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Original Research Article

Cyclophane-lectin Conjugates as a New Class of Water-soluble Host

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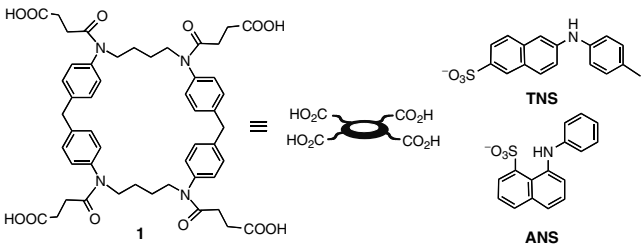
Abstract

A conjugate composed of tetraaza[6.1.6.1]paracyclophane bearing carboxylic acids and lectin, a carbohydrate binding protein, was prepared. The specific saccharide-binding abilities as well as the secondary structural features of the lectin were not disturbed, when the cyclophane were covalently bound to the lectin. The conjugate was found to act as a water-soluble host for inclusion of anionic guest molecules such as 6-*p*-toluidino-naphthalene-2-sulfonate (TNS) and 8-anilinonaphthalene-1-sulfonate (ANS) in aqueous acetate buffer (pH 4.0) with binding constants of 4.2×10^4 and 1.5×10^4 dm^3 mol^{-1}, respectively. The obtained binding constants were much larger than those by untethered water-soluble cyclophane. A highly desolvated microenvironment was provided by the cyclophane cavity on the protein surfaces so that the tight host–guest interaction, which brought about the marked motional repression of the entrapped guests, became effective. The conjugate also showed molecular discrimination capabilities toward the anionic guests through electrostatic repulsion mechanism originating from acid-dissociation equilibrium of carboxylic acids of the cyclophane branches.

Introduction

Currently, the development of water-soluble artificial hosts [1] capable of performing sophisticated molecular recognition has been attracting much attention. On these grounds, macrocyclic hosts exhibiting guest-inclusion capability, such as cyclodextrins [2] and cyclophanes [3], as well as other host compounds based on phenylboronic ester formation [4] or hydrogen-bonding [5] force, etc., were widely investigated as an artificial host. On the other hand, conjugation of artificial host molecules with naturally occurring proteins is a feasible strategy to enhance their recognition abilities and increase their solubility in aqueous media. For instance, when a phenylboronic acid-type host molecule capable of binding 1,2- or 1,3- diol derivatives is covalently attached to a carbohydrate binding protein (lectin), the resulting conjugate acts as a biosensor for oligosaccharides with enhanced binding affinity and saccharide selectivity [6]. To the best of our knowledge, however, few studies have so far been made on covalently bound conjugates of native proteins with macrocyclic hosts such as cyclophanes and cyclodextrins. On these grounds, we decided to develop conjugates composed of a cyclophane and proteins in order to create a new-class of water-soluble semi-artificial hosts. We have now designed a cyclophane protein conjugate composed of tetraaza[6.1.6.1]paracyclophane [7] bearing carboxylic acids (**1**) and wheat germ agglutinin (WGA) [8] lectin which recognizes specific carbohydrates such as *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-neuraminic acid (NeuNAc). Interestingly, Wirth and co-workers [9] reported that WGA specifically bound prostate cancer cells through saccharide-lectin interactions as confirmed by flow cytometry. Therefore, the cyclophane-WGA conjugate with guest (drug) has a potential to act as a cancer-cell targeted drug delivery system. In this context, we report the preparation of cyclophane-WGA conjugate and the affinity of the resulting conjugate to the hydrophobic-guests, as compared with those for untethered water-soluble cyclophane, as well as the carbohydrate ligands.



