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Establishment and validation of an alternative automated synthesis of [68Ga]Ga-DOTA-Siglec-9 in an independent laboratory for clinical use

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Abstract

Background Siglec-9, a member of the Siglec family of receptors, plays a crucial role in modulating immune cell trafficking and inflammation, with significant clinical implications. It is predominantly expressed on immune cells such as neutrophils, monocytes, and dendritic cells –key components of both innate and adaptive immunity. Siglec-9 binds to sialic acid residues on glycoproteins, commonly found on endothelial cells, a mechanism central to immune regulation during inflammation and tissue injury. Notably, its interaction with vascular adhesion protein 1 (VAP-1), an endothelial adhesion molecule, is of particular interest for therapeutic development in chronic inflammatory diseases, autoimmune disorders, and cancer. Recent studies have demonstrated that a Siglec-9 motif-containing peptide conjugated with 1,4,7,10-tetraazacyclododecane-N, N′,N′′,N′′′-tetraacetic acid (DOTA) and radiolabelled with gallium-68 ([^{68}Ga]Ga-DOTA-Siglec-9) enables effective positron emission tomography (PET) imaging of these pathological conditions. This study aimed to develop a new automated radiolabelling protocol for the preparation of [^{68}Ga]Ga-DOTA-Siglec-9 for clinical use. The synthesis was carried out using a fully automated module with real-time monitoring of key parameters including time, temperature, and radioactivity.

Results Following optimization of labelling conditions and assessment of peptide stability, [^{68}Ga]Ga-DOTA-Siglec-9 was successfully synthesized with a radiochemical yield (RY) of 55.04%, radiochemical purity (RCP) of 99.48%, and molar activity (Am) of 23.15 GBq/µmol at 65 °C in 6 min. Process validation yielded consistent mean values of RY (56.16%), RCP (99.40%). and Am (20.26 GBq/µmol). Stability testing at room temperature over 3 h demonstrated that [^{68}Ga]Ga-DOTA-Siglec-9 maintained acceptable RCP (mean99.29%), pH, appearance, and sterility.

Conclusion The final product meets *Ph. Eur.* quality requirements and is suitable for clinical application.

Keywords [^{68}Ga]Ga-radiopharmaceuticals, [^{68}Ga]Ga-DOTA-Siglec-9, VAP-1, PET imaging

Introduction

Siglec-9 (sialic acid-binding immunoglobulin-type lectin 9), a member of the CD33-related Siglec family, plays a pivotal role in regulating immune cell migration and modulating inflammatory responses. It is predominantly expressed on innate immune cells—particularly neutrophils, monocytes, and certain subsets of dendritic cells—which are essential mediators of both innate and adaptive immunity (Zhang et al. 2000; Pillai et al. 2012; Schauer 2009). Functionally, Siglec-9 acts as an inhibitory receptor by engaging sialylated glycoconjugates, typically present on endothelial or epithelial cells, thereby modulating leukocyte activation, trafficking, and survival (Miralda et al. 2023; Yu et al. 2017).

The binding of Siglec-9 to sialic acid residues on glycoproteins is highly context-dependent and crucial during inflammation and tissue injury (Khatua et al. 2013; Macauley et al. 2014). In particular, Siglec-9 has been identified as a natural ligand for vascular adhesion protein-1 (VAP-1), an endothelial adhesion molecule and monoamine oxidase (AOC3). VAP-1 is stored intracellularly and rapidly translocated to the luminal surface of blood vessels in response to inflammation. The interaction between Siglec-9 and VAP-1 - mediated by both enzymatic and non-enzymatic domains of VAP-1 - plays a significant role in facilitating leukocyte adhesion and transmigration into inflamed tissues (Aalto et al. 2011; Salmi and Jalkanen 2019).

Clinically, the VAP-1/Siglec-9 axis presents a promising target for diagnostic imaging and therapeutic intervention. VAP-1 expression is significantly upregulated in a various chronic inflammatory diseases, including rheumatoid arthritis (Wang; Wang et al. 2017; Mahajan and Pillai 2016; Danielli et al. 2022), inflammatory bowel disease (Danielli et al. 2022; Brzezicka and Paulson 2023), and acute respiratory distress syndrome (Bhowmik et al. 2024), as well as in the vasculature of several tumor types (Amani et al. 2017; Wang et al. 2023; Kinoshita et al. 2021; Chen et al. 2023), where it contributes to the development of an immunosuppressive tumor microenvironment. Accordingly, molecular imaging agents targeting this interaction could enable non-invasive assessment of inflammatory activity, disease progression, and therapeutic efficacy.

To visualize this interaction, a Siglec-9-derived peptide has been conjugated to 1,4,7,10-tetraazacyclododecane-N, N′,N′′,N′′′-tetraacetic acid (DOTA) and radiolabelled with gallium-68, yielding [^{68}Ga]Ga-DOTA-Siglec-9, a PET radiotracer with high specificity for VAP-1. Preclinical studies have demonstrated that this tracer accumulates in inflamed tissues in animal models of synovitis (Marttila-Ichihara et al. 2009), lung inflammation (Virtanen et al. 2015), colitis (Brzezicka and Paulson 2023), and certain tumors (Retamal et al. 2016; Viitanen et al. 2022). Furthermore, first-in-human clinical trials (Viitanen et al. 2021) have shown that [^{68}Ga]Ga-DOTA-Siglec-9 is safe, well-tolerated, and capable of visualizing inflammatory lesions with high contrast.

