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Article

Fluorescence "Turn-on" Lectin Sensors Fabricated by Ligand-Assisted Labeling Probes for Detecting Protein–Glycoprotein Interactions

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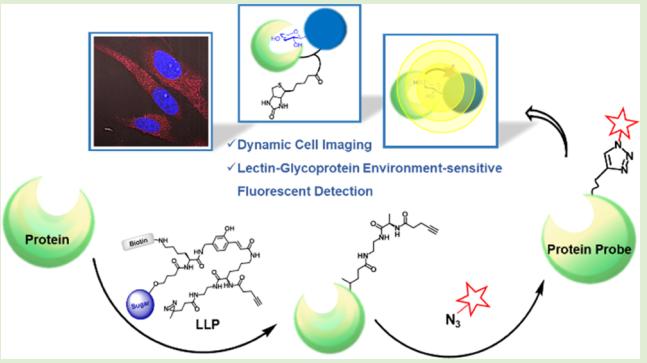
ABSTRACT: Elucidation of protein–protein interactions (PPIs) is often very challenging and yields complex and unclear results. Lectin–glycoprotein interactions are especially difficult to study due to the noncovalent nature of the interactions and inherently low binding affinities of proteins to glycan ligands on glycoproteins. Here, we report a "ligand-directed labeling probe (LLP)"-based approach to fabricate protein probes for elucidating protein–glycoprotein interactions. LLP was designed with dual photoactivatable groups for the introduction of an alkyne handle proximal to the carbohydrate-binding pocket of lectins, *Ricinus communis* agglutinin 120 (RCA_{120}) and recombinant human Siglec-2-Fc. In proof-of-principle studies, alkynylated lectins were conjugated with a photoreactive diazirine cross-linker and an environment-sensitive fluorophore, respectively, by the bioorthogonal click reaction. The modified RCA_{120} or Siglec-2-Fc was used for detecting the interaction with the target glycoprotein in the solution or endogenously expressed glycoproteins on live HeLa cells. We anticipate that the fabrication of these protein probes will accelerate the discovery of novel PPIs.



INTRODUCTION

Proteins exert their biological functions in cells via interactions with other proteins. Thus, investigation of protein–protein interactions (PPIs) is crucial for uncovering molecular mechanisms and the physiological basis of diseases and developing next-generation therapies.[1] In particular, the specific cell surface interactions between lectins and glycans are responsible for the mediation of various cellular functions and disease states.[2] However, these interactions are generally weak and challenging to detect. Although many approaches are widely used for studying PPIs, there are many inherent limitations associated with these methods, such as requiring PPIs with a high binding affinity (low dissociation constants (K_d))[3,4] or involving complex genetic engineering.[5] In contrast, chemical cross-linking methods[6,7] enable direct labeling of the target protein but is not amenable for real-time detection in living cells.[7] Moreover, the characterization of lectin and glycoprotein interactions is challenging because of the transient, noncovalent, and reversible nature of these weak interactions with low affinities (K_d = 10^{-4}–10^{-7} M).[8,9] In addition, most of the above strategies are not suitable for observing PPI dynamics. To address these challenges, here, we report a strategy for the functionalization of an intact protein via site-selective modification. The modified protein can be easily converted to protein probes for lectin–glycoprotein cross-linking and real-time cell imaging.

The chemical reporter strategies[10,11] are attractive methods for investigating weak binding interactions due to their abilities to introduce a desired functional group onto the bait protein for assisting the identification or purification of the interacting proteins. Currently, there are two main approaches for the assembly of a chemical reporter at the site near the ligand-binding epitope of the bait protein. One method is a genetic mutation,[12] which involves the incorporation of an unnatural amino acid at a specific position of the protein by genetic engineering.[13,14] Although this approach is suitable for proteins with known three-dimensional structures, it faces steep technical barriers and is impossible to site-selectively modify nonengineered natural proteins. The other approach is ligand-assisted labeling,[15−18] which can selectively modify the target protein in the vicinity of the ligand-binding site. However, in this strategy, depending on the distance from the nucleophilic residues to the protein binding site, different spacer lengths

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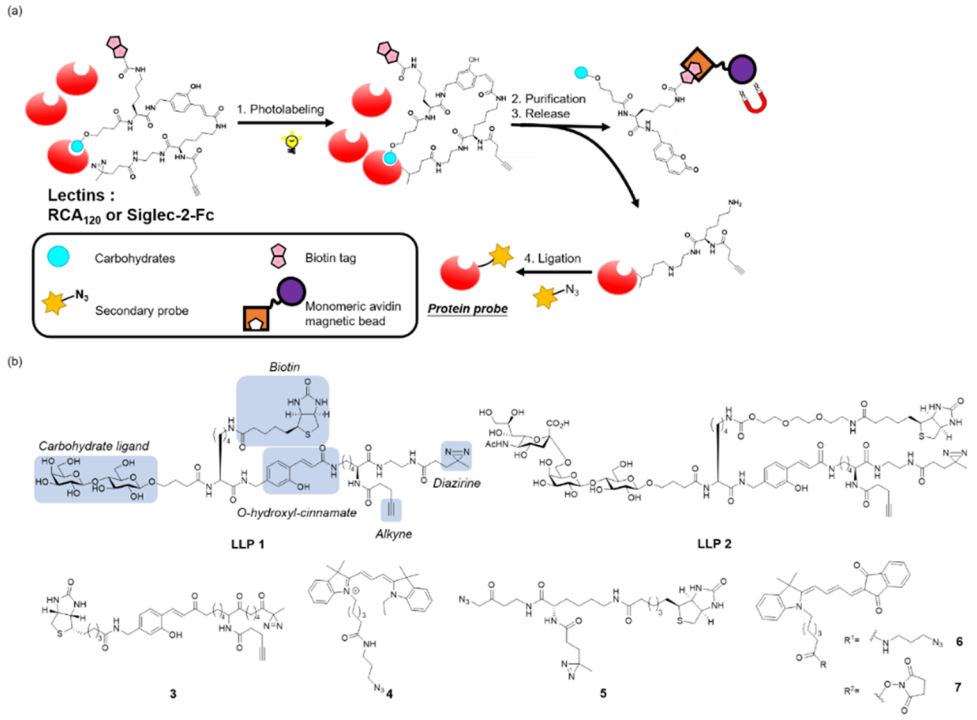

Figure 1. Protein probes were fabricated by the LLP strategy. (a) Workflow of the LLP strategy designed for protein labeling, enrichment, selective release, and postmodification. (b) **LLP 1** and **2** contain five key functional elements that are highlighted in blue. The structures for postmodification are shown as the photoreactive biotin control probe **3**, Cy3 probe **4**, trifunctional probe **5**, and environment-sensitive fluorophore **6**. Probe **7** was used for the control experiments of PPIs.

have to be evaluated to find the most suitable fit.[19] In contrast, we envisioned a ligand-directed photoaffinity labeling technique[20] that would be an attractive choice for site-selective assembly of a desired group at the closest location to a low-affinity carbohydrate-binding pocket.

