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RESEARCH ARTICLE

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[^{11}C]Carboxylated Tetrazines for Facile Labeling of Trans-Cyclooctene-Functionalized PeptoBrushes

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Functionalization of macromolecules (antibodies, polymers, nanoparticles) with click-reactive groups greatly enhances the versatility of their potential applications. Click chemistry based on tetrazine – trans-cyclooctene (TCO) ligation is especially promising and is already widely applied for pretargeted imaging and therapy. Indirect radiolabeling of TCO-functionalized macromolecules with substoichiometric amounts of radioactive tetrazines is a convenient way to monitor the fate of those macromolecules by means of positron emission tomography (PET) imaging after their administration into the test subject. In this work, the preparation is reported of TCO-containing graft copolymers, namely PeptoBrushes (polyglutamic acid-graft-polysarcosine), novel [^{11}C]carboxylated tetrazines, and their combined use in radiolabeling the polymer by inverse electron demand Diels Alder reaction, to investigate it is potential for an application in pretarget imaging or injectable brachytherapy. The procedure for [^{11}C]tetrazine production is easy and scalable, while indirect TCO-PeptoBrushes labeling with these [^{11}C]tetrazines is mild, fast, and quantitative. This strategy allows facile ^{11}C-labeling of diverse TCO-functionalized macromolecules, so that their localization and distribution shortly after injection can be assessed by PET.

1. Introduction

Engineered macromolecules such as antibodies, polymeric nanoparticles and gels have shown great potential as theranostic (i.e., diagnostic and therapeutic) agents in cancer and infectious diseases.[1]

Antibodies and polymeric nanoparticles can be used as targeting vectors that accumulate in tumors or infected sites by passive or active targeting.[2–4] Polymers and polymeric gels can be used as injectable biodegradable depots for controlled release of therapeutic payload or long-term retention of drugs or therapeutic radiation sources (brachytherapy).[5–8]

Theranostic applications of engineered macromolecules can be greatly enhanced by bio-orthogonal click chemistry, since it enables side specific labeling or drug release inside the body. Modification with click-reactive chemical tags gives macromolecules the capability to capture, retain or release small-molecular payload, which can be a radioactive or fluorescent label for diagnostic imaging, a therapeutic radionuclide or a drug.[9–11] Click chemistry based on inverse-electron demand Diels–Alder cycloaddition between tetrazines (Tzs) and trans-cyclooctenes (TCOs) is an especially versatile modality, which has proven its effectiveness in pretargeted imaging and externally controlled drug release.[12,13] Usually, macromolecules are modified with TCOs while Tzs are used as payload carriers or releasers. The ultrafast kinetics of the Tz-TCO ligation (up to 10^6 $M^{-1} \times s^{-1}$) means that it proceeds efficiently even at low concentrations.[14] Therefore, TCO sites on a macromolecule can be used for different modalities in a fractional manner: for example, a low dose of a radiolabeled Tz can be preclicked to the macromolecule to follow its early fate post-injection via diagnostic imaging, while a high-dose of another Tz optimized for click-to-release reaction will be used later to trigger therapeutic drug release.

Several groups, including our, have recently demonstrated that graft copolymer architectures, namely PeptoBrushes, based on a polyglutamic acid backbone to which polysarcosine (pSar) side chains and TCO molecules are grafted to, are well applicable to pretargeted imaging.[15–20] The unique microstructure of such graft copolymers facilitates the reaction rate of the Tz-TCO ligation by two orders of magnitude, while enhancing TCO stability in vivo.[20] Moreover, polypept(o)ids are in general well

