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Long-circulating non-toxic blood pool imaging agent based on hyperbranched polyglycerols

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ABSTRACT

Purpose: Currently, *in vivo* or *in vitro* ^{99m}Tc-radiolabelled red blood cells are the standard blood pool imaging agents. Due to risks associated with handling of blood and the problems with the current ^{99m}Tc shortage, we were interested in a long-circulating biocompatible synthetic macromolecule that would be simple to prepare and could also be used for PET imaging.
Methods: A high molecular weight hyperbranched polyglycerol (HPG) of 500 kDa was derivatized to coordinate radioactive gallium and to establish its labelling efficiency, stability and pharmacokinetics.
Results: The resulting radiopharmaceutical in kit form was labelled rapidly within a couple of minutes at room temperature, was stable in transferrin and EDTA challenge tests, and was non-toxic in both cell viability and different hemocompatibility assays. A pharmacokinetic biodistribution study showed that the ^{67}Ga–HPGN was confined to the blood compartment with a biological half life of 50.7 h.
Conclusion: ^{67}Ga–HPGN is thus a simple to prepare blood pool imaging agent for applications where a long biological half-life is essential, i.e., the diagnosis of internal bleeding. Since radiolabelling of the same kit with ^{68}Ga was also confirmed, we plan to evaluate it shortly as a PET blood pool imaging agent for cardiac applications.

1. Introduction

Cardiac blood pool imaging is a technique used in Nuclear Medicine to evaluate cardiac contractile function with the help of radioactive probes. More specifically, cardiac blood pool imaging is used for the evaluation of ventricular wall motion, ejection fraction, and other parameters of systolic and diastolic function at rest and/or during exercise stress or pharmacologic intervention. The same technique is also employed in gastrointestinal blood loss and hemangioma studies. The radioactive probe for blood pool imaging consists normally of the patient's own red blood cells (RBC) labelled with the gamma emitter ^{99m}Tc (^{99m}Tc-RBC), whose radioactive signal is detected by planar equilibrium radionuclide angiography (ERNA) or single photon emission computed tomography (SPECT).

Disadvantages of this method have to do with the red blood cell labelling. The labelling involves the handling of blood with all its associated risks (e.g., possible contact with infectious agents such as hepatitis C and HIV), it is sensitive to the amounts of tin used for the reduction of the ^{99m}Tc (Greenberg et al., 1977), it is associated with inconvenient waiting periods between the labelling steps and can thus not be prepared in advance, and finally, it is not just a single protocol but consists of 3 methods from which the physician has to choose one. The reason for the use of 3 protocols is that the substances used in RBC labelling potentially interact with other substances used in the treatment of patients (e.g., heparin (Wilson and Hung, 1992), citrate dextrose solutions (Wilson and Hung, 1992), catheter materials) and also contrast agents (Finkel et al., 1988; Parker et al., 1983), immunosuppressants (Reisdorff et al., 1992), and especially chemotherapeutic drugs (Ballinger et al., 1988; Lee et al., 1983), leading to lower than optimal labelling efficiencies (duCret et al., 1988). Lower labelling efficiencies in turn can lead to a large uptake of activity in non-target areas such as the stomach lining or thyroid, which may result in suboptimal imaging quality that can alter the results of ejection fraction calculation. Inaccurate ejection fraction calculations are particularly common

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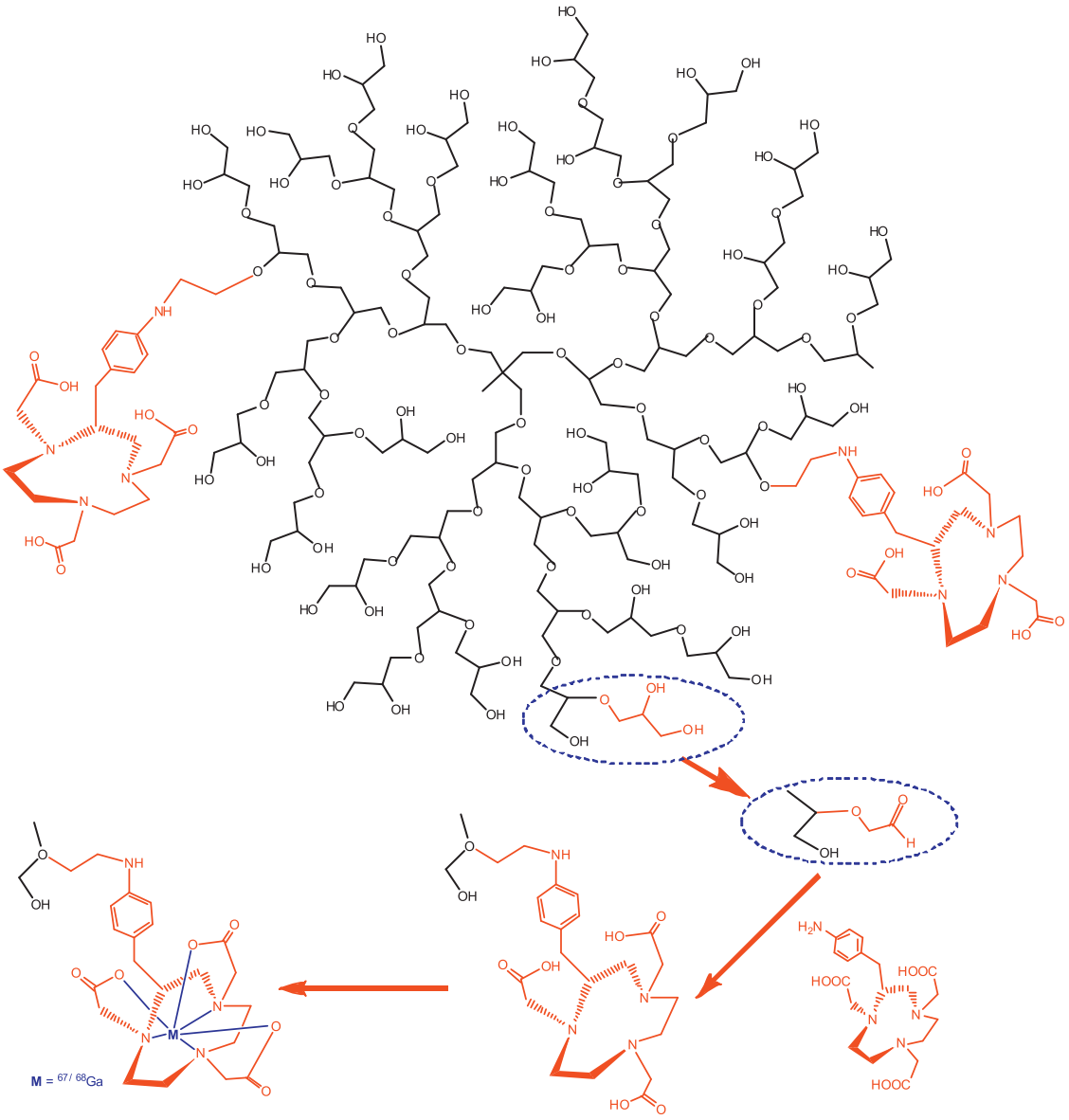

Fig. 1. Synthesis and radiolabelling scheme for ^{67}Ga/^{68}Ga–HPGN.

when assessing myocardial function in patients who have recently received chemotherapy and can lead to inappropriate administration or withholding of potentially cardiotoxic chemotherapy drugs to cancer patients.