To fabricate a useful protein probe, some essential factors need to be considered such as (1) purification of the labeled protein to avoid contamination with nonlabeled proteins and (2) removal of the directing ligand and purification tag to prevent interference with the labeled moiety during PPIs. Recently, we reported a method that used photoactivable and photocleavable probes for labeling proteins[20] and releasing labeled proteins from solid supports.[18] Encouraged by these results, herein, we combined two photosensitive groups in a molecule to design a ligand-directed labeling probe (LLP) for the fabrication of a lectin probe to study protein−glycoprotein interactions. The LLP consists of five main components (Figure 1): a ligand for directing the probe to the binding site of the protein, a photoactivatable cross-linking group to covalently attach the probe to the protein, a biotin tag to purify the labeled protein, a controlled-release linker to remove the ligand and biotin tag after protein purification, and an alkyne group to act as a clickable handle for installing the desired functional reporter on the bait protein for probing lectin−glycoprotein interactions. RCA_{120}, a plant lectin, was selected as the model protein for the construction of the desired protein probe. RCA_{120} is a heterodimer, and each monomer is composed of an A chain and a B chain. The B chain contains a carbohydrate-binding domain and recognizes the lactose (Lac) ligand (K_d = 5.6 × 10^{-5} M)[21] of the probe. Previously, we successfully demonstrated a galactose-directed photoaffinity probe to label RCA_{120} mainly at the B chain.[20] Thus, during incubation, **LLP 1** is guided to the carbohydrate-binding site of RCA_{120}. Upon photoirradiation, the diazirine group of **LLP 1** is activated by generating a carbene and then forms a covalent bond with lectin at the position near the sugar-binding site (Figure 1a, step 1). In addition, the *E* form of the cinnamoyl group of **LLP 1** is photoisomerized during UV irradiation to yield the *Z* isomer.[22] Then, monomeric avidin magnetic beads (MAMBs) are used to enrich the labeled RCA_{120} via interaction with the biotin tag (Figure 1a, step 2). Acid-assisted lactonization via an intramolecular nucleophilic acyl substitution to form coumarin results in the release of the labeled protein from beads (Figure 1a, step 3). After this process, only an alkyne group remains on the **LLP 1**-labeled RCA_{120}. To demonstrate the advantage of our method for the fabrication of desired protein probes, a fluorescent dye, a photoaffinity labeling (PAL) reagent, and an environment-sensitive dye were, respectively, assembled by Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC)[23] (Figure 1a, step 4). Furthermore, the environment-sensitive probe **6**-RCA_{120} was used for the study of fluorescence turn-on activity with an interacting glycoprotein. In addition, this protein probe was used for rapid washing-free imaging of cell surface glycoproteins of live cells.

EXPERIMENTAL SECTION

Materials. All solvents were dried and distilled by standard techniques. Dichloromethane (DCM), toluene, and acetonitrile (ACN) were distilled from calcium hydride under N_2. Tetrahydrofuran (THF) was distilled from sodium under N_2 prior to use. All chemicals for the synthesis were obtained from Acros Organics, Merck, Fluka, or Sigma-Aldrich and used without further purification unless otherwise noted. Bovine serum albumin (BSA, A9418), *Ricinus communis* agglutinin 120 (RCA120, L7886), ovalbumin (OVA, A5378), and monoclonal antibiotin antibody conjugated with horseradish peroxidase (HRP) (A0185) were purchased from

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Sigma-Aldrich. Protein deglycosylation Mix II (P6044S), which contains PNGase F, *O*-glycosidase, α2-3,6,8,9 neuraminidase A, β1−4 galactosidase S, and β-*N*-acetylhexosaminidase, was purchased from New England Biolabs. Neuraminidase from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque. LysoTracker Green DND-26 was purchased from Thermo Fisher Scientific.

All reactions were carried out in oven-dried glassware (104.0 °C) and performed under anhydrous conditions with N_2 unless indicated otherwise. The reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F_{254} plates (0.25 mm). Detection was accomplished by examination under UV light (254 nm) and by staining with *p*-anisaldehyde, ninhydrin, cerium molybdate, or potassium permanganate staining solution. Silica gel column chromatography was performed using a forced flow of the indicated solvent on silica gel 60 (Merck). C18 reverse-phase silica column chromatography cartridges were purchased from Waters (SepPak Vac C18 cartridge 35 c.c./10 g, 55−105 μm). ^{1}H and ^{13}C NMR spectra were recorded by Bruker AV-400, AV-600, Varian MR-400, or VNMRS-700 NMR spectrometers. Chemical shifts are expressed in ppm using residual $CDCl_3$ (7.24 ppm) and CD_3OD (3.31 ppm) as internal standards in ^{1}H NMR spectra. ^{13}C NMR spectra were recorded in either $CDCl_3$, CD_3OD, or D_2O at 100 MHz using the central resonances of $CDCl_3$ (77.0 ppm) and CD_3OD (49.0 ppm) as the internal references. Multiplicities are reported using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. *J* = coupling constant and its values are expressed in hertz. High-resolution mass spectra (HRMS) were recorded on Varian 901-FTMS. Fluorescence spectra were recorded using a TECAN Infinite M200Pro multimode plate reader. Fluorescence imaging was carried out using a laser scanning confocal microscope (LSM 700, Zeiss, Germany). For the Cy5 channel, images were taken using a 555 nm excitation laser with emission from 590 to 700 nm. For the Hoechst channel, a 405 nm laser with an SP490 emission filter was used. For LysoTracker Green DND-26, images were taken using a 488 nm laser with a BP490−555 emission filter. All of the protein solutions were prepared in phosphate-buffered saline (PBS) buffer (pH 7.4) unless otherwise stated.

Methods. *Synthesis.* All of the synthetic procedures and compound characterizations are described in the Supporting Information.

Photoisomerization of ***LLP 1*** *and Acid-Assisted Coumarin Formation.* **LLP 1** (1.8 mM in CD_3OD) in an NMR tube was irradiated by a UV lamp (365 nm, 18.7 mW/cm^2 at 4 cm) (Blak-Ray B-100AP high-intensity UV lamp) at 4 °C for 10 min. The progress of the reaction was monitored by ^{1}H NMR spectroscopy. When all of the *E*-form **LLP 1** was converted to *Z* form, the solution was further irradiated for 1 h and heated at 33 °C for another 5 h. The resulting solution was analyzed by ^{1}H NMR spectroscopy. In addition, to a solution of *Z*-form **LLP 1** (300 μL CD_3OD) was added AcOH (20 μL). The reaction mixture was incubated at room temperature (rt) for 2 h. The progress of the reaction was monitored by ^{1}H NMR spectroscopy.

