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Automated synthesis of [^{68}Ga]oxine, improved preparation of ^{68}Ga -labeled erythrocytes for blood-pool imaging, and preclinical evaluation in rodents†

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Radiolabeled erythrocytes have multiple applications in nuclear medicine, including blood pool imaging. Historically they have been labeled with SPECT radionuclides. A PET blood pool imaging agent is highly desirable as it would improve clinical applications with better image quality and resolution, higher sensitivity, and dynamic scanning capabilities. With the coming of age of modern $^{68}\text{Ge}/^{68}\text{Ga}$ generator systems, gallium-68 is now widely accessible. In this paper we describe an updated method for the preparation of ^{68}Ga -labeled erythrocytes and their preliminary use in rodent blood pool imaging. A novel automated synthesis of [^{68}Ga]oxine using a $^{68}\text{Ge}/^{68}\text{Ga}$ generator and automated synthesis module is reported. [^{68}Ga]Oxine was synthesized in $50 \pm 5\%$ ($n = 3$) non-decay corrected radiochemical yield and $>99\%$ radiochemical purity. Rat and human erythrocytes were successfully labeled with the complex in 42% RCY, and the ^{68}Ga -labeled erythrocytes have been shown to clearly image the blood pool in a healthy rat. Human erythrocytes labelled with [^{68}Ga]oxine were shown to be viable up to 2 hours post-labelling, and washout of the radiolabel was minimal up to 1 hour post-labelling. Further optimization of the labeling method to translate for use in human cardiac and oncologic blood pool PET imaging studies, is underway.

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Introduction

Blood pool imaging by positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been used for the investigation of infection,¹ gastrointestinal bleeding,² cardiac function,^{3–5} and to measure changes in vascularity and perfusion of tumors and organs.^{6,7} Radiolabeled small molecules and blood proteins (such as human serum albumin (HSA)) have been widely used for this purpose,^{8–10} but radiolabeled erythrocytes in particular have been demonstrated to have numerous applications in nuclear medicine.^{11–13} Such uses include measurement of total erythrocyte volume and erythrocyte survival time, identification of sites of erythrocyte destruction, selective spleen imaging with damaged erythrocytes, and general blood pool imaging (BPI) studies.¹⁴ Technetium-99m (as the UltraTag™ kit) is the most widely used γ -emitting isotope for labeling erythrocytes for

imaging with single photon emission computed tomography or scintigraphy.^{15,16} A positron-emitting variant of the $^{99\text{m}}\text{Tc}$ -based agents is desirable as it would have comparable clinical applications with improved image quality and resolution, higher sensitivity, and the dynamic scanning capability offered by positron emission tomography imaging. The potential instability in the future supply of $^{99\text{m}}\text{Tc}$,¹⁷ coupled to the superior imaging quality of PET, indicates that there is need for the development of a simple PET agent for blood pool imaging.

There are examples of erythrocytes radiolabeled with PET isotopes being used to image the blood pool. Carbon monoxide labeled erythrocytes (using [^{15}O]CO or [^{11}C]CO) have been demonstrated as suitable agents for this purpose,^{18–20} however the short half-life of oxygen-15 and carbon-11 agents necessitates an onsite cyclotron and specialist facilities, and such agents cannot be widely distributed from centralized nuclear pharmacies to satellite imaging centers. Fluorine-18 ($t_{1/2} = 110$ min) or gallium-68 ($t_{1/2} = 68$ min) are therefore the most attractive PET radionuclides for labeling PET blood pool agents due to their longer half-lives. There are numerous advantages to using gallium-68, including its availability from a (relatively) low-cost commercial generator that has a long shelf life (6 months to 1 year) and radiochemical syntheses