To stay away from these disadvantages we decided to investigate a long-circulating macromolecule, hyperbranched polyglycerol (HPG), for its usefulness as a blood pool agent. HPGs with narrow size distribution and sizes between 1.8 and 30 kDa were originally developed by Frey et al. (Frey and Haag, 2002; Sunder et al., 1999). The larger molecular weight HPGs with similarly narrow size distribution became then available in 2006, when Kainthan et al. developed a simple one-pot method (Kainthan et al., 2006b). HPGs have a large number of derivatizable hydroxyl groups (Fig. 1) and are thus highly water soluble, have a low intrinsic viscosity, and are highly biocompatible. Using several *in vitro* techniques and animal toxicity studies, HPG were found to be as biocompatible as polyethylene glycol (PEG) therefore making them potentially useful for drug delivery applications (Kainthan and Brooks, 2007; Kainthan et al., 2007). No signs of drug related toxicity were observed even when the HPG were administered to mice in doses as high as 1 g/kg. Furthermore, the plasma half-life was found to be 30 h for a HPG with a 100 kDa molecular weight, or about 52 h with a 500 kDa molecular weight.

To prepare a blood pool imaging agent, a radioisotope must be bound to the HPG with good stability. In this work, we decided to replace the gamma emitter ^{99m}Tc with current supply issues (Guerin et al., 2010) with the positron emitting isotope ^{68}Ga (Bandoli et al., 2009). The PET isotope ^{68}Ga has similar patient dose and imaging properties as ^{99m}Tc and can be produced from its parent isotope ^{68}Ge in an on-site ^{68}Ge/^{68}Ga-generator (Fani et al., 2008; Zhernosekov et al., 2007). Cyclotron produced by a ^{69}Ga(p,2n)^{68}Ge reaction, ^{68}Ge has a half-life of 270.8 days, which allows it to be easily shipped over long distances and obviates the need for an on-site cyclotron at hospitals and clinics (Naidoo et al., 2002). Its long half-life also means that these generators can be used for about a year, thus rendering them very cost effective. The daughter nuclide ^{68}Ga which is eluted from these generators has a half-life of 68 min with a high positron yield of 89%, properties which make it highly useful for PET imaging.

To modify HPG for Ga coordination a suitable hexadentate ligand needs to be attached to the macromolecule. Appropriate chelators for bioconjugation with gallium are azomacrocycles which form highly stable compounds and are easily available commercially. For gallium, the ligand NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) has been found to contain the proper sized cavity (Moore et al., 1990) leading to extremely strong N_3O_3 coordination with a stability constant log *K* of 31.9 (Clarke and Martell, 1991; Sun et al., 1996). Labelling with ^{68}Ga is quick and easy at ambient temperature as reported by several authors (Ferreira et al., 2010; Velikyan et al., 2008).

Herein we report the modification of a 500 kDa HPG for fast and stable Ga coordination. The radiolabelling optimization and the relevant *in vitro* toxicity and blood compatibility results are reported. The *in vivo* biodistribution, stability and pharmacokinetics are also discussed and have been determined with ^{67}Ga as a long-lived stand in for ^{68}Ga.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich. Both $^{67}GaCl_3$ and $^{68}GaCl_3$ was purchased from MDS Nordion (Ottawa, ON, Canada). *p*-NH_2–Bn–NOTA was purchased from Macrocyclics (Dallas, TX, USA). HPG (~500 kDa) was synthesized according to published procedures in the Brooks laboratory (Kainthan et al., 2006b). H NMR spectra were recorded on a Bruker AV-300 or AV-400 at 300.13 or 400.13 MHz using deuterated solvents (99.8% D; Cambridge Isotope Laboratories, Andover, MA, USA) with the solvent peak as a reference. ICP analyses were submitted to Exova (Surrey, BC, Canada). PD10 columns (Sephadex G-25M) were from GE Healthcare, Piscataway, NJ, USA. Instant thin layer chromatography (ITLC) was performed on green TEC-Control strips (Cat# 150-771; Biodex, Shirley, NY, USA) using a 33.3% mixture of saline, HCl (0.1 M) and Na_2EDTA (0.1 M) as the mobile phase. Dialysis tubing was from Spectrum Laboratories (Rancho Dominguez, CA, USA) with a molecular weight cut off (MWCO) of 1000. The radioactive TLCs were made visible by phosphor imaging (Cyclone storage phosphor imager with 20 cm × 25 cm phosphor screen, Perkin Elmer, Waltham, MA, USA) and analyzed using OptiQuest software. Ultracel-YM100 microconcentrators were purchased from Millipore Corporation (Billerica, MA, USA). All experiments were repeated at least three times. Fresh human blood was obtained from volunteers on an ethics approved protocol.

2.2. Synthesis of chelating HPG ($HPGN_{50}$)

HPG (500 mg, 1 μmol) was dissolved in 10 mL of water. A solution of H_5IO_6 (27 mg, 100 μmol) in 1.5 mL water was added drop wise under stirring. After 1 h the solution was dialyzed against water (2.5 L, 1000 MWCO) over night and water changed once. *p*-NH_2–Bn–NOTA (35 mg, 50 μmol) was dissolved in 1 mL water and added to the reaction mixture and the solution stirred for 1 h. Ethanolamine (60 μL, 10% v in water) was added and solution stirred for another 1 h. $NaBH_3CN$ (15 mg, 238 μmol) was dissolved in 200 μL water, added and stirred for >6 h. The reaction mixture was dialyzed for 5 days against water (2.5 L) with daily changes for fresh water and then lyophilized. For $HPGN_{200}$, the stoichiometry was modified to get a HPG:NOTA ratio of 1:200 following the same synthetic procedure. HPGN used in this work contained 50 chelating groups per HPG (=$HPGN_{50}$), with the exception of some of the toxicity tests, where we also analyzed the response to $HPGN_{200}$. ^{1}H NMR (D_2O, 300 MHz): δ 2.5–4.5 (m); 7.2–7.6 (m).

2.3. Synthesis of Ga–HPGN

A 0.2 mM solution of HPGN (50 μL) was added to 0.15 M NH_4OAc (100 μL) with mixing, followed by 5 μL of $GaNO_3 \cdot 6H_2O$ (9 mg/90 μL H_2O). After 10 min shaking at 1000 rpm at room temperature Ga-HPGN was purified by either dialysis (1000 MWCO) or using a microfilter (100,000 MWCO) or by simple precipitation using acetone. ICP analysis confirmed coordination of Ga (calculated 0.19 μg/mL, found 0.20 μg/mL).

