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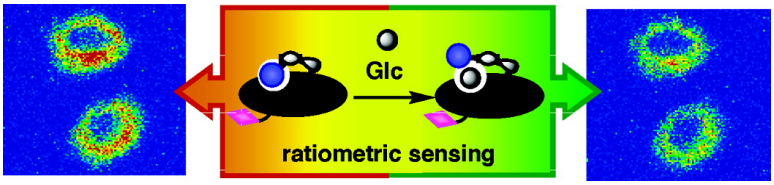

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Double-Modification of Lectin Using Two Distinct Chemistries for Fluorescent Ratiometric Sensing and Imaging Saccharides in Test Tube or in Cell

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Abstract: The site-selective incorporation of two different fluorophores into a naturally occurring protein (lectin, a sugar-binding protein) has been successfully carried out using two distinct orthogonal chemical methods. By post-photoaffinity labeling modification, Con A, a glucose- and mannose-selective lectin, was modified with fluorescein in the proximity of the sugar binding site (Tyr100 site), and the controlled acylation reaction provided the site-selective attachment of coumarin at Lys114. In this doubly modified Con A, the fluorescein emission changed upon the binding to the corresponding sugars, such as the glucose or mannose derivatives, whereas the coumarin emission was constant. Thus, the doubly modified Con A fluorescently sensed the glucose- and mannose-rich saccharides in a ratiometric manner while retaining the natural binding selectivity and affinity, regardless of the double modification. On the benefit of the ratiometric fluorescent analysis using two distinct probes, the sugar trimming process of a glycoprotein can be precisely monitored by the engineered Con A. Furthermore, the doubly modified Con A can be used not only for the convenient fluorescent imaging of saccharides localized on a cell surface, such as the MCF-7, a breast cancer cell having rich high-mannose branch, but also for the ratiometric fluorescent sensing of the glucose concentration inside HepG2 cells. These results demonstrated that the semisynthetic lectin modified doubly by two distinct chemistries is superior to the singly modified one in function, and thus, it may be potentially useful in cell, as well as in test tube.

Introduction

A recent advance in proteins engineering has provided us with several new methodologies to incorporate unnatural molecules, including unnatural amino acids into a native protein scaffold with a site-specific manner.[1] In combination with these orthogonal methods, natural proteins can be modified with two distinct unnatural functionalities.[2] It is naturally anticipated that the introduction of two or more unnatural molecules is capable of conferring a more sophisticated function upon a native protein, although an experimental demonstration has not yet been sufficiently achieved. Although elegant examples using the single modification have already been reported in protein functionalization,[3] the site-selective incorporation of two or more synthetic molecules into proteins is very limited.[4] Furthermore, the introduction of new functions to native proteins by the double modification with artificial molecules has been rarely reported.[4e,f]

Among the unnatural molecules, artificial fluorophores are some of the promising candidates to enrich the structure and function of the original native proteins, especially as useful biosensors. In molecular biology, many proteins were fused with GFP (green fluorescent protein), a representative protein-based fluorophore, and its derivatives for various objectives. In particular, two protein-based fluorophores, such as GFP or YFP, were conjugated with a natural protein scaffold, successfully demonstrating that a FRET (fluorescent resonance energy transfer)-type of communication between two fluorescent protein modules is quite useful for sensing and imaging targeted biological events.[5] This GFP-based technology gave us an

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important clue for protein functionalization by artificial molecules.[6] In practice, it has been pointed out that the attachment of large proteins induces protein denaturation in some cases since such protein-based fluorophores usually have a molecular weight higher than 20 000 per one protein.[1a] Thus, the incorporation of small molecular fluorophores (M_w are less than 500) into natural proteins is considered to be a desirable and unique strategy to minimize incorporation-induced protein perturbation. As a pioneering approach along this direction, Hahn and coworkers reported that one GFP unit can be replaced with a synthetic fluorophore to produce a GFP-fused protein bearing the fluorophore as a powerful bioimaging tool.[7]

During our effort in chemistry-based protein engineering, we developed a method for the active-site directed modification (so-called P-PALM: post-photoaffinity labeling modification) of native proteins and initially applied this method to a sugar-binding protein (lectin).[8] Using P-PALM, we recently attached a fluorophore or an artificial receptor unit to the proximity of a sugar-binding site of Concanavalin A (Con A), an α-linked glucose or mannose binding protein,[9] so that the modified Con A was converted to a fluorescent saccharide biosensor.[10] We describe herein that a controlled acylation reaction is combined with P-PALM in order to carry out the site-selective incorporation of two distinct fluorophores into Con A. The doubly modified Con A can selectively sense sugar derivatives including glucose- or mannose-rich oligosaccharides and glycoproteins in a ratiometric fluorescence manner. More significantly, the engineered Con A can clearly visualize not only glycoproteins and/or -lipids located on a cell surface but also the glucose concentration inside a cell by the benefit of ratiometry.

Results and Discussion

A Strategy for Site-Specific Incorporation of Two Distinct Fluorophores into Con A. In addition to P-PALM, we employed another reaction for tethering two distinct fluorophores to Con A, that is, the acylation of the ϵ-amino group of Lys. It is reported that Con A bears a loose binding site for hydrophobic substances distinct from the sugar-binding pocket.[11] Thus, it may be reasonably expected that an appropriately hydrophobic fluorophore is bound there so that it reacts at the vicinal site of the hydrophobic domain. To test this hypothesis, a coumarin-appended active ester (AMCA-SE; Figure 1a) was used to react

