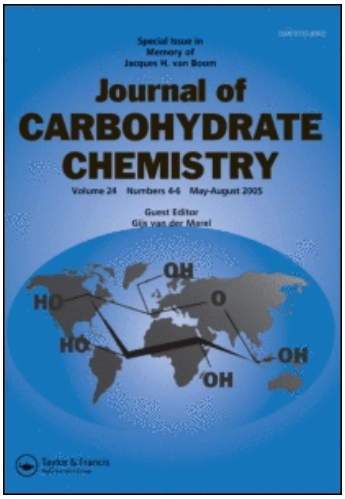

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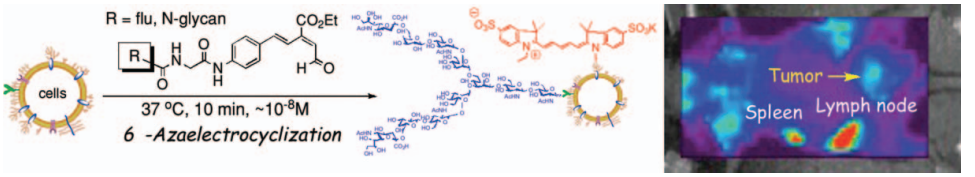

Graphical Abstract

Combined azaelectrocyclization and Staudinger ligation allowed proteins and living cells to be modified by small molecules (i.e., biotin or *N*-glycans). Chemically engineered lymphocytes modified by complex-type *N*-glycan targeted DLD-1 tissues implanted in nude mice at the whole-body level.

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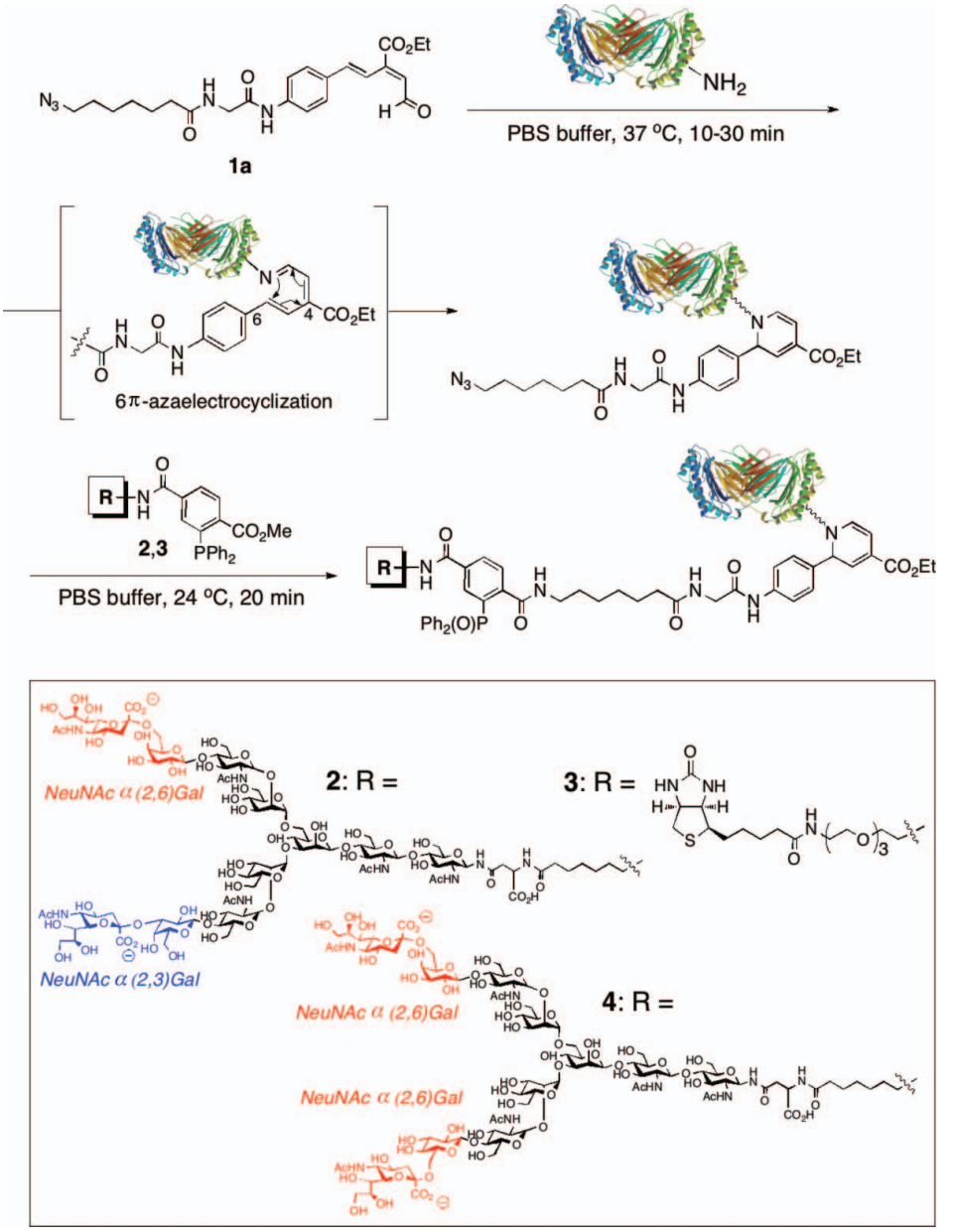
Keywords Cancer; Chemical Engineering; *N*-Glycan; Lymphocytes; Noninvasive Imaging; 6π-Azaelectrocyclization

