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Helical Assembly of Azobenzene-Conjugated Carbohydrate Hydrogelators with Specific Affinity for Lectins

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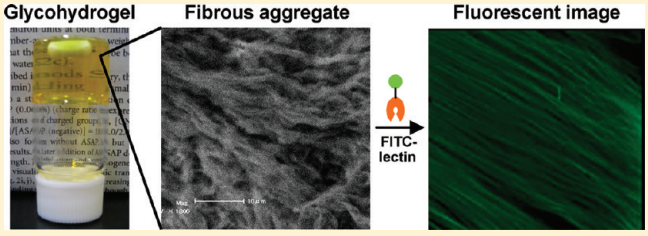
ABSTRACT: Carbohydrate-mediated interactions are involved in various biological processes via specific molecular assembly and recognition. Such interactions are enhanced by multivalent effects of the sugar moieties, and thus supramolecular sugar-assembly, i.e., spontaneous association of glycoamphiphiles, is a promising approach to tailor glycocluster formation. In this study, novel sugar-decorated nanofibers were successfully prepared by self-assembly of low molecular weight hydrogelators composed of azobenzene and disaccharide lactones. Circular dichroism measurement of the as-prepared hydrogels indicated that the azobenzene amphiphile containing a lactose moiety possessed (*R*)-chirality, while the maltose−azobenzene conjugate exhibited (*S*)-chirality, even though the cellobiose-conjugated azobenzene existed in an achiral form. This suggests that the chiral orientation of the chromophoric azobenzene depended on both the glycosidic linkages and the steric arrangement of hydroxyl groups in the conjugated carbohydrates. Lectin-binding and cell adhesion assays revealed that the nonreducing ends of the conjugated sugar moieties were exposed on the surfaces of self-assembled nanofibrous hydrogels, allowing them to be effectively recognized by the corresponding lectins. In addition, photoisomerization of azobenzene under ultraviolet irradiation induced the sol−gel transitions of the hydrogels. These results demonstrate that the reversibly transformed fibrous glycohydrogels show potential for application as carbohydrate-decorated scaffolds for cell culture engineering.



INTRODUCTION

Carbohydrates play essential roles in all living organisms, and interactions between carbohydrates and cellular proteins govern a wide variety of life processes including bacterial and viral attachment, immune systems, transplant rejection, fertilization, and cell proliferation/differentiation.[1,2] Despite such significant functions, monovalent carbohydrates generally have a low affinity for most proteins, with dissociation constants ranging from 10^{-4} to 10^{-3} M. Nevertheless, carbohydrate−protein interactions result in strong binding power and the specificity required to mediate biological processes through multivalent contact, i.e., the glycocluster effect.[3−5]

In all living systems, glycolipid conjugates form sugar assemblies on cell surfaces via morphological changes such as rafts and caveolae, and glycoproteins also possess sugar-clustered architectures.[6] Based on biomimetic approaches, many studies have been carried out to develop sugar-planted arrays, aiming at using cluster formation to overcome weak monovalent sugar−protein binding. Glycoconjugated polymers,[7−9] glycodendrimers,[10] glycoliposomes,[11,12] and sugar microarrays using self-assembled monolayers (SAM)[13,14] have been reported. Conventional approaches used to prepare glycoconjugated materials usually involve the formation of covalent bonds between sugar residues and designed substrates. Recently, amphiphilic glycolipids found *in vivo* have attracted attention because of their spontaneous association to form micelles, bilayers, and vesicles where dense populations of glyco moieties are present on their surfaces.[15−17] Herein, we are interested in the self-assembly of low molecular weight glycoconjugates into nanostructures to design multivalent sugar clusters.

Carbohydrate-based hydrogelators can form porous, three-dimensional entangled networks in water by hierarchical self-assembly.[18−25] Supramolecular hydrogelators are generally amphiphilic low molecular weight compounds and have received attention because of their unique self-assembly features. Such small organic molecules, even at low concentration (less than ca. 0.05 wt %), spontaneously form various nanostructures, e.g., nanofibers, nanosheets, nanoribbons, helices, and bundles, that can trap water molecules in interstitial spaces.[18−39] The driving forces for hierarchical self-assembly include intermolecular interactions such as $\pi-\pi$ stacking, hydrogen bonding, van der Waals interactions, hydrophobic association, and electrostatic attraction. In consequence, supramolecular glyco-based amphiphiles that form self-assembled nanostructures are expected to have the following advantages: (1) spontaneous hydrophobic association is possible in aqueous environments, and sugar moieties are exposed on the surfaces of the assemblies as hydrophilic head groups; (2) supramolecular aggregations form via physicochemical interactions, and thus a reversible sol−gel transition is expected in response to external stimuli; (3)

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multiple hydroxyl groups in carbohydrate moieties can both donate and accept protons to form various hydrogen bonds; and (4) most supramolecular gels are composed of nanofibers, which allow molecular recognition by proteins. These features can be used for biological applications such as a lectin array for saccharide detection,[21] wound healing,[23] and stimuli-responsive biomaterials.[37,39]

In carbohydrate-mediated interactions, the nonreducing ends of carbohydrate head groups bind with protein receptors.[40] Although studies have been carried out on monosaccharide-based hydrogelators,[19−25,39] oligosaccharide head groups[18] and their specificity for protein recognition have seldom been investigated. We have chosen disaccharide lactones as a hydrophilic, biofunctional headgroup because they are the simplest unit to examine the diversity of glycosidic linkages, anomeric configuration, and positions of hydroxyl groups in bioactive sugars. In addition, some natural homopolysaccharides are known to form structures with secondary order. For example, with regard to glucose (Glc) polymers, amylose (Glc(α1→4)Glc) forms a helical structure, and cellulose (Glc(β1→4)Glc) forms sheetlike structures.[41,42] Delicate intra- and intermolecular associations between polysaccharides induce the regular formation of higher-order structures; as a result, we are interested in the effects of the noncovalent assembly of saccharide moieties of sugar-based amphiphiles on the characteristics of ordered structures formed in supramolecular hydrogels.

