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Post-photoaffinity labeling modification using aldehyde chemistry to produce a fluorescent lectin toward saccharide-biosensors†

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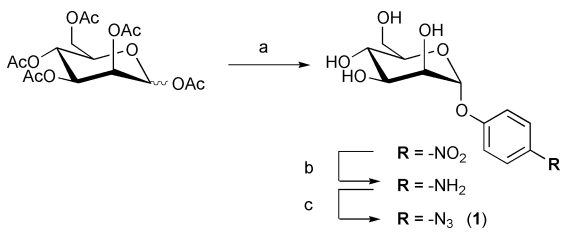
A new method to construct a fluorescent saccharide biosensor based on a lectin protein is successfully proposed using post-photoaffinity labeling modification coupled to aldehyde chemistry.

A biosensor is regarded as one of the ideal systems to monitor ions and molecules of biological importance both *in vivo* and *in vitro*,[1,2] since a protein framework can provide a superior molecular recognition scaffold. To carry out the rational coupling of a signal transduction device with the molecular recognition events on a protein scaffold, most examples rely upon site-directed mutagenesis,[3] GFP (green fluorescent protein) fusion strategy[4] and protein total-[5] or semi-[6]synthesis. In contrast to genetic technologies, however, chemistry-based versatile methods for the manipulation of biomacromolecules have not been satisfactorily developed. In fact, there are still limited numbers of procedures that are capable of the site-directed introduction of unique thiol[3] or ketone[7] groups into native proteins in order to tag unnatural signaling molecules.

We describe herein a new method named as '*post-photoaffinity labeling modification*' to construct a fluorescent saccharide-biosensor based on a naturally occurring saccharide-binding protein, concanavalin A (Con A). Con A, a kind of lectin family, can bind α-D-mannoside and α-D-glucoside derivatives selectively.[8] An active site-directed modification of the lectin was conducted by the photoaffinity-labeling technique using *p*-azidophenyl-α-D-mannopyranoside **1**. The subsequent oxidative degradation of the mannose part to produce aldehyde tags, followed by the chemical modification with a hydrazine derivative yields a fluorescent lectin.

The affinity ligand **1** which has both a α-mannoside unit, the strongest monosaccharide ligand for Con A, and a phenylazide group as a photoaffinity labeling group was synthesized as shown in Scheme 1. Peracetylmannose was glycosylated by *p*-nitrophenol in the presence of $BF_3{\cdot}Et_2O$,[9] followed by deacylation with NaOMe and conversion of the nitro-group to azide in the conventional manner to afford the target **1**.[10]



Scheme 1 Synthesis of photoaffinity labeling reagent **1.** *Reagents and conditions*: (a) (i) *p*-nitrophenol, $BF_3{\cdot}Et_2O$ (1 eq.), dry CH_2Cl_2, rt, 3 days; (ii) NaOMe, dry MeOH, rt, 2 h (55% for 2 steps). (b) 10% Pd/C, MeOH, rt, 30 min (95%). (c) (i) $NaNO_2$, 0.4 M HCl, 0 °C, 30 min; (ii) NaN_3, 0 °C, 20 min (61%).