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Results and discussion

The 1,6,20,25-tetraaza[6.1.6.1]paracyclophane ring, prepared by Koga and Odashima, has been frequently used as a fundamental molecular skeleton for functionalized macrocyclic hosts, because durene, a guest molecule, was accommodated in its cavity as confirmed by X-ray structural analysis [7]. By introducing hydrophilic groups such as amino acids [10] and carbohydrates [11] into the skeleton, various water-soluble hosts have been developed. In order to prepare a cyclophane moiety for the conjugates, a cyclophane having four carboxylic acids (**1**) was derived from 1,6,20,25-tetraaza[6.1.6.1]paracyclophane by a reaction with succinic anhydride and isolated as a tetracarboxylic acid form. Nonionic cyclophane **1** has only a very limited solubility in aqueous acetate buffer (pH 4.0), whereas the anionic form of **1** is soluble in aqueous media above pH 7 due to its hydrophilic carboxylate groups.

WGA is a lectin composed of two identical 17 kDa (m/z, 16990 ± 10 confirmed by MALDI-TOF MS spectroscopy) subunits and is stable even in acidic aqueous solution at pH 4 [8]. Etzler and co-workers [8] reported that chemical modification such as acetylation or succinylation of amino groups located on the surface of WGA did not markedly change the lectin dimeric subunit structure or erythrocyte agglutinating ability. Therefore, we adopted WGA as a protein framework which was attached to cyclophane **1** in order to develop cyclophane-lectin conjugates. It is expected that the amino groups of either lysine or *N*-terminal of WGA react with activated residues of **1** by *N*-hydroxysuccinimide (NHS) and 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide (EDC), as shown in Scheme 1. MALDI-TOF MS spectroscopy clearly showed that 1:1 conjugate of **1** to WGA (m/z 17920 ± 10) was predominantly obtained by the conjugation procedure (Figure 1). The native WGA shows positive circular dichroism (CD) signals around 224 nm, reflecting their secondary structures [12]. An indistinguishable CD spectrum was also observed for **1**-WGA conjugates in acetate buffer (pH 4.5), as shown in Figure 2. These results suggest that the structural features of native WGA were not disturbed, when cyclophane **1** were covalently attached to WGA.

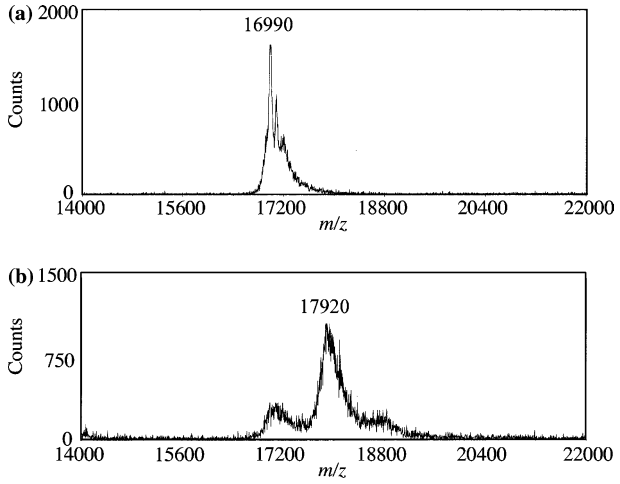


Figure 1. MALDI-TOF MS spectra of WGA (a) and **1**-WGA conjugate (b).

