Epitope-Based Specific Antibody Modification

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ABSTRACT

Modified antibodies play essential roles in various fields, such as analysis, diagnosis, and therapy, which require modified antibodies with optimal physical and biological properties. To this aim, the development of methods for site-selective antibody modification is crucial. Herein, we introduce a Fab region–selective antibody modification method using epitope-based affinity labeling. Although affinity labeling exploiting the high affinity between an antibody and its epitope

may sound straightforward, it remains as a challenge probably due to target affinity loss caused by modification around the epitope binding site. By thoroughly screening of the modifying agent structure, reaction conditions, and purification methods, we achieved to develop an efficient method for the selective modification of the Fab region of the antibody while maintaining its high affinity for the epitope.

INTRODUCTION

In contemporary life science, antibodies are employed for various purposes, such as diagnosis, therapy, and biological research. In some cases, antibodies are used without chemical modification[1]; however, depending on the applications, chemical modification of the antibody with functional groups such as dyes[2], polyethylene glycols[3], drugs[4,5], and radioisotopes[6,7] is usually required. For the chemical modification of antibodies, various methods targeting amino acid side chains such as the amino group in lysine[8], the sulfanyl group in cysteine[9], the hydroxyphenyl group in tyrosine[10,11], the imidazolyl group in histidine[12], and the indolyl group in tryptophan[13,14] have been developed. However, when the modification target is an abundant amino acid such as lysine, site-selective modification is difficult because the modification generally occurs at multiple positions.

Particularly for therapy applications, antibodies must be homogeneously modified to avoid the presence of suboptimal antibody derivatives with undesired bioactivity or biodistribution[15], which are commonly found in randomly modified antibodies. Additionally, homogeneity of the modified antibody is important for other applications such as diagnostics and biological analysis to minimize noise derived from heterogeneity.

Recent research efforts directed toward producing homogeneously modified antibodies have focused on the development of methods for the site-selective antibody modification, such as

modification of the cysteine thiols derived from specific disulfide bonds in the antibody[16,17], enzymatic modification of a specific amino acid residue[18], enzymatic modification of *N*-glycan[19], oxidative functionalization of a tryptophan indolyl group[13,14], and Fc-binding peptide–guided affinity labeling[20,21,22]. Most of these methods have been designed to modify the Fc region of an antibody because, in contrast to the Fab region, the Fc region is not involved in target binding. Additionally, because the Fc structure is identical in all antibodies of the same class, methods targeting the Fc region can be applied to a whole antibody class regardless of the variable region structure of the Fab region. However, although the effect of the modification of the Fc region on the target binding is low, the Fc region is responsible for important functions involving the interaction with the Fc receptors, such as effector and recycling functions[23], which might be affected by modification of the Fc region. Moreover, the affinity of an Fc ligand is specific to an antibody subtype and, therefore, different Fc ligands are required to modify different antibody subtypes.

In contrast to the selective modification of the Fc region, the selective modification of the Fab region has been scarcely explored owing to concern for target affinity loss. Nevertheless, in terms of the labeling efficiency, Fab could be a promising target for specific labeling using affinity labeling methods because it has a specifically high affinity to its epitope. Thus, the binding constant (K_b) between an antibody and its epitope could be up to $>10^{10}$ M^{-1}, which is much higher than that between an Fc and its binding peptide ($K_b = 10^7$–10^8 M^{-1}), despite the latter being exploited for specific Fc labeling[21,22].

Previously, our group developed a catalytic affinity labeling method for protein modification. The specific modification of avidin and streptavidin was achieved using a biotin-derived labeling agent. This method exploited the extremely high affinity between biotin and avidin or streptavidin

($K_b > 10^{15}$ M^{-1}) to achieve a high labeling yield with only 1.0 equivalent of the ligand-derived labeling agent relative to the target protein. The results suggested the high efficiency of the affinity labeling using a high affinity ligand–protein pair[24,25,26,27].

Actually, there are some reports about epitope-based affinity labeling of antibodies[28,29]; however, in these examples, epitope affinity of the labeled antibody was lost or not determined. To the best our knowledge, epitope-based affinity labeling that can retain original target affinity has never been reported to date. These facts might further induce researchers to think that the epitope-based affinity labeling is not suitable for antibody modification.

During the epitope-based affinity labeling reaction, amino acid residues of the antibody responsible for the epitope association should be temporarily masked by the epitope structure. The labeled amino acid residue should be located close to the epitope binding site. However, this labeled amino acid would not essentially contribute to epitope binding. Therefore, we hypothesized that the modified antibody could preserve its affinity for the epitope if the epitope-derived structure is removed from the antibody after the reaction. The epitope-based antibody modification would offer several advantageous features. Firstly, it is expected to provide highly selective and efficient modification around the epitope binding site due to the extremely high binding constant between the antibody and the epitope. Additionally, the function of the Fc region, such as effector function and recycling function, would be retained because only Fab region would be specifically modified, while Fc region would remain unmodified. Moreover, this method would be applicable to any antibody subtypes, including fragment antibodies such as scFv and VHH, that cannot be modified using Fc region–specific modification method.

There are two critical factors for the success of the epitope-based affinity labeling method. The first is the design of the epitope-based modifying agent. It must specifically target and modify the

antibody directed by the association between the antibody and the epitope. Additionally, the epitope part needs to be cleaved after the reaction, as seen in certain ligand-directed affinity labeling of proteins[30,31]. The second is establishing a removal procedure for the cleaved epitope that strongly binds to the epitope binding site without disrupting the 3D structure of the site. In this report, we present a fundamental study pioneering the epitope-based affinity labeling, with a focus on these key factors.