Chemical methods for labeling or modification of proteins and living cells with functional molecules, such as oligosaccharides or biofunctional peptides (termed "bioconjugation"), have garnered significant attention, especially in the fields of molecular imaging and bioengineering. These methods offer the advantages of easy operation[1] and general applicability.[2] The chemical modification of biomolecules or living cells should be performed at low concentrations under mild conditions, with the goal of preserving the native bioactivities of the biomolecules in addition to attaining new functions from the conjugated molecules. Recently, a variety of new methods, which can also be combined with biological techniques, have been actively investigated,[3] for example, the Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition reaction (Sharpless/Meldal click reaction) with the azide moiety genetically introduced at desired positions within proteins.[4] Cell-friendly versions of the Cu(I)-free click reaction using strained acetylenes,[5–7] as well as the Staudinger reaction,[8,9] were developed by Bertozzi and coworkers. These reactions were successfully used for fluorescence imaging on living cell surfaces[10] and in living animals.[7c] The use of transition metal catalysts in the conjugation of biomolecules has also been reported. Davis and coworkers introduced mono- and disaccharides onto proteins by employing Ru-catalyzed cross-metathesis[11] and Pd-catalyzed Suzuki-Miyaura coupling.[12] Chemistry-based unique bio-orthogonal approaches have been extensively investigated by Hamachi and coworkers.[13] Hamachi's group developed a single molecule containing the fluorescent labels, the anchoring functional groups, and the protein ligands to selectively label regions near ligand-binding sites. After cleavage of the ligands from labeled proteins, proteins with recovered activity were used as sensitive reporter molecules for fluorescent imaging (e.g., for visualization of glycolysis rates inside cells). Hamachi's group also developed many efficient variants to this strategy, which involves post affinity labeling modification (P-ALM).

We recently developed a primary amine-based labeling of peptides, proteins (antibodies),[14,15] and even living cells,[16] which is based on a rapid 6π-azaelectrocyclization (Sch. 1).[17] This method was used to efficiently and selectively introduce both fluorescent groups and DOTA (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid), a metal-chelating agent, to amino groups. Amines in target proteins react with unsaturated aldehyde probes, such as probe **1a** (Sch. 1), at low concentrations ($\sim 10^{-8}$ M) within a short time (10 to 30 minute) at room temperature. Our method could be applied to visualization of the in vivo dynamics of glycoproteins[14] and cellular trafficking of lymphocytes[16] by means of noninvasive PET and fluorescence



Scheme 1: Two-step procedure for protein engineering through azaelectrocyclization-Staudinger ligation. (Figure available in color online.)

imaging. Our new method precisely controls the introduction of DOTA or fluorescence labels onto amines, such as lysines or ethanolamines, in target proteins or cells by adjusting the probe concentration so that the activity of the biomolecules can be retained.

Based on these initial results, we speculated that our azaelectrocyclization chemistry could also be used to covalently modify proteins or cells with functional molecules other than labels under mild physiological conditions. Herein, we report a combined azaelectrocyclization/Staudinger method useful as a new bioconjugation procedure to install biotin or complex-type *N*-glycans on proteins or living cells. Remarkable effects of chemical engineering with *N*-glycan were discovered during whole-body imaging of lymphocyte trafficking in living animals.