In this study, three kinds of low molecular weight amphiphilic conjugates composed of disaccharide lactones and azobenzene with glycine as a connecting spacer were synthesized. The azobenzene chromophore was used as a hydrophobic group, aiming to allow strong $\pi-\pi$ interactions derived from the planar *trans*-azobenzene unit as well as reversible association via photoisomerization. Herein, we show that this supramolecular self-assembly strategy offers great potential form tailoring glycocluster formation for specific protein recognitions. Secondary and higher-order structures were investigated by spectroscopic and microscopic analyses. The ultraviolet (UV)-induced gel-to-sol transition, lectin binding, and cell adhesion behavior of the glyco-based structures are also discussed in detail.

EXPERIMENTAL SECTION

Chemicals. The following reagents were used as received: lactose, glycine, β-cyclodextrin, urea, chloroform, methanol (Wako Pure Chemicals, Osaka, Japan), maltose, cellobiose, di-*tert*-butyldicarbonate, 4-aminoazobenzene (Sigma-Aldrich), and fluorescein isothiocyanate-labeled *Ricinus communis* agglutinin 120 (FITC-RCA120), RCA120, concanavalin A (ConA), FITC-ConA (Seikagaku Co., Tokyo, Japan). *N*-(*tert*-Butoxycarbonyl)glycine (Boc-Gly) was synthesized by the conventional method.[43] A rat liver cell line (IAR-20) was provided by the Human Science Research Resources Bank (Osaka, Japan). William's medium E, penicillin−streptomycin, and trypsin were obtained from Invitrogen Corp. (Carlsbad, CA). Collagen gel (Cell Matrix Type I-P) and phosphate buffered saline (PBS) were purchased from Nitta Gelatin Inc. (Osaka, Japan), and Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. Tissue culture polystyrene (TCPS) dishes and plates (24-well) were obtained from Sumitomo Bakelite Co. Ltd. (Tokyo, Japan). Other chemical reagents were reagent grade and used without further purification.

General Analysis. Nuclear magnetic resonance (NMR) spectra were recorded on a JMN-AL400 spectrometer (JEOL Ltd. Tokyo, Japan) in a solution of DMSO-d_6 or $CDCl_3$. Melting point (T_m) measurement was performed at a heating rate of 5 °C min^{-1} using a differential scanning calorimeter (Pyrus 1, PerkinElmer Inc., San Jose, CA). Elemental analysis was performed with a CHN corder (MT-6, YANACO Co. Ltd., Kyoto, Japan).

Synthesis of Glycine−Azobenzene Conjugate. 2-Amino-*N*-*p*-phenylazophenyl acetamide (Gly-Azo): To Boc-Gly (0.37 g, 2.1 mmol) in chloroform (10 mL) cooled to 0 °C was added 1-ethyl-3-(*N*,*N*′-dimethylaminopropyl)carbodiimide hydrochloride (0.40 g; 2.1 mmol) and 1-hydroxbenzotriazole (0.30 g, 2.2 mmol), followed by 4-aminoazobenzene (0.40 g, 2.0 mmol) in chloroform (20 mL). The mixture was stirred for 2 h in an ice bath. The reaction medium was allowed to gradually reach ambient temperature overnight. The mixture was concentrated; the residue was redissolved in the minimum amount of chloroform and then washed sequentially with deionized water, 0.1 mol L^{-1} aqueous hydrochloric acid, deionized water, and finally saturated aqueous sodium hydrogen carbonate. The organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated. The concentrate was distilled under reduced pressure to afford Boc-Gly-Azo. Finally, the Boc protecting group was removed by treatment in trifluoroacetic acid/dichloromethane (1:1 by volume) for 1 h. The resulting trifluoroacetic salt was neutralized with triethylamine/chloroform and evaporated to dryness *in vacuo* to give Gly-Azo as a yellowish solid. Yield: 0.457 g (85.6%). ^{1}H NMR ($CDCl_3$): 9.65 (s, 1H), 7.96−7.80 (m, 7H), 7.51−7.45 (m, 3H), 3.52 (s, 2H). ^{13}C NMR ($CDCl_3$): 170.9, 152.7, 140.2, 130.7, 129.1, 124.1, 122.7, 119.4, 45.2. MALDI-TOF-MS (m/z): [M + H] = 255.12 (calcd), 255.58 (found); [M + Na] = 277.11 (calcd), 277.56 (found).

Synthesis of Disaccharide Lactone Derivatives. *N*-*p*-Phenylazophenyl acetamide-[*O*-β-D-galactopyranosyl-(1→4)]-D-gluconamide (LL-Gly-Azo): lactonolactone (LL) was prepared from lactose according to a reported protocol.[8,44] As-prepared LL (0.68 g, 2.0 mmol) and Gly-Azo (0.51 g, 2.0 mmol) were dissolved in methanol and heated under reflux for 5 h and then allowed to stand at room temperature to yield an orange-yellow crystal precipitate. The precipitate was filtered and then washed with methanol and chloroform to remove unreacted reagents. The purified product was dried under vacuum to give 0.98 g (82.7%) of yellowish crystals. The purity of the sample was confirmed by TLC (silica gel F254 precoated plates). T_m: 217 °C with decomposition. ^{1}H NMR (DMSO-d_6): 10.00 (s, 1H), 8.11 (t, 1H), 7.79−7.91 (m, 6H), 7.53−7.60 (m, 3H), 5.47 (d, 1H), 5.32 (d, 1H), 4.96 (d, 1H), 4.85 (d, 1H), 4.61 (t, 1H), 4.35−4.41 (dd, 2H), 3.97 (d, 2H). ^{13}C NMR (DMSO-d_6): 172.8, 168.0, 151.9, 147.5, 141.6, 130.9, 129.3, 123.6, 122.2, 119.3, 104.5, 82.97, 62.17, 60.48. Elemental analysis calculated for $C_{26}H_{34}N_4O_{12}$: C, 52.52; H, 5.76; N, 9.42%. Found: C, 52.52; H, 5.76; N, 9.39. MALDI-TOF-MS (m/z): 617.21 (calcd), 617.19 (found). The chemical structure of LL-Gly-Azo is shown in Chart 1.

