Molecular Imaging

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Labeling and Glycosylation of Peptides Using Click Chemistry: A General Approach to ^{18}F-Glycopeptides as Effective Imaging Probes for Positron Emission Tomography**

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In the field of molecular imaging, positron emission tomography (PET) has emerged as an imaging modality with excellent sensitivity for in vivo studies.[1] PET labeling is challenging since short-lived positron-emitting isotopes such as ^{18}F and ^{11}C are used as labeling agents.[2] The optimization and efficient application of rapid and reliable labeling strategies are prerequisites for obtaining access to new radiopharmaceuticals for both research and clinical trials.

Bioactive peptides that specifically address molecular targets in vivo represent an important class of PET tracers to facilitate predictive imaging and PET-guided therapy. Diverse strategies for the synthesis of peptide-based radiopharmaceuticals using ^{18}F-labeled prosthetic groups have been elaborated, including chemoselective oxime conjugation[3] and the use of ^{18}F-labeled maleimide derivatives as cysteine-reactive reagents.[4,5] Following the concept of click chemistry introduced by Sharpless et al.,[6] the Huisgen [3+2] azide–alkyne cycloaddition has been adapted to ^{18}F-radiosynthetic methods in order to take advantage of its selectivity, reliability, and speed under aqueous mild Cu^{I}-promoted reaction conditions.[7]

The versatility of peptide imaging agents is frequently hampered by their instability in vivo because of rapid degradation by endogenous peptidases. As an example, the synthesis of radiolabeled peptide-based imaging agents for the neurotensin receptor-1 (NTR-1), which is overexpressed in a number of human cancers, requires modifications to improve the metabolic stability.[8]

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Synthetic approaches to RGD tracers targeting $\alpha_v\beta_3$ integrin, which plays a key role in angiogenesis, capitalize on the pioneering studies by Kessler et al., who successfully developed cyclic pentameric RGD peptides that selectively recognize integrin $\alpha_v\beta_3$.[9] Various radiolabeled cyclic RGD peptides have been described.[10] Among these, [^{18}F]galacto-RGD[11] has been extensively evaluated in clinical studies.

Since glycosylation of peptides is known to frequently improve the biokinetic and in vivo clearance properties, [^{18}F]galacto-RGD and further radiopeptides have been approached.[12,13] However, the multistep radiosynthesis of [^{18}F]galacto-RGD is time-consuming and laborious. In proposals to overcome this drawback, ^{18}F-labeling by 2-deoxy-2-[^{18}F]fluoroglucose (FDG) has been discussed.[5,14,15]

The major disadvantages of the ^{18}F-peptide-labeling strategies currently used are 1) harsh reaction conditions, 2) laborious multiple-step syntheses with a limited decay-uncorrected radiochemical yield (RCY), which would complicate the automation for large-scale production, and 3) lipophilic derivatization, which impair the biokinetic properties of the tracer.

Based on our previous work on click chemistry in drug discovery[16] and the synthesis of β-mannosyl azides,[15] we herein present an efficient strategy toward ^{18}F-labeling with concomitant glycosylation for the synthesis of ^{18}F-glycopeptides as imaging agents for PET. We combined this strategy with the development of a metabolically stable glycopeptoid analogue of NT(8–13), which is the highly potent C-terminal hexapeptide of the natural agonist neurotensin (NT). As a proof of concept, two ^{18}F-glycopeptides derived from NT(8–13) and c(RGDfPra), respectively, were applied to biodistribution studies and μPET for imaging NTR and $\alpha_v\beta_3$-integrin expression in vivo using xenograft nude mice models.

In detail, 2-deoxy-2-fluoroglucosyl azide (**3**) could be obtained starting from tetraacetylated 2-deoxy-2-fluoroglucose.[15] The glucosyl azide **3** was applied for the Cu^{I}-catalyzed azide–alkyne coupling with a series of alkyne-functionalized peptides to evaluate the influence of the appended glycosyl residue on receptor recognition. Commercially available propargylglycine (Pra) was introduced by solid-phase-supported synthesis at position X into the sequence of the bioactive peptide c(RGDfX) and at the N terminus of NT(8–13) and metabolically stabilized derivatives thereof. Considering our studies on the influence of peptide backbone modifications and ligand conformation on affinity changes for a series of NT(8–13) analogues,[17] metabolic stabilization was envisioned by alteration of three amino acids in the sequence

