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Article

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The label matters: μ PET imaging of the
biodistribution of low molar mass ^{89}Zr and ^{18}F -
labeled poly(2-ethyl-2-oxazoline)

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Abstract. Poly(2-alkyl-2-oxazoline)s (PAOx) have received increasing interest for biomedical applications. Therefore it is of fundamental importance to gain an in-depth understanding of the biodistribution profile of PAOx. We report the biodistribution of poly(2-ethyl-2-oxazoline) (PEtOx) with a molar mass of 5 kDa radiolabeled with PET isotopes ^{89}Zr and ^{18}F . ^{18}F -labeled PEtOx is prepared by the strain-promoted azide-alkyne cycloaddition (SPAAC) of [^{18}F]fluoroethylazide to bicyclo[6.1.0]non-4-yne (BCN) functionalized PEtOx as many common labeling strategies were found to be unsuccessful for PEtOx. ^{89}Zr -labeled PEtOx is prepared using desferrioxamine end-groups as a chelator. 5 kDa PEtOx shows a significantly faster blood clearance compared to PEtOx of higher molar mass while uptake in the liver is lower indicating a minor contribution of the liver in excretion of the 5 kDa PEtOx. While [^{18}F]-PEtOx displays a rapid and efficient clearance from the kidneys, [^{89}Zr]-Df-PEtOx 5 kDa is not efficiently cleared over the time course of the study which is most likely caused by trapping of ^{89}Zr -labelled metabolites in the renal tubules and not the polymer itself demonstrating the importance of selecting the appropriate label for biodistribution studies.

Keywords: poly(2-oxazoline); radiolabeling; biodistribution; end-group modification

Introduction

The conjugation of synthetic polymers to physiologically active (bio)molecules as a strategy to improve the pharmacokinetic (PK) and pharmacodynamic (PD) profile of these compounds has gained increasing interest in recent decades.^{1, 2} Poly(ethylene glycol) (PEG; also known as poly(ethylene oxide) [PEO]) whose covalent attachment to bioactive substances is known as PEGylation is the most frequently used polymer for this purpose.^{3, 4} Although PEG is currently considered as gold standard in the field, intensive studies of its physiological behavior also revealed some drawbacks including vacuolization in the kidneys,^{5, 6} formation of toxic oxidative breakdown products and the induction of immune responses.⁷ Therefore, alternatives to PEG in biomedical applications are investigated.^{3, 8, 9} In addition, the structure of PEG permits the covalent attachment of molecules only at the chain ends generating the demand for chemically more versatile polymers with equal or superior *in vivo* behavior compared to PEG. Poly(2-alkyl-2-oxazoline)s (PAOx) represent a promising class of such polymers. Their preparation by living cationic ring-opening polymerization (CROP) of 2-oxazolines generates well-defined polymers whose properties can be tuned by variation of the side chain structure.¹⁰ In particular PAOx with short aliphatic side chains such as methyl (poly(2-methyl-2-oxazoline), PMeOx) or ethyl (poly(2-ethyl-2-oxazoline), PEtOx) are hydrophilic polymers with similar biocompatibility, hydration and stealth behavior to PEG.¹¹⁻¹³ PAOx with synthetic handles at both chain termini can be easily prepared using functional initiators or terminating agents (Scheme 1).¹⁴

Scheme 1. General scheme of the CROP of 2-oxazolines

Therefore PAOx have received increasing attention as potential alternative to PEG in biomedical applications.^{10, 15, 16} It is crucial for the further development of such applications to gain an in-depth understanding of the biodistribution profile of PAOx.