The aim of this study was to develop and validate an alternative automated radiosynthesis method for the standardized production of [^{68}Ga]Ga-DOTA-Siglec-9-clinical batches. The process was designed to ensure that all procedures, materials, equipment,

and quality control steps used in the synthesis consistently yield a product that meets regulatory requirements for clinical application.

Materials and methods

Materials

The precursor for the preparation of [^{68}Ga]Ga-DOTA-Siglec-9, namely Siglec-9 motif-containing peptide, was sourced from ABX (Radeberg, Germany). A sterile aqueous stock solution at a concentration of 0.5 mg/mL was prepared under aseptic conditions and stored at −20 °C until use.

All reagents employed in the radiolabelling process—including sodium chloride (NaCl), ethanol, 2-[4-(2-hydroxyethyl)−1-piperazinyl]-ethanesulfonic acid (HEPES) buffer, phosphate-buffered saline (PBS), and water for injection (WFI)—were of the highest pharmaceutical purity and provided as a single-use synthesis kit (SC-01, ABX, Radeberg, Germany), ensuring batch-to-batch consistency and compliance with current Good Manufacturing Practice (cGMP) requirements.

Analytical-grade, metal-free reagents used for quality control procedures, including trifluoroacetic acid (TFA), water, and acetonitrile for radioactivity and ultraviolet-coupled high-performance liquid chromatography (Radio-UV-HPLC), as well as ammonium acetate and methanol for radiochemical purity assessment, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All medicinal products referenced in this study are authorized for clinical use and sourced from certified suppliers.

Radiolabelling was carried out using an automated synthesis module (Scintomics GRP®, Fürstenfeldbruck, Germany) equipped with a single-use disposable cassette (SC-01, ABX). The module operated within a GMP-compliant, ISO Class 5 (Grade A) hot cell (NMC Ga-68, Tema Sinergie, Faena, Italy), enabling aseptic production conditions.

A ^{68}Ge/^{68}Ga generator (GalliaPharm®, Eckert & Ziegler, Berlin, Germany; nominal activity 1850 MBq), certified for GMP compliance and aligned with the European Pharmacopoeia monograph [Gallium (^{68}Ga) Chloride solution for radiolabelling, Monograph 2464], was used. The levels of metallic impurities and ^{68}Ge breakthrough were confirmed by the manufacturer to be within the specified pharmacopeial limits.

Radioactivity measurements were performed using a dose calibrator (CRC® 25-PET, Capintec, Florham Park, NJ, USA). Radio-UV-HPLC was conducted on a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Milan, Italy) fitted with a BioBasic-c18 reverse-phase column (5 μm, 300 Å, 250 mm × 4.6 mm) and coupled to both a UV and a γ-detector (Berthold Technologies, Milan, Italy). Radiochemical purity was also assessed via thin-layer chromatography (TLC) using a Cyclone® Plus Storage Phosphor System (PerkinElmer, Milan, Italy). Endotoxin testing was performed using the Nexgen PTS system (Charles River, Wilmington, MA, USA), in accordance with pharmacopeial guidelines.

Solubility of Siglec-9 upon thermal treatment

Prior to radiosynthesis procedures, the solubility of Siglec-9 was evaluated under various potential labeling conditions. A solution of 0.5 mg/mL was prepared by resuspending the peptide powder in ultrapure water, then subjected to thermal treatment at 65 °C, 95 °C, and 100 °C for 6, 10, and 15 min, respectively. An untreated sample was used as a

control. Following heat exposure, samples were centrifuged, and protein content in the supernatants was assessed using a Bradford assay. Absorbance was measured at 550 nm with a HaloLED 96-well microplate reader (Control Tecnica, Padova, Italy). Protein quantification was performed using a calibration curve generated with bovine serum albumin (BSA) standards. All measurements were conducted in triplicate.

Mass spectrometry of Siglec-9

To assess the chemical stability of the peptide after thermal treatment, three representative samples were prepared as described above. Post-treatment, the samples were centrifuged at maximum speed, and the supernatants were analyzed by mass spectrometry using an Agilent InfinityLab LC/MSD XT system (Agilent Technologies, Santa Clara, CA, USA). Samples were diluted to a final concentration of 0.16 µg/µL in a 1:1 (v/v) mixture of water (0.1% formic acid) and acetonitrile (0.1% formic acid). Direct infusion analyses were performed in full scan mode using positive electrospray ionization (ESI + AJS), with a capillary voltage of 3.5 kV. A volume of 1 µL was injected per sample at a flow rate of 0.2 mL/min, with a scan range of m/z 250–2500. Data acquisition and processing were carried out using OpenLab CDS software (Agilent Technologies, Santa Clara, CA, USA).