N-*p*-Phenylazophenyl acetamide-[*O*-α-D-glucopyranosyl-(1→4)]-D-gluconamide (MAL-Gly-Azo): maltose was oxidized to yield maltonolactone (MAL) in the same manner as LL, and the respective conjugate was prepared from MAL and Gly-Azo using the method described above. MAL-Gly-Azo 0.78 g (65.2%) was obtained as a yellowish crystalline precipitate. T_m: 206 °C with decomposition. ^{1}H NMR (DMSO-d_6): 9.91 (s, 1H), 8.20 (t, 1H), 7.78−7.93 (m, 6H), 7.54−7.60 (m, 3H), 5.77 (d, 1H), 5.70 (d, 1H), 5.00 (d, 1H), 4.95 (d, 1H), 4.53 (t, 1H), 4.45−4.49 (m, 2H), 3.89−4.05 (m, 2H). ^{13}C NMR (DMSO-d_6): 172.8, 168.0, 151.9, 147.5, 141.6, 130.9, 129.3, 123.6, 122.2, 119.3, 100.9, 83.1, 69.8, 62.4, 60.5. Elemental analysis calculated for $C_{26}H_{34}N_4O_{12}$: C, 52.52; H, 5.76; N, 9.42%. Found: C, 52.52; H, 5.76; N, 9.39. MALDI-TOF-MS (m/z): 617.21 (calcd), 617.31 (found). The chemical structure of MAL-Gly-Azo is shown in Chart 1.

N-*p*-Phenylazophenyl acetamide-[*O*-β-D-glucopyranosyl-(1→4)]-D-gluconamide (CL-Gly-Azo): CL-Gly-Azo was synthesized from cellobionolactone (CL) and Gly-Azo using the procedure described above. CL-Gly-Azo 0.93 g (78.4%) was obtained as a yellowish crystalline precipitate. T_m: 211 °C with decomposition. ^{1}H NMR (DMSO-d_6): 10.10 (s, 1H), 8.11 (t, 1H), 7.80−7.91 (m, 6H), 7.53−7.60 (m, 3H), 5.48 (d, 1H), 5.32 (d, 1H), 5.00 (d, 1H), 4.95 (d, 1H), 4.61 (t, 1H), 4.35−4.41 (m, 2H), 3.97 (m, 2H). ^{13}C NMR (DMSO-d_6): 173.0, 167.9, 151.9, 147.5, 141.6, 130.9, 129.3, 123.6, 122.2, 119.3, 104.0, 82.7, 62.2, 61.2. Elemental analysis calculated for $C_{26}H_{34}N_4O_{12}$: C, 52.52; H, 5.76; N, 9.42%. Found: C, 52.52; H, 5.78; N, 9.39. MALDI-TOF-MS (m/z): 617.21 (calcd), 617.27 (found). The chemical structure of CL-Gly-Azo is shown in Chart 1.

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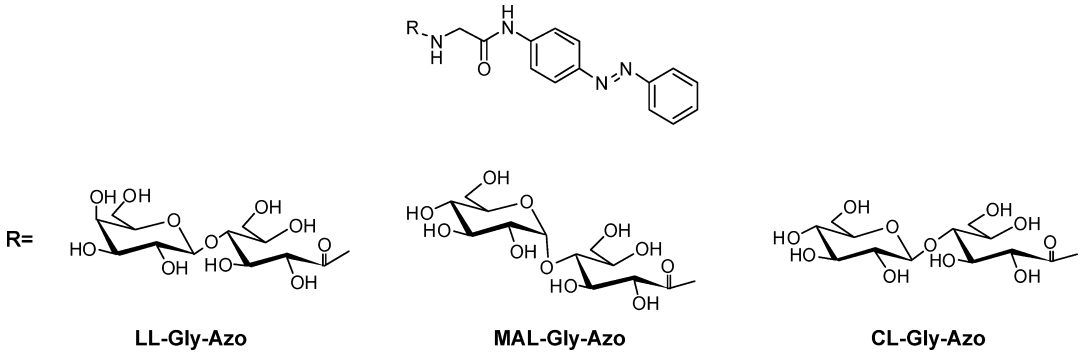
Chart 1. Amphiphilic Disaccharide–Azobenzene Conjugates[a]



[a]LL-Gly-Azo: *N*-*p*-phenylazophenyl acetamide-[*O*-*β*-D-galactopyranosyl-(1→4)]-D-gluconamide; MAL-Gly-Azo: *N*-*p*-phenylazophenyl acetamide-[*O*-*α*-D-glucopyranosyl-(1→4)]-D-gluconamide; CL-Gly-Azo: *N*-*p*-phenylazophenyl acetamide-[*O*-*β*-D-glucopyranosyl-(1→4)]-D-gluconamide.