PROTEIN ENGINEERING

Electrocyclization probes such as aldehydes 2–4 (Sch. 1) are difficult to prepare, with the synthetic route involving oxidation of an allylic alcohol to the conjugated aldehyde as the last step.[14a] According to our preliminary trials, biotin or oligosaccharide moieties with numerous hydroxyl groups were incompatible with various oxidation conditions, and the reactions only provided decomposition products. To overcome this challenge, we developed a two-step engineering procedure shown in Scheme 1. Proteins are first tagged with an azide residue through an azaelectrocyclization. The cyclized products are then treated with phosphine reagents to complete the chemical bioconjugation via Bertozzi's Staudinger ligation.[8,9]

Human serum albumin (HSA)[15] was first investigated as a model protein (MW = 66,000 and containing 59 Lys). HAS was incubated with azide probe **1a**[14a,16] in 0.1 M phosphate buffer at 25°C for 30 min (HSA: 1.6×10^{-5} M, probe **1a**: 3.2×10^{-3} M). We "tagged" several azide groups onto HSA (*vide infra*) using the electrocyclization procedure performed at high concentrations to maximize the number of the azide functions on the protein template. Because the reactivity of the Staudinger ligation was previously shown to be relatively low, increasing the number of azide groups on the protein was crucial for the success of the two-step procedure. The excess of probe **1a** was removed by size-partitioning gel filtration, using the Microcon (Millipore, filter: MW = 10,000) under centrifugal conditions.[14a,16] Based on the MALDI-TOF-MS spectrum of the azide-tagged HSA, approximately 12 molecules of the probe were conjugated to each molecule of HSA (Fig. 1b). The azide-tagged HSA was then treated with Bertozzi's 2-methoxycarbonylphenyldiphenylphosphine reagents **2** and **3**,[8d] derived from the complex-type *N*-glycan and biotin (Sch. 1),[8] respectively, at 25°C for 3.5 h (N_3-modified HSA: 1.6×10^{-5} M, phosphines **2** and **3**: 2.0×10^{-3} M) to complete the protein engineering. The success of the bioconjugation was analyzed by using MALDI-TOF-MS after centrifugal separation with the Microcon. Although the broadening signals showed a mixture of reacted and unreacted N_3-HSAs from the Staudinger ligation, 1 molecule of *N*-glycan and 4 molecules of biotin were introduced onto HSA under these conditions on average, as shown in Figure 1c and d.

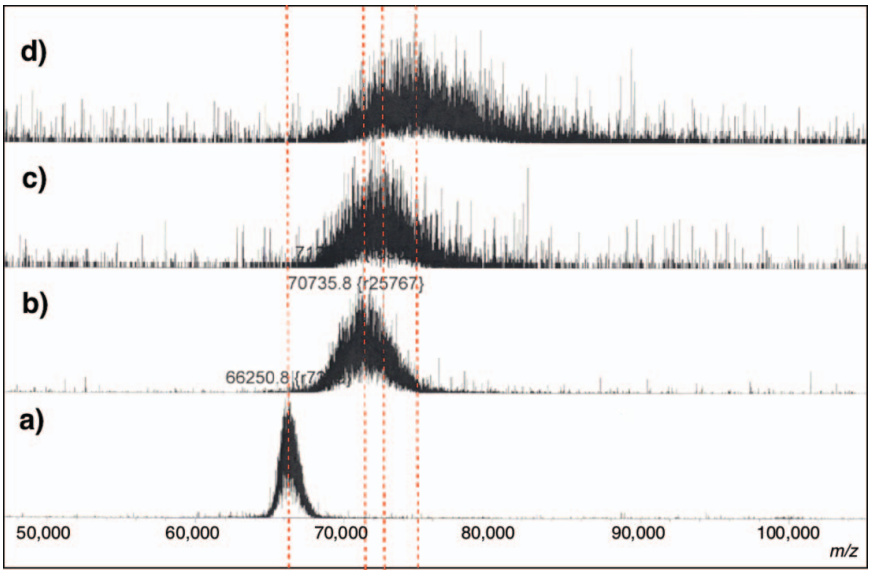
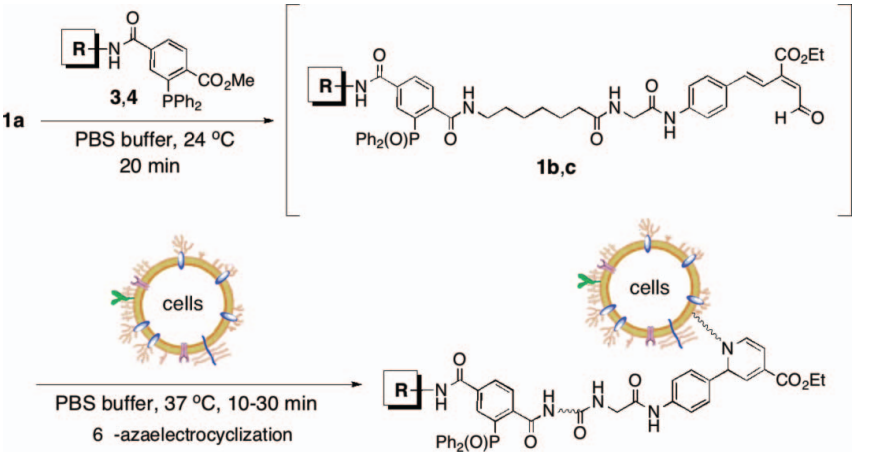