2.4. Radiolabelling of HPGN with $^{67/68}$Ga

In a first method, 5 μL of an aqueous solution of HPGN (5 mg/50 μL) was added to 1.2 MBq of $^{67}GaCl_3$. After 10 min shaking at room temperature 450 μL of 0.1 M Na_2EDTA was added to coordinate excess $^{67}Ga^{3+}$ and the solution eluted through a PD10 column with saline (for ^{68}Ga labelling the HPGN was added to NH_4OAc (0.15 M) solution before adding the activity). Aliquots (30× 500 μL) were collected and their activity measured using a gamma-counter. Instant thin layer chromatography (ITLC) was also performed with 100 μL of a 33.3% mixture of saline, HCl (0.1 M), Na_2EDTA (0.1 M) as the mobile phase. In this ITLC system, ^{67}Ga–EDTA moves to $R_f = 1$ and ^{67}Ga–HPGN to $R_f = 0$. ITLC was performed on the original solution and developed using a phosphor imager, followed by cutting the ITLCs in the middle and counting them in a gamma-counter. In a second method, PBS or saline was added to the reaction vial as solvents after mixing HPGN and Ga to a total volume of 500 μL (i.e., no EDTA was added to the reaction mixture before running the PD10 column).

2.5. Stability tests with ^{67}Ga–HPGN

Two different methods were used. *EDTA challenge*: To a 0.2 mM solution of HPGN (5 mg/50 μL) was added $^{67}GaCl_3$ (5 μL, 1.2 MBq). After 10 min shaking at room temperature Na_2EDTA (450 μL, 0.1 M) was added and the solution was heated at 37 °C while shaking. At 2 and 24 h post-mixing, 250 μL samples of this solution eluted through a PD10 column as described in 2.4. *Transferrin challenge*: HPGN (1 mg/10 μL H_2O (0.2 mM)) was mixed with 15 μL $^{67}GaCl_3$ (40.7 MBq) and 85 μL of water was added with mixing for 10 min at room temperature. To 50 μL of this solution was added 950 μL of apotransferrin solution in PBS (2.5 mg/mL). The solution was shaken on an Eppendorf thermomixer at 1000 rpm and 37 °C. After 1 and 24 h, 500 μL of the solution was eluted through a microconcentrator (100,000 MWCO) and the activity of both the supernatant and the Ga–HPGN was measured.

2.6. Cell viability assay (MTT assay)

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) (Pieters et al., 1989), a quantitative analysis of cell viability was performed with HPG and HPGN in human vascular endothelial cells (HUVECs). For this assay, 100 μL of the cells (5000 cells/mL) were plated into 96-well plates and incubated for 2 days. The test materials at a concentration of 10, 2, 1, 0.5, 0.1 and 0 mg/mL were added in 100 μL of medium to each well and incubated for 1 day. Twenty microliters of the MTT stock solution (5 mg/mL) was added to each well, incubated for 3 h, the supernatant gently removed and 150 μL DMSO added to every well. The plate was shaken at 1000 rpm and at 37 °C for 1 h and the optical density measured at 540 nm on a Multiscan Ascent plate reader.

2.7. RBC aggregation test

Blood was incubated with HPGN, HPG and Ga–HPGN to test their influence on red blood cell aggregation. For this purpose, 10 μL of

the polyglycerol solutions (10 mg/mL) were added to 40 μL citrate anticoagulated whole blood and incubated at 37 °C for 30 min. The RBC were then isolated by spinning down the blood for 30 s. About 4 μL of plasma was resuspended and diluted such that single RBC or aggregations were well visible under the bright field light microscope (Zeiss Axioskop 2plus). A drop was placed on a wet mounted slide and images were taken with a mounted black and white CCD camera (Qimaging Retiga 1300).

2.8. Clinical coagulation assays

Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured to evaluate the influence of the HPG solutions on extrinsic and common coagulation pathway of blood. For both assays, an ST4 Diagnostica Stage device was used. A polymer solution (1 mg/mL) was prepared in platelet poor plasma (obtained by spinning down whole blood at 1500 × *g* for 15 min). Three times 100 μL of it was heated to 37 °C and 200 μL of innovin (for the PT assay) or 100 μL of actin incubated with platelet poor plasma and 100 μL of calcium chloride (for the APTT assay) was added and the time to fibrin clot formation recorded.

2.9. Thrombelastograph

The platelet function and mechanical clot stability was analyzed in a thrombelastograph (TEG) haemostasis system 5000 (Haemoscope Corp, Niles, IL, USA). The TEG measures the physical properties of the clot in an oscillating cylindrical cup holding the blood sample, at an angle of 4°45′. Each of the rotational cycles lasted 10 s and the properties were measured by a stationary pin. Fresh citrated blood (360 μL) was mixed with 40 μL of the polymer test solutions (1 mg/mL) and 20 μL of 0.2 M $CaCl_2$ added in the thrombelastograph and the resulting curves analyzed by TEG's proprietary software.

2.10. Complement activation

The complement activation pathway of the body upon exposure to the polymers was investigated using the Quidel C3a enzyme immunoassay kit (Alberts et al., 2008). For this purpose, samples (1 mg/mL) and controls were diluted 1:100 with sample buffer and added to a 96 microassay plate coated with C3a antibody coated strips. After 60 min of incubation at room temperature, 100 μL of C3a conjugate was added into each well followed another 60 min later by adding the substrate and incubating for another 15 min. The reaction was then stopped by the addition of 100 μL of 1 M HCl solution and read at 450 and 650 nm on a plate reader.

2.11. Platelet activation

Platelet activation, which is the tethering of platelets occurring on sites of vascular injuries, was investigated by measuring the CD62 expression level in the blood by marking it with CD62 mouse human antibodies (Patrono and Davi, 2008). Fresh donated citrate anti-coagulated blood was centrifuged at 800 × *g* (900 rpm) for 30 s to separate the red blood cells from the plasma without spinning down the platelets to get platelet rich plasma (PRP). PRP (150 μL) was then incubated at 37 °C with 15 μL of the polymer solutions to obtain a final concentration of 1 mg/mL. Aliquots were withdrawn after 10, 30 and 60 min, 5 μL of it were added to 45 μL of PBS and 5 μL of CD62 mouse human antibodies and analyzed in the flow cytometer. As a positive control, 45 μL of PBS was mixed with 5 μL of PRP, 5 μL of CD62 mouse human antibodies and 5 μL of Gly-Pro-Arg-Pro (GPRP). To prove that platelets were existent in the PRP, CD42 antibodies were added instead of CD62.

2.12. Pharmacokinetics and biodistribution study

The use of animals for this study was approved by the University of British Columbia's Animal Care Committee and all experimental protocols conformed to the Canadian Council on Animal Care guidelines. Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 350–400 g were kept under a 12 h light/dark cycle with free access to water and food (regular rat chow).