Circular dichroism (CD) of Siglec-9

To investigate conformational changes associated with thermal exposure, circular dichroism (CD) spectra were recorded using a Jasco™ J-1500 spectropolarimeter equipped with a Peltier temperature control unit set at 20 °C. The peptide was prepared at a concentration of 150 µM in 10 mM potassium phosphate buffer, pH 7.0. Spectra were acquired in the far-UV region (250–180 nm) and represent the average of three accumulations. Buffer baselines were subtracted from all spectra. Secondary structure estimation was performed using the BeStSel web server (https://bestsel.elte.hu/index.php). For thermal unfolding studies, the CD signal at 200 nm was monitored continuously as the temperature increased from 20 to 100 °C at a ramp rate of 1 °C/min to estimate the melting temperature (Tm).

Radiosynthesis procedure

The radiolabelling procedure followed a synthesis template identical to that previously established for [^{68}Ga]Ga-PSMA (Migliari et al. 2017)and [^{68}Ga]Ga-DOTA-ECL1i (Migliari et al. 2023), ensuring protocol consistency and validated operational conditions. Radiolabelling was conducted using the GRP module 3 V automated synthesis platform (Scintomics GRP®, Fürstenfeldbruck, Germany).

The ^{68}Ge/^{68}Ga generator (GalliaPharm®, Eckert & Ziegler) was eluted with 0.1 M HCl. A preparatory elution was performed 24 h prior to labelling to remove built-up stable Zn-68 resulting from Ga-68 decay. The eluted [^{68}Ga]$GaCl_3$ was pre-concentrated using a strong cation exchange (SCX) cartridge (ABX, Radeberg, Germany), which functions by selectively retaining cationic species based on total surface charge interactions. Gallium-68 was subsequently eluted from the SCX cartridge using a 5 M NaCl/0.1 M HCl solution.

The recovered eluate was transferred into a reaction vessel pre-loaded with DOTA-Siglec-9 precursor (40 µg) dissolved in 1.5 M HEPES buffer, adjusted to a pH of 4.0–4.5. The radiolabelling reaction was carried out at 65 °C for 6 min. Following completion of

the labelling step, the reaction mixture was allowed to cool and subsequently passed through a Sep-Pak® C18 reverse-phase cartridge (Agilent Technologies, Santa Clara, CA, USA). The cartridge was washed with water for injection (Ph. Eur.), and the radiolabelled compound was eluted using 2 mL of a 1:1 ethanol/water solution. The eluted product was then diluted with phosphate-buffered saline (PBS) and sterile-filtered through a 0.2 µm Millex-GV membrane filter (Sigma-Aldrich (St. Louis, MO, USA) into a sterile, capped 25 mL glass vial. A final dilution with PBS was performed to achieve the desired formulation volume and isotonicity. The total synthesis time, including purification and formulation, was approximately 35 min.

Quality control and process validation

To ensure compliance with regulatory standards concerning potential contaminants, rigorous control over both production and quality assessment of the final injectable radiopharmaceutical is essential, in accordance with the European Pharmacopoeia (European Pharmacopoeia Commission 2013).

Following synthesis, the radiopharmaceutical [^{68}Ga]Ga-DOTA-Siglec-9 underwent comprehensive quality control evaluation. The synthesis efficiency and release criteria were evaluated following the *Ph. Eur.* guidelines for all final products including radiochemical yield (RY), radiochemical purity (RCP), detection of free gallium (via Radio-UV-HPLC), gallium colloids (via Radio-TLC), molar activity (Am), chemical purity, pH, and Limulus Amebocyte Lysate [LAL] assay. After optimizing the method, the entire process was validated through three consecutive syntheses, with stability monitored over time. RCP and product stability were monitored over 3 h at room temperature using both Radio-TLC and Radio-UV-HPLC. For Radio-TLC analysis, ITLC-SG strips (8 cm length, 1 cm thickness (Agilent Technologies, Santa Clara, CA, USA), were used as the stationary phase, with a 1:1 mixture of ammonium acetate and methanol as the mobile phase. Quantitative analysis of the chromatograms was performed using Opti-Quant™ software, with each radiochemical fraction expressed as a percentage of total detected radioactivity.

Radio-UV-HPLC was performed using a validated gradient method. The mobile phases consisted of (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile, at a flow rate of 0.6 mL/min. The gradient was as follows: the Radio-UV-HPLC analysis: 0–12 min from 18% B to 50% and 12–14 min from 50 to 18% B. The chromatographic system was maintained at 25 °C, and detection was carried out using both a UV detector (220 nm) for chemical impurities and a γ-detector for radiochemical species. Data acquisition and interpretation were performed using Chromeleon™ 7 software. Reference solutions of [^{68}Ga]$GaCl_3$, DOTA-Siglec-9, and the final product [^{68}Ga]Ga-DOTA-Siglec-9 were analyzed under the same chromatographic conditions to verify product identity and purity. Chemical purity, with a specific focus on residual HEPES content, was evaluated according to the European Pharmacopoeia Monograph 2482 (Gallium radiopharmaceuticals), using a previously validated HPLC method, following our validated HPLC method (Migliari et al. 2022). This assay employed a Waters XBridge® C18 column (150 mm × 4.6 mm, 3.5 µm) operated in isocratic mode with 20 mM ammonium formate buffer at pH 9.5 as the mobile phase, at a flow rate of 0.7 mL/min. Detection was carried out via UV absorption at 195 nm and γ-detection (Berthold Technologies, Milan, Italy).

Sterility testing was performed in accordance with the European Pharmacopoeia (EMA/CHMP/ICH/645592/2008), and endotoxin levels were determined using the Nexgen PTS™ system (Charles River, Wilmington, MA, USA).

