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A Submicrogram-Scale Protocol for Biomolecule-Based PET Imaging by Rapid 6π-Azaelectrocyclization: Visualization of Sialic Acid Dependent Circulatory Residence of Glycoproteins**

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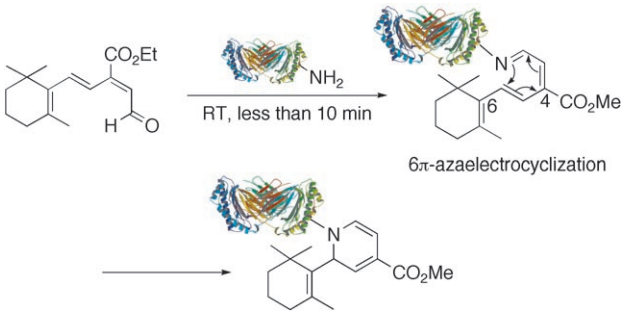
In memory of Yoshihiko Ito

Molecular imaging is an important topic that has gained significant attention in the fields of chemical biology, drug discovery, and diagnosis. Positron emission tomography (PET) is a widely used, noninvasive method that quantitatively visualizes the locations and levels of radiotracer accumulation with high imaging contrast.[1] 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) has become an important small-molecule-based PET tracer used especially in the detection of primary and metastatic cancers. Current efforts focus on the development of biomolecule-based tracers, which are composed of peptides, monoclonal antibodies (mAbs), and oligonucleotides.[1] Generally, PET imaging of these biomolecules is achieved by conjugation to metal-chelating agents, such as dota (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid) or dtpa (diethylenetriaminepentaacetic acid), prior to radiolabeling with a suitable β^+-emitting metal. The chelating agents can be introduced either during the solid-phase synthesis of peptides or more directly by the reaction of lysine residues or N-terminal amino groups with dota-*O*-sulfosuccinimidyl ester.[2] However, the latter method, using the activated dota ester, usually proceeds slowly and requires as much as a few milligrams of sample to keep the reaction concentrations high. Because PET experiments require only small amounts of the tracers, and because important biomolecules are sometimes obtained only in small amounts, a submicrogram-scale conjugation methodology would greatly expand the applicability of PET imaging. Herein, we describe the submicrogram-scale labeling of lysine residues by a rapid 6π-azaelectrocyclization, which led to an efficient PET protocol; a first visualization of sialic acid dependent circulatory residence of glycoproteins was realized.

Although various labeling methods for biomolecules have been reported,[3] an efficient, rapid, and mild protocol that can be applied in various buffer solutions remains to be found. Recently, Katsumura and co-workers reported that unsaturated (*E*)-ester aldehydes quantitatively react with primary amines, including lysine, within five minutes in water, thus providing 1,2-dihydropyridines in irreversible reactions (Scheme 1).[4] These reactions proceed by smooth azaelectrocyclization of the intermediary Schiff bases (1-azatrienes), which is strongly accelerated owing to the efficient interactions between the highest occupied and lowest unoccupied molecular orbitals (HOMO and LUMO) within the 1-azatriene systems; these interactions are favored by the electron-withdrawing substituent at C4 and the conjugation at C6.[4d] Inspired by Katsumura's findings, we designed and synthesized a new dota labeling probe, dota-(*E*)-ester aldehyde **4a** (Scheme 2). (*Z*)-Vinyl bromide **2** and glycine-linked (*E*)-stannane **1** were heated to 110 °C in the presence of [$Pd_2(dba)_3$] (dba = *trans*,*trans*-dibenzylideneacetone), P(2-furyl)$_3$, and LiCl in DMF to provide the Stille coupling product,[4d] which was subsequently treated with 3 M hydrochloric acid to give aminoalcohol **3** in 73 % yield for two steps.



Scheme 1. Rapid reaction with lysine through 6π-azaelectrocyclization.