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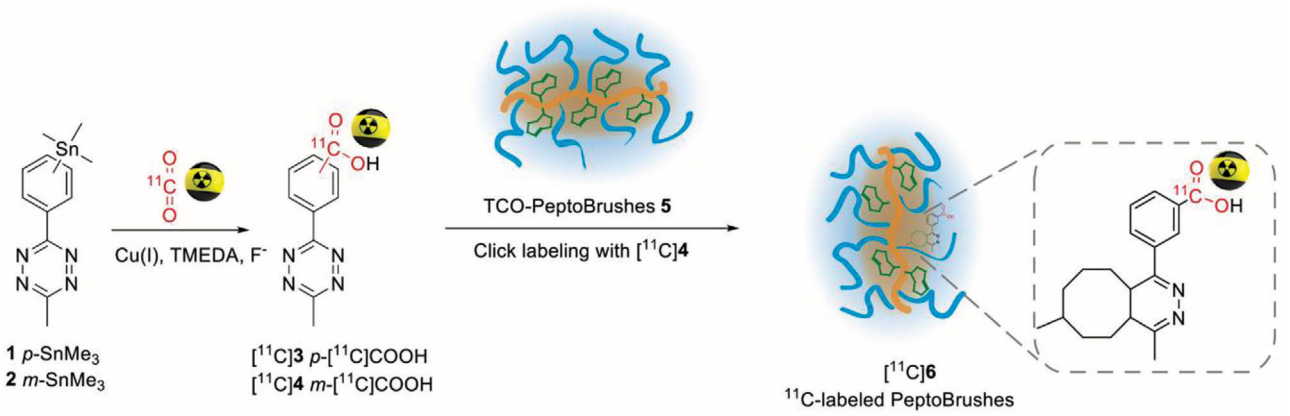
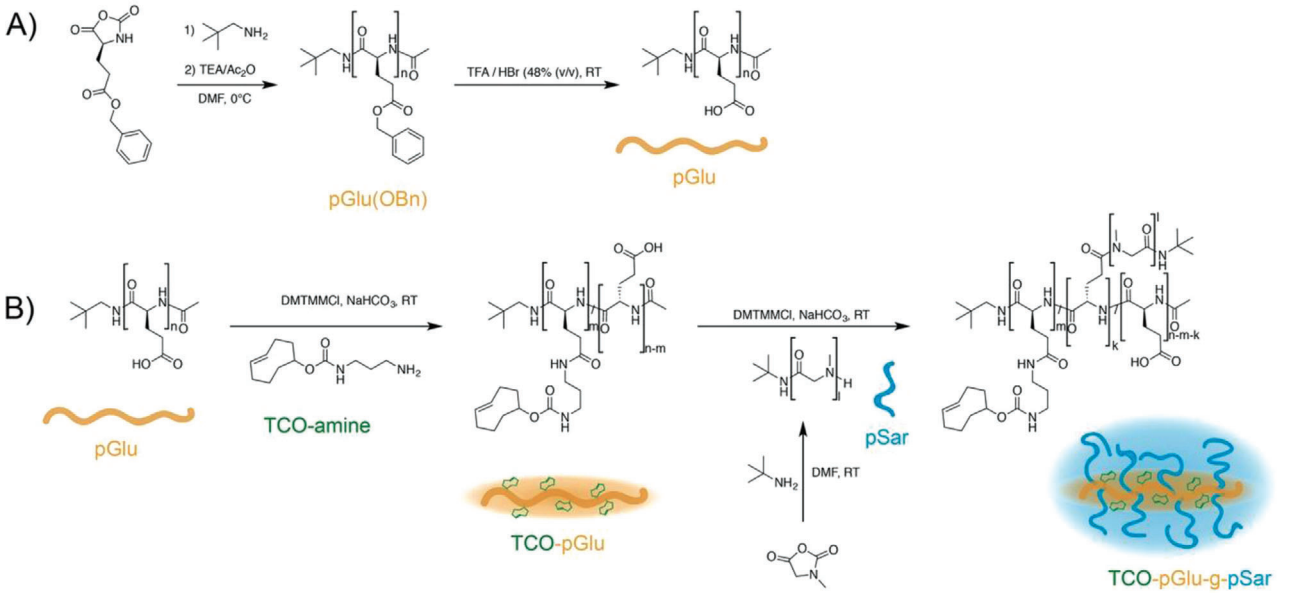


Figure 1. General strategy for application of [^{11}C]carboxylated tetrazines to ^{11}C-labeling of TCO-containing PeptoBrushes.



Scheme 1. Schematically overview of the synthesis of PeptoBrushes by ring-opening polymerization and attachment of pSar side chains and TCO moieties onto the pGlu backbone: A) Synthesis of pGlu backbone. B) Functionalization of pGlu with TCO and grafting-onto polymerization of pSar on the pGlu backbone; $n = 94$, $k =$ pSar grafting density (20%), $m =$ TCO loading (30%), $l = 122$.

tolerated in various in vivo models and even can provide an improved safety profile compared to PEG based systems.[19,21–27]

While our former work was focused on the development of probes for pretargeted imaging using indium or fluorine based tetrazine markers, carbon-11 (^{11}C) based Tz are at least equally interesting. Short-lived positron-emitting isotopes such as ^{11}C are especially well suited to follow the early pharmacokinetics of intravenously injected macromolecules or ensure correct location of injectable theranostic gels by positron emission tomography (PET) imaging.[28,29] In a research setting, the short half-life of ^{11}C (20.4 min) ensures minimal carryover radiation and allows subsequent imaging of other relevant targets in the test subject to be started with minimal delay. In a clinical setting, it ensures minimal radiation dose for the patient.