RESULTS

Initial study of the selective antibody modification

As a model antibody, we selected anti-fluorescein antibody **X** (mouse IgG₁ anti-fluorescein antibody purchased from Jackson ImmunoResearch) because both the antibody and the corresponding epitope (fluorescein) are widely used and easily available. We targeted an ε-amino group of lysine because amino group–based bioconjugation methods are well established and lysine would locate around the epitope binding site with high probability according to the general abundance of lysine in the variable region of the antibody[32]. As a reacting group, we selected a thioester because it shows sufficient reactivity toward the amino group and moderate stability in water. The epitope (fluorescein) structure was incorporated into the thiol part so that it is cleaved after the antibody modification. Furthermore, the 7-hydroxycoumarin structure was incorporated to the thioester to evaluate the modification rate of the antibody via fluorescence imaging. According to this design, we prepared alkyl thioester **1** and aryl thioester **2** as model affinity labeling agents (**Fig. 1a**) and performed the labeling reaction of anti-fluorescein antibody **X** using the following reaction conditions: [antibody] $= 6 \times 10^{-6}$ M, [labeling agent] $= 6 \times 10^{-5}$ M, 10 mM

sodium phosphate (pH 7.6), and 250 mM NaCl. After 24 h incubation, the reaction mixture was quenched with excess cysteine to remove any remaining labeling agents. The relative modification levels with **1** and **2** were evaluated using the SDS-PAGE method. As a control experiment, dummy IgG antibody **Y** with no fluorescein affinity was subjected to the same reaction conditions. Additionally, to evaluate the occurrence of nonspecific modification, blocking experiments with excess fluorescein were performed. The 7-hydroxycoumarin fluorescence SDS-PAGE image is shown in **Fig. 1b**. For **1**, no coumarin-derived fluorescence was observed for anti-fluorescein antibody **X** with/without blocking and dummy antibody **Y**. In contrast, for **2**, coumarin-derived fluorescence was observed for antibody **X**, and the fluorescence was blocked in the presence of excess fluorescein. For dummy antibody **Y**, almost no fluorescence was observed.

These results suggest that **2** specifically modifies anti-fluorescein antibody **X** directed by specific binding between the epitope binding site of **X** and fluorescein part of **2**. On the other hand, **1** was not sufficiently reactive to modify the antibody, although the distance between fluorescein and the thioester moiety is almost the same for **1** and **2**. This difference can be attributed to the inherent reactivity of the thioesters, since aryl thioesters are generally more reactive than alkyl thioesters. In the antibody labeled with **2**, the fluorescence intensity in the heavy chain was more intense than that in the light chain. It would be because the distance between the modified residue in the heavy chain and the fluorescein recognition site matches the distance between fluorescein and the thioester in **2**.
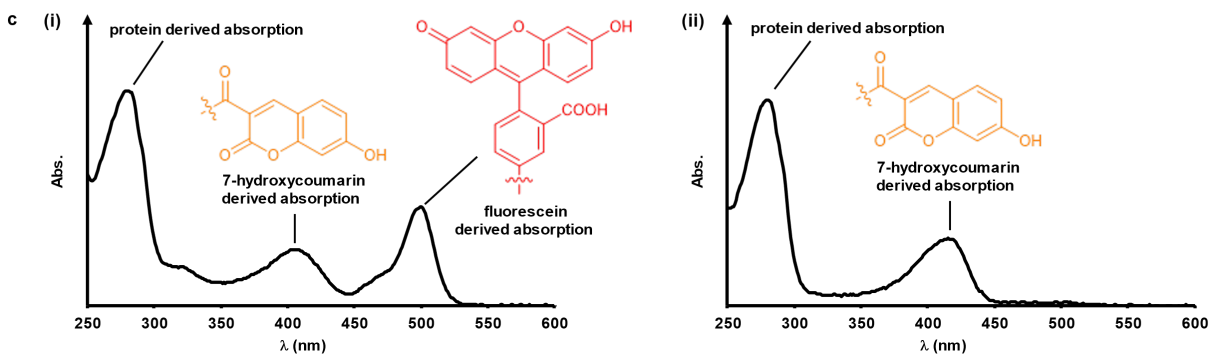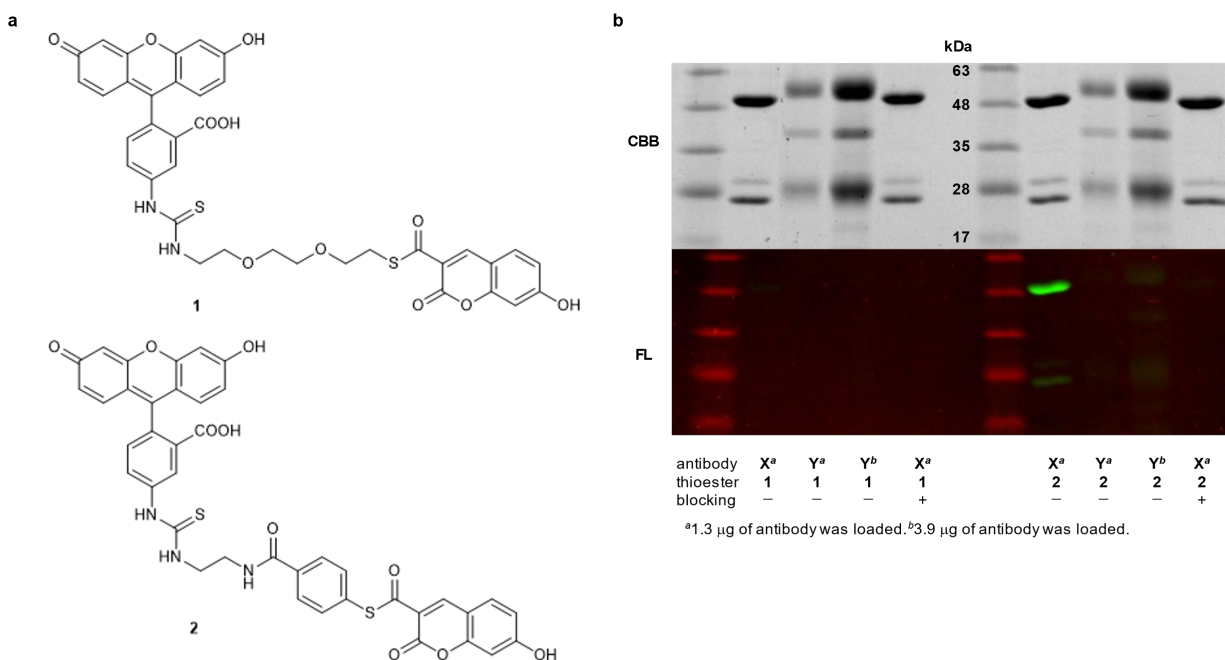

Removal of the antigen-derived product from the antibody

The recovered antibody after the modification with **2** was analyzed via UV–vis spectroscopy. A representative spectrum is shown in **Fig. 1c(i)**. Three main absorptions were observed, i.e., an

absorption at 280 nm (Abs$_{280\,nm}$), which stems from the antibody, an absorption at 412 nm (Abs$_{412\,nm}$) due to 7-hydroxycoumarin[33], and an absorption at 498 nm, which is specific to fluorescein and indicated that fluorescein was still bound to the epitope-binding site. These results support the hypothesis that the epitope binding site is masked by the epitope during the reaction, which would help retain the affinity to the epitope after the affinity labeling.