To enable blood sampling the right external jugular vein was cannulated on the day before the PK study with a two-part catheter consisting of PE-50 connected to silastic tubing. The surgery was carried out under inhalational isoflurane anesthesia. The cannula was exteriorized at the dorsal part of the neck. Following the surgery animals were housed individually and allowed to recover for 24 h. The next morning ^{67}Ga–HPGN (1.20 ± 0.05 MBq of ^{67}Ga bound to 1 mg of HPGN in phosphate buffered saline (PBS pH 7.4) was administered intravenously via jugular vein cannula. Systemic blood (0.25 mL) was sampled 5 min pre-dose and 5, 15, and 30 min post-dose, and then 1, 2, 4, 8, 24, 48 and 72 h post-dose. After each sampling, the catheter was flushed and the withdrawn blood samples with equal volumes of heparinized saline (100 IU/mL).

The blood was also collected 24 h before sacrificing the animals (0.25 mL, 144 h after administration) and immediately before the sacrifice for biodistribution and separated into plasma for lipoprotein distribution purposes (terminal blood collection as completely as possible before tissue collection from vena cava under isoflurane anesthesia, 10–12 mL, 168 h post-dose). For the biodistribution study, whole blood, heart, liver, right kidney, lungs, brain, spleen and quadriceps muscle were harvested, weighed and their activity determined using a Packard Cobra II auto gamma-counter. Results were expressed as the percentage of the injected dose per organ and per gram of tissue (%ID/g), and the organ-to-blood ratios calculated from the %ID/g values.

WinNonlin 5.0.1 Professional (Pharsight, Sunnyvale, CA, USA) was used for pharmacokinetic analysis of the data. The compartmental analysis was applied to fit the intravenous bolus data of 72 h after the administration of ^{67}Ga–HPGN. The 144 h and 168 h (terminal) blood collections were not used in PK modeling due to the large gap between the 72 h and later time points.

2.13. Interaction of HPGN with plasma components

From 4 animals, the plasma taken at the terminal endpoint was separated by step gradient density ultracentrifugation into the high density lipoprotein (HDL), the low density lipoprotein (LDL), the very low density lipoprotein (VLDL) and the lipoprotein deficient plasma (LPDP) fractions which contains albumin and alpha-1-glycoprotein, according to a previously reported method (Cassidy et al., 1998). In short, 3 mL of the plasma were placed in an ultracentrifugation vial and 2.8 mL density solutions of 1.21, 1.063 and 1.006 g/mL layered on top. The vials were centrifuged at 40,000 rpm (∼285,000 × *g*) for 18 h at 15 °C in a Beckman L8-80M Ultracentrifuge using the SW 41 Ti swinging bucket rotor (Beckman Instruments Inc.) and the fractions then analyzed in a gamma counter for activity distribution.

3. Results and discussion

For a radiopharmaceutical to be clinically useful, it has to be highly stable *in vivo* and should at the same time be easy and quick to prepare. Simple kits that require the addition of the radioactivity and can then be directly injected without further purification are thus ideal. In this investigation, we prepared and tested such

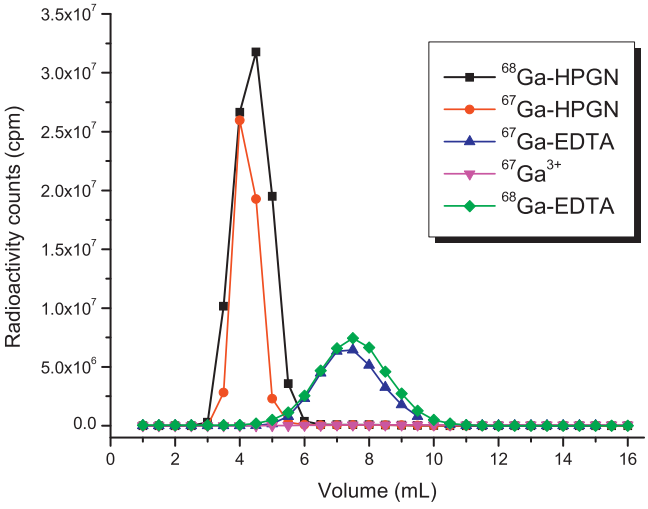

Fig. 2. Labelling efficiency of ^{67}Ga–HPGN and ^{68}Ga–HPGN measured on a size exclusion column.

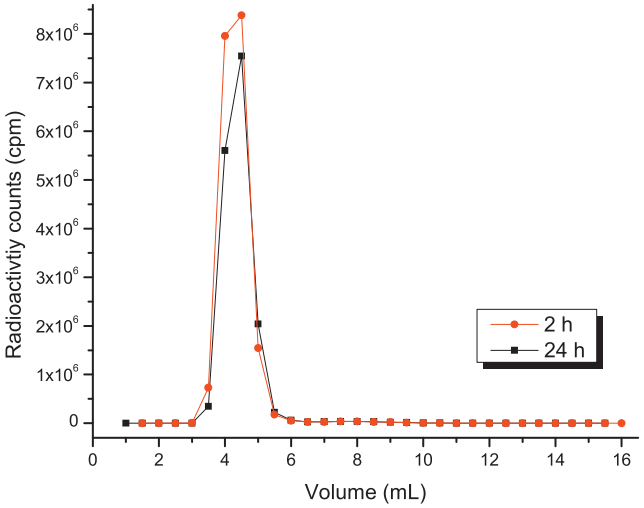

Fig. 3. Stability challenge of ^{67}Ga–HPGN in 0.1 M EDTA as measured by size exclusion over 24 h.

a kit containing a long circulating gallium-binding polyglycerol for cardiac blood pool imaging.

3.1. Radiolabelling of HPG and its radiochemical stability

NOTA was chosen as the ligand of choice for HPG modification due to its established fast and extremely stable Ga coordination that is well suited for biological applications and *in vivo* applications (Boros et al., 2010). The cavity of this triazocycle is perfectly sized for Ga binding and forms a neutral gallium complex with its three side-arm carboxylates. NOTA is reported to have a very wide range of kinetic and thermodynamic stability towards acid- and base-catalysed hydrolysis (Prata et al., 2000). The Ga-NOTA metal complex has been reported to survive a pH range of 0.7–12 and is thus stable under all physiological conditions.

For our investigation we modified a high molecular weight HPG (500 kDa) with NOTA in various ratios (50–200). While the toxicity of the $HPGN_{200}$ was tested, all other radiolabelling and biodistribution studies reported here were done with the $HPGN_{50}$. The synthesis of NOTA-modified HPG (HPGN) started by a periodate oxidation of diols on the original HPG, followed by further modification of the formed aldehyde functionality with *p*-NH_2–Bn–NOTA using reductive amination (Fig. 1). The appearance of ^{1}H NMR signals of aromatic protons from the benzyl backbone of NOTA in the formed HPGN was indicative of a successful modification.