Photolabeling of RCA_{120} *by* ***LLP 1.*** To evaluate the imprinting efficiency, 60 μL of solution containing RCA_{120} (final concentration 6 μM) and **LLP 1** (final concentrations 50, 150, 500, 1000, and 1500 μM, respectively) was briefly agitated at 4 °C for 60 min and then irradiated by a UV lamp at 4 °C for 10 min. Unreacted **LLP 1** was removed from the reaction solution via spin concentration (Microcon centrifugal filter 10 000 MWCO, Millipore, MA). An aliquot of this partially purified mixture was treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.3 M Tris−HCl, 10% SDS, 30% glycerol, 9.3% dithiothreitol (DTT), pH 6.8) containing 100 mM DTT under room temperature (rt) for 10 min followed by electrophoresis. Samples were visualized by InstantBlue staining, and the photolabeled protein was verified by Western blot analysis using an HRP-conjugated antibiotin antibody. Negative control experiments were performed by compound **3**.

Purification of ***LLP 1***-RCA_{120} *by MAMB.* An aliquot of the above partially purified mixture (200 μL) was incubated with monomeric avidin−MNP (BcMag monomeric avidin magnetic beads, MAMB) at rt for 60 min with vortexing. The **LLP 1**-RCA_{120}−MAMB complex was isolated by applying a magnet and the resulting complexes were washed with 50 μL of washing buffer (PBS with 0.05% Tween 20, pH 7.4) 3 times. The samples were then treated with SDS-PAGE loading buffer (0.3 M Tris−HCl, 10% SDS, 30% glycerol, 9.3% DTT, pH 6.8) containing 100 mM DTT at rt for 10 min followed by electrophoresis. Samples were visualized by InstantBlue staining and verified by Western blotting. The labeling efficiency was estimated by gel image analysis (UN-SCAN-IT gel 6.1 software).

Preparation of Alkynylated RCA_{120}. The pH of **LLP 1**-RCA_{120}−MAMB complex solution was adjusted to 3 by adding citric acid−Na_2HPO_4 buffer solution, and the resulting solution was incubated at rt for 1 h. The samples were treated with SDS-PAGE loading buffer (0.3 M Tris−HCl, 10% SDS, 30% glycerol, 9.3% DTT, pH 6.8) containing 100 mM DTT under rt for 10 min followed by electrophoresis. Samples were visualized by InstantBlue staining and verified by Western blotting. Similarly, alkynylated Siglec-2-Fc was prepared by following the same protocols as described in the fabrication alkynylated RCA_{120} but using **LLP 2**.

Modification of Alkynylated RCA_{120} *by CuAAC.* To a solution of 270 μL of 3.1 μM alkynylated RCA_{120} was added 10 μL of 60 mM $CuSO_4$, 50 μL of 60 mM THPTA, 20 μL of 200 mM sodium ascorbate, and 1.0 μL of 50 mM probe **4**. The reaction mixture was incubated at 4 °C for 12 h. The desired **4**-RCA_{120} was purified by a PD Minitrap G-25 column by following the protocol suggested by the manufacturer. Similarly, **5**-RCA_{120}, **6**-RCA_{120}, and **6**-Siglec-2-Fc were prepared by following the same reaction conditions.

Photo-Cross-Linking of ***5***-RCA_{120} *with OVA.* **5**-RCA_{120} (50 μL, 5.0 μM) was incubated with 2 μL of 12.5 mM ovalbumin (OVA). The mixture was briefly agitated at 4 °C for 60 min and then irradiated by a UV lamp (365 nm, 18.7 mW/cm^2 at 4 cm) at 4 °C for 30 min. The mixture was treated with SDS-PAGE loading buffer (0.3 M Tris−HCl, 10% SDS, 30% glycerol, 9.3% DTT, pH 6.8) containing 100 mM DTT for 10 min under rt followed by electrophoresis. Samples were visualized by InstantBlue staining and **5**-RCA_{120}−OVA, cross-linking was verified by Western blot analysis using an HRP-conjugated antibiotin antibody. The negative control experiment was performed at the aforementioned conditions but using BSA.

Interaction between ***6***-RCA_{120} *and OVA.* To a 195 μL of 0.1 μM **6**-RCA_{120} was added 5 μL of 500 μM OVA with (or without) pretreating with 20 μL of protein deglycosylation Mix II (from NEB) at rt for 60 min. The fluorescence emission spectrum of the solution was recorded from 590 to 750 nm with an excitation wavelength of 570 nm by a TECAN Infinite M200Pro multimode plate reader. In the competition experiments, **6**-RCA_{120} was incubated with OVA at rt for 60 min followed by adding 28.5 μL of 1 M lactose and then incubating at rt for 6 h or adding 20 μL of protein deglycosylation Mix II and then incubating at 37 °C for 1.5 h.

Random Modification of RCA_{120} *by Compound* ***7.*** To a solution of merocyanine acid (40.0 mg, 0.09 mmol), *N*-hydroxysuccinimide (10.1 mg, 0.09 mmol), and *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (16.8 mg, 0.09 mmol) in dry DCM (1.8 mL) was stirred at rt under a nitrogen atmosphere for 10 h. The solvent was removed under reduced pressure to give crude **7**, which was dissolved in DMSO as a 50 mM stock solution. HRMS (ESI-TOF, *m*/*z*) calculated for $C_{33}H_{33}N_2O_6$ $[M + H]^+$ 553.2339, found: 553.2338. To a 200 μL of 2 μM RCA_{120} was added 0.8 μL of 50 mM compound **7**, and the resulting mixture was incubated at rt for 12 h. The dye conjugated protein (7-RCA_{120}) was purified by a PD Minitrap G-25 column. 7-RCA_{120} (175 μL, 0.5 μM) was incubated with 25 μL of 500 μM OVA or BSA at rt for 2 h. The fluorescence emission spectrum of the solution was recorded from 590 to 750 nm with an excitation wavelength of 570 nm by a TECAN Infinite M200Pro multimode plate reader.

Microarray Analysis of Binding Affinities with RCA_{120} *and Modified* RCA_{120}. The glycan microarray was fabricated by Cu(I)-catalyzed alkyne−azide cycloaddition as described previously.[39] The glycan slide was incubated with an RCA_{120} (5 μg/mL) solution (0.1% BSA in PBST) at 25 °C. After incubation for 1 h, the glycan slide was washed with PBST 3 times. After that, it was incubated with the anti-

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RAC antibody (EY Lab, AL-2003-1) (2 μg/mL) solution (0.1% BSA in PBST) at 25 °C for 1 h followed by washing with PBST 3 times and then staining with the antirabbit IgG-Cy3 (Sigma, C2306, 1/200) solution (0.1% BSA in PBST) at 25 °C. After being stained for 1 h, the glycan slide was washed with PBST 3 times to visualize the binding signal. The fluorescence intensity was measured using VIDAR Revolution 4550 with an excitation wavelength of 532 nm, and image analysis was performed using ArrayEase software. The average over the printed replicates was calculated using Array Vision version 8.0 (Imaging Research, Inc.).

