentile (Dv_{90}) was 109 μm, whereas the median volume
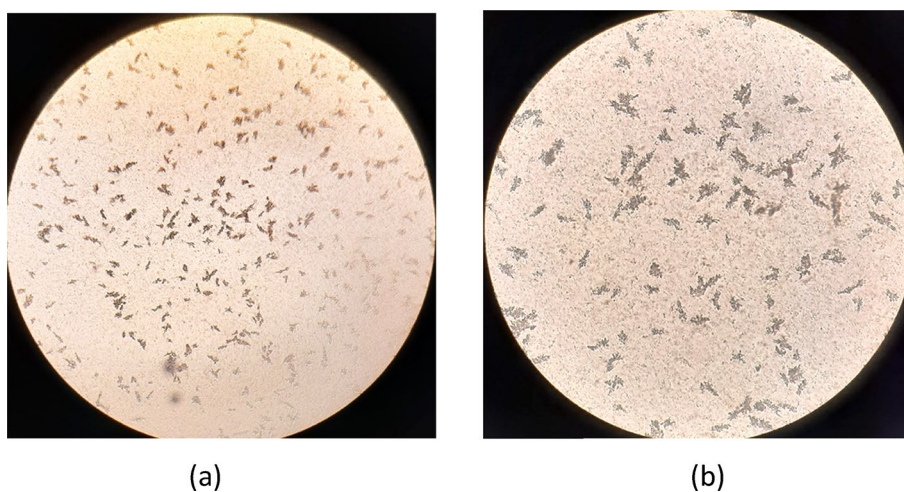
Fig. 6 Microscope images of [^{68}Ga]Ga-Pulmocis sample **a** 100 × and **b** 200 x) after the labelling procedure
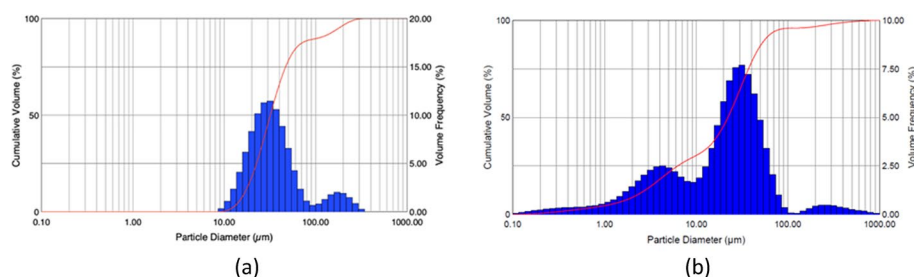

Fig. 7 Particle size distribution analysis reported as cumulative undersize volume diameter distribution (red line) or percentage of frequency of particles by volume diameter (blue histogram) of Pulmocis® **a** and [^{68}Ga] Ga-MAA **b**

diameter (50th percentile, Dv_{50}) was 32.5 μm. These data are in good agreement with the particle size range described in the Pulmocis® data sheet, reported between 10 and 100 μm.

[^{68}Ga]Ga-MAA obtained at a labelling temperature of 95 °C showed a trimodal distribution with a small population < 10 μm, one with diameter between 10 and 100 μm, by far the most abundant, and a very small one above 100 μm (Fig. 7b). The distribution parameters were: $Dv_{10} = 2.25$ μm, $Dv_{50} = 22.25$ μm, $Dv_{90} = 56$ μm. The only substantial difference compared to Pulmocis® immediately after reconstitution is the presence of the particle population < 10 μm and the consequent reduction of the percentage of the particles in the other two populations, confirming what was already observed by microscope analysis (Fig. 6).

Circular dichroism

To investigate the relationship between labelling temperature and selectivity towards MAA vs free HSA, we carried out circular dichroism measurements. Spectra of HSA analysed with the dichroweb algorithm indicated a 69.5% α helix content, consistent with the predicted secondary structure of native HSA (Fig. S9A, solid line and Table S4). Only 0.7% of the secondary structure was identified as β strands. A temperature ramp

between 20 and 100 °C resulted in a transition centred at 73.34 ± 0.2 °C (Fig. S9B), which—however—did not produce full denaturation. Indeed, at 100 °C, residual secondary structure was observed, with 26% α helix and 21.7% β strands (Fig. S9A, dotted line, and Table S4), indicating a partially preserved structure at high temperatures.

Materials and methods

Reagents and chemical synthesis

All chemicals used for the radiolabelling reaction, i.e. saline (NaCl), ethanol, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer solution, PBS buffer solution and water were of the highest available purity grade and commercially obtained as a single disposable kit (reagents and cassettes for synthesis of [^{68}Ga]Ga-peptides using cationic purification ABX, Advanced Biochemical Compounds, Radeberg, Germany).

