^{18}F -Labeling of Mannan for Inflammation Research with Positron Emission Tomography

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S Supporting Information

ABSTRACT: Recently mannan from *Saccharomyces cerevisiae* has been shown to be able to induce psoriasis and psoriatic arthritis in mice, and the phenotypes resemble the corresponding human diseases. To investigate the pathological processes, we set out to label mannan with fluorine-18 (^{18}F) and study the ^{18}F -labeled mannan *in vitro* and *in vivo* with positron emission tomography (PET). Accordingly, mannan has been transformed into ^{18}F -fluoromannan with ^{18}F -bicyclo[6.1.0]nonyne. In mouse aorta, the binding of [^{18}F]fluoromannan to the atherosclerotic lesions was clearly visualized and was significantly higher compared to blocking assays ($P < 0.001$) or healthy mouse aorta ($P < 0.001$). In healthy rats the [^{18}F]fluoromannan radioactivity accumulated largely in the macrophage-rich organs such as liver, spleen, and bone marrow and the excess excreted in urine. Furthermore, the corresponding ^{19}F -labeled mannan has been used to induce psoriasis and psoriatic arthritis in mice, which indicates that the biological function of mannan is preserved after the chemical modifications.

KEYWORDS: Fluorine-18, inflammation, mannan, positron emission tomography, tetrazine ligation

Inflammatory diseases (e.g., rheumatoid arthritis) affect large populations and cause significant morbidity and healthcare costs.^{1,2} Inflammation is a critical process in many diseases including cardiovascular, metabolic, oncological, and neurodegenerative conditions. Our research has long been focused on the diagnostics and pathogenesis of inflammatory diseases.^{3–6} Recently, we have generated a new psoriasis and psoriatic arthritis mouse model.⁷ After being administered with a single dose of mannan from *Saccharomyces cerevisiae* (*S. cerevisiae*), mice develop an acute inflammation in skin and articular joints. The disease phenotypes resemble the human psoriasis and psoriatic arthritis, which has implications in clinical inflammation research. Mannan is a naturally occurring polysaccharide, and it may bind to mannose receptors as well as other mannan-binding lectins.⁸ Structurally, mannan from *S. cerevisiae* is quite different from other polysaccharides (e.g., dextran) and even differs from mannans from other sources (e.g., plants).⁹ We have proposed that mannan can activate tissue macrophages and that interleukin-17A secretion from $\gamma\delta$ T cells is triggered,

causing the acute inflammation.⁷ This opens perspectives in using mannan as a probe for investigating the pathological processes and developing corresponding therapies. In addition, mannan has great potential for other types of medical use. To mention a few, (1) mannan-conjugated protein mucin 1 has been in several clinical trials for studying immunotherapy in patients with early stage breast cancer or other cancers;⁸ (2) mannan-conjugated adenovirus can enhance gene therapy effects in tumor-bearing mice;¹⁰ (3) mannan and its conjugates have been used for development of vaccines against cancers and fungal infections.^{11,12} Thus, we set out to label mannan with a radionuclide, fluorine-18 (^{18}F), for positron emission tomography (PET) imaging and study the biodistribution of [^{18}F]fluoromannan *in vivo*. PET is a medical diagnosis technique, and it can monitor functional changes in a

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noninvasive and quantitative manner with extremely high sensitivity.¹³ As a prerequisite for PET, a suitable positron-emitting pharmaceutical (PET tracer) needs to be administered into patients prior to imaging. ¹⁸F is the most often used positron emitter in clinical PET, due to its favorable physical properties (e.g., clean positron emitting process and low positron energy). Another favorable physical property of ¹⁸F is its suitably long half-life (109.8 min), although ¹⁸F is a short-lived radionuclide. This enables multistep radiosynthesis feasible^{14,15} and distribution to hospitals distanced from production sites possible.