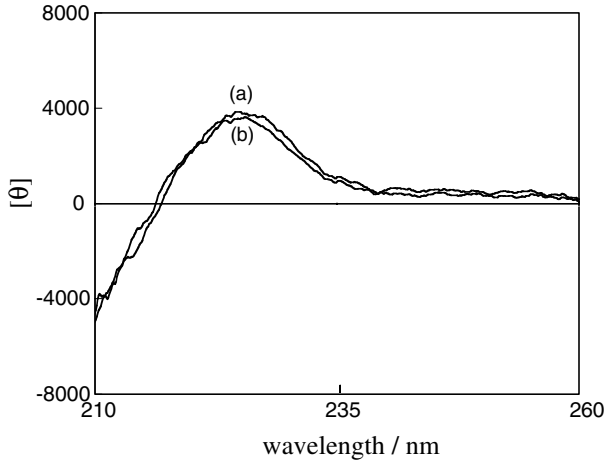
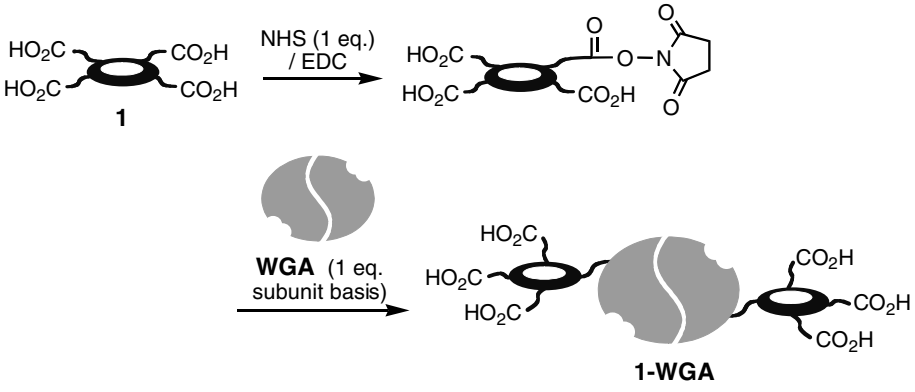


Figure 2. CD spectra of native WGA (a) and **1**-WGA (b) in acetate buffer (pH 4.5) at 298 K.

WGA recognizes specific carbohydrates such as GlcNAc and NeuNAc, as mentioned above. We investigated the specific saccharide-binding abilities of **1**-WGA by the following turbidity experiments [13] with



Scheme 1. Preparation of a cyclophane conjugate with WGA.

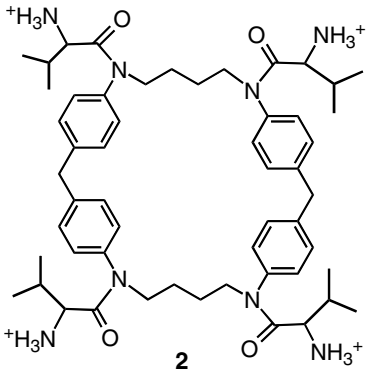
glycoproteins. Bovine fetuin, the predominant glycoprotein of fetal calf serum, is an α-globulin of 48 kDa [14], which has three *O*-glycosidically linked carbohydrate chains having terminal α-NeuNAc residues. It was found that the terminal multivalent ligands of fetuin were specifically recognized by WGA composed of several subunits, each having a saccharide-binding site, so that those interactions were conveniently monitored by a turbidity of the solution due to a cross-linked agglutination of these components. Agglutination behavior of the present **1**-WGA conjugates toward fetuin was examined by turbidity monitoring in aqueous 2-[4-(2-hydroxy-ethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) buffer (0.01 M, pH 7.0, μ 0.1 with KCl) (1 M = 1 mol dm^{-3}) at 298 K. Upon addition of fetuin to the HEPES buffer containing **1**-WGA conjugates, the solution becomes turbid very readily to give an insoluble material. In addition, minimum concentration of **1**-WGA to induce the agglutination of fetuin (500 μg/ml) was estimated to 20 μM which was comparable to that for native WGA (20 μM). Moreover, the turbid solution obtained from **1**-WGA conjugates and fetuin becomes again clear showing deagglutination, upon addition of a large excess amount of *N*,*N*′-diacetylchitobiose (GlcNAc disaccharide) (100 eq.) as a competitive inhibitor [14], while mannose never show such a deagglutination. These results indicate that the specific saccharide-binding abilities as well as the above-mentioned structural features of WGA were retained, when cyclophane **1** were covalently bound to the lectin.