All the medicinal products used in this study are commercially available and authorized for clinical use.

Macroaggregate of human serum albumin colloidal particles is a commercialized radiopharmaceutical kit available as Pulmocis® (Curium, UK). Each vial contains between 2×10^6 and 4×10^6 of the ligand, human serum albumin macroaggregates (macrosalb, 2 mg), with a declared diameter ranging between 10 and 100 μm (95% of the population). It also contains excipients with known function: non-denatured human albumin (7 mg) as adjuvant, stannous (II) chloride dihydrate (0.2 mg) as reducing agent, sodium chloride (8.7 mg) as isotonic agent and sodium octanoate (sodium caprylate) (Summary of Product Characteristics (SPC) xxxx).

The 68Ge/68 Ga generator (1850 MBq, GalliaPharm® Eckert & Ziegler, Berlin, Germany) is pharmaceutical grade, GMP certified and compliant with European Pharmacopoeia, and the amount of detected metal impurities as provided by the manufacturer is less than the defined limit in the European Pharmacopeia monograph (Gallium Chloride xxxx).

The aseptic production was conducted in a GMP grade A hot cell (NMC Ga-68, Tema Sinergie). Both 68Ge/68 Ga generator and automated synthesis module (Scintomics GRP® module, Germany) were placed in the hot cell.

All chemicals used to perform quality controls, i.e. trifluoroacetic acid (TFA), water and acetonitrile used for Radio-UV-HPLC, as well as ammonium acetate and methanol, were metal-free and purchased from Sigma Aldrich (Saint Louis, Missouri, USA).

All chemicals used as reference solutions were certified: albumin (Proalbumin®) and the Ph. Eur. products sodium octanoate (sodium caprylate) and sodium N-acetyltryptophan, purchased from Grifols (Italy) and Merck Millipore (Italy) respectively.

Radiosynthesis

The elution of a pharmaceutical grade, GMP certified and compliant with European Pharmacopoeia 68Ge/68 Ga generator (GalliaPharma®, Eckert and Ziegler) was carried out using the GRP module 3 V automated synthesis system (Scintomics GmbH, Fürstenfeldbruck, Germany) from Scintomics GmbH. The generator was eluted with 0.1 M HCl 24 h before labelling to remove the accumulated stable Zn-68 from Ga-68 decay and the elute [^{68}Ga]GaCl$_3$, obtained from the generator elution, was collected and pre-purified

on a SCX cartridge, which separates the ions based on their net total surface area change, and then eluted with 1.5 mL of 5 M NaCl.

[^{68}Ga]GaCl$_3$ was added manually into a reaction vial containing the entire available commercial kit Pulmocis® (MAA 2.0 mg), previously dissolved with 1 mL of NaCl and 1.6 mL of 1.5 M HEPES to a final pH of 4–4.5. After vigorous mixing with MAA, the reactor containing [^{68}Ga]Ga-MAA suspension was incubated in a thermoblock at different temperatures (25°–40°–75°–95 °C) for 20 min.

After incubation, quality controls of the crude product were performed without previous purification. The [^{68}Ga]Ga-MAA activity was measured in a Capintec CRC 25-PET dose calibrator and the entire radiopharmaceutical production takes 30 min.

The final [^{68}Ga]Ga-MAA was filtered through a 0.22 µm low-protein-binding filter and the [^{68}Ga]Ga-MAA was then transferred into the final vial by passing 7 mL of saline from the top to the bottom of the filter.

Plastic needles were used to prevent the presence of metal traces that could seriously affect the synthesis efficiency.

The key steps of [^{68}Ga]Ga-MAA production are presented in Fig. 8.

Radiochemical purity

To ensure that the final injectable radiopharmaceutical product fulfils regulatory requirements relating to contaminants, suitable production and quality control are crucial (Commission and Council of Europe 2013).

