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Interactions of *N*-acetyl-D-glucosamine-conjugated silk fibroin with lectins, cytoskeletal proteins and cardiomyocytes

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Abstract

We have reported that cytoskeletal proteins such as desmin and vimentin are expressed on the surface of muscle, mesenchymal and cancer cells, and possess *N*-acetyl-β-D-glucosamine (β-GlcNAc) residue-binding properties. As cell-recognizable β-GlcNAc residue-bearing biopolymer, we prepared glycoconjugates (SF-GlcNAc) composed of silk fibroin (SF) and monosaccharide *N*-acetyl-D-glucosamine (GlcNAc) by chemical modification using cyanuric chloride. The covalent immobilization of GlcNAc into SF was assessed by ^{1}H-NMR measurements. The ^{1}H-NMR spectrum of SF-GlcNAc conjugates showed new peaks attributed to the methyl protons of the *N*-acetyl group in GlcNAc, and the integration of these peaks revealed that the GlcNAc content in the conjugates was 9 wt%. The existence of β-GlcNAc residues in SF-GlcNAc was examined by the criteria using lectins such as wheat germ agglutinin (WGA). Addition of WGA to SF-GlcNAc solution caused an increase in the turbidity of the solution due to lectin-mediated aggregation. Solid-phase lectin binding assay based on the biotin-avidin interaction showed that biotinylated succinylated WGA bound more strongly onto SF-GlcNAc conjugate-coated wells compared to SF-coated well. Following the establishment of the existence of β-GlcNAc residues in SF-GlcNAc, the interaction of SF-GlcNAc with desmin was examined by enzyme-linked immunosorbent assay using anti-desmin antibody. The stronger binding of desmin was observed for SF-GlcNAc conjugate-coated wells compared to SF-coated wells. The use of SF-GlcNAc conjugates as a substrate for culturing desmin-expressing human cardiac myocytes demonstrated an increase in the numbers of attached cells and proliferating cells on the conjugate-coated wells compared to SF-coated wells. These results suggest that the immobilization of monosaccharide GlcNAc is a useful method for the versatile functionalization of SF as an application in tissue engineering.

Keywords: *N*-acetyl-D-glucosamine, silk fibroin, glycoconjugates, desmin, cardiomyocytes

1. Introduction

Silk fibroin (SF) is a natural fibrous protein produced by the *Bombyx mori* silkworm and SF fibers have been historically used for textiles and surgical sutures. It has been recently found that an aqueous SF solution prepared by dissolving SF fibers in highly concentrated salt solution can be reformed into hydrogels, tubes, sponges, fibers, microspheres, and thin films [1-5]. The reformed SF has been applied in the biomedical field, in particular as substrates for cell culture and tissue engineering due to its mechanical properties and biocompatibility [1-3].

Since *Bombyx mori* SF protein does not have selective cell binding sites, the introduction of cell recognition peptide motifs, namely, Arg-Gly-Asp (RGD) into SF has been performed by genetic or chemical modification to enhance cell attachment [3,6,7]. It is also known that β-galactose sugar residues are specifically recognized by the asialoglycoprotein receptor, a hepatic lectin, on the surfaces of hepatocytes [8,9] and promote hepatocyte attachment [10,11]. Therefore, synthetic or natural polymers endowed with β-galactose residues have been developed as hepatocyte-specific culture substrates [10-15]. We previously prepared lactose-SF glycoconjugates (Lac-CY-SF) by chemical modification of amino acid residues of SF with lactose bearing the β-galactose residue using cyanuric chloride (CY) as a coupling reagent [16]. In order to change drastically the properties of SF, it was necessary to introduce as much lactose into SF as possible. Since the tyrosine residue is the most abundant among reactive amino acid residues in SF, CY, which can react with both the tyrosine residue and saccharides, was selected as a crosslinking reagent [16,17]. Studies on cell culture using Lac-CY-SF conjugates and hepatic cells, including rat primary hepatocytes [16,18] and human hepatocellular carcinoma-derived cells [19], revealed improved cell attachment and spheroid formation, suggesting that Lac-CY-SF conjugates are promising candidates as a substrate for hepatic cells.