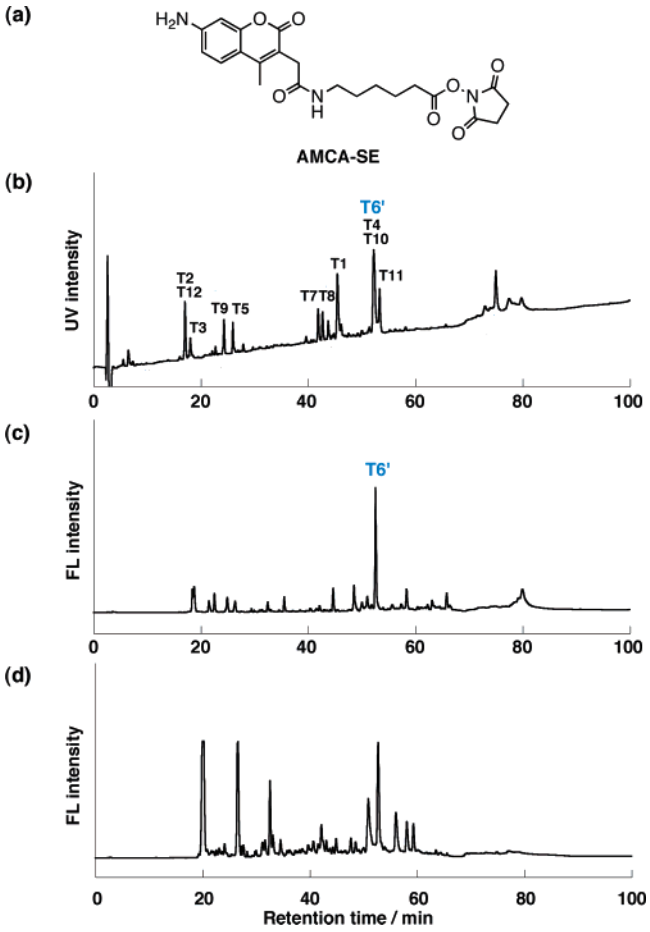

Figure 1. (a) Molecular structure of AMCA−SE. (b and c) Reverse-phase HPLC chart of AMCA−Con A (prepared under the acidic conditions (pH 5.0)) after trypsin digestion detected by (b) UV mode (220 nm) and (c) fluorescence mode (350 nm for excitation, 450 nm for emission). (d) Reverse-phase HPLC chart of AMCA−Con A (prepared under the basic conditions (pH 8.5)) after trypsin digestion detected by fluorescence mode (350 nm for excitation, 450 nm for emission).

with the native Con A, and the site selectivity was examined by the conventional peptide-mapping technique using trypsin digestion. The HPLC analysis of the digested mixture clearly showed that the almost single peak was monitored by a fluorescence mode detector for the reaction under acidic pH conditions (pH 5.0) (as shown in Figure 1c). Subsequently, the fluorescent peptide fragment was collected and assigned to the peptide 102−116 (T6′) based on MALDI-TOF MS. The T6′ peptide was further analyzed by the tandem mass−mass technique. As shown in Figure S-1b, each difference in the mass peaks of T6′ showed good agreement with the mass unit of the corresponding natural amino acid with the exception of Lys114. The difference mass of the Lys114 site was determined to be 456.24 Da, the value of which is completely coincident with the sum of the M_w of Lys and AMCA (the calculated value of 456.24 Da). Thus, the labeling site was undoubtedly assigned to Lys114, indicating that the reaction selectively occurred at pH 5.0 to tether AMCA at 114 Lys of Con A. In contrast, multiple peaks appeared for the reaction under basic conditions (pH 8.5) (as shown in Figure 1d), suggesting that the reaction proceeded with a rather low selectivity at pH 8.5.

On the basis of the above acylation result, the scheme for the double modification of Con A was established as shown in Figure 2b. The labeled Con A prepared by photoaffinity labeling

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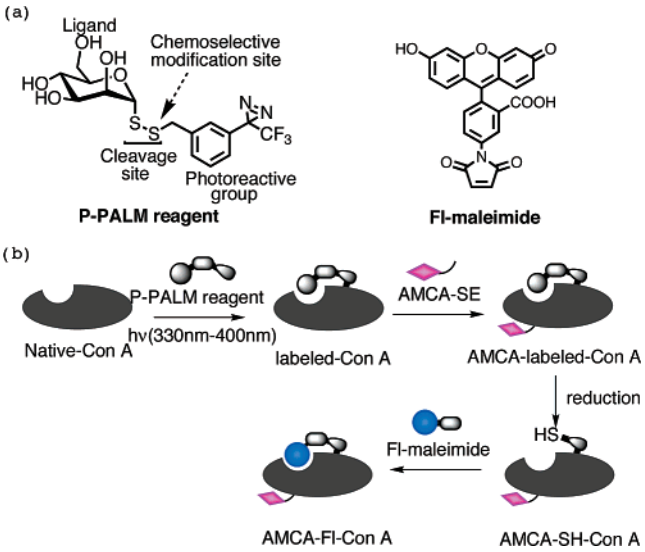

Figure 2. (a) Molecular structure of a P-PALM reagent and Fl-maleimide. (b) Scheme for double modification of Con A by coupling P-PALM with the controlled acylation to afford semisynthetic AMCA−Fl−Con A.

was selectively acylated with AMCA−SE to afford the AMCA-labeled Con A. Using the previously reported P-PALM method,[8] the labeled ligand unit was cleaved off and fluorescein was attached to the produced thiol site via the maleimide/SH reaction to yield the doubly modified AMCA−Fl−Con A. Each step was followed by MALDI-TOF MS spectroscopy, and the obtained AMCA−Fl−Con A was characterized by UV−visible, fluorescence (excitation and emission), and mass spectroscopies (see Supporting Information Figure S-2). The UV−visible spectrum was the simple sum of Con A (at 280 nm), AMCA (350 nm), and the Fl (492 nm) chromophores (Figure 3a). On the other hand, the fluorescence spectrum was not the simple sum of them, but showed the occurrence of a fluorescent resonance energy transfer (FRET) between AMCA and Fl. For the simple sum of the fluorescence of AMCA−Con A and Fl−Con A, the peak ratio of 513 nm (due to Fl) over 450 nm (due to AMCA) was 0.58, which was significantly lower than 2.5, the ratio of AMCA−Fl−Con A (Figure 3b). It is clear that the intramolecular FRET effectively took place in the doubly modified AMCA−Fl−Con A. This is supported by the fluorescence lifetime measurement. When we compared the fluorescence lifetime of the AMCA unit, a FRET donor, of AMCA−Fl−Con A (3.1 ns) to that of AMCA−Con A (4.0 ns), the shortened lifetime by 1 ns was observed in the case of AMCA−Fl−Con A (Figure 3c).[12] This may be ascribed to the intramolecular energy transfer from AMCA to Fl in AMCA−Fl−Con A.

Ratiometric Sensing to Saccharide and Its Derivatives. These two distinct fluorescence peaks of AMCA−Fl−Con A may be potentially useful for the ratiometric sensing of saccharides. Figure 4a shows a typical fluorescence change of AMCA−Fl−Con A upon the addition of 1,3- and 1,6-mannotriose (Man-3). It was found that the fluorescence of AMCA (450 nm) was not altered, but the fluorescence intensity of Fl (513 nm) decreased with the addition of Man-3. Such a change did not take place in the case of the randomly modified Fl−Con A (for random Fl−Con A, see Supporting Information Figure S-4). Therefore, the fluorescence change may be attributed to the microenvironmental change of Fl caused by the sugar binding because Fl was tethered at the proximity of the sugar-binding site of Con A.[13] We previously demonstrated that