The Radiochemical purity of [^{68}Ga]Ga-MAA was evaluated with both Radio-TLC and Radio-UV-HPLC.
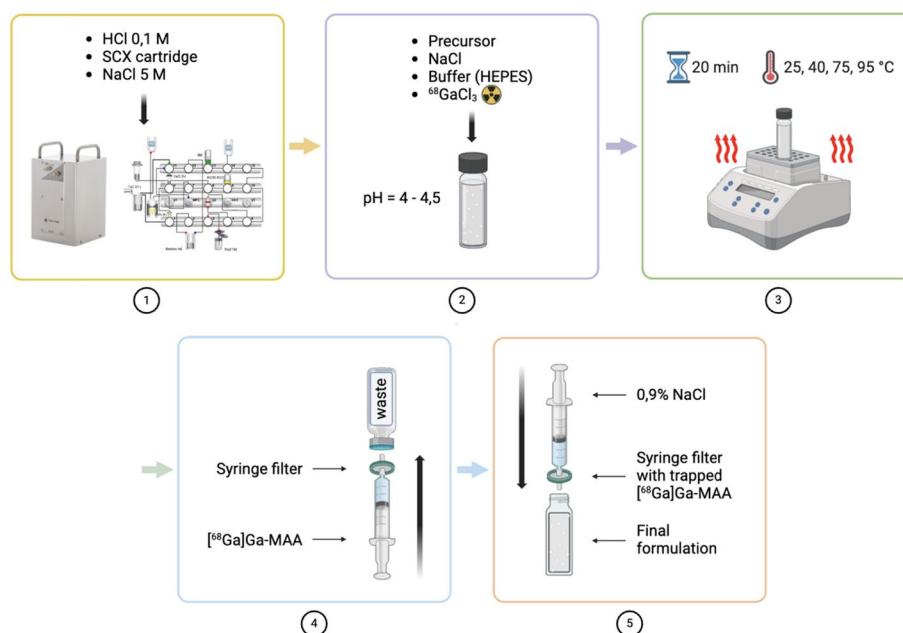
Fig. 8 Synthesis production scheme of [^{68}Ga]Ga-MAA: 1. 68Ge/68 Ga generator elution and [^{68}Ga]GaCl$_3$ eluate purification. 2. Mixing the [^{68}Ga]GaCl$_3$ eluate with the MAA. 3. Heat the reaction solution at (25°–40°–75°–95 °C). 3. Filtration of [^{68}Ga]Ga-MAA. 4. Collection of [^{68}Ga]Ga-MAA for the final formulation

For Radio-TLC, a 5 μL sample was dripped onto ITLC-SG, Agilent Technologies (8-cm length, 1 cm thick) and carried out with NaCl 0.9% as mobile phase.

MAA labelled with Ga-68 remained at the point of application (Rf:0), while free Ga-68 migrated with the solvent front (Rf:0.8–1). ITLC-SG paper strips, used a stationary phase, were counted with a scanner (Cyclone® Plus Storage Phosphor system, Perkin Elmer) and the chromatograms were analysed with OptiQuantTM software. The percentages of each fraction were determined relative to the total activity of the chromatogram.

Reference solutions of $[^{68}Ga]GaCl_3$ and $[^{68}Ga]Ga$-MAA were assessed using the same analytical conditions.

The Radio-UV-HPLC was additionally used to determine accurately the chemical and radiochemical purity of $[^{68}Ga]Ga$-MAA.

For Radio-UV-HPLC, a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) equipped with a BioBasic-18 column 5 μm 300 Å (250 mm × 4.6 mm) and a UV and a γ-detector (Berthold Technologies, Milan, Italy). The used solvents were A) 0.1% TFA in water and B) 0.1% TFA in acetonitrile.

The flow rate of the mobile phase was set at 1 mL/min, with a total run of 40 min. The following phase gradient was used in the Radio-UV-HPLC analysis: $0-2$ min from 0 to 10% B, 2–30 min from 10 to 90% B, 30–32 min 100% B, from 32 to 45 min 100% B, 45–48 min from 100 to 10% B and 48–58 min 10% B. The column temperature was kept at 25 °C and the samples were also monitored with UV detector at 220 nm to detect chemical impurities in the final product. Activity corresponding to Ga-68 ion and $[^{68}Ga]$ Ga-MAA was measured by Radio-UV-HPLC γ-detector. The software system Chromeleon 7 was used to assemble the information.

Reference solutions of HSA, MAA, sodium octanoate and sodium N-Acetyl-L-tryptophan, $[^{68}Ga]Ga$-Cl_3 were assessed in the same way.

Chemical purity

In this study, we determined the residual HEPES content, according to Ph. Eur. Monograph (Gallium 2482), following our validated HPLC method (Migliari et al. 2022). For HPLC a Waters Xbridge® column C18 (150 mm × 4.6 mm, 3.5 μm), as stationary phase was used, connected to an UV detector set to a wavelength of 195 nm and a γ-detector (Berthold Technologies, Milan, Italy) and ammonium formate 20 mM pH 9.5, as mobile phase, at an isocratic flow of 0.7 mL/min.