Gelation Studies. In a typical gelation experiment, disaccharide amphiphile (10 mg) was put into a sealed sample tube with one of 10 different polar/nonpolar solvents (distilled water, methanol, ethanol, chloroform, acetone, *N*,*N*-dimethylacetamide, *N*,*N*-dimethylformamide, dimethyl sulfoxide, *n*-hexane, and toluene) to result in a concentration of 1% (w/v). Likewise, aqueous solutions of *β*-cyclodextrin (11 mM), and urea (4 M) or saline solutions, such as physiological saline or PBS, were subjected to gelation tests. The mixture was first stirred at ambient temperature and then heated around the corresponding boiling temperature until the solid material dissolved. The solution was set aside and allowed to cool to 25 °C for 24 h. The hydrogelation behavior of all disaccharide derivatives was tested with a mixture of designated amounts of samples (0.25–5.0 mg) in hot distilled water (0.5 mL), then allowed to cool, and stabilized at 25 °C for 24 h. The minimum hydrogelation concentration was determined using the inverted test tube method.

UV–vis and Circular Dichroism (CD) Spectroscopy. UV–vis and CD spectra were obtained using a J-720 CD spectrometer (JASCO, Japan) with a water-jacketed cylindrical quartz cell with a path length of 0.1 mm. Each sample (5.0 mg) was suspended in distilled water (1 mL), heated until it became clear, and then injected into the quartz cell, which was then carefully sealed. Thermoresponsive UV–vis and CD spectra were recorded from 20 to 70 °C. Data were collected from 600 to 250 nm, at 0.5 nm intervals with a scan speed of 200 nm min^{-1} and bandwidth of 1 nm.

LVSEM Observation. Low-voltage scanning electron microscopy (LVSEM; Wet-SEM, Shimadzu SS-550, Japan) was used to observe the *in situ* morphology of the hydrogels. A small piece of each hydrogel (0.5 wt %) was fixed on the sample holder without any additional treatment. Measurements were performed under a low vacuum of 20–100 Pa and acceleration voltage of 5–20 kV.

FITC-Labeled Lectin Binding Assay. In this study, two kinds of FITC-labeled lectins were used for lectin binding assays: one from *Ricinus communis* (RCA120) and another from *Canavalia ensiformis* (ConA). LL-Gly-Azo and MAL-Gly-Azo were dissolved in PBS solution and heated until a clear solution was obtained. The resulting hydrogels were cut into small pieces and then washed 5 times in PBS solution. Each piece of hydrogel was then immersed in FITC-RCA120 or FITC-ConA (20 $\mu g\ mL^{-1}$) in PBS solution for 30 s. The gel pieces were washed with PBS solution at least 3 times and then sandwiched between two sterile coverslips. Fluorescence microscopic images of the gel samples were captured using a Nikon 2000 confocal laser scanning microscope (CLSM; Nikon, Japan). Conjugated FITC moieties were visualized using excitation and emission wavelengths of 488 and 515 nm, respectively, at room temperature.

Cell Adhesion Test. Rat liver cells (IAR-20, JCRB Cellbank) were dispersed in a serum-free culture of William's medium E containing 5% antigen (penicillin and streptomycin). LL-Gly-Azo (0.5 mL, 0.5% w/v), and the collagen matrix solution (0.5 mL, 0.3% w/v) dissolved in William's medium E was poured into 24-well microtiter plates. A TCPS 24-well plate was used as a control. After gel formation and equilibration at 37 °C, IAR-20 cells (2.0×10^5 cells per well) were seeded on the top of the gels and then incubated at 37 °C for a designated time from 3 to 48 h in an incubator containing 5% CO_2. After the removal of suspended (unattached) cells by thorough rinsing with PBS, adhered cells were detached from the matrix surface by digestion treatment with 0.05% trypsin in PBS at 37 °C for 15 min, and then counted by a cell counter. Each cell assay test was repeated three times.

UV-Induced Sol–Gel Transition of Hydrogels. A high-pressure mercury lamp (500 W, Ushio, Tokyo, Japan) with the appropriate glass filter (UV-35+UVD-36C (365 nm), Asahi Technoglass, Tokyo, Japan) was used as an irradiation source. Hydrogel samples (0.3 wt %) were sandwiched to a thickness of 0.5 mm using two quartz plates covered with a photomask and set vertically onto a stand. UV light was then irradiated vertically on the sample cell through the photomask with a rectangular window (5 × 10 mm).

RESULTS AND DISCUSSION

Gelation Properties of Azobenzene-Conjugated Carbohydrates. All disaccharide derivatives synthesized in this report were insoluble in nonpolar solvents but readily soluble in aprotic polar solvents (*N*,*N*-dimethylacetamide, *N*,*N*-dimethylformamide, and dimethyl sulfoxide). However, the disaccharide conjugates consisting either of lactose (Gal(*β*1→4)Glc, LL-Gly-Azo) or maltose (Glc(*α*1→4)Glc, MAL-Gly-Azo) dissolved well in hot water (with or without saline), and clear gels were successfully formed after standing at room temperature for several hours. These hydrogels exhibited a thermoreversible sol–gel transition and were stable in the gel state for more than 10 months. In particular, LL-Gly-Azo possessed excellent hydrogelation ability, resulting in obvious water gels at a low concentration of 1.0 mg mL^{-1} (1.68 mM). However, no gelation occurred in an aqueous solution containing either *β*-cyclodextrin or urea. The former is well-known to form an inclusion complex with azobenzene,[45,46] and the latter can eliminate intermolecular hydrogen bonds between polysaccharides.[47] These results strongly suggest that both the hydrophobic effect of stacking azobenzene groups and the multiple hydrogen bonds of sugar moieties made a significant contribution to hydrogel formation.