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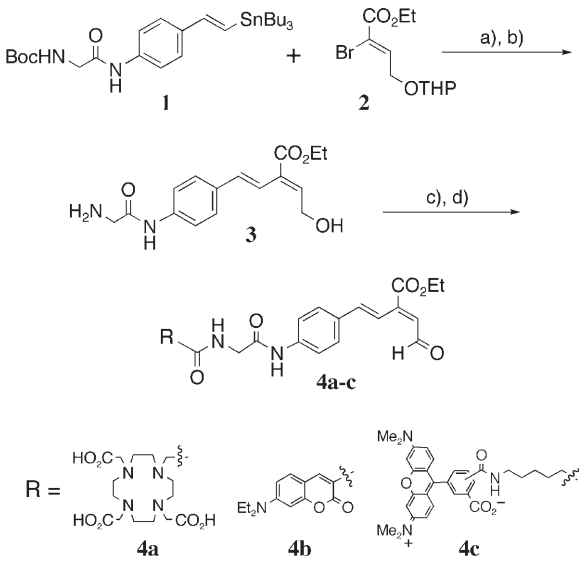
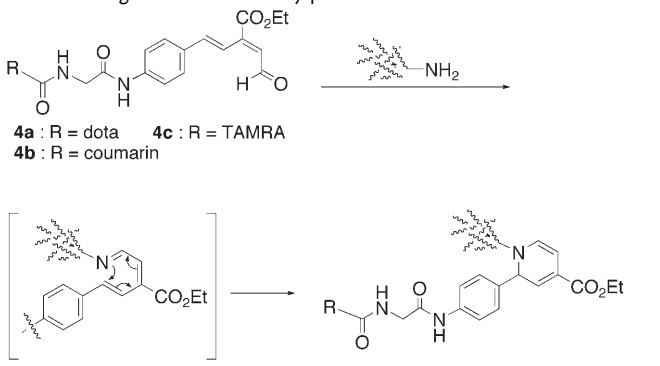
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 2. Synthesis of dota and fluorescent labeling probes: a) $[Pd_2(dba)_3]$, P(2-furyl)$_3$, LiCl, DMF, 110 °C, 73 %; b) 3 M HCl, MeOH/H_2O (1:1), RT, quantitative yield; c) dota-OSu, Et_3N, DMF, RT, 58 % (for **4a**); coumarin-OSu, CH_2Cl_2, RT, 68 % (for **4b**); TAMRA-OSu, DMF/CH_2Cl_2 (1:1), RT, 74 % (for **4c**); d) Dess–Martin periodinane, DMF/CH_2Cl_2 (3:10), RT for **4a**; polystyrene-supported *o*-iodoxybenzoic acid (IBX), DMF/CH_2Cl_2 (1:1), RT for **4b** and **4c**.

Table 1: Labeling of biomolecules by probes **4a–c**.[a]



Entry	Probe	Biomolecule (*m* [μg])	Biomolecule conc [M]	Equiv probe	*t* [min]	Labeled Lys[c]
1[b]	**4a**	somatostatin (170)	5.5×10^{-4}	100	30	1
2	**4a**	albumin (100)	5.0×10^{-5}	25	10	5
3	**4a**	albumin (100)	5.0×10^{-5}	10	10	2
4	**4a**	albumin (100)	5.0×10^{-5}	500	10	20
5	**4a**	orosomucoid (62)	4.5×10^{-6}	10	30	2
6	**4a**	asialoorosomucoid (62)	4.5×10^{-6}	10	30	3
7[b]	**4a**(Gd)[d]	somatostatin (32)	3.6×10^{-4}	31	30	1
8	**4b**	albumin (120)	1.7×10^{-5}	7	30	1[e]
9	**4c**	albumin (12)	2.1×10^{-5}	7	30	1[e]
10	**4c**	anti-GFP mAb (2.0)	1.1×10^{-6}	20	30	2[e]

[a] Unless otherwise noted, reactions were performed in 0.1 M phosphate buffer (pH 7.4) at 24 °C. [b] in H_2O. [c] The number of labeled lysine residues was calculated by γ counting of ^{57}Co introduced to dota (see the Supporting Information) [d] Gd was introduced by the reaction of **4a** with 0.1 M $GdCl_3$ in H_2O. [e] Estimated by emission spectra at 470 nm (coumarin) and 555 nm (TAMRA).