Vimentin and desmin are type III intermediate filament proteins expressed in

mesenchymal cells and muscle cells such as skeletal, cardiac and smooth muscle cells, respectively [20]. These cytoskeletal proteins play an important role in the stabilization of cell architecture and structure [21-23]. Recent studies indicated that cytoskeletal proteins are highly expressed in lesion sites of various chronic diseases, for example amyloidosis and cancer, and can be used as a diagnostic marker [24-26]. Additionally, we reported that vimentin and desmin are expressed on the surface of mesenchymal, muscle and cancer cells, and possess *N*-acetyl-β-D-glucosamine (β-GlcNAc) residue-binding lectin-like properties [27-29]. It was found that these vimentin- and/or desmin-expressing cells specifically interact with β-GlcNAc residue-bearing synthetic polymers [27-30]. Based on the above findings, we aimed to apply β-GlcNAc residue-bearing SF to a substrate for mesenchymal cells including muscle cells in this study.

We previously reported the preparation of conjugates of SF with β-GlcNAc oligomers, namely *N*-acetyl-chito-oligosaccharides (NACOS) using CY as a coupling reagent [17]. However, it was clarified that a terminal β-GlcNAc monosaccharide residue has an essential role in the interaction of glycopolymers with vimentin and/or desmin-expressing cells while the repetition of β-GlcNAc residue is not essential for the interaction [29]. In the present study, we carried out chemical modification of SF with an easily available monosaccharide, *N*-acetyl-D-glucosamine (GlcNAc), using CY to prepare new glycoconjugate (SF-GlcNAc). Although SF-GlcNAc was considered to be a glycoconjugate endowed with both α-GlcNAc and β-GlcNAc residues based on our previous study [17], the covalent immobilization of the essential β-GlcNAc residue into SF was confirmed by examinations using lectins, wheat germ agglutinin (WGA), specific for β-GlcNAc residues. We then assessed the interaction of SF-GlcNAc with cytoskeletal protein, desmin. Moreover, attachment and growth of human cardiac myocytes (HCM), that express desmin on their surfaces, were investigated on SF-GlcNAc conjugate-coated wells of microplates.

2. Materials and methods

2.1. Materials

GlcNAc, CY, *N,N'*-diacetylchitobiose ($(GlcNAc)_2$) and phosphate buffer powder (1/15 M, pH 7.0) used for the preparation of 150 mM NaCl-67 mM phosphate buffer of pH 7.0 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals used for chemical modification were commercial reagent-grade products. WGA and biotinylated succinylated WGA (biotin-sWGA) were obtained from Seikagaku Co. (Tokyo, Japan) and Vector Laboratories, Inc. (Burlingame, CA), respectively. Neutravidin-horseradish peroxidase (HRP) conjugate and 3,3',5,5'-tetramethylbenzidine (TMB) substrate for HRP (TMB/E Substrate) were purchased from Thermo Fisher Scientific (Waltham, MA) and Merck Millipore (Billerica, MA), respectively. Recombinant desmin was prepared as previously described in our report [29]. Rabbit polyclonal anti-desmin antibody and HRP-conjugated anti-rabbit antibody were purchased from Thermo Fisher Scientific and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), respectively. HCM and Myocyte Growth Medium were supplied by PromoCell (Heidelberg, Germany). Millex-HV syringe-driven filter unit (pore size 0.45 μm) was obtained from Merck Millipore. Cell Count Reagent, that contains a water-soluble tetrazolium salt, WST-8, was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Preparation of SF-GlcNAc and SF

An aqueous solution of SF was prepared from *Bombyx mori* silkworm cocoons as previously described [16-19]. Briefly, the cocoons were degummed with boiling 0.5% (w/v) Na_2CO_3 solution and rinsed with hot water to remove sericin protein from SF fiber. The purified SF fiber was dissolved in 9M LiBr aqueous solution at 60 °C over 30 min. Then, the

dissolved solution was dialyzed against distilled water to obtain 1~2% (w/v) SF solution. It is known that degumming with alkaline solution and dissolution in concentrated salt solution induced degradation of SF [31,32]. Since low molecular weight SF fragments were removed by subsequent dialysis, the solution contained SF having the heterogeneous molecular weight of more than 20~30 kDa [31]. An aqueous solution containing depolymerized SF can be preserved without protein association in the refrigerator for several months. Amino acid analysis of the lyophilized product from the obtained SF solution indicated that the content of the tyrosine residue was 5.2 mol% [17]. This is exactly the same value as calculated for the intact SF protein based on amino acid sequences of the heavy chain fibroin (H-chain), the light chain fibroin (L-chain) and glycoprotein P25, and their composition, H-chain : L-chain : P25 = 6:6:1 [33].