The dissociation of a protein and its ligand can be performed using various conditions, such as acidic conditions, basic conditions, and the use of chaotropic agents. For the dissociation of an antibody from protein A, acidic glycine buffer has been generally used. Thus, to remove the fluorescein-derived small molecule from the antibody **X** modified with thioester **2**, we investigated several conditions for protein denaturation, including the acidic glycine buffer conditions. First, we tackled the removal of the small molecule via ultrafiltration and size exclusion after denaturation, followed by refolding of the antibody using pH 7.6 phosphate buffer. However, the UV–vis analysis of the recovered samples revealed poor results in terms of antibody recovery yield and fluorescein removal efficiency. Under acidic conditions [0.1 M Gly–HCl buffer (pH 2.0)], which are generally used for the dissociation of an antibody from protein A, the fluorescein-derived absorption disappeared; however, the protein recovery yield was less than 10% most likely due to protein aggregation after neutralization of the antibody as reported previously[34]. Meanwhile, under basic conditions [0.1 M Gly–NaOH buffer (pH 10.0)], although the antibody was recovered in high yield (86%), a large amount of fluorescein remained. Using chaotropic agent conditions (5 M LiCl) resulted in a low antibody recovery of only 13% and an incomplete removal of the fluorescein derivative (**Supplementary Table S2**). Then, we tried to remove the fluorescein derivative using a highly concentrated arginine solution, which has been reported to enable the effective recovery of the antibody from a protein A column without denaturation[35]. Accordingly,

the sample after the reaction was mixed with a 2 M arginine–HCl solution (pH 3.6), followed by ultrafiltration and size exclusion to remove small molecules. The solvent of the sample was replaced with phosphate buffer (pH 7.6). After this process, the antibody was recovered in good yield (67%) and the fluorescein derivatives were completely removed from the antibody [**Fig. 1c(ii)**].

antibody	X^a	Y^a	Y^b	X^a		X^a	Y^a	Y^b	X^a
thioester	1	1	1	1		2	2	2	2
blocking	−	−	−	+		−	−	−	+

^{a}1.3 μg of antibody was loaded. ^{b}3.9 μg of antibody was loaded.

c (i) protein derived absorption
7-hydroxycoumarin derived absorption
fluorescein derived absorption
Abs.
λ (nm)

(ii) protein derived absorption
7-hydroxycoumarin derived absorption
Abs.
λ (nm)

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Fig. 1: Labeling of antibodies with thioesters 1 and 2. a, Structures of labeling agents **1** and **2**. **b,** SDS-PAGE analysis after labeling. CBB indicates a gel image stained with CBB. FL indicates a fluorescence image of a gel (excitation: 365 nm, emission > 410 nm). **c,** UV–vis spectra of antibody **X** modified with thioester **2** (i) before removal of fluorescein derivatives and (ii) after removal of fluorescein derivatives.
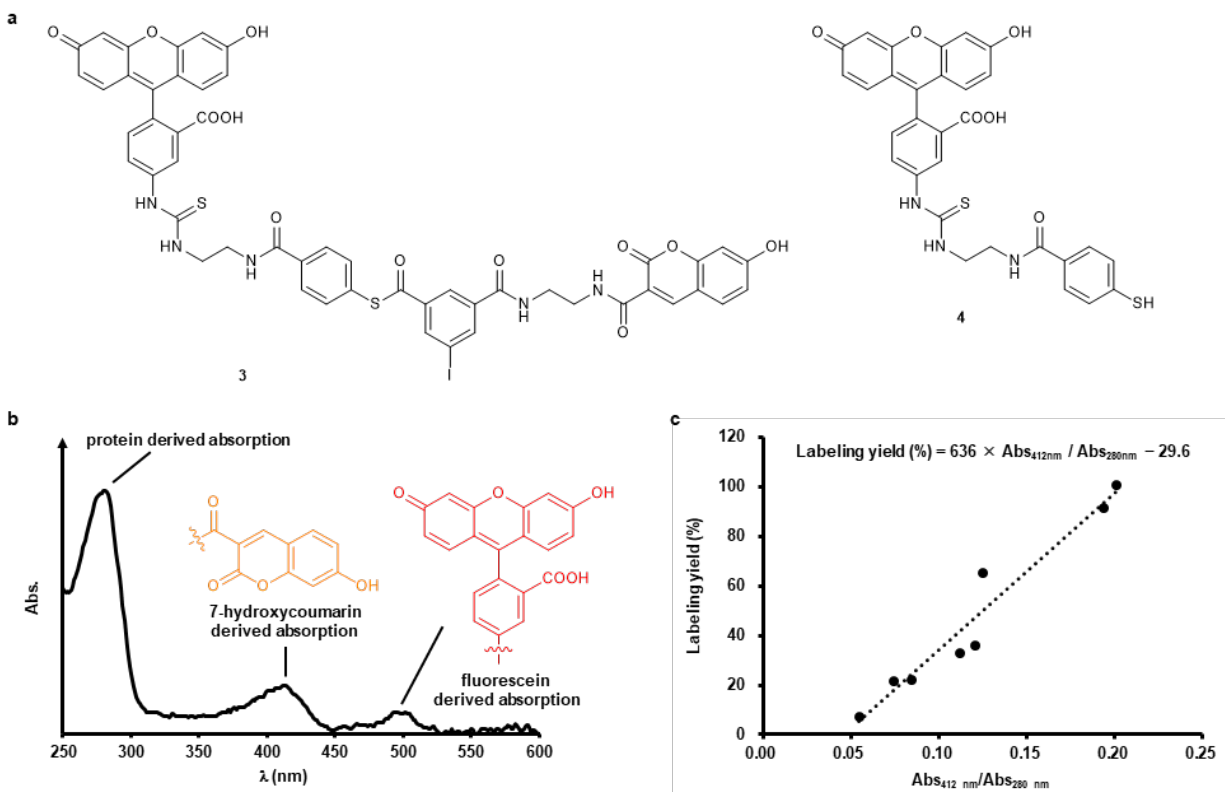
Quantification of the modification yield

The physical properties of the modified antibody depend on the modification yield, which can be estimated according to the ratio of $Abs_{412\,nm}$ (as an indicator of coumarin) to $Abs_{280\,nm}$ (as an indicator of protein). However, the UV absorbance of dyes may vary after they are introduced into the antibody, hindering a precise quantification of the modification yield only on the basis of the UV–vis spectra. Therefore, to obtain an accurate modification yield, we designed and synthesized thioester **3** bearing a 7-hydroxycoumarin moiety as a fluorescent dye and an iodophenyl group as a tag for ICP-MS quantification (**Fig. 2a**).