Cell Culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

*Interaction of **6**-RCA$_{120}$ with Glycoproteins on Cell Surfaces of HeLa Cells.* About 1×10^3 cells were maintained in a culture medium supplemented with 10% fetal bovine serum and seeded in 8-well chamber slides. The cells were cultured at 37 °C in air with 5% CO_2 for 2 days. Cells were treated with 20 μL of 400 nM **6**-RCA$_{120}$, and the resulting mixture was incubated at 37 °C under air with 5% CO_2 for 90 min followed by the acquisition of live-cell images without any washing steps. Moreover, cells were treated with 10 μL of protein deglycosylation Mix II (NEB) or 0.01 U of neuraminidase from *A. ureafaciens* [EC 3.2.1.18] (Nacalai Tesque) under cell culture conditions at 37 °C for 2 h. Then, 20 μL of 400 nM **6**-RCA$_{120}$ was added and the resulting mixtures were incubated for 90 min under the same conditions. Without removal of the excess probe, cell images were taken by a laser scanning confocal microscope (LSM 700, Zeiss, Germany). The images were taken using an excitation wavelength of 555 nm and emission wavelength from 590 to 750 nm.

*Dynamic Observation of **6**-RCA$_{120}$ Interacting with Glycoproteins on Cell Surfaces of HeLa Cells.* About 1×10^3 cells were seeded in 8-well chamber slides and cultured at 37 °C in air with 5% CO_2 for 2 days. Cells were treated with 10 μL of protein deglycosylation Mix II (NEB) and then incubated under above cell culture conditions for 1.5 h. Then, the medium was replaced with the freshly prepared growth medium 200 μL containing 20 μL of 400 nM **6**-RCA$_{120}$ and 0.01 U neuraminidase. Cell images were taken, without removal of the excess probe, using a laser scanning confocal microscope for 40 min (Video S1). Then, the medium containing excess probe was replaced by the freshly prepared medium (without **6**-RCA$_{120}$ and neuraminidase) and images were taken for an additional 30 min (Video S2). In addition, **4**-RCA$_{120}$ was used as the probe for control experiments to dynamically observe probe−glycoprotein interactions on the cell surface, as shown in Video S3.

*Investigation of Endocytosis of **6**-RCA$_{120}$.* About 1.5×10^3 cells were seeded in 8-well chamber slides and cultured at 37 °C in air with 5% CO_2 for 15 h. Then, cells were treated with 0.01 U of neuraminidase and 0.5 μL of 20 mM LysoTracker Green DND-26 under cell culture conditions. After incubation for 2 h, cells were cooled down to 4 °C for 10 min and then 20 μL of 400 nM **6**-RCA$_{120}$ was added. The resulting mixture was incubated at 4 °C for 10 min and then at 25 °C for 30 min. Cell images were taken without removal of the excess probe using a laser scanning confocal microscope.

RESULTS AND DISCUSSION

Our LLP strategy for fabricating a lectin probe involves protein photoaffinity labeling (PAL), followed by postmodification. The fabrication starts with the chemical synthesis of **LLP 1** and **2** (Figure 1b). **LLP 1** was synthesized by coupling the building blocks (see Schemes S1−S8), mainly by amide bond formation. An *O*-hydroxycinnamoyl group[24] was introduced as a controlled cleavable linker by photoisomerization and acid-assisted cyclization to remove the redundant moieties (ligand and purification tag) of LLP after labeling.

To fabricate a lectin probe, RCA$_{120}$ was first incubated respectively with various concentrations of **LLP 1** in PBS (pH 7.4) at 4 °C for 60 min followed by UV irradiation (λ_{max} = 365 nm) for 10 min. The biotin purification tag on the protein was employed for the detection and purification (by MAMB) of a covalently cross-linked RCA$_{120}$ adduct (**LLP 1**-RCA$_{120}$) and measurement of labeling efficiency. Thus, after the removal of unreacted **LLP 1**, the protein solutions were resolved on SDS-PAGE gel and the photolabeled protein was verified by Western blot analysis using an HRP-conjugated antibiotin antibody (Supporting Information Figure S1). A saturated biotin signal was noticed when **LLP 1** was used at 1000 μM (concentration ratio, protein/**LLP 1** = 6:1000) (Supporting Information Figure S1, lane 4). The labeling specificity of **LLP 1** was further confirmed by control probe **3** (lacking the carbohydrate ligand) under the same labeling conditions, resulting in no detectable biotin signal (Supporting Information Figure S1, lane 8). Notably, the faint band between A and B chains is attributed to an isomer/impurity, which is characteristic of the variety of the lectin.[25] After MAMB purification and gel image analysis, approximately 34% of protein was labeled by **LLP 1** (Supporting Information Figure S2). The labeling sites were identified by LC−MS/MS analysis of trypsin-digested **LLP 1**-RCA$_{120}$ to show that residues Thr239 on the A chain and Tyr478 on the B chain of RCA$_{120}$,[25] respectively, were selectively labeled by **LLP 1** (Supporting Information Figure S3). Due to the presence of potential galactose binding sites on the surface of RCA$_{120}$,[26] the different labeled sites may result in the **LLP 1** binding to different sites of RCA$_{120}$. It should be noted how many probes were labeled on RCA$_{120}$ was not determined. Moreover, to investigate whether thiol is a potential nucleophile to react with **LLP 1**, free cysteine was incubated with **LLP 1** under the same protein labeling conditions (molar ratio, cysteine/**LLP 1** = 1:4) and no Michael addition adduct was observed in electrospray ionization (ESI) mass analysis, indicating that the thiol group of the protein is not a nucleophile to trap **LLP 1**.