Selective acylation of the amino group in **3** was achieved in 58 % yield by the reaction with dota-*O*-succinimidyl ester (dota-OSu)[5] in the presence of Et_3N in DMF. Finally, the allylic alcohol was oxidized by Dess–Martin periodinane in DMF/CH_2Cl_2 (3:10) to afford the desired aldehyde **4a**, which was used for labeling as its DMF solution after size-partitioning filtration and removal of CH_2Cl_2. By applying the established route in Scheme 2, various functionalities can be introduced at the amino group in **3**, thereby providing access to general probes; the coumarin (**4b**) and the carboxytetramethylrhodamine (TAMRA, **4c**) derivatives have been prepared for fluorescent labeling (Scheme 2).

With probes **4a–c** in hand, we tested the reactivity toward lysine residues of various biomolecules, namely, somatostatin as a peptide as well as albumin, orosomucoid, and anti-GFP antibody (GFP = green fluorescent protein) as proteins (Table 1). To our satisfaction, the reaction of somatostatin (170 μg) with 100 equivalents of dota probe **4a** at room temperature for 30 min provided mono-dota labeled somatostatin in 96 % yield based on the HPLC analysis (Table 1, entry 1). Interestingly, under these labeling conditions, only one of the two lysine residues in somatostatin, namely that not involved in receptor binding, was modified (see the Supporting Information).[6] In contrast, under conventional labeling conditions, using the dota-activated ester required a much longer reaction time (over 24 h), which resulted in indiscriminant modification at both lysine residues and the N terminus as well as the recovery of the intact peptide. Thus, the high reactivity of **4a** enabled the lysine azaelectrocyclization modification to be completed in a very short time, which resulted in selective labeling of the more accessible lysine residue. Similarly, the incubation of human serum albumin (100 μg) with 25 equivalents of probe **4a** for just 10 min and subsequent quick size-partitioning gel filtration successfully provided the modified protein with five incorporated dota units (Table 1, entry 2). Noteworthy is that labeling by azaelectrocyclization proceeds in nearly quantitative yields based on the lysine residues, both at high (greater than 10^{-3} M) and at low (less than 10^{-5}–10^{-6} M) concentrations of the probe **4a**. As a result, the number of dota units introduced to a protein can be precisely controlled by adjusting the number of equivalents of **4a** used. For example, with a reaction time of ten minutes, ten equivalents of **4a** provided the labeled protein with two molecules of dota, while use of 500 equivalents resulted in the introduction of 20 dota molecules (Table 1, entries 3 and 4).

To further demonstrate the performance of this labeling method, proteins that are available in only small amounts (62 μg of glycoproteins, orosomucoid, and asialoorosomucoid), were labeled successfully with the incorporation of two or three units of dota by incubating the respective protein with ten equivalents of **4a** for 30 min (Table 1, entries 5 and 6). Furthermore, the paramagnetic metal Gd^{3+} can be chelated by the dota unit of **4a** prior to peptide labeling (Table 1, entry 7). This new process could allow for the labeling of valuable or unstable materials.

The present electrocyclization protocol is also applicable to rapid fluorescent labeling; 10–100 μg albumin was labeled

with probes **4b** and **4c**(Table 1, entries 8 and 9). As little as 2 μg of anti-GFP mAb (ca. 10 pmol) was successfully labeled by **4c** within 30 min (Table 1, entry 10); 20 equivalents of **4c** resulted in preferential labeling at the more accessible Fc fragment with two molecules of fluorophore, thereby retaining its GFP recognition activity with 90% of the intact mAb (see the Supporting Information).