Validation of radio-UV-HPLC analytical method to determine chemical purity

The analytical method for determining the chemical purity of $[^{68}Ga]Ga$-MAA was validated following ICH Q2 (R1) and EDQM guidelines ((ICH Guideline 1995); ICH Q8 2017; EDQM Guidelines xxxx). Key parameters assessed included specificity, linearity, precision, accuracy, limit of quantification (LOQ), and limit of detection (LOD). The acceptance criteria for each parameter are listed in Table S2. Specificity was assessed by analyzing a mixture of critical components in the $[^{68}Ga]Ga$-MAA solution, demonstrating the method' ability to distinguish between HSA, $[^{68}Ga]Ga$-HSA, and free Ga-68.

Linearity was determined using standard solutions of varying concentrations (20, 10, 5, 3, 2, 1 mg/mL) of albumin reference solution (Proalbumin®, "mother" solution 200 mg/mL) and evaluated via linear regression, calculating the curve equation and R^2.

Precision was assessed as repeatability or intermediate precision, calculated using the coefficient of variation (CV%) or relative standard deviation (RSD). The formula is CV% = s/m × 100, where s is the standard deviation of peak areas and m is the average peak area; CV% must be ≤ 2% for all concentrations.

Accuracy was measured by bias%, which evaluates the difference between experimental values and nominal values. The acceptance criterion is bias% > 95%, with deviations not exceeding 5%. CV% and bias% were determined by injecting samples three times at the concentrations used in the linearity test.

The experimental LOQ was established by analyzing diluted standard HSA solutions until achieving > 95% precision, confirmed through a precision analysis at the LOQ concentration, with an acceptance criterion of CV% < 5%. LOD and LOQ were calculated using:

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where S is the slope of the calibration curve and σ is the standard deviation of the response, assessed during the linearity test at concentrations of 1–20 mg/mL (ICH Guideline 1995).

Validation of Radio-UV-HPLC analytical method to determine radiochemical purity

Validation of the analytical method for the determination of radiochemical purity is presented here. In the validation of the methods for radioactive compounds, some of the ICH guidelines of validation parameters may not be of concern and do not apply (Table S3).

Considering the radioactive nature and the short half-life of Ga-68, the typical experimental approach based on the preparation of a series of solutions with different concentrations does not apply. On the contrary, in this case, one sample solution with suitable radioactive concentration, was analyzed five times, at defined time intervals (20 min). Indeed, the radioactivity being the physical parameters of concern for radiochemical detectors, the radionuclide decay itself provides the necessary linear series of values. Determination of linearity was done analyzing five different concentrations of $[^{68}Ga]Ga$-MAA, produced at 45 °C, a calibration curve was calculated and R^2 extrapolated from it.

Precision may be considered at different levels as a measure of repeatability or intermediate precision.

The same considerations described for linearity, can be apply also for repeatability. The decay of the radionuclide Ga-68 inevitably leads to a decrease over time of the radioactivity, however repeatability was evaluated analyzing a series of HPLC runs obtained with repetitive injections of a single $[^{68}Ga]Ga$-MAA sample and by recalculating the obtained peak area values with the decay equation: $\ln A_0 = \ln A + \lambda t$, where $\lambda = 0.693/t_{1/2}$, A_0 = corrected peak area, A = measured peak area, t = time interval between the considered injection and the first one, $t_{1/2}$ = half-life (Ga-68 = 67.63 min).

The peak area values normalized for decay were compared and yielded a consistent analysis. Average, standard deviation and CV% were calculated.

Radio-SDS-PAGE

Pulmocis® was reconstituted according to the manufacturer' instructions and filtered using a 0.22 μm low-protein-binding filter to separate free HSA, expected in the permeate, from MAA, expected in the retentate. The as-is Pulmocis® suspension, the permeate and the retentate solutions were denatured with standard sample buffer for SDS-PAGE under three different conditions, i.e. at 100 °C for 20 min in the presence of 2 mM mercaptoethanol, at 100 °C for 45 min presence of 2 mM mercaptoethanol and at 37 °C for 20 min with no 2 mM mercaptoethanol. Samples were diluted to achieve similar protein concentration and finally separated by 12% SDS-PAGE. The gels were stained using Coomassie Brilliant Blue and analysed using a ChemiDoc™ XRS + imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Morphological structure and size distribution of the MAA and [^{68}Ga]Ga-MAA particles