The post-photoaffinity labeling modification for Con A is briefly outlined in Scheme 2. When the ligand **1** was bound to the binding pocket of Con A, photolabeling *via* nitrene generation was carried out by UV light irradiation (λ = 254 nm).[10] The labeled Con A was purified by gel chromatography (Biogel-P-30), followed by affinity chromatography (Sephadex G-100). Two fractions bearing the lessened affinity to the Sephadex column than native Con A were obtained.† The analysis by MALDI-TOF mass spectroscopy shows that the first fraction showing no affinity to Sephadex is a Con A homodimer, the monomer of which is modified with 1 mole of **1** (a mass peak appeared only at 25 850 ± 10 due to Con A plus **1**), and the second one showing moderate affinity is a heterodimer that comprises the modified Con A monomer (1 mole of **1** per 1 mole of Con A**)** and the unmodified monomer [two mass peaks appeared at 25 850 ± 10 and 25 583 (native Con A)]. The total yield of the modified Con A is about 30%. To determine the labeled site, the labeled Con A was digested by lysyl-endopeptidase (at 37 °C, pH 9.0 for 15 h in the presence of 3 M urea).[11] The HPLC (ODS column) and MALDI-TOF mass analyses of the digested peptides showed that only two fragments [the peptide 1 (A1-K30) and the peptide 6 (R60-K101)] among 10 fragments of the digested peptide were labeled with 1 mole of the ligand **1**. The crystal structure of Con A[12] shows that these two peptides are strongly involved in the sugar binding. Especially, Tyr12 in fragment 1 and Tyr100 in fragment 6 form a hydrophobic fence of the binding pocket. These reasonably suggest that the photoaffinity labeling reaction site-selectively proceeds under the present conditions. Subsequently, the mannose unit of **1** introduced by photoaffinity labeling can be converted by oxidation with periodate (IO_4^-) under mild conditions into unique aldehyde groups (CHO-Con A),[13] that can be chemoselectively labeled with hydrazine- or aminooxy-appended fluorophores [DCCH-Con A in the case of DCCH (a coumarin derivative)-hydrazine].[14]

Secondary structure of the modified Con As was monitored by CD spectroscopy. A negative Cotton peak at 218 nm, characteristic of the typical β-sheet, in all of the modified Con As (the labeled, CHO-, and DCCH-Con A), is comparable to that of native Con A,[15] suggesting that the secondary structure is not significantly disturbed by the present modification.

DCCH-Con A showed an absorption at 435 nm[16] (Fig. 1a) and a strong fluorescence at 485 nm (excitation at 435 nm), due to the coumarin unit, an environmentally sensitive fluorophore. Thus, we monitored emission spectral changes of DCCH-Con A by the addition of methyl-α-D-mannoside (Me-α-Man), the strongest mono-saccharide ligand for native Con A (inset of Fig. 1b). With increasing concentration of Me-α-Man, the emission intensity at 485 nm is gradually lessened and the change is saturated at less than 10 mM. The saturation curve (Fig. 1b) obtained by the fluorescent titration can be analyzed by a Benesi–Hildebrandt plot to give a binding constant of 1.9×10^3 M^{-1} for Me-α-Man. Similar saturation curves were observed in the fluorescent titration of other types of saccharides and the association constants are determined as summa-

† Electronic supplementary information (ESI) available: experimental and spectral data for **1**, and a colour version of Scheme 2. See http://www.rsc.org/suppdata/cc/b0/b008323m/

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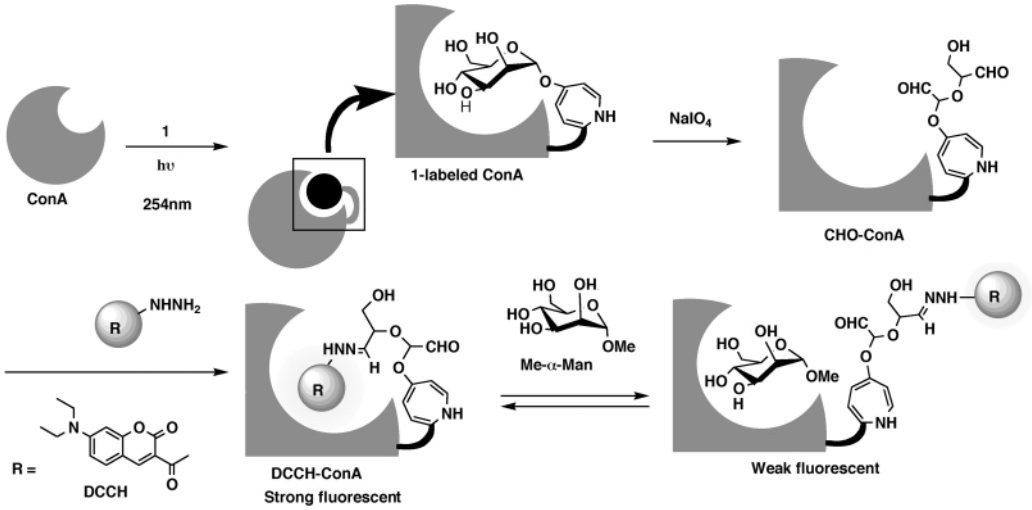

