

**Title: Specific imaging of bacterial infection:
A translational approach using positron emission tomography
and gallium-68-labeled maltohexaose**

Authors:

Sophie M. Wegener^{1*}, Desiree Weiberg¹, James T. Thackeray¹, Christoph P. Czerner¹,
Jasmin S. Hanke², Jan D. Schmitto², Geerd-Jürgen Meyer¹, Jens P. Bankstahl¹, Arjang
Ruhparwar², Frank M. Bengel¹ and Tobias L. Ross¹

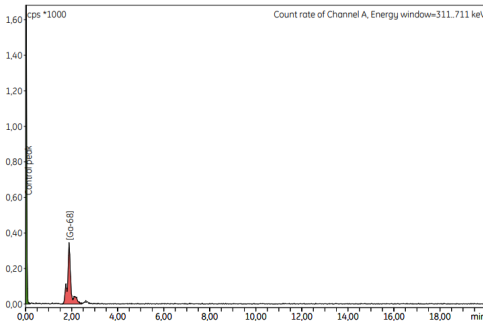
SUPPLEMENTARY FIGURES

Validation of the analytical method for quality control of [⁶⁸Ga]Ga-DMALTO via radio-HPLC

Radio-TLC was validated with radio-HPLC [NUCLEODUR NH2-RP HPLC column from Macherey-Nagel (Düren, Germany)] with a gradient of 0 – 10 min of 50 – 100% citrate buffer and 50 – 0% acetonitrile and 10 – 13 min 100% citrate buffer. The retention time of [⁶⁸Ga]Ga-DMALTO is 8.1 min and the retention time of free [⁶⁸Ga]Gallium is 1.9 min. The control peak at a retention time of 0.1 min is a reference peak, which comes from a first activity detector pass of the full sample without any retention on the HPLC column. The subsequent time course then reflects radioactivity after retention on the HPLC column. Due to retention and

dilution, the following peaks are not exactly identical to the early control peak, but the timing, height, and width convincingly show that no [⁶⁸Ga]Gallium-complexes remained on the HPLC column.

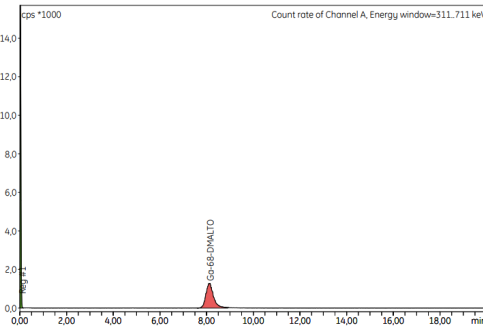
A



Integration Count rate of Channel A, Energy window=311..711 keV

| Substanz | R/T min | Typ | Fläche Counts | %Fläche % |
|--------------|---------|-------|---------------|-----------|
| Control peak | 0,08 | DD(M) | 4267,000 | 55,21 |
| [Ga-68] | 1,88 | DD(M) | 3461,000 | 44,79 |

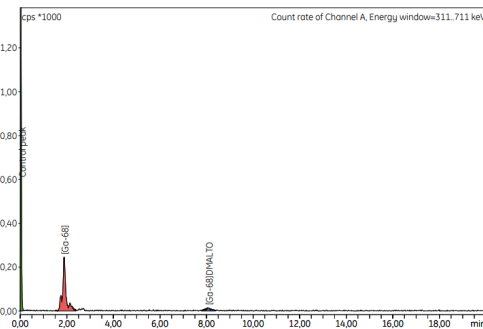
B



Integration Count rate of Channel A, Energy window=311..711 keV

| Substanz | R/T min | Typ | Fläche Counts | %Fläche % |
|--------------|---------|-------|---------------|-----------|
| Reg #1 | 0,07 | DD(M) | 37036,00 | 56,22 |
| Ga-68-DMALTO | 8,12 | DD(M) | 28842,00 | 43,78 |

C



Integration Count rate of Channel A, Energy window=311..711 keV

| Substanz | R/T min | Typ | Fläche Counts | %Fläche % |
|---------------|---------|-------|---------------|-----------|
| Control peak | 0,05 | DD(M) | 3439,000 | 55,24 |
| [Ga-68] | 1,88 | DD(M) | 2535,000 | 40,72 |
| [Ga-68]DMALTO | 8,08 | DD(M) | 251,000 | 4,03 |