^a Department of Radiology, University of Michigan Medical School, Ann Arbor, MI 48109, USA. E-mail: bviglia@med.umich.edu; Tel: (+1) 919 451 0813^b Department of Veterans Administration, Ann Arbor, MI 48105, USA† Electronic supplementary information (ESI) available: Experimental procedures for the synthesis of [^{68}Ga]oxine (including chromatographic data) and preparation of ^{68}Ga -labeled erythrocytes, as well as details of pre-clinical PET imaging, cell viability and [^{68}Ga]oxine washout studies. See DOI: 10.1039/c7md00607a

with the isotope that are amenable to automation and kit type preparation. The convenient half-life of 68 min is also long enough to allow commercial distribution, but short enough that repetitive syntheses and imaging studies can be conducted within the same day.^{21,22}

Radioisotopes of gallium and indium, most often *via* their oxine (tris(8-quinolinolato)) complexes, have been widely explored for labeling blood products, including erythrocytes, leukocytes and platelets.^{23–27} The synthesis of ⁶⁸Ga-labeled erythrocytes was first reported by Welch *et al.* in 1977, where it was demonstrated that ⁶⁸Ga-labeled erythrocytes provided PET images comparable to those obtained with [¹¹C]CO-labeled erythrocytes in a dog model.²⁶ Their reported manual synthesis of the agent for erythrocyte labeling, [⁶⁸Ga]oxine, required a number of evaporation steps and extraction of the lipophilic complex into chloroform, a class II solvent per ICH.²⁸ With the coming of age of modern ⁶⁸Ge/⁶⁸Ga generator systems, many of these steps were no longer necessary and, if pursued, would raise safety concerns and require additional quality control testing in the current regulatory environment. In addition, the original study by Welch *et al.* reported high liver uptake of the ⁶⁸Ga-labeled erythrocytes, which was unanticipated, as the known vascularity of the liver compared to that of the heart does not support the observation of equivalent uptake. Syntheses of other radioactive isotopologues of Ga-oxine, including [⁶⁶Ga]oxine²⁹ and [⁶⁷Ga]oxine²⁷ have also been previously reported, and [^{nat}Ga]oxine (KP46, tris(8-hydroxyquinolato)gallium) has recently shown promise as a therapeutic agent for melanoma³⁰ and renal carcinoma.³¹

As an alternative to the older ⁶⁸Ga-based agents, there have been recent literature reports describing the synthesis and evaluation of ¹⁸F-labeled erythrocytes labeled using *N*-succinimidyl 4-[¹⁸F]fluorobenzoate ([¹⁸F]SFB),³² [¹⁸F]sulfonamide derivatives,³³ or [¹⁸F]fludeoxyglucose ([¹⁸F]FDG).^{34,35} [¹⁸F]FDG is the overt tracer to pursue given its commercial availability and “FDA approved” status for metabolic imaging. Jinzaki labeled erythrocytes with [¹⁸F]FDG and reported high retention of the radiotracer in the cardiovascular system and clear visualization of extravascular blood in intraabdominal bleeding models.³⁴ Choi conducted a similar study with [¹⁸F]FDG-labeled erythrocytes and reported high activity accumulation in the cardiac chambers and surrounding pulmonary vasculature.³⁵ Terman and Viglianti also demonstrated that [¹⁸F]FDG-labeled erythrocytes were excluded from the tumor vasculature when pre-treated with sickle cells.^{36,37} These studies provided promising evidence for the clinical development of [¹⁸F]FDG-labeled erythrocytes as a PET BPI agent. However, active GLUT-1 mediated-labeling of erythrocytes with a glucose-derivative is dependent on the metabolic state of the cells. To control for this, the cells or patients were subjected to glucose deprivation/fasted (30 min–6 hours) to ensure optimal labeling efficiency. This procedure can be subjective and is difficult to accurately reproduce due to variability across subjects. Additionally, for certain clinical applications such as GI bleeding studies, such deprivation can be problematic. Moreover, there is a need for a cyclotron within

a deliverable range to provide the [¹⁸F]FDG. The widespread adoption of ¹⁸F-labeled erythrocytes is complicated by the need to undertake careful pre-preparation steps (like prolonged fasting) before radiolabeling of the erythrocytes.^{34,35}

With renewed interest in a readily accessible PET BPI agent, we wanted to capitalize on the advantages of gallium-68 as a PET radionuclide and set out to develop an automated synthesis of [⁶⁸Ga]oxine without the use of hazardous solvents such as chloroform. Herein, we describe an automated synthesis of [⁶⁸Ga]oxine and subsequent re-evaluation of the preparation and application of ⁶⁸Ga-labeled erythrocytes.³⁸ With appropriate modifications, we demonstrate in a rat model that ⁶⁸Ga-labeled erythrocytes meet the requirements for an operationally simple, widely applicable PET BPI agent.