To validate the robustness and reproducibility of the production and analytical processes, three independent batches of [^{68}Ga]Ga-DOTA-Siglec-9 were manufactured on separate days under routine conditions. Each batch was subjected to full analytical characterization to confirm that all quality attributes met the pre-defined acceptance criteria, ensuring the reliability of the overall production process for clinical application.

Results

Peptide stability

Upon treatment at different temperatures envisaged for possible labelling and for the corresponding incubation times, and after centrifugation, the residual concentration of the peptide in solution upon was assessed by Bradford assay. In all cases, no decrease in concentration was detected, indicating that thermal denaturation and precipitation does not occur, unlike reported for several other peptides (Fig. 1A) (Zapadka et al. 2017).

Chemical stability

To assess potential chemical modifications upon heating, we performed mass spectrometry analyses (Fig. 1B). Samples of peptide treated at controlled temperatures were analyzed in full scan mode to identify the characteristic ions. Under positive electrospray ionization (ESI + AJS), the peptide exhibited an extended charge state distribution, with ions detected at 1211.1 *m/z* $[M+2\,H]^{2+}$, at 807.9 *m/z* $[M+3\,H]^{3+}$, at 606.3 *m/z* $[M+4\,H]^{4+}$, and at 485.2 *m/z* $[M+5\,H]^{5+}$. This multicharged pattern reflects the presence

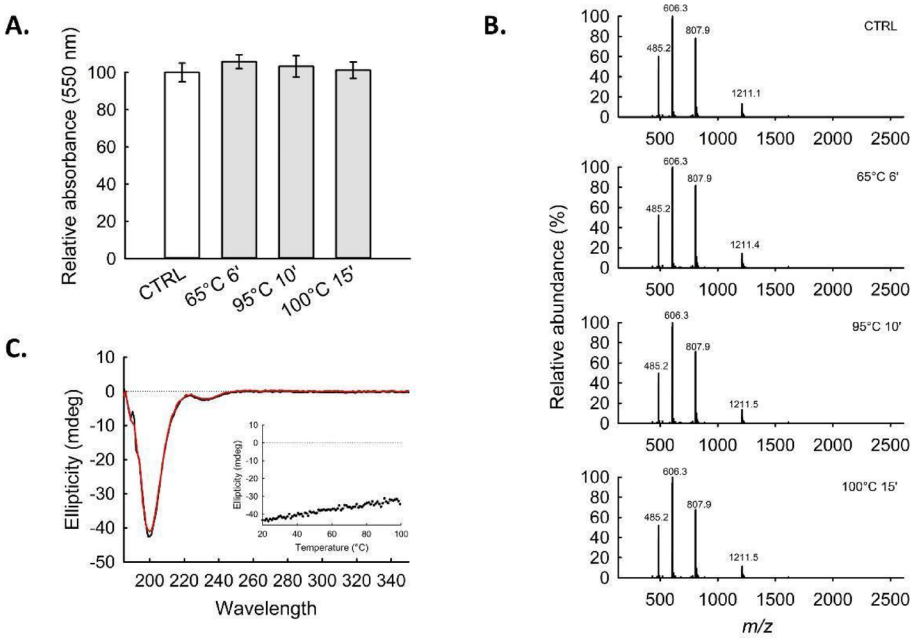


Fig. 1 **A** Bar plot of the Bradford assay after thermal treatment at 65 °C, 95 °C, and 100 °C for 6, 10, and 15 min. An untreated sample was used as a control and the measurements were conducted in triplicate. All samples were centrifuged after temperature treatment. **B** MS spectra of the peptide after thermal treatment compared with the untreated peptide. Ions were detected from $[M+H]^{+}$ to $[M+5\,H]^{5+}$. **C** CD spectra recorded before (black line) and after heating at 100 °C (red line). The insert shows the temperature ramp experiment between 20 and 100 °C monitored in circular dichroism at 200 nm

of several protonatable basic residues and suggests a conformation that remains sufficiently flexible to accommodate multiple positive charges. Thermal treatment of the peptide up to 100 °C had no impact on the ionization profile, indicating substantial conformational stability and excluding thermal-induced effects such as charge suppression or aggregation into insoluble forms. In all cases, no detectable differences were observed among the samples, effectively ruling out heat-induced chemical alterations. Notably, peptide heating has been associated with various chemical modifications, including deamidation (particularly of Asn and Gln), oxidation (affecting Met, Cys, Trp, and Tyr), and racemization of amino acid residues. Such modifications can significantly compromise peptide structure, stability, and biological activity.

Conformational stability

Circular dichroism (CD) spectra recorded before and after heating at 100 °C were virtually superimposable (Fig. 1C), indicating preserved secondary structure. Quantitative analysis using BestSel revealed that the peptide exhibited 30.0% antiparallel β-sheet, 19.7% turns, and 50.4% unordered content prior to heating, compared to 30.7% antiparallel β-sheet, 19.8% turns, and 49.5% unordered content post-heating at 100 °C. These results confirm that the peptide maintained its conformational integrity upon thermal treatment. Temperature ramp experiments (Fig. 1C, insert) did not reveal any distinct thermal transition, although a slight non-specific slope was observed. This further supports the conclusion that the peptide undergoes no significant conformational change upon heating.