Scheme 2 Schematic illustration for semisynthesis of DCCH-ConA. The typical structures of the oxidative degradation of mannose are shown.

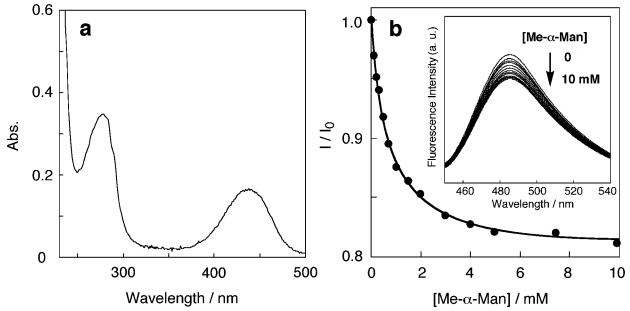

Fig. 1 (a) UV-vis spectrum of DCCH-ConA; (b) fluorescence titration curve of DCCH-ConA with Me-α-Man. [DCCH-ConA] = 3 μM, 50 mM HEPES buffer (pH 7.0), 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.1 M NaCl at 15 ±1 °C, λ_{ex} = 435 nm; inset: fluorescence spectral change of DCCH-ConA by the addition of Me-α-Man.

Table 1 The binding constants (*K*) of DCCH-ConA for saccharides

	K/M^{-1}	
Saccharide	DCCH-ConA	Native ConA
Me-α-mannoside	1900	11 000[a]
Me-α-glucoside	1450	3 000[a]
Me-β-glucoside	520	—[b]
Me-α-glactoside	570	—[b]

[a] Ref. 16. [b] The binding constants for Me-α-galactoside and Me-β-glucoside have not been reported because of their low affinity.

rized in Table 1. It is clear that the order of the affinity constants for various saccharides is the same as that of native Con A (Me-α-Man > Me-α-Glc > Me-α-Gal, Me-β-Glc), although the affinity constants are lower than the literature values of native Con A[17] determined by ITC (isothermal titration calorimetry) measurement. Significantly these results imply that the molecular recognition event occurring in the binding pocket of DCCH-Con A can be directly transduced by the fluorescence signal. This is the first step toward the rational design of fluorescent saccharide biosensors based on lectins. The reduced selectivity of DCCH-ConA might be due to the partial blocking of the sugar-binding pocket by the appended fluorophore and/or the structural disturbance of the binding site by the unnatural groups. Details are now under investigation in our laboratory.

We believe that this strategy is so general that other fluorescent biosensors may be produced showing different saccharide specificities by the simple replacement of the sugar part of **1** and the usage of the corresponding lectin.

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Notes and references

1 For reviews, see; (*a*) D. A. Zachrias, G. S. Baird and R. Y. Tsien, *Curr. Opin. Neurobiol.*, 2000, **10**, 416; (*b*) H. W. Hellinga and J. S. Marvin, *Trends Biotechnol.*, 1998, **16**, 183; (*c*) K. A. Giuliano and D. L. Taylor, *Trends Biotechnol.*, 1998, **16**, 135.

2 Synthetic chemosensors for saccharides have been actively developed in recent years. T. D. James, S. Samankumara and S. Shinkai, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1910.