Pulmocis® kit was reconstituted with NaCl 0.9% to a final concentration of 1 mg/mL and compared to [^{68}Ga]Ga-MAA obtained after the incubation at 95 °C for 20 min. One hundred μL of the samples were placed on a glass slide and covered by a coverslip. A microscope (Nikon Optiphot2-pol) at 100 or $200 \times$ magnification was used to evaluate the morphological structures of both samples, then images were acquired by a digital camera (Apple IPhone 13, Apple inc. Cupertino, USA) fixed with a holder to the ocular of the microscope. A Spraytec laser diffraction system (Malvern Instruments Ltd.) equipped with a 300 mm focal lens was used to analyze size distribution of both samples and to investigate the MAA integrity at the end of the labelling procedure. The measurement was carried out with an obscuration threshold of at least 10%. Particle size data are presented as Dv_{10}, Dv_{50}, and Dv_{90}, indicating equivalent volume diameters at 10%, 50%, and 90% of cumulative volume distribution, respectively. The width of the particle size distribution was expressed by Span, calculated as $(Dv_{90}\text{-}Dv_{10})/Dv_{50}$.

Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jasco J-1500 spectropolarimeter equipped with a Peltier thermostatic unit set at 20 °C and employing 0.1 mm quartz cells. JASCO Spectra Manager II software was used for data acquisition (Fig. S10) and Table S4. HSA concentration was 3.1 μM in 10 mM K_2HPO_4 buffer at pH 7. Spectral scans were collected from 250 to 180 nm with a data interval of 0.5 nm, an 8 s integration time (DIT), a 2 nm bandwidth, and a scanning speed of 50 nm/min. Each reported spectrum is the average of 3 accumulations. All CD spectra were baseline-corrected for buffer contributions. Secondary structure analysis was performed using the Dichroweb server (Whitmore and Wallace 2004). Changes in the far-UV CD signal at 222 nm were monitored during a temperature ramp from 20 to 100 °C, with 5 °C increments and a 1 s equilibration time at each temperature before measurement. Thermal unfolding transitions were analyzed using a sigmoidal equation.

Discussion

Radiolabelled MAA is a tracer for PET and single-photon emission tomography (SPECT). It can accurately localize the tumor and assess perfusion. It also provides better cross-sectional images for preoperative or pre-treatment planning (Persico et al. 2020). Thanks to the great advantages of PET imaging over SPECT imaging, such as higher sensitivity, spatial and temporal resolution, superior quantitative capability, and easier respiratory gated acquisition, many efforts have been dedicated to switch from conventional scintigraphy to PET imaging, as well as from Tc-99 m to Ga-68 (Blanc-Béguin et al. 2022).

The commercial product Pulmocis® consists of macroaggregated albumin (MAA) obtained by heat denaturation of stannous chloride-treated HSA under controlled conditions. Because of this production process, the kit contains the excipients octanoate (caprylate, CA) as a stabilizer of HSA against thermal stress (Yu and Finlayson 1984) and N-AcTrp, which has a protecting effect on sulfhydryl groups, diminishing the oxidation of HSA (Anraku et al. 2004). However, these kits were produced and optimized for technetium labelling, so they contain many auxiliary substances that are not required for labelling with other metals such as Ga-68, including the reducing agent stannous chloride and free HSA.

Here, we have developed a synthesis method to produce [^{68}Ga]Ga-MAA using a non-modified MAA from a commercially available kit. We have also developed a quality control (QC) system, which includes a new radio-UV-HPLC method in addition to the radio-TLC, to be used rountinly to assess the RCP of [^{68}Ga]Ga-MAA.

For the radiosynthesis process, we have evaluated the composition of the kit and optimized the labelling conditions, defining a protocol for [^{68}Ga]Ga-MAA purification, with no need to pre-wash MAA. The protocol provided a RY > 95%, high RCP (100%) and high As (425.66 GBq/g). No difference was detected in the labelling efficiency upon removing stannous chloride (Shanehsazzadeh et al. 2017) and free HSA (Amor-Coarasa et al. 2014) through a pre-wash step of MAA.

To assess the synthesis parameters, including temperature, reaction time and solvent, the behaviour of HSA under different labelling conditions was determined by a novel QC system.

We initially considered the use of size exclusion chromatography (SEC) to separate the radiopharmaceutical from the radionuclide and evaluate the RCP of [^{68}Ga]Ga-MAA (Vallabhajosula et al. 1982; Schmitt et al. 2021; Lazar et al. 2005). However, we did not detect any peaks, unlike the case of radiolabelled antibodies or their derivates, regardless of the radionuclide (Lazar et al. 2005; Brady et al. 2008).