We previously reported a ^{11}C-labeled Tz, which we successfully conjugated to TCO-functionalized polyglutamic acid and confirmed by PET imaging that the latter polymer is retained in tumors after intratumoral injection.[30,31] However, the above-mentioned [^{11}C]Tz required a multi-step synthesis from the ^{11}C source [^{11}C]CH_4 and was prone to side product formation upon purification.[30] In this work, we report a family of new, more stable ^{11}C-labeled tetrazines, which can be obtained in one step via copper-mediated ^{11}C-carboxylation from [^{11}C]CO_2, a more widely available ^{11}C source.[32] In order to demonstrate their applicability to the radiolabeling of TCO-macromolecules, we conjugated one of the new ^{11}C-labeled tetrazines to a TCO-PeptoBrush polymer (**Figure 1**).[20] This class of polymers has shown promising results as a targeting vector for pretargeted imaging, and, being based on the polyglutamic acid backbone, may also be used as a vehicle for injectable brachytherapy.[20,30] Our work thus presents the first example of copper-mediated ^{11}C-carboxylation of the tetrazine scaffold and opens the path towards easy ^{11}C-labeling of TCO-modified theranostic agents to follow their early fate postinjection.

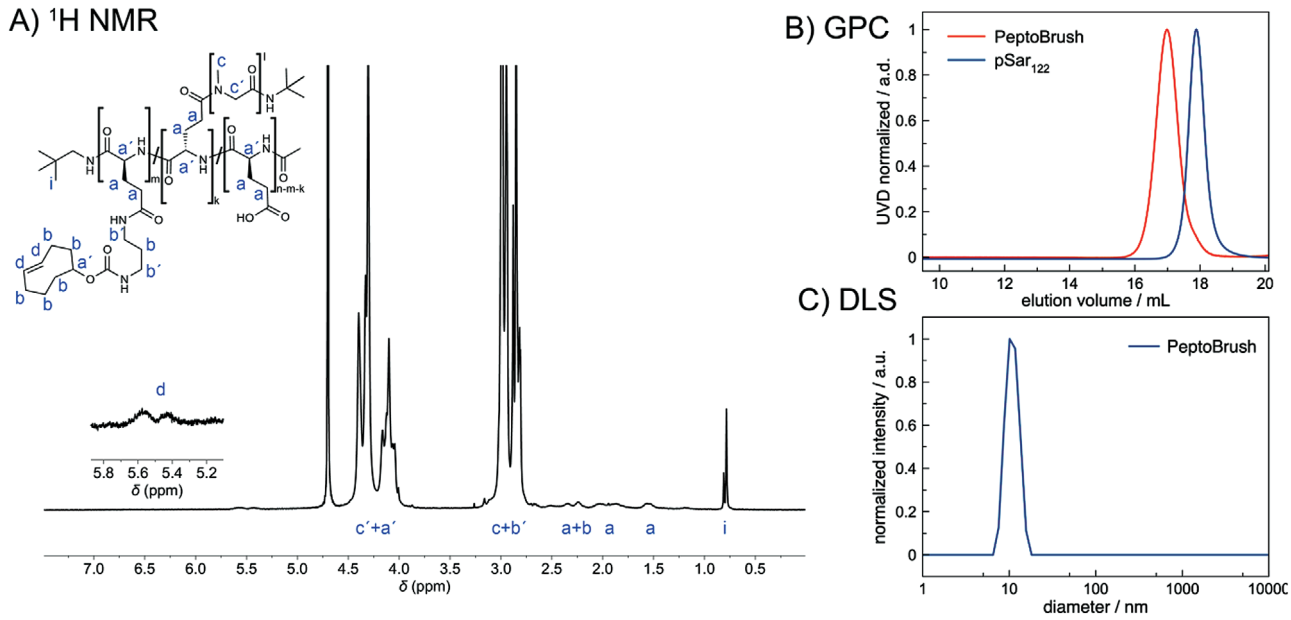
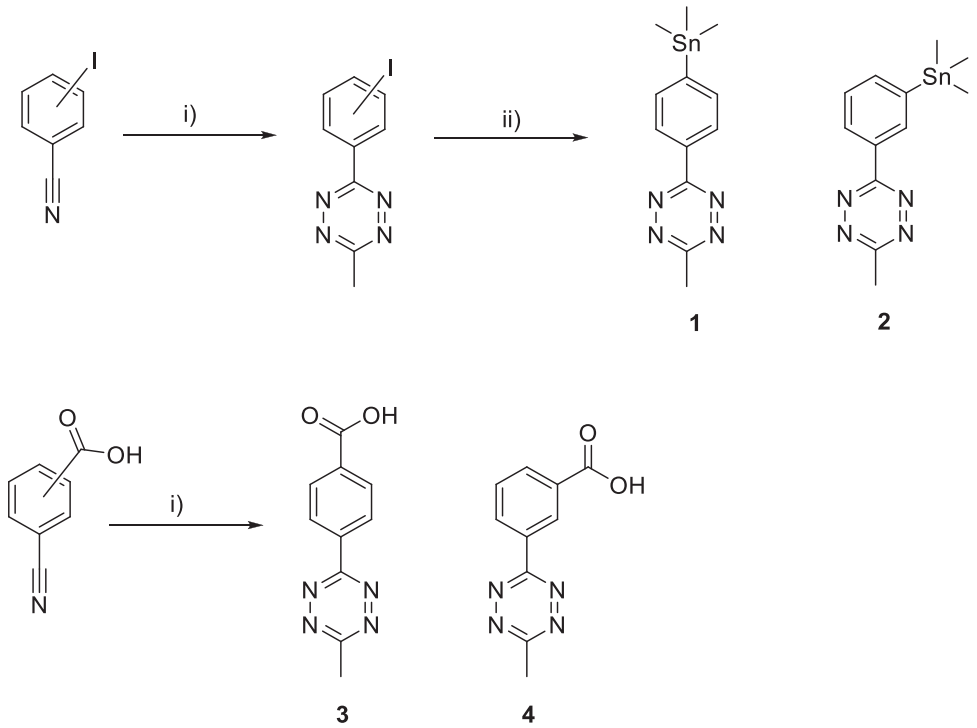