Micro positron emission tomography (μ PET) is a non-invasive molecular imaging technique which allows longitudinal follow-up of the *in vivo* behavior of radiolabeled molecules in small animals. For this purpose molecules can be radiolabeled via covalent attachment of a nonmetal PET isotope of which ^{11}C (half life = 20 min) and ^{18}F (half life = 110 min) are the most frequently utilized, or they can be radiolabeled by incorporation of a PET radiometal like ^{68}Ga (half life = 68 min), ^{64}Cu (half life = 12.7 h) or ^{89}Zr (half life = 78.4 h) through chelation. The choice of PET radioisotope mostly depends on the biological half life of a molecule which should match the physical half life of the radioisotope. By intravenous injection of the radiolabeled molecule into an animal and subsequent μ PET imaging, the pharmacokinetic behavior and biodistribution of the molecule in the body can be qualitatively and quantitatively studied over time in a non-invasive manner. We have recently published the first systematic study of the biodistribution of low dispersity PEOx in a molar mass range of 20 to 110 kDa by μ PET imaging employing ^{89}Zr -labeled PEOx.¹⁷ That study revealed that the blood clearance of PEOx decreases with increasing molar mass as previously described for other non-ionic polymers. The cut off for glomerular filtration of PEOx was found to be around 40 kDa compared to a previously described cut off of 30 kDa for PEG. Furthermore, no unspecific adsorption nor kidney retention was observed for PEOx with molar masses of 20 kDa up to 40 kDa. Herein we report an expansion of the previous study, investigating the biodistribution of PEOx with a lower molar mass (5 kDa) radiolabeled with PET isotopes ^{89}Zr or ^{18}F . We aim to extend the understanding of the *in vivo* behavior to a wider molar mass range on the one hand

and examine the influence of the radiolabeling methodology on the observed biodistribution on the other hand. Such lower molar mass PEtOx is often used as building block for constructing larger assemblies, including polymer-protein conjugates, and, thus, its clearance profile is of interest. During the course of this studies, unexpected difficulties in modification of the PEtOx end-group were encountered, which may be important for designing future modification protocols.

Experimental Section

Materials. Acetonitrile and triethylamine (Aldrich) were dried in a solvent purification system (J. C. Meyer). 2-Ethyl-2-oxazoline (EtOx; Aldrich) was distilled over barium oxide and stored under argon. Methyl tosylate (MeOTs) was distilled and stored under argon. Piperazine was purified by sublimation and stored under argon. (1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (BCN-PNP) was purchased from SynAffix and stored at -20°C. All other chemicals were purchased from Sigma-Aldrich or Acros Organics and used as received.

Instrumentation. Polymerizations were performed in a capped vial in a microwave reactor (Biotage Initiator Sixty) equipped with an IR temperature sensor. ¹H NMR spectra were recorded in CDCl₃ on a Bruker Avance 300 MHz spectrometer. Size-exclusion chromatography (SEC) was performed on a Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostatted column compartment (TCC) at 50°C equipped with two PLgel 5 μm mixed-D columns and a precolumn in series, a 1260 diode array detector (DAD) and a 1260 refractive index detector (RID) using *N,N*-dimethylacetamide containing 50 mM of LiCl (flow rate of 0.59 mL min⁻¹) as solvent. Molar masses were calculated against polymethylmethacrylate standards.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was performed on an Applied Biosystems Voyager De STR MALDI-TOF mass spectrometer equipped with 2 m linear and 3 m reflector flight tubes. All mass spectra were obtained with an accelerating potential of 20 kV in positive ion mode and in reflectron mode (matrix: trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB), 2-(4'-hydroxybenzeneazo)benzoic acid (HABA) was used for PEtOx-pip, salt: sodium trifluoroacetate).

Synthesis of PEtOx-pip. A solution of EtOx (496 mg, 5.00 mmol) and MeOTs (15.1 μ L, 0.10 mmol) in acetonitrile with an initial monomer concentration of 4 M was prepared in a microwave vial and closed with a crimp cap under argon. The polymerization mixture was heated to 140 $^{\circ}$ C for 7.5 min under microwave irradiation and subsequently cooled to ambient temperature. The polymerization mixture was diluted with 0.5 mL acetonitrile, added to a solution of piperazine (86.1 mg, 1 mmol) in 1 mL acetonitrile and stirred at ambient temperature for 1 h. The solution was concentrated under reduced pressure and the residue dissolved in dichloromethane. The solution was filtered through a short pad of alumina and the polymer was precipitated in diethyl ether. DMac-SEC: M_n = 8.0 kDa, $\bar{D}$ = 1.08; MALDI-TOF MS: M_n = 4.9 kDa, $\bar{D}$ = 1.02.