As shown in Fig. 1, SF-GlcNAc conjugates were synthesized by the chemical modification of amino acid residues in SF with monosaccharide GlcNAc using CY as a coupling reagent. Based on our previous study on the modification of SF with NACOS using CY [17], it is suggested that CY-activated GlcNAc was prepared by the reaction between the anomeric hydroxyl group of GlcNAc and a chlorine atom of CY, after which a second chloride atom of CY-activated GlcNAc reacted with the phenolic hydroxyl group of the tyrosine residue (5.2 mol%) and the ε-amino group of lysine residue (0.3 mol%) in SF (Fig. 1).

A solution of 61 mg (280 μmol) GlcNAc in 4 mL distilled water was cooled in an ice bath. To the GlcNAc solution, 52 mg (280 μmol) CY in 1.3 mL 1,4-dioxane was slowly added at 4 °C while the pH of the mixture was adjusted to about 9 with 20% (w/v) Na_2CO_3 solution. The reaction mixture was further stirred at 4 °C and pH 9 for 2 h to prepare CY-activated GlcNAc. The solution of CY-activated GlcNAc (280 μmol assuming 100% reaction efficiency) was used for the following reaction with SF without purification. To the CY-activated GlcNAc solution, 2.7 mL of 1.48% (w/v) SF aqueous solution containing 40 mg SF

was added. Since the average molecular weight of the amino acid unit constituting SF is 75 [16,17], it was estimated that 40mg SF contained 28 μmol (= 0.040 × 0.052/75) of tyrosine residues. The mixture was then incubated at 37 °C and pH 9 for 3 h to make tyrosine and lysine residues of SF react with CY-activated GlcNAc. The mixture was neutralized with 1N HCl solution to stop the reaction. The resulting product SF-GlcNAc was purified from a large excess of unreacted CY-activated GlcNAc by dialysis against distilled water, followed by concentration using ultrafiltration in the same way as described previously [17]. The concentrated SF-GlcNAc solution was cast onto polyethylene film and dried in an ambient relative humidity at room temperature. The prepared SF-GlcNAc films were further dried in a desiccator containing P_2O_5 for several days and 43 mg SF-GlcNAc product was obtained.

2.3. ^{1}H-NMR measurements

^{1}H-NMR spectra were recorded in D_2O at 750 MHz with a Bruker Avance 750 NMR spectrometer (Bruker, Billerica, MA). The residual HDO peak was used as an internal chemical shift standard. NMR samples of SF-GlcNAc, SF and a mixture consisting of SF and monosaccharide GlcNAc (the weight ratio of GlcNAc to SF was 0.25) were prepared by dissolving SF-GlcNAc films or SF films, which were obtained in a similar manner to that of SF-GlcNAc films, in D_2O respectively. These NMR samples included 1~2% (w/v) SF. In order to improve the quality of ^{1}H-NMR spectra of SF-GlcNAc and the mixture, the WATERGATE method was used for suppression of the water peak [34,35].

2.4. Lectin-induced agglutination assay

The lectin-induced agglutination of SF-GlcNAc was detected by the change in absorbance at 350 nm of SF-GlcNAc solution at room temperature using a JASCO V-550 UV/vis spectrophotometer (JASCO Inc., Tokyo, Japan) [36,37]. Both sample and reference

semimicro cuvettes were filled with 350 μL of 250 μg/mL SF-GlcNAc solution in 150 mM NaCl-67 mM phosphate buffer of pH 7.0, and inserted in the cuvette holder to record a base line. Then, 10 μL of 2.0 mg/mL WGA solution in 150 mM NaCl-67 mM phosphate buffer of pH 7.0 was added to the sample cuvette and quickly mixed using a micro spatula. Agglutination of SF-GlcNAc was estimated by the time-dependent increase in absorbance at 350 nm. The reversibility of agglutination was assessed by addition of 50 μL of 40 mg/mL $(GlcNAc)_2$ solution in 150 mM NaCl-67 mM phosphate buffer of pH 7.0 [36-38].