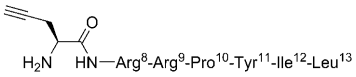
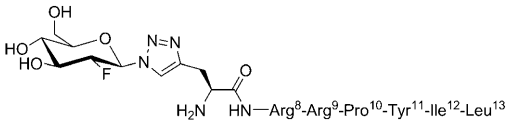
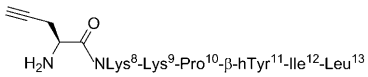
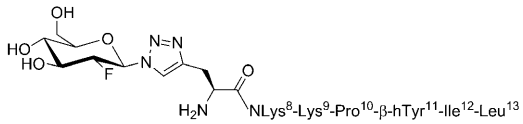
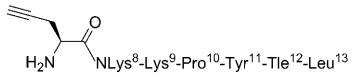
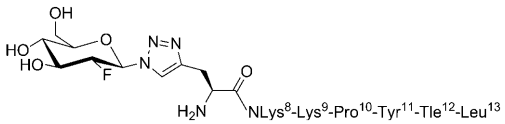
of NT(8–13) (Table 1). Receptor binding studies using CHO cells, stably expressing the human NTR-1,[18] provided evidence that introduction of Pra at the N terminus (NT1) did not significantly influence the NTR-1 affinity, whereas β-homo-Tyr in position 11 (NT2 and NT3) of the peptoid-like *N*-(4-aminobutyl)Gly-Lys (= NLys-Lys) derivative strongly decreased the affinity. The replacements of Ile to Tle in position 12 and Arg-Arg to NLys-Lys (NT4) were motivated by an anticipated stabilization towards peptidase-catalyzed degradation.

The ligation of NT1, NT3, NT4, and c(RGDfPra) with 2-deoxy-2-fluoro-β-glucopyranosyl azide (**3**) was accomplished in the presence of $CuSO_4$ and sodium ascorbate to give the desired peptide conjugates FGlc-NT1, FGlc-NT3, FGlc-NT4, and FGlc-RGD in yields of 80–90% and purities of >98%. The retention of the β-anomeric configuration of the glycosyl moiety of FGlc-NT1 was elucidated by ^{19}F NMR spectroscopy.[15]

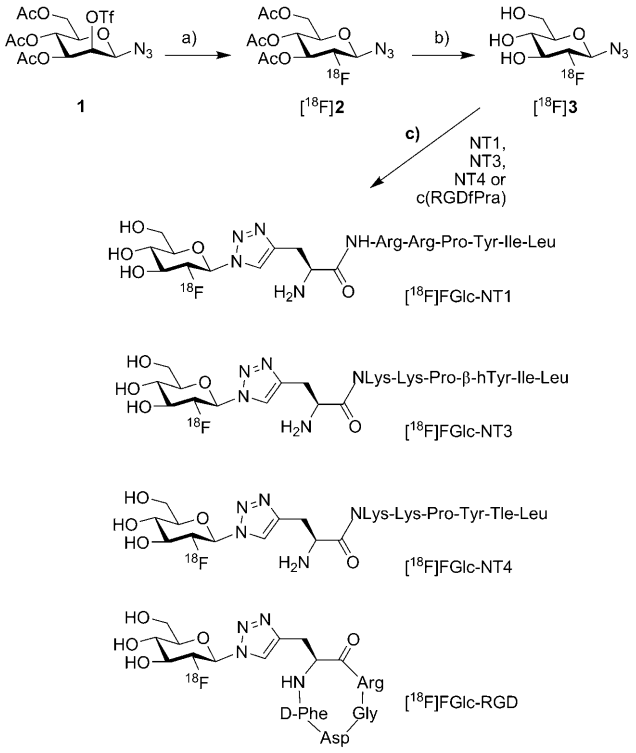
According to our ligand binding experiments, the glycosylated NTR-1 ligands proved to be almost equipotent to the respective nonglycosylated congeners (Table 1). In particular FGlc-NT4 revealed an excellent inhibition constant in the low double-digit nanomolar range, thereby offering promise for ^{18}F-labeling.