Before further modification of **LLP 1**-RCA$_{120}$, ^{1}H NMR spectroscopy was utilized to monitor the photoisomerization of *E* to *Z* and the following cyclization process of **LLP 1** (Figure 2). **LLP 1** (1.8 mM in CD_3OD) was UV-irradiated (λ_{max} = 365 nm) at 4 °C for 10 min. The photoisomerization was very efficient and all of the E-form **LLP 1** was converted to the *Z* form. As evident from the ^{1}H NMR spectrum, the characteristic doublet signals of the transolefinic protons at δ_H = 7.78

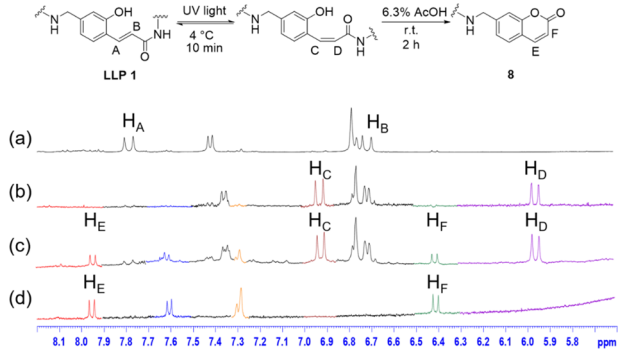

Figure 2. Monitoring of *E*−*Z* photoisomerization of the *O*-hydroxycinnamide unit in **LLP 1** (1.8 mM in CD_3OD) and photochemical cyclization to the coumarin derivative by ^{1}H NMR spectroscopy. Only protons of unsaturated carbons are shown. (a) Without UV irradiation, (b) UV irradiation for 10 min, (c) UV irradiation for 10 min followed by sealing and incubation at 33 °C for 5 h, and (d) UV irradiation for 10 min followed by addition of 6.3% AcOH and incubation for 2 h.

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ppm (H_A) and 6.71 ppm (H_B) with a coupling constant (J) of 15.8 Hz (Figure 2a) disappeared and shifted upfield to δ_H = 6.92 ppm (H_C) and 5.95 ppm (H_D), respectively, with J = 12.6 Hz (Figure 2b). In conjugation with previously PAL results, it should be noted that during the PAL process the diazirine forms carbene to cross-link the protein, while the *E* form of the cinnamoyl linker was isomerized to the *Z* form. Unlike previous studies,[23] prolonging irradiation (up to 1 h) with/without heating (33 °C for 5 h) resulted in only a little cyclization (formation of coumarin), as indicated by the appearance of two small new peaks centered at δ_H 7.95 and 6.41 ppm with J = 9.6 Hz, corresponding to H_E and H_F of coumarin derivative **8** (Figure 2c). After many attempts, we found that the cyclization rate could be enhanced by the addition of acetic acid. Thus, after UV irradiation for 10 min, AcOH was added to the solution (V_{CD_3OD}/V_{AcOH} =15:1) and the reaction mixture was incubated at room temperature for 2 h. **LLP 1** could be easily converted to **8** as evidenced by ESI-MS analysis ($[M + Na]^+$: m/z = 962.3674) and ^1H NMR (Figure 2d). It should be noted that the neutral pH and a little increase of solution acidity do not promote cyclization efficiency. Then, the above-optimized conditions were applied to fabricate alkynylated RCA_{120}. Thus, MAMBs were used to capture **LLP 1**-RCA_{120} and the resulting particle complexes were incubated in buffer solution at pH 3 or pH 4 to initiate the cyclization−release process. As shown in Figure 3a, the

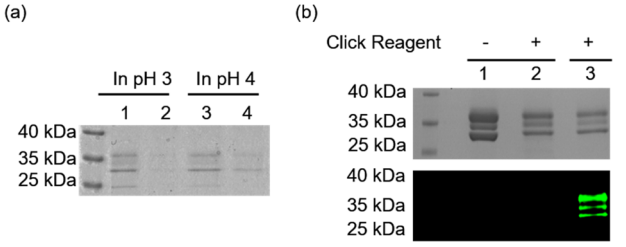

Figure 3. Release of labeled RCA_{120} from MAMB and modification of alkynylated RCA_{120} with Cy3 **4**. (a) **LLP 1**-RCA_{120} particle complexes were incubated in buffer solutions at pH 3 or 4 for 1 h. Proteins were visualized by InstantBlue staining. Lanes 1 and 3: supernatant; lanes 2 and 4: beads. (b) Fabrication of a fluorescent protein probe by modifying the alkynylated RCA_{120} with azido-Cy3 **4** by CuAAC, InstantBlue stain (top) and in-gel fluorescence image (bottom). Lane 1: native RCA_{120} only. Lane 2: native RCA_{120} with click reagents. Lane 3: CuAAC of alkynylated RCA_{120} with azido-Cy3 **4**.

cyclization efficiency was almost quantitative at pH 3 buffer solution to release **LLP 1**-RCA_{120} from MAMBs. Moreover, the coelution of intrinsically biotinylated proteins can be avoided by this cleavable *O*-hydroxycinnamate linker. This process essentially leaves labeled RCA_{120} equipped only with an alkyne functional group, which can be utilized for further modification to yield the desired protein probe.

Protein photo-cross-linking[27] is an appealing technique for studying PPIs, particularly for weak or transient interactions that are usually difficult to detect with traditional methods. To demonstrate the advantage of our method, we fabricated a photoaffinity labeling protein probe by CuAAC of alkynylated RCA_{120} with a heterotrifunctional cross-linker **5** for cross-linking protein interaction. Successful incorporation of **5** onto RCA_{120} (denoted **5**-RCA_{120}) was indicated by the strong biotin signal on the labeled protein, as evidenced by Western blot analysis (Figure 4b, lane 2). This protein probe was then used

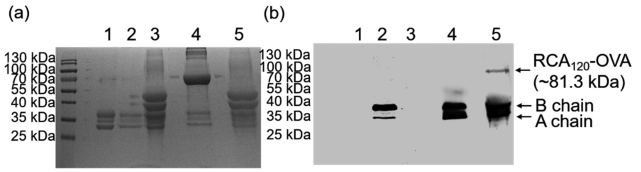

Figure 4. **5**-RCA_{120} and OVA were cross-linked via PAL. (a) Total protein staining with InstantBlue and (b) Western blot analysis with an antibiotin antibody. Lane 1: native RCA_{120}. Lane 2: CuAAC of alkynylated RCA_{120} with **5**. UV irradiation of Lane 3, native RCA_{120} with OVA; lane 4, **5**-RCA_{120} with BSA; and lane 5, **5**-RCA_{120} with OVA.

to cross-link an interacting glycoprotein, ovalbumin (OVA), by the PAL strategy. OVA (~43 kDa) contains neutral glycans, including terminal LacNAc (Galβ(1→4)GlcNAc) residues, as one of the major glycoforms.[28] Thus, OVA can be labeled via interactions with the carbohydrate recognition domain of RCA_{120}. Incubation of **5**-RCA_{120} with OVA followed by UV irradiation (λ_{max} = 365 nm) at 4 °C for 30 min resulted in a biotin signal at ~81.3 kDa, corresponding to the RCA_{120}−OVA conjugate (Figure 4b, lane 5), which demonstrated successful labeling of the interacting protein using the photoaffinity protein probe. By contrast, bovine serum albumin (BSA, ~66 kDa) was included as a nonglycosylated control protein to determine the specificity of the lectin−glycoprotein cross-linking process. No band of the corresponding RCA_{120}−BSA conjugate was observed by SDS-PAGE or Western blot analysis (Figure 4, lane 4). Furthermore, UV irradiation of the mixture containing native RCA_{120} and OVA (Figure 4b, lane 3) resulted in no additional band observed. These results confirm that photo-cross-linking is specifically driven by glycan-mediated interactions between glycoproteins and RCA_{120} and support that the alkynylated protein is a convenient precursor for the fabrication of protein probes for tackling PPIs. However, the low efficiency of cross-linking may be due to the steric hindrance of redundant biotin on **5**-RCA_{120}.