In order to clarify characteristic features of the cyclophane-WGA conjugate as a host, we examined guest-binding behavior of **1**-WGA conjugate toward fluorescent probes such as 6-*p*-toluidino-naphthalene-2-sulfonate (TNS) and 8-anilinonaphthalene-1-sulfonate (ANS) in acetate buffer (0.01 M, pH 4.0, μ 0.1 with KCl) at 298 K, whose fluorescence emission is extremely sensitive to change in microenvironmental polarity [15] experienced by the molecule. Upon addition of TNS to an acetate buffer containing the **1**-WGA conjugates, the fluorescence intensity at 420 nm originated from TNS increased with saturation behavior (Figure 3a). On the other hand, control experiments employing native WGA did not show fluorescence changes (Figure 3b). These results indicate that TNS molecules were captured into the hydrophobic cyclophane cavities of **1**-WGA. The microenvironmental polarity experienced by the entrapped guest molecule was evaluated on the basis of a correlation between λ_{max} and solvent polarity parameter (E_T^N) [16] in a manner as described previously [10a, 10b]. The E_T^N value for TNS placed in the cyclophane cavity was estimated to 0.50 that was equivalent to values of *sec*-butyl alcohol and was smaller than that for the identical guest upon complexation with peptide cyclophane **2** [8a, 17] ($E_T^N = 0.84$) (Table 1). Under the conditions in an acidic buffer (pH 4.0), the microenvironment of the guest-binding site of **1**-WGA is hydrophobic on account of the carboxylic acids (non-ionic form) of the cyclophane. Binding constant (*K*) for complexes of **1**-WGA with TNS was calculated on the basis of Benesi–Hildebrand relationship in a manner

Table 1. Binding constants (*K*) for host–guest complexes of hosts with TNS and ANS, microenvironmental polarity parameter (E_T^N), and steady-state fluorescence polarization (*P*) for guest incorporated into cyclophanes in aqueous acetate buffer (0.01 M, pH 4.0, μ 0.1 with KCl)

Host	Guest	K, M^{-1}	E_T^N	P
1-WGA	TNS	4.2×10^4	0.50	0.27
2[a]	TNS	1.4×10^4	0.84	0.09
1-WGA	ANS	1.5×10^4	0.70	0.26
2[a]	ANS	1.4×10^3	0.73	0.10

[a] See also Ref. [10]a.



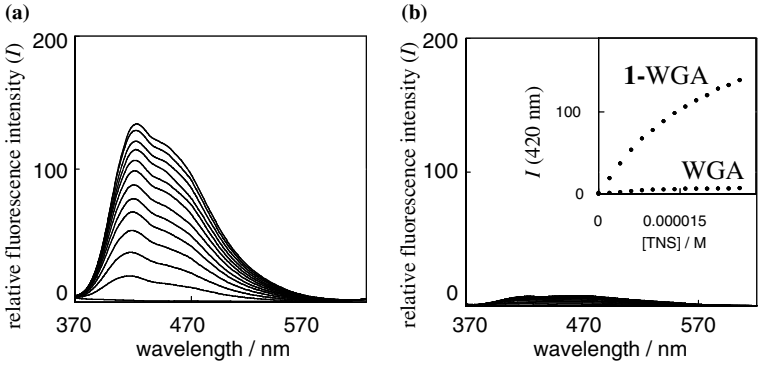


Figure 3. Fluorescence spectra of TNS at various concentrations (2–26 μM) (from bottom to top) in the presence of **1**-WGA conjugates (a) or WGA (b) in pH 4.0 acetate buffer. Ex, 326 nm. Inset: the corresponding fluorescence titration curves.