2.5. Solid-phase lectin binding assay

Methods in which the high affinity biotin-avidin interaction is combined with enzymatic detection [4,5,30] were used for the detection of lectin binding to SF-GlcNAc. We measured the binding of biotin-sWGA to SF-GlcNAc conjugate-coated microplates by means of HRP-conjugated neutravidin (Fig. 2) based on our previous study [30]. To each well of the 96-well microtiter plates 100 μL of 2 mg/mL SF-GlcNAc or SF aqueous solution was added and dried in air. The sample-coated wells and uncoated wells were blocked with 0.1% (w/v) solution of bovine serum albumin (BSA) in phosphate buffered saline (PBS). After being left for 2 h at room temperature, the blocking solution was removed and the wells were rinsed with 100 μL of PBS-0.05% Tween 20 (pH 7.0). Subsequently, 100 μL of 0.5 μg/mL biotin-sWGA (dilution: 1:10,000 in PBS) was added to each well and incubated for 1 h at room temperature. The wells were then washed three times with PBS-0.05 % Tween 20 (pH 7.0), and 100 μL of HRP-conjugated neutravidin solution (dilution: 1:10,000 in PBS) was added to each well. After 2 h of incubation at room temperature, the plates were washed and detection was performed by the addition of 100 μL of TMB/E Substrate solution. After 30 min incubation with the substrate, the reaction was stopped with 100 μL of 0.3 M H_2SO_4. The absorbance was measured at 450 nm with a microplate reader (iMark, Bio-Rad Laboratories, Inc., Hercules,

CA). As a blank value, the absorbance for uncoated wells was subtracted from the data of sample-coated wells.

2.6. Enzyme linked immunosorbent assay (ELISA) for detection of desmin binding

The binding of desmin to SF-GlcNAc conjugate-coated microplates was determined by ELISA using anti-desmin antibody. Recombinant desmin was prepared using a KRX *Escherichia coli* protein expression system according to our previous procedure [29], and dissolved in 6 M urea-PBS. Since the recombinant desmin was denatured by 6 M urea-PBS, the protein was renatured by diluting with PBS just before reacting with SF-GlcNAc or SF. In the same manner as described above, 96-well microtiter plates were coated with SF-GlcNAc or SF, and the sample-coated wells and uncoated wells were blocked in 0.1% (w/v) BSA blocking buffer. After blocking, 100 μL of 10 μg/mL desmin solution was added to each well and incubated for 2 h at room temperature. Next, 100 μL of rabbit polyclonal anti-desmin antibody (dilution: 1:5,000) was added to each well as a primary antibody and incubated for 24 h at 4 °C. This was followed by incubation with 100 μL of HRP-conjugated anti-rabbit antibody (dilution: 1:20,000) as a second antibody for 2 h at room temperature. After incubation, the plates were washed and detection was performed by the addition of TMB/E Substrate solution as described above. The absorbance was measured at 450 nm with a microplate reader (iMark, Bio-Rad Laboratories, Inc., Hercules, CA). The absorbance for uncoated wells was subtracted from the data of sample-coated wells.

2.7. Culture of HCM

Primary HCM were cultured in Myocyte Growth Medium containing fetal calf serum (0.05 mL/mL), recombinant human epidermal growth factor (0.5 ng/mL), recombinant human basic fibroblast growth factor (2 ng/mL) and recombinant human insulin (5 μg/mL) at 5% CO_2

and 37 °C. The cells were grown in T75 flasks until there was a sufficient number of cells for use in experiments and trypsinized. Sample-coated microtiter plates for cell culture were prepared as described previously [16,18]. An aqueous solution of 0.5% (w/v) SF-GlcNAc or SF was sterilized by filtration using Millex-HV syringe-driven filter unit. An aliquot (130 μL) of the filtrate was placed in each well of 96-well culture plates at 4 °C for 24 h to permit SF proteins to be absorbed on the surface of the well. After the removal of the sample solution, 130 μL of PBS was added to each well. Sample-coated plates were stored in PBS until use at 4 °C.