Fig. S1. Radio-HPLC of [⁶⁸Ga]Ga-DMALTO

Comparison of μ PET/CT evaluation procedures

The evaluation of the activity in the affected muscles was defined manually via μ PET/CT. The PET scans were fused with the CT scans. A volume of interest (VOI) was first positioned via the CT image so that the VOI did not reach into the tibia or knee joint. Secondly, the VOI was adjusted via PET images around the fibula or above the fibula when the leg was significantly swollen. Because soft tissue in the hindlimbs is not well represented in CT scans and the mice did not lie at exactly the same position for each scan, it is not possible to match the total and only muscle mass with the positioned VOI. Furthermore, [^{18}F]FDG uptake in the bone marrow of the tibia and knee joints could interfere with the selected VOI. To judge these effects, two independent people analyzed the data. Each evaluator kept the size of the VOI constant for all mice and muscles. Comparison of the evaluation of both people is shown in Fig. S2. The results show that even with a VOI that does not match perfectly the muscle mass, the results of both evaluation processes show no significant difference. Consequently, the basic statement of all experiments is not influenced by the evaluation procedure or person.

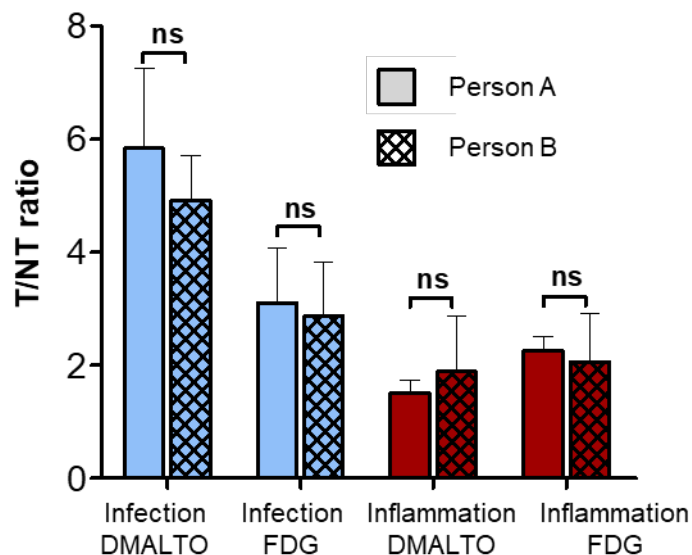


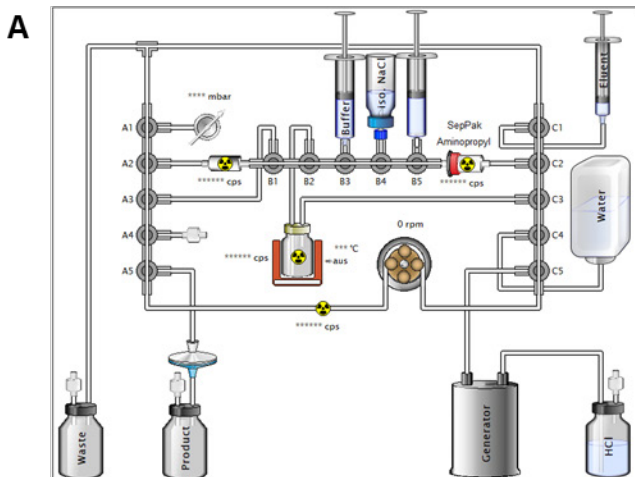
Fig. S2. Comparison of the evaluation procedures. Two independent people (A + B) evaluated for infection or inflammation with [^{68}Ga]Ga-DMALTO or [^{18}F]FDG in both hindlimbs of mice.

GMP-production of [^{68}Ga]Ga-DMALTO

Radiosynthesis was performed on a qualified GAIA GMP automated radiosynthesizer (Elysia-raytest, Straubenhardt, Germany). Operational qualification of the radiosynthesizer, including testing and calibration of the electrical heating and cooling module, radiation detectors, pumps, valve mechanisms, and reactor, is performed periodically.