similar to that reported previously [10]; 4.2×10^4 M^{-1}. The binding affinity was 3-fold larger than that of **2** for the identical guest [10] in aqueous media (Table 1). The enhanced guest-binding affinity of **1**-WGA suggests that overall non-covalent interactions such as hydrogen-bonding and electrostatic forces, etc., between the guest and **1**-WGA become effective on the protein surfaces. In consideration of poor solubility of **1** itself in acetate buffer (pH 4.0), the conjugate affords advantages of water-solubilities as well as host-functions to **1** in acetate buffer. In addition, relatively large fluorescence polarization values (*P*) were obtained for entrapped guest molecule by **1**-WGA conjugates (0.27) and the obtained *P* value was much larger than that of **2** (0.09) [10]a and was comparable to those of albumin-bound guest probes [15] (0.26–0.28). A similar fluorescence feature on the guest-binding behavior of **1**-WGA was observed when ANS was employed as a guest (Table 1). These results indicate that a guest molecule incorporated into the cyclophane cavity of the conjugate is effectively desolvated and its molecular motion is remarkably repressed relative to those captured by **2**.

It is noteworthy that the fluorescence intensity originating from TNS decreased with increasing pH value of the buffers (Figure 4). Acid-dissociation equilibrium of carboxylic acids originating from cyclophane side-chains was reflected in the observed pH-dependent fluorescence spectra. The pK_a value for the carboxylic acid residues of **1**-WGA was evaluated to be 4.30 from the pH-fluorescence intensity profile and is almost comparable to those of phenyl propionic acid and butyric acid (pK_a = 4.40 and 4.57, respectively). Naturally, anionic TNS was not incorporated into the cavity of anionic host **1** in HEPES buffer (pH 7.0), reflecting an electrostatic repulsion [10]. The fluorescence spectral changes were also negligible upon addition of TNS to the aqueous HEPES buffer (pH 7.0) containing **1**-WGA. These results indicate that the characteristic molecular discrimination through hydrophobic and electrostatic interactions of **1** was effectively performed, when cyclophane **1** was covalently bound to WGA(see Scheme 2).

In conclusion, a cyclophane-WGA conjugate was successfully prepared by the simple procedures. The present cyclophane-WGA conjugates demonstrate enhanced guest-binding abilities toward fluorescent guests with marked motional repression in comparison with those of untethered peptide cyclophane as well as specific saccharide-binding abilities in aqueous media. By combining these capabilities, the WGA conjugates having host-function are expected to take an advantage for sophisticated molecular recognition by using cooperatively [6]. In addition, development of saccharide-directed molecular/drug (guest) delivery sys-

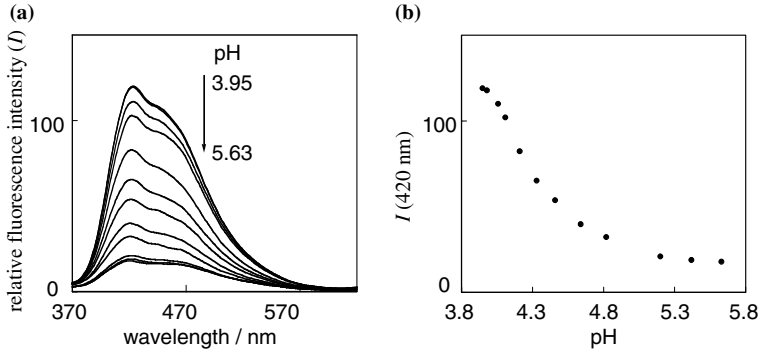
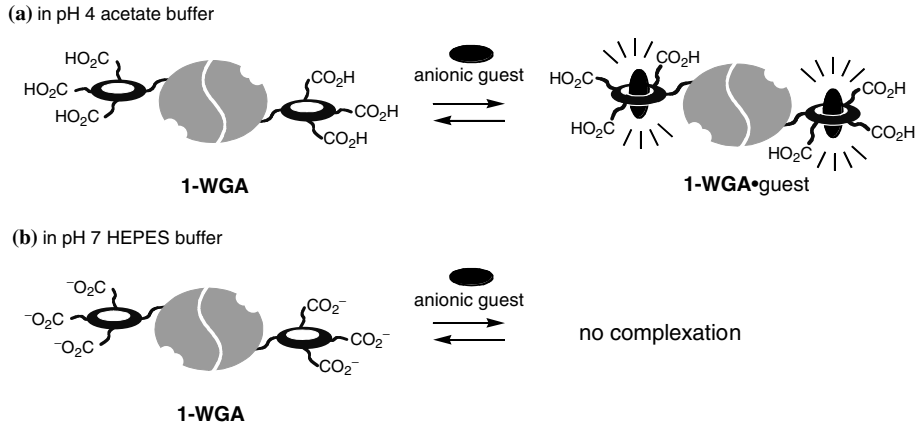


