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Cell surface biotinylation by azaelectrocyclization: Easy-handling and versatile approach for living cell labeling

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ABSTRACT

Versatile method for living cell labeling has been established. Cell surfaces are initially biotinylated by azaelectrocyclization, and then treated with the fluorescence-labeled avidin or the anti-biotin antibody.

1. Introduction

Covalent chemical labeling of living cells has garnered significant attention in the fields related to molecular imaging,[1] and has broad and general applicability to both in vitro and in vivo studies of living cells.[2] Undesirable modification of key functions on cell surfaces should be avoided in order to retain the cell's native functions. Therefore, bioorthogonal approaches, which can be combined with biological techniques, have actively been investigated.[3] Successful examples include Bertozzi's Staudinger ligation[4–6] and strain-accelerated Huisgen 1,3-cycloaddtion reaction.[7–10] These methods have been applied to labeling of cells expressing oligosaccharides with azido-containing sugar residues on the cell surfaces through biosynthetic pathways,[8] which subsequently reacted with the methoxycarbonyl phenyldiphenylphosphine or cyclooctyne derivatives. Variety of the reactive cyclooctyne derivatives have continuously been developed in pursuit of the efficient bioconjugation on the living cell surfaces by this strategy.[10b]

On the other hand, we recently developed an amine-based labeling through rapid 6π-azaelectrocyclization.[11,12] We have used this method to efficiently and conveniently introduce the fluorescent groups to the amino groups on the cell surfaces[13] via a reaction with unsaturated aldehyde probes (such as probe **1a** in Scheme 1a) at very low concentrations (can be decreased down to 10^{-8} M) within a short time (10 min) at room temperature. Although our reaction is non-bioorthogonal to the primary amino groups on cell surfaces, under such mild reaction conditions the most exposed and densely expressed amines that is, lysines of membrane proteins and/or ethanol amine derivatives at cell surfaces react rapidly and selectively; hence minimizing the indiscriminative amino modification as well as the significant probe internalization, which cause the interferences with their native functions. Pyridine derivatives as the electrocyclization products, which retain the cationic charges as those of the inherent lysines, might also contribute to the retention of the cell functions. In vivo imaging of the Cy5 (cyanine fluorescence dye)-labeled lymphocytes was also achieved as an application of the present protocol; the trafficking of the cells into the immune-related organs was clearly visualized with markedly high imaging contrasts.[13,14]

In widely applying our electrocyclization chemistry to the cell surface labeling by various fluorescence dyes and/or the other small molecules,[14] however, a 'generality problem', in a sense of the convenient use of our chemistry in both organic chemistry and biology laboratories, still remains unsolved. Namely, the unsaturated aldehydes **1a** are to be synthesized for each different fluorescence probe. Although **1a** with TAMRA or Cy5 dyes could readily be prepared, some fluorescence structures, such as Alexa Fluor® series and fluoresceins, were also found to decompose during the oxidation process from the precursor alcohol to the probes **1a**. Because the structures of most of the fluorescence dyes are not even disclosed, the stability during the preparation of the probes cannot be predicted. In order to generalize the cell-surface labeling, herein we report a two-step protocol by combining our electrocyclization chemistry with the biotin/

