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Modification of Fab Fragments by Dibromopyridazinediones Carrying Mono- and Double-Biotin Functionalities

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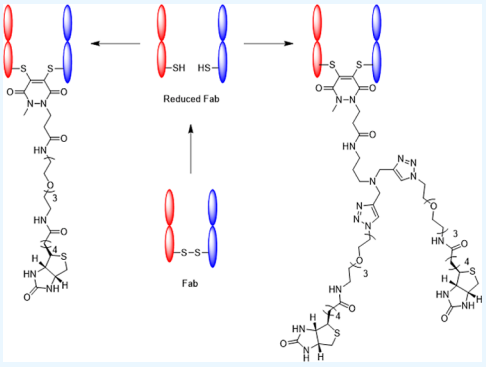
ABSTRACT: To verify the potencies of dibromopyridazinediones with mono- and double-biotin groups, the functions as cysteine-selective biotinylation reagents were evaluated through conjugation with a goat anti-mouse IgG Fab fragment as a functional protein model. The starting Fab was reduced with tris(2-carboxyethyl)-phosphine to cleave the disulfide bond and then treated with the reagents. These reagents simultaneously introduced biotin groups into the reduced Fab and re-bridged the disulfide moiety. Furthermore, we demonstrated that the biotin-labeled Fabs were reactive to an antigen and streptavidin.



INTRODUCTION

Numerous reagents and methods for regioselective and homogeneous protein modifications have been developed. Among them, maleimide reagents targeting cysteine residues are one of the most versatile reagents. Indeed, antibody-drug conjugates and therapeutic applications have been developed in the life science field by introducing functions of interest into proteins via maleimides.[1,2] Cysteine is a small fraction of the amino acid composition of proteins.[3,4] Therefore, cysteine residues are more favorable than other amino acid residues as regioselective modification target sites.[5] A sulfhydryl group on a cysteine side chain can react with a maleimide under mild conditions around neutral pH. If the target sulfhydryl group is not present in the protein because it is used in a disulfide bond, the sulfhydryl group is made available by cleaving the disulfide group through a reduction reaction. One disulfide bond yields two sulfhydryl groups. Maleimide reacts with one or both of the sulfhydryl groups, which may cause heterogeneity in the number of modifications. In addition, the loss of cross-linking by disulfide bonds upon labeling can lead to instability of the protein structure, making it more susceptible to metabolism and possible loss of activity. Recently, dibromopyridazinediones have been developed as new cysteine-selective modification reagents that can compensate for the loss of cross-linking, a drawback of maleimide-mediated modification.[6] The substitution of two vicinal bromine atoms on a dibromopyridazinedione with a pair of sulfhydryl groups derived from a disulfide bond provides a cross-linked structure. Therefore, the proteins are expected to retain their structural stability and activity after labeling. Dibromopyridazinediones with terminal alkyne and strained alkyne groups facilitate the introduction of the desired functional group into the proteins of interest by designs intended for click chemistry.[7−19]

Among the many functional modification groups that have been introduced into proteins, biotin is one of the simple and most versatile labels. Biotin binds strongly to avidin and streptavidin in equilibrium dissociation constants of 1×10^{-15} and 4×10^{-14} M, respectively, one of the most robust non-covalent interactions found in nature.[20] Numerous applications using biotin−avidin (or streptavidin) systems including the immobilization of antibodies and detection systems (e.g., enzyme-linked immunosorbent assay, immunohistochemistry, and immunofluorescence) have been reported and have proven effective in a wide range of fields. If biotin is made divalent, it would form a tight complex by divalent binding to a single avidin molecule rather than monovalent biotin or form oligomers by cross-linking multiple avidin molecules, which are expected to expand applications of avidins.[21] In our previous work, the double biotin group provided a divalent sugar ligand linked to it with a favorable orientation for binding to wheat germ agglutinin lectin, resulting in interaction with high binding affinity.[22] Even if the divalent sugar ligand is replaced by other biomolecules, it is expected that their functionality will be well retained. Here, we present new biotinylation reagents **1** and **2**, which have a design with mono

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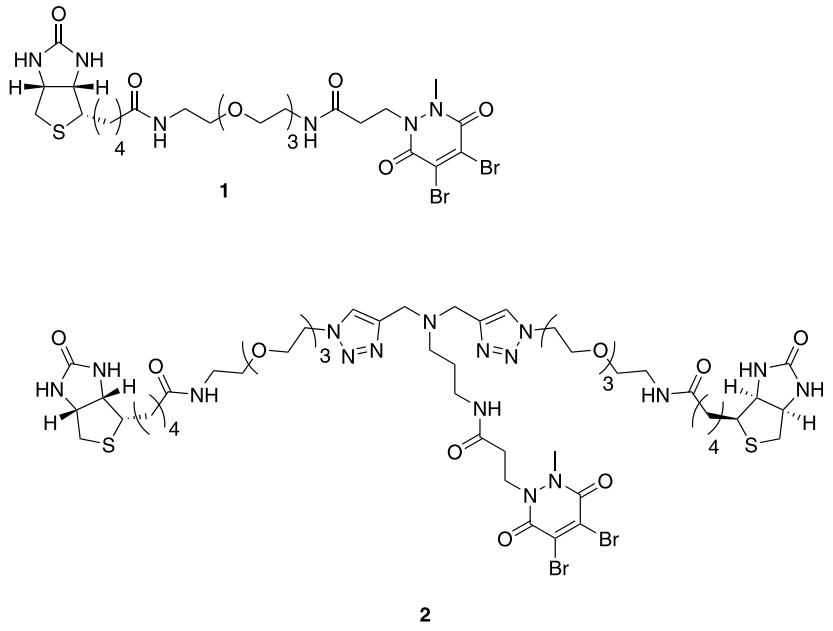
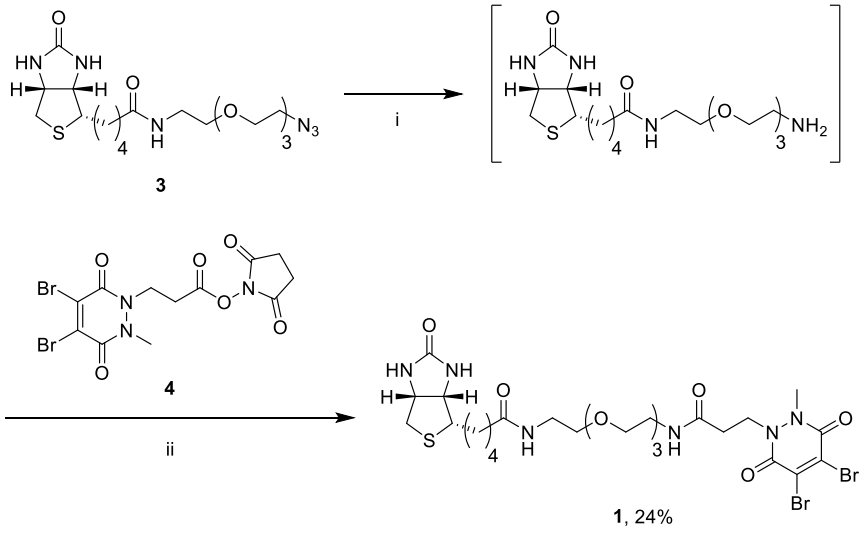


Figure 1. Mono-biotin PD **1** and double-biotin PD **2** synthesized in this study.