For attachment study, the cells were seeded onto sample-coated or uncoated wells of a 96-well plate at a density of 1×10^4 cells/well with 100 μL of Myocyte Growth Medium, and cultured in a humidified incubator for 2 h. Alternatively, the cells at a density of 1×10^3 cells/well were seeded onto each well with 100 μL of Myocyte Growth Medium and cultured for 1 day and 3 days for cell growth study. Cell morphologies at 2 h, 1 day and 3 days of culture were observed by phase-contrast microscope (CKX31, Olympus, Tokyo, Japan). Cell attachment and growth were assessed using a Cell Count Reagent based on the conversion of a water-soluble tetrazolium salt, WST-8, to a water-soluble formazan dye upon reduction by dehydrogenase [39,40]. The number of viable cells was found to be directly proportional to the metabolic reaction product obtained in WST-8 assay [39,40]. After the cultivation, the medium was replaced by 100 μL of fresh medium containing 10 μL of Cell Count Reagent and incubated. The absorbance of the medium was measured at a wavelength of 450 nm with a reference wavelength of 650 nm using a microplate reader (ARVO MX, PerkinElmer Inc., Waltham, MA) after 2 h incubation for attachment study or 4 h incubation for growth study. The absorbance for the blank wells was subtracted from the data.

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD) from more than 5 samples. Statistical analyses were performed using an unpaired Student's *t* test or a one-way ANOVA followed by Tukey-Kramer test with KaleidaGraph software (HULINKS Inc., Tokyo, Japan) to compare different material samples. Differences with $p < 0.05$ were considered significant.

3. Results

3.1. Preparation of SF-GlcNAc conjugates

Our previous ^{1}H- and ^{13}C-NMR spectral analysis of the reaction product of monosaccharide GlcNAc, that is the monomer unit and model compound of NACOS, and CY clarified that the terminal anomeric hydroxyl group of GlcNAc reacted with a chlorine atom of CY, and both α- and β-anomers of the product, CY-activated GlcNAc were then formed [17]. Based on NMR spectral analysis and amino acid analysis of SF-NACOS conjugates, it was suggested that a second chloride atom of CY-activated GlcNAc reacted with phenolic hydroxyl group of the tyrosine residue and ε-amino group of the lysine residue in SF [17]. Therefore, the chemical structure of SF-GlcNAc is estimated as shown in Fig. 1.

To confirm the covalent immobilization of GlcNAc into SF and determine the content of GlcNAc in the conjugates, the ^{1}H-NMR spectra of SF-GlcNAc, a mixture of SF and monosaccharide GlcNAc, and SF were measured (Fig. 3). The ^{1}H-NMR spectrum of SF-GlcNAc showed peaks attributed to GlcNAc in addition to the SF peaks. Weak peaks at 3.4~3.8 ppm were assigned to the methine and methylene protons of GlcNAc, whereas the peaks at 1.93~2.07 ppm were assigned to the methyl protons of the *N*-acetyl group of GlcNAc. This indicates the successful introduction of GlcNAc into SF by chemical modification. The spectrum of monosaccharide GlcNAc showed two peaks attributed to the anomeric protons of β and α forms at 4.70 and 5.20 ppm, respectively (Fig. S1) although the spectrum of the mixture of SF and GlcNAc recorded using WATERGATE did not show the peak attributed to the

anomeric proton of β form of GlcNAc at 4.70 ppm, that was very close to the water peak [34,35]. The spectrum of SF-GlcNAc included many weak peaks attributed to the anomeric protons of GlcNAc at 5.25~6.5 ppm. Therefore, downfield shifts caused by deshielding of the triazine ring of CY [16,17] were observed for anomeric protons of GlcNAc in SF-GlcNAc conjugates. Moreover, two peaks at 6.77 and 7.05 ppm attributed to aromatic protons of the tyrosine residues appeared in the spectrum of SF, but two broad peaks appeared at 7.05 and 7.25 ppm instead in the spectrum of SF-GlcNAc. Thus, the downfield shifts caused by CY [16,17] were observed for the aromatic protons of the tyrosine residue of SF in SF-GlcNAc as well as the anomeric protons of GlcNAc in SF-GlcNAc. The downfield shifts of both the anomeric protons of GlcNAc and the tyrosine residue protons of SF supported the fact that the hydroxyl group at the anomeric position of GlcNAc and the phenolic hydroxyl group of the tyrosine residue of SF were bound to the triazine ring of CY (Fig. 1). The downfield shift of the tyrosine residue was proof that the reaction product SF-GlcNAc was not a physical mixture but conjugate formed via covalent bonding. Although the peaks of the lysine residue could not be detected by ^{1}H-NMR measurement owing to its low abundance (0.3 mol%), the nucleophilic ε-amino group of the lysine residue was estimated to be another reaction site of SF based on a previous study on amino acid analysis of SF-NACOS conjugates [17].