Modification of antibody **X** was conducted using 2–10 equivalents of **3** and 1 or 24 h of incubation time. After the reaction, the mixture was quenched with excess cysteine to remove any remaining reagents. The recovered antibody was analyzed via UV–vis spectroscopy, which showed a weak absorption at 498 nm due to the fluorescein derivative (representative data shown in **Fig. 2b**). This contrasts with the result of the antibody modification with **2**, in which the fluorescein derivative was completely removed. For thioesters **2** and **3**, after quenching the reaction, thiol **4** would be generated as a fluorescein-derived small molecule (**Fig. 2a**). The different efficiency in the removal of **4** from the antibody between the antibodies modified with **2** and **3** suggests that the antibody modification could influence the denaturation efficiency of the epitope

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recognition site to different extents depending on the structure of the compound introduced into the antibody.

In the UV–vis spectrum of a sample after the reaction with thioester **3**, a specific absorption of 7-hydroxycoumarin was observed at 412 nm, as in the case of the sample modified with **2**. The ratio between the absorptions at 412 and 280 nm ($Abs_{412\ nm}/Abs_{280\ nm}$) increased upon increasing the reagent amount and the reaction time, suggesting that the $Abs_{412\ nm}/Abs_{280\ nm}$ value can be associated to the modification yield. For the recovered samples, precise modification yields were quantified via iodine ICP-MS determination. The obtained yields showed good correlation with the $Abs_{412\ nm}/Abs_{280\ nm}$ values, confirming that these values can be used to determine the modification yields.

Although the modification yield with **3** was accurately quantified via ICP-MS, the fluorescein derivative could not be completely removed from the antibody modified with **3**. Therefore, the antibody modified with **2** was used for further investigation of the physical properties of the modified antibody. The modification yield with **2** was calculated using the standard curve of the modification yield vs. $Abs_{412nm}/Abs_{280\ nm}$ as shown in **Fig. 2c**.

Fig. 2: Quantification of the labeling yield via ICP-MS and UV–vis spectroscopy. a, Structures of thioester **3** and thiol **4**. **b**, UV–vis spectrum of antibody **X** modified with thioester **3**. The reaction conditions were as follows: [antibody **X**] = 6×10^{-6} M, [thioester **3**] = 6×10^{-5} M, 10 mM sodium phosphate (pH 7.6), 250 mM NaCl, reaction time: 24 h. **c,** Correlation between $Abs_{412\,nm}/Abs_{280\,nm}$ and the labeling yield. The labeling yield was determined via iodine quantification using ICP-MS and the protein concentration quantified via UV–vis spectroscopy.

Optimization of the reaction conditions

To optimize the reaction conditions of the modification with thioester **2**, the reaction was conducted with 2–40 equivalents of **2**. According to the investigation with thioester **3**, the reaction time was fixed to 24 h. After quenching the reaction with excess cysteine, small molecules were

removed by ultrafiltration and size exclusion using 2 M arginine–HCl (pH 3.6). Then, the buffer was exchanged to phosphate buffer (pH 7.6) and the sample was analyzed via UV–vis spectroscopy.

The yields gradually increased as the reagent amount increased until reaching 233% for 25 equivalents of reagents. No further increase in the yield was observed upon increasing the reagent equivalents from 25 to 40 (**Fig. 3a**). These results suggest that the reaction specifically occurs at the nucleophile having a specific distance from the fluorescein recognition site of the anti-fluorescein antibody. Moreover, the nonspecific reaction with excess reagents hardly proceeds under the reaction conditions.

Considering that antibodies generally have high binding constants (K_b = 10^8–10^{12} M^{-1}), most antibody molecules could be expected to form a complex with fluorescein derivatives under the reaction conditions ([antibody] = 6.0 μM, [acylating agent] > 60 μM). Therefore, we speculated that the reaction could proceed under lower concentration conditions. The reaction was conducted with two different antibody concentrations (6.0 and 0.6 μM) and 25 equivalents of **2**, and the modification yield for each condition was determined at 3, 6, 12, and 24 h. As shown in **Fig. 3b**, the modification yields were similar between both antibody concentration conditions, with the modification yield increasing in a time-dependent manner. These results suggest that the rate-determining step is a pseudo-intramolecular reaction between the antibody and **2** bound to the antibody rather than a paratope–epitope complexation step between the antibody and **2**.

Since the rate-determining step of the nonspecific modification is most likely an intermolecular reaction, the extent of the nonspecific modification could be reduced under low concentration conditions. To confirm this hypothesis, we analyzed the modification yield of the non-fluorescein-binding dummy IgG at concentrations of 6.0 and 0.6 μM. As shown in **Fig. 3c**, UV–vis

spectroscopy revealed that 41% of nonspecific modification was observed in the presence of 6.0 μM dummy IgG, whereas almost no nonspecific modification occurred under 0.6 μM dummy IgG conditions. Additionally, blocking experiments with fluorescein were performed for the modification of the anti-fluorescein antibody with 6.0 and 0.6 μM concentrations. Almost no modification was observed under both conditions. These results suggest that our method is effective for specific antibody modification, with low concentration conditions being conducive to the specificity.