Scheme 1. Synthesis of Mono-Biotin PD 1[a]



[a]Reagents and conditions: (i) H_2, $Pd(OH)_2/C$, MeOH, rt, 6 h; (ii) DMF, rt, 14 h, 24%.

and double-biotin functionality directly linking to a pyridazinedione (PD) scaffold (Figure 1). Our reagents allowed cysteine-selective biotin labeling and disulfide re-bridging simultaneously in one step.

RESULTS AND DISCUSSION

Synthesis of Biotinylated PDs. Synthesis of the mono-biotinylated dibromopyridazinedione **1** is shown in Scheme 1. Hydrogenolysis of biotin-PEG3-azide **3** proceeded smoothly to give the corresponding amine, which was coupled with NHS-activated dibromopyridazinedione **4** to afford mono-biotin PD **1** in 24% yield. Next, the double-biotinylated dibromopyridazinedione **2** was synthesized, as shown in Scheme 2. Biotin-PEG3-azide **3** was coupled with 3-(dipropargyl)propanol **5** by copper-catalyzed azide-alkyne cycloaddition reaction to give **6** in 58% yield. Compound **6** was converted into its azide derivative **7** via mesylate in 47% yield. Hydrogenolysis of azide **7** gave amine **8**, which was coupled with **4** to afford double-biotin PD **2** in 35% yield.

Synthesis of Fab−PD Conjugates. With the mono- and double-biotin PDs in hand, we attempted biotin labeling of the Fab fragment, as shown in Scheme 3. As the target protein for labeling, we chose a polyclonal goat Fab fragment that reacts with the whole mouse IgG. Antibodies and their fragments are used not only for fundamental research such as functional analysis of cells and proteins and screening of gene expression but also for immunological measurement and analysis, making them one of the essential tools in life science research. For some applications, polyclonal antibodies are preferred instead of monoclonal antibodies. Polyclonal antibodies are mixtures of different antibodies that recognize multiple epitopes on a single antigen and have varying affinities. They can be produced more rapidly and inexpensively than monoclonal antibodies. Previous studies using PD derivatives have used

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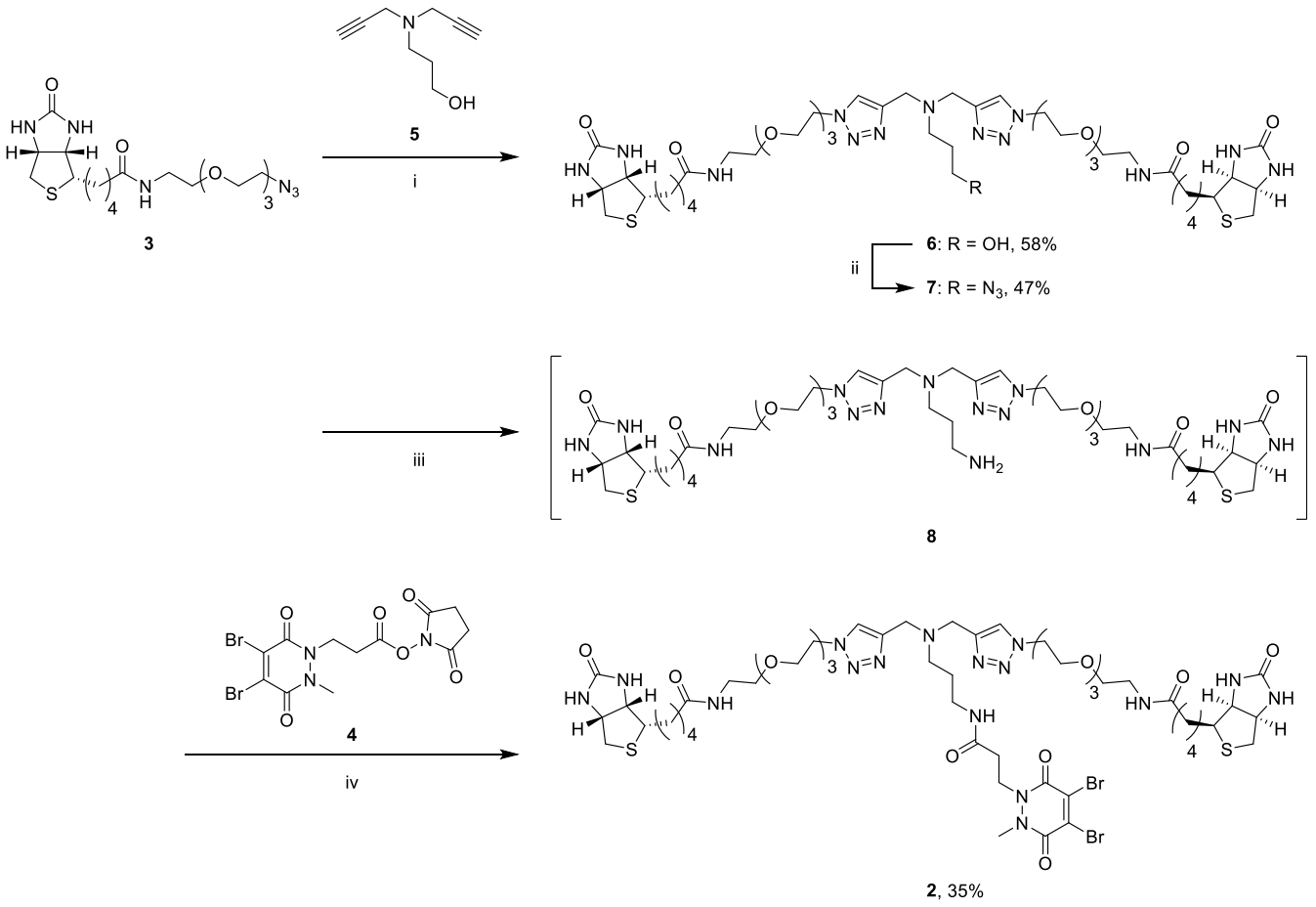
Scheme 2. Synthesis of Double-Biotin PD 2[a]



