Effect of chloroacetyl modification on the suppression of dissociation of a fluorescent molecule from cells for antigen-specific cell staining

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Abstract

We previously developed a hydrolase-based fluorescence amplification method for antigen-specific cell labelling, in which fluorescent substrates stained cells by non-covalent hydrophobic interaction. To improve the substrates retention in cells, we examined here the effect of a chloroacetyl group modification on the substrate retention. We found that the chloroacetyl group suppressed the dissociation of the substrate after forming a covalent bond with intracellular proteins. However, slow reaction speed of the chloroacetyl group allowed dissociation for cells in the early stage of staining reaction.
Introduction

Cells are identified based on kinds and copy numbers of expressing membrane proteins. To label the membrane proteins, fluorophore-modified antibodies have been used. In this case, autofluorescence of cells resulting from intracellular molecules decides the limit of detection of the copy numbers of membrane proteins, which is sometimes not satisfactory especially when a fluorophore with a shorter wavelength is used.\(^1\)\(^,\)\(^2\) To improve the limit of detection, enzymatic reaction of horse radish peroxidase has been utilized to accumulate large number of fluorophores modified with enzymatic substrate domains.\(^3\)\(^,\)\(^5\)

We previously extended the enzyme-based fluorescence signal amplification method to hydrolases such as alkaline phosphatase and β-galactosidase (CARP method).\(^6\)\(^,\)\(^7\) The CARP method is based on the increment of hydrophobicity of the fluorescence substrate upon removal of a hydrophilic group by the enzymatic hydrolysis. The hydrophobic fluorescent substrate stained cells by non-covalent hydrophobic interaction. Although the stability of the hydrophobic interaction is relatively strong, dissociation of the fluorescent substrate is inevitable during the long-time incubation.

Covalent bond formation with cellular components is generally used to avoid dissociation of dyes in the fluorescent staining of cells. Reactive groups with thiol and amine groups in intracellular proteins have been utilized so far.\(^8\)\(^,\)\(^9\) Among these groups, we employed here a chloroacetyl group\(^10\) which has a good balance of stability and reactivity, i.e., being stable to afford diffusion of dyes into cells and reactive to rapidly form covalent bond with a thiol group. We examined effect of the chloroacetyl group on the suppression of dissociation of a fluorescent substrate (Fig. 1).
**Experimental**

**Reagents and chemicals**

NovaSyn TGR resin was purchased from Novabiochem (Darmstadt, Germany). Dichloromethane (DCM) and trifluoro acetic acid (TFA) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). N,N-Dimethylformamide (DMF) was obtained from Kanto Chemical (Tokyo, Japan). Chloroacetyl chloride and triisopropylsilane (TIS) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Methyl-β-cyclodextrin (M-β-CD) was obtained from Sigma Aldrich (St. Louis, MO). NHS-rhodamine (TAMRA SE) and streptavidin protein were obtained from Thermo Fisher Scientific (Waltham, MA).

**Synthesis of 2**

Substrate 2 was synthesized on NovaSyn TGR resin using Fmoc compounds following the reported procedure with some modification. For introduction of chloroacetyl group and rhodamine on to each lysine residue, Mtt and ivDde protected lysines have been used, respectively. After the synthesis of main chain of the substrate on the resin, the ivDde group of the second lysine residue was removed by a deprotecting reagent (hydrazine : DMF = 2 : 98). Then, DMF/0.1 M NaHCO₃ (pH 8.3–8.5) (1/1) solution containing 1.5 equivalents of TAMRA SE was added to the resin and allowed to react for 10 hours. Next, the Mtt group on the first lysine residue was removed by a deprotecting reagent (TFA/TIS/DCM = 1:1:18). Subsequently, DMF containing three equivalents of chloroacetyl chloride was added to the resin and placed for 6 hours. Cleavage from the resin and purification of the compound were conducted following the previous report. The compound 2 was identified by matrix-assisted laser
desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Auto- flex III, Bruker Daltonics).

**Cell culturing**

Daudi and K562 cells were cultured in RPMI 1640 medium (Wako) containing 20% (Daudi) or 10% (K562) fetal bovine serum (FBS), 100 U/mL penicillin, 100μg/mL streptomycin, and 0.25μg/mL amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY). Cells were cultured in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C.

**Cell staining by 1 or 2**

Daudi cells were washed twice with a serum-free RPMI 1640 medium, and suspended in 50 µL of the serum-free medium to a concentration of 4.0 x 10⁵ cells/50 µL in a 1.5-mL tube. Substrate solution (0.2 or 1 µM substrate containing 4 mM M-β-CD) was prepared by mixing a DMSO solution of the substrate (0.4 or 2 µM) with a solution of M-β-CD (8 mM) in the serum-free medium for 30 minutes. Then, 50 µL of the substrate solution was added to the cell suspension, and the cells were incubated at 37°C for 2 hours. Then, the cells were washed with RPMI 1640 medium containing 10% FBS. Finally, the cells were resuspended in an appropriate amount of the serum-free medium and analyzed by a BZ-8000 fluorescence microscope (KEYENCE, Japan).