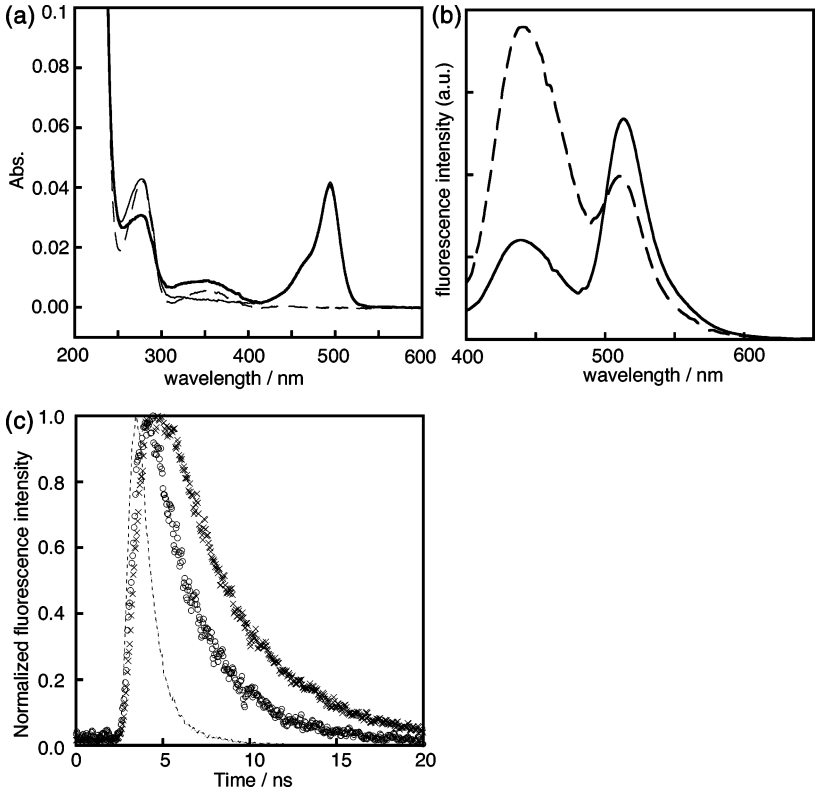

Figure 3. (a) UV−visible spectra of AMCA−Fl−Con A (bold line), AMCA−Con A (broken line), and Fl−Con A (plane line). (b) Fluorescence spectra of AMCA−Fl−Con A (bold line) and a simple mixture of AMCA−Con A and Fl−Con A (broken line). (c) Time-dependent fluorescence decay curve of AMCA was recorded from 440 to 460 nm after laser excitation at 337 nm for AMCA−Fl−Con A (○) and AMCA−Con A (×), and the temporal response (TR: broken line).

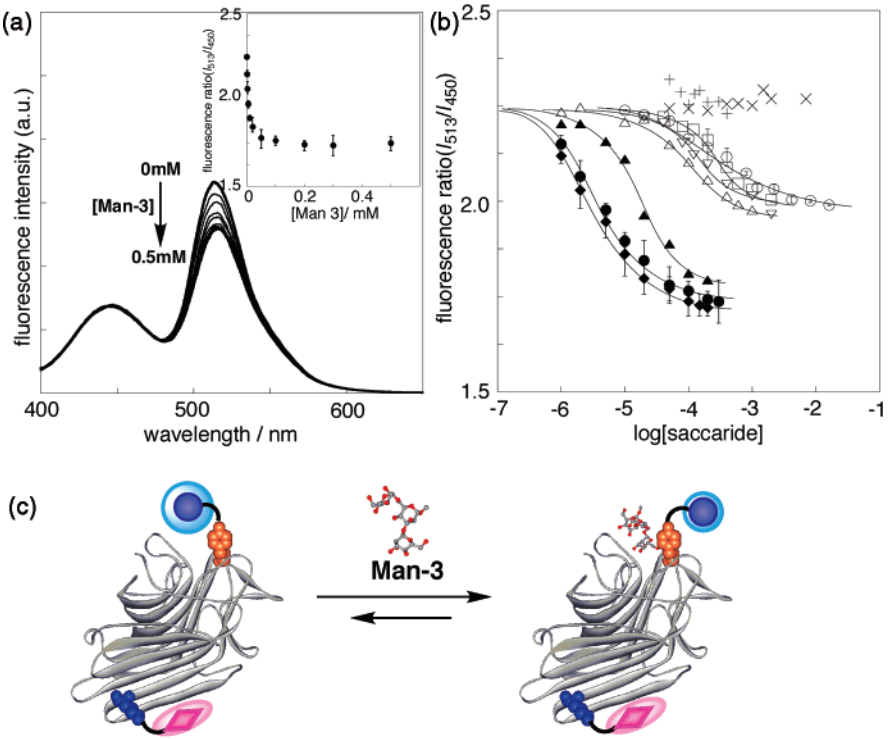

Figure 4. (a) Fluorescence spectral change of AMCA−Fl−Con A upon addition of Man-3 (0−0.5 mM; inset: fluorescent titration plots of the intensity ratio (I_{513}/I_{450}) versus Man-3 concentration (●)). (b) Fluorescent titration plots of the emission intensity ratio (I_{513}/I_{450}) versus the saccharide concentration (log [saccharide]): Man-1 (○), 12-Man-2 (△), 13-Man-2 (□), 16-Man-2 (▽), Man-3 (●), Man-4 (◆), Man-5 (▲), Gal (×), Cel (+) in 50 mM HEPES buffer (pH 7.5), 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1 M NaCl, $T = 20 \pm 1$ °C, $\lambda_{ex} = 350$ nm. (c) Schematic illustration of the present ratiometric sensing mode of AMCA−Fl−Con A.

the dansyl fluorophore was released from the sugar-binding pocket upon the sugar binding in the dansyl-appended Con A, which was prepared by P-PALM. It is considerable that the similar event occurred in the case of AMCA−Fl−Con A. A seesaw-type of change in the AMCA and Fl emission was never observed, indicating that the FRET efficiency between AMCA and Fl was not influenced by the sugar binding. In fact, no difference was detected when we compared the fluorescence lifetime of the AMCA part of AMCA−Fl−Con A in the absence or presence of the sugar (10 mM of Man-1) (see Supporting Information Figure S-3). This may suggest that the distance change between two fluorophores induced by the saccharide binding is not significant for FRET.

Table 1. Comparison of the Association Constants of AMCA−Fl−Con A with Those of Native Con A toward Various Saccharide and Glycoproteins

saccharide	log K	
	AMCA–Fl–Con A[d]	native Con A[b]
Man-1	4.62	4.04
12-Man-2	4.99	4.62
13-Man-2	4.75	4.48
16-Man-2	4.41	4.13
Man-3	5.39	5.40
Man-4	5.62	5.30
Man-5	4.70	5.30
Gal	*a*	*a*
Cel	*a*	*a*
Ribo B (Man-5 derivative)	5.11	*c*
Ribo A (Man-5 nonderivative)	*a*	*c*

[a] Precise values cannot be determined because of the low affinity. [b] Determined by ITC.[9b] [c] No data were reported in previous literatures. [d] The averaged values of at least three independent titration experiments.