Synthesis of PEtOx-BCN. To a solution of PEtOx-pip (200 mg, 0.04 mmol) and BCN-PNP (15.8 mg, 0.05 mmol) in 2 mL dichloromethane was added triethylamine (17 μ L, 0.12 mmol) and the reaction mixture was stirred at ambient temperature overnight. The solution was passed through a plug of basic alumina to remove the *p*-nitrophenol byproduct and the polymer was precipitated in diethyl ether. The polymer was redissolved in milli-Q water and lyophilized. DMac-SEC: M_n = 8.0 kDa, $\bar{D}$ = 1.08. ; MALDI-TOF MS: M_n = 5.0 kDa, $\bar{D}$ = 1.02.

Radiosynthesis of [^{89}Zr]-Df-PEtOx. ^{89}Zr -labelled PEtOx with a molar mass of 5 kDa was prepared by chelation of ^{89}Zr into desferal modified PEtOx (Df-PEtOx) as previously described.¹⁷ Following purification over a Sephadex G25 PD-10 desalting column (GE Healthcare) eluted with a solution of 5 mg/mL gentisic acid in 0.25 M aqueous sodium acetate the ^{89}Zr -labeled polymer was sterile filtered over a 0.2 μm filter (Acrodisc syringe filter, Pall Life Sciences) into a sterile vial for *in vivo* evaluation.

Radiosynthesis of ^{18}F -PEtOx. No carrier-added [^{18}F] F^- was produced in a Siemens Eclipse HP cyclotron by bombardment of [^{18}O] H_2O (Rotem Industries, Israel) by the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction. ^{18}F -labeling of PEtOx-BCN was performed in an automated module (Fluorsynthon II, Comecer Netherlands) using a two-step procedure. First, [^{18}F]-fluorethylazide (FEA) was prepared by reaction of 2-azidoethyl-4-toluenesulfonate (2 mg in 0.5 mL acetonitrile) with azeotropically dried [^{18}F] F^- at 130 $^{\circ}\text{C}$ for 10 min. The obtained [^{18}F]-FEA was distilled into a second reactor, and reacted with PEtOx-BCN (1 mg in 0.8 mL water) for 15 min at 100 $^{\circ}\text{C}$. The reaction mixture was dried under a stream of N_2 to remove acetonitrile, redissolved in water and purified using a Sephadex G25 PD-10 cartridge eluted with phosphate buffered saline (PBS). Quality control was performed using a Zenix-C80 C-18 column (Sepax Technologies Inc., USA) and 10mM sodium phosphate buffer, pH 7.0 as mobile phase.

In vitro plasma stability. [^{89}Zr]-Df-PEtOx and [^{18}F]-PEtOx were diluted in mouse plasma (1/10) and incubated at 37 $^{\circ}\text{C}$ up to 24 h for [^{89}Zr]-Df-PEtOx and up to 2 h for [^{18}F]-PEtOx. At different time points aliquots were analyzed for radiochemical purity. For [^{89}Zr]-Df-PEtOx, 5 μL of analyte was spotted onto an iTLC-SG strip, the strip was developed in 90/10 aqueous 20mM citric acid - MeCN, dried, cut in halves and each part was measured in an automated gamma counter. The RCP was expressed as percentage bound radioisotope and calculated as: $100 \times$

[Radioactivity bottom strip half_(CPM)/(Radioactivity bottom strip half_(CPM) + Radioactivity top strip half_(CPM))]. For [¹⁸F]-PEtOx plasma proteins were precipitated with acetonitrile, and the supernatant was analyzed by analytical radio-HPLC, using a Zenix-C80 C-18 column and 10mM sodium phosphate buffer, pH 7.0 as mobile phase. The RCP was assessed by comparing the radioactivity peak corresponding to intact [¹⁸F]-PEtOx to the total activity registered in the radiochromatogram.