Figure 3d shows the SDS-PAGE analysis after papain digestion of antibody **X** modified with **2**. The 7-hydroxycoumarin-derived fluorescence was specifically observed in the $F(ab')_2$ region and not in the Fc region, suggesting that the thioester-derived acyl group was specifically introduced near the antigen recognition site of the antibody via an affinity labeling mechanism.
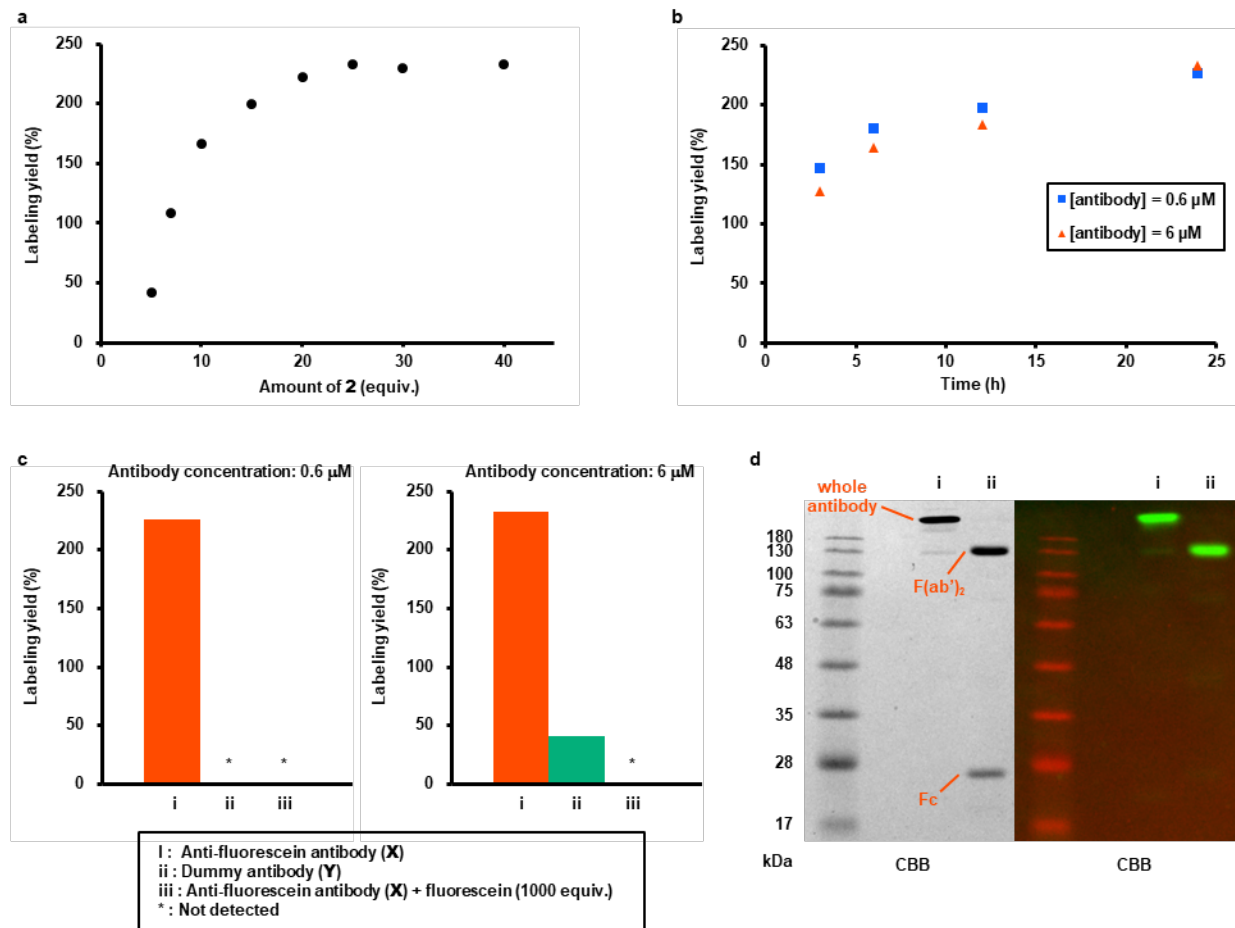
Fig. 3: Effect of reagent amount, reaction time, and reaction concentration on the labeling reaction. a, Relationship between the amount of thioester **2** and the labeling yield. **b,** Relationship between the reaction time and the labeling yield with an antibody concentration of 0.6 μM (blue squares) and 6 μM (orange triangles). **c,** Influence of the antibody concentration on the nonspecific modification. **d,** SDS-PAGE analysis of antibody **X** modified with **2** (i) before papain digestion and (ii) after papain digestion. SDS-PAGE was conducted under nonreducing conditions.

Mechanistic insights into the reaction

Considering the high affinity between antibody and epitope, no excess amount of labeling agent should be required for the reaction. Initially, we attributed the fact that an excess of labeling agent

was required to the low stability of **2** under aqueous conditions; however, HPLC monitoring of **2** during the reaction allowed disregarding this hypothesis (**Supplementary Fig. S13**). Alternatively, antibody **X** could promote the decomposition of labeling agent **2** via hydrolysis or by producing unstable intermediates that would be eventually broken by excess cysteine in the quenching step. This could be the case of the imidazole group of histidine, which could form a reactive acyl imidazole that would cause a decrease in the labeling efficiency. However, this hypothesis cannot be confirmed because the sequence of the antibody is confidential and not disclosed.

Since an excess of thioester **2** would be required to convert free thiol **4** to thioester **2** via thioester exchange or ligand exchange from **4** to **2**, the labeling reaction of 6 and 0.6 μM of antibody **X** was conducted using thioester **2** in the absence or presence of thioester **5** lacking a fluorescein structure (**Fig. 4**). Using 6 μM of antibody **X**, the labeling yield was 250% with 2.0 equivalents of **2** and 23 equivalents of **5**, which is comparable to the yield obtained using 25 equivalents of **2**, whereas no antibody modification was observed with only 2.0 equivalents of **2**. Under the 0.6 μM antibody conditions, the labeling yield was 123% with 2.0 equivalents of **2** and 23 equivalents of **5**. Again, no modification was observed with 2.0 equivalents of **2** in the absence of **5**. Meanwhile, using 23 equivalents of thioester **5** without **2** afforded a labeling yield of 76% for an antibody concentration of 6 μM and 31% for an antibody concentration of 0.6 μM. Although the background reaction without **2** was observed, the combination of **2** (2.0 equivalents) and **5** (23 equivalents) resulted in a significantly higher yield compared with using **2** (2.0 equivalents) or **5** (23 equivalents) individually. These results support the hypothesis that thiol **4** acts as an acyl transfer catalyst and suggests that an excess of thioester is required due to its nonproductive decomposition induced by antibody **X**.
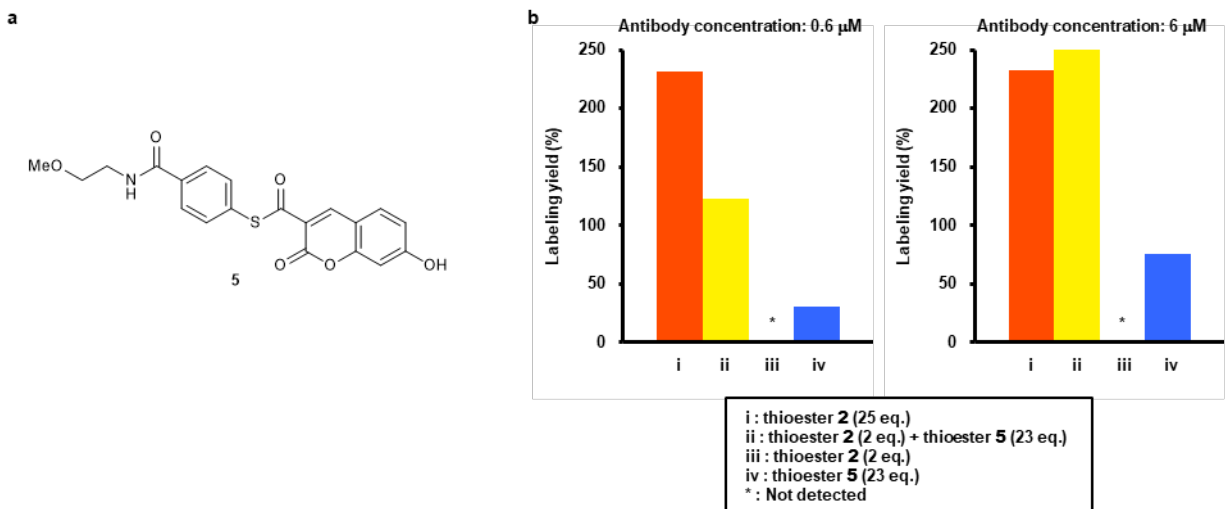
Fig. 4: Investigation of antibody labeling with epitope-conjugated thioester in the absence or presence of epitope-unconjugated thioester. a, Structure of thioester **5**. **b,** Yields of the labeling reaction using thioester **2** in the absence or presence of **5**. Antibody **X** (6×10^{-6} M or 6×10^{-7} M) was incubated with specific amounts of thioester **2** in the absence or presence of **5** in 10 mM sodium phosphate (pH 7.6) and 250 mM NaCl for 24 h.