**Evaluation of transfer of 1 or 2 from stained cells to unstained cells**

K562 cells were washed twice with a serum-free RPMI 1640 medium, and suspended in 50 µL of the serum-free medium to a concentration of 4.0 x 10⁵ cells/50 µL in a 1.5-mL tube. The cells were stained following the above-mentioned procedures at the final
concentration of substrate of 0.5 μM and M-β-CD of 2 mM. Then, the cells were washed with RPMI 1640 medium containing 10% FBS and suspended in 500 μL of the serum-free medium. Then, fresh K562 cells in 50 μL of the serum-free medium (4.0 x 10^5 cells/50 μL) were mixed with the stained cells and kept at 4 or 37 °C. The fluorescence intensity of the cell mixtures was analyzed by CytoFLEX S (Beckman Coulter).
Results and Discussion

We designed a chloroacetyl group-modified fluorescent substrate 2 based on a substrate 1 which was previously found to be a suitable substrate for β-galactosidase-responsive staining of cells (Fig. 2). Because of the two hydrophilic galactosyl groups in 1, penetration of cell membrane can be suppressed comparing with a substrate with single galactosyl group. The chloroacetyl group was modified on ε-amine group of the extra lysine residue. Fluorescent substrates 1 and 2 are the molecules after enzymatic hydrolysis of β-galactosyl groups modified on tyrosine residues, which allowed us to evaluate the cell staining behavior of the substrates after the hydrolysis.

We stained cells with the fluorescent substrate 1 or 2 following the procedure we established previously. Mixture of the substrate and M-β-CD was added to cell suspension at 37°C for 2 hours. The unbound substrate was removed by washing the cells with a serum containing medium. As shown in Fig. 3, 2 required a higher concentration (0.5 μM) to stain the cells with equivalent intensity to 1 (0.1 μM). However, intracellular region stained by 2 was similar to 1; whole cytosol except for nucleus seemed to be stained. The weaker staining ability of 2 will result from the hydrophilicity of the terminal chloroacetyl lysine residue, which impedes the cell membrane permeation of the substrate.

We evaluated the transfer of the substrates 1 and 2 bound to the cells to unstained cells during the incubation for extended time. First, the cells were stained by the substrate, then the stained cells were mixed with the same number of the unstained cells. The transfer of the substrates between the stained and the unstained cells were evaluated by flow cytometry (Fig. 4). In the case of 1, the fluorescence intensities of the stained and the unstained cells were almost constant at 4 °C during 12 hours incubation, indicating the negligible transfer of the substrate 1 from the stained cells to the
unstained cells. However, at 37 °C, the fluorescence intensity of the stained cells decreased and that of the unstained cells increased with time, showing that the substrate 1 transferred from the stained cells to the unstained cells. The suppression of the transfer of 1 at 4 °C indicates that the transfer includes an energy-dependent process. Transporters expressed on the cell membrane which pump out hydrophobic molecules from cytosol to outer medium may be responsible for the dissociation process of the substrate from the stained cells.11 Another possible energy-dependent process to release the substrate from the stained cells would be exosome-mediated release.12,13

In the case of 2 at 37 °C, the fluorescence increase of the unstained cells was observed in the first one hour, then the increment was suppressed later. The cells stained by 2 showed minor reduction in the fluorescence comparing with those stained by 1. These results indicated that fraction of 2 which did not form the covalent bond with intracellular proteins dissociated from the stained cells in the first one hour, while the fraction of 2 bound covalently did not dissociate any more.

Thus, the covalent bond formation of 2 with intracellular proteins is advantageous to suppress the transfer of the substrate to the unstained cells. However, because a mild reactivity of the chloroacetyl group required a time for the covalent bond formation. As a solution of this issue, we recently reported a fluorescent substrate which acquires a quite high reactivity upon the enzymatic hydrolysis.14

Conclusion
We designed a fluorescent substrate 2 with a chloroacetyl group to suppress the transfer of the substrate to unstained cells. The substrate achieved the suppression of dissociation from the stained cells, leading to the reduction in the transfer to the unstained cells. However, the slow reaction speed of 2 with intracellular proteins
allowed the dissociation in the early stage of staining reaction.

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Fig. 1. Schematic illustration of cell staining by a fluorescent substrate with a thiol reactive group for CARP method.
Fig. 2. Chemical structures of fluorescent substrates.
Fig. 3. Microscopic images of Daudi cells stained by fluorescence substrates at indicated concentrations. The upper and lower rows are bright field and fluorescence images, respectively.
Fig. 4. Evaluation of transfer of fluorescent substrates from stained to unstained K562 cells at each temperature