[a]Reagents and conditions: (i) $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, DMF, rt, 42 h; (ii) methanesulfonyl chloride, Et_3N, DMF, rt, 15 h; then NaN_3, DMF, rt, 24 h; (iii) H_2, $Pd(OH)_2/C$, MeOH, rt, 20 h; and (iv) DMF, rt, 21 h.

monoclonal antibodies or their fragments as target proteins, and none have used polyclonal antibodies or their fragments. If polyclonal antibody fragment-PD complexes could be prepared, they would be useful for a wide range of experiments, including Western blotting and immunohistochemistry. The Fab fragment consists of one light chain and one heavy chain, and both chains are cross-linked by a single disulfide bond. To insert the mono- and double-biotin PDs between two sulfur atoms forming the disulfide bond, the Fab fragments were first reduced by tris(2-carboxyethyl)phosphine (TCEP) in borate buffer saline containing ethylenediaminetetraacetic acid, pH 8.0 (BBS) at 37 °C and separated into light and heavy chains. After removing the unreacted TCEP and its oxide by ultrafiltration (MWCO 3000), the mono-biotin PD **1** or double-biotin PD **2** was reacted to the recovered light and heavy chains at 21 °C. After removing the unreacted PD and salts by ultrafiltration, the resulting Fab was recovered as a solution in BBS.

Starting Fab, reduced Fab, and resulting Fab were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions followed by Western blotting with Cy3-conjugated whole mouse IgG and with Cy3-conjugated streptavidin. Images of Coomassie-stained gels for the conjugation of Fab with mono-biotin PD **1** and double-biotin PD **2** are shown in Figure 2a,d, respectively. We observed that starting Fab produced two major bands: one was a broad band due to the overlap of two bands of 46 and 43 kDa, and the other was a band of 35 kDa (lane 1 in Figure 2a,d). Nelson et al. reported that freshly prepared Fabs were monovalent but aged Fabs spontaneously formed bivalent complexes susceptible to proteolysis rather than monovalent Fabs.[25] Proteolysis of aged Fab chains specifically occurred during SDS-PAGE analysis under non-reducing conditions, producing a pattern that favors faster mobility bands. The band patterns we observed were consistent with their report. We used the same lot of starting Fab for both experiments, but densitometric analysis revealed differences in the ratios of the two bands: 60:40 (Figure 2a) and 75:25 (Figure 2d). The faster mobility bands (43 and 35 kDa) preferentially observed in Figure 2a may be due to an increase in the Fab dimer fraction as the Fab ages. The experiment for which results are shown in Figure 2a was performed 27 days after the experiment for which results are shown in Figure 2d. The starting Fab used for the reaction with mono-biotin PD **1** should have contained a higher fraction of bivalent complex than that used for the reaction with double-biotin PD **2**. Upon treatment with TCEP, the two major bands shifted to 23 and 20 kDa (lane 2 in Figure 2a,d). These were band patterns that corresponded to the full-length light and heavy chains of Fabs. Truncated chains by proteolysis were not observed after reduction, consistent with SDS-PAGE analysis of aged Fab under reducing conditions.[25] The ratios of the

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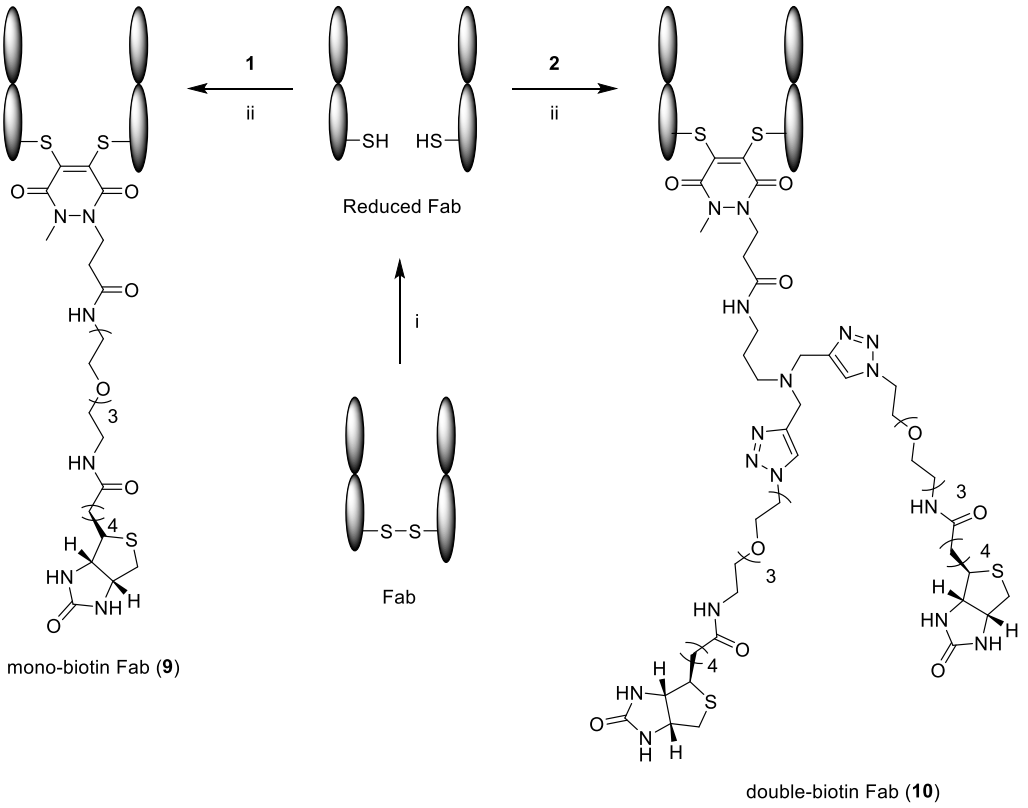
Scheme 3. Biotin Labeling and Simultaneous Disulfide Re-Bridging for Fab Fragments[a]



[a]Reagents and conditions: (i) TCEP·HCl, BBS (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0), 37 °C, 1.5 h; (ii) DMF/BBS (5:95, v/v), 21 °C, 18 h; and (iii) DMF/BBS (7:93, v/v), 21 °C, 21 h.