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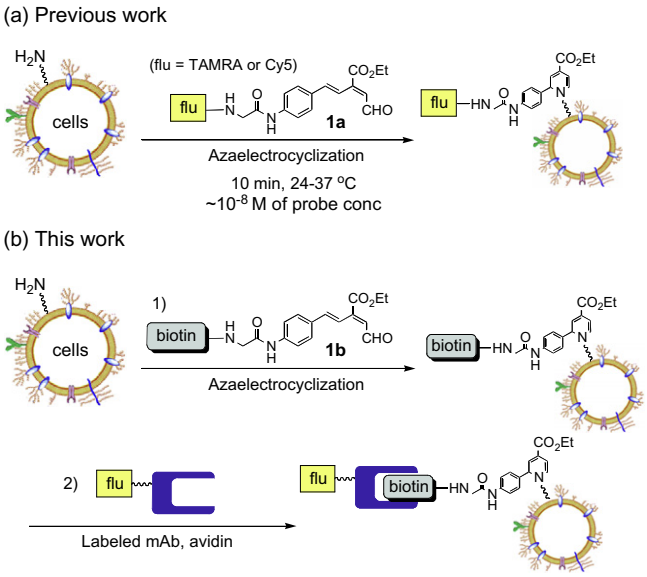
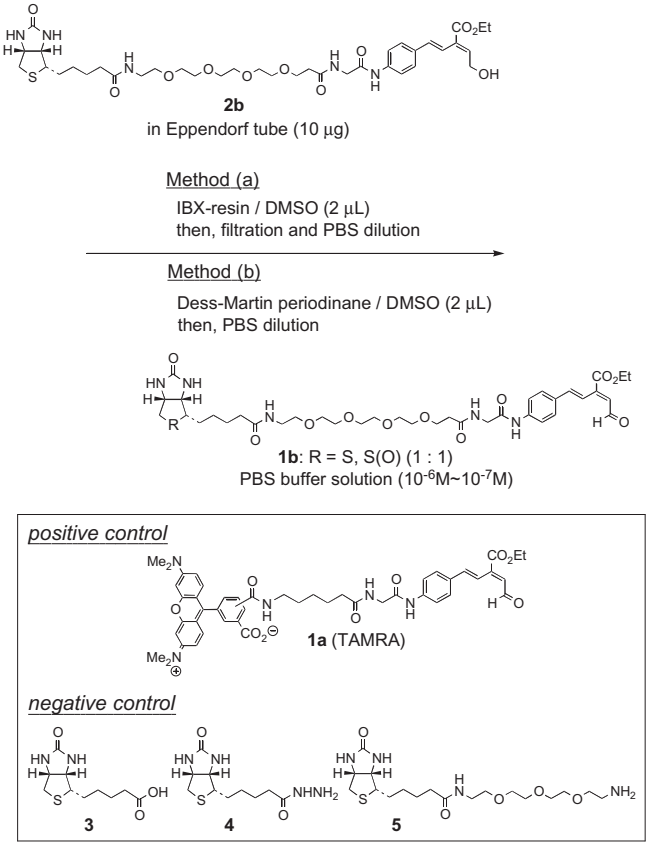


Scheme 1. Schematic presentation of cell surface labeling by azaelectrocyclization. (a) Previous work. (b) This work.

avidin and/or biotin/anti-biotin monoclonal antibody (mAb) interactions, as shown in Scheme 1b. Thus, the cell surfaces are initially biotinylated by azaelectrocyclization using the probe **1b**, and then treated with the fluorescence-labeled avidin or the anti-biotin antibody. Considering the commercial availability of variously labeled avidins and mAbs, and the numerous reports on the biotin-based techniques in biology-directed research, the present method offers the general and versatile approach to the living cell surface labeling and/or engineering.



Scheme 2. Oxidation of biotin-alcohol to **1b** and the other biotin derivatives as control for labeling experiments.

2. Results and discussion

2.1. Probe preparation

Although we have previously reported in situ generation of the biotin-containing unsaturated adehyde[14] through the Staudinger ligation using the 2-methoxycarbonylphenyldiphenylphosphine reagent,[5d] the facile air oxidation of the phosphine sometimes caused the reproducibility problems during the cell labeling experiments. We therefore used in this experiment the probe **1b**, where the biotin is covalently introduced to the unsaturated aldehyde in advance through the amide bond. Oxidation of the alcohol **2b**, which is now commercialized[15] as the precursor of **1b**, was achieved by the improved procedures using a few μg-scale in Eppendorf-tube (Scheme 2). Thus, the alcohol **2b** was treated with IBX-resin (2-iodoxybenzoic acid) in small amount of DMSO for 30 min.[13] After the resin was filtered off, the resulting aldehyde probe **1b** was directly used by diluting with PBS buffer solution (final PBS solution containing 0.01% of DMSO). Alternatively, the alcohol **2b** could be oxidized by Dess–Martin periodinane in DMSO (see details in Section 4); the reaction solution was diluted by PBS buffer and directly treated with the living cells. Any significant effects on cell viability and/or functions could not be observed by the latter procedure (vide infra). For both cases, the sulfur atom in a half amount of **2b** was oxidized to the sulfoxide as judged by MALDI-TOF-MS analysis; although the oxidized derivatives of biotin are known to show the 10-fold reduced interaction with the avidins,[16] they still retained very small K_d value of 10^{-14} M, and therefore used for cell labeling without separating the two compounds. In addition to the biotin-aldehyde probe **1b**, the TAMRA (carboxytetramethylrhodamine)-fluorescence probe **1a**,[13] as well as biotin **3**, hydrazone linked **4**, and PEG-amine linked **5**, were used as the positive and negative controls for the labeling experiments (Scheme 2).