Regarding ¹⁸F-labeling of polysaccharides and polysaccharide-derived substance, two prosthetic groups have been used previously.^{16,17} Compound **1** (Figure 1) is an alkyne for

Figure 1. Previously used prosthetic groups for ¹⁸F-labeling of dextran and dextran-derived nanoparticles.^{16,17}

conjugation of dextran-derived (or decorated) nanoparticles with copper-catalyzed 1,3-dipolar cycloaddition reactions.¹⁶ In another work, tetrazine-bearing dextrans have been conjugated to ¹⁸F-*trans*-cyclooctene ([¹⁸F]TCO, Figure 1) based on inverse electron demand Diels–Alder (IEDDA) reaction.¹⁷ IEDDA reaction has emerged as the fastest chemistry scheme, and tetrazine-*trans*-cyclooctene ligation has been widely applied in different technologies.¹⁸ Tetrazine-*trans*-cyclooctene ligation may generate products with multiple isomers.^{19,20} This might complicate the interpretations of PET imaging results and drug approval processes, if it is not possible to exclude different pharmacokinetic patterns caused by different isomeric products. However, we must emphasize that *trans*-cyclooctene ligation is absolutely favorable in a number of aspects. In this context, we have proposed a ¹⁸F-labeled bicyclo[6.1.0]nonyne ([¹⁸F]BCN) as an alternative ligation partner for tetrazines,²¹ in the cases where simpler isomeric profile is preferred. BCN-based conjugation has been used for labeling of antibodies, living cells, etc., with a number of dienophiles.^{22,23} [¹⁸F]BCN ligates to tetrazines in a near-stoichiometric manner, which is especially useful for labeling of large molecules.²¹ To conjugate [¹⁸F]BCN or [¹⁹F]BCN to mannan, the molecular structure of mannan needs to be functionalized with tetrazine moieties. In literature, strategies for activation of mannan include periodate oxidation, cyanation and carbamoylation.^{24–27} In periodate oxidation reactions, part of the hexasaccharide rings is opened to form aldehydes, and new epitopes may be generated in the resulted conjugates.²⁸ Cyanation or carbamoylation activate some of the hydroxyl groups without breaking the sugar ring structures. We envisaged that cyanation or carbamoylation could be a method of choice for our purposes to better preserve the biological functions of labeled mannan. According to the previous method,^{25,26} mannan from *S. cerevisiae* (Sigma-Aldrich, #M7504) was subjected to cyanation with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and amination with 1,6-diaminohexane in one pot of reaction (Figure 2). Amino-functionalized mannan **2** was purified by dialysis against water at 4 °C overnight. Subsequently, *N*-hydroxysuccinimide-activated tetrazine (tetrazine-NHS) was used to react with **2** at pH 9.0 in the presence of dimethyl sulfoxide as a

Figure 2. Preparation of amino-functionalized mannan **2**,^{25,26} tetrazine-modified mannan **3**, and [¹⁹F]fluoromannan.

cosolvent (Supporting Information, SI). After a centrifugal filtration, tetrazine-modified mannan **3** was formulated in water and stored at −20 °C for next step conjugations.

In the previous studies in mice and in this work, we used the mannan product supplied by Sigma-Aldrich.⁷ This mannan has been prepared by alkaline extraction from yeast *S. cerevisiae*, and the molecular weight is in the range of 34.0–62.5 kDa as measured by low-angle laser light scattering technique.²⁹ In high-performance liquid chromatography (HPLC) analyses with a size-exclusion column, relatively broad peaks were observed for mannan and its derivatives (SI). To estimate the number of tetrazine loading, the absorbance of **3** was measured at 534 nm by using 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid as a reference. At this wavelength, the absorbance of mannan was negligible (e.g., 0.002 A for mannan at 1.9 mg/mL). Accordingly, the loading of tetrazines was 115 nmol/mg.