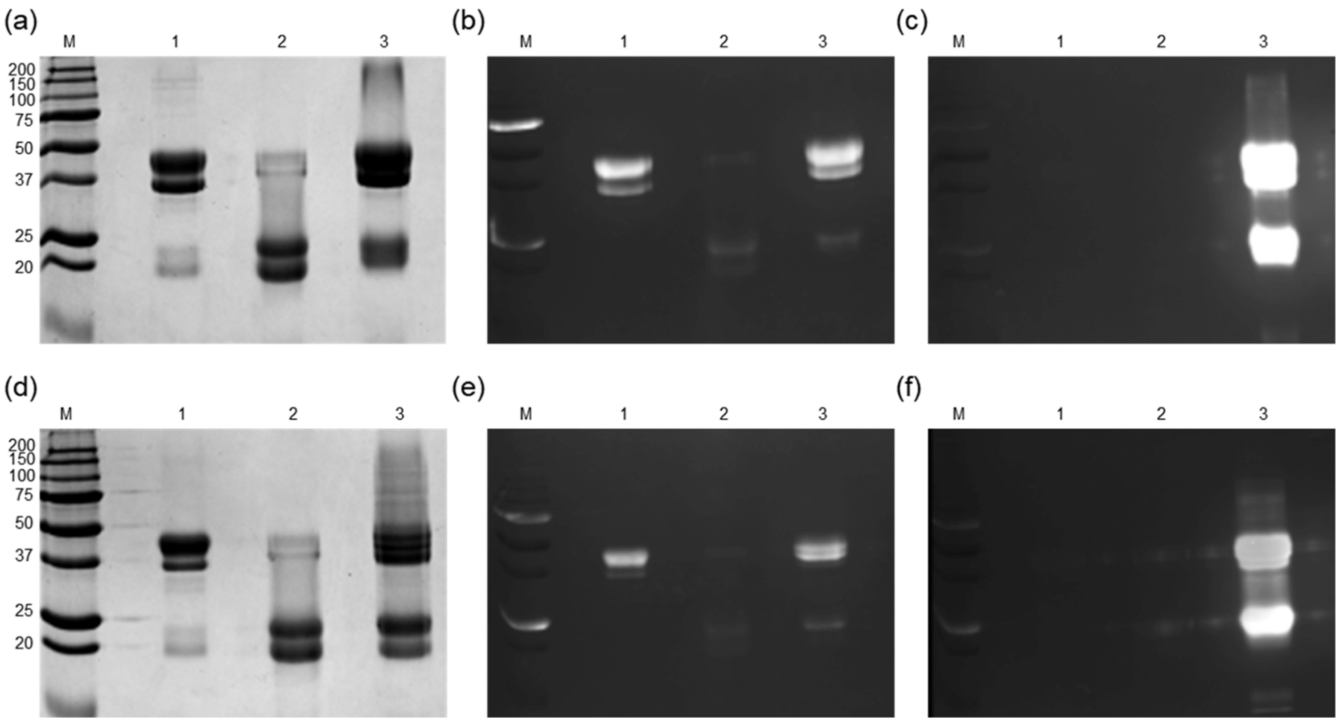


Figure 2. Preparation of mono-biotin Fab and double-biotin Fab according to Scheme 3. The top three images (a–c) show the results for mono-biotin Fab **9** and the bottom three images (d–f) show the results for double-biotin Fab **10**: non-reducing SDS-PAGE with Coomassie staining (a,d), Western blots with Cy3-conjugated antigen (b,e), and Western blots with Cy3-conjugated streptavidin (c,f). Lanes 1, 2, and 3 are starting Fab, reduced Fab, and biotin-labeled Fab, respectively. Lane M shows the molecular size standard with masses given in kDa.

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bands corresponding to reduced Fab that was generated and starting Fab that remained were 90:10 (Figure 2a) and 92:8 (Figure 2d), respectively. These suggest that the reduction reaction proceeded smoothly to separate light and heavy chains in both cases. The differences in starting Fab status seemed to have little effect on reduction efficiency. Incubation of the reduced Fab with mono-biotin PD **1** generated two major bands (45 and 37 kDa) such as starting Fab (lane 3 in Figure 2a,d). The pattern of the bands generated by conjugation of reduced Fab and double-biotin PD **2** was similar, but three bands (48, 44, and 39 kDa) were observed in clear separation. The conjugations of the reduced Fabs and both PDs successfully re-bridged the light and heavy chains, yielding mono-biotin Fab **9** and double-biotin Fab **10**. The ratios of the bands corresponding to re-bridged Fabs and unbridged Fabs were 68:32 (Figure 2a) and 55:45 (Figure 2d), respectively. Mono-biotin PD **1** with less steric hindrance resulted in higher complexation efficiency than did double-biotin PD **2**.

Fluorescent Western blots provided a functional insight into the resulting Fabs. Apparent reactivity to the antigen (whole mouse IgG) was detected on the bands corresponding to starting Fab and mono- and double-biotin Fabs. In contrast, much less reactivity was found on the bands corresponding to reduced Fab (Figure 2b,e). If mouse IgG, used as an antigen, has a binding activity to Fab, it should show reasonable binding ability against reduced Fab. However, mouse IgG had poor reactivity against both light and heavy chains, suggesting that the positive bands for mouse IgG were basically due to the binding ability of Fab, not mouse IgG. Reactivity against streptavidin was detected only after the treatment of reduced Fab with PDs as expected (lane 3). Both bands corresponding to the molecular size of reduced and re-bridged Fab were positive, indicating that biotin was introduced not only to re-bridged Fabs but also to unbridged light and heavy chains.

In general, there are five disulfide bonds in a single Fab molecule: two intrachain disulfide bonds in the light chain, two intrachain disulfide bonds in the heavy chain, and one interchain disulfide bond between the light and heavy chains.[26] If all disulfide bonds were reductively cleaved, five thiol groups would be generated on each of the light and heavy chains. If two random thiols generate interchain disulfide bonds during re-bridging, chain interchange and many "incorrect" interchain linkage modes will occur. The result is that there can be 25 different combinations of light chain−light chain, light chain−heavy chain, and heavy chain−heavy chain, respectively. This situation is further complicated by the heterogeneity of the Fab. Because the Fab used in this study is derived from a polyclonal antibody, it is not a single Fab, but a mixture of multiple Fabs. Because chains can be exchanged even between different Fab molecules, the number of possible combinations is extremely large. However, Western blotting results show that light chains alone and heavy chains alone have little or no antigen binding activity. Therefore, it is reasonable to assume that light chain−light chain and heavy chain−heavy chain combinations do not exhibit antigen-binding activity. Even in the light chain−heavy chain pair, if both chains are linked by incorrect disulfide bonds, the antigen-binding activity will still be low because the respective antigen-binding sites of the light and heavy chains will be oriented improperly. It is known that even if the disulfide bond linking the light and heavy chains is lost, they continue to form correct pair association between light and heavy chains and function in solution.[27] Western blotting results also showed that the antigen-binding activity of the re-bridged Fab was comparable to that of the starting Fab. This suggests that most of the Fabs produced by re-bridging are light chain−heavy chain pairs linked by correct disulfide bonds.