The use of environment-sensitive fluorescent probes has become a popular technique for selective detection and imaging of interacting proteins in vitro and in vivo.[29−31] The environment-sensitive fluorescent signatures are often weak in polar and aqueous buffer solutions but when positioned in close proximity to a hydrophobic environment of proteins or membranes could alter to show strong emission.[31] The unique solvatochromic off−on feature has been applied for monitoring protein conformation dynamics and protein interactions.[30−32] Despite their prevalent applications, there were no protein probes that were developed to selectively detect lectin−glycoprotein interactions and to visualize interacting glycoproteins in living cells. Protein glycosylation is abundant and one of the most critical post-translational modifications, and these modifications have been implicated in the control of the stability and function of a wide range of proteins.[33] Therefore, environment-sensitive lectin probes that can be used to selectively profile endogenous glycoproteins via glycan interactions are undoubtedly important for monitoring cell surface protein−glycoprotein interactions. For this purpose, among various environment-sensitive small-molecule-based chemical probes, merocyanine dyes[34] are especially well suited as chromophores for live-cell imaging and thus selected for conjugation with RCA_{120}.

Herein, we produced an environment-sensitive meroxzcyanine-linked RCA_{120} probe (**6**-RCA_{120}) in which probe **6** was

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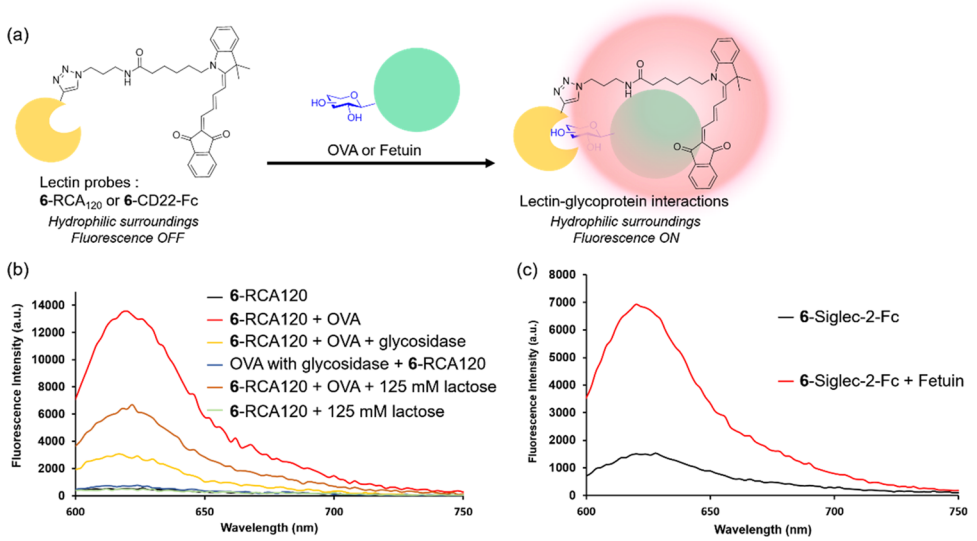

Figure 5. Environment-sensitive lectin probe for lectin−glycoprotein interaction. (a) Schematic diagram of PPI between a lectin probe and a glycoprotein. (b) Fluorescence spectra of **6**-RCA_{120} and **6**-RCA_{120} with OVA under different conditions. (c) Fluorescence spectra of **6**-Siglec-2-Fc and **6**-Siglec-2-Fc with fetuin.

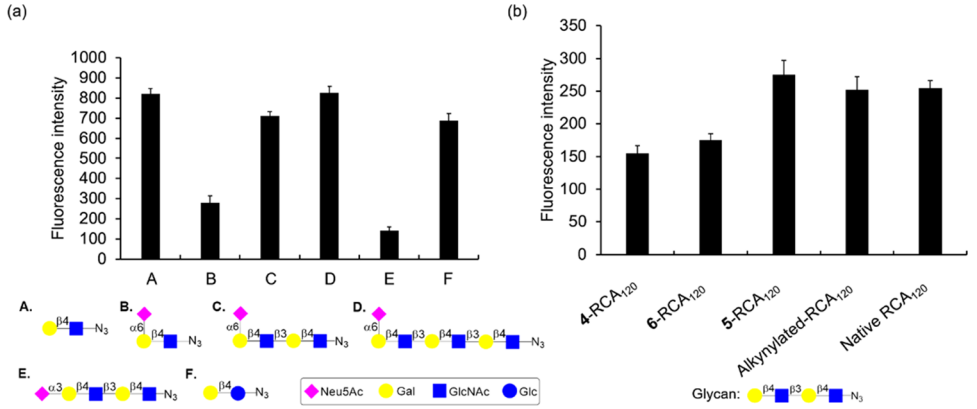

Figure 6. Binding affinities of RCA_{120} and modified RCA_{120} were analyzed by microarray analysis. (a) Relative binding affinities of potential glycan ligands toward RCA_{120}. (b) Relative binding affinities of RCA_{120} and different modified RCA_{120} proteins toward di-LacNAc.

covalently attached at a site proximal to the carbohydrate-binding pocket of RCA_{120} by a CuAAC reaction. We reasoned that because hydrophobic interactions dominate many protein−ligand interactions,[35] once the glycoprotein was bound to RCA_{120}, a net change of the hydrophobic surroundings would occur, resulting in detection of the carbohydrate−protein interaction (Figure 5a). As shown in Figure 5b, **6**-RCA_{120} exhibited weak emission, suggesting that the fluorescent probe involved little or no interaction with RCA_{120}. Upon incubation of **6**-RCA_{120} with OVA, specific binding between the glycan moiety on the OVA surface with RCA_{120} occurred, resulting in a marked 30-fold enhancement of original intensity in the presence of a high concentration of Lac (125 mM), demonstrating the regulation of the interaction by Lac and a preference for the glycan component of the glycoprotein over free Lac. Notably, RCA_{120} can bind both Lac (K_d = 56 μM)[36] and LacNAc (K_d = 4.1 μM),[37] with a higher affinity for the latter. In addition, natural LacNAc residues on the N-linked glycans of the glycoproteins provide an opportunity for multivalent interaction with RCA_{120}, making it difficult for mono-Lac to completely dissociate RCA_{120}−OVA interaction. However, incubation of **6**-RCA_{120} with deglycosylated OVA, which was obtained by treatment with deglycosylation enzymes prior to incubation, resulted in the absence of emission. In contrast, mixing of **6**-RCA_{120} with OVA followed by incubation with deglycosylation enzymes for 1.5 h significantly abolished the fluorescence signal to 0.2-fold of original intensity. Moreover, a mixture of **6**-RCA_{120} and nonglycosylated BSA only exhibited weak fluorescence emission (Supporting Information Figure S4). Taken together, these results demonstrate that fluorescence activation occurs specifically due to glycan-mediated interaction, indicating that the **6**-RCA_{120} protein probe can be harnessed as a sensitive probe to directly detect endogenous glycoprotein binding events on living cells (see below).