Figure 2. Characterization of the TCO-containing PeptoBrushes 1: A) 1H-NMR of in D_2O. B) GPC characterization of pSar side chains and final graft copolymers in hexafluoroisopropanol (HFIP), C) DLS in PBS buffer.



Scheme 2. Reaction conditions: (i) a) MeCN, $Zn(OTf)_2$, $NH_2NH_2 \cdot H_2O$, EtOH, 60 °C, 24 h, b) $NaNO_2$, AcOH, 0 °C, 20 min, (ii) $(Me_3Sn)_2$, $Pd(PPh_3)_4$, THF, 65 °C, MW, 2 h.

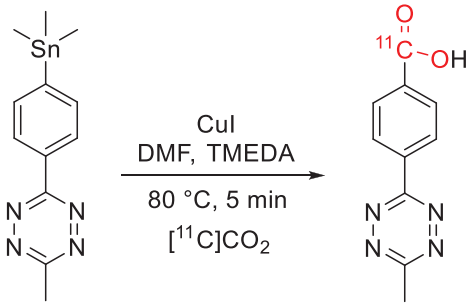
2. Results and Discussion

2.1. Synthesis of TCO-PeptoBrushes by a Grafting onto Strategy

In contrast to our recent report on the synthesis of TCO-containing PeptoBrushes we used a polyglutamic acid backbone and slightly longer pSar side chains (20% longer) and a TCO content of 30 % to improve the balance between stability and accessibility further (see **Scheme 1**).

The synthesis of the pGlu(OBn) backbone proceeded under our recently published condition using purified pGlu(OBn)-NCA, neopentylamine initiator, and purified solvents under N_2

Table 1. Optimization of the Copper(I)-Mediated ^{11}C-Carboxylation of stannane precursor **1** to [^{11}C]**3**.