Figure 1: MALDI-TOF-MS spectra of azide-, biotin-, and *N*-glycan-engineered HSA: (a) Intact HSA (MW = 66,000); (b) Electrocyclization-modified HSA with an average of 12 molecules of azide; (c) Staudinger-modified HSA with an average of 1 molecule of *N*-glycan; (d) Staudinger-modified HSA with an average of 4 molecules of biotin. (Figure available in color online.)

CELL ENGINEERING

The engineering procedure was next applied to living cells, specifically to rat C6 glioma cells.[18] Unfortunately, application of the conditions shown in Scheme 1 led to the death of the cells, presumably because of exposure of the living cells to the harsh chemical reagents (aldehydes, phosphines, or DMSO solvent, see Experimental section) for a prolonged time (total reaction time over 4h). However, the cell engineering was successfully realized when the procedure was performed in the alternative reaction order, that is, by performing the Staudinger ligation of the probe and phosphine reagents first followed by azaelectrocyclization with the cells (Sch. 2). The azide-containing aldehyde **1a** was initially treated with phosphines **3** and **4** in PBS buffer at 24°C (reaction conc. for both **1a** and phosphines: 1.2×10^{-3} M). The *N*-glycan structures of **2** and **4** are slightly different in terms of the sialic acid linkages to the galactose residues. The glycan in **4** has two NeuNAcα(2,6)Gal nonreducing ends, whereas that in **2** has both NeuNAcα(2,6)Gal and NeuNAcα(2,3)Gal moieties (Sch. 1).

Based on the MALDI-TOF-MS spectra, the Staudinger ligation proceeded about 50% within 20 min, accompanied by the CHO-reduced byproduct (alcohol derivative of **1a**). A reaction concentration greater than 10^{-3} M and a prolonged reaction time led to the oxidation of the phosphine and the reduction



Scheme 2: One-pot procedure for cell engineering. (Figure available in color online.)

of the aldehyde in **1a**. Unpurified intermediates **1b** (biotin) and **1c** (*N*-glycan) were subsequently reacted with the C6 glioma cell in a one-pot procedure at 37°C for 10 to 30 min (engineering concentration: 2.0×10^{-5} M for **1b** and **c**). The modified cell surfaces were evaluated by treatment with TRITC (rhodamine)-labeled avidin and TRITC-labeled SNA (Sambucus nigra, Elderberry, Neuα(2,6)Gal-specific lectin)[19] (Fig. 2).

Although TRITC-derived red fluorescence was observed for the **1b** (biotin)-labeled cell (Fig. 2a), only a negligible level of fluorescence was detected for the control cell, which was treated with phosphine reagent **3** instead of **1b** (Fig. 2b). The covalent modification of the cells with biotin was achieved through a combined sequence of azaelectrocyclization/Staudinger ligation. Similarly, confocal microscopy detected red fluorescence on the **1c** (*N*-glycan)-labeled C6 cell upon treatment with TRITC-labeled SNA (Fig. 2c). Red fluorescence was also observed for the intact cell (Fig. 2d) because the C6 glioma cell itself had native *N*- and/or *O*-glycans with a nonreducing end Neuα(2,6)Gal motif on the cell surface. The SNA lectin was able to interact with these native glycans. However, the **1c**-modified glial cell showed higher fluorescence intensity than that of the intact cell, thereby confirming the modification by *N*-glycan.