For the radiosynthesis of the desired ^{18}F-labeled glycopeptides, the prosthetic group 2-deoxy-2-[^{18}F]fluoroglucosyl azide [^{18}F]**3**[15] was prepared by cryptate-mediated ^{18}F-for-triflate substitution in the mannosyl triflate **1** followed by basic hydrolysis with NaOH (Scheme 1). Subsequent glycosylation was realized in the same reaction vial in a mixture of phosphate-buffered saline (PBS)/ethanol (10:1) at 60 °C; only 100 μg of the alkyne-bearing peptide was required in a total volume of 500 μL. Starting from [^{18}F]fluoride, high RCYs were achieved for this three-step, two-pot procedure, and the ^{18}F-glycopeptides [^{18}F]FGlc-NT1, [^{18}F]FGlc-NT3, [^{18}F]FGlc-NT4, and [^{18}F]FGlc-RGD were obtained in excellent purities and 17–20% uncorrected RCY ($n = 71$ individual experiments) with adequate specific activities (55–210 GBq μmol^{-1}) in a total synthesis time of 75 min. Thus, this protocol for the introduction of an ^{18}F-glucopyranoside label into peptides is unique and well-suited for the ^{18}F-labeling of peptides since it was performed 1) in an aqueous environment, 2) with high chemoselectivity, 3) in reliably high RCY, and 4) at low reactant concentrations. Notably, the ultimate reliability of the "^{18}F-click" glycosylation was reflected by a record of 71 successive radiosyntheses without a single unsuccessful attempt.

Table 1: Receptor binding data of NT analogues and of the reference NT(8–13) as determined by displacement of [^{3}H]NT using hNTR-1 expressing CHO cells.[a]

Peptide	Sequence	K_i [nM]
NT(8–13)	Arg^8-Arg^9-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}	0.23 ± 0.042[b]
NT1		0.29 ± 0.046[b]
FGlc-NT1		0.32 ± 0.11[c]
NT2	NLys^8-Lys^9-Pro^{10}-β-hTyr^{11}-Ile^{12}-Leu^{13}	130 ± 140[c]
NT3		350 ± 78[c]
FGlc-NT3		400 ± 21[c]
NT4		5.2 ± 0.92[b]
FGlc-NT4		16 ± 2.8[c]

[a] K_i values are based on the means of two to seven individual experiments each done in triplicate. [b] K_i ± standard error of the mean (SEM) [nM]. [c] K_i ± SD [nM].



Scheme 1. a) K^{18}F, Kryptofix 2.2.2, K_2CO_3, KH_2PO_4, acetonitrile, 2.5 min, 85 °C, 71% RCY; b) NaOH, 5 min, 60 °C; c) 1. HCl (0.1 M); 2. alkynyl peptide, PBS (10% ethanol), $CuSO_4$, sodium ascorbate, 20 min, 60 °C, 70–80% RCY.

The stability of the ^{18}F-labeled NT analogues was investigated in human serum at 37 °C. In contrast to [^{18}F]FGlc-NT1, which was completely degraded after 30 min (see Figure S2 in the Supporting Information), the peptoids [^{18}F]FGlc-NT3 and [^{18}F]FGlc-NT4 showed dramatically enhanced metabolic stability of > 90 % (Figure 1 a). Saturation binding and internalization studies with [^{18}F]FGlc-NT3 and [^{18}F]FGlc-NT4 were performed using NTR-expressing HT29 cells. Specific binding was demonstrated for [^{18}F]FGlc-NT4 with a B_{max} value of 403 fmol mg^{-1}, a K_d value of (8.5 ± 2.9) nM (Figure 1 b), and an internalization rate of 60–80 % after 30 min. In vitro autoradiography using rat brain slices further demonstrated specific binding of [^{18}F]FGlc-NT4 in NTR-rich areas, which was completely inhibited in the presence of 1 μM NT (see Figure S6 in the Supporting Information).

We then investigated the biodistribution of [^{18}F]FGlc-NT4 in HT29 xenografted nude mice at 10, 30, and 65 min p.i. (postinjection; see Figure S7 in the Supporting Information). [^{18}F]FGlc-NT4 demonstrated metabolic stability in vivo (>98 %, Figure 1 a), fast blood clearance, high uptake in the kidneys, and low uptake in other organs. [^{18}F]FGlc-NT4 uptake in HT29 tumors was 2.0 and 1.2 % ID g^{-1} (ID = injected dose) after 30 and 65 min, respectively, with tumor/blood ratios rapidly increasing from 1.7 to 3.5 (see Figure S8 in the Supporting Information); this suggests a suitable signal/noise ratio for PET imaging at early time points after injection. In fact, μPET studies successfully demonstrated specific visualization of NTR expression by [^{18}F]FGlc-NT4 in vivo (Figure 1 c).