Since the AMCA emission was invariable in various saccharide concentrations, the ratiometric sensing may be carried out using the AMCA emission as the internal standard.[7,14] The emission ratio of Fl versus AMCA showed a typical saturation behavior as in the inset of Figure 4a, producing a binding affinity (log *K*) of 5.39, the value of which is almost identical with that of native Con A (Table 1). This result suggested that the ratiometric saccharide sensing was precisely carried out in the present AMCA−Fl−Con A using the unchanged AMCA fluorescence as the internal standard. A similar ratiometric fluorescence titration was conducted for other saccharides and glycoproteins (for glycoprotein, see Supporting Information

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(13) This is supported by the experiment using singly labeled Fl−Con A prepared by P-PALM. In the titration experiments of Fl−Con A with saccharides, we found that the fluorescence intensity of Fl decreased, similar to the results obtained in AMCA−Fl−Con A (see Supporting Information Figure S-4a). Consistently, the fluorescence anisotropy decreased in the presence of sugar, relative to that in the absence of sugar (see Supporting Information Figure S-5), suggesting that the rather restricted Fl mobility was relaxed by kicking out Fl upon sugar binding. In contrast, the randomly modified Fl−Con A did not show any fluorescence response to the corresponding saccharides (see Supporting Information Figure S-4b), which indicates that tethering of Fl to the proximity of the binding pocket is crucial to this fluorescent sensing. As demonstrated in our previous papers (refs 8a and 8c), the sugar-binding pocket of Con A is rather hydrophobic because of the proximal two Tyr residues and Ile. Similar to the previous DANS−Con A, Fl was kicked out so as to suppress the fluorescence intensity upon the saccharide binding. In addition to this microenvironmental effect, the UV−visible titration experiments (Supporting Information Figure S-6) suggested that the pH change depending on the location of Fl is also an additional factor for the quenching. In the case of Fl, it is well-known that the pH-dependent change takes place between the strong fluorescent form and the weak fluorescent form. By comparing the UV−visible spectral change, it is suggested that the weak fluorescent form slightly increases upon sugar binding in Fl−Con A.

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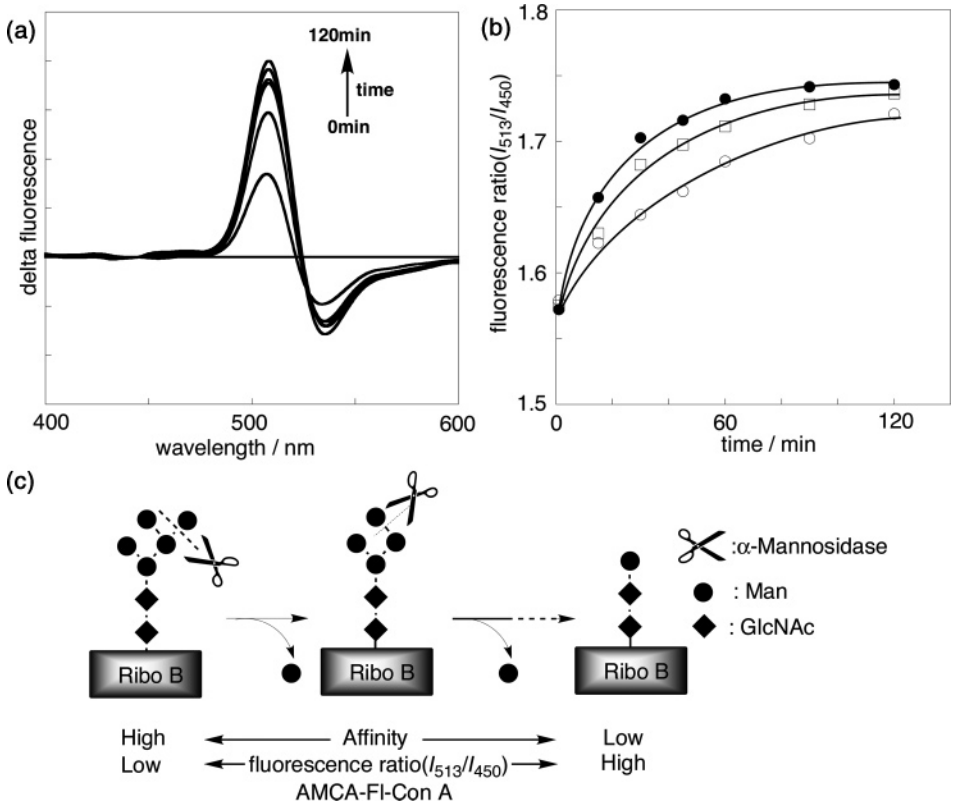

Figure 5. (a) Time-dependent differential fluorescence spectral change of AMCA−Fl−Con A during the enzymatic trimming of Ribo B (0.50 mM) catalyzed by α-mannosidase (0 → 120 min). (b) Time profile of the emission intensity ratio (I_{513}/I_{450}) at various Ribo B concentrations (0.25 mM (○), 0.33 mM (□), 0.50 mM(●)). (c) Fluorescent sensing scheme of the enzymatic trimming process toward a branched mannoside attached to Ribo B by AMCA−Fl−Con A.

Figure S-7). The semilog plot of the ratio value versus the saccharide concentration shown in Figure 4b gave binding constants for various saccharides and glycoproteins that are summarized in Table 1. The emission ratio changes by the addition of all the α-linked mannose derivatives, whereas Me-α-galactose or a *β*-linked glucose derivative, such as cellobiose, does not cause the ratio change. The evaluated selectivity and binding affinity (shown in Table 1, that is, Man-5, -4, -3 > several types of Man-2, Man-1 > Cel, ≫ Me α-Gal) are almost identical with the reported values of the native Con A determined by the ITC (isothermal titration calorimetry) experiment,[9b] indicating that AMCA−Fl−Con A is a ratiometric fluorescent biosensor retaining a natural selectivity despite the double modification (Figure 4c).

Fluorescent Monitoring of a Saccharide Trimming Process of Glycoprotein. Since the present AMCA−Fl−Con A can sense a high-mannose-type of branch attached to a protein surface, such as ribonuclease B (Ribo B, Figure S-7),[15] we next attempted to fluorescently monitor the trimming process of the high-mannose branch on Ribo B by α-mannosidase.[16] Figure 5a shows the difference spectra of the fluorescence depending on the reaction time. Clearly, the emission at 513 nm due to Fl gradually intensified in AMCA−Fl−Con A relative to the AMCA peak, along with a slight wavelength shift. Such a slight emission shift that was observed during the titration experiment of AMCA−Fl−Con A with Ribo B produced a seesaw-type of the fluorescence spectral change. The time-dependent Fl intensification is reasonably ascribed to the affinity difference of Con A between the high-mannose branch and the simple mannose unit. That is, prior to the hydrolysis by α-mannosidase, AMCA−Fl−Con A was predominantly bound to the high-mannose of Ribo B, and as a result, the emission peak of Fl was suppressed. After the high-mannose branch was enzymatically degraded into a simple mannose unit, which shows only a low affinity to Con A, the Fl emission recovered during the trimming process (see Figure 5c). As shown in Figure 5b, this reaction was accelerated by the increase in the Ribo B concentration, and it is important to note that the ratiometric sensing by AMCA−Fl−Con A successfully monitored the time course of the enzymatic trimming. By the fluorescent titration using AMCA−Fl−ConA in the previous section, we confirmed that the spectral change was completed within less than 1 min for each step, suggesting the rapid equilibrium in this binding. On the other hand, the mannosidase-catalyzed trimming of Ribo B is rather slow (longer than 1 h). Thus, it is conceivable that AMCA−Fl−Con A is successfully applied to the present monitoring system without being inferred by the sensing process. Artificial fluorogenic or chromogenic substrates have been usually employed for such a glycosidase activity assay, which unfortunately cannot give a real rate for naturally abundant substrates, such as glycoproteins or glycolipids. The present AMCA−Fl−Con A method does not need any modification of the substrates, so that it may give us a unique tool for the investigation of the enzyme activity toward natural substrates.