The process validation of the labeling sequence in Figure S3 was done under GMP-conditions with a quality risk management approach. Briefly, 30 nmol of DMALTO precursor in 2.1 mL 3M sodium acetate buffer and 1 mL ethanol (95%) is transferred into the reaction vessel and warmed to 40 °C. [^{68}Ga]GaCl₃ in 1.1 mL 0.1N HCl is eluted from the generator directly into the

reaction vessel, and heated to 100 °C for 12 min. The reaction is quenched by addition of 5 – 8 mL of water. The reaction mixture is passed over a NH₂ Sep-Pak plus cartridge (Waters AG, Germany) to remove excess of free [⁶⁸Ga]GaCl₃. The solution is further transferred via a sterile filter into a final product vial.



B

| | A1 | A2 | A3 | A4 | A5 | B1 | B2 | B3 | B4 | B5 | C1 | C2 | C3 | C4 | C5 | Pump | Heater | Wait Condition | Wait Timeout | Measurement | Description (English) |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|------|----------------|---------------------|--------------|---|---|
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | off | off | | | | Initial State |
| 1 | 0 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | off | Fixed delay | 15 | | NH2 Purge |
| 2 | 1 | 2 | 2 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 3 | 2 | 0 | 0 | 0 | 250 | off | Pressure > 2000mbar | 180 | | Kit Integrity Test: Pressurizing |
| 3 | 1 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | off | off | Fixed delay | 5 | | Kit Integrity Test: Equilibration |
| 4 | 1 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | off | off | Fixed delay | 15 | Gaia-Pressure changes less than 200.0mbar | Kit Integrity Test: Measuring |
| 5 | 2 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | off | off | Fixed delay | 5 | | Kit Integrity Test: Venting |
| 6 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 100 | off | Fixed delay | 10 | | NaCl Syringe Preparation |
| 7 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 200 | off | Fixed delay | 15 | | Buffer Addition: Preparation |
| 8 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | off | off | Fixed delay | 2 | | Buffer Addition: Preparation Venting |
| 9 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | -250 | off | Fixed delay | 30 | | Buffer Addition |
| 10 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | off | off | Fixed delay | 3 | | Buffer Addition: Venting |
| 11 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | -150 | off | Fixed delay | 30 | | Cartridge Activation: SCX + NH2 |
| 12 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | -150 | off | Fixed delay | 10 | | Cartridge Activation: NH2 |
| 13 | 1 | 1 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 250 | off | Fixed delay | 5 | | Cartridge Activation: Purge Preparation |
| 14 | 1 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 250 | off | Fixed delay | 5 | | Cartridge Activation: Purge 1 |
| 15 | 0 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | 40 | Fixed delay | 10 | | Cartridge Activation: Purge 2 |
| 16 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | 40 | Fixed delay | 10 | | Cartridge Activation: Purge 3 |
| 17 | 0 | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | 40 | Fixed delay | 3 | | Cartridge Activation: Venting |
| 18 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 3 | -20 | 60 | Flow > 1000cps | 300 | | Generator Elution: Waste (1000 cps 300s) | |
| 19 | 0 | 0 | 3 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 3 | 0 | 3 | 3 | -20 | 90 | Fixed delay | 120 | | Generator Elution: Collect | |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 3 | -20 | 90 | Fixed delay | 10 | | Generator Elution: Waste | |
| 21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | 100 | Fixed delay | 10 | | SCX Washing: Line | |
| 22 | 0 | 3 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | 100 | 100 | Fixed delay | 10 | | SCX Washing |
| 23 | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | 100 | Fixed delay | 5 | | SCX Washing: Line Purge Preparation |
| 24 | 0 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | 100 | Fixed delay | 15 | | SCX Washing: Line Purge |
| 25 | 0 | 3 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 250 | 100 | Fixed delay | 10 | | SCX Washing: Cartridge Purge |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | -70 | 100 | Fixed delay | 12 | Gaia-Manifold Detector 1 | SCX Elution: Preparation |
| 27 | 0 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | -20 | 100 | Fixed delay | 15 | | SCX Elution 1 |