Target affinity analysis of the modified antibody

According to the abovementioned results, the specific modification of the Fab region was achieved in high yield. Additionally, the fluorescein derivative was completely removed from the antibody. Next, we investigated the epitope affinity of the modified antibody.

Using the antibody modified under the best conditions (0.6 μM **X**, **2** 25 equivalents, 24 h of reaction time), we performed fluorescein quenching experiments to evaluate the fluorescein affinity on the basis of the quenching of the fluorescence of fluorescein upon binding to the anti-fluorescein antibody[36,37]. A fluorescein solution with a specific concentration was mixed with different concentrations of an anti-fluorescein antibody solution to achieve a final concentration of fluorescein of 1.0×10^{-9} M, and the IC$_{50}$ value for fluorescein quenching was evaluated. The

IC$_{50}$ values of the antibody were determined to be 2.3×10^{-9} and 2.0×10^{-8} M before and after chemical modification, respectively (**Fig. 5a**). Although a slight decrease in the affinity was observed, it can be concluded that the modified antibody retained a high affinity to fluorescein.

Application of the modification method to a different antibody

To evaluate the generality of the epitope-based affinity labeling method, it was applied to the modification of an anti-fluorescein antibody with a different structure to that of antibody **X**, i.e., a mouse IgG$_{2a}$ anti-fluorescein antibody (antibody **Z**) produced by a hybridoma (purchased from DSHB; antibody registry ID: AB_10804672). Thioester **2** was used for the modification. Under the optimized conditions for the modification of **X** (0.6 μM **Z**, 25 equivalents of **2**, 24 h), a labeling yield of 180% was achieved. An SDS-PAGE analysis after papain digestion suggested that the modification specifically occurred around the antigen recognition site of the Fab region (**Supplementary Fig. S15**). The fluorescein affinity of antibody **Z** was almost retained after the modification. IC$_{50}$ values of 5.3×10^{-9} and 1.1×10^{-8} M were obtained before and after modification of antibody **Z**, respectively (**Fig. 5b**). These results suggest that a single modifying agent could be applied to modify any antibody type using our method, provided the antibodies exhibit high affinity to a common epitope.
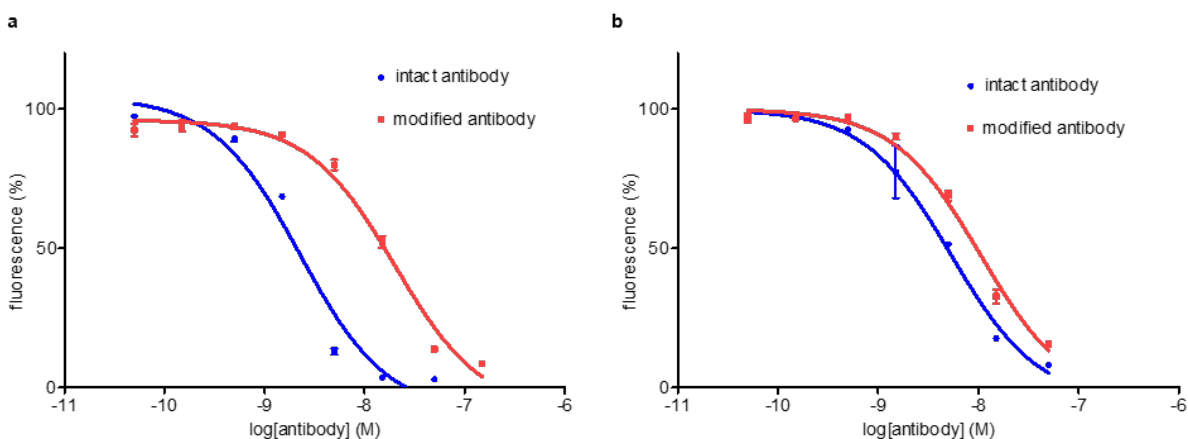
Fig. 5: Binding assay of modified and unmodified antibodies. a, Binding assay of intact antibody **X** (blue circles) and antibody **X** modified with thioester **2** in 235% labeling yield (red squares). **b,** Binding assay of intact antibody **Z** (blue circles) and antibody **Z** modified with thioester **2** in 180% labeling yield (red squares). Error bars represent standard deviation.

Labeling of epitope-conjugated protein with the modified antibody

Although the above experimental results strongly suggest that the antibody modified with our method retains its target affinity, both modified and unmodified antibodies could be present in the material obtained after the reaction. In that case, the affinity could be due to the unmodified antibody. To exclude this possibility, the modified antibody was bound to an FITC-modified protein and the fluorescence property of the antibody–protein complex was analyzed. As a model substrate, BSA was labeled with 1.0 equivalent of FITC. FITC-labeled BSA (Flu-BSA) was mixed with the anti-fluorescein antibody **Z** modified with thioester **2** in 180% labeling yield, and the mixture was analyzed via HPLC using a size exclusion column equipped with UV–vis detector and fluorescence detectors. The complex formed between the modified antibody and Flu-BSA was sufficiently stable to afford a distinct retention time compared with the individual retention times of antibody **Z** and Flu-BSA (**Supplementary Fig. S16**). The complex fraction exhibited

fluorescence derived from 7-hydroxycoumarin. Therefore, it can be concluded that the antibody modified with thioester **2** retains high target affinity and can deliver a modified compound to the antigen site.