Imaging a Cell Surface Decorated with High-Mannosides. In addition to sensing the corresponding saccharides in test tubes, AMCA−Fl−Con A is capable of imaging sugar derivatives

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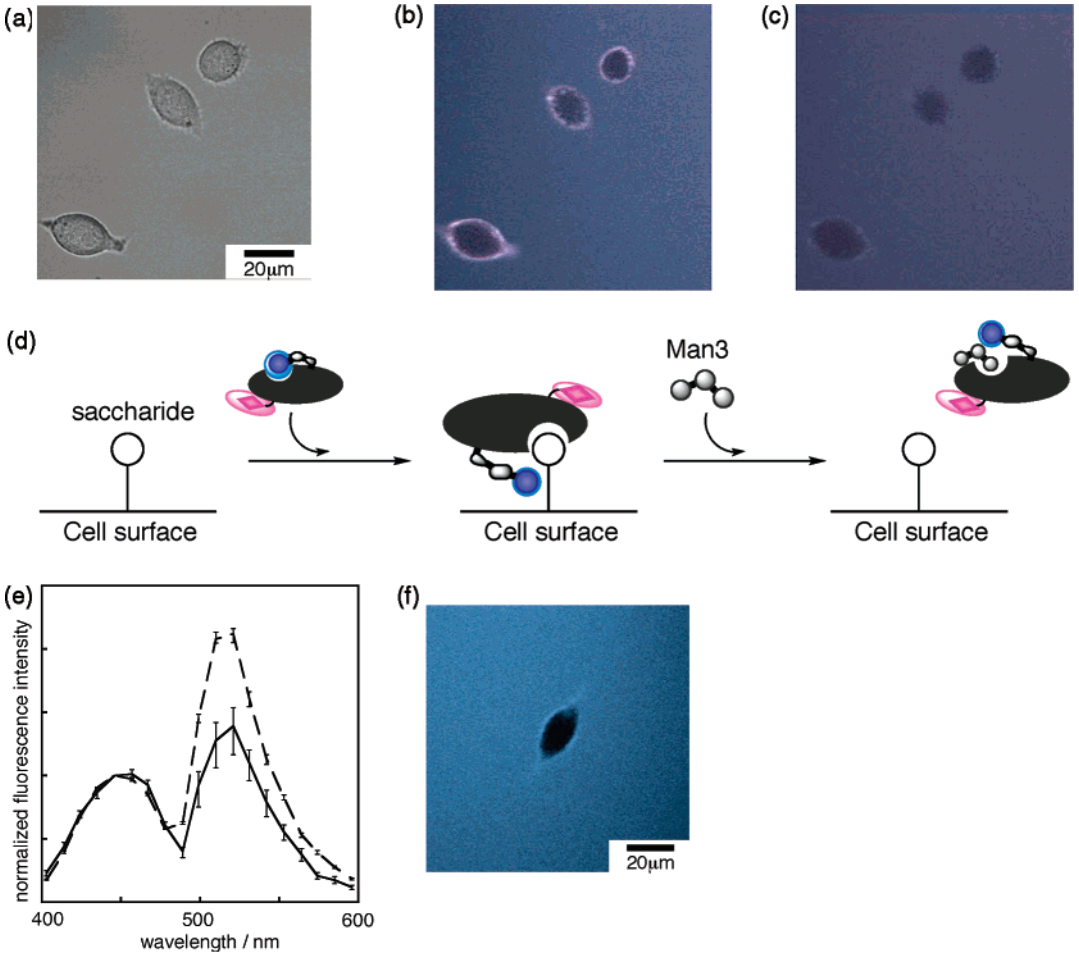

Figure 6. CLSM images of MCF-7 cells. (a and b) The images of the samples stained with 1 μM AMCA−Fl−Con A by transmission channel (a) and fluorescence channel (b). (The red color and the blue color were assigned for AMCA and Fl, respectively.) (c) The image (fluorescence channel) of the sample (b) after the treatment by Man-3 (100 μM). (d) Schematic illustration of the present CLSM image change probed by AMCA−Fl−Con A on the MCF-7 cell surface. (e) Fluorescence spectra of AMCA−Fl−Con A normalized at 446 nm on the cell surface (plane line) and in the bulk solution (dashed line). These spectra were collected from the cell image of Figure 6b. (f) Fluorescence image of MCF-7 cells stained with 1 μM randomly modified Fl−Con A.

localized on a cell surface by the ratiometric technique. As a proof-of-principle experiment, the fluorescent imaging MCF-7 cell line, a breast cancer cell decorated with a high-mannose-type of saccharide, was conducted.[17] Figure 6a−c shows photographs obtained by confocal laser scanning microscopy (CLSM). Upon treatment of the MCF-7 cell with AMCA−Fl−Con A, the stronger pinkish fluorescence due to AMCA was observed on the surface of the MCF-7 cells, relative to that of the bulk solution (Figure 6a and b). When Man-3 (100 μM, Figure 6c) or endoglycosidase H[18] (0.2 unit mL^{-1}; see Supporting Information Figure S-9) was added to the resulting solution, the contrast between the cell surface and the bulk became smeared.[19] This is reasonably explained by the fact that AMCA−Fl−Con A is concentrated on the MCF-7 cell surface by the binding to the high-mannose types of glycosides, and the concentrated AMCA−Fl−Con A was released into the bulk solution upon competitive binding to the excess amount of Man-3 or the saccharide trimming by glycosidase (Figure 6d). This is strongly supported by the fluorescence spectra of the localized space of the cell. As shown in Figure 6e, the fluorescence intensity coming from Fl was weaker on the cell surface based on the fluorescence from AMCA than the Fl intensity of the bulk solution (the ratio of Fl over AMCA was 1.5 and 2.2 for the cell surface and for the bulk, respectively). Such a ratiometric change between the two emission peaks indicates that AMCA−Fl−Con A binds to the saccharides on the cell surface, but not in the bulk solution. In addition, the advantage provided by the ratiometric spectral analysis was undoubtedly displayed when the photograph obtained by the ratiometry was compared to that by the simple intensification system. Apparently, a clearer color change was detected at the cell surface in the image of the ratiometric system using AMCA−Fl−Con A than in the image by a simple intensity change system using the random Fl−Con A (Figure 6f), the

(17) (a) Edwards, D. P.; Grzyb, K. T.; Dressler, L. G.; Mansel, R. E.; Zava, D. T.; Sledge, G. W.; McGuire, W. L. *Cancer Res.* **1986**, *46*, 1306. (b) Sugahara, T.; Ohama, Y.; Fukuda, A.; Hayashi, M.; Kawakubo, A.; Kato, K. *Cytotechnology* **2001**, *36*, 93.