On the other hand, the cellobiose derivative CL-Gly-Azo demonstrated different gelation behavior. CL-Gly-Azo did not form hydrogels up to 10% w/v within 24 h when dissolved in hot water (just below the boiling point), although it was readily soluble in water at room temperature, forming a clear gel. Considering the glycosidic linkage, cellobiose units in cellulose are connected to each other in a "*trans*" form; however, several experimental and theoretical investigations into the conformational preference of

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cellobiose and their derivatives have revealed that isolated and hydrated cellobiose units adopt a "*cis*" conformation bound by inter-ring hydrogen bonds.[48,49] These results suggest that water dynamics could play a critical role in determining the most stable structure of the cellobiose conjugate. This implies that the thermal history critically affected the formation of intra- and intermolecular hydrogen bonds of the cellobiose−azobenzene amphiphile so that it exhibited irregular gelation behavior. In addition, the CL-Gly-Azo hydrogel formed was fragile around the gel-to-sol transition temperature of ca. 30 °C, making this gel unsuitable for subsequent experiments. The most noteworthy point is that the difference in gelation behavior is caused by the change in position of just one hydroxyl group; LL-Gly-Azo is a C4-epimer of the nonreducing end of CL-Gly-Azo. These results indicate that the affinity of these compounds for water depends on the rotation angle of the sugar headgroup, directly affecting their gelation behavior.

Morphology of Self-Assembled Hydrogels. Hydrogels consisting either of LL-Gly-Azo or MAL-Gly-Azo are representative physical gels that form through noncovalent associations such as hydrophobic and hydrogen bonding interactions. It is well-known that low molecular weight gelators, namely nonpolymeric molecular ones, enable construction of nanoscale suprastructures, such as planar ribbons, helical ribbons, nanofibrils, and nanosheets. A variety of microscopic techniques have been used to visualize hydrogels, such as SEM, transmission electron microscopy, and atomic force microscopy.[18−20,22−26,31−39] In this research, the gel-state morphology of LL-Gly-Azo and MAL-Gly-Azo was directly observed using LVSEM. This method allows us to observe a quasi-actual hydrogel in a wet state, without any conductive coating. Therefore, LVSEM images are regarded as being essentially reflected *in situ* images of the hydrogels in water. As expected, these hydrogels formed the entangled structures of nanofibrous assemblies, as shown in Figure 1. Both gelators appeared to have

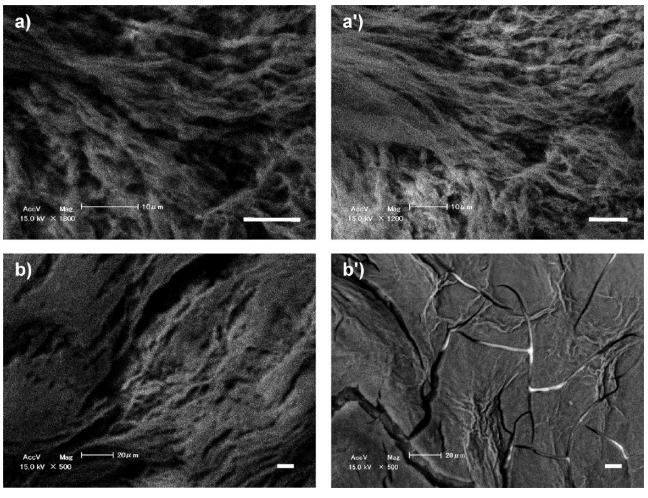

Figure 1. LVSEM images of (a, a′) LL-Gly-Azo and (b, b′) MAL-Gly-Azo. [Sample] = 0.5% w/v. Scale bars correspond to 10 μm.

a twisted structure but did not form well-defined helices. Interestingly, the LVSEM images of the LL-Gly-Azo hydrogel (Figure 1a,a′) and MAL-Gly-Azo (Figure 1b,b′) suggested that numerous thin fibrils aligned in the same direction were intertwined into bundles of larger fibrils, which conserved the tertiary structure of the hydrogels. The secondary structure of aggregates of amphiphilic molecules in water has been investigated previously; e.g., simple micelles can be converted to ellipsoidal and then cylindrical micelles when their concentration is increased.[27,28] Similar phenomena may occur for both LL-Gly-Azo and MAL-Gly-Azo, which possibly form cylindrical micellar fibrils in which hydrophobic azobenzene moieties are directed to the inside of micelles and the hydrophilic carbohydrate head groups exposed outward are in contact with surrounding water molecules. In other words, it is presumed that the sugar moieties self-assemble on the surfaces of the thin micellar fibrils, so they are present on the surface of the entangled nanofibers.

Supramolecular Structures of Sugar-Conjugated Hydrogelators. The geometrically rigid and longitudinal structures of azobenzene molecules make them ideal mesogens. Several studies have been carried out investigating the ordered formation of azobenzene-containing amphiphiles,[20,26,33−37] and their light absorption spectra reflect the secondary structure of their aggregates. Thus, UV−vis absorption and CD spectral examinations were performed in solution and gel states to obtain direct evidence for the stacking of azobenzene moieties and formation of chiral aggregates in water. Figure 2 shows the

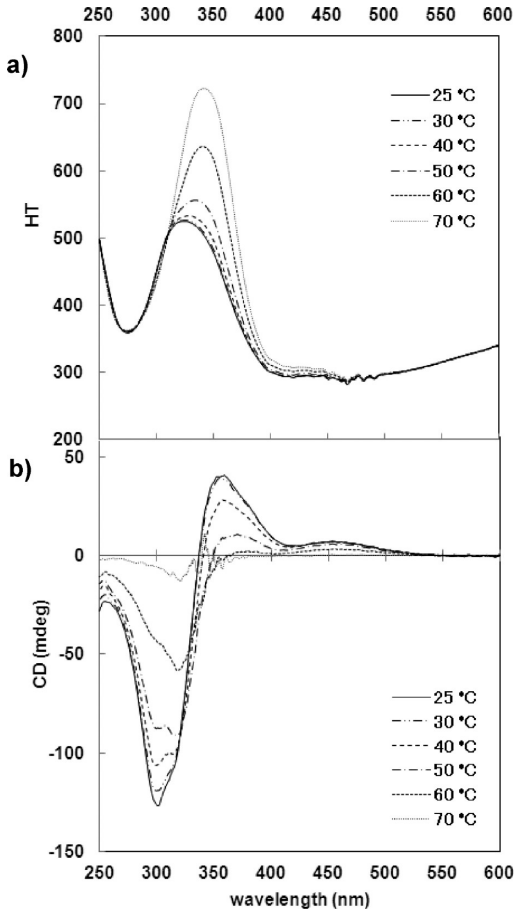

Figure 2. Temperature dependence of (a) UV−vis absorbance profiles and (b) corresponding CD spectra for 0.5% w/v aqueous LL-Gly-Azo.