1 [^{11}C]**3**

Precursor	Temperature	Time	Solvent	Catalyst	Catalystamount	Base/Ligand	Base amount	F source	RCC
3.3 mg (10 µmol)	100	5	DMF (400 µL)	CuTC	760 µg (0.4 eq)	TMEDA[a)]	63 µL (44 eq)	–	36%
3.3 mg (10 µmol)	80	5	DMF (400 µL)	CuTC	380 µg (0.2 eq)	TMEDA	63 µL (44 eq)	–	53%
3.3 mg (10 µmol)	80	5	DMF (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	20 µL (13 eq)	K-222[b)]/KF	0%
3.3 mg (10 µmol)	80	5	DMF (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	20 µL (13 eq)	TBAT[c)]	16%
3.3 mg (10 µmol)	80	5	NMP (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	20 µL (13 eq)	TBAT	45%
3.3 mg (10 µmol)	80	5	NMP (400 µL)	$Cu(PPh_3)_3Br$	380 µg (0.2 eq)	TMEDA	20 µL (13 eq)	TBAT	0%
3.3 mg (10 µmol)	80	5	NMP (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	10 µL (6.5 eq)	TBAT	21%
3.3 mg (10 µmol)	80	5	NMP (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	45 µL (30 eq)	TBAT	39%
3.3 mg (10 µmol)	100	5	NMP (400 µL)	CuI	380 µg (0.2 eq)	TMEDA	20 µL (13 eq)	TBAT	68%
3.3 mg (10 µmol)	100	5	NMP (400 µL)	CuI	1.14 mg (0.6 eq)	TMEDA	20 µL (13 eq)	TBAT	52%

[a)] Tetramethylethylenediamine; [b)] 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (Kryptofix 222); [c)] Tetrabutylammonium difluorotriphenylsilicate.

atmosphere.[33,34] Complete deprotection was achieved using acidic conditions (HBr/TFA) to obtain the polyglutamic acid backbone which was purified by dialysis and lyophilization.[35]

First, TCO amines were grafted onto the polyglutamic acid backbone using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholiunium chloride (DMTMM Cl) before the $pSar_{122}$ side chains were grafted onto the backbone using the same coupling chemistry.[36] We grafted the TCO to the backbone first for two reasons. First and most important, only when the TCO is grafted first to the backbone a loading of 30% and above is possible. Starting with the pSar chains first limits the TCO content to less than 10% presumable due to the fact that the after addition of the pSar side chains the backbone is sterically hindered by polar polymer chains, permitting access for the TCO amine to the carboxylic acids of the polyglutamic acid backbone. Second, starting with the TCO first enables a better characterization of the actual TCO content (see the Supporting Information). The characteristic ^{1}H-NMR double bond signal of the TCO remains visible but displays the expected peak broadening after attachment to the polymer backbone (see **Figure 2**A). We cannot exclude partial trans-cis isomerization of the TCO completely, but we observed an increased reaction rate with tetrazines (45–77 fold) compared the unimolecular TCO, which underlines that the majority of TCO remains in the reactive trans form.[20]

The final graft copolymers showed a narrow molecular weight (see Figure 2B) and size distribution of 11 ± 0.2 nm in aqueous buffer and thus are comparable to the previously synthesized PeptoBrushes (see Figure 2C).

In conclusion, the desired PeptoBrush with slightly elongated backbone, TCO content and pSar side chains could be successfully synthesized leading to unimolecular nanoparticles of 11 nm in diameter.

2.2. Synthesis of Tetrazine Derivatives for ^{11}C-Labeling

Recently, we reported several new methodologies to radiolabel Tzs with fluorine-18.[37–40] In particular, aryl stannane Tz derivatives were employed as precursors allowing for the first-time direct aromatic ^{18}F-fluorination of this scaffold.[38] At the same time, the preparation of ^{11}C-labeled carboxylic acids from aryl stannane precursors and [^{11}C]CO_2 via a copper-mediated carboxylation was reported by other authors.[41–43] We decided to explore if this reaction could be applied to stannane Tz derivatives as well. Aryl stannane Tzs **1** and **2** and the corresponding carboxylic acid derivatives **3** and **4** were synthesized as previously reported from the corresponding nitriles with overall yields of 20%–40% (Section S3, Supporting Information) (**Scheme 2**).[38,44]