(18) (a) Tarentino, A. L.; Maley F. *J. Biol. Chem.* **1974**, *249*, 811. (b) Tarentino, A. L.; Plummer, T. H., Jr.; Maley, F. *J. Biol. Chem.* **1974**, *249*, 818. (c) Arakawa, M.; Muramatsu, T. *J. Biochem.* **1974**, *76*, 307.

(19) The darkness difference in the background is mainly due to the released AMCA−Fl−Con A from the cell surface to the bulk solution upon Man-3 addition. The released AMCA−Fl−Con A should bind to Man-3 so that the reddish fluorescence is stronger than that of AMCA−Fl−Con A in the bulk in Figure 6b. Since the background of Figure 6c is more reddish than that of Figure 6b, the darkness seems to be different between them. The difference of the fluorescence spectra of the background was shown by the spectrum mode of CLSM (see Figure S-8).

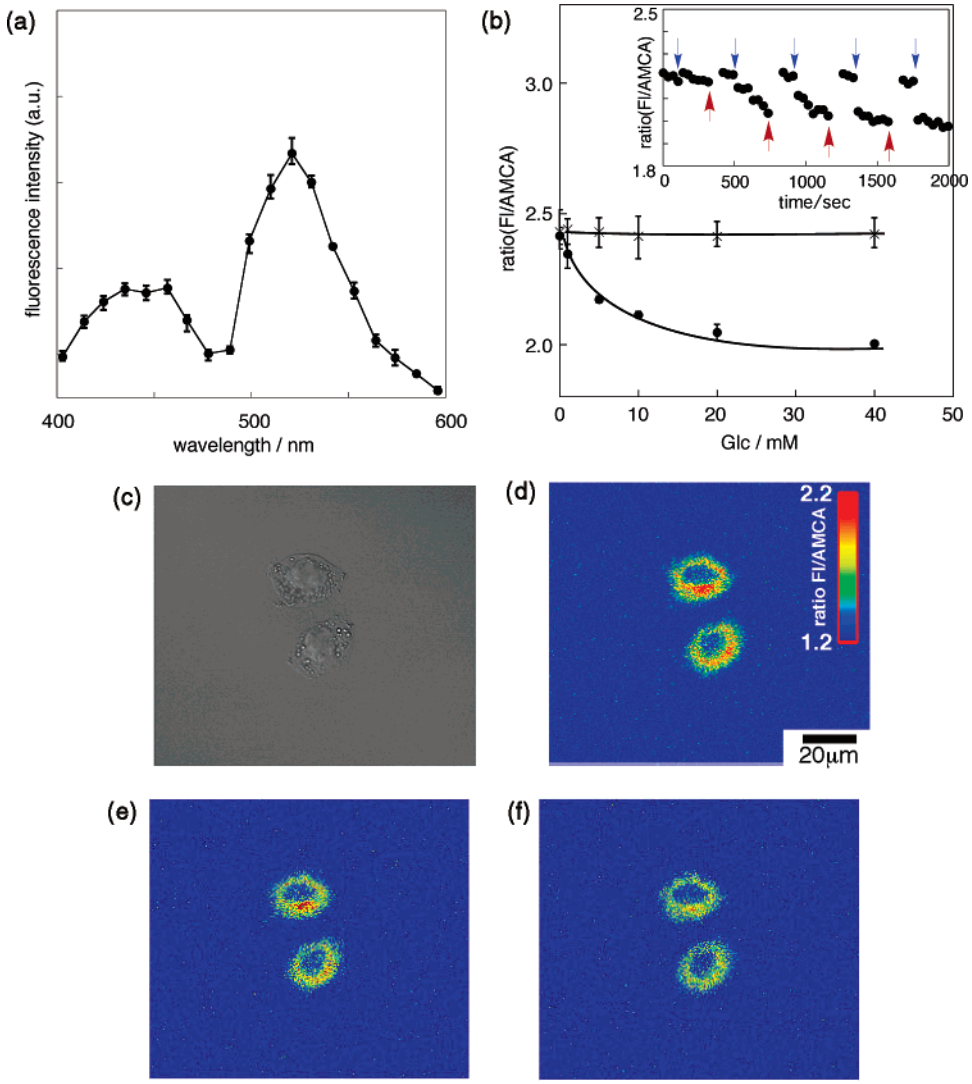

Figure 7. (a) Fluorescence spectra of AMCA−Fl−Con A in cytosol of HepG2. (b) The ratiometric intensity (I_{Fl}/I_{AMCA}) change depending on various extracellular saccharide concentrations. (Glc (●) and Gal (×)) [inset: the time course of the ratiometric intensity change (I_{Fl}/I_{AMCA}) upon the Glc addition. The blue arrows and the red arrows show the Glc addition (1, 5, 10, 20, and 40mM (from the left to the right)) and the washing process, respectively]. (c−f) Intracellular ratiometric image of HepG2 cells probed by AMCA−Fl−Con A. CLSM: (c) Transmission channel. (d−f) Pseudocolor fluorescence ratio image. The extracellular concentration of Glc: 0 mM (d), 5 mM (e), 40 mM (f).

spatial distribution of which induced the increase or decrease in the fluorescence intensity.

Fluorescent Imaging of Glucose Concentration inside Cell. The ratiometric fluorescence analysis should be more powerful if it is conducted inside a cell. To evaluate the applicability of AMCA−Fl−Con A for intracellular glucose imaging, AMCA−Fl−Con A was transported into the HepG2 cell, a hepatic cell line, based on the conventional procedure using surfactant (see the Experimental Section in detail). As shown in Figure 7d, the CLSM study showed that a strong fluorescence appeared inside the HepG2 cell. From the spectral analysis of the CLSM image (Figure 7a), it is clear that the fluorescence is due to both AMCA (450 nm) and Fl (513 nm), indicating that AMCA−Fl−Con A was localized inside the cell. The emission ratio (*I* (Fl) over *I* (AMCA)) was not practically altered during the five scans for observation over 20 min, showing that the troublesome photobleaching was not crucial under such conditions using the ratio value in the present AMCA−Fl−Con A. In the more detailed CLSM observations, AMCA−Fl−Con A was not inside the nuclei, but predominantly localized in the cytosol of HepG2. When the glucose (Glc) concentration of the external bulk solution increases, it is known that the HepG2 cell uptakes Glc, depending on the Glc concentration. Figure 7d−f shows the ratiometric image by CLSM for the various Glc concentrations (from 0, 5, and 40 mM, respectively). With the increase in the Glc concentration outside the cell, a reddish-colored image changed into a yellow-to-bluish one in cells, implying that the relative fluorescence intensity coming from Fl was gradually reduced. Such a ratiometric change indicates that AMCA−Fl−Con A located in the cytosol is gradually bound to Glc with the increase in the Glc concentration.[20] In contrast, the addition of galactose (Gal) did not influence the CLSM image because