2.2. Labeling experiments

Labeling experiments were performed using MDCK (Madin-Darby canine kidney) and HeLa (cervical cancer cell line) cells (see details in Section 4): We initially treated the cells (2×10^5/700 μL), which were cultured on glass cover slips coated with poly-L-lysine, with biotin-probe **1b** (1.0×10^{-6} M in PBS) for 5 min at 37 °C, 5% CO_2 (Scheme 1b). After the cells were washed with culture medium twice in order to remove the excess aldehyde probe, they were subsequently treated with Cy3-labeled anti-biotin mAb (excitation: 547 nm, emission: 563 nm) or Alexa594-labeled streptavidin (590 nm/617 nm) in medium (dilution of 1:100 and 1:200, respectively) for 30 min, under the identical conditions performed for the biotin-labeling conditions. As seen in Figure 1, any significant differences in morphology were not found between the labeled cells (Fig. 1a and b) and the control cells (Fig. 1c and d). Cell division could also be observed in some labeled cells; thus any notable effects on cell viability could not be observed during a two-step labeling procedure, but the prolonged subjection of cells to the PBS solution of **1b**, that is, more than 20 min, caused round-shaped outward appearance. The fluorescence labeled cells were also treated with Hoechst33342 as a blue dye to visualize the nuclei and fixed with formaldehyde. All the control experiments using **1a** (TAMRA) and **3**–**5** were also performed according to the procedures described above, and analyzed by confocal microscopy (Fig. 1).

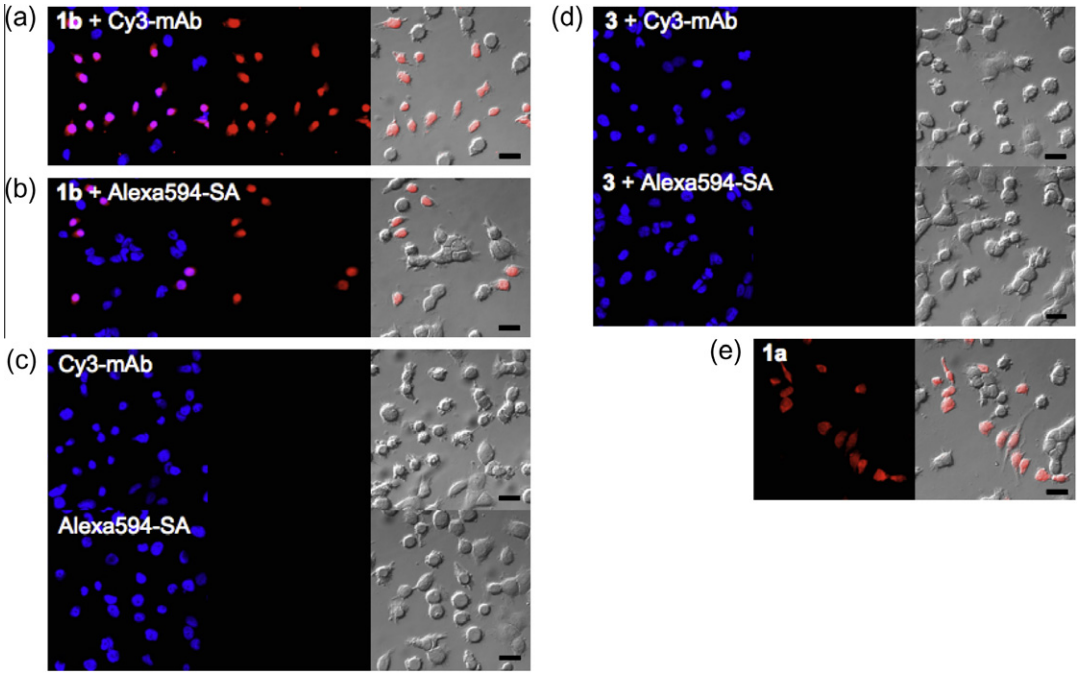


Figure 1. Detection of biotin-labeled HeLa cells by azaelectrocyclization; bars: 20 μm. (a, b) Cells treated by the method using **1b** to label the cell surface. (c, d) Cells treated with either PBS (c) or biotin (d) instead of method using **1b**. For each condition, cells were further labeled with either Cy3-conjugated anti-biotin mouse monoclonal antibody (a, c, d) or Alexa594-conjugated streptavidin (SA) (b, c, d). Nuclei and chromosomes were labeled with Hoechst33342 (Blue). (e) Cells treated by the method using **1a** to label the cell surface with TAMRA (red). (a–d) Left panels: merge of Cy3 or Alexa594 and Hoechst33342, middle panels: Cy3 or Alexa594, right panels: merge of phase contrast and Cy3 or Alexa594. (e) Left panel: TAMRA, right panel: merge of phase contrast and TAMRA.