Labeling and quality control results for DOTA-Siglec-9 precursor and the validated synthesis

The fully automated synthesis of [^{68}Ga]Ga-DOTA-Siglec-9 was performed using 40 µg of the peptide precursor DOTA-Siglec-9 for radiolabelling with gallium-68. Following each production run, comprehensive quality control (QC) analyses were carried out on the final product to assess and refine synthesis parameters. These analyses were essential for identifying the optimal reaction conditions to ensure consistent high RY, purity, and overall process robustness. The production process was validated using the same precursor amount (40 µg), in accordance with applicable regulatory standards. This validation was aimed at demonstrating the reproducibility and reliability of the [^{68}Ga]Ga-DOTA-Siglec-9 labelling process. Selected QC parameters were evaluated according to the specifications outlined in the European Pharmacopoeia monograph 11.0/0125 (Table 1). RCP was calculated as the percentage of the labelled compound relative to the total radioactivity (RCP = 100% – free gallium – gallium colloids). The presence of free gallium-68 was assessed by radio-UV-HPLC, while gallium colloids were analyzed by radio-TLC. Free [^{68}Ga]Ga^{3+} eluted at a mean retention time (Rt) of 1.433 min ± 0.5 (Fig. 2 A), whereas [^{68}Ga]Ga-DOTA-Siglec-9 was observed at Rt = 8.293 min (Fig. 2B), yielding a RCP exceeding 99%.

The reference solution of unlabelled DOTA-Siglec-9 exhibited a slightly shorter retention time (Rt = 7.877 min) compared to the radiolabelled compound [^{68}Ga]Ga-DOTA-Siglec-9, which eluted at Rt = 8.293 min, as shown in Fig. 2C. This shift in retention time is primarily attributed to differences in detector systems (UV–VIS for the reference compound and radioactivity detection for the radiolabelled product), as well as structural

Table 1 Summary data of three consecutive validation batches of [^{68}Ga]Ga-DOTA-Siglec-9 (40 µg)

Test	Batch 1	Batch 2	Batch 3	Acceptance criteria
Radiochemical purity (Radio-UV-HPLC)	99.48%	99.30%	99.40%	> 95%
Radiochemical purity (Radio-TLC)	100%	100%	100%	> 95%
pH	7	7	7	4–8.5
Radiochemical yield (n.d.c)	55.04%	56.01%	57.42%	> 40%
Radioactivity concentration	50.21	50.00	52.10	> 50 MBq/mL
Radioactivity	502	500	521	> 150 MBq
Volume	10	10	10	2–10 mL
Colour	Colourless	Colourless	Colourless	Colourless
Molar activity	23.15 GBq/µmol	18.18 GBq/µmol	19.45 GBq/µmol	1–60 GBq/µmol
Radionuclidic purity	100%	100%	100%	> 99.9%
Ge-68 breakthrough	0.00000034%	0.00000036%	0.00000037%	< 0.001%
EtOH amount	3.70%	3.68%	3.54%	< 10% (V/V) (< 2.5 g)
HEPES content	9.45 µg/mL	9.45 µg/mL	9.45 µg/mL	Less than 200 µg/V of HEPES in test solution
Endotoxins	< 17.5 IU/mL	< 17.5 IU/mL	< 17.5 IU/mL	< 17.5 IU/mL
Sterility test	Sterile	Sterile	Sterile	Sterile
Stability over 3 h (RCP%)	100%	100%	100%	> 95%

and charge-related changes resulting from gallium-68 complexation. Upon coordination with Ga^{3+}, the DOTA chelator undergoes a conformational and electronic modification, reducing the number of free carboxylic acid groups from three to none. This coordination decreases the overall hydrophilicity of the molecule and alters its interaction with the stationary phase, resulting in a longer retention time. Radio-TLC analysis confirmed the absence of gallium-68 colloidal species, with no radioactive signal observed at Rf = 0.2. The radiopharmaceutical product was clearly detected at Rf = 0.8, indicating successful radiolabelling and high chemical purity (Fig. 3).

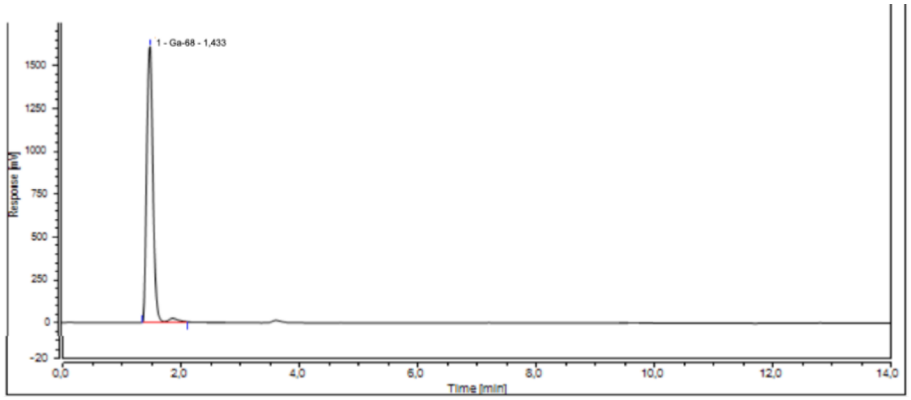
HPLC performed on the final radiopharmaceutical solution showed that the residual content of HEPES in the final preparation was lower in the HEPES test solution (12.5 µg/mL) (Fig. 4).