NONINVASIVE IMAGING OF N-GLYCAN-ENGINEERED LYMPHOCYTES IN A CANCER MOUSE MODEL

Finally, we investigated how *N*-glycan engineering affects the trafficking properties of lymphocytes in living animals. Lymphocytes (1 mL, 1.0×10^5 cells) directly extracted from the abdominal cavity of nude mice were fluorescently labeled by azaelectrocyclization (Cy5-fluorescence),[16,20] and the labeled lymphocytes (100 μL/mouse, 10^4 cells) were administrated to the tail vein of a

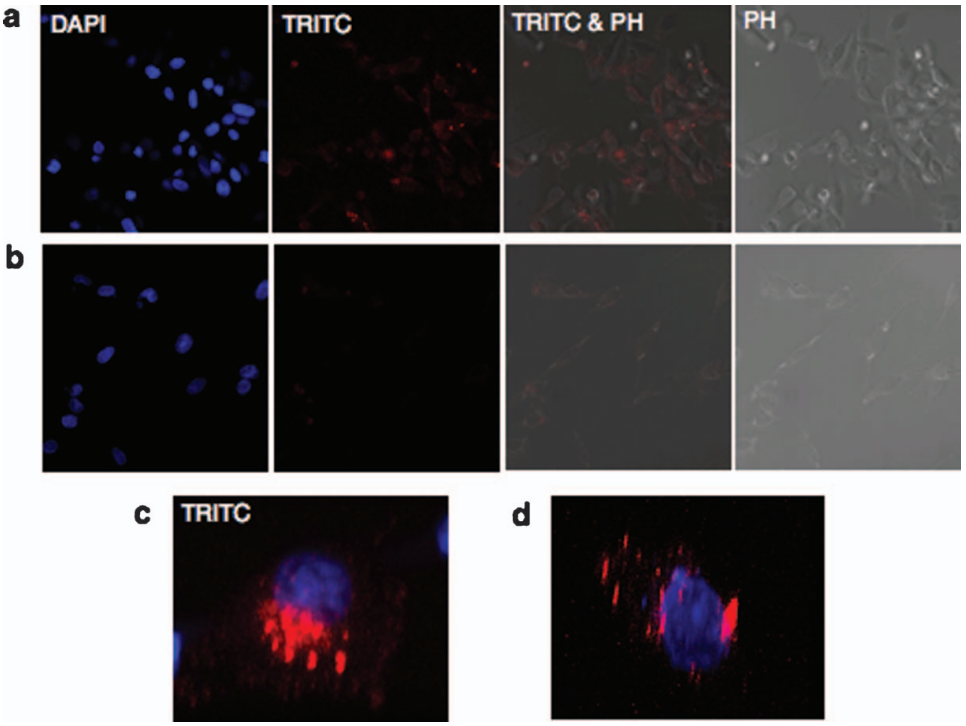


Figure 2: Confocal microscopy of biotin- and *N*-glycan-modified C6 glioma cells (DAPI-, TRITC, and PH detections). (a) **1b**-Modified cell on TRITC-avidin treatments; (b) control cell treated with biotin-containing phosphine **3** on TRITC-avidin treatments; (c) **1c**-modified cell on SNA lectin (Neuα(2,6)Gal-specific) treatments; (d) intact cell on SNA lectin treatments. PH: phase contrast. (Figure available in color online.)

mouse that had a DLD-1 human colon carcinoma implanted to the dorsal division (Experimental Section). The dynamic fluorescence images were recorded over a week by using eXplore Optix, GE Healthcare, Bioscience (Fig. 3).[21] The lymphocytes gradually accumulated over 6 h, mainly in the spleen and intestinal lymph nodes, and the fluorescence intensity in the spleen was found to decrease over time (Fig. 3a). These trafficking properties were similar to our earlier findings in the normal nude mice,[16] although the accumulation to the spleen was slightly slower in the cancer model. However, no fluorescence was detected in the tumor tissues over the course of a week. On the other hand, we made a remarkable observation when the lymphocytes were simultaneously labeled by the Cy5-fluorophore and engineered by *N*-glycan **1c** under the conditions shown in Scheme 2. The fluorescence intensity of the engineered cells remained strong in the spleen after 48 h (Fig. 3b), and the cells gradually began to accumulate into DLD-1 (TM). The fluorescence intensity in the tumor regions became even stronger over a week.