μPET imaging of [¹⁸F]-PEtOx and [⁸⁹Zr]-Df-PEtOx. All animal studies were ethically performed in accordance with European Directive 86/609/EEC Welfare and Treatment of Animals. In addition, all animal experiments were approved (ECD 2013-34) by the ethical committee of the University of Antwerp (Belgium).

To evaluate the pharmacokinetic profile, C57BL/6 mice (n = 4 for per polymer) were anesthetized with isoflurane (5% for induction, 2% for maintenance) and intravenously injected with purified [¹⁸F]-PEtOx (14.48 ± 2.66 Mbq, ~100 μg) or [⁸⁹Zr]-Df-PEtOx (10.63 ± 1.51 MBq, ~100 μg) via a tail vein catheter. Following injection, a dynamic scan of 120 min was acquired (Siemens Inveon PET-CT) and rebinned in static frames as follows: 16 frames of 30 s, 5 frames of 90 s and 20 frames of 300 s. Thereafter the mouse was removed from the scanner and allowed to regain consciousness. At the time corresponding to 4 h post tracer injection, mice were again anesthetized and a static scan of 10 min was acquired. Furthermore, the mice injected with [⁸⁹Zr]-Df-PEtOx underwent an additional static scan at 8 h and 24 h post tracer injection given the longer half life of ⁸⁹Zr. For quantitative analysis PET data were reconstructed with 2-dimensional ordered subset estimation maximisation (OSEM2D) using 4 iterations and 16 subsets¹⁹ following Fourier rebinning (FORE).²⁰ The PET images were reconstructed on a 128 × 128 × 159 grid with a pixel size of 0.776 × 0.776 × 0.776 mm. Normalization, dead time,

random, CT-based attenuation and single scatter stimulation (SSS)²¹ scatter corrections were applied. Subsequent to all PET scans, a 5 min CT scan was acquired using a 220 degree rotation with 12-rotation steps at 80 keV and 500 μ A.

Volumes of interest (VOI) were delineated for liver, kidneys and heart in PMOD v3.3 (PMOD Technologies, Switzerland) to allow quantification of the in vivo behavior of [¹⁸F]-PEtOx and [⁸⁹Zr]-Df-PEtOx. Standard uptake values (SUV) were calculated as follows:

$$SUV = (tissue\ uptake[kBq/cc] * body\ weight[g]/injected\ dose[kBq] * 100).$$

Time-activity curves (TACs) for the average radioactivity in these organs, expressed as standardized uptake values (SUVs) as a function of time, were determined. For visual comparison of polymer uptake, normalized images were scaled according to SUV.

Autoradiography. To further investigate accumulation of activity in the kidneys for animals injected with [⁸⁹Zr]-Df-PEtOx, autoradiography of kidney slices was performed. Therefore, animals were sacrificed after the last scan at 24 h pi by cervical dislocation under isoflurane anesthesia and the kidneys were removed and processed for autoradiography. Kidneys were snap-frozen and subsequently sectioned on a cryostat (Leica CM1950). Sections (100 μ m) were dried and exposed to Fujifilm BAS IP MS 2025 plates for 5 min. The plates were imaged using a Fuji Phosphoimager system (FLA7000).

Results and Discussion

Synthesis of ⁸⁹Zr-and ¹⁸F-labeled PEtOx

In our previously reported study¹⁷ on the biodistribution of PEtOx with a molar mass between 20 and 110 kDa, the long living PET isotope ⁸⁹Zr was selected for radiolabeling and consecutive μ PET imaging because of the expected longer blood circulation time of the higher molar mass