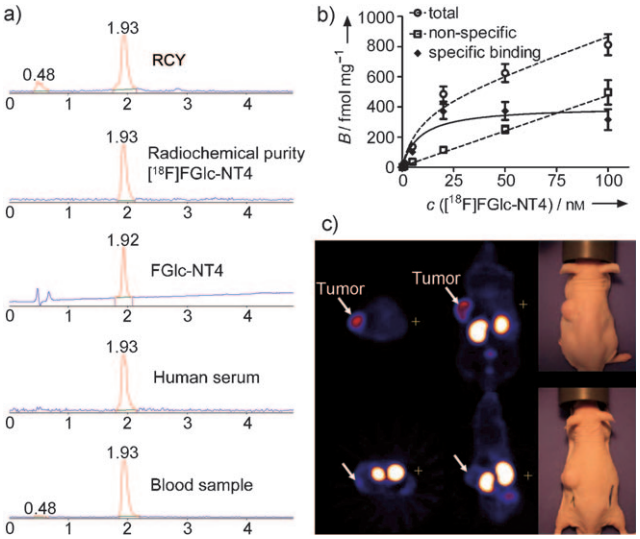


Figure 1. Purity, stability, and specific binding in vitro and μPET imaging with [^{18}F]FGlc-NT4. a) Radio-HPLC traces showing the RCY of [^{18}F]FGlc-NT4 after 20 min, the radiochemical purity after isolation by HPLC, the purity of the reference FGlc-NT4 (UV), and the stability of [^{18}F]FGlc-NT4 in human serum (3 h) and in mouse blood (30 min p.i.). b) Saturation binding curve of [^{18}F]FGlc-NT4 in HT29 cells in vitro. c) Static μPET images (left: transaxial, middle: coronal) of nude mice bearing HT29 tumors at 45–65 min p.i. of 5.7 MBq [^{18}F]FGlc-NT4 with (bottom) and without (top) coinjection of NT4 (100 μg per mouse).

The successful application of our sequential strategy of ^{18}F-labeling and glycosylation was also demonstrated by the in vitro and in vivo data on [^{18}F]FGlc-RGD. In vitro binding experiments were conducted with the disintegrin [^{125}I]echistatin using a solid-phase $\alpha_v\beta_3$ binding assay (Figure 2 b) and $\alpha_v\beta_3$-expressing human U87MG glioblastoma cells, confirming a double-digit nanomolar affinity of FGlc-RGD for $\alpha_v\beta_3$ (see Table S2 in the Supporting Information).