Results and discussion

Synthesis of [⁶⁸Ga]oxine

We envisaged that the synthesis of [⁶⁸Ga]oxine could be automated using a configuration commonly used for radiolabeling peptides with gallium-68. The key difference between Welch's manual synthesis²⁶ and our proposed automated synthesis, beyond the use of a ⁶⁸Ge/⁶⁸Ga generator, is the use of a C₁₈ cartridge to replace the chloroform extraction step.

Using an automated Scintomics GRP Cassette Module, [⁶⁸Ga]Ga³⁺ was trapped on an ion-exchange column, before being eluted with aqueous NaCl as [⁶⁸Ga]GaCl₃. [⁶⁸Ga]Oxine was synthesized from this [⁶⁸Ga]GaCl₃ and 8-hydroxyquinoline in a 2 M pH 5.5 NaOAc buffer for 10 minutes at ambient temperature (Scheme 1). [⁶⁸Ga]Oxine was trapped on a C₁₈ cartridge, and the cartridge washed with water to remove excess buffers and salts. The product was eluted from the cartridge with either ethanol, or an ethanol:water mixture (1 : 1). This was performed either manually, or under automation, dependent on the age of the generator and reformulation requirements. When the generator was new (~1110–1850 MBq (30–50 mCi) [⁶⁸Ga]Ga³⁺ per elution), radioactivity and ethanol concentrations in the final reformulated product were suitable for radiolabeling of small volumes of rat and human erythrocytes. As the generator aged, and the radioactivity at the start of the synthesis dropped below 1110 MBq (30 mCi) per elution, manual reformulation was necessary to obtain solutions with sufficient radioactivity concentrations suitable for radiolabeling small volumes of cells in radiochemical yields suitable for use in microPET imaging experiments. When manual reformulation was required, the complex was

Scheme 1 Synthesis of [⁶⁸Ga]oxine.

manually eluted from the C₁₈ cartridge, through a sterile filter, with ethanol, before reformulation with a small volume of saline to provide [⁶⁸Ga]oxine in a 33% EtOH in saline solution in 35 ± 9% (*n* = 6) non-decay corrected radiochemical yield in approximately 50 min from generator elution (see ESI† for details). We subsequently automated the reformulation step to provide [⁶⁸Ga]oxine in a 12% EtOH in saline solution in 50 ± 5% (*n* = 3) non-decay corrected radiochemical yield in ~42 min.

In each case, the radiochemical purity of [⁶⁸Ga]oxine was >99%, determined by both radio-HPLC and C₁₈ trapping experiments (see ESI† for details). The identity of the product was verified by comparison of the retention time of the radiolabeled product with that of an authentic [^{nat}Ga]oxine reference standard on HPLC (Fig. S1†).

MicroPET imaging with unwashed ⁶⁸Ga-labeled erythrocytes in rat

Our initial labeling and imaging experiment attempted to repeat the experiments conducted by Welch,²⁶ to establish whether the modified synthesis affected erythrocyte labeling efficiency or the PET images obtained with the agent. Following the protocol described by Welch *et al.* washed human and rat erythrocytes were incubated with [⁶⁸Ga]oxine. Labeling yields (decay corrected) were found to be slightly lower than those reported by Welch *et al.* (84 ± 5% (*n* = 6) for human erythrocytes, 70 ± 7% (*n* = 12) for rat erythrocytes, compared to 93 ± 5% (*n* = 5) for dog erythrocytes). ⁶⁸Ga-labeled erythrocytes prepared by this method (17.6 MBq, 476 μCi, *n* = 1) were administered to a rat (290 g, *n* = 1) and imaging showed distribution of the ⁶⁸Ga-labeled erythrocytes predominantly in the heart and liver, with uptake in these regions appearing similar (see Fig. S3†). The images from the original study (in a dog, Fig. S3A†) and a summed image (0–25 min) from this study (Fig. S3B†) appeared similar, where apparent equivalent uptake in both the liver and heart is observed.