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PEtOx. Although shorter circulation times were anticipated for the 5 kDa PEtOx evaluated in the current study, we included ^{89}Zr labeling of the low molar mass PEtOx as well for consistency. As previously reported, PEtOx 5 kDa was labeled with ^{89}Zr by modification with a desferal chelator followed by incubation with a buffered [^{89}Zr]Zr(III) oxalate solution for 20 min at room temperature. Using this strategy, ^{89}Zr -labelled PEtOx could be obtained in a radiochemical yield (RCY) of 70% and a radiochemical purity (RCP) of > 90%. To use a radiolabel which better fits the expected faster clearance of a low molar mass polymer as well as to determine the impact of the radiolabel on the observed biodistribution of the polymer, PEtOx 5 kDa was also labeled with shorter living PET isotope ^{18}F . Before engaging into radiochemistry, different labeling strategies were evaluated using cold fluorination reactions with ^{19}F with 2 kDa PEtOx to facilitate easy end-group analysis by ^1H NMR spectroscopy and MALDI-TOF MS. To date, only a few examples of ^{18}F labeled synthetic polymers have been reported, mostly via the side chains.²² Copolymers based on *N*-(2-hydroxypropyl)-methacrylamide (HPMA) were labeled via reaction of 2- [^{18}F]fluoroethyl-1-tosylate with phenolic tyramine moieties.^{23, 24} Akai *et al.* reported the synthesis of ^{18}F labeled PEG by fluorination of PEG tosylates with [^{18}F]tetrabutylammonium fluoride ([^{18}F]TBAF) (Scheme 1a).²⁵ We attempted to transfer this strategy to the synthesis of ^{18}F labeled PEtOx as shown in Scheme 1b as hydroxyl terminated PEtOx (PEtOx-OH) can be easily prepared by termination of the living polymerization mixture with tetramethylammonium hydroxide.²⁶ The labeling precursor PEtOx-OTs was prepared by tosylation of PEtOx-OH (2 kDa) using tosyl chloride and triethylamine (see Supporting Information for experimental details and characterization).

a) Previously reported fluorination of PEG

b) Attempted fluorinations of PEtOx

Scheme 1 a) Previously reported fluorination of PEG. b) Attempted fluorinations of PEtOx.

The fluorination of PEtOx-OTs (2 kDa) was subsequently investigated by reacting it with TBAF or KF/Kryptofix[2.2.2] in acetonitrile at 80 °C for 1 h. Analysis by MALDI-TOF MS revealed that no reaction had occurred with both fluorinating agents although 90% conversion after 20 min has been reported for PEG under those conditions.²⁵ The fluorination reaction of PEtOx-OTs was equally unsuccessful employing DMF or DMSO as solvent. To ensure that the reaction conditions were chosen properly the fluorination of PEG-OTs (2 kDa) was also tested employing identical reaction conditions. ¹H NMR analysis showed close to quantitative fluorination of PEG-OTs (see Figure S5) confirming the results of Akai *et al.* indicating an unanticipated difference in reactivity of PEtOx-OTs compared to PEG-OTs. Therefore, a tosylated PEtOx with a diethylene glycol spacer (PEtOx-DEG-OTs) that resembles the chain-end structure of PEG-OTs was synthesized by termination of the living CROP of EtOx with 1-[2-(2-hydroxyethoxy)ethyl]piperazine as termination agent followed by tosylation of the hydroxy group (see Supporting Information for experimental details). However, MALDI-TOF MS

monitoring (Figure 1) of the fluorination reaction using the conditions described above revealed that unreacted PEtOx-DEG-OTs was the solely present species after 1 h of reaction. This result clearly indicated that the difficulty in the end-group modification is not related to a difference in reactivity of the tosylate end-group, but apparently is related to the different nature of the polymer chains. Even though we do not fully understand how the PEtOx lowers the reactivity compared to PEG, it may be speculated that the polymer chain ends are less accessible in the PEtOx, *i.e.* they are hidden inside the polymer coil, hindering modification.

Figure 1 MALDI-TOF MS spectra of PEtOx-DEG-OTs before (top) and after 1 h reaction with TBAF (middle) and KF/K[2.2.2] (bottom) at 80 °C in MeCN. The peak assignments refer to the sodium adduct $[M+Na]^+$. PEtOx with a tosyl end-group (**1** and **1'**) is the only detected species. The minor distribution **1'** originates from a chain transfer reaction during polymerization.²⁷

An alternative strategy to nucleophilic aliphatic substitution is ^{18}F labeling by nucleophilic aromatic substitution of arenes with a strong electron-withdrawing group in *ortho* or *para* position to the leaving group.²⁸ To examine this strategy for the fluorination of PEtOx,

3,4-dinitrobenzoate (DNB) was used as termination agent for the living CROP of EtOx forming the ester terminated PEtOx-DNB (Scheme 2) as confirmed by ^1H NMR spectroscopy (see Figure S4).