At this point, it became critical to evaluate whether the labeling method could preserve the desired biological function of mannan for inducing inflammation or not. In the original studies, mannan (10 mg per mouse) was intraperitoneally injected into mice to induce psoriasis and psoriatic arthritis.⁷ To perform the biological evaluations in similar experimental settings, [¹⁹F]fluoromannan is needed. Accordingly, [¹⁹F]-fluoromannan was prepared by the IEDDA ligation of **3** to [¹⁹F]BCN in phosphate-buffered saline (PBS, pH 7.4). [¹⁹F]fluoromannan was formulated in PBS after purification by centrifugal filtration and stored at −20 °C when necessary (SI). To perform the *in vivo* studies, [¹⁹F]fluoromannan (10 mg per mouse) was injected intraperitoneally into mice with a neutrophil cytosolic factor 1 gene mutation (Ncf1^{m1j/m1j}),⁷ and mannan (10 mg per mouse) was administered into control mice for comparison. The disease phenotypes were followed by visual observation, and the disease severity was quantified by blinded scoring. The onset of inflammation was clearly observed starting from day 2 postadministration (Figure 3). The mice were judged by redness and swelling of the paws (1 point per knuckle or toe, and 5 points per ankle), and a total of 60 points were scored. They were also judged upon psoriatic lesions on paws and ears, and a total of 15 points were scored by giving one point from 0–3 lesions per paw or ears. On day 3 postadministration, the frequency of arthritis reached 100% in all the mice injected with either [¹⁹F]fluoromannan or mannan.

Figure 3. Evaluation of the function of [¹⁹F]fluoromannan for inducing inflammation in mice. Representative photos from mice injected with [¹⁹F]fluoromannan (A,B), mannann (C,D) and naïve (E), arthritis severity (F), and the corresponding frequency (G), severity (H), and frequency (I) of psoriasis.

In addition, the frequency (66%) of psoriasis was also considered to be high in the limited number of studied mice that were injected with [¹⁹F]fluoromannan, even though higher frequency (100%) was observed in the control group of mice. These data indicated that the labeling did not change the critical function of mannann to induce arthritis or skin inflammation.

Next we developed the production methods for [¹⁸F]-fluoromannann, the radioactive “hot” counterpart of [¹⁹F]-fluoromannann, for preclinical PET studies in animals. The prosthetic group [¹⁸F]BCN was produced by a nucleophilic substitution reaction as previously described, with a remote-controlled radiosynthesis device.^{21,30} In the initial tests with little amount (e.g., 2 MBq) of radioactivity, the conjugation product [¹⁸F]fluoromannann was clearly observed in PBS at r.t. This encouraged us to scale up the conjugation under similar conditions. In routine productions, we added tetrazine 3 to [¹⁸F]BCN in PBS at pH 7.4. After 3–5 min at r.t., the reaction mixture was loaded onto a NAP-25 size-exclusion cartridge for purification (SI). The eluted [¹⁸F]fluoromannann was formulated in PBS ready for injection. Starting from 12.6 GBq of [¹⁸F]fluoride at end of bombardment, [¹⁸F]fluoromannann was obtained in $7.9 \pm 2.2\%$ ($n = 10$) of decay-corrected radiochemical yield over the two steps of radiosynthesis, and the total synthesis time was about 118 ± 6 ($n = 10$) min. The specific (radio)activity was 1.7 ± 0.4 ($n = 10$) GBq/mg. Quality control of [¹⁸F]fluoromannann was performed with size-exclusion HPLC analyses (Figure 4) and with thin-layer chromatography (TLC) analyses (SI). The radiochemical purity was >95%. As expected from the common property of

Figure 4. Radiosynthesis and quality control of [¹⁸F]fluoromannann. Preparation of [¹⁸F]fluoromannann with [¹⁸F]BCN as the prosthetic group (A), HPLC chromatograms of the conjugation product with radioactive detection (B), and the reference [¹⁹F]fluoromannann with UV detection (C).

hydrophilicity of polysaccharides, the octanol–PBS distribution coefficient (logD) of [¹⁸F]fluoromannann was -1.48 . In PBS at r.t., [¹⁸F]fluoromannann was stable over 4 h (longer time was not tested), thus being suitable for *in vivo* applications.