Despite the replication of the results, the near equivalent uptake of ⁶⁸Ga-labeled erythrocytes in the heart and liver seemed erroneous. The hepato-splanchnic cardiac output fraction in anesthetised rats is ~20%, and a similar fraction is reported for humans.³⁹ Assuming the heart represents a 100% vascular space, we anticipated that the SUV of the ⁶⁸Ga-labeled erythrocytes in the liver would be lower than the SUV in the heart. In addition, recently reported BPI agents tend to show high concentrations of the BPI tracers in the heart/blood, while concentrations in the liver tend to be significantly lower (17–30% of the activity in the heart, depending on the radiotracer).^{32–34} As Welch did not report additional washing steps after labeling, we hypothesized that radiolabeling of the erythrocytes did not proceed to completion, and that residual [⁶⁸Ga]oxine may be responsible for the observed liver uptake.

⁶⁸Ga-labeled erythrocyte washing studies

A cell washing study was performed with the [⁶⁸Ga]oxine radiolabeled human and rat erythrocytes, immediately after

labelling. The data showed that there was a decrease in the activity associated with the cells after each washing step, corresponding to removal of excess, or unbound [⁶⁸Ga]oxine (Fig. S2†). After four washing cycles, the activity associated with erythrocytes from both species plateaued at ~42%. For rat erythrocytes, activity associated with the cells stabilized after three wash cycles, and this was deemed optimal for studies moving forward.

Washed ⁶⁸Ga-labeled erythrocytes MicroPET imaging in rat

After demonstrating that a significant quantity of the radioactivity was washed from the cells, further microPET imaging experiments were conducted in rats (378 ± 31 g, *n* = 3) using ⁶⁸Ga-labeled erythrocytes where three washing cycles were performed (9.1 ± 1.2 MBq, 245 ± 33 μCi). Representative summed microPET images from a single rat, and the averaged time activity curves (*n* = 3) of region of interest (ROI) in the heart and liver of a rats administered ⁶⁸Ga-labeled erythrocytes are shown in Fig. 1.

Repetition of the imaging study in the rat with washed ⁶⁸Ga-labeled erythrocytes revealed strikingly different uptake results. In contrast to the first imaging experiment with unwashed ⁶⁸Ga-labeled erythrocytes, ⁶⁸Ga-labeled erythrocytes that had been washed three times were found to distribute primarily in the vascular spaces. The heart showed higher

Fig. 1 A. Sagittal (sag) and coronal (cor) summed (0–55 min) microPET image of washed ⁶⁸Ga-labeled erythrocytes in a rat, showing high uptake in the heart and descending aorta/vena cava, and minimal liver uptake. B. Time activity curve (*n* = 3) showing peak SUV of 10.5 in the heart and 4 in the liver, and equilibrium SUV of 9 in the heart and 3.4 in the liver.

uptake ($SUV = 9$ at equilibrium), while the large blood vessels, including the aorta/vena cava and carotid arteries/jugular veins, and lungs were also visible. Distribution in the liver was lower ($SUV = 3.7$ at equilibrium) than previously observed, and more consistent with the vascularity of the liver compared to the '100%' vascularity of the left ventricle of the heart. Closer quantitative analysis of the time-activity curves of the two ROIs showed that ^{68}Ga -labeled erythrocytes equilibrate rapidly (within 300 s), and that the radioactivity present in the heart and liver remain relatively constant throughout the remainder of the imaging experiment.