All product samples were tested for bacterial endotoxins, with concentrations consistently below 17.5 EU/mL, meeting acceptance criteria. Sterility testing confirmed that all samples were sterile. The radiochemical stability of [^{68}Ga]Ga-DOTA-Siglec-9 in buffer at room temperature was assessed over a 3-hour period using Radio-UV-HPLC, radio-TLC, and pH measurements. As illustrated in Fig. 5, [^{68}Ga]Ga-DOTA-Siglec-9 remained stable throughout the testing period. No radiolytic degradation products or free [^{68}Ga]Ga^{3+} were observed, and the radiochemical purity (RCP) consistently exceeded 99%.

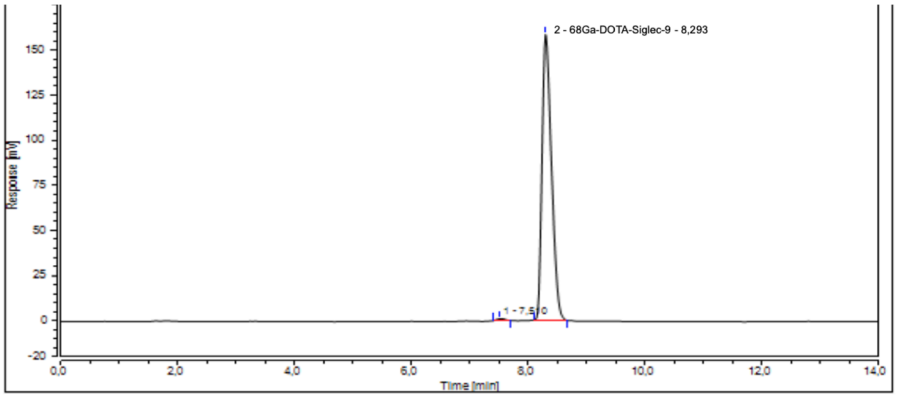
The RCP% was also assessed and confirmed by Radio-TLC (Fig. 6), while the pH value remained stable at 7 throughout the 3-hour period.

Discussion

Siglec-9, expressed on key innate immune cells, interacts with sialyted ligands and VAP-1 to regulate leukocyte trafficking in inflammation. Targeting this pathway with gallium-68-labeled peptide [^{68}Ga]Ga-DOTA-Siglec-9 offers a promising approach for PET imaging of inflammatory conditions. In this study, we developed and optimized an



A.



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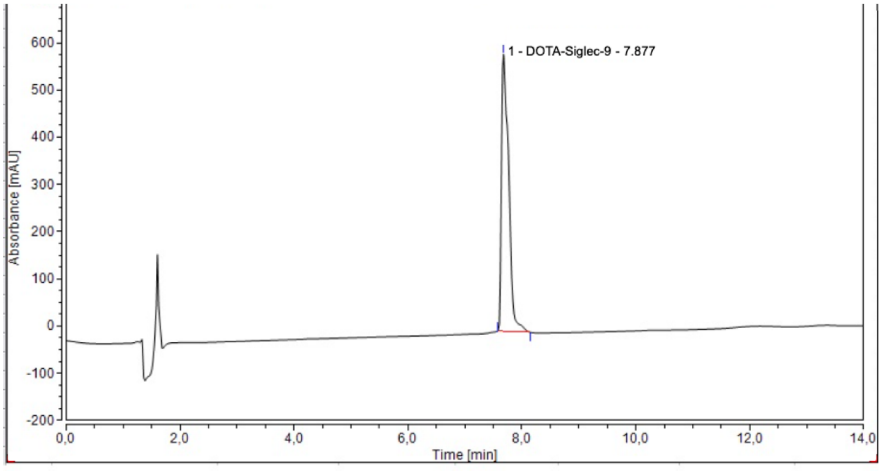

C.

Fig. 2 Radio-UV-HPLC chromatogram of the eluate [^{68}Ga]$GaCl_3$; **B.** Radio-UV-HPLC chromatogram of [^{68}Ga]Ga-DOTA-Siglec-9; **C.** Radio-UV-HPLC chromatogram of DOTA-Siglec-9

alternative automated radiolabelling protocol aimed at improving synthesis efficiency, reproducibility, and clinical applicability. Our approach also simplifies existing protocols by validating the use of a lower precursor mass (40 µg) and confirming the optimal labeling temperature (65 °C) (Käkelä et al. 2018; Jensen et al. 2017).