Surface Plasmon Resonance Analysis. To further study the binding of Fab−PD conjugates to the antigen, we performed surface plasmon resonance (SPR) analyses. First, pH scouting for the immobilization of mouse IgG (antigen) on a CM5 sensor chip by using 10 mM sodium acetate buffer with pH ranging from 4.0 up to 5.5 was carried out. We selected pH 5.0 showing the highest response as an appropriate immobilization pH value. Using amine coupling by the standard protocol, three CM5 sensor chips were immobilized with mouse IgG under the same conditions. The resulting immobilized levels were 704, 784, and 635 RU, from which the theoretical R_{max} (maximum change in the SPR angle) values of 235, 264, and 217 RU, respectively, were calculated (Table 1).

Table 1. Summary of the Parameters Obtained by SPR Analyses[a]

	Fab	mono-biotin Fab **9**	double-biotin Fab **10**
$K_{D,app}$ (M)	7.066×10^{-9} (100%)	9.907×10^{-9} (72%)	1.061×10^{-8} (66%)
$k_{a,app}$ (1/Ms)	1.978×10^{4} (100%)	1.434×10^{4} (72%)	1.306×10^{4} (66%)
$k_{d,app}$ (1/s)	1.398×10^{-4} (100%)	1.425×10^{-4} (98%)	1.443×10^{-4} (97%)
experimental R_{max} (x)	635	2214	1494
theoretical R_{max} (y)	235	264	217
R_{max} ratio (x/y)	2.7	8.4	6.9

[a]Apparent dissociation constant $K_{D,app}$, apparent association rate constant $k_{a,app}$, apparent dissociation rate constant $k_{d,app}$, and experimental R_{max} were calculated by ising a 1:1 interaction model.

Each chip was used for SPR binding analysis for starting Fab, mono-biotin Fab **9**, and double-biotin Fab **10** with five concentrations of 62.5, 125, 250, 500, and 1000 nM (Figure 3). The apparent dissociation constant $K_{D,app}$, apparent association rate constant $k_{a,app}$, apparent dissociation constant $k_{d,app}$, and experimental R_{max} were calculated by using a 1:1 interaction model. These parameters are summarized in Table 1. Starting Fab showed the highest binding affinity with an apparent dissociation constant ($K_{D,app}$) value of 7.1 nM. When this affinity was set to 100%, the mono-biotin Fab and double-biotin Fab showed 72 and 66% of the affinity, respectively. A comparison of rate constants of the three analytes showed that there was little difference in apparent dissociation rate constants ($k_{d,app}$), whereas there was a similar difference in apparent association rate constants ($k_{a,app}$) as in $K_{D,app}$. The antigen-binding site of the Fab−PD conjugate is on the opposite side of the biotin, so dissociation from the antigen is not significantly affected, but the steric bulk of whole Fab that was increased by conjugation with PDs appears to prevent access to the antigen.

It should be noted that the SPR data presented here are only a rough indication of the trend of binding to the antigen. There are three reasons for the lack of clarity. The first is the purity of analytes. In our cases, Fab−PD conjugates used as analytes were a mixture of Fab−PD, heavy chain−PD, and light chain−PD conjugates. The second reason is clonality. We used not monoclonal but polyclonal Fab to prepare the Fab−PD

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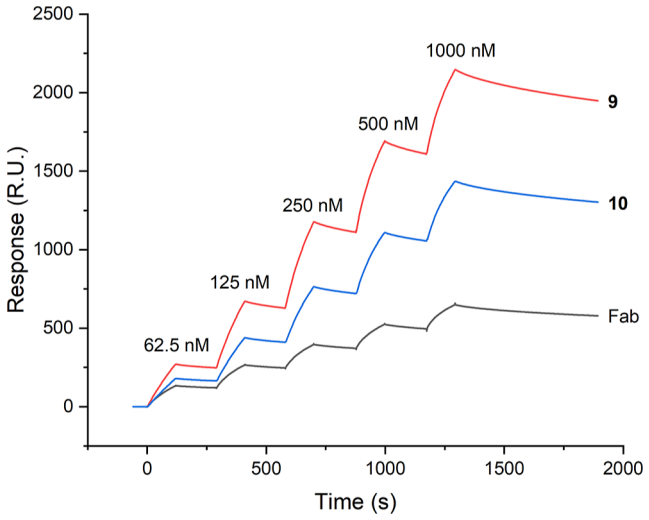

Figure 3. SPR single-cycle kinetic sensorgrams for starting Fab, mono-biotin Fab **9**, and double-biotin Fab **10** (62.5−1000 nM, five concentrations) against mouse IgG (antigen) immobilized on a CM5 sensor chip by amine coupling.

conjugate. The Fab and Fab−PD conjugate may recognize multiple epitopes on the antigen. In other words, multiple analytes (Fab or Fab−PD conjugates) may bind to one ligand (antigen) simultaneously. The third reason is the aggregative propensity of Fab itself. Even if we could prepare 100% pure Fab and Fab−PD conjugates, it is difficult to exclude the possibility of dimer formation during the SPR experiment. These concerns are manifested by the fact that experimental R_{max} exceeds theoretical R_{max} (Table 1). In a typical SPR interaction analysis, experimental R_{max} is the same or less than theoretical R_{max} because the response is saturated below theoretical R_{max} when the analyte concentration is continuously increased. However, starting Fab showed an experimental R_{max} that was 2.6-fold higher than theoretical R_{max}, suggesting the possibilities of Fab dimer formation and multiple binding to the antigen. Mono-biotin Fab and double-biotin Fab showed experimental R_{max} values that were 8.4 and 6.9-fold higher than theoretical R_{max}, more than twice the values observed for the starting Fab. The formation of aggregates larger than dimers or nonspecific binding is suspected; the presence of light chain− and heavy chain−PD conjugates contained in the Fab−PD conjugates may accelerate these phenomena.

CONCLUSIONS

We presented the syntheses of mono- and double-biotinylation reagents and their capabilities by using a Fab fragment as a model protein. Both reagents were capable of simultaneously achieving cysteine-selective biotin labeling and re-bridging of the reduced Fab fragment in a single step. The resulting Fab conjugates were demonstrated to have the ability to bind to an antigen and streptavidin. These simple reagents and protocols will provide a significant advantage for biotin labeling of functional proteins having disulfide bonds.