The content of GlcNAc in SF-GlcNAc was calculated by comparing the integral values of the resonance peaks due to methyl protons of *N*-acetyl group of GlcNAc at around 2.0 ppm in the spectra of SF-GlcNAc and the mixture of SF and GlcNAc (the weight ratio of GlcNAc to SF was 0.25), that served as a standard for the calculation. In each spectrum, the integral value of the peak due to the β-methyl protons of the alanine residue in SF at 1.35 ppm, which was not modified with CY-activated GlcNAc, was used as an integration reference (1.00). While the integral value of the peak at 2.05 ppm was 0.235 in the spectrum of the mixture, the integral value of the peaks at 1.93~2.07 ppm was 0.115 (= 0.059 + 0.056) in the spectrum of SF-GlcNAc.

However, the spectrum of SF showed a weak peak due to the β-methine proton of the valine residue at 2.05 ppm (its integral value was 0.024), that overlapped with the peak due to the methyl protons of *N*-acetyl group in the spectra of SF-GlcNAc and the mixture. Thus, the net integral value of the peak due to the methyl protons of the *N*-acetyl group was calculated to be 0.211 (= 0.235 − 0.024) in the spectrum of the mixture, whereas the net integral value of the peak due to *N*-acetyl group was calculated to be 0.091 (= 0.115 − 0.024) in the spectrum of SF-GlcNAc. From these results, the weight ratio of GlcNAc to SF in SF-GlcNAc was calculated to be 0.11 (= 0.25 × 0.091/0.211). Since the molecular weight of the CY moiety (C_3N_3Cl: 113.5) is almost half of the GlcNAc moiety ($C_8H_{14}NO_6$: 220.2), the weight ratio of the CY moiety to SF can be calculated to be 0.06 (= 0.11 × 113.5/220.2). Consequently, the GlcNAc content in SF-GlcNAc was determined to be 9 wt% (= 0.09 = 0.11/(1 + 0.11 + 0.06)).

Assuming that all tyrosine residues, whose content is 5.2 mol% in SF, are completely conjugated with GlcNAc, about one out of 20 amino acid residues is conjugated with one GlcNAc molecule via CY. Since the average molecular weight of the amino acid unit constituting SF is 75 [16,17], the molecular weight of 20-amino acid peptide is 1400 (= 74 × 20). Taking the molecular weights of GlcNAc moiety and CY moiety into consideration, the GlcNAc content in SF-GlcNAc is estimated to be 13 wt% (= 0.13 = 220.2/(1400 + 220.2 + 113.5)). A comparison of the expected value of the GlcNAc content and the experimental value obtained from the integrated intensities of NMR peaks suggests that about 70% (= 9/13) of the tyrosine residue reacted with CY-activated GlcNAc despite the addition of a large excess of CY-activated GlcNAc.

3.2. Recognition of SF-GlcNAc by lectins

Although ^{1}H-NMR spectrum of SF-GlcNAc supported the covalent immobilization of GlcNAc into SF, we further studied interactions between SF-GlcNAc and WGA lectins to

confirm that the β-anomer of GlcNAc was immobilized into SF. WGA selectively binds to β-1,4-GlcNAc and *N*-acetylneuraminic acid (sialic acid) residues of glycoproteins and glycolipids [41,42]. Fig. 4 shows the typical turbidity changes observed by the addition of WGA to SF-GlcNAc or SF solution. Since little change in absorbance at 350 nm was observed for SF solution, there was no lectin-mediated aggregation of SF. In contrast, rapid increase in absorbance at 350 nm was observed for SF-GlcNAc solution after the addition of WGA, suggesting the cause of the aggregation of SF-GlcNAc. By the addition of excess free dimeric GlcNAc, $(GlcNAc)_2$ the lectin-induced aggregation was promptly reversed (Fig. 4).

Succinylated WGA (sWGA) is more specific than inherent WGA and has only a binding affinity for the β-1,4-GlcNAc residue [42]. The specific interaction of biotin-sWGA with SF-GlcNAc was investigated by solid-phase lectin binding assay based on the biotin-avidin interaction [4,5]. The absorbance values of SF-GlcNAc conjugate-coated wells were very high compared to those of SF-coated wells (Fig. 5), indicating that the conjugates showed a strong lectin-binding property.