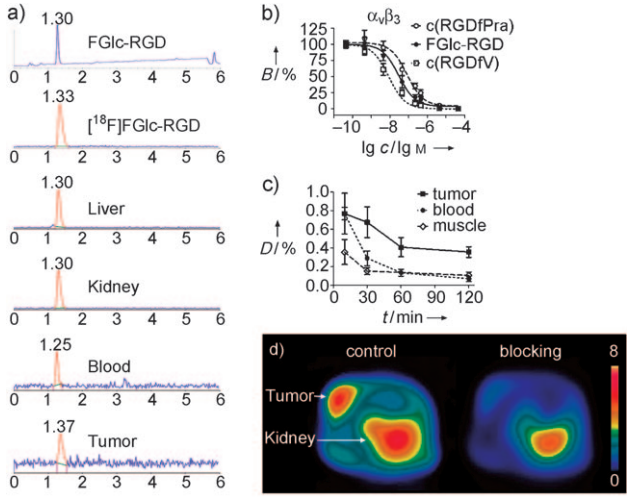


Figure 2. Purity, $\alpha_v\beta_3$-binding in vitro, metabolic stability in vivo, and μPET imaging of $\alpha_v\beta_3$ expression with [^{18}F]FGlc-RGD. a) Purity of FGlc-RGD and [^{18}F]FGlc-RGD, and HPLC analysis of radioactivity extracted from mouse organ homogenates (40 min p.i.). b) Competition binding curve between [^{125}I]echistatin and FGlc-RGD, c(RGDfPra), and c(RGDfV). The fraction of bound radioactivity to $\alpha_v\beta_3$ integrin (*B* in %) is shown. Data are expressed as mean values ± standard deviation (SD) from three experiments each performed in triplicate. c) Time–radioactivity curves of [^{18}F]FGlc-RGD in tumor, blood, and muscle tissue of U87MG-bearing nude mice. Tissue radioactivity is expressed as the percentage of injected dose per gram of tissue (*D*, mean value ± SD, $n = 3$–6). d) μPET using [^{18}F]FGlc-RGD at 30–40 min p.i.: Transaxial images from U87MG-bearing mice injected with the tracer alone (control) and coinjection of c(RGDfV) (12.5 mg kg^{-1}, blocking).

Biodistribution experiments with [^{18}F]FGlc-RGD in U87MG-bearing mice showed a similar blood clearance, but a threefold increased uptake in liver and kidney (see Figure S9 in the Supporting Information), when compared to [^{18}F]galacto-RGD.[13] The high hepatic and kidney uptake could not be explained by differences in lipophilicity between [^{18}F]FGlc-RGD and [^{18}F]galacto-RGD, since their lg $D_{7.4}$ values were comparable (lg $D_{7.4} = -3.8$ ($n = 3$) vs. −3.2[13]). The specific tumor uptake of [^{18}F]FGlc-RGD was 0.49 % ID g^{-1} (60 min p.i.). Our studies confirmed excellent in vivo metabolic stability of [^{18}F]FGlc-RGD and fast tumor accumulation combined with non-variant tumor/blood and tumor/muscle ratios of 2.3 and 4.4, respectively, early at 30 min p.i. (Figure 2 a,c). This allowed successful imaging of $\alpha_v\beta_3$-integrin expression in vivo (Figure 2 d), indicating high potential of [^{18}F]FGlc-RGD for future application in PET imaging studies.

Applying our click-chemistry-based glycosylation method for ^{18}F-labeling, we considerably improved the overall yield of [^{18}F]FGlc-RGD (20%) and the total synthesis time (70 min) in comparison with the clinically applied [^{18}F]galacto-RGD (10%, 200 min[13]). Moreover, we successfully developed [^{18}F]FGlc-NT4 as the first peptoidic tracer for imaging NTR expression in vivo by PET.

The presented procedure is highly amenable to automation since carbohydrate deprotection and the peptide ligation step by click chemistry were performed in a one-pot reaction. The reliable accessibility of ^{18}F-glycopeptides will significantly facilitate the further evaluation of these tracers in longitudinal μPET studies on animal models. Future efforts also aim at increasing the kidney clearance by varying the glycosyl residue of the presented ^{18}F-glycopeptides.

We anticipate that the ligation of ^{18}F-labeled glycosyl azides and alkyne-bearing peptides should be generally applicable to a wide variety of target peptides, providing a series of ^{18}F-glycopeptides as new imaging agents with improved biokinetics for both experimental research and early clinical trials by PET.

Experimental Section

Typical procedure for ^{18}F-labeling with concomitant glycosylation of peptides: The mannosyl precursor **1**[15] (9 mg, 15 μmol) in anhydrous acetonitrile (450 μL) was added to the dried K^+/Kryptofix 2.2.2/$^{18}F^-$ complex and the solution was stirred for 2.5 min at 85 °C. [^{18}F]**2** was isolated by semipreparative HPLC (Kromasil C8, 125 × 8, 4 mL min^{-1}, acetonitrile (0.1% trifluoroacetic acid (TFA)/water (0.1% TFA) 30:70) and trapped on a C18 cartridge (Lichrosorb, Merck, 100 mg). After elution with ethanol (0.8 mL) and evaporation of the solvent, a solution of NaOH (10% ethanol, 60 mM, 250 μL) was added. After 5 min at 60 °C (formation of [^{18}F]**3**), HCl (0.1 M, 13.5 μL) was added, followed by a solution of alkyne-functionalized peptide (100 μg, see the Supporting Information) dissolved in 250 μL PBS (10% ethanol), sodium ascorbate (0.6 M, 10 μL), and $CuSO_4$ (0.2 M, 10 μL). After the reaction mixture had been stirred for 20 min at 60 °C, the ^{18}F-glycopeptides were isolated by semipreparative HPLC and subsequent SPE (solid phase extraction), allowing formulation in PBS for in vitro and in vivo use.

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