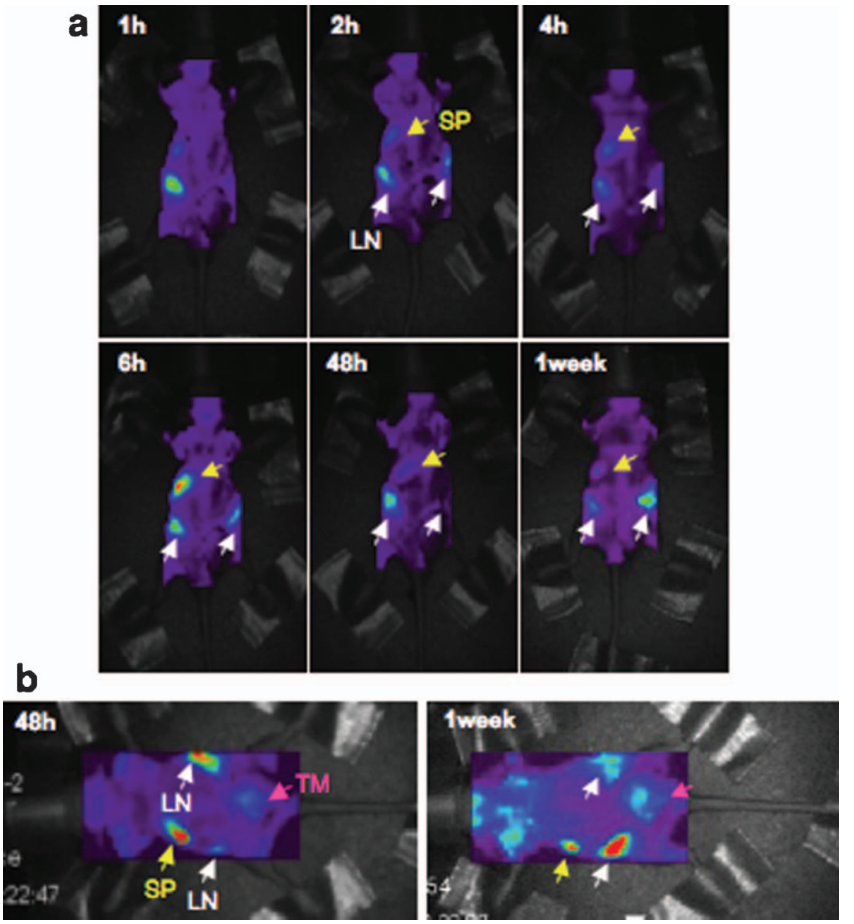


Figure 3: Fluorescence imaging of lymphocytes in mice. Labeled and/or engineered cells were administrated intravenously (n = 3, 100 μL/mouse, 10^4 cells) and the whole body was scanned from the back side by eXplore Optix, GE Healthcare, Bioscience (excitation at 646 nm, emission 663 nm), 1 h, 2 h, 4 h, 6 h, 48 h, and 1 week after injection. Data were normalized. SP: spleen; LN: lymph node of epidermal intestinal tract; TM: DLD-1 human colon carcinoma. (a) Cy5-labeled cells injected to nude mice that were implanted with DLD-1 at the dorsal division; (b) both Cy5-labeled and *N*-glycan-engineered lymphocytes in the tumor model. (Figure available in color online.)

In order to explain the imaging results in Figure 3, further investigations are needed to examine the mechanisms at the molecular level. Nevertheless, since neither the native lymphocytes nor the complex-type *N*-glycan[22] traffics to the cancer regions on their own, the engineering of the lymphocyte surfaces is key to the tumor targeting. The low efficiency of lymphocyte-based cancer immunotherapy, such as using NK, LAK, CTL, and TIL as the immune effector cells, is well documented, and in order to circumvent the problems, (1) coinjection with cytokines, costimulatory molecules or cancer antigens, and more

recently, (2) engineering of the lymphocytes by the chimeric antigen receptors have widely been applied.[23]

The reason for *N*-glycan-enriched lymphocyte trafficking to tumor is unclear. There are two possible explanations: (1) the *N*-glycan-enriched lymphocytes might interact with the tumor via additional interaction with lectins on cancer cells or (2) the interaction of Siglec (Sialic acid-binding, immunoglobulin-like lectin) and internal sialoglycans on the lympocytes, which stimulates the immunosuppressive signals through the ITIM (immunoreceptor tyrosine-based inhibitory motif) molecules,[24] could be interrupted by the *N*-glycan **1c** externally introduced by chemical engineering. This eventually might activate the lymphocytes to target the tumor. These preliminary results indicate the potential for the electrocyclization-based engineering to be applied to whole cell-based targeting to specific cancers or immune-related organs, and therefore highlight the present glycan-engineering protocol.