To prove the necessity of the site-selective modification described above, RCA_{120} was randomly modified by the NHS-activated compound **7** (Figure 1b) via amide bond formation with the amines of Lys residues to yield 7-RCA_{120}. Surprisingly, upon incubation of 7-RCA_{120} with OVA and BSA, the results were different from those of previous experiments. It was difficult to observe fluorescence emission corresponding to the formation of the 7-RCA_{120}−OVA complex, but the fluorescence signal for the 7-RCA_{120}−BSA complex was strong,

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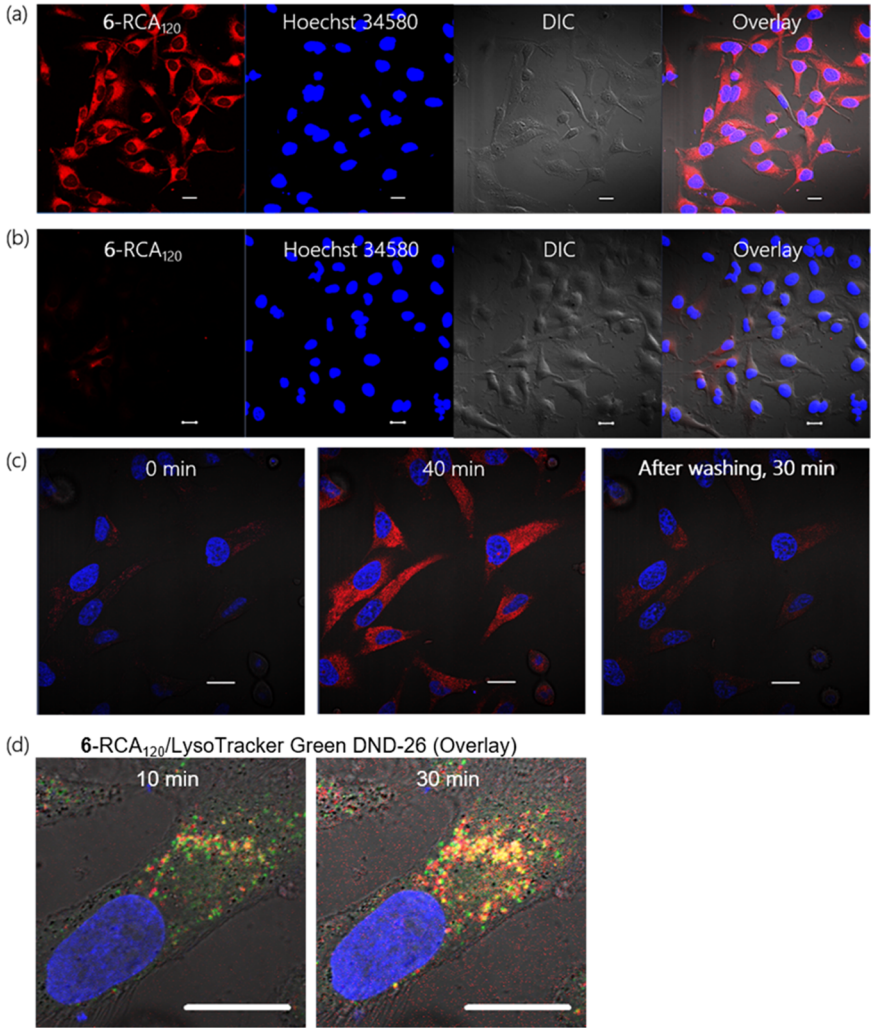

Figure 7. Confocal images of modified RCA_{120} uptake by cell surface glycoprotein interaction-mediated endocytosis. HeLa cells were (a) pretreated with neuraminidase followed by incubation of **6**-RCA_{120} and (b) pretreated with deglycosylation enzymes and then incubated with **6**-RCA_{120}. The cell nuclei were stained by Hoechst 34580. (c) Overlay of the images of cell nuclei and **6**-RCA_{120}. (d) Overlay of the images showing yellow fluorescence, indicating that **6**-RCA_{120} colocalized with LysoTracker Green DND-26 in late endosomes and/or lysosomes. Scale bar, 20 μm.

indicating the presence of nonspecific binding (Supporting Information Figure S5). The results clearly demonstrate that the environment-sensitive molecule should be assembled at the site close to the binding pocket to efficiently sense changes in environmental polarity. To show the generality of using the LLP strategy for the fabrication of a protein probe, the developed method was applied to produce **6**-Sigelec-2-Fc by **LLP 2**, Siglec-2-Fc fusion protein, and probe **6**. Siglec-2 is a sialic acid-binding immunoglobulin (Ig)-like lectin and recognizes α-2,6-sialyl glycans.[38] Similar to **6**-RCA_{120}, **6**-Sigelec-2-Fc only shows weak emission, as shown in Figure 5C, indicating that the length between the dye and the protein may need to be further adjusted to suppress the background emission. However, the addition of fetuin (a glycoprotein that contains sialyl glycans)[39] to interact with **6**-Sigelec-2-Fc results in the enhancement of fluorescent emission, demonstrating the success of fabricating the environment-sensitive protein probe.

We then explored the application of probe **6**-RCA_{120} for imaging endogenous glycoproteins on living cells for the investigation of RCA_{120}−endogenous cell surface glycoprotein interactions by the fluorescence turn-on **6**-RCA_{120} probe. Because both N- and O-linked sialic acid (NeuAc)-containing glycoproteins were abundantly displayed on the HeLa (human cervical cancer) cell surface,[40] a glycan microarray[41] was first used to evaluate the relative binding affinities between native RCA_{120} and various glycans, as shown in Figures 6a and S6. RCA_{120} exhibited similar affinities to Lac (**A**) and LacNAc (**F**), whereas $\alpha(2\rightarrow6)$-sialylated LacNAc (**B**) showed a significantly decreased binding affinity, indicating that the presence of sialic acid at the nonreducing end blocks the interaction. However, extended LacNAc chains could recover the binding affinity, indicating that RCA_{120} can recognize internal LacNAc units (**C** and **D** vs **B**). Interestingly, $\alpha(2\rightarrow3)$ sialylation of the nonreducing end (**E**) had a stronger blocking effect on lectin binding than $\alpha(2\rightarrow6)$ sialylation (**C** and **D**). The effect of modification on the RCA_{120} binding affinity was then investigated using di-LacNAc as a ligand, as shown in Figure 6b. Alkynylated RCA_{120} and **5**-RCA_{120} exhibited similar affinities to those of native RCA_{120}, indicating that such modifications do not affect the original binding activity. However, when modified with a hydrophobic dye (**4**-RCA_{120} and **6**-RCA_{120}), the modified proteins exhibited decreased binding activity toward di-LacNAc. Nevertheless, these lectin

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probes still show affinities to the interacting glycans and thus can be used to tackle lectin−glycoprotein interactions.