2.3. Optimization of Cu-Mediated ^{11}C-Carboxylation of Tetrazines

Compounds **1** and **2** were reacted with [^{11}C]CO_2 using the same conditions for arylstannane ^{11}C-carboxylation reported by Duffy et al.[41] Both compounds were successfully radiolabeled to give respectively [^{11}C]**3** and [^{11}C]**4**. However, in order to use these compounds for the radiolabeling of TCO-modified polymers, we

had to solve two issues. First, the original methodology of Duffy et al. used copper(I) thiophene-2-carboxylate (CuTC) as a Cu (I) source. This compound is extremely air sensitive and requires the reaction to be performed under glove-box conditions, which is impractical. For this reason, we set out to find a different, more stable Cu (I) source. Second, we aimed to maximize the radiochemical yield (RCY) of tetrazine ^{11}C-labeling. Therefore, we screened different Cu (I) sources, base/ligand amounts, temperatures, and solvents (**Table 1**). We also tested the effect of fluoride ion source addition since fluoride ions are known to promote ^{11}C-carboxylation of boronic esters.[45] CuI was eventually selected as an alternative Cu (I) source since it is stable, widely available, cheap and had been employed for carboxylation reactions starting from boronic ester intermediates.[42,46] The final optimized reaction conditions provided [^{11}C]**3** and [^{11}C]**4** in a radiochemical conversions (RCC) of 68% and 85% respectively (Table 1).[47] To our knowledge, this is the first report of a direct ^{11}C-carboxylation on a Tz scaffold.

The radiolabeling procedure based on the optimized ^{11}C-carboxylation conditions (CuI, TMEDA, [^{11}C]CO_2, NMP, TBAT, 5 min, 100 °C) was fully automated, including labeling, purification, and formulation of the [^{11}C]-tetrazines (**Figure 3**). The automated synthesis, including [^{11}C]CO_2 release, trapping and labeling, HPLC purification and formulation was carried out within 30 min. The HPLC-purified product [^{11}C]**3** was obtained in a radiochemical purity (RCP) of >99%, a radiochemical yield (RCY) of 10 ± 3% (d.c.), and an A_m: 9 ± 6 GBq μmol^{-1} (d.c) (n = 3) (Table S1, Supporting Information). The same conditions were applied to the tetrazine [^{11}C]**4** which was obtained in 15 ± 5% (d.c.) RCY, RCP of 99% and an A_m: 11 ± 7 GBq μmol^{-1} (d.c.). The stability of the tetrazine moieties was investigated by reacting them with an excess of TCO-PNB carbonate as shown for [^{11}C]**4**.[38] Full consuption of the compound was observed suggesting that the tetrazine core is fully intact (Figure 3). Tetrazine [^{11}C]**4** was chosen for further experiments because of the higher activity yield (≈250 MBq isolated at the end of the synthesis, starting from 3-min-long target irradiation).

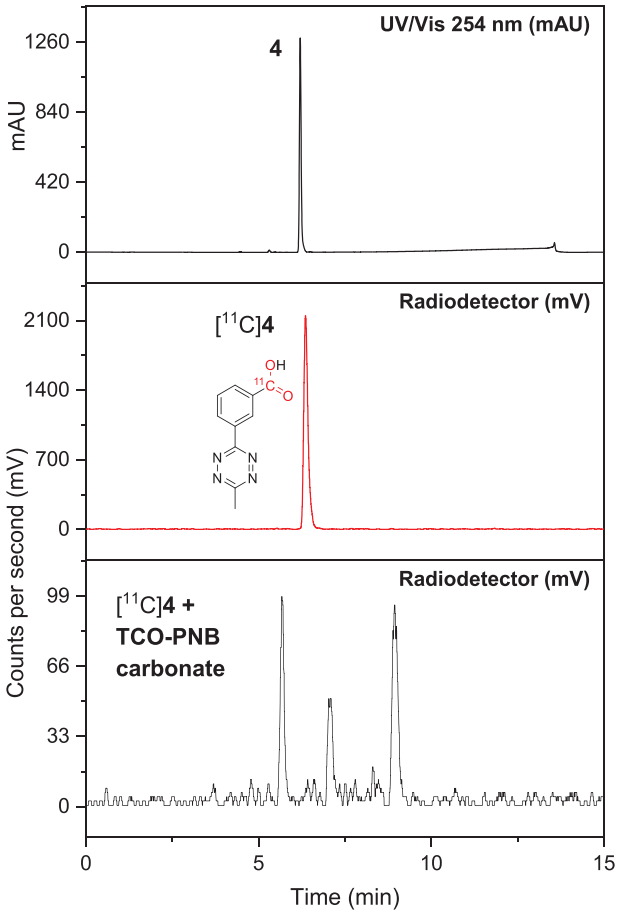


Figure 3. Analytical-HPLC of reference compound **4** (UV–vis, 254 nm) (upper panel), and radio-HPLC of the purified [^{11}C]**4** (middle panel) and ligation product after click reaction with the TCO-*p*-nitrophenyl carbonate (lower panel, click generates several isomers/tautomers). Analytical HPLC conditions: Luna 5 μm C18(2) 100 Å, 150 × 4.6 mm; eluents: A) H_2O with 0.1% TFA; B) MeCN with 0.1% TFA; gradient from 100% A to 100% B over 12 min, back to 100% A over 3 min, flow rate 2 mL min^{-1}.