EXPERIMENTAL PROCEDURES

Materials and Methods. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. *N*,*N*-Dimethylformamide (DMF) was stored over molecular sieves (MS4A) and methanol (MeOH) was stored over MS3A prior to use. Compounds **3**,[23] **4**,[14] and **5**[24] were synthesized according to methods reported previously. Reactions leading to the synthesis of compounds **1** and **2** were monitored by thin-layer chromatography on a pre-coated plate of silica gel 60 F254 (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). Column chromatography was performed on silica gel (Silica Gel 60; 63-200 μm, E. Merck). IR spectra were recorded by using a SHIMADZU IR Prestige-21 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a BRUKER AVANCE 400 spectrometer equipped with a cryoprobe, at 400 MHz for ^{1}H and 100 MHz for the carbon ^{13}C, in $CDCl_3$, $(CD_3)_2SO$, or D_2O. NMR signals were assigned by ^{1}H, ^{13}C, and H−H COSY measurements. Matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOFMS) were recorded by using 2,5-dihydroxybenzoic acid as a matrix on a BRUKER autoflex III-2S spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 355 nm nitrogen laser. Prior to the experiment, the instrument was calibrated using an external standard calibration mixture composed of angiotensin I, angiotensin II, substance P, bombesin, adrenocorticotropic hormone clips 1−17 and clips 18−39, and somatostatin 28 from Bruker Daltonics.

Mono-Biotin PD (1). To a solution of **3** (1.2 g, 2.3 mmol) in methanol (10 mL) was added 20% w/w $Pd(OH)_2/C$ (1.25 g) at room temperature. After stirring for 6 h at room temperature under a H_2 atmosphere, the reaction mixture was passed through a Celite pad. The filtrate was concentrated under reduced pressure. The residue was dissolved in DMF (4 mL) and **4** (778 mg, 1.86 mmol) was added at room temperature. After stirring for 14 h at room temperature, the reaction mixture was concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel (chloroform/methanol = 6:1) to give **1** (292 mg) in 24% yield as a yellowish oil. Analytical data for **1**: R_f = 0.30 [4:1 (v/v) chloroform/methanol]; ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 4.57−4.41 (m, 3H, NHC(O)NHC*H*CH$_2$, NCH$_2$C*H*$_2$CO), 4.31 (dd, J = 7.9, 4.4 Hz, 1H, NHC(O)-NHC*H*CH), 3.68 (s, 3H, NC*H*$_3$), 3.66−3.62 (m, 4H, OC*H*$_2$CH$_2$NH), 3.62−3.46 (m, 8H, OC*H*$_2$C*H*$_2$O), 3.41−3.30 (m, 4H, OCH$_2$C*H*$_2$NH), 3.21 (ddd, J = 8.9, 5.9, 4.4 Hz, 1H, NHC(O)NHCHC*H*), 2.93 (dd, J = 12.7, 5.0 Hz, 1H, NHC(O)NHCHC*H*$_2$), 2.69 (dd, J = 12.7, 5.0 Hz, 1H, NHC(O)NHCHC*H*$_2$), 2.60 (t, J = 6.8 Hz, 2H, NCH$_2$C*H*$_2$CO), 2.22 (t, J = 7.4 Hz, 2H, COCH$_2$C*H*$_2$CH$_2$CH$_2$), 1.81−1.53 (m, 4H, COCH$_2$C*H*$_2$C*H*$_2$CH$_2$), 1.51−1.38 (m, 2H, COCH$_2$CH$_2$C*H*$_2$CH$_2$); ^{13}C NMR (100 MHz, CD_3OD): δ (ppm) 176.2 (COCH$_2$CH$_2$CH$_2$CH$_2$), 176.1 (CH$_2$CH$_2$CONH), 172.2 (COCH$_2$CH$_2$CH$_2$CH$_2$), 166.1 (NHC(O)NHCHCH), 154.8 (CH$_3$NCO), 154.5 (CH$_2$NCO), 136.8 (CH$_3$NCOCBr), 136.5 (CH$_2$NCOCBr), 71.6 (OCH$_2$), 71.2 (OCH$_2$), 70.6 (OCH$_2$), 70.4 (OCH$_2$), 63.4 (NHC(O)NHCHCH), 61.6 (NHC(O)NHCHCH$_2$), 57.0 (NHC(O)NHCHCH), 45.5 (C), 41.1 (C), 40.5 (C), 40.3 (C), 36.8 (C), 35.6 (NCH$_3$), 34.7 (C), 29.8 (C), 29.5 (C), 26.8 (C), 26.3 (C), 18.4 (C); MALDI-TOFMS: m/z: calcd for $C_{26}H_{40}Br_2N_6NaO_8S$ $[M + Na]^+$, 777.08873; observed,777.0530.

Double-biotin Alcohol (6). To a stirred solution of biotin-PEG3-azide **3** (24.4 g, 54.9 mmol) in DMF (220 mL) were added 3-[di(prop-2-yn-1-yl)amino]propan-1-ol **5** (4.15 g, 27.5 mmol), a solution of sodium L-ascorbate (5.99 g, 30.2 mmol) in water (20 mL), and a solution of $CuSO_4{\cdot}5H_2O$ (0.75 g, 3.02

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mmol) in water (5 mL), and the reaction mixture was stirred in the dark for 42 h. After concentration under reduced pressure, the resulting material was purified by column chromatography on silica gel (ethyl acetate/methanol = 1:0 → 1:2) to give **6** (16.5 g) in 58% yield as a yellowish oil. Analytical data for (6): R_f = 0.30 [methanol]; ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 7.99 (s, 2H, triazole−C*H*), 4.61−4.56 (m, 4H), 4.49 (ddd, J = 7.8, 5.0, 0.9 Hz, 2H, NHC(O)NHC*H*CH_2), 4.30 (dd, J = 7.9, 4.5 Hz, 2H, NHC(O)NHC*H*CH), 3.94−3.88 (m, 4H), 3.77 (s, 4H), 3.61 (q, J = 1.3 Hz, 9H), 3.58 (d, J = 4.6 Hz, 10H), 3.52 (t, J = 5.5 Hz, 4H, $NCH_2CH_2CH_2OH$), 3.34 (d, J = 1.7 Hz, 7H), 3.31 (s, 2H), 3.20 (ddd, J = 8.8, 5.8, 4.4 Hz, 2H, NHC(O)NHCHC*H*), 2.92 (dd, J = 12.8, 5.0 Hz, 2H, NHC(O)NHCHCH_2), 2.70 (d, J = 12.7 Hz, 2H, NHC(O)-NHCHCH_2), 2.57 (t, J = 7.1 Hz, 2H, $NCH_2CH_2CH_2OH$), 2.20 (t, J = 7.4 Hz, 4H, $CH_2CH_2CH_2CH_2CO$), 1.82−1.53 (m, 11H, $NCH_2CH_2CH_2OH$, $CH_2CH_2CH_2CH_2CO$), 1.43 (p, J = 7.4 Hz, 4H, $CH_2CH_2CH_2CH_2CO$); ^{13}C NMR (100 MHz, CD_3OD): δ (ppm) 176.1 ($CH_2CH_2CH_2CH_2CO$), 166.1 (NHC(O)NHCHCH_2), 145.2 (triazole−*C*CH), 126.1 (triazole−C*C*H), 71.6, 71.5, 71.5, 71.3, 70.6, 70.4, 63.4 ($NCH_2CH_2CH_2OH$), 61.6, 61.6, 57.0, 51.4 ($NCH_2CH_2CH_2OH$), 41.1, 40.3, 36.7, 30.8, 29.8, 29.5, 26.8; IR (neat): 3333, 2928, 1651, 1557, 1454, 1121, 552 cm^{-1}; MALDI-TOFMS: m/z: calcd for $C_{45}H_{77}N_{13}NaO_{11}S_2$ [M + Na]$^+$, 1062.5199; observed, 1062.4728.