temperature-dependent profiles of the CD spectra and high-tension (HT) voltage of a solution of LL-Gly-Azo. Upon heating, the CD intensity gradually decreased, possibly implying the dissociation of chiral aggregation.[50] Moreover, the CD intensity approached zero above 70 °C, which implies the absence of regular structures (isotropic state). The HT

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signal, which provides a direct measure of the quantity of light being produced and passed through the sample, is roughly proportional to the absorbance. At 75 °C in a sol state, LL-Gly-Azo exhibited a typical absorption spectrum of a monomeric azobenzene derivative with a weak n → π^* transition at 450 nm and an intense $\pi \rightarrow \pi^*$ transition at 330 nm. Upon cooling to ambient temperature, the main peak broadened, decreased in intensity, and exhibited a blue shift ($\Delta\lambda_{max}$ = 16 nm) compared with that for the sol state (Figure 2a). This spectral change provides evidence for H-type aggregation (card-pack array) of the chromophore, which has indeed been observed for most amphiphilic azobenzene derivatives in aqueous solution.[20,33,36,51] A similar shift was also observed for a solution of MAL-Gly-Azo, although no significant profile was displayed for a solution of CL-Gly-Azo dissolved in hot water (sol state) (Figure 3). Such blue shifts of the absorption spectra of fibrillar

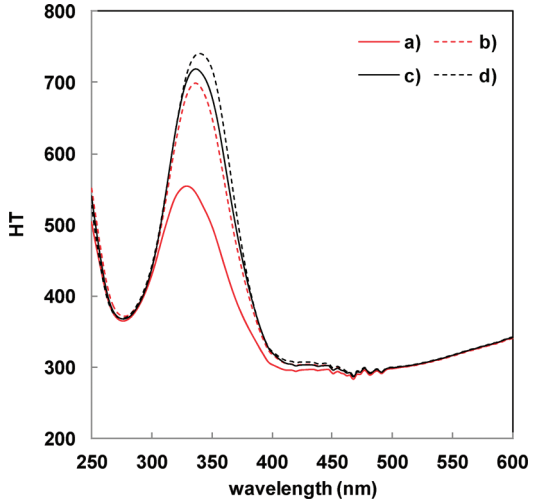

Figure 3. UV−vis absorbance spectra of an aqueous solution of MAL-Gly-Azo at (a) 25 °C and (b) 50 °C and for a solution of CL-Gly-Azo at (c) 25 °C and (d) 50 °C. [Sample] = 0.5% w/v.

assembly inside the hydrogels are explained by taking into consideration the H-type π-stacking of azobenzene chromophores in the conjugate.

Further evidence for nanofiber formation is provided in the CD spectra of aqueous solutions of LL-Gly-Azo and MAL-Gly-Azo in a gel state (25 °C) shown in Figure 4. Both amphiphiles were CD-active, and their zero-crossing point was close to their absorption maximum (around 330 nm), indicating the CD Cotton effect was possibly caused by exciton coupling bands forming between the stacked azobenzene chromophores. The CD intensity of the same concentration of LL-Gly-Azo and MAL-Gly-Azo was relatively similar although the polarity was reversed. This stark contrast means they exhibit opposite helical chirality in an aggregated state. LL-Gly-Azo gel shows a positive first Cotton effect and negative second Cotton effect, indicating a right-handed helical structure, i.e., (*R*)-chirality. In contrast, the inverted CD spectrum was observed for the hydrogel of MAL-Gly-Azo, indicating a left-handed chiral arrangement, i.e., (*S*)-chirality. Several chiral glycohydrogelators[20,22,25] and glycopolymers[52−54] with different sugar head groups have been reported; however, there are few investigations regarding helical inversion of aggregates induced only by the structure of the sugar head groups.[55]

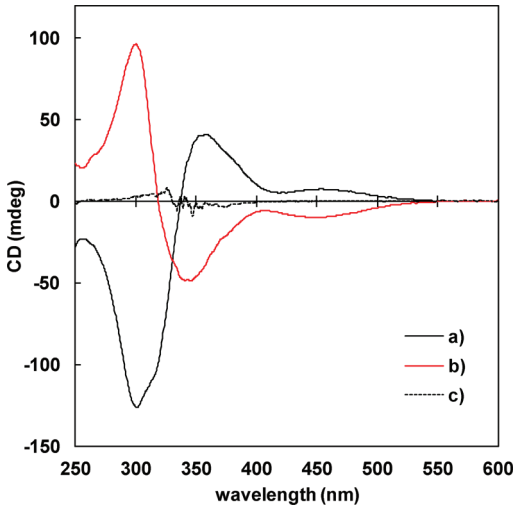

Figure 4. CD spectra of 0.5% w/v of (a) LL-Gly-Azo, (b) MAL-Gly-Azo, and (c) CL-Gly-Azo at 25 °C. All samples were dissolved in hot water and stored overnight at room temperature.