2.3. Confocal microscopic analysis

Red fluorescence derived from the Cy3 and Alexa594 dyes was observed on both HeLa and MDCK cells reacted with the aldehyde **1b** (Data shown only for HeLa cells in Figure 1a and b, and see Supplementary data for MDCK cells), whereas no fluorescence was observed in cells treated with PBS (without **1b**) and fluorescence-labeled anti-biotin mAb or streptavidin (Fig. 1c). Furthermore, any fluorescence could not be observed by using the biotin derivatives **3–5**, instead of **1b**, which lack the unsaturated aldehyde structures necessary for azaelectrocyclization (Fig. 1d and Supplementary data). Detailed 2D and 3D microscopic analysis of the labeled cells by the biotin/avidin and the biotin/antibody conjugates, clearly showed the fluorescence localization on the cell membrane. These results clearly show that biotin-probe **1b** rapidly reacts with the amino groups of the lysines and/or other amino-containing cell membrane constitutes, such as phosphatidyl ethanolamines, to covalently anchor the biotin molecule through azaelectrocyclization,[13] which the antibody or the streptavidin interacted with.

It should be noted from Figure 1a and b that not all cells were equally labeled, therefore showed selective cell surface labeling. Namely, rather than the cells with cell–cell contact showing colonization, the cells that do not contact with neighboring cells are preferentially labeled possibly by more exposed cell surface to the probes. Such labeling properties were same as those performed by directly reacted with TAMRA-fluorescence probe **1a** (Fig. 1e). Based on previous results on the reactivity of **1a** and other unsaturated aldehyde probes,[12,13] azaelectrocyclization selectively proceeds at the most exposed surface regions to the probes and/or at the high protein assembly (high concentration of Lys) on the cell surface. Taking these into consideration, the biotin-labeling might also proceed most probably at the accessible cell surfaces rather than those inside the aggregates under the mild labeling conditions, that is, at 37 °C for 5 min. Alternatively, the cell lines are still individually heterogeneous in functional and physical properties and those of amino-riched domain could preferentially be labeled, of which investigation is currently in progress.

3. Summary

In summary, we established labeling of whole cells through chemical biotinylation followed by the anti-biotin mAb or avidin treatments. With our hands, the labeling by biotin-probe **1b** was still effective at a 10^{-7} M concentration (Supplementary data), providing one of the highly diluted chemical methods realized so far through a covalent bond formation. Because variously labeled and functionalized biotin antibodies and avidins are commercially available, and easily prepared if necessary, the present two-step protocol offers versatile and convenient chemical approaches to label and engineer the living cells. Based on the results described herein, it may further be possible to label living cells with the metal-incorporated DOTA,[12] which would expand the method to the radiotracer-based targeting or imaging of whole cells for PET, MRI,[17] or other radiopharmaceutical purposes. Research in this direction is currently under way in our laboratory.

4. Experimental

4.1. Preparation of biotin probe 1b

Dess–Martin periodinane (22 μg, 52 nmol) in 2 μL of DMSO was added at room temperature to allylic alcohol **2b** (10 μg, 13 nmol) in an Eppendorf tube. After the reaction mixture was shaken at room temperature for 10 min, the resulting DMSO solution of **1b** was diluted by adding the 198 μL of PBS (ca. 6×10^{-5} M). Various concentrations of solution of probe **1a** were then prepared by further dilution by PBS; the 1.0×10^{-6} M solution of **1b** contains 0.01% DMSO, by this procedure.

4.2. Cell culture and cell labeling procedure

MDCK and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum. Cells of 2×10^5 were seeded on 13 mm diameter glass cover slips pre-coated with 0.05 mg/mL poly-L-lysine/0.15 M Borate buffer solution (pH 8.4) and grown overnight at 37 °C, 5% CO_2. Cells were washed twice with PBS (pH 7.2–7.4) to exclude residual labeling inhibitors. Washing buffer was replaced with either biotin-conjugated cell labeling probe or biotin derivative diluted in PBS at 1×10^{-6}–1×10^{-7} M concentration. After incubation for 5 min at 37 °C, 5% CO_2, cells were washed twice with cultured medium to stop the labeling reaction. Biotinylated cells were further incubated with Cy3-conjugated anti-biotin mouse monoclonal antibody (Jackson ImmunoResearch Inc.) or Alexa Fluor® 594-conjugated streptavidin (Invitrogen) in culture medium for 30 min at 37 °C, 5% CO_2. Nuclei and chromosomes were labeled with 2 μg/mL Hoechst33342 (Invitrogen) in addition to the secondary antibody. Labeled cells were fixed with 2% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature. They were subsequently washed with PBS and mounted with Prolong Gold (Invitrogen). Images were captured by confocal laser microscopy Fluoview FV1000 system (Olympus) using 20× objective lens 4× zoom.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.043.

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