There are notable differences between these rodent imaging results washed erythrocytes and the results obtained by Welch using dogs, as well as our initial rodent experiment using unwashed cells. The expected distribution between heart and liver is consistent with the idea that residual ^{68}Ga oxine is responsible for higher than expected liver uptake when working with unwashed cells. To investigate this further, we conducted separate imaging studies with both ^{68}Ga oxine and a mix of ^{68}Ga oxine + ^{68}Ga -labeled erythrocytes (*vide infra*). An additional difference was high spleen uptake observed in Welch's dog images, but minimal uptake in the rodent spleen in our work. There are two potential explanations for this difference. Firstly, we employed isoflurane anesthesia while Welch utilized sodium pentobarbital, and there is early literature suggesting that use of the latter increases the number of circulating red blood cells

found in the spleen, although this effect could be mitigated with a vasoconstrictor such as epinephrine.⁴⁰ Secondly, the dog spleen differs from rodent and human spleen in that it can store large amounts of blood for rapid release in an emergency.⁴¹ Either or both of these scenarios would lead to the high spleen uptake in dogs observed by Welch. Notably, we expect this will not be an issue in human studies with ^{68}Ga -labeled erythrocytes, which we expect to be similar to blood pool imaging using $^{99\text{m}}\text{Tc}$ -based radiotracers.

^{68}Ga oxine microPET imaging in a rat

As a control experiment, ^{68}Ga oxine, the agent used for radiolabeling of the erythrocytes (14.8 ± 2.1 MBq, 400 ± 57 μCi), was administered to healthy rats (391 ± 40 g, $n = 2$) to assess the imaging profile and determine if the radiotracer leached from the erythrocytes *in vivo*. The resultant summed microPET images, and the averaged time activity curves (including standard deviation, $n = 2$) of ROIs in the heart and liver are shown in Fig. 2. In contrast to washed ^{68}Ga -labeled erythrocytes, ^{68}Ga oxine accumulated rapidly in the liver and spleen (equilibrium $SUV = 7$), although some uptake in the heart was also observed ($SUV = 3.5$). It is likely that excretion of the tracer through the hepatobiliary system is also observed by the presence of radioactivity in the lower abdomen, however renal clearance cannot be discounted, as the bladder was not in frame.

This data suggests that the liver uptake observed in the initial experiments where the cells were not washed prior to administration to the rat, was likely due to free ^{68}Ga oxine in the sample. To investigate further whether ^{68}Ga oxine was responsible for the increased liver uptake observed in the unwashed ^{68}Ga -labeled erythrocytes, a chasing experiment was performed, where ^{68}Ga oxine (13.7 MBq, 369 μCi) was administered to a healthy rat (372 g, $n = 1$) 30 min post-administration of washed ^{68}Ga -labeled erythrocytes (6.4 MBq, 173 μCi). The results of this study were in line with previous experiments. At the beginning of the scan, ^{68}Ga -labeled erythrocytes were primarily observed in the vascular spaces, with the heart and major blood vessels clearly visible (Fig. S4A†). After administration of ^{68}Ga oxine 30 minutes into the experiment, an immediate increase in the radioactivity in the liver was observed (Fig. S4B†). This change is clearly reflected in the TACs (Fig. S4C†) and offers further evidence that ^{68}Ga oxine preferentially accumulates in the liver.

Cell viability and washout studies

An essential property of an erythrocyte-based PET BPI agent is that the labelling process does not perturb the viability of the erythrocytes. Damaged erythrocytes accumulate in the spleen for re-processing, and as such, radiolabeled, heat-damaged erythrocytes are commonly used for imaging the spleen.⁴² To investigate the viability of erythrocytes labeled and washed as described above, samples of the labelled erythrocytes, and samples of unlabeled control erythrocytes were taken immediately after, 1 hour post-, and 2 hours post-

Fig. 2 A. Sagittal (sag) and coronal (cor) summed (0–55 min) microPET image of ^{68}Ga oxine in a rat, showing high uptake in the liver, but minimal cardiac uptake. B. Time activity curve ($n = 2$) showing peak SUV of 7 in the liver and 9 in the heart, and equilibrium SUV of 3.7 in the heart 6.5 in the liver.