The development of a new radiopharmaceutical requires a robust synthesis protocol and thorough quality control. Preliminarily to radiosynthesis, we evaluated the stability

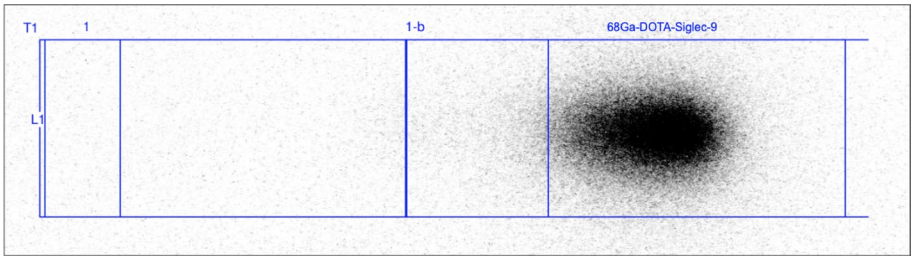

Fig. 3 Radio-TLC chromatogram of [^{68}Ga]Ga-DOTA-Siglec-9

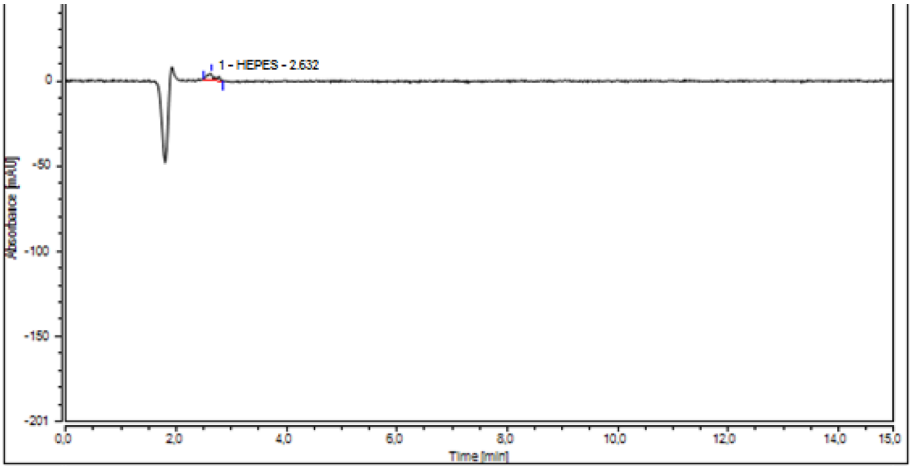

Fig. 4 HPLC chromatogram of HEPES content in [^{68}Ga]Ga-DOTA-Siglec-9

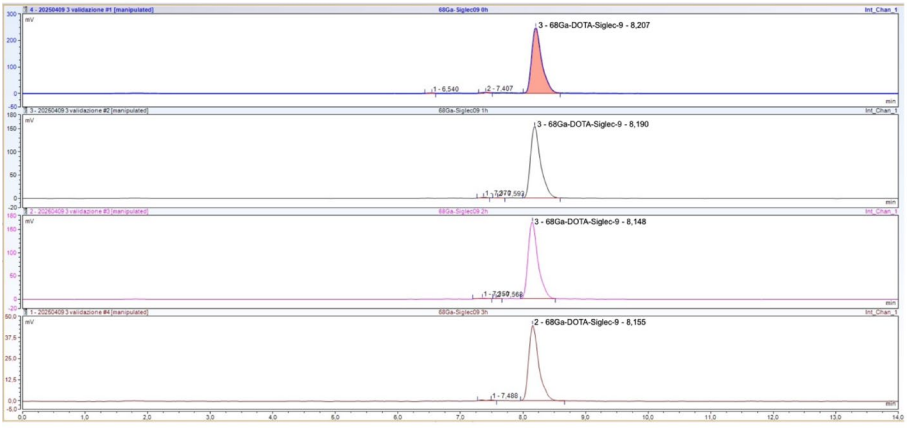

Fig. 5 Stability of [^{68}Ga]Ga-DOTA-Siglec-9 tested with Radio-UV-HPLC (blue dash: 0 h, grey dash: 1 h, pink dash: 2 h, red dash: 3 h)

of Siglec-9 in terms of solubility, chemical stability and conformational stability, at various temperatures, also following the synthesis protocols typically employed in our routine radiopharmaceutical production of [^{68}Ga]Ga-PSMA (European Pharmacopoeia Commission 2013), [^{68}Ga]Ga-DOTATATE (Sammartano et al. 2020), [^{68}Ga]Ga-Pentixafor (Migliari et al. 2021), [^{68}Ga]Ga-DOTA-ECL1i (Migliari et al. 2023)(95 °C for 10 min), [^{68}Ga]Ga-NODAGA-Exendin-4 (Migliari et al. 2022)(100 °C for 15 min) and the proposed method (Käkelä et al. 2018), reaching the conclusion that it's stable in all respects.

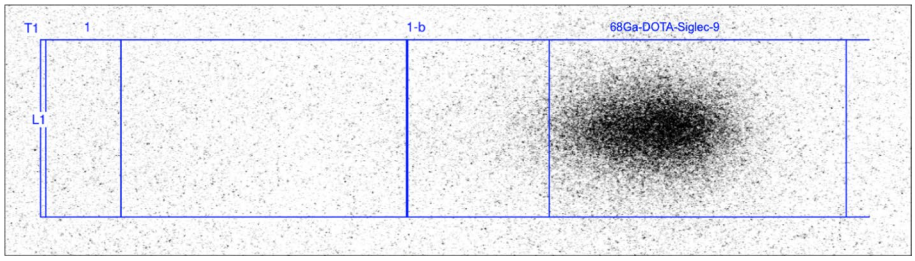

Fig. 6 Radio-TLC chromatogram of [^{68}Ga]Ga-DOTA-Siglec-9 over 3 h

The chemical and conformational stability of Siglec-9 at high temperatures contrasts with the reported instability of several therapeutic peptides, which are often prone to a variety of degradation pathways, including aggregation into amorphous or highly structured fibrillar species, chemical modifications such as deamidation and oxidation, and conformational changes induced by environmental factors such as heat, pH, peptide concentration, ionic strength, and interactions with surfaces or excipients (Zapadka et al. 2017). Despite the stability in a wide range of conditions, we opted for a temperature of 65 °C to yield a better control over the reaction kinetics. However, we showed that – if needed – labelling could be carried out at higher temperatures without compromising the peptide stability.