Figure 4. pH-titration of fluorescence spectra of TNS (20 μM) in the presence of **1**-WGA conjugate (a) and the corresponding pH profile (b).



Scheme 2. Schematic repesentation for guest-binding behavior of **1**-WGA with anionic guest in acetate buffer (a) and its molecular discrimination through hydrophobic and electrostatic interactions in HEPES buffer (b).

tems toward a cell surface displaying specific glycoproteins is quite promising by using this strategy.

Experimental

General methods. Elemental analyses were performed at the Microanalysis Center of Kyushu University. IR spectra were recorded on a Perkin-Elmer spectrum one spectrometer and ^{1}H NMR spectra were taken on a Bruker DRX 600 spectrometer. Fluorescence spectra were recorded on a JASCO FP-750 spectrophotometer by excitation at 326 and 365 nm for TNS and ANS, respectively, while CD spectra were run on a JASCO J-700 spectropolarimeter. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and FAB mass spectrometry were recorded on AppliedBiosystems Voyager DE-RP and JEOL JMS-HX110A spectrometer, respectively.

N,N′,N″,N‴-Tetrakis(3-carboxy-propionyl)-1,6,20,25-tetraaza[6.1.6.1]paracyclophane (**1**).
Succinic anhydride (160 mg, 1.6 mmol) was added to a solution of 1,6,20,25-tetraaza-[6.1.6.1]pacacyclophane (0.1 g, 0.20 mmol) and triethylamine (166μl, 1.2 mmol) in dry dichloromethane (10 ml) at room temperature, and the mixture was stirred for 12 h. Ethylenediamine (210μl, 3.2 mmol) was added to the mixture to quench the reaction. The solution was then washed with 0.1 N aqueous hydrochloride acid (30 ml) and saturated aqueous sodium chloride (30 ml) in this sequence. After being dried (Na_2SO_4), the solution was evaporated to dryness under reduced pressure. The resulting crude product was purified by gel filtration chromatography on a column of Sephadex LH-20 with methanol as eluent. The product fraction was dried in vacuo to give a white solid. 0.12 g (70%). **1**: 600 MHz, (DMSO-d_6, 298 K) δ 1.29 (m, 8H), 2.02 (m, 8H), 2.32 (m, 8H), 3.51 (m, 8H), 3.95 (s, 4H), 7.02 (d, J = 7 Hz, 8H) and 7.31 (d, J = 7 Hz, 8H). Found: C, 65.26; H, 6.24; N, 6.03%. Calcd. for $C_{50}H_{56}N_4O_{12}$•H_2O: C, 65.06; H, 6.33; N, 6.07%. HRFAB-MS: calcd. for $C_{50}H_{57}N_4O_{12}$: 905.3973 [MH^+]; Found 905.3973.

Preparation of **1**-WGA conjugate. WGA (0.1 mM (subunit basis)) was added to an aqueous HEPES buffer of **1** (0.1 mM), NHS (0.1 mM) and EDC (0.1 mM) and the mixture was stirred for 2 days at 293 K, and then ethanolamine was added to the mixture to quench the reaction. With further purification with dialysis (8 kDa cut-off) for 1 day, **1**-WGA conjugate was obtained.

Acknowledgements

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17. Cyclophane **2** having valine residues was used as an untethered reference host, because **2** was soluble in the acetate buffer and capable of providing a guest-binding hydrophobic cavity surrounded by valine residues.