| 28 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | -20 | 100 | Fixed delay | 60 | | SCX Elution 2 |
| 29 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | -250 | 100 | Fixed delay | 10 | | SCX Elution: Purge |
| 30 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | 100 | Fixed delay | 10 | Gaia-Reactor Detector | Labeling / Measuring Reactor Activity |
| 31 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | 100 | Fixed delay | 240 | | Labeling Part 1 |
| 32 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | -40 | 100 | Fixed delay | 10 | | Labeling / Lift Purge |
| 33 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | 100 | Fixed delay | 340 | | Labeling Part 2 |
| 34 | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 | 150 | 40 | Fixed delay | 10 | | Preparation Trapping |
| 35 | 0 | 0 | 0 | 1 | 0 | 3 | 1 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 | 250 | 40 | Fixed delay | 30 | | Preparation Trapping 2 |
| 36 | 0 | 0 | 0 | 1 | 1 | 3 | 1 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | -80 | 40 | Fixed delay | 30 | | NH2 Trapping: Preparation |
| 37 | 0 | 0 | 0 | 1 | 1 | 3 | 1 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | -150 | 40 | Fixed delay | 10 | | NH2 Trapping |
| 38 | 0 | 1 | 1 | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 0 | 1 | 3 | 0 | 0 | -250 | 40 | Fixed delay | 8 | | Washing Reactor |
| 39 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 1 | 3 | 0 | 150 | 40 | Fixed delay | 5 | | NH2 Washing Preparation |
| 40 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 3 | 0 | 1 | 3 | 0 | 250 | 40 | Fixed delay | 30 | | NH2 Washing |
| 41 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | -100 | off | Fixed delay | 20 | | NH2 Preparation Elution |
| 42 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | -150 | off | Fixed delay | 20 | | NH2 Elution |
| 43 | 1 | 0 | 0 | 3 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | -150 | off | Fixed delay | 60 | | Formulation |
| 44 | 1 | 0 | 1 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | -200 | off | Fixed delay | 15 | Gaia-Manifold Detector 2 | Formulation: Purge / Measuring NH2 Post_Elution |
| 45 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | off | Fixed delay | 5 | Gaia-Manifold Detector 1 | Venting Preparation / Measuring SCX Post_Elution |
| 46 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | off | off | Fixed delay | 2 | Gaia-Reactor Detector | Measuring Reactor Activity |
| 47 | 1 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | off | off | User prompt | 1 | | Remove Product, Connect Waste |
| 48 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | -250 | off | Fixed delay | 30 | | Filter Integrity Test: Filter Purge |
| 49 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 250 | off | Fixed delay | 5 | | Filter Integrity Test: Line Purge Preparation |
| 50 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 250 | off | Fixed delay | 15 | | Filter Integrity Test: Line Purge 1 |
| 51 | 0 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 250 | off | Fixed delay | 15 | | Filter Integrity Test: Line Purge 2 |
| 52 | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | -250 | off | Fixed delay | 10 | | Filter Integrity Test: Line Purge 3 |
| 53 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | -250 | off | Pressure > 2500mbar | 120 | | Filter Integrity Test: Pressurizing |
| 54 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | -60 | off | Pressure > 2500mbar | 200 | | Filter Integrity Test: Approaching Bubble Point Value |
| 55 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | -30 | off | Sterile Filter Test | 600 | | Filter Integrity Test: Measuring Bubble Point Value |
| 56 | 2 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | off | off | Fixed delay | 5 | | Venting |
| 57 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | off | off | Stop Synthesis | 5 | | Stop |

Fig. S3. Specific operating parameters of the automated radiosynthesizer

Synthesis of the precursor DOTA-maltohexaose (DMALTO)

To obtain a new radiotracer which, on one hand targets bacteria, and on the other hand is detectable by PET, we used maltohexaose and coupled it to the bifunctional chelator DOTA to allow radiolabeling with [⁶⁸Ga]Gallium. The synthesis of precursor DOTA-maltohexaose (DMALTO) followed the scheme described in Figure S4. An alkyne-DOTA derivative (3.75 μmol, 1.4 equivalent) was conjugated to azide-functionalized maltohexaose (2.67 μmol, 1 equivalent) using Cu(I)-catalyzed alkyne-azide 1,3-dipolar (click) cycloaddition (CuAAC).