Scheme 2 Attempted fluorination of PEtOx via nucleophilic aromatic substitution.

Fluorination by $\text{S}_{\text{N}}\text{Ar}$ commonly requires high temperatures and polar aprotic solvents.²⁸ PEtOx-DNB was therefore reacted with TBAF or $\text{KF/K}[2.2.2]$ in DMSO at 150 °C. However, no PEtOx-FNB could be detected after 1 h reaction time. This observation further supports the hypothesis that the failure of these direct fluorinations is due to the limited accessibility of the chain ends that may be hidden in the polymer globules.

Due to the failure of these direct fluorination approaches an indirect fluorination of PEtOx exploiting an ^{18}F -labeled prosthetic group was designed. It was previously demonstrated that bicyclo[6.1.0]non-4-yne (BCN) functionalized PAOx undergo rapid strain-promoted azide-alkyne cycloaddition (SPAAC) reactions with various azides.^{29, 30} Hence the SPAAC reaction between PEtOx-BCN, prepared via a previously reported route,²⁹ and $[^{18}\text{F}]$ fluoroethylazide depicted in Scheme 3 was selected as a promising strategy for the radiosynthesis of ^{18}F -labeled PEtOx. The living CROP was terminated by adding the polymerization mixture to a solution containing excess of piperazine to avoid chain coupling as was confirmed by comparison with a fraction of the polymer that was terminated with a monofunctional terminating agent. Subsequently, the BCN-moiety was introduced by reacting the piperazine end-capped polymer

with (1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (BCN-PNP). The synthesis of [¹⁸F]fluoroethylazide followed by distillation and click reaction with PEtOx-BCN was performed in an automated synthesis module and [¹⁸F]-PEtOx could be successfully obtained in a RCY of 4.30% (n=4, decay corrected to end of bombardment). HPLC analysis of [¹⁸F]-PEtOx purified by Sephadex G-25 (PD-10 column) revealed a RCP of 100%. The analytical data of the two polymers that were used for radiolabeling and *in vivo* imaging are summarized in Table 1.

Table 1. Analytical data of the synthesized poly(2-ethyl-2-oxazoline)s used in this study.

Polymer	radiolabel	M_n	$\bar{D}$
Df-PEtOx	⁸⁹ Zr	5.1 kDa ^a	1.01
PEtOx-BCN	¹⁸ F	5.0 kDa ^b	1.08

^a by SEC-MALS in DMAc (50 mM of LiCl)

^b determined by SEC in DMAc (50 mM of LiCl) corrected according to ref. 18

iTLC analysis of [^{89}Zr]-Df-PEtOx following incubation in mouse plasma demonstrated only minor loss of radiolabel with $87.35 \pm 1.04\%$ of intact [^{89}Zr]-Df-PEtOx remaining at 24 h post incubation (note that *in vivo* PET imaging was performed for 4 h). For [^{18}F]-PEtOx only intact tracer was detected by radio-HPLC analysis following a 2h incubation in mouse plasma. This high plasma stability of both tracers was also observed in the PET imaging study. The long half-life of ^{89}Zr allowed *in vivo* evaluation of the pharmacokinetic profile of [^{89}Zr]-Df-PEtOx up to 24 h post intravenous injection. For [^{18}F]-PEtOx, PET imaging was performed up to 4 h post injection (pi). Representative $\mu\text{PET}/\text{CT}$ images are presented in Figure 2. ^{18}F or ^{89}Zr are known to be sequestered by the bone when released in the plasma. For both tracers, bone uptake was very limited ($\text{SUV} = 0.02 \pm 0.02$ at 4h pi for [^{18}F]-PEtOx, and $\text{SUV} = 0.01 \pm 0.00$ at 4h pi and