Bearing in mind that an aim of this work was to investigate biodistribution of [¹⁸F]fluoromannann, a set of biological studies was carefully designed. Since mannann was supposed to bind to mannose receptors in macrophages in inflamed tissues, we reasoned that aorta from atherosclerotic mice could be appropriate tissues for this type of study. These mice do not have vulnerable plaques, i.e., the plaques do not rupture like human plaques may do. However, these mice have highly inflamed plaques, and most of the inflammatory cells are macrophages. We incubated mice aorta sections bearing atherosclerotic plaques with [¹⁸F]fluoromannann. Indeed, the accumulation of [¹⁸F]fluoromannann in the lesions was clearly visualized (Figure 5A). Comparison with hematoxylin-eosin staining (Figure 5B) revealed that the binding was most prominent in plaques and calcified areas, whereas binding in normal appearing vessel wall was low. Furthermore, the

Figure 5. [^{18}F]fluoromannan PET studies. (A) *In vitro* autoradiography of an atherosclerotic mouse aorta section (P plaque, C calcified area, W vessel wall). (B) Hematoxylin-eosin staining of the same section. (C) PET image of a rat (L liver, S spleen, K kidney, B urinary bladder).

[^{18}F]fluoromannan binding specificity was confirmed by competitive blocking assays with 100-fold excess of unlabeled mannan as well as with healthy C57BL6N mice aorta (SI). To study the biodistribution *in vivo*, healthy Sprague–Dawley rats ($n = 6$) were intravenously administered with [^{18}F]fluoromannan (20.0 ± 1.3 MBq per rat), and 3 h dynamic PET imaging was performed with a High Resolution Research Tomograph device (Siemens). Images were iteratively reconstructed with the ordered-subsets expectation maximization 3D algorithm. As shown in the Figure 5C, the radioactivity accumulated largely in the macrophage-rich organs such as liver, spleen, and bone marrow and the excess excreted in urine. The *ex vivo* measurements of the excised organs/tissues at 3 h postinjection confirmed PET imaging results (SI).

In conclusion, an IEDDA-based ligation strategy has been devised for ^{18}F -labeling of mannan, a polysaccharide substrate, under biocompatible conditions. To the best of our knowledge, this is the first attempt to ^{18}F -label the mannan itself and explore its fate *in vivo* by PET. The corresponding ^{19}F -labeled mannan has been used to induce psoriasis and psoriatic arthritis in mice, which indicates that the biological function of mannan is preserved after the chemical modifications. Furthermore, ^{18}F -labeled mannan has been shown to bind macrophage-rich tissues *in vitro* and *in vivo*. Further animal studies with [^{18}F]fluoromannan are warranted to set up protocols for monitoring mannan distribution in the context of using mannan as therapeutics or carriers for drug delivery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00160.

Experimental details for the preparation and analysis of compounds 2, 3, [^{19}F]fluoromannan, and [^{18}F]fluoromannan. Animal experimental details for [^{19}F]fluoromannan studies *in vivo*. *In vitro* and *in vivo* experiments with [^{18}F]fluoromannan (PDF)

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Author Contributions

X.-G.L., A.R., and R.H. have designed the studies. C.H., R.S., H.V., O.S., H.L., and J.T. have done the biological experiments. X.-G.L. and J.B. have performed the chemistry work. All the aforementioned authors and J.K. have participated in preparing, editing, and approving the content of the manuscript.

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Notes

Animal studies were conducted with approval from the Lab-Animal Care and Use Committee of the State Provincial Office of Southern Finland and in compliance with the European Union directive related to conduct of animal experimentation. The authors declare no competing financial interest.

■ ABBREVIATIONS

[^{18}F]BCN, ^{18}F -bicyclo[6.1.0]nonyne; CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; HPLC, high-performance liquid chromatography; IEDDA, inverse electron demand Diels–Alder reaction; Ncf1^{m1/m1}, neutrophil cytosolic factor 1 gene mutation; PBS, phosphate-buffered saline; PET, positron emission tomography; [^{18}F]TCO, ^{18}F -trans-cyclooctene; TLC, thin-layer chromatography

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