Because LL-Gly-Azo and MAL-Gly-Azo have different molecular structures only for the disaccharides, the dominant factor determining helicity must be the steric structure of the saccharide headgroup. Assuming that hydrophobic forces have no influence on the precise formation of hydrogen bonding,[56] the steric position of hydroxyl groups are of great importance to stabilize the chiral organization through hydrogen bonding interactions. Furthermore, the intermolecular hydrogen bonds derived from the amide groups of the Gly linkers as well as $\pi-\pi$ stacking of the azobenzene groups possibly assisted the formation of microscopic fibrous nanostructures of both hydrogels.

Surface Glyco-Decoration Determined by FITC-Labeled Lectin Assay. Spectroscopic and microscopic studies suggested that as-synthesized amphiphilic sugar−azobenzene conjugates form micelle-like aggregations, with the sugar residues gathered closely together on the surfaces of the twisted cylindrical fibers. Hence, a lectin-binding assay was carried out to elucidate the presence of sugar moieties on the surfaces of the self-assembled glycohydrogels that the lectin can access to bind. Lectin has considerable specificity for binding to carbohydrates; RCA120 can react with β-D-galactosyl residues, and ConA can interact with α-D-mannosyl and α-D-glucosyl residues.[40,57] In this research, CLSM observation was applied using FITC-labeled lectins to visualize the sugar assembly on the hydrogels. Figure 5 shows fluorescent CLSM images of hydrogels of LL-Gly-Azo and MAL-Gly-Azo bound to respective FITC-lectins. A clear fibrous fluorescent image was observed for the LL-Gly-Azo gel treated with FITC-RCA120 (Figure 5a), and MAL-Gly-Azo with FITC-ConA (Figure 5d) was also visualized clearly. Thus, the nonreducing end group of the carbohydrate structure of each gelator, either Gal on the LL-Gly-Azo fiber or Glc on the MAL-Gly-Azo fiber, were effectively recognized by the corresponding lectins for a very short time within 30 s, in comparison to the lectin assay system using hydrogelators as proposed by Hamachi et al., e.g. 30 min.[21] It was presumed that the sugar-clustering effects via azobenzene packing possibly led to such a higher affinity for the corresponding lectin. By contrast, undesirable nonspecific binding was not observed because no fluorescent signal was detected for ConA−LL-Gly-Azo and RCA120−MAL-Gly-Azo pairs (Figure 5b,c). Consequently, it is expected that these sugar assemblies could act as sugar−protein recognition

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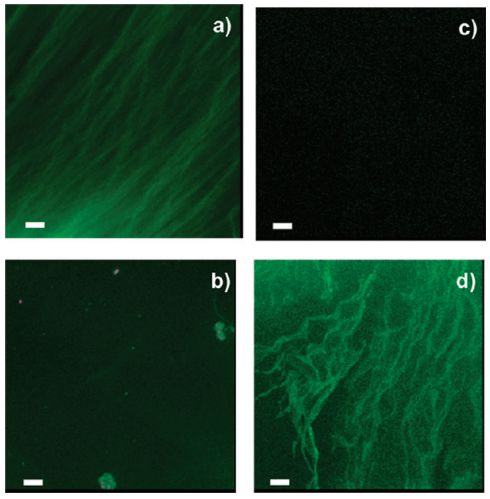

Figure 5. Fluorescent images of LL-Gly-Azo hydrogels treated with (a) FITC-RCA120 and (b) FITC-ConA and MAL-Gly-Azo hydrogel treated with (c) FITC-RCA120 and (d) FITC-ConA. Each compound was dissolved in PBS (0.5% w/v, pH 7.2). Scale bars correspond to 10 μm.

sites through their highly packed and intertwined structures, which results in their high affinity and selectivity for lectins.

Cell Adhesion Assay. The presence of a Gal-specific receptor on the surfaces of rat hepatocytes is responsible for the selective uptake of partially deglycosylated glycoproteins.[44,58−60] The LL-Gly-Azo hydrogel designed in this study has dense Gal residues; thus, a cell adhesion assay was carried out to elucidate the function of the hydrogel as a biointerface. Figure 6 shows

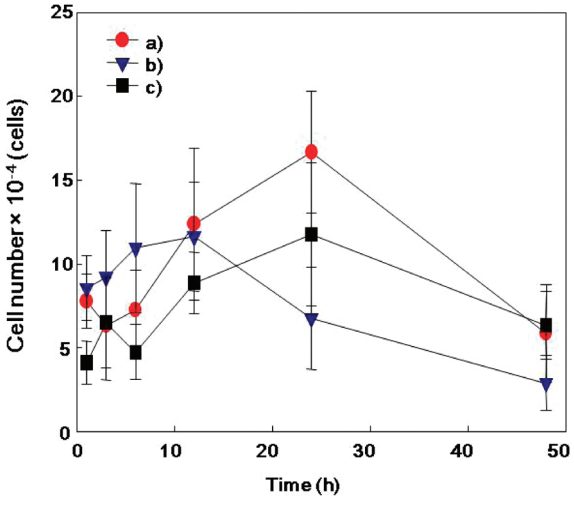

Figure 6. Cell adhesion behavior on (a) 0.5% w/v LL-Gly-Azo gel, (b) 0.3% w/v collagen gel, and (c) TCPS plate following incubation for 1−48 h.

the cell adhesion behavior of rat hepatocytes in serum free culture. The LL-Gly-Azo gel demonstrated higher cell adhesion than the collagen gel and TCPS plate. After incubation for 24 h, cell attachment reached up to 83% on LL-Gly-Azo, although it was less than 60% and 35% for TCPS and collagen gel, respectively. As mentioned above, a lectin binding assay with RCA-120 suggested that the Gal residues were effectively clustered on the surfaces of the self-assembled fibers, and thus such initial hepatocyte attachment was possibly dominated by the clustered Gal residues directly interacting with the asialoglycoprotein receptors on the rat liver cell surfaces.[61−63] Azobenzene may have some kinds of cytotoxic influences on cell culture applications; however as-synthesized sugar-conjugated azobenzenes were densely stacked in a stable gel state to form the fibrous micelles entangled each other, resulting in negligible cytotoxicity and good cell adhesion behavior. Therefore, an increasing trend in the cell attachment on the biocompatible LL-Gly-Azo hydrogel was attributed to the direct, biological recognition of Gal residues on the fibrous aggregates. Other types of biological cells having maltose- and/or glucose-recognizing receptors would interact with MAL-Gly-Azo gel, and such a tunable combination is regarded as promising in biointerface applications.