SUV = 0.01 ± 0.00 at 24h pi for [^{89}Zr]-Df-PEtOx) indicating minimal release of radiolabel in the plasma *in vivo*. The radioactivity was quantified in the heart as a measure of blood pool activity as well as in the major excretory organs liver and kidneys. Compared to [^{18}F]-PEtOx, [^{89}Zr]-Df-PEtOx displayed an initial higher radioactivity in the heart (peak SUV of 7.71 ± 1.14 for [^{89}Zr]-Df-PEtOx and 3.31 ± 0.49 for [^{18}F]-PEtOx) and a slightly slower blood clearance, yet both tracers were almost completely cleared from the blood pool within 1 h pi (SUV = 0.19 ± 0.04 for [^{89}Zr]-Df-PEtOx and SUV = 0.10 ± 0.01 for [^{18}F]-PEtOx at 62 min pi) (Figure 3 A). As expected, the blood clearance is significantly faster compared to that of the previously reported higher molar mass [^{89}Zr]-Df-PEtOx (SUV = 1.61 ± 0.07 for 20 kDa [^{89}Zr]-Df-PEtOx and 5.34 ± 0.33 for 110 kDa [^{89}Zr]-Df-PEtOx at 62 min pi).¹⁷

Figure 2 Representative μ PET/CT images of C57BL/6 mice injected with [^{18}F]-PEtOx (A and B) or [^{89}Zr]-Df-PEtOx (C and D) at 1 min (A and C) or 60 min (B and D) pi.

Figure 3 Time-activity curve up to 4 h pi for the heart uptake (A) and for kidney and liver uptake (B) extracted from in vivo μPET scans of C57BL/6 mice injected with $[^{89}\text{Zr}]\text{-Df-PEtOx}$ or $[^{18}\text{F}]\text{-PEtOx}$ 5 kDa. Results are expressed as $\text{SUV} \pm \text{SD}$ ($n = 4$).

Likewise, the uptake of PEtOx 5 kDa in the liver was significantly lower compared to the previously studied higher molar mass PEtOx ($\text{SUV} = 0.07 \pm 0.01$, 0.26 ± 0.12 , 1.16 ± 0.29 and 2.71 ± 0.33 at 62 min pi for $[^{18}\text{F}]\text{-PEtOx}$ 5 kDa, $[^{89}\text{Zr}]\text{-Df-PEtOx}$ 5 kDa, $[^{89}\text{Zr}]\text{-Df-PEtOx}$ 20 kDa and $[^{89}\text{Zr}]\text{-Df-PEtOx}$ 110 kDa, respectively). The low uptake and fast clearance of PEtOx 5 kDa from the liver indicates a minor contribution of the liver in the excretion of the 5 kDa polymer from the body and most likely mainly reflects blood circulation in the liver. As was seen for the heart uptake, the use of ^{18}F as radiolabel had only a minor impact on the pharmacokinetic profile with a slightly faster clearance for $[^{18}\text{F}]\text{-PEtOx}$ compared to $[^{89}\text{Zr}]\text{-Df-PEtOx}$ 5 kDa (Figure 3 B). However, the uptake of both $[^{18}\text{F}]\text{-PEtOx}$ and $[^{89}\text{Zr}]\text{-Df-PEtOx}$ in the kidneys was remarkably higher compared to the liver uptake pointing to a dominant renal excretion of PEtOx 5 kDa (Figure 3 B). For the previously studied higher molar mass PEtOx, SUV in the kidney remained low (< 3.5) indicating a limited participation of the kidneys to clearance of higher molar mass PEtOx. In fact, the cut off for glomerular filtration was found to be around 40 kDa.¹⁷