To verify the applicability of the new labeling method to PET imaging, the dota–somatostatin adduct was treated with $^{68}Ga^{3+}$ as a β^+ emitter.[7] The resulting ^{68}Ga-labeled adduct was investigated in rabbits (Figure 1). Somatostatin receptors are expressed on pancreas, kidney, gastrointestinal tract, and endocrine tumors. Somatostatin receptor imaging has been used for the diagnosis of endocrine tumors by using radiolabeled octreotide, a metabolically stable analog of somatostatin. In our case, the microPET imaging of [^{68}Ga]dota–somatostatin successfully visualized accumulation of the tracer in the pancreas two to four hours after the injection, thus establishing this method as a powerful PET labeling protocol.[7,8]

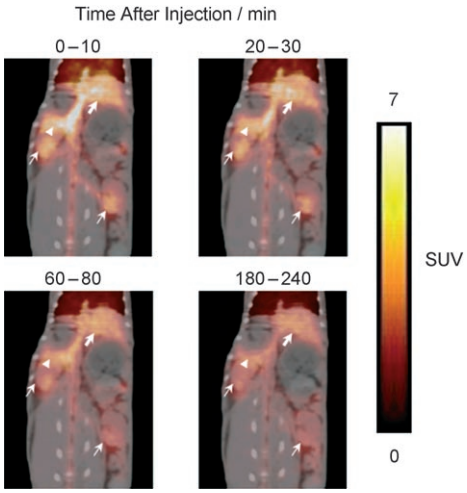


Figure 1. MicroPET images of [^{68}Ga]dota–somatostatin in rabbits (axial view, overlaid with computed tomography (CT) images). The images represent time-dependent changes of the accumulation in the liver (large arrow), the kidney (small arrow), and the pancreas (arrowhead) after injection of [^{68}Ga]dota–somatostatin. SUV = standardized uptake value.

Furthermore, microPET images of [^{68}Ga]dota–orosomucoid and asialoorosomucoid adducts in rabbits (Figure 2) successfully visualized the asialo glycoprotein being cleared through the kidneys faster than orosomucoid,[9] thus achieving the first visualization of sialic acid dependent circulatory residence of glycoproteins.[8]

In summary, we have developed a new lysine-based labeling method for biomolecules on the basis of rapid 6π-azaelectrocyclization. Both dota as a metal-chelating agent (either for MRI, PET, or other radiopharmaceutical purposes, such as single photon emission computed tomography (SPECT) with γ emitters) and fluorescent groups were efficiently and selectively introduced to lysine residues in either phosphate or Tris–HCl buffer solutions (Tris = tris(hydroxymethyl)aminomethane)[4b] within 30 min at room temperature. The dota-labeled somatostatin and glycoproteins were subsequently radiometallated with ^{68}Ga, and the receptor-mediated accumulation of somatostatin in the pancreas was observed. Furthermore, oligosaccharide-dependent circulatory residence of glycoproteins was visualized for the first time by microPET. There are two distinct features of this labeling method: 1) Labeling requires samples of only a few micrograms, or even less, if desired. 2) Near-quantitative reaction at the lysine residues of biomolecules enables the use of a simple isolation procedure (i.e. quick gel filtration or centrifugal separation). The protocol developed herein will expand the applicability of PET on peptides, proteins, and other amine-containing molecules. In vivo kinetic studies of proteins such as the cytokines is currently in progress.