labeling and subjected to staining with trypan blue. Erythrocyte viability was then determined using a hemocytometer. While some lysed cells were detected in samples of the erythrocytes labeled with [^{68}Ga]oxine, likely due to the repeated washing steps, the number of lysed cells observed was similar to that of unlabeled erythrocytes, with at least 95% of the erythrocytes remaining viable up to 2 hours post-labeling. Light microscopy of the samples confirmed that the erythrocytes maintained their biconcave morphology and appeared identical to unlabeled erythrocytes at all time points (Fig. S5†).

Finally, the washout of $^{68}\text{Ga}^{3+}$ from the radiolabeled erythrocytes was investigated. Immediately after the standard labelling and washing procedure, aliquots of the ^{68}Ga -labeled erythrocyte suspension in saline were centrifuged, and the activity in the supernatant and pellet measured. At this point, it was determined that >96% of the activity was associated with the erythrocytes. A second aliquot of the cell suspension was analyzed in the same manner 1 hour later, and it was found that >96% of the activity still remained associated with the erythrocyte pellet. This suggests that, once $^{68}\text{Ga}^{3+}$ is trapped inside the erythrocyte, the radiolabel is not readily released from the cell. The *in vitro* stability of the radiolabel corroborates what was observed in the TAC in the microPET imaging experiment with well washed erythrocytes (Fig. 1B). In that experiment, the activity in the blood pool did not decrease and activity in the liver did not increase during the imaging experiment confirming *in vivo* stability of the radiolabel once trapped in the erythrocyte.

Overall, our results suggest that [^{68}Ga]oxine-labeled erythrocytes demonstrate potential as a BPI PET agent, provided that the cells are suitably washed prior to use. The use of generator-based BPI agents, predominantly with $^{99\text{m}}\text{Tc}$, remains the most popular method for blood pool imaging, and this study serves to provide an analogous PET agent for this purpose. In the context of other blood pool PET imaging agents, including radiolabeled CO, [^{18}F]FDG, [^{18}F]SFB- and [^{18}F]carbonic anhydrase inhibitor-labeled red blood cells, the optimization and streamlined development of ^{68}Ga -labeled erythrocytes represents a potentially attractive alternative to such agents. The fact that the isotope itself is generator produced is advantageous, as an expensive cyclotron and radiochemistry facility is no longer required and such labeling studies can be performed in any traditional nuclear pharmacy. In addition, the longer half-life of ^{68}Ga (compared to ^{15}O and ^{11}C), and the use of non-gaseous radiolabels, offer significant technical advantages. One limitation of this method is the need for manual washing steps of the blood both prior to labeling, and after incubation with [^{68}Ga]oxine. We are presently investigating methods to reduce handling of the blood and practical methods for washing the blood cells which can easily be conducted in a sterile environment.

Experimental

Full details of experimental procedures as well as associated analytical data can be found in the ESI†

Conclusions

We have developed a novel automated synthesis of [^{68}Ga]oxine using a $^{68}\text{Ge}/^{68}\text{Ga}$ generator and automated synthesis module. Rat and human erythrocytes were successfully labeled with the complex, remained viable, and the ^{68}Ga -labeled erythrocytes have been shown to clearly image the blood pool in a healthy rat. It was found that repeated washes of the labeled erythrocytes were required to remove excess [^{68}Ga]oxine, otherwise anomalous liver uptake was observed. Further optimization of the labeling methods to reduce handling steps, and translation of this to larger scale for use in human cardiac and oncologic blood pool PET imaging studies is ongoing.

Ethics statement

All animal experiments were conducted under the supervision of the University of Michigan and its Institutional Animal Care and Use Committee (IACUC) and were conducted according to approved protocols in accordance with all applicable federal, state, local and institutional laws or guidelines governing animal research. All experiments with human blood were approved by the University of Michigan Institute Review Board (IRB).

Conflicts of interest

The authors declare no competing interest.

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