Then, we developed an HPLC method with a C18 column to evaluate the RCP of [^{68}Ga]Ga-MAA more accurately, even if we were aware that the method is unsuitable to detect MAA particles because of their large dimension (10–100 μm). This method allowed us to separate radioactive impurities such as free Ga-68 and [^{68}Ga]Ga-HSA, evaluating the real RCP% of[^{68}Ga]Ga-MAA together with the best labelling conditions to ensure patient safety and enable the daily production and transferability of our production method in other radiopharmaceutical laboratories. We validated our implemented radio-UV-HPLC analytical method for HSA by evaluating a standard reference solution of albumin reference solution (Proalbumin®) standard solutions at different

concentrations (20, 10, 5, 3, 2, 1 mg/mL). The developed chromatographic method complied with all International Conference of Harmonisation (ICH) requirements over time. These validation results demonstrated the suitability of the method in determining the radiochemical and chemical purity of [^{68}Ga]Ga-HSA, providing accuracy, robustness, and repeatability also for the routine clinical application.

Radio-UV-HPLC proved capable to monitor [^{68}Ga]Ga^{3+}, labelled HSA and non-labelled HSA. However, it did not directly detect MAA. To achieve the separation and detection of MAA and free HSA, either labelled or unlabelled, we carried out a filtration step followed by SDS-PAGE. Under denaturing conditions, a single band corresponding to HSA was observed for both MAA (the retentate of the filtration step) and free HSA (the permeate of the filtration step). A single band was also detected in the absence of reducing agents, indicating the absence of intermolecular bonds in Pulmocis®, although intramolecular bonds could be inferred by the altered electrophoretic mobility. Filtration followed by radio-SDS-PAGE was then applied to MAA labelled at the highest temperature. Radioactivity was observed only in the band corresponding to HSA derived from MAA—the retentate of the filtration step—with no signals from free HAS—the permeate. Therefore, unlike TLC, filtration followed by SDS-PAGE achieved efficient separation of peptide chains based on their molecular weight and this approach can be used to efficiently monitor the concentration of radioactivity in complex protein mixtures. Moreover, the comparison of SDS-PAGE under reducing and non-reducing conditions can distinguish between intermolecular and intramolecular disulphide bonds, a key factor in MAA formation. Indeed, the complete blocking of the free -SH groups inhibits the irreversible denaturation of serum albumin after heating to 75 °C and excludes to a large extent any aggregation. Aggregation due to the formation of S–S bridges, would be obviously irreversible (Wetzel et al. 1980).

Once the QC system was put in place, we addressed several issues associated with the labelling process.

Firstly, we addressed the requirement for metals in the reaction mixture. Upon radiolabelling with sodium pertechnetate Tc-99 m injection solution, the stannous ions reduce Tc-99 m, which binds aggregated albumin to yield [^{99m}Tc]Tc-MAA (Monograph on Technetium (99Tc) xxxx; Summary of Product Characteristics xxxx; Gallium Chloride xxxx). The [^{68}Ga]Ga-labelled MAA can be effectively obtained by hydrolysis and precipitation of [^{68}Ga]Ga^{3+} ions in the presence of albumin particles, or by covalent conjugation of the macroaggregates with a high affinity [^{68}Ga]Ga-chelating ligand. The chemical structure of the complex between Ga-68 and MAA particles is unknown. In the reaction mechanism, Ga-68 is adsorbed to insoluble gallium hydroxide on the surface of MAA particles; Ga(III) ion was evaluated as a mechanism for capturing specific interactions with proteins on the particle surface (Ayşe et al. 2021). The presence of metal ion contaminants such as zinc (Zn), tin (Sn), nickel (Ni), lead (Pb), aluminium (Al), silicon (Si), and titanium (Ti), as well as high acidity of the eluate can interfere with the complexation of Ga-68 and chelators (Whitmore and Wallace 2004; Lepareur 2022). Zn, the decay product of Ga-68 from pollutant metals from the generator, is a very strong competitor that can complex with chelators (Uğur et al. 2020). To improve the RY without compromising an efficient radioactive labelling with Ga-68, we performed a pre-purification of the Ga-68 eluate by adsorption/elution with NaCl from the PSH + cartridge in

all our radiopharmaceutical productions to remove the metallic impurities and Ga-68, as well as to limit excess protons from hydrochloric acid used for elution (Amor-Coarasa et al. 2014; Ament et al. 2013a.The contaminant radiometal ions can adversely affect also RCP% and, indirectly, the molar activity, by necessitating higher amounts of peptide precursor to achieve high radionuclide incorporation. However, the pre-purification step of the elute Ga-68 is also necessary to reduce the volume, as required for molecule radiolabelling and for obtaining high As (Gillings et al. 2021; Luurtsema et al. 2021).