While [^{18}F]-PEtOx displayed a rapid and efficient clearance from the kidneys ($\text{SUV} = 7.10 \pm 0.74$ at 2 min pi, 1.76 ± 0.36 at 10 min and 0.28 ± 0.02 at 62 min pi) (Figure 3 B), [^{89}Zr]-Df-PEtOx 5 kDa unexpectedly showed a somewhat delayed peak uptake of 7.88 ± 2.54 at 4 min pi and was not efficiently cleared over the time course of the study ($\text{SUV} = 4.63 \pm 1.62$ at 10 min pi, 3.83 ± 1.24 at 62 min pi, 3.58 ± 1.11 at 4 h pi and 2.36 ± 0.84 at 24 h pi). Unexpectedly, the retention of radioactivity in the kidneys even exceeded that of all other tested higher molar mass ^{89}Zr -labeled polymers evaluated previously up to 24 h pi (Figure 4).¹⁷

Figure 4 Uptake of [^{89}Zr]-Df-PEtOx 5 kDa in the kidneys at several time points post intravenous injection in comparison to previously reported kidney uptake for [^{89}Zr]-Df-PEtOx 20 – 110 kDa.¹⁷ Results are expressed as $\text{SUV} \pm \text{SD}$ ($n = 4$).

The rapid and efficient renal clearance that was witnessed for [^{18}F]-PEtOx has been demonstrated before for ^{125}I and ^{18}F labelled low molar mass PEG.^{25, 31} A study by Gaertner *et al.* using 4.5 kDa ^{111}In -labeled PEtOx and PMeOx on the other hand also demonstrated high uptake of activity in the kidneys (max 3.64 %ID/g at 30 min pi) followed by kidney retention

(2.39 %ID/g at 3 h pi) with almost no washout up to 24 h pi (2.38 %ID/g).³² The authors explain the kidney retention by trapping of the tracer into the renal tubular cells. As in that study radiometal ¹¹¹In was used for labeling of the polymer, the unusually high kidney retention witnessed for [⁸⁹Zr]-Df-PEtOx 5 kDa in the current study might also be related to the use of a radiometal for radiolabeling. Non-specific retention in the kidneys of radiometallated (eg ¹¹¹In, ⁹⁰Y, ¹⁷⁷Lu) peptides and proteins is a known issue. Molecules with a molar mass < ~12 kDa are freely filtered by glomerular filtration.³³ The major part of the filtered load is reabsorbed in the proximal renal tubules. Endocytosis in the proximal tubules of radiometallated peptides and proteins followed by lysosomal degradation can result in trapping of radiolabelled metabolites in the proximal tubules.²¹ Indeed, autoradiography of the kidneys indicated accumulation of the [⁸⁹Zr]-Df-PEtOx 5 kDa in the renal cortex where renal tubules are located (Figure 5). The low molecular weight of both [¹⁸F]-PEtOx and [⁸⁹Zr]-Df-PEtOx 5 kDa thus results in a more efficient glomerular filtration compared to the previously studied high molecular weight PEtOx. However, while ¹⁸F-labeled metabolites formed in the proximal tubules are efficiently cleared via the urine, ⁸⁹Zr-labeled metabolites probably become trapped in the lysosomes, resulting in accumulation of radioactivity in the renal cortex. The kidney uptake witnessed in the μ PET imaging can therefore most likely be related to the use of ⁸⁹Zr for radiolabeling and not to the polymer itself.

Figure 5 Representative autoradiography images of kidney sections showing dominant cortical uptake of [^{89}Zr]-Df-PEtOx 5 kDa at 24 h pi.

Conclusions

Herein, we have reported the biodistribution of PEtOx with a molar mass of 5 kDa radiolabeled with PET isotopes ^{89}Zr or ^{18}F . Direct fluorination attempts of PEtOx by nucleophilic aliphatic or aromatic substitution were unsuccessful proposedly due to inaccessibility of the reactive chain ends in the polymer globules. ^{18}F -labeled PEtOx was prepared by the SPAAC of [^{18}F]fluoroethylazide to BCN functionalized PEtOx. Both polymers showed a significantly faster blood clearance compared to PEtOx of higher molar mass.¹⁷ While [^{18}F]-PEtOx displayed a rapid and efficient clearance from the kidneys [^{89}Zr]-Df-PEtOx 5 kDa was not efficiently cleared over the time course of the study which is most likely caused by trapping of ^{89}Zr -labeled metabolites in the renal tubules and not the polymer itself.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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