Discussion

In this study, we explored the potential of epitope-based affinity labeling as a novel method for specific antibody modification with high precision. The Fab region of the antibody was specifically modified using an epitope-linked thioester, and the modified antibody retained high affinity to the epitope.

Removal of the epitope derivative after the modification reaction was unexpectedly difficult due to the high affinity between the epitope and the antibody and probable aggregation during the epitope removal process. After careful screening of the epitope removal conditions, such as solute and pH, ultrafiltration and size exclusion (gel filtration) using an acidic arginine solution was found to enable the removal of the epitope derivative from the antibody with minimal loss of the antibody quantity and activity.

Our results suggest that an epitope derivative having a sulfanyl group could work as an acyl transfer catalyst for specific modification around the epitope binding site of the antibody. Although the current method requires a stoichiometric or even an excess amount of a ligand-based reagent, this amount could be reduced to a catalytic amount by optimizing the affinity of the reagent with the antibody and improving the activity of the catalytic site.

In contrast with conventional Fc modification methods, the epitope-based affinity labeling method can be expected to preserve the functions of the Fc region, such as effector functions and antibody recycling via Fc receptor. Additionally, this method can be applied to diverse types of

antibodies having an identical epitope structure, including antibody fragments such as scFv and VHH, using a single modifying agent. Therefore, it could provide a complementary tool to the widely known Fc-selective modification. The developed epitope-based affinity labeling method can be expected to broaden the structure and function scope of modified antibodies, thus leading to new applications.

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Methods

Materials. A mouse IgG$_1$ anti-fluorescein antibody (antibody **X**, code number: 200-002-037) and a bovine IgG polyclonal antibody (dummy IgG, antibody **Y**, code number: 001-000-003) were purchased from Jackson ImmunoResearch. A mouse IgG$_{2a}$ anti-fluorescein antibody (antibody **Z**, IgG$_{2a}$) was produced by a hybridoma purchased from DSHB (antibody registry ID: AB_10804672) and purified with protein G column (KanCap G Prepacked Column, 1mL, Kaneka). Papain was purchased from Wako. Bovine serum albumin (BSA) was purchased from Sigma, and BSA modified with fluorescein (Flu-BSA, the fluorescein/BSA ratio = 1.0) was synthesized according to the procedure reported by Barbero.[38]

General procedure for antibody modification. 975 μM thioester solution in pH 7.6 10 mM sodium phosphate buffer with 250 mM NaCl (25 equiv.) was added to 0.61 μM solution of anti-fluorescein antibody in pH 7.6 10 mM sodium phosphate buffer with 250 mM NaCl (1 equiv.) at room temperature. The resulting solution was shaken by a seesaw shaker at room temperature for 24 h in the dark. The concentrations of the solutes in the resulting solution were as follows: anti-fluorescein antibody 0.6 μM; thioester 15 μM. The solution was quenched with 39 mM cysteine solution in pH 7.6 10 mM sodium phosphate buffer with 250 mM NaCl (1000 equiv.). The resulting solution was shaken by a seesaw shaker at room temperature for 1 h in the dark. The small molecules were removed by successive treatment with ultrafiltration (pH 3.6, 2 M Arginine-HCl, 50 μL × 3) on a 50 kDa cutoff filter (Vivacon 500, Sartorius) and size exclusion (centrifugation, 1690 × g, 2 min) on Sephadex G-50 fine (Cytiva) swelled in pH 3.6, 2 M Arginine-HCl. Then the buffer was exchanged to 10 mM sodium phosphate buffer, pH 7.6, 250 mM NaCl by ultrafiltration (pH 7.6, 10 mM sodium phosphate buffer with 250 mM NaCl, 50 μL × 3) on a 50 kDa cutoff filter (Vivacon 500).

Control experiment with dummy IgG with no affinity to fluorescein. The experiment was performed in the same manner as described above for antibody modification with 0.61 µM solution of dummy IgG antibody **Y** in 10 mM sodium phosphate buffer, pH 7.6, 250 mM NaCl (1 equiv.) instead of anti-fluorescein antibody.

Competitive inhibition of modification by addition of fluorescein. The experiment was performed in the same manner as described above for antibody modification except for the addition of 39 mM fluorescein solution in 10 mM sodium phosphate buffer, pH 7.6, 250 mM NaCl (1000 equiv.).

SDS-PAGE experiment. SDS-PAGE was carried out according to the standard procedure of Laemmli.[39]

Reducing sample buffer: 0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% sucrose, 10% 2-mercaptoethanol, 0.004 % bromophenol blue

Gel: 3% stacking gel, 12% resolving gel

Running buffer: 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS

   261 or 783 µg/mL solution of antibody resulting from labeling experiments in pH 7.6 10 mM sodium phosphate buffer with 250 mM NaCl (6 µL) was added to the reducing sample buffer (6 µL). The resulting solution was heated at 95 °C for 2 min, loaded on the gel, and resolved by SDS-PAGE. The gel was run at constant voltage (200 V) in 1 × SDS running buffer. All gels were stained with 0.25% Coomassie brilliant blue (CBB) R-250 dye in staining solution (5% ethanol, 7.5% acetic acid). Fluorescence images (excitation: 365 nm, emission: > 410 nm) and photographs of gels were acquired using a CCD camera (LAS-4000, Fujifilm).

Papain digestion of labeled antibodies. Papain digestion was carried out according to the procedure of Parham *et al.*[40]

   100 mM cysteine solution in digestion buffer (pH 5.5, 0.1 M sodium acetate with 3 mM ethylenediaminetetraacetic acid, 10 µL) was added to 8 mg/mL papain solution in digestion buffer (10 µL) at room temperature. The resulting solution was incubated at 37 °C for 30 min. The cysteine in the resulting solution was removed by ultrafiltration on a 10 kDa cutoff filter (Vivacon 500). The papain concentration was calculated based on the absorbance at 278 nm on UV-vis spectroscopy (NanoDrop, Thermo Scientific), on the premise that the molar extinction coefficient ($M^{-1}cm^{-1}$) of papain at 278 nm is 58570.[41]

   The activated papain was immediately diluted with digestion buffer to a concentration of 0.3 mg/mL, and the papain solution (0.5 µL) was added to 0.75 mg/mL solution of labeled antibody diluted in digestion buffer (4 µL) at room temperature. The resulting solution was incubated at 37 °C for 1.5 h or 3 h for the digestion of labeled antibodies **X** or **Z**, respectively. The digestion was terminated by adding 100 mM iodoacetamide solution in the digestion buffer (4.5 µL) to the

digestion solution at room temperature, and the solution was shaken by seesaw shaker at room temperature for 30 min in the dark.