Metal coordination was confirmed first using cold gallium. An excess of metal solution (Ga^{3+}) was added to coordinate all metal binding sites. By inductive coupled plasma (ICP) analysis, a Ga concentration of 0.20 μg/mL was found (calculated 0.19 μg/mL), confirming that all NOTA groups had been saturation coordinated with Ga.

Radiolabelling of HPGN with ^{67}Ga carried out at different concentrations (1.11–111 MBq/mg HPGN) and different incubation times (up to 60 min), but always at room temperature showed a labelling efficiency always exceeding 97%. Fig. 2 shows a typical PD10 size exclusion profile of the radiolabelled HPG. Almost all activity elutes in the first radioactive fraction around 4 mL, while only traces of activity are visible at 7.5 mL where Ga-EDTA would elute. The reaction kinetics was investigated by adding ^{67}Ga (48.1 MBq) to HPGN (1 mg) in 500 μL of saline and taking aliquots (1 μL) from the mixture at various time points (0, 30, 60, 90, 120, 180, 240 and 360 s post-mixing) and running an ITLC. From the 60 s sample on, labelling efficiency always exceeded 98%.

The stability of Ga–HPGN for *in vivo* use was predicted by challenging the bound Ga with the competitive ligands EDTA (Fig. 3) and the physiologically present transferrin. Transferrin is a blood plasma protein with suitable binding sites for Fe^{3+} coordination and is physiologically responsive for iron transport. Since Ga^{3+} is the same size and charge as Fe^{3+} (0.620 Å *vs* 0.645 Å), it has a high affinity for binding to transferrin which usually contains about 33% vacant binding sites. Transferrin is referred to as the metal scavenger in the blood and its high concentration (0.25 g/100 mL) favours *in vivo* exchange, so it is very important to avoid trans-chelation of any radioisotope to transferrin. Both EDTA and transferrin bind Ga^{3+} with similar stability (Boros et al., 2010). At pH 7.4 and 25 °C, their log *K* for Ga is 21.7 and 20.3 (p*M* of 18.3 and 21.3), respectively. Despite these high binding constants, only very little ^{67}Ga was transchelated from the ^{67}Ga–HPGN, something that was expected due to NOTA's high log *K* of 30.98 (and p*M* of 27.9) (Boros et al., 2010). The transferrin challenge resulted in 99.3% and 98.9% being HPGN bound at the 1 and 24 h time points.

3.2. Toxicity of Ga–HPGN

For a diagnostic radiopharmaceutical, biocompatibility is of utmost importance. Specifically, no toxicity to the blood components such as red blood cells is allowed, and there should be no negative interaction with the endothelial cell lining of the blood vessels or with any other cells. For this reason, we performed first a general cell viability test with HUVEC cells, and then investigated the polymer's short term interaction with different blood components.

Endothelial cell toxicity: The HUVEC cell viability upon incubation with up to 10 mg/mL of HPG and HPGN is shown in Fig. 4. Both the non-derivatized HPG and HPGN behaved similarly, even though the HPGN has free carboxyl groups on their surface. There was only a slight – and statistically non-significant – decrease in cell viability at the highest concentration. This highest test concentration was chosen as 100-fold higher than the concentration planned to be used in patients. The MTT assay of the novel HPG derivatives also confirmed the earlier found results by Kainthan et al. (2006a, 2007). They found no cytotoxicity in HPG and considered the material thus comparable to linear polyethylene glycols (PEG).

RBC aggregation: Erythrocytes make up the vast majority of cells in the human blood and are able to aggregate naturally with molecules like fibrin as a natural protection mechanism from bleeding. Other macromolecules such as antibodies and high molecular

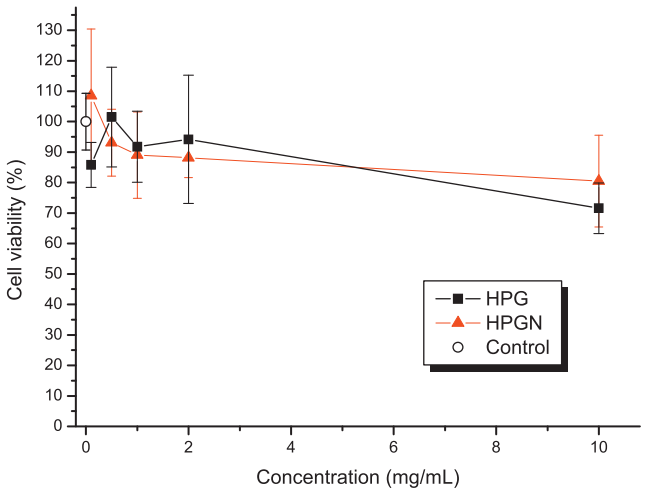

Fig. 4. Cell viability of HUVECs upon incubation with HPG and derivatives after 24 h, expressed in % of healthy control cells.

weight dextran can also trigger this aggregation (Alberts et al., 2008; Kainthan et al., 2007). Diagnostic molecules like the here tested blood pool imaging agents, however, are not allowed to involve aggregation. We tested the HPGN's hemocompatibility at 10 mg/mL whole blood by incubation for 30 min and inspection under the microscope. No aggregation of RBC occurred (Fig. 5), and all RBC maintained their boat like shape for HPG, HPGN and Ga–HPGN. Infrequent stacking, known as rouleaux, occurred because of the stop of motion, but could be disassembled easily by resuspension. The RGB structure thus was not changed indicating that the cells retain their functionality (Kobuchi et al., 1988).

Clinical coagulation assays: HPGN was then tested for its effect on changing the clotting times in the common extrinsic (PT) and intrinsic (APTT) coagulation pathway (Fig. 6). All results, even at the highest concentrations, were within the range of the saline control and the HPG control. The HPGN molecules thus do not influence the fibrin clot formation, which is again confirming results by Kainthan et al. which tested different concentration of HPG (Kainthan et al., 2006a).

Mechanical blood clot properties: The thrombelastograph is often used after surgery to analyze other factors of the coagulation pathway than the PT and APTT assays. Although the results in Fig. 7 show a much broader variance than the other blood compatibility tests, they still are comparable to blood controls and normal values reported from data from US hospitals. One reason for the large variance is that the test takes relatively long and requires fresh donor blood. It was thus necessary to use different donors with their individual clotting kinetics, and that complicated the comparison of values.

Complement activation: The nonspecific immune response expressed by complement activation was also investigated. This cascade of reactions involves C3 and a fragment C3a which act as diffusible signals for inflammatory response leading to cell lysis, clumping, phagocytosis and chemotaxis (Alberts et al., 2008). Measuring the concentration of C3a at two different time points and using insulin as a positive and EDTA as a negative control, both HPG and HPGN were shown to produce similar concentrations of C3a (Fig. 8). The chelating molecules and surface charge present in HPGN thus do not seem to induce additional complement activation over the underivatized HPG. The low level of C3a activation, potentially through the alternative pathway (Nilsson et al., 2007), does not seem to lead to any acute toxicity, but should be monitored in the future for longer term effects that might include inflammation and innate immune responses.