(20) It is known that several glucose-dependent pathways generate the fluorescent cofactor NAD(P)H from the nonfluorescent NAD(P). Using this phenomenon, Evans et al. reported the glucose-dependent increase of the fluorescence due to NAD(P)H (so-called the autofluorescence of the cell at 400−500 nm by excitation at 340 nm: Evans, N. D.; Gnudi, L.; Rolinski, O. J.; Birch, D. J. S.; Pickup, J. C. *Diab. Technol. Ther.* **2003**, *5*, 807). In our study, such an autofluorescence was not significantly measured under the various glucose concentrations (from 0 to 40 mM, without AMCA−Fl−Con A), confirming that the autofluorescence by the glucose uptake did not influence the present ratiometric fluorescence change of AMCA−Fl−Con A in the HepG2 cell.

Gal is not bound to Con A. Figure 7b shows the change of the intracellular Glc monitored by the emission ratio (I (Fl)/I (AMCA)) by various Glc concentrations of the external solution. Importantly, it shows that the typical saturation curve and the evaluated binding constant for Glc is roughly identical to the value obtained in the test tube experiment described in the previous section. The Glc uptake rate monitored by the emission ratio change is accelerated by stepwise increments of the external Glc concentration (from 1 to 40 mM), as shown in the inset of Figure 7b and Figure S-10. In the case of 40 mM, the change becomes constant within 1 min, whereas longer than a few minutes is needed for 5 mM. It is also noteworthy that the ratio value returned back to almost the original one when the bulk Glc was washed out, suggesting the reversibility of the present sensing process using AMCA−Fl−Con A. As a control experiment, we confirmed that the Glc-dependent imaging was not clearly distinguishable using random Fl−Con A. This is ascribed to the fact that the random Fl−Con A cannot respond to the Glc concentration.

These results clearly demonstrated that the semisynthetic AMCA−Fl−Con A, which tethers two distinct small fluorophores, can respond to the Glc concentration change inside the HepG2 cell. It is clear that the present ratiometric biosensor system is more powerful for the imaging of the Glc concentration inside a cell than the intensity-based sensing systems which frequently yield problematic artifacts due to photobleaching and/or the spatial redistribution of a single-modified probe protein.

Conclusion

The site-selective incorporation of two different fluorophores into a naturally occurring protein was successful using two orthogonal chemical modification methods. The doubly modified Con A retains the natural sugar-binding selectivity and affinity, regardless of such chemical modifications, and can sense the corresponding sugars and glycoproteins in the ratiometric fluorescence mode. Using the benefit of the ratiometric fluorescence analysis, the engineered Con A can be successfully applied not only to sensing the structurally complicated saccharide of biological significance in a test tube but also to sensing and imaging these saccharides localized on a cell surface or the glucose concentration inside a cell. In particular, such an in-cell glucose imaging may potentially lead to a cell-based drug screening assay for type II diabetes.[21] Despite the many reports on the fusion protein using two protein-based fluorophores, examples of the controlled incorporation of two artificial fluorescent probes with low molecular weights into proteins are very limited. To the best of our knowledge, this is the first example demonstrating that a semisynthetic lectin doubly modified with unnatural small molecules is useful in its function in a cell as well as in a test tube. It is expected that this example will stimulate the relevant research fields in which various orthogonal chemical methodologies applicable to engineering proteins and enzymes will be established.

Experimental Section

Preparation of AMCA−Con A: To a solution of native Con A (20 μM) in 10 mM acetate buffer (pH 5.0 or 8.5 (10 mM Tris buffer)) was slowly added AMCA−SE (a final concentration of AMCA−SE is 20 μM, commercially available from Molecular Probes) in DMSO, and the reaction mixture was incubated for 12 h at 25 °C in the dark. Then, the solution was submitted to Mono S cation exchange chromatography (Applied Biosystems) at pH 5.0 (10 mM acetate buffer) and eluted by NaCl gradient (from 0 to 1 M for 15 CV). The eluted AMCA−Con A fraction was collected. The labeling efficiency was estimated from the ratio of the corresponding absorbance to be 0.80−0.95. AMCA−Con A was identified by MALDI-TOF MS (Voyager RP): m/z calculated, 25 911; found (SA), 25 922 ± 10. UV spectra: AMCA $\epsilon_{348} = 16\,300\ M^{-1}\ cm^{-1}$, $\epsilon_{280} = 4200\ M^{-1}\ cm^{-1}$ (H_2O). Con A: $\epsilon_{280} = 35\,000\ M^{-1}\ cm^{-1}$.

Trypsin Digestion: The solution of AMCA−Con A (67 μM, 200 μL) was mixed with in 100 mM ammonium bicarbonate buffer (pH 8.5) containing 2 M urea, and the mixture was treated with trypsin (commercially available from Sigma) at 37 °C for 20 h. The digestion reaction was stopped by TFA addition (the final concentration: 0.1% (v/v)). The digested peptides were separated by reverse-phase HPLC (column; YMC-pack ODS-A, 250 × 10 mm), and each fraction was analyzed by MALDI-TOF MS (SA or CHCA as matrix). The HPLC conditions were as follows: CH_3CN (containing 0.1% TFA)/H_2O (containing 0.1% TFA) = 5/95−55/45 (linear gradient over 100 min), flow rate = 3 mL/min, detection by UV (220 nm) or FL (excitation wavelength = 350 nm, emission wavelength = 450 nm).

Peptide Sequencing by Tandem Mass: The peptide fraction including fragment T6′ collected by HPLC was used for MALDI-TOF MS analysis (Bruker autoflex). The target peak ([T6′ + H]: m/z calculated, 2083.08; found, 2083.07) was sequenced by a tandem mass−mass method, and the pattern was analyzed by BioTool software (manual mode).

Photoaffinity Label of Con A with P-PALM Reagent 1: The photoaffinity labeling process using **1** was conducted according to our recipe reported previously.[8] The first fraction of the labeled Con A was collected and used in the following study.