Double-Biotin Azide (7). To a stirred solution of alcohol **6** (16.2 g, 15.6 mmol) in DMF (234 mL) were added Et3N (1.74 g, 17.2 mmol) and methanesulfonyl chloride (1.97 g, 17.2 mmol) in the dark at room temperature. After stirring in the dark for 15 h at room temperature, sodium azide (5.17 g, 79.5 mmol) was added to the reaction mixture. After stirring for 24 h at room temperature, the reaction mixture was concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel (ethyl acetate/methanol = 1:2) to give **7** (7.87 g) in 47% yield as a yellowish oil. Analytical data for (7): R_f = 0.20 [1:2 (v/v) ethyl acetate/methanol]; ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 8.00 (s, 2H, triazole−C*H*), 4.59 (t, J = 5.1 Hz, 4H), 4.51−4.46 (m, 2H, NHC(O)NHC*H*CH_2), 4.30 (dd, J = 7.9, 4.5 Hz, 2H, NHC(O)NHC*H*CH), 3.91 (t, J = 5.1 Hz, 4H), 3.79 (s, 4H), 3.64−3.55 (m, 18H), 3.52 (t, J = 5.5 Hz, 4H, $NCH_2CH_2CH_2OH$), 3.34 (d, J = 1.7 Hz, 9H), 3.20 (ddd, J = 8.9, 5.9, 4.4 Hz, 2H, NHC(O)NHCHC*H*), 2.92 (dd, J = 12.8, 5.0 Hz, 2H, NHC(O)NHCHCH_2), 2.70 (d, J = 12.7 Hz, 2H, NHC(O)NHCHCH_2), 2.59 (t, J = 7.1 Hz, 2H, $NCH_2CH_2CH_2OH$), 2.20 (t, J = 7.4 Hz, 4H, $CH_2CH_2CH_2CH_2CO$), 1.84−1.52 (m, 10H, $NCH_2CH_2CH_2OH$, $CH_2CH_2CH_2CH_2CO$), 1.43 (p, J = 7.2 Hz, 4H, $CH_2CH_2CH_2CH_2CO$); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 176.1 ($CH_2CH_2CH_2CH_2CO$), 166.1 (NHC(O)NHCHCH_2), 144.9 (triazole−*C*CH), 126.2 (triazole−C*C*H), 71.5, 71.5, 71.5, 71.3, 70.6, 70.4, 63.4 ($NCH_2CH_2CH_2N_3$), 61.6, 61.6, 57.0, 51.4 ($NCH_2CH_2CH_2N_3$), 41.1, 40.3, 36.7, 30.7, 29.8, 29.5, 26.8; IR (neat): 3317, 2932, 2872, 2099, 2031, 1682, 1557, 1456, 1211, 1141, 725 cm^{-1}; MALDI-TOFMS: m/z: calcd for $C_{45}H_{76}N_{16}NaO_{10}S_2$ [M + Na]$^+$, 1087.5264; observed, 1087.4129.

Double-Biotin PD (2). To a solution of azide **7** (400 mg, 375 μmol) in methanol (4 mL) was added 20% w/w $Pd(OH)_2/C$ (500 mg) at room temperature. After stirring for 20 h at room temperature under an H_2 atmosphere, the reaction mixture was passed through a Celite pad. The filtrate was concentrated under reduced pressure. MALDI-TOFMS and IR spectrum of the residue suggested generation of the corresponding amine **8**. The residue was dissolved in DMF (10 mL) and **4** (127 mg, 280 μmol) was added at room temperature. After stirring for 21 h at room temperature, the reaction mixture was concentrated under reduced pressure. The resulting material was purified by column chromatography on silica gel (ethyl acetate/methanol = 1:0 → 0:1) to give **2** (134 mg) in 35% yield as a yellowish oil. Analytical data for **8**: R_f = 0.10 [methanol]; IR (neat): 3439, 3005, 2936, 1639, 1402, 1200, 1074, 789, 567, 534, 482 cm^{-1}; MALDI-TOFMS: m/z: calcd for $C_{45}H_{79}N_{14}NaO_{10}S_2$ [M + Na]$^+$, 1062.5437; observed, 1062.5152. Analytical data for **2**: R_f = 0.30 [methanol]; ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 7.98 (s, 2H, triazole−C*H*), 4.59 (t, J = 5.1 Hz, 4H), 4.54−4.39 (m, 4H, NHC(O)NHC*H*CH_2, NCH_2CH_2CO), 4.30 (dd, J = 7.9, 4.5 Hz, 2H, NHC(O)NHC*H*CH), 3.91 (t, J = 5.1 Hz, 4H), 3.74 (s, 3H, NCH_3), 3.67 (s, 2H), 3.65−3.54 (m, 18H), 3.52 (t, J = 5.4 Hz, 5H), 3.31 (s, 11H), 3.19 (ddt, J = 8.9, 6.7, 4.5 Hz, 2H, NHC(O)NHCHC*H*), 2.92 (dd, J = 12.8, 5.0 Hz, 2H, NHC(O)NHCHCH_2), 2.79 (d, J = 2.1 Hz, 1H), 2.70 (d, J = 12.7 Hz, 2H), 2.66−2.58 (m, 2H, NHC(O)NHCHCH_2), 2.52−2.48 (m, 2H), 2.21 (t, J = 7.4 Hz, 4H, $COCH_2CH_2CH_2CH_2$), 1.65 (dddt, J = 38.7, 24.3, 13.9, 6.8 Hz, 8H, $COCH_2CH_2CH_2CH_2$), 1.43 (p, J = 7.3 Hz, 4H, $COCH_2CH_2CH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 176.1 ($COCH_2CH_2CH_2CH_2$), 172.1 (CH_2CH_2CONH), 166.1 (NHC(O)NHCHCH), 154.8 (CH_3NCO), 154.5 (CH_2NCO), 145.3 (triazole−*C*CH), 136.7 ($CH_3NCOCBr$), 136.5 ($CH_2NCOCBr$), 126.1 (triazole−C*C*H), 71.6 (OCH_2), 71.5 (C), 71.5 (C), 71.3 (C), 70.6 (C), 70.4 (C), 63.4 (NHC(O)NHCHCH), 61.6 (NHC(O)NHCHCH_2), 57.0 (NHC(O)NHCHCH), 51.9 (C), 51.4 ($NHCH_2CH_2CH_2N$), 49.9 (C), 45.5 (C), 41.1 (C), 40.4 (C), 38.9 (C), 36.8 (C), 35.7 (NCH_3), 35.0 (C), 29.8 (C), 29.5 (C), 27.3 (C), 26.9 (C), 26.3 (C); MALDI-TOFMS: m/z: calcd for $C_{53}H_{84}Br_2N_{16}NaO_{13}S_2$ [M + Na]$^+$, 1399.4104; observed, 1399.4133.