In summary, a combined electrocyclization/Staudinger protocol enables functional groups, such as biotin and complex-type *N*-glycans **2** and **4**, to be loaded onto proteins and living cells. The present method was applied to modification of lymphocytes with *N*-glycan and the artificial lymphocytes targeted a tumor in living animals due to the synergistic effects of both functions of lymphocytes and oligosaccharides. The current method is not yet generally applicable to most systems because of the low reactivity of the Staudinger ligation and the facile oxidation of phosphine reagents under physiological conditions. Nevertheless, combining our electrocyclization with other conjugation reactions, such as the Cu(I)-mediated Huisgen 1,3-dipolar cycloaddition or strain-releasing reactions, and/or generating the aldehyde function at the last stage of the probe synthesis (i.e., enzymic oxidation or protecting groups conversion under the mild conditions), may significantly broaden the scope of this approach to chemical engineering. The development of more efficient engineering probes and the biological evaluation of the *N*-glycan-engineered proteins and living cells are now in progress in our laboratory.

EXPERIMENTAL SECTION

Ethyl (E,E)-4-Hydroxy-2-{4-[2-(7-azidoheptanamide) acetamide]styryl}but-2-enoate (alcohol precursor of 1a)

To a solution of ethyl (*E,E*)-[2-(*N-tert*-butoxycarbonyl-2-aminoacetamide) styryl-4-(tetrahydro-2*H*-pyran-2-yloxy)]but-2-enoate (40.0 mg, 81.9 μmol) in MeOH (1.25 mL) was added 6N aqueous HCl (1.25 mL) dropwise at 0°C. After the reaction mixture was stirred at rt for 1 h, the mixture was extracted with hexane. The aqueous layer was neutralized with 1N aqueous NaOH and

desalted through a column filled with HP-20 to afford ethyl (*E*,*E*)-4-hydroxy-2-[4-(2-aminoacetamide)styryl]but-2-enoate as a yellow solid, which was used without further purification.

To a solution of the crude aminoalcohol obtained above (20.1 mg, 66.1 μmol) in DMF (1 mL) was added the succinimidyl ester of 7-azidoheptanoic acid (17.7 mg, 66.1 μmol) at rt. After the mixture was stirred at rt for 2 h, the solution was concentrated in vacuo. The residue was directly purified by using preparative chromatography on silica gel (CH_3Cl: CH_3OH = 30:1) to give the title compound (8.1 mg, 22% for two steps) as a white solid: mp 105°C; IR (neat, cm^{-1}) 3350, 1748, 1707; ^1H NMR (500 MHz, CD_3OD) δ 7.55 (2H, d, J = 8.7 Hz), 7.43 (2H, d, J = 8.9 Hz), 6.86 (1H, d, J = 16.3 Hz), 6.77 (1H, d, J = 16.3 Hz), 6.76 (1H, t, J = 6.0 Hz), 4.51 (1H, br, s), 4.46 (2H, d, J = 6.1 Hz), 4.26 (2H, q, J = 7.2 Hz), 3.99 (2H, s), 3.28 (2H, t, J = 6.9 Hz), 2.30 (2H, t, J = 7.5 Hz), 1.69–1.63 (2H, m), 1.62–1.57 (2H, m), 1.42–1.39 (4H, m), 1.32 (3H, t, J = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 176.75, 169.66, 168.40, 142.57, 139.61, 135.43, 134.31, 131.58, 128.21, 121.15, 120.88, 62.10, 59.99, 52.38, 44.03, 36.69, 29.75 (x2), 27.51, 26.63, 14.53; ESI HRMS m/z calcd for $C_{23}H_{31}N_5O_5Na$ $(M+Na)^+$ 480.2223, found 480.2204.