2.4. Application of the Tetrazine [^{11}C]4 to TCO-PeptoBrushes Labeling

Before exploring [^{11}C]**4** click-labeling of the TCO-PeptoBrushes **5**, we assessed the availability of the TCO groups by titrating the TCO polymer with unlabeled **4**. Consumption of **4** after 20 min of incubation with **5** at room temperature agreed well with previous estimates of TCO loading of **5**, namely ≈50 nmol TCO / mg polymer were available for labeling.[20] For example, at 20 min after adding 0.5 equivalents of TCO-PeptoBrush (assuming 50 nmol TCO / mg polymer) to Tz **4**, the peak area of **4** decreased by 48% (**Figure 4**).

After proving that the TCOs of 5 can click to 4, we proceed to apply [^{11}C]4 for the indirect labeling of 5. Titration of 5 with an excess of [^{11}C]4 produced results similar to the titration with unlabeled 4: the radioactive [^{11}C]4 peak decreased by 40% when mixed with 0.5 equivalents of 5 and disappeared altogether when mixed with 1 equivalent of 5. Reaction kinetics test was performed using an excess of 5 relative to [^{11}C]4 (20 and 16 × 10^{-6} M, respectively, 1.2-fold excess of TCO). [^{11}C]4 fully clicked on TCO-Peptobrush within 5 min of reaction at room temperature, without the need for stirring, in a formulated solution of 15% EtOH in PBS (**Figure 5**). This corresponds to a bimolecular reaction rate constant of 1600 M^{-1} × s^{-1}. The RCP of the labeled polymer was >95%, so purification of the ^{11}C-polymer was not necessary.

3. Conclusion

We have developed a reproducible, simple and fast method for direct ^{11}C-labeling of tetrazines, through stannanes precursors to label PeptoBrushes efficiently, without the need of glove-box conditions. Besides the optimization with PeptoBrushes, we have demonstrated a short, clean, and high-yielding procedure for the ^{11}C-labeling of TCO-functionalized polymer with the prepared [^{11}C]tetrazines. Our indirect ^{11}C-labeling approach can be used to radiolabel other macromolecules, such as peptides, antibodies, nanoparticles, or engineered polymers, as it relies on a chemically orthogonal Tz-TCO ligation, proceeds at ambient temperature in neutral aqueous solution and reaches