Secondly, we addressed temperature as a factor affecting complex formation between HSA and Ga-68 (Whitmore and Wallace 2004; Chao et al. 1998). To evaluate the optimal temperature, we tested various labelling temperatures (25°–40°–75°–95 °C) using a thermoblock. Quality control results demonstrated that the best radiolabelling temperature to produce [^{68}Ga]Ga-MAA is 95 °C. Radio-TLC showed the complete absence of unbound Ga-68 at this temperature, whereas Radio-UV-HPLC confirmed not only that the radiopharmaceutical product does not contain free Ga-68, but also the absence of Ga-68 radiolabelled HSA, despite the great excess of HSA in the kit, as well as of secondary radiolabelled products. Probably, the temperature dependence of the complex formation is associated to a conformational change at higher temperatures that facilitates labelling. Indeed, temperature ramps monitored by circular dichroism at 222 nm confirmed that HSA undergo a transition at 73 °C, with part of the α helix secondary structure still retained at 100 °C. Compared to HSA before heating, the percent of β strands increases, adding up to around 20% at 100 °C. This result is fully consistent with the observed labelling selectivity at temperatures higher than 70 °C.

Thirdly, we assessed a final purification step after labelling. Many authors have used different purification steps at the end of the synthesis to improve the RCP of the final product, such as centrifugation (Mathias and Green 2008; Maziere et al. 1986) suspension through a Sep-Pak C18 cartridge (Maus et al. 2011) and filtration (Blanc-Béguin et al. 2021). Potential radiolysis products, such as ions and excited molecules need to be investigated and removed because of the undesired and serious side effects potentially caused by them (Mu et al. 2013; Eppard et al. 2017). Radiolysis may be reduced by utilizing compounds insensitive to radiation or extenuating the process with additives such as radical scavengers like ascorbic acid or HEPES used in the reaction mixture. HEPES and acetate buffers are biocompatible with no toxicity issue, and they have demonstrated better characteristics to stabilize and prevent Ga-68 (III) precipitation and colloid formation. Nevertheless, in contrast to sodium acetate, HEPES is not approved for human use, and thus, purification and additional quality control analyses are required, resulting in further time and resource consumption (Velikyan 2015). Therefore, at the end of the MAA synthesis, we performed a filtration from the bottom using a low protein-binding filter (0.22 µm) to remove almost all HEPES buffer to make it ready to be used in clinical setting rather than to improve the RPC of the final product. Moreover, this final step confirmed that all MAA have been correctly radiolabelled with Ga-68, because the measured radioactivity was only on the filter, where [^{68}Ga]Ga-MAA is retained because of its large size.

Upon defining the optimal labelling temperatures, we assessed the MAA size and size distribution before and after incubation at 95 °C. Since MAA particles are in the µm range, final product sterility cannot be guaranteed by filtration. However, we guaranteed

the maintenance of the kit sterility by avoiding preliminary manipulation steps such as the lyophilization of MAA and conducting the radiosynthesis in a clean (sterile) environment, in accordance with Italian rules of Good Preparation of Radiopharmaceuticals in Nuclear Medicine (Gültekin et al. 2020) and European directives and guidelines (Gillings et al. 2021; Norme di Buona Preparazione dei radiofarmaci per medicina nucleare xxxx; EU Guidelines for Good Manufacturing Practice xxxx). The dimensions of MAA are critical to evaluate and maintain, as organ selectivity depends on particle size. The administered aggregates should be homogeneous and standardized, since MAA perfusion works via capillary blockage. MAA does not penetrate lung parenchyma (interstitial or alveolar) but temporarily occludes capillaries. After i.v. injection, most MAA particles are retained in the arterioles and capillaries of the lungs during the first passage. Pulmocis® company data states that over 95% of particles are between 10 and 100 μm, with none above 150 μm. The monograph on Technetium (Tc-99 m) macrosalb injection of the European Pharmacopoeia (Ph. Eur. 11th Ed.) (Monograph on Technetium (xxxx) requires that 90% of particles fall within the range of 10–100 μm but does not specify a measurement method. This limit minimizes the risk of vessel occlusion, particularly in patients with pulmonary artery hypertension, where vasoconstriction may be present (NucMedTutorials 2022). EANM guidelines for ventilation/perfusion scintigraphy (Bajc et al. 2009; Bajc et al. 2019) recommend particle sizes between 15 and 100 μm, different from the Pharmacopoeia' range (Jensen et al. 2022).