UV-Induced Sol−Gel Transition Behavior. In an isotropic state, azobenzene derivatives in solution undergo photoinduced *trans*/*cis* isomerization under UV irradiation, while *cis*-to-*trans* back-isomerization can be activated thermally or by visible light. The azobenzene moiety in an H-type aggregate state, however, is known to show remarkable resistance to UV-induced isomerization. Suppression of photoisomerization of azobenzene moieties has been observed in tightly packed, rigid aggregates, so in this case the reversible photoresponse of azobenzene is limited.[26,33−36] Therefore, the hydrogels were irradiated with high-power UV light to examine the effect of photoisomerization of azobenzene moieties of sugar conjugates. The hydrogels were exposed to high-intensity UV light (365 nm, ca. 10 mW cm^{-2}) through a rectangular pattern in a photomask.

A typical example of UV-exposure testing for the LL-Gly-Azo gel is shown in Figure 7. Upon irradiation with UV light, only the exposed area of the gel gradually melts to the bottom of the sample cell. This observation implies the disruption of aggregation caused by *trans*-to-*cis* photoisomerization of the azobenzene moieties. Moreover, the resulting sol gelated again within 12 h under weak visible light (Figure 7) because of back-isomerization that promotes the reaggregation of azogelator molecules in a *trans* form. A similar reversible sol−gel transition phenomenon was also observed for the hydrogel of MAL-Gly-Azo, and such a reversible sol−gel transition behavior is expected to be useful for practical cell culture applications.

Because photoisomerization of azobenzene moieties occurs with a large change in molecular geometries, this process is allowed to proceed only when there is sufficient space present around the azobenzene units.[26,64] In the case of our disaccharide−azobenzene hydrogelators, the bulky structure of the sugar headgroup, which can form higher order structure in the hydrogen bonding network, possibly caused the azobenzene mesogenic cores to be loosely packed, so they had enough room to undergo a conformational transition to the *cis* form. During photoisomerization of azobenzene, molecular polarity changes as well as molecular shape. Therefore, two possible mechanisms can be considered: one is the steric effect of the conjugated azobenzene, and another is the increasing solubility of the gelator induced by variation in molecular polarity, resulting in reduced hydrogen bonding between sugar moieties. As mentioned above, because these hydrogelators slowly form a gel again, it is possible to tailor heterogeneous glyco-patterned gels by the combined use of other hydrogelators with different sugar head groups under controlled UV irradiation. Such reversible, tunable glyco-based interfaces are promising for cell culture applications.

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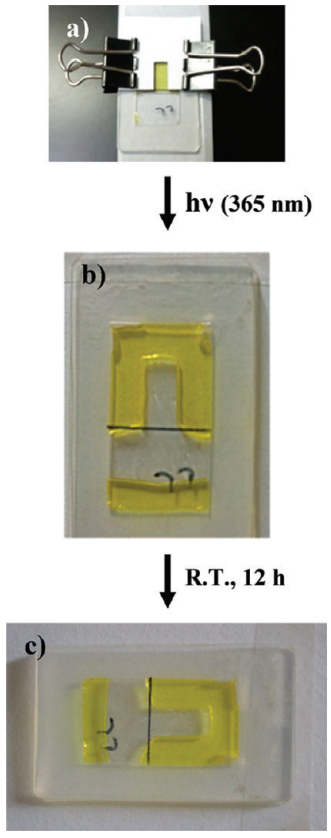

Figure 7. UV induced gel−sol patterning of LL-Gly-Azo hydrogel (0.3% w/v); photographs of hydrogel (a) with a photomask containing a rectangular window of 5 × 10 mm before UV irradiation, (b) after UV light irradiation, and (c) after leaving at room temperature for 12 h.

CONCLUSION

Novel sugar-decorated nanofibrous hydrogels were successfully prepared via self-assembly of low molecular weight supramolecular amphiphiles composed of azobenzene and disaccharide lactones. LVSEM observation roughly suggested that LL-Gly-Azo and MAL-Gly-Azo self-assembled to form helical thin nanofibers entangled each other. The intermolecular interactions of azobenzene conjugated to disaccharides in a gel state were evidenced by UV−vis absorption and CD measurements. Upon cooling to form hydrogels, azobenzene moieties stacked to form an H-type aggregate. CD measurements also revealed that the azobenzene moieties of LL-Gly-AZO were organized with right-handed helicity, and thus the eventual chirality of the sugar−azobenzene conjugates depended greatly on both the nonreducing end structure of the sugar moieties and the type of glycosidic linkages. FITC-labeled lectin assays indicated that Gal and Glc residues were exposed on the surfaces of self-assembled nanofibers composed of LL-Gly-Azo and MAL-Gly-Azo, respectively. Furthermore, a rat hepatocyte adhesion study using LL-Gly-Azo gel revealed that the glyco-based hydrogelator system provided a bioactive interface for cell attachment. These hydrogels also exhibited a reversible sol−gel transition in response to temperature and UV irradiation. These glycohydrogels have unique nanostructures with sugar clusters, and are expected to promote the functional design of cell culture scaffolds via glycoreceptor-mediated cell−cell communication.

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Notes

The authors declare no competing financial interest.

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