Platelet activation: The effect of HPG and derivatives on blood platelets was investigated by incubation with plasma followed by detection of the CD62 level, which is increased upon platelet activation (Patrono and Davi, 2008). Both HPG and HPGN slightly activated platelets, as shown in Table 1, to similar levels. The values, however, showed fibrinogen binding levels below 10% (which is similar to the one caused by saline) (Kainthan et al., 2007). These

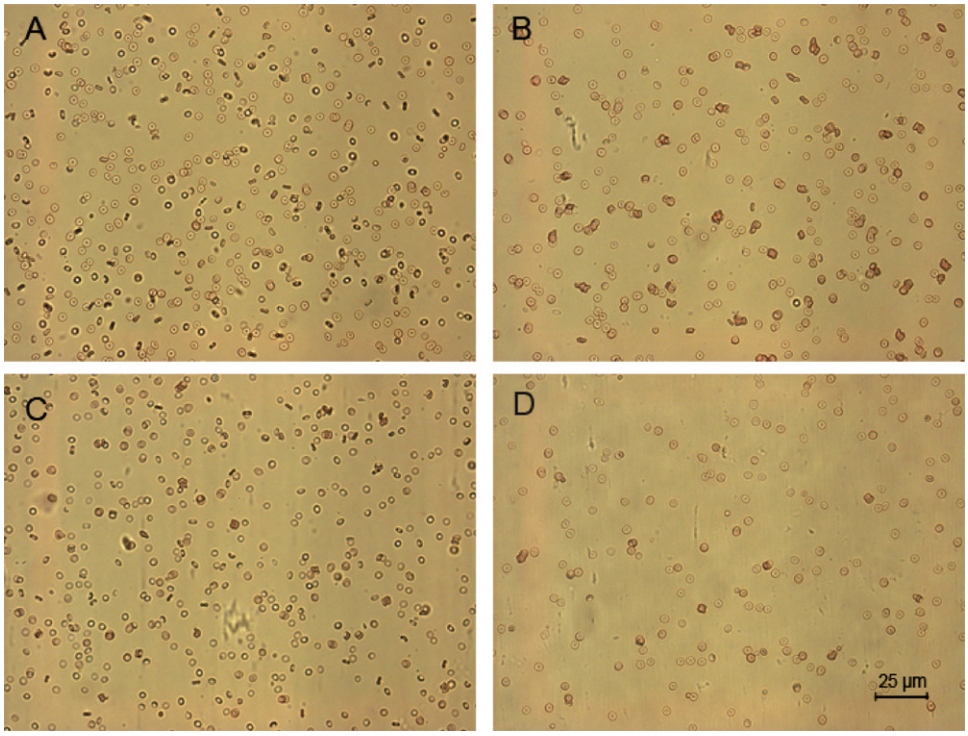

Fig. 5. Microscopy pictures of red blood cells in the RBC aggregation assay: (A) HPG, (B) Ga–$HPGN_{50}$, (C) $HPGN_{50}$, and (D) $HPGN_{200}$.

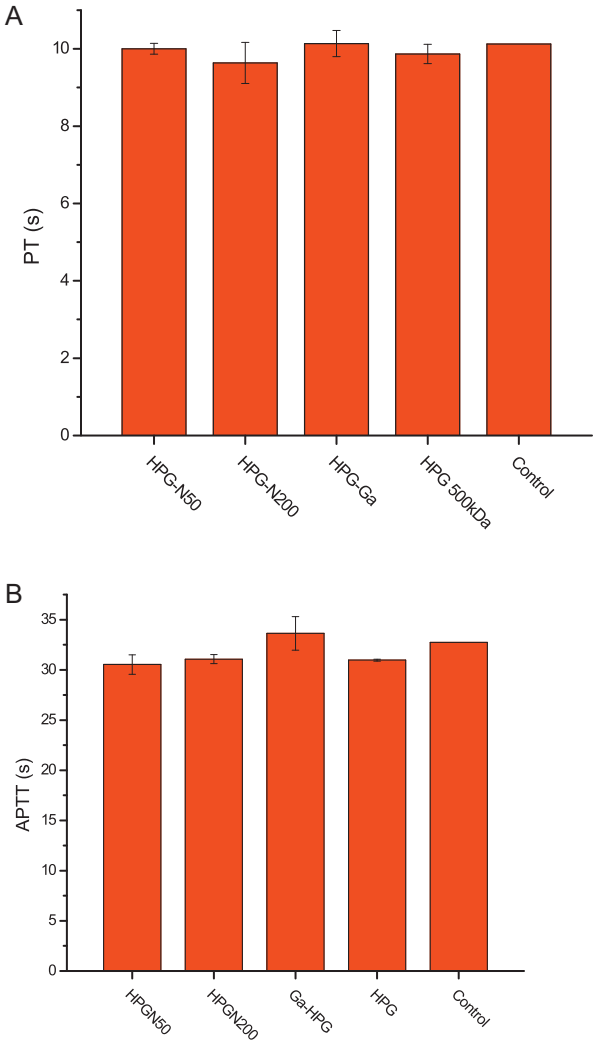

Fig. 6. Clotting time of plasma with added HPG and derivatives (1 mg/mL): (A) prothrombin time PT; (B) activated partial thromboplastin time APTT.

values are still within the normal range, and could have been triggered by stress from shaking or pipetting.

3.3. Pharmacokinetics

The 72 h whole blood concentration–time profile of ^{67}Ga–HPGN following the intravenous bolus administration to rats is shown in Fig. 9. A two-compartment model was implemented for fitting these data for each individual animal as determined by regression analysis using WinNonlin software. The derived pharmacokinetic parameters are summarized in Table 2. The intravenous administration of ^{67}Ga–HPGN resulted in a long circulation time with a mean residency time (MRT) of 50.7 h. The radiolabelled compound was still detectable in the blood as late as 144 h and 168 h (terminal sample) following the administration.

Table 1
Platelet activation caused by HPG and derivatives (1 mg/mL) in whole blood as measured by the level of CD62 expression, in percent. Experiments were done in triplicates.

	10 min	±SD	30 min	±SD	60 min	±SD
Positive control	97.0	0.33	96.5	0.10	95.4	0.57
Negative control	0.0	0.00	0.0	0.00	0.0	0.00
Platelet measurement	99.8	0.00	99.8	0.00	99.8	0.05
HPG	5.9	0.65	11.0	1.06	6.9	0.90
Ga–HPGN$_{50}$	6.5	0.29	10.6	0.69	7.4	0.73
HPGN$_{50}$	6.9	0.26	10.9	0.62	6.0	0.84
HPGN$_{200}$	6.6	0.45	11.3	1.28	7.3	0.54

Table 2
The pharmacokinetic parameters derived from whole blood concentration–time profile of ^{67}Ga–HPGN following intravenous administration of 1.2 MBq per rat (n = 8).