Heat treatment for Ga-68 labelling can cause MAA fragmentation, reducing embolic efficacy and affecting diagnostic applications (Velikyan 2015; Persico et al. 2020; Yu and Finlayson 1984; Anraku et al. 2004; Vallabhajosula et al. 1982; Schmitt et al. 2021; Lazar et al. 2005; Brady et al. 2008; Wetzel et al. 1980; Ayşe et al. 2021; Lepareur 2022; Uğur et al. 2020; Gillings et al. 2021; Luurtsema et al. 2021; Chao et al. 1998; Gültekin et al. 2020; Blanc-Béguin et al. 2021; Mu et al. 2013; Eppard et al. 2017; Norme di Buona Preparazione dei radiofarmaci per medicina nucleare xxxx; NucMedTutorials. 2022; Bajc et al. 2009; Bajc et al. 2019; Jensen et al. 2022; Canziani et al. 2022). Fragments smaller than 10 μm can escape the capillary filter and be absorbed by the spleen, liver, and bone marrow, deteriorating the target-to-background ratio and hampering a quantitative approach. Although most [^{68} Ga]Ga-MAA particles measure 25–50 μm, an increase in particles < 10 μm results from the high temperature (95 °C) used during labelling, potentially fragmenting MAA without compromising diagnostic efficacy. Despite their importance, particle size and morphology after Ga-68 labelling has not been exhaustively elucidated in the literature, with only six studies showing mixed results (Mathias and Green 2008; Shanehsazzadeh et al. 2017; Mueller et al. 2017; Maus et al. 2011 Jan; Pandey et al. 2014; Ahn and Boros 2018). Some found no changes in particle size or morphology (Mathias and Green 2008; Mueller et al. 2017; Maus et al. 2011 Jan; Ahn and Boros 2018) using optical microscopy or SEM (Maus et al. 2011), while two others reported MAA fragmentation (Shanehsazzadeh et al. 2017; Norme di Buona Preparazione dei radiofarmaci per medicina nucleare xxxx). Interestingly, the Ph. Eur. and EANM do not suggest the same particle size range.

The ranges described in Ph. Eur. are too wide and are unable to identify RCP% of [^{99m}Tc]Tc-MAA. The Ph. Eur. describes only the TLC as method to assess the quality of [^{99m}Tc]Tc-MAA (Monograph on Technetium (99Tc) xxxx; Gallium Chloride xxxx)

which, however, differentiates only between [^{99m}Tc]Tc-MAA and free [^{99m}Tc]NaO$_4$. Although TLC is a very simple and convenient technique, it has low sensitivity for some compounds, it does not differentiate between enantiomers and some isomers and especially it is rather a qualitative method. Current nomenclature rules defined RCY as the ratio of the activity of a radionuclide of a specific element before and after its radiochemical separation or labelling (Jensen et al. 2022; Coenen et al. 2017; Blois et al. 2019) and it is well defined in Ph. Eur. (Chapter 5, Radiopharmaceutical preparations), (European Pharmacopeia 11.0, 2024). It is one of the release criteria of radiopharmaceuticals according to QC parameters based on the European Pharmacopoeia (11.0/0125).

Conclusions

The determination of the RCP implies the quantitative identification of individual impurities and is based on separation of (radio)chemical forms (radioactive impurities) other than the intact labelled radiopharmaceutical. RCP ensures clinical suitability. Here, we have provided a reliable QC method to evaluate and assess the radiochemical purity of [^{68}Ga]Ga-MAA. Our QC demonstrated that the NaCl-based labelling procedure provides for convenient and rapid preparation of high specific activity [^{68}Ga]Ga-MAA. This method allows the use of most of the eluted Ga-68 activity from the generator and delivers a high radiochemical yield of [^{68}Ga]Ga-MAA. Furthermore, the procedure does not require the use of organic solvents. The method can make [^{68}Ga]Ga-MAA easily accessible for routine use in centres with high demand as well.

Abbreviations

As	Specific activity
CA	Octanoate, caprylate
CT	Computed tomography
GMP	Good manufacturing practice
GRP	Good radiopharmaceutical practices
EANM	European association of nuclear medicine
Eur. Ph.	European pharmacopeia
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HPLC	High pressure liquid chromatography
HSA	Human serum albumin
MAA	Macroaggregated human serum albumin
N-AcTrp	Acetyltryptophanate
NBP-MN	Norme di Buona Preparazione in Nuclear Medicine
TLC	Thin layer chromatography
TFA	Trifluoroacetic acid
PET	Positron emission tomography
QC	Quality control
RCY	Radiochemical yield
RPC	Radiochemical purity
SDS-Page	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SEC	Size exclusion chromatography
SPECT	Single-photon emission tomography

Supplementary Information

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Supplementary file 1

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