The catalytic amounts of Cu(I) typically used for CuAAC reactions could not be applied because the DOTA moiety preferentially complexed copper ions [Bock VD, Hiemstra H, van Maarseveen JH. CuI-Catalyzed Alkyne–Azide “Click” Cycloadditions from a Mechanistic and Synthetic Perspective. *Eur J Org Chem.* 2006; 2006(1): 51–68; Struthers H, Mindt TL, Schibli R. Metal chelating systems synthesized using the copper(I) catalyzed azide-alkyne cycloaddition. *Dalton transactions (Cambridge, England: 2003).* 2010; 39(3): 675–696; Meldal M, Tornøe CW. Cu-Catalyzed Azide - Alkyne Cycloaddition. *Chem Rev.* 2008; 108: 2952–3015]. As a result, catalytic quantities of Cu(I) were sequestered by DOTA and were unavailable for the cycloaddition reaction. Only upon addition of a stoichiometric excess of copper (2.8 equivalents) was a rapid and quantitative reaction achieved. Subsequently, copper complexed to the DOTA moiety was removed by precipitation using a 31-fold molar excess of sodium sulfide, enabling quantitative copper removal (CuS solubility product: $K_{sp} = 6 \cdot 10^{37}$). In a third step, acid-mediated deprotection was performed to obtain the final precursor, DMALTO. Following purification, DMALTO was analyzed by electrospray ionization mass spectrometry (ESI-MS),

which confirmed the absence of copper–DMALTO complexes. This is a critical prerequisite for efficient [⁶⁸Ga]Gallium radiolabeling and for meeting precursor purity requirements under GMP conditions.

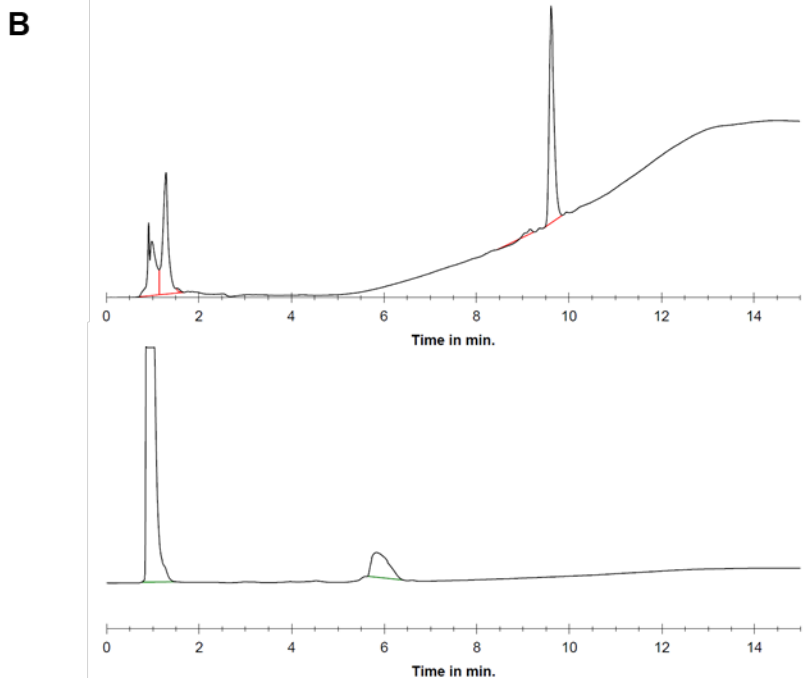
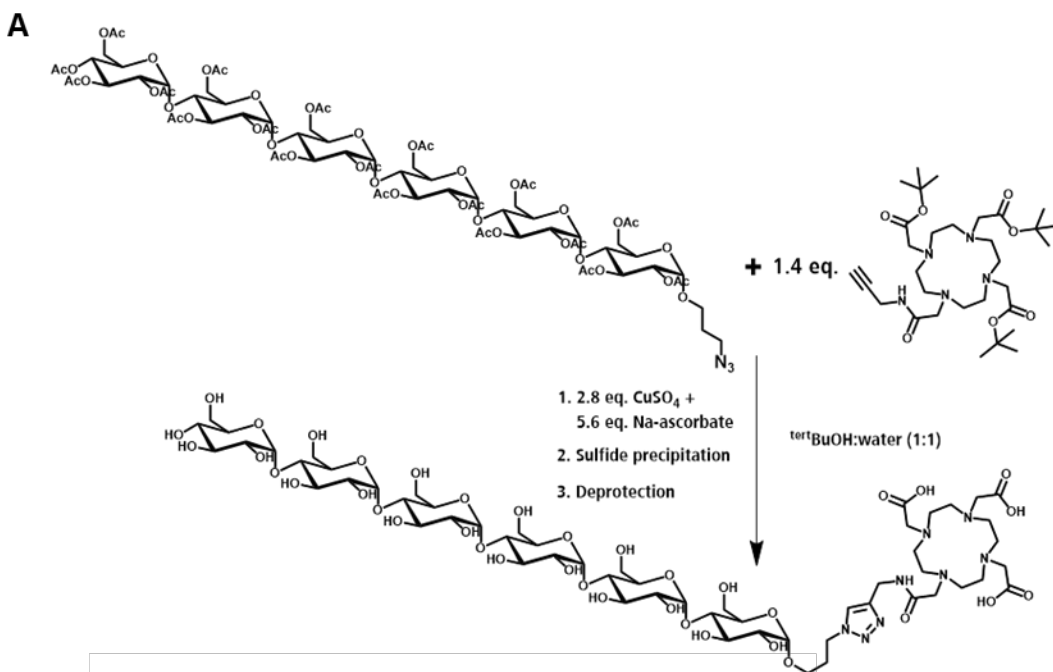