PK parameter	Mean ± SD
A (μg/mL)	1.11 ± 0.54
B (μg/mL)	3.84 ± 0.22
α (h^{-1})	0.45 ± 0.18
β (h^{-1})	0.02 ± 0.002
V_1 (mL/kg)	741 ± 38
V_2 (mL/kg)	177 ± 52
V_{ss} (mL/kg)	918 ± 49
AUC (h μg/mL)	201.8 ± 15.7
MRT (h)	50.7 ± 5.2
CL (mL/(h kg))	18.2 ± 2.1

The long retention time of ^{67}Ga–HPGN imaging agent within the systemic blood compartment of the animal maybe due to the agent's small hydrodynamic size (~10 nm in diameter (Kainthan et al., 2006b)). Furthermore, the limited surface charge at physiologic pH might result in an initial decrease in interaction with blood components and circulating macrophages. The pharmacokinetic profile of ^{67}Ga–HPG in the first 72 h following administration is very similar to the previously determined ones in mice by Kainthan and Brooks (2007) although we used completely different doses and different species. For example, if one calculates the percentage of dose injected per gram of tissue for heart and kidneys after 7 days, then our data in rats are 5.8% and 2.9%, while Kainthan et al. measured in mice 5.5% and 3.0%, respectively. Even the biological half lives in blood are similar, 50.7 h for the current investigation, and 57.5 h for the one by Kainthan and Brooks (2007). These data also confirm the high *in vivo* stability of the ^{67}Ga-labelled polyglycerol.

3.4. Biodistribution

As determined by the terminal biodistribution study 7 days after injection, there was still some activity measurable in the blood compartment (Table 3 and Fig. 10). None of the other organs contained significant amounts of ^{67}Ga–HPGN except the organs of the reticuloendothelial system (RES) liver, spleen and lungs.

HPG is not biodegradable and thus may have long-term effects which will not be apparent in our short experiments (and toxicity assays). Polyglycerols are chemically similar to polyethylene glycol (PEG). Although not biodegradable, the FDA has approved many pegylated compounds and polymers and has them on their GRAS list, which means "generally regarded as safe" (Hoffman, 1998). Both PEG and HPG can degrade by oxidative enzymatic processes starting at the terminal hydroxyl-groups (Kawai, 2002) and are, if larger than 50 kDa, mainly cleared through the reticuloendothelial system of the liver and spleen (Kaminskas et al., 2008). Our biodistribution data from the 7 day time point support their hypothesis that the remaining compound was ultimately sequestered by the reticuloendothelial system and/or metabolized mainly in the liver and spleen. More detailed investigations, however, should be done to further elucidate exact mechanisms and investigate the very large variation between the animals after 7 days.

3.5. Interaction with plasma components

The separation of the plasma into the different lipoprotein fractions showed that only a very small amount of less than 2% of the ^{67}Ga–HPGN was found in the combined HDL, LDL or VLDL fractions (Fig. 11). The rest of the radioactivity was found in the lipoprotein deficient plasma fraction, the same fraction that contains the serum albumin, alpha-1 glycoprotein and majority of the

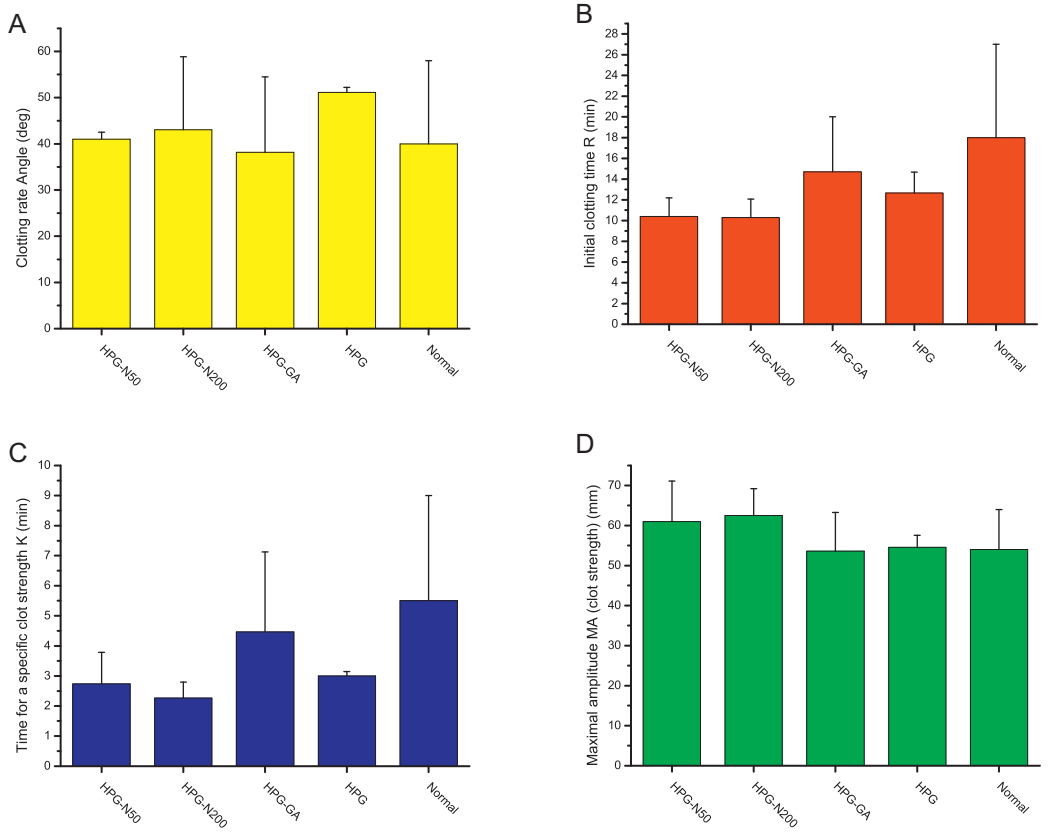

Fig. 7. Different blood clotting parameters were measured in whole blood with added HPG and derivatives (1 mg/mL) using a thrombelastograph (triplicates). The parameters included (A) clotting rate angle, (B) initial clotting time, (C) time to obtain a specific clot strength, and (D) the maximal amplitude.

aqueous plasma components. Since ^{67}Ga–HPGN is hydrophilic in nature these results appear consistent with the fact that ^{67}Ga–HPGN may be fully solubilised within the aqueous component of lipoprotein deficient plasma fraction.