Reduced FAB. To a solution of AffiniPure Fab Fragment Goat Anti-Mouse IgG (H + L) (Jackson Immunoresearch Laboratories, Inc.) in BBS buffer (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) (1.3 mg/mL, 28.3 μL) was added a solution of TCEP·HCl in BBS buffer (26.1 mM, 21.8 μL), and the reaction mixture was incubated at 37 °C for 15 h. Excess reagents were removed by ultrafiltration by using an Amicon Ultra-0.5 mL centrifugal filter device (Ultracel-3k) to give a solution of reduced Fab.

Mono-Biotin Fab (9). To a solution of reduced Fab were added a solution of mono-biotin PD **1** in DMF (15 mM, 3.4 μL) and BBS buffer (66.6 μL) and the reaction mixture was incubated at 21 °C for 18 h. Excess reagents were removed by ultrafiltration by Ultracel-3k. Analysis of the retentate by SDS-PAGE and Western blotting confirmed mono-biotin Fab **9**.

Double-Biotin Fab (10). To a solution of reduced Fab were added a solution of double-biotin PD **2** in DMF (6 mM, 4.9 μL) and BBS buffer (66.6 μL) and the reaction mixture was incubated at 21 °C for 21 h. Excess reagents were removed by ultrafiltration by Ultracel-3k. Analysis of the retentate by SDS-PAGE and Western blotting confirmed double-biotin Fab **10**.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Non-reducing SDS-PAGE at 12% acrylamide running gel with a 4% acrylamide stacking gel on a Mini-

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Protean Tetra Cell (Bio-Rad) and Power pack Basic (Bio-Rad) was performed following standard lab procedures. Precision Plus Protein Dual Xtra Prestained Protein Standards molecular weight marker (Bio-Rad) was co-run to estimate protein weights. Samples (15 μL) were mixed with 2× Laemmli Sample Buffer (Bio-Rad) (15 μL) and 30 μL of each mixture was loaded in the wells. All gels were run with a running buffer solution [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS] under a constant current of 30 mA until bromophenol blue reached the bottom of the running gel. The gels were stained using Bullet CBB Stain One(Ready To Use) (Nacalai Tesque, Inc.) at room temperature for 15 min. Gels were imaged using WSE-5300 Printgraph CMOS I (ATTO Corp.) without destaining. Densitometric evaluations were performed using the image analysis software CS Analyzer 4 (ATTO Corp.).

Detection of Biotin-Labeled Proteins with Cy3−Streptavidin Conjugate. Samples were separated by SDS-PAGE as above. Protein bands were transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad) by electroblotting at a constant current of 1.3 A for 7 min on Trans-Blot Turbo (Bio-Rad). After transfer, biotin-labeled proteins were detected using Cy3−streptavidin conjugate. Blots were blocked against nonspecific reactions by soaking in Bullet Blocking One for Western Blotting (Nacalai Tesque, Inc.) (50 mL/gel) at room temperature for 30 min. The blots were incubated with Cy3−streptavidin (Jackson ImmunoResearch Laboratories, Inc.) (1.0 mg/mL, 10 μL/gel) in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.6) (50 mL) at room temperature for 1 h on a shaker. The blots were then washed with TBS-T (50 mL) at room temperature for 5 min. This washing procedure was repeated three times. Fluorescent gel images were acquired on a Typhoon 9400 scanner (Amersham Biosciences/GE Healthcare) using a 532 nm laser and an emission filter of 570 nm BP20.

Detection of Anti-mouse IgG Proteins with Cy3−Mouse IgG Conjugate. SDS-PAGE and subsequent Western blotting were performed as described above except that Cy3-ChromPure Mouse IgG, whole molecule (Jackson ImmunoResearch Laboratories, Inc.) (1.0 mg/gel) was used instead of Cy3−streptavidin to detect protein bands exerting anti-mouse IgG activity.

Surface Plasmon Resonance. All SPR analyses were performed with the Biacore X100 instrument (GE Healthcare) on dextran-coated gold sensor chips (CM5, GE Healthcare) at 25 °C in HBS-EP+ (10×, Cytiva, final concentration of 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20) as a running buffer. At first, immobilization pH was optimized by 10 mM acetate buffer (pH 4.0, 4.5, 5.0, and 5.5) according to software instructions. The most effective preconcentration of ChromPure Mouse IgG, whole molecule (Jackson Immunoresearch Laboratories, Inc.), used as an antigen in our experiment, was observed at pH 5.0. Flow cells were activated by injection of a 1:1 (v/v) of 100 mM *N*-hydroxysuccinimide and 400 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at a constant flow rate of 5 μL/min. The original solution of ChromPure Mouse IgG (5.7 mg/mL of 10 mM sodium phosphate, 250 mM NaCl, pH 7.8) was diluted with 10 mM acetate buffer, pH 5.0 to a concentration of 9.5 μg/mL. This solution was injected at a constant flow rate of 5 μL/min until reaching the target level of 500 RU. In three independent experiments using a fresh CM5 sensor chip, the immobilization levels yielded 704, 784, and 635 RU. After sufficient protein coupling, the surface was deactivated by 1 M ethanolamine−HCl, pH 8.5. Protein concentrations of analytes (starting Fab, mono-biotin Fab, and double-biotin Fab) were estimated by measurements of ultraviolet absorbance at 280 nm and adjusted to 62.5, 125, 250, 500, and 1000 nM using a running buffer. SPR single-cycle kinetic experiments were performed using five concentrations of the analytes (each 60 μL) at a constant flow rate of 30 μL/min with 120 s as a contact time and 600 s as a dissociation time. Kinetic constants were calculated from the Sensorgrams using the 1:1 fit model with Biacore X100 Evaluation Software (Version: 2.0.1 Plus Package, GE Healthcare).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04379.

NMR spectra, MALDI-TOF MS, and IR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Fab, fragment antigen-binding; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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