To perform live-cell imaging of the interactions between our protein probe and cell surface glycans,[42] HeLa cells were incubated with **6**-RCA_{120} at 37 °C under 5% CO_2 for 1.5 h, followed by the acquisition of live-cell images by confocal microscopy with excitation at 550 nm and emission at wavelengths greater than 620 nm without any washing steps. However, due to the presence of NeuAcs at nonreducing ends of glycans on the surface glycoproteins, related weak fluorescence was observed (Supporting Information Figure S7). In contrast, significant enhancement of red fluorescence was observed with cells pretreated with neuraminidase, as shown in Figure 7a. Neuraminidase treatment removes NeuAcs and exposes inner galactoses that interact with **6**-RAC_{120}. Furthermore, due to the cytoadhesive and cytoinvasive properties of lectins,[43,44] instant cellular uptake and internalization of **6**-RCA_{120} resulted in increased intracellular fluorescence. The specific lectin−glycan interaction that led to fluorescence activation was further confirmed by incubation of **6**-RCA_{120} with cells pretreated with deglycosylation enzymes. Due to the lack of available glycan ligands on cell surfaces, very less fluorescence was observed (Figure 7b). Importantly, all of the fluorescence images were obtained without any conventional washing step after incubation because the fluorescence of **6**-RCA_{120} is activated only when the protein probe interacts with glycan ligands. This property of the environment-sensitive lectin probes strongly validates its utility for real-time detection of lectin−glycoprotein binding events on living cells, which is impossible using traditional probes that require washing procedures or fixing cells.

Dynamic observation of the interactions between **6**-RCA_{120} and cell surface glycoproteins was performed by incubating cells (pretreated with deglycosylation enzymes) with **6**-RCA_{120} in the presence of neuraminidase. Due to the regeneration of cell surface glycans, the fluorescence intensity gradually increased, reaching a maximum value after 40 min (Figure 7c-2 and Supporting Information Movie S1). After the removal of probes in the culture medium, the red fluorescence (**6**-RCA_{120}) intensity was continuously monitored. As the incubation time increased, a significant decrease in the fluorescence intensity after 30 min was observed, most likely due to the cellular metabolism of these proteins that decomposed the interaction (Figure 7c-3 and Supporting Information Movie S2). In contrast to the results obtained by **6**-RCA_{120}, the use of **4**-RCA_{120} did not lead to an obvious change in the fluorescence intensity under the above conditions but exhibited a high background signal (Supporting Information Movie S3). Traditional dyes show emission upon excitation. Thus, the washing step to remove unbound dyes is required. Our results demonstrate the advantage of using an environment-sensitive protein probe for real-time tracing of dynamic changes in cell surface glycoproteins. To confirm that the probe-modified RCA_{120} was recruited by endocytosis after glycoprotein binding, cells were cooled to 4 °C for 10 min and then treated with **6**-RCA_{120}. Because glycoprotein receptors rapidly enter endocytic vesicles[45,46] and are recycled back to the cell surface, **6**-RCA_{120} was not located on the cell membrane under low-temperature incubation conditions. To further identify the destination of **6**-RCA_{120} after endocytosis, LysoTracker Green DND-26 was used to label late endosomes and lysosomes. After treating cells with **6**-RCA_{120} in the presence of neuraminidase for 10 or 30 min, confocal microscopy images showed an increase in yellow fluorescence, indicating the colocalization of red (**6**-RCA_{120}) and green fluorescence (LysoTracker Green DND-26) (Figures 7d and S8). Notably, within 30 min of incubation, some of the red complex escaped from the endosomes and lysosomes. It should be noted that the merocyanine dye (acid form of **6**) is not sensitive to the pH change (Figure S9). Thus, fluorescence emission from endosomes and lysosomes indicates the remained protein complex.

CONCLUSIONS

In this work, we have developed a feasible method for site-selective chemical modification of RCA_{120} and Siglec-2-Fc by using our LLPs. The small molecules, **LLP 1** and **2**, are composed of a ligand, two photosensitive groups, a biotin tag, and an alkyne group, which were assembled via continuous amide bond formation chemistry. The developed labeling strategy can be used for various lectins because the LLP can be guided to the proximal carbohydrate-binding site using an appropriate ligand. Due to the introduction of a biotin tag for purification, which can be easily removed, purified labeled protein probes could be obtained. Finally, the presence of an alkyne group at the proximal ligand-binding site of the labeled protein allowed easy derivatization for the introduction of a desired functional group by the bioorthogonal click reaction. As a proof of concept, we successfully fabricated a photo-labeling protein probe for cross-linking an interacting protein and an environment-sensitive protein probe for real-time live-cell imaging of glycoproteins without washing step.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.9b01495.

Synthetic procedures, characterization, and NMR spectra of LLPs; labeled protein purification, conditions for cell culture and binding assays; and images of glycan microarray (PDF)

Cell images taken without removal of the excess probe, using a laser scanning confocal microscope for 40 min (AVI)

Images taken for an additional 30 min with freshly prepared medium (without **6**-RCA_{120} and neuraminidase) (AVI)

Observation of probe−glycoprotein interactions on the cell surface using **4**-RCA_{120} (AVI)

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Author Contributions

P.-J.L., T.-C.C., and C.-C.L. provided the conception and designed experiments. P.-J.L., M.T.A., S.K.K., H.-J.C., and K.-T.T. performed chemical synthesis and in vitro biochemical experiments. P.-J.L. and D.S.J. carried out cell-based experiments. C.-Y.F. fabricated the glycan microarray and analyzed binding results. H.-Y.L. and Y.-J.C. identified the labeled sites of protein by MS analysis. The manuscript was written by P.-J.L. and C.-C.L., and edited by all the co-authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PPI, protein−protein interaction; PAL, photoaffinity labeling; LLP, ligand-directed labeling probe; RCA_{120}, *Ricinus communis* agglutinin 120; Siglec, sialic acid-binding Ig-like lectin; OVA, ovalbumin; BSA, bovine serum albumin; NHS, *N*-hydroxysuccinimide

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