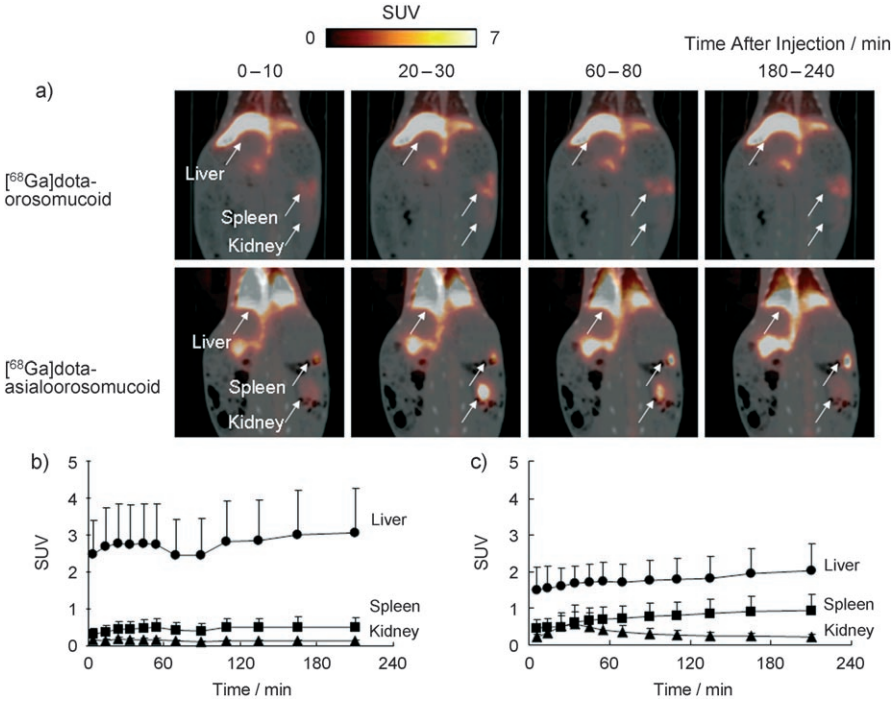


Figure 2. Dynamic microPET images and time–activity curves of [^{68}Ga]dota glycoproteins in rabbits. a) Time course of accumulation of [^{68}Ga]dota–orosomucoid (top) and [^{68}Ga]dota–asialoorosomucoid (bottom) in some peripheral organs (axial views). These PET images were overlaid on anatomical images obtained by CT. b, c) Changes in the standardized uptake value (SUV) of [^{68}Ga]dota–orosomucoid (b) and [^{68}Ga]dota–asialoorosomucoid (c) in the liver (●), the spleen (■), and the kidney (▲). SUV was used as a quantitative evaluation of PET data and normalized by radioactivity in the tissue region, the injected dose, and the weight of the subjects. These values represent the mean and standard deviation.

Experimental Section

Representative procedure for labeling with probe **4a** (Table 1, entry 5): Probe **4a** (14 nmol, 1.5 μL) in DMF was added to orosomucoid (62 μg, 1.4 nmol, 295 μL, pH 7.4) in phosphate-buffered saline (PBS) at room temperature (concentration: 4.5×10^{-6} M for orosomucoid, 4.5×10^{-5} M for **4a**). After the mixture had stood at room temperature for 30 min, the dota-labeled protein was purified by ultrafiltration (30000 cut). Solutions of ammonium acetate (0.25 M, 20 μL, pH 7.0) and $CoCl_2$ (4.0 nmol, 400 μM, 5 μL, containing more than 350000 cpm μL^{-1} $^{57}CoCl_2$) were added to a PBS solution of the labeled protein (6.67×10^{-10} mol, 5.74 mg mL^{-1}, 5 μL, pH 7.4). After the mixture was incubated at 40 °C for 3 h, a solution of dota (40 μg, 100 nmol, 10 mM, 10 μL) was added, and the mixture was incubated at 40 °C for 30 min. A 1.0-μL aliquot of the resulting solution was spotted on a TLC plate and developed until the solvent (MeOH/H_2O = 1:1) reached 7 cm from the origin. The plate was cut into seven fractions, for each of which ^{57}Co was counted by a gamma counter. The number of the ^{57}Co chelates per orosomucoid was calculated by the ratio of ^{57}Co at the bottom of TLC plate (R_f = 0–0.14, corresponding to ^{57}Co–dota–protein) to the rest of the plate, which contained ^{57}Co chelated by free dota.

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