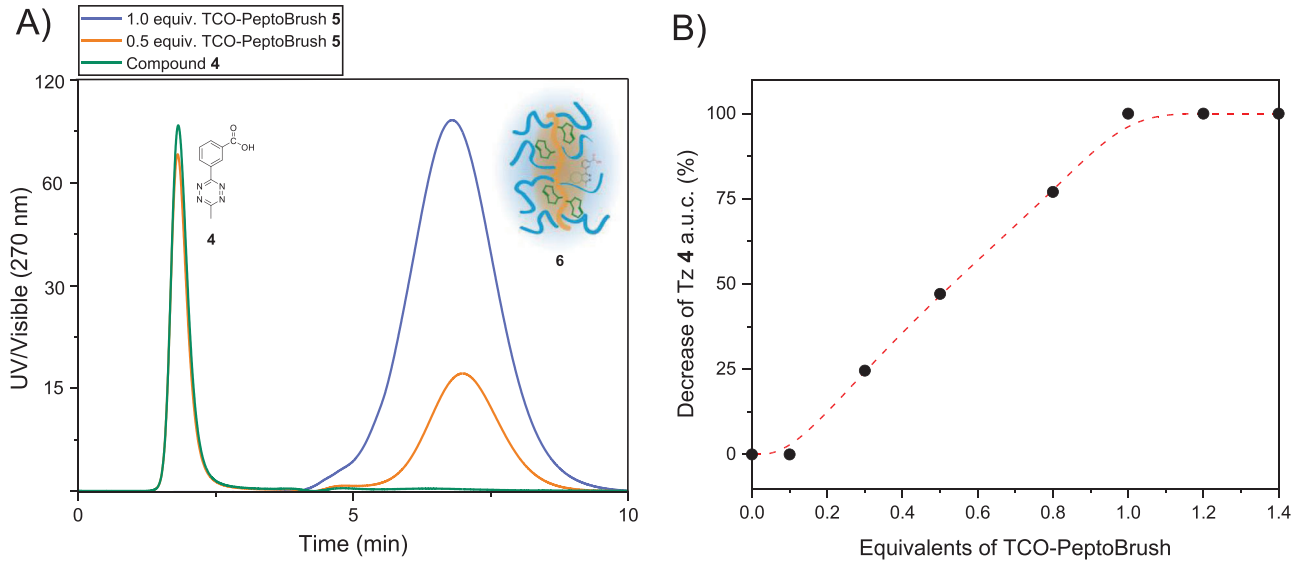


Figure 4. Fi Titration of the TCO-PeptoBrushes with reference compound 4. A) Analytical-HPLC (UV–vis normalized, 270 nm) of reference compound 4 and ligation product **6** after click reaction with the TCO-PeptoBrush **5** using 0.5 and 1 equivalents. Analytical HPLC conditions: HiTrap Desalting column with Sephadex G-25 Superfine resin; eluent: 0.1 M phosphate buffer pH = 7.4, over 10 min, flow rate 1 mL min^{-1}. B) Decrease of Tz-4 area under the curve (a.u.c.) (%) versus TCO-PeptoBrush equivalents used in the click reaction.

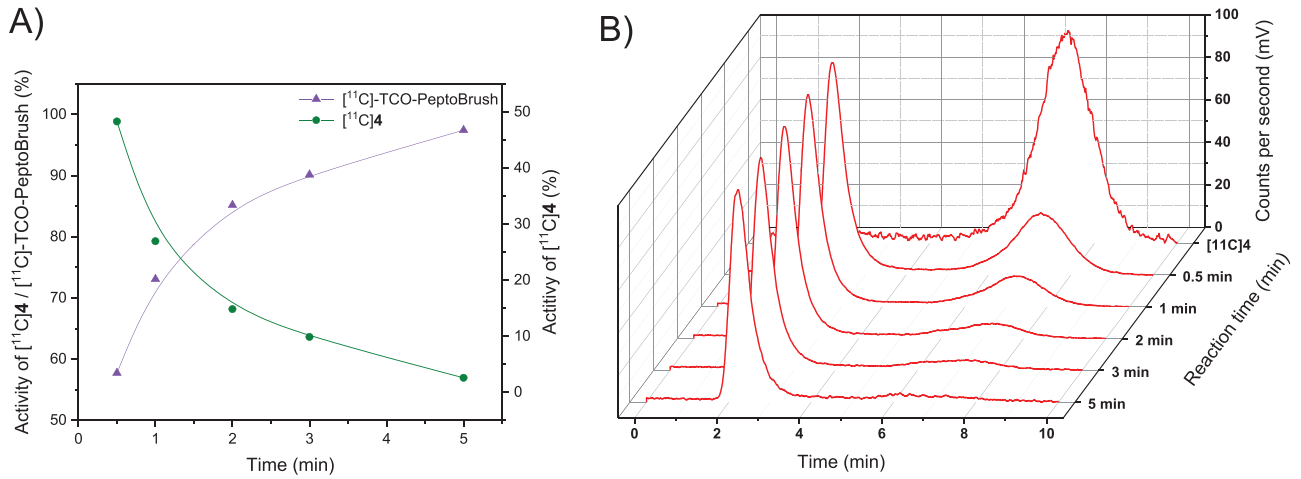


Figure 5. A) Graph comparing the activity of [^{11}C]**4** (%) versus the activity of [^{11}C]-TCO-PeptoBrush ([^{11}C]**6**) (%) during time. B) Radio-HPLC of the purified [^{11}C]**4** and ligation product [^{11}C]**6** after click reaction with the TCO-PeptoBrushes at different time-points. Analytical HPLC conditions: HiTrap Desalting column with Sephadex G-25 Superfine resin; eluent: 0.1 M phosphate buffer pH = 7.4, over 10 min, flow rate 1 mL min^{-1}.

full conversion within a few minutes. Thus, the in vivo fate of TCO-functionalized PeptoBrushes shortly after injection can be easily followed by means of PET imaging.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

^{11}C labeling, PeptoBrush, PET, polymers, tetrazine ligation, theranostics

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