General Procedure to Chemically Engineer Cell Surfaces by Modification with Biotin and N-Glycans

IBX-resin (10 mg, 8.0 μmol) was added at rt to a solution of the alcohol precursor of **1a** (200 μg, 440 nmol) in DMF (25 μL) and CH_2Cl_2 (25 μL) in an Eppendorf tube. After the reaction mixture was shaken at rt for 30 min, the IBX-resin was removed by using a centrifugal filtration tube (Cosmospin Filter H, 0.45 μm, Nacalai Tesque), and the solvents were removed by centrifugal concentration at rt. The aldehyde probe **1a** remaining in the Eppendorf tube was dissolved in the appropriate volume of the PBS containing 20% DMSO, and the solution concentration of probe **1a** was adjusted to 3.14×10^{-3} M. To the resultant solution (140 μL, 3.14×10^{-3} M) was added a PBS solution of the biotin/phosphine reagent **3**[8d] (220 μL, 2.0×10^{-3} M, cont. 20% of DMSO) (reaction concentrations: 1.22×10^{-3} M for both **1a** and **3**). The resulting solution was kept at rt for 20 min (model Staudinger product with the stable alcohol precursor of **1a**; MALDI-TOF HRMS m/z calcd for $C_{61}H_{78}N_7NaO_{13}PS$ $(M+Na)^+$ 1202.5, found 1202.9). The mixture was then treated with the suspended C6 glioma cells (cell number: 1.0×10^5/mL, final labeling concentrations of the probe: 2.0×10^{-5} M, cont. 0.2% of DMSO, 1.0 mL). After maintaining the mixture at 37°C for 10 min under a carbon dioxide atmosphere, the resulting cells were washed three times with the culture medium to remove excess probe.

Rat Glioma Cell Culture and Cell Engineering Procedure

Rat glioma C6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells (1×10^5) were trypsinized and washed with PBS three times to exclude any inhibitors for engineering probes, such as amines. The washing buffer was replaced with new buffer containing probe (2.0×10^{-5} M). After incubation at 37°C, the cells were washed with cultured medium to stop the reaction. Then engineered cells were seeded into culture dishes to estimate the reaction efficiency by viewing the cell surface by microscopy.

Preparation of Lymphocytes, Cancer Model, and in vivo Fluorescence Imaging

A wild-type mouse (BALB/cAJcl, CLEA Japan, Inc.) was euthanized by using excess anesthetic (Isoflurane). After creating an incision into the abdomen, about 1 mL of blood was collected from the aorta descendens. Heparin sodium (100 μL, 100 units/mL) was added to the collected blood and the blood was stored at rt for 2 h, whereupon the lymphocytes were extracted. Mouse peripheral blood diluted with medium was added onto the lymphosepar II layer, and the mixture was centrifuged. The lymphocyte fraction in the middle layer (suspended cell culture) was collected, medium was added, and the mixture was centrifuged again. The pellet was then suspended in cell culture medium and the number of cells was counted. By repeating this procedure, a total of 3.6×10^6 cells were collected from 3.5 mL of the blood sample. After adjusting the cell number using culture medium, the cells were immobilized on antibody-coated plates and cultured at 37°C under a 5% carbon dioxide atmosphere. The cultured lymphocytes were stored for 2 h at rt in a physiological salt solution before performing the labeling and engineering procedures described above. A total of 1×10^5 cells/1 mL PBS buffer of the lymphocytes were labeled with a Cy5-probe[16] (incubation concentration: 2.0×10^{-5} M), and these lymphocytes were engineered with the *N*-glycan probe **1c** at an incubation concentration of 5.1×10^{-5} M.

Eight-week-old mice (BALB/cAJcl–nu/nu, CLEA Japan, Inc.) were used for the in vivo fluorescence imaging study. The cancer model was prepared by subcutaneously injecting DLD-1 (10^7 cells/100 μL) to the dorsal division of the mouse (BALB/cAJcl–nu/nu, CLEA Japan, Inc.). A tumor was grown up to a proper size for imaging purposes over a 2- to 3-week period from the injection. Therefore, 10-week-old mice (2 weeks from the injection of DLD-1) were used for imaging. Fluorescence-labeled or *N*-glycan-engineered lymphocytes (1×10^4 cells/100 μL of a physiological salt solution) were injected from the caudal vein without anesthesia, and a whole-body scan was performed by using eXplore Optix, GE Healthcare, Bioscience, over 1 week after injection.

The in vivo fluorescence image was taken under inhalation anesthesia with Isoflurane, with the concentration of Isoflurane kept at 4% from 15 min to 1 h after the injection and then at 1.5% to 2% during the rest of the measurements.

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