It is, however, not clear if ^{67}Ga–HPGN interacts directly with albumin. No such interactions have been reported previously. The initial chelated HPG has a negative charge at physiological pH, as was confirmed by a slightly negative zeta potential of −4.9 mV. After binding of ^{67}Ga, it was still negatively charged, but at a slightly lower level of −1.5 mV. This was expected, as the gallium binding produces a neutral metal–chelate complex, while the number of chelating groups is in excess and will thus maintain the overall negative charge of the ^{67}Ga–HPGN. Interactions between albumin and other compounds are mainly with positively charged macromolecules, i.e., PAMAM dendrimers (Lee et al., 2010). Furthermore, even with this interaction, ^{67}Ga–HPGN would still be an excellent blood pool imaging agent, as producing a compound that binds to

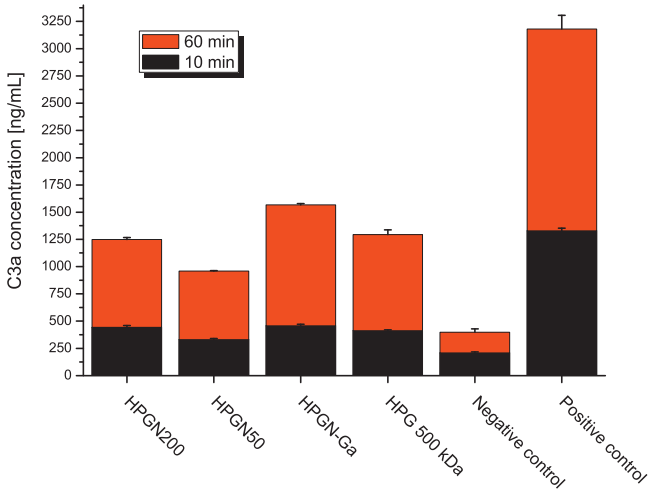

Fig. 8. Complement activation in plasma with added HPG and derivatives (1 mg/mL) measured by C3a concentration.

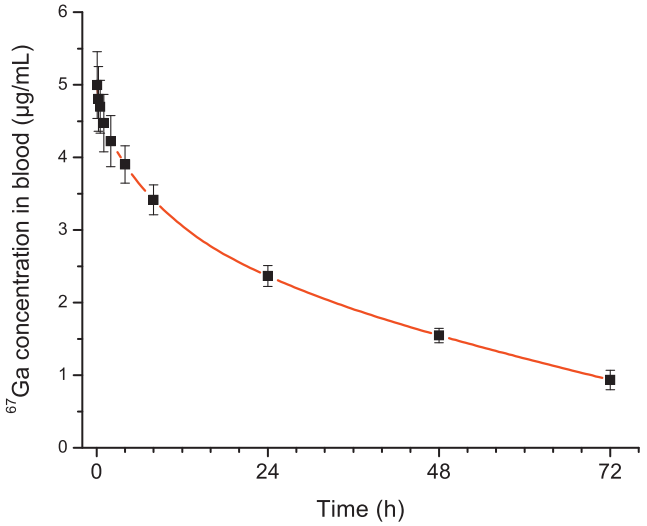

Fig. 9. The whole blood concentration–time profile of ^{67}Ga–HPGN in rats following intravenous administration of 1.2 MBq of ^{67}Ga bound to 1 mg of the polymer (n = 8).

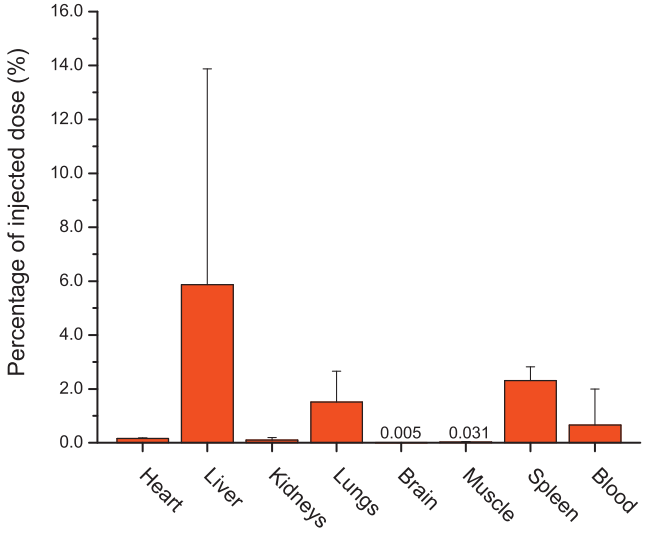

Fig. 10. Biodistribution of ^{67}Ga 7 days after intravenous injection of ^{67}Ga–HPGN ($n = 8$).

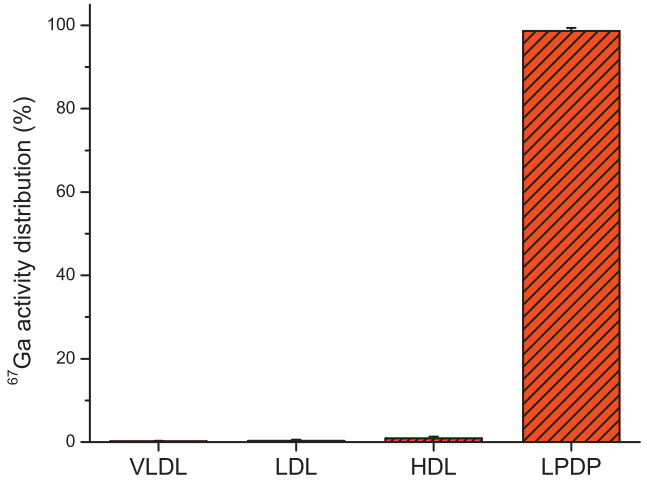

Fig. 11. ^{67}Ga distribution into the different plasma fractions: very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein deficient plasma (LPDP) which contains albumin and alpha-1-glycoprotein.

albumin *in vivo* is one way of making a blood pool agent (Mohs and Lu, 2007). These agents, however, normally contain hydrophobic regions to reversibly bind serum albumin in a non-covalent fashion. Our compound ^{67}Ga–HPGN, though, does not contain any hydrophobic regions.

Table 3
Biodistribution of ^{67}Ga–HPGN at the terminal time point 7 days after intravenous injection given in time corrected (to the time of injection) organ activity concentrations and organ to blood ratios ($n = 8$).

	Organ/blood ratio	±S.D.	Organ concentration (kBq/g)	±S.D.
Heart	13.6	14.5	2.55	0.39
Liver	132.2	147.3	20.84	3.07
Kidneys	6.3	6.9	1.27	0.30
Lungs	158.1	195.6	27.46	15.50
Brain	0.3	0.3	0.06	0.02
Muscle	2.3	2.5	0.45	0.08
Spleen	232.6	275.2	38.04	9.05
Blood	1.0	0.0	1.00	1.06

4. Conclusion

The tested hyperbranched polyglycerol with a molecular weight of approximately 500 kDa behaves like the ideal blood pool agent. Its biological half-life in blood, 50.7 h, is a bit longer than the 29 h (Atkins et al., 1980) of the currently used blood pool agent, radiolabelled red blood cells. Due to its high radiolabelling efficiency it is very simple to prepare in a "shake and use" kit and does not require any blood handling. Furthermore, no toxicity has been found in a battery of cell viability and blood compatibility assays. The ^{68}Ga–HPGN radiopharmaceutical might thus be the preferred choice for a blood pool imaging agent. Based on biological half life, the gamma-emitting ^{67}Ga–HPGN would also be directly useful in a blood pool imaging application which requires a radioisotope with longer half life, such as the detection of internal bleeding.

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