Upon radiosynthesis, other parameters were evaluated. Among the key ones, Am is particularly critical, as it influences receptor binding, tracer efficacy, and potential side effects. An optimal Am ensures minimal receptor saturation while maintaining sufficient peptide mass for efficient radiolabelling and reproducible biodistribution. In this work, we optimized the synthesis conditions to achieve a balanced Am, ensuring both high radiochemical yield and favorable in vivo imaging characteristics.

The decision to use 40 µg of DOTA-Siglec-9 for radiosynthesis was guided by a translational scale-down approach based on preclinical data in rats. This dosage corresponds to the extrapolated human equivalent dose derived from a safety study involving a structurally similar peptide DOTA-Siglec-9, administered intravenously to Sprague Dawley rats (Chrusciel et al. 2019). This strategy ensures a peptide mass that is appropriate for effective radiolabelling while minimizing the potential for pharmacological effects in humans.

The setup of the radiosynthesis process, as mass and labelling temperature, lead us to validate theentire radiopharmaceutical production process. In Table 1 the three consecutive validation syntheses and QC results, demonstrate that our synthesis protocol allows a high RCP (>99%), good RY (54.38%) (n.d.c.) as well as good Am (20.26 GBq/µmol). Moreover, we achieved an endotoxin-free, sterile and stable solution of [^{68}Ga]Ga-DOTA-Siglec-9 maintaining a RCP greater than 99% over a period of 3 h.

Although the literature commonly reports optimal temperatures between 80 and 95 °C for the complexation of ^{68}Ga with DOTA, due to the slow coordination kinetics of this macrocyclic chelator (Nelson et al. 2022; Tsionou et al. 2017), the use of a lower temperature can be advantageous under specific conditions. In particular, preliminary experimental data from this study demonstrate that efficient complexation of Ga-68 with the DOTA-conjugated peptide can still be achieved at 65 °C, without compromising the radiochemical yield. Furthermore, this milder thermal condition ensures better preservation of the peptide's conformational and chemical integrity, minimizing the risk

of heat-induced degradation such as denaturation, deamidation, or oxidation. Therefore, although slightly lower than the standard temperatures reported in the literature, the use of 65 °C represents an optimal compromise for this specific system, balancing labeling efficiency, radiocomplex stability, and structural preservation of the peptide.

The successful implementation of an automated and simple synthesis method is essential for ensuring that radiopharmaceutical research can be reliably tested and transferred across laboratories. The robustness and efficiency of the method make it suitable for routine radiopharmaceutical production, potentially facilitating broader preclinical and clinical use of [^{68}Ga]Ga-DOTA-Siglec-9 as an imaging biomarker for immune-mediated diseases. Moreover, the ability to achieve efficient complexation at a lower temperature, such as 65 °C, not only preserves the structural integrity of the peptide but also opens the possibility for the development of cold-kit formulations. Such kits would enable rapid, on-site radiolabeling without the need for complex instrumentation or high-temperature conditions, thus enhancing the practicality and accessibility of [^{68}Ga]Ga-DOTA-Siglec-9 for decentralized clinical applications.

Conclusions

The synthesis of [^{68}Ga]Ga-DOTA-Siglec-9 was successfully achieved using a fully automated protocol on a GRP Scintomics module. The final radiopharmaceutical met all quality control criteria in accordance with the European Pharmacopoeia, including RCP, pH, endotoxin levels, and sterility. Furthermore, the product demonstrated stability for at least 3 h post-synthesis, as verified by radio-UV-HPLC analysis. By enabling reliable and scalable production of [^{68}Ga]Ga-DOTA-Siglec-9, it opens new opportunities for non-invasive evaluation of inflammation and immune activation in various pathological conditions, including autoimmune diseases, chronic inflammation, and cancer. Future studies will be required to validate this tracer in larger cohorts and further explore its diagnostic and theranostic applications.

Abbreviations

Am	Molar activity
CD	Circular dichroism
GMP	Good manufacturing practice
GRP	Good radiopharmaceutical practice
Eur. Ph.	European Pharmacopeia
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HPLC	High-performance liquid chromatography
n.d.c.	Not decay corrected
TLC	Thin-layer chromatography
TFA	Trifluoroacetic acid
PET	Positron emission tomography
QC	Quality control
RCY	Radiochemical yield
RPC	Radiochemical purity

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Authors' contributions
SM, AG, AG, SB, RG, SB and LV have contributed to the organization of the content for this manuscript. SM, AG, AG, RG and SB collected relevant information and prepared the draft. SM, AG, AG, RG, SB, MS and GB drafted and SB, AR, SF and LV revised the manuscript.

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Declarations

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Not applicable.

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