Preparation of AMCA−Fl−Con A: To a solution of the labeled Con A (a homodimer consisting of the labeled monomer, that is, first fraction in the affinity column, 16.5 μM/20 mL) in 10 mM acetate buffer (pH 5.0) was added AMCA−SE (the final concentration is 16.5 μM), and it was incubated for 12 h at 25 °C in the dark. The eluted AMCA-labeled−Con A was purified according to the same method as AMCA−Con A. The AMCA-labeled−Con A solution thus obtained was dialyzed against 10 mM phosphate buffer (pH 7.5). TCEP (the final concentration = 0.1 mM) was added to the solution of AMCA-labeled−Con A (6.5 μM, 12 mL), and the reaction mixture was incubated for 5 h at 4 °C under nitrogen atmosphere. The AMCA−SH−Con A thus prepared was treated with fluorescein-5-maleimide (Fl-maleimide, the final concentration of Fl-maleimide is 0.5 mM, Molecular Probes) in 10 mM phosphate buffer (pH 7.5) at 25 °C for 12 h in the dark. The excess amount of Fl-maleimide was removed by gel filtration chromatography [TOYO PEARL, 1 cm × 15 cm, eluent; 10 mM acetate buffer (pH 5.0)], and the eluted protein fraction was collected, concentrated by ultrafiltration, followed by dialysis against 50 mM HEPES buffer solution (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1 M NaCl, to afford AMCA−Fl−Con A (0.5 μM, 5 mL). The labeling efficiency was estimated from the ratio of [Fl]/[Con A] to be 0.60−0.75, and [AMCA]/[Con A] to be 0.50−0.70. Each step was followed by MALDI-TOF MS: AMCA-labeled−Con A (SA): m/z calculated, 26 310; found, 26 312 ± 5, AMCA−SH−Con A: m/z calculated, 26 115; found. 26 123 ± 9. AMCA−Fl−Con A: m/z calculated, 26 542; found, 26 580 ± 10. UV−visible spectral data: Fl $\epsilon_{492} = 80\,000\ M^{-1}\ cm^{-1}$, $\epsilon_{280} = 23\,500\ M^{-1}\ cm^{-1}$ (pH 7.5), AMCA $\epsilon_{348} = 16\,300\ M^{-1}\ cm^{-1}$, $\epsilon_{280} = 4200\ M^{-1}\ cm^{-1}$ (H_2O), Con A: $\epsilon_{280} = 35\,000\ M^{-1}\ cm^{-1}$. Fluorescence data: excitation and emission spectra: $\lambda_{ex} = 350$ and 488 nm, $\lambda_{em} = 450$ and 513 nm.

Fluorescence Lifetime Study: The fluorescence lifetime was measured with a fluorescence lifetime instrument (Hamamatsu photonics, streak camera, Model C4334, optically coupled to a change-

(21) (a) Schwartz, M. W.; Porte, D., Jr. *Science* **2005**, *307*, 375. (b) Lowell, B. B.; Shulman, G. I. *Science* **2005**, *307*, 384.

coupled-device (CCD) array detector); 0.5 μM AMCA−Fl−Con A solution [50 mM HEPES buffer solution (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1 M NaCl at 20°C] was measured. N_2 laser (337 nm) was used for AMCA excitation.

Fluorescence Titration with Saccharide Derivatives: Fluorescent spectra were recorded on a Perkin-Elmer LS55 spectrometer. Saccharide solution was added dropwise to a 0.1 μM AMCA−Fl−Con A solution [50 mM HEPES buffer solution (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1 M NaCl at 20 °C], and then the fluorescence spectra were measured. The slit widths for the excitation and emission were fixed to 10 and 10 nm, respectively. The excitation wavelength used was $\lambda_{ex} = 350$ nm. Fluorescence titration curves were analyzed with a nonlinear least-squares curve-fitting method or the Benesi−Hildebrandt plot.

α-Mannosidase Activity Assay by AMCA−Fl−Con A: After 0.05 μmol of ribonuclease B (Ribo B) in 0.2 mL of pH 6.5 100 mM Tris/bis-Tris buffer (0.25 mM) was mixed with 1 unit of α-mannosidase (from Jack Bean, Seikagaku Corporation) in distilled water, the reaction mixture was incubated at 37 °C. At a corresponding reaction time, the reaction solution was collected and mixed with 0.2 μM AMCA−Fl−Con A (the final concentration of Ribo B = 10 μM, 50 mM HEPES buffer (pH 7.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1 M NaCl), and the fluorescence spectrum was measured. The trimming process of Ribo B was also monitored by MALDI-TOF MS.

Confocal Laser Scanning Microscopy Study: Confocal laser scanning microscopy (CLSM, Carl Zeiss LSM 510 META equipped with a UV laser (351/364 nm) was measured as the following conditions: the excitation wavelength: $\lambda_{ex} = 351$ nm, all images by the emission fingerprinting. The fluorescence spectra of the CLSM images were obtained by the averaged value of at least five independent points of the photos. The AMCA and Fl spectra were separated from each other by the automatic calculation. The scanning speed and the laser intensity were adjusted to avoid photobleaching of the fluorescent probes because the photobleaching of AMCA−Fl−Con A occurred under the severe conditions of CLSM experiments.

Imaging a Cell Surface using AMCA−Fl−Con A: MCF-7 cell (human breast adenocarcinoma: 1×10^4 cells. Provided by Cell Resource Center for Biomedical Research, Tohoku University) was seeded in a 35 mm glass-bottom dish and incubated overnight in MEM supplemented with 10% of FBS at 37 °C under 5% CO_2 atmosphere. After washing with PBS three times, the cells were treated with 1 μM AMCA−Fl−Con A (or randomly modified Fl−Con A) in PBS and were observed with CLSM. After the initial observation, Man-3 (final concentration = 100 μM) was added to the resultant solution, and the cells were again observed with CLSM, or the cells were treated with 0.2 unit mL^{-1} of endoglycosidase H (from *Streptomyces griseus*, Seikagaku Corporation) for 2.5 h at 37 °C and observed with CLSM.

Fluorescent Sensing of Glucose Concentration inside Cell: HepG2 cell (human a hepatic cell: 1×10^4 cells) was seeded in a 35 mm glass-bottom dish and incubated in DMEM supplemented with 10% of FBS at 37 °C under 5% CO_2 atmosphere. After the overnight incubation, the cells were washed with PBS, fixed for 15 min at room temperature in a solution containing 4% (w/v) *para*-formaldehyde, and washed again. Subsequently, the fixed cells were treated with TBP solution [0.1% (w/v) Triton X-100, 1% BSA, 0.1% $NaBH_4$ in PBS] for 30 min, and then treated with TBP solution [0.1% (w/v) Triton X-100, 1% BSA in PBS] containing 0.3 μM AMCA−Fl−Con A for 1 h. The fixed cells were then washed and observed by CLSM under various Glc concentrations.

Acknowledgment. E.N. thanks JSPS for his JSPS predoctoral fellowship.

Supporting Information Available: Experimental details, including characterization of AMCA−Fl−Con A, preparation procedure of Fl−Con A, several spectral data for elucidating the fluorescence quenching mechanism, control experiment data for cell imaging, and chemical structures of the used saccharide. This material is available free of charge via the Internet at http://pubs.acs.org.

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