3 (*a*) J. S. Marvin and H. W. Hellinga, *J. Am. Chem. Soc.*, 1998, **120**, 7; (*b*) G. Gilardi, G. Mei, N. Rosato, A. F. Agro and A. E. G. Cass, *Protein Eng.*, 1997, **5**, 479; (*c*) G. Gilardi, L. Q. Zhou, L. Hibbert and A. E. G. Cass, *Anal. Chem.*, 1994, **66**, 3840.

4 A. Miyawaki, J. Llopis, R. Heim, J. M. McCaffery, J. A. Adams, M. Ikura and R. Y. Tsien, *Nature*, 1997, **388**, 882.

5 (*a*) J. R. Sydor, C. Herrmann, S. B. H. Kent, R. S. Doody and M. Engelhard, *Proc. Nat. Acad. Sci. USA*, 1999, **96**, 7865; (*b*) G. K. Walkup and B. Imperiali, *J. Am. Chem. Soc.*, 1996, **118**, 3053.

6 (*a*) G. J. Cotton and T. W. Muir, *Chem. Biol.*, 2000, **7**, 253; (*b*) I. Hamachi, R. Eboshi, J. Watanabe and S. Shinkai, *J. Am. Chem. Soc.*, 2000, **122**, 4530; (*c*) I. Hamachi, T. Nagase and S. Shinkai, *J. Am. Chem. Soc.*, 2000, **122**, 12 065.

7 V. W. Cornish, K. M. Hahn and P. G. Schultz, *J. Am. Chem. Soc.*, 1996, **118**, 8150.

8 (*a*) H. Bittiger and H. P. Schnebli, *Concanavalin A as a Tool*, John Wiley and Sons, New York, 1976; (*b*) H. Lis and N. Sharon, *Chem. Rev.*, 1998, **98**, 637 and references therein.

9 E. Smits, J. B. F. N. Engberts, R. M. Kellogg and H. A. Doren, *J. Chem. Soc., Perkin Trans. 1*, 1996, 2873.

10 M. Beppu, T. Terao and T. Osawa, *J. Biochem.*, 1975, **78**, 1013. The labeling reagent **1** was identified by FTIR and ^{1}H-NMR.†

11 Y. Shikata, M. Kuwada, Y. Hayashi, A. Hashimoto, A. Koide and N. Asakawa, *Anal. Chim. Acta*, 1998, **365**, 241.

12 J. H. Naismith, C. Emmerich, J. Habash, S. J. Harrop, J. R. Helliwell, W. N. Hunter, J. Raftery, A. J. Kalb and J. Yarib, *Acta Crystallogr., Sect. D*, 1994, **50**, 847.

13 G. A. Lemieux and C. R. Bertozzi, *Trends Biotechnol.*, 1998, **16**, 506.

14 Chemical modification of the aldehyde group produced by the sugar oxidation with $NaIO_4$ is a traditional method to modify glycoproteins (*e.g.* antibody) and RNA with hydrazine derivatives. It has been reported that the mild oxidation does not affect protein function. See the following review articles; (*a*) D. J. O'Shannessy and M. Wilchek, *Anal. Biochem.*, 1990, **191**, 1; (*b*) D. J. O'Shannessy and R. H. Quarles, *J. Immunol. Methods*, 1987, **99**, 153.

15 M. S. Herrmann, L. M. Richardson, L. M. Setzler and W. D. Behnke, *Biopolymers*, 1978, **17**, 2107.

16 UV-vis spectrum of DCCH-ConA showed that 0.5 mol of DCCH was labeled for 1 mol of ConA monomer. This may be due to the incomplete oxidation of the mannose unit. Although the further oxidation for longer than 20 h to complete sugar oxidation was attempted, it was unsuccessful.

17 (*a*) F. P. Schwarz, K. D. Puri, R. G. Bhat and A. Surolia, *J. Biol. Chem.*, 1993, **268**, 7668; (*b*) D. K. Mandal, N. Kishore and C. F. Brewer, *Biochemistry*, 1994, **33**, 1149.