Analysis of papain digestion products by non-reducing SDS-PAGE. Non-reducing SDS-PAGE was carried out according to the procedure of Schumacher *et al*.[42]

Sample buffer: 0.167 M Tris-HCl, pH 6.8, 3.3% SDS, 1.37 M glycerol, 0.008 % CBB R-250 dye

Gel: 3% stacking gel, 12% resolving gel

Running buffer: 0.025 M Tris-HCl, pH 8.3, 0.192 M glycerol, 0.1% SDS

174 μg/mL solution of labeled antibody before/after papain digestion in digestion buffer (6 μL) was added to the sample buffer (6 μL). The resulting solution was heated at 65 °C for 2 min, loaded on the gel, and resolved by SDS-PAGE. The gel was run at constant voltage (200 V) in 1 × SDS running buffer. The CBB staining and the imaging of gels were performed in the same manner described above for SDS-PAGE experiment.

Quantification of labeling yield by ICP-MS. Modification of antibody **X** using thioester **3** was performed in the same manner described above except for the reaction concentration (antibody: 6 μM), the reaction time (1 h or 24 h), and the equivalents of thioester **3** (2, 3, 5, 10, or 20 equiv.). The labeling yield of antibody was determined by measuring the iodine quantified by ICP-MS (Element 2, Thermo Scientific) and the antibody concentration was quantified by UV-vis spectroscopy (NanoDrop), on the premise that the molar extinction coefficients ($M^{-1}cm^{-1}$) of antibody at 280 nm is 210000.[43]

For the iodine quantification by ICP-MS, 1.27–2.87 mg/mL solution of labeled antibody (7 μL) was diluted with H_2O (993 μL) and 2% tetramethylammonium hydroxide in H_2O containing 4ppb In as an internal standard (1000 μL). This solution was analyzed directly by ICP-MS. Linear range of calibration by potassium iodide was 0.03–30 ppb. The operating parameters of the ICP-MS are as follows.

Power: 1200 V
Resolution: low resolution
Auxiliary gas flow rate: 0.80 L/min
Additional gas flow rate: 0.255 L/min
Cool gas flow rate: 15.88 L/min
Sample gas flow rate: 1.000 L/min
Dead time: 26 ns
Sample time: 0.0100 s
Mass window: 150%

The labeling yield of antibody ($L_{antibody}$) was calculated by

$L_{antibody}$ (%) = 100 × C_{iodine} / $C_{antibody}$

where $C_{antibody}$ and C_{iodine} are concentration (M) of antibody and iodine connected to antibody, respectively.

Correlation between $Abs_{412\,nm}$/$Abs_{280\,nm}$ and labeling yield quantified by ICP-MS. Quantification of labeling yield was performed in the same manner as described above (**Supplementary Fig. S8**). The absorption of the labeled antibody was measured by UV-vis spectroscopy (NanoDrop, **Supplementary Fig. S9**).

The $Abs_{412\,nm}$/$Abs_{280\,nm}$ was calculated by

$Abs_{412\,nm}$/$Abs_{280\,nm}$ = the absorbance at 412 nm ($Abs_{412\,nm}$) / the absorbance at 280 nm ($Abs_{280\,nm}$).

The correlation between $Abs_{412\,nm}$/$Abs_{280\,nm}$ and labeling yield is described in **Fig. 2c**. According to this data, labeling yields of antibody modification with thioester **2** were calculated by

$L_{antibody}$ (%) = 636 × $Abs_{412\,nm}$/$Abs_{280\,nm}$ − 29.6

Binding assay of antibody anti-fluorescein antibodies. Anti-fluorescein antibodies with/without modification were diluted to 1×10^{-10}–1×10^{-7} M in 20 mM HEPES buffer with 150 mM NaCl, pH 7.5. This antibody solution (100 μL) and 2×10^{-9} M fluorescein solution in 20 mM HEPES buffer with 150 mM NaCl, pH 7.5 (100 μL) were mixed in microplate (ELISA plate, 96 well, flat base, PS, black, Medium Binding, Sarstedt). The concentrations of solutes in the resulting solution were as follows: antibody 5×10^{-11}–5×10^{-8} M; fluorescein 1×10^{-9} M. After the solution were shaken by seesaw shaker at room temperature for 1 h in the dark, the fluorescent intensities at 535 nm with excitation at 485 nm at room temperature were measured by microplate reader (Spark 10M, Tecan).

The half maximal inhibitory concentration (IC_{50}) values were calculated by curve fitting of the observed fluorescence intensities, using a sigmoidal dose response model with GraphPad Prism 5.04 (GraphPad Software Inc., San Diego, CA).

HPLC monitoring of compounds in the solution of antibody modification reaction. RP-HPLC was performed with LC-2050C 3D (SHIMADZU) equipped Mightysil RP-18 GP Aqua 150-4.6 (5 μm, Kanto chemical). All runs used linear gradients of H_2O containing 0.05% TFA (solvent A) and 0.05% MeOH containing 0.05% TFA (solvent B). The column was eluted with solvent A/solvent B = 65/35-(8 min)-65/35-(10 min)-0/100-(5 min)-0/100 at a flow rate of 1.0 mL/min. The effluent was monitored by its absorbance at 370 and 446 nm using PDA. 20 μL of the reaction solution of antibody **X** using **2** was analyzed.

Analysis of complex of the modified antibody and Flu-BSA. The HPLC apparatus comprised a PU-4080i pump, a UV-4075 UV-vis detector, an FP-4025 fluorescence detector, and an LC-

NetII/ADC interface box (JASCO). The size exclusion 300 Å, 2.7 μm column (4.6 × 150 mm, AdvanceBio SEC, Agilent) was eluted with a 150 mM sodium phosphate buffer, pH 7.0 at a flow rate of 0.2 mL/min. The effluent was monitored by its absorbance at 220 nm and fluorescence at 445 and 515 nm with excitation at 412 and 498 nm, respectively. 2 μM solution of antibody **Z** with/without modification using thioester **2** in 150 mM sodium phosphate, pH 7.0 (11 μL) and 4 μM solution of BSA or Flu-BSA in 150 mM sodium phosphate, pH 7.0 (11 μL) were mixed and allowed to react at room temperature for 12 min. Then, 20 μL of the reaction mixture was analyzed by HPLC.

Acknowledgements

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