Fig. S4. Synthesis of precursor DMALTO. (A) Synthesis scheme of DMALTO. (B) Analytical HPLC chromatogram at the beginning (top) and end (bottom) of reaction: quantitative conversion of maltohexaose (9.5 min).

Imaging aseptic inflammation with [¹⁸F]FDG over time

To the best of our knowledge, the time point when inflammation peaks after LPS injection into the hindlimb muscle has not been described. Therefore, we chose to investigate inflammation using [¹⁸F]FDG PET imaging. We evaluated the uptake of [¹⁸F]FDG over time between two hours and seven days after injection of LPS. Fig. S5 indicates that the inflammation peaked at day 3.

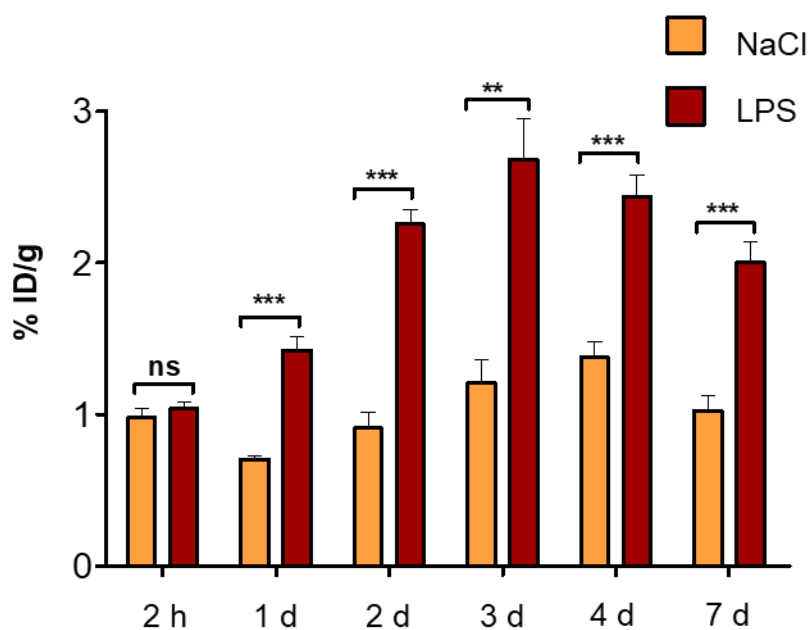


Fig. S5. Aseptic inflammation over time. Relative activity accumulations in %ID/g unit of [¹⁸F]FDG at different time points (2h – 7d).