3.3. Interaction of SF-GlcNAc with desmin

Detection of the interaction between SF-GlcNAc and desmin was carried out by ELISA. The absorbance values of SF-GlcNAc conjugate-coated wells were higher than those of SF-coated wells (Fig. 6), demonstrating that the interaction of SF-GlcNAc with desmin was strong while the interaction of SF with desmin was weak. It was demonstrated that desmin and vimentin have a β-GlcNAc residue-binding activity, however, these proteins could bind to multivalent GlcNAc derivatives but not monovalent GlcNAc derivatives [27,29]. Therefore, these results suggest that multiple GlcNAc molecules were conjugated to one SF molecule and recognized by desmin.

3.4. Morphologies of HCM

The morphologies of HCM cultured on SF-GlcNAc conjugate-coated wells, SF-coated wells and uncoated wells were observed at 2 h, 1 day and 3 days after seeding (Fig. 7). Most of the cells were round on all kinds of wells at 2 h. At 1 day after seeding, a considerable number of the cells became flat and revealed an elongated morphology, while some of the cells remained round-shaped, regardless of the kind of well. Most of the cells on any kind of well had an elongated morphology at 3 days. No morphological difference was observed for the cells cultured on 3 different kinds of wells when the cells were cultured for the same period of time. Time dependent morphological change from round to flat was observed for the cells cultured on all kinds of wells.

3.5. Attachment and proliferation of HCM on SF-GlcNAc conjugate-coated wells

Attachment of HCM on the wells coated with SF-GlcNAc and SF was examined at 2 h after seeding as shown in Fig. 8a. The number of cells attached on SF-coated wells was lower than that on uncoated wells. The number of cells attached on SF-GlcNAc conjugate-coated wells tended to be lower than that on uncoated wells, however, no significant difference between them was observed.

Cell proliferation on the coated wells at day1 and day 3 after seeding is shown in Fig. 8b. At day 1 as well as 2 h after seeding, the number of cells cultured on SF-GlcNAc conjugate-coated wells was comparable to that on uncoated wells, and higher than that on SF-coated wells. On uncoated wells, cell numbers at day 3 were very slightly higher than those at day 1, and cell proliferation was hardly observed in spite of an additional 2 days of incubation. In contrast, since the numbers of cells cultured on the conjugate-coated wells and SF-coated wells for 3 days were 1.3 times and 1.6 times higher than those cultured for 1 day, respectively, it is considered that slow cell proliferation probably occurred on the conjugate-coated wells and SF-

coated wells. At day 3, the number of cells cultured on the conjugate-coated wells was higher than that on uncoated wells while the number on SF-coated wells was almost the same as that on uncoated wells.

4. Discussion

Based on our previous study [17], we prepared glycoconjugates, SF-GlcNAc by chemical modification of amino acid residues in SF with monosaccharide GlcNAc using CY. The covalent immobilization of GlcNAc into SF was supported by ^{1}H-NMR measurements. The ^{1}H-NMR spectrum of SF-GlcNAc showed new peaks due to the methyl protons of the *N*-acetyl group of GlcNAc, and the GlcNAc content in SF-GlcNAc was calculated to be 9 wt% from the integrated intensities of these peaks (Fig. 3). Since we previously reported that α- and β-anomers of CY-activated GlcNAc were prepared by the reaction of GlcNAc with CY [17], it was estimated that SF-GlcNAc conjugates were endowed with both α- and β-GlcNAc residues. To confirm the immobilization of the essential β-GlcNAc residue, lectin recognition studies of SF-GlcNAc were carried out.

Mixing SF-GlcNAc solution with WGA lectins resulted in the increase in absorbance at 350 nm (Fig. 4) due to the recognition of the β-GlcNAc residue in SF-GlcNAc by WGA and subsequent aggregation of SF-GlcNAc mediated by WGA [36]. The reversibility of the aggregation by the addition of free $(GlcNAc)_2$ provided evidence for the dissociation of SF-GlcNAc conjugates cross-linked with WGA lectins that have a higher selectivity for free $(GlcNAc)_2$ [38]. These results suggest that SF-GlcNAc conjugates were at least endowed with β-GlcNAc residues which induced the aggregation with WGA. Because of a lack of a binding affinity for sialic acid residues [42], sWGA can bind more specifically to β-GlcNAc residues than WGA. Thus, biotin-sWGA was used for recognition studies as a more specific lectin. Very strong binding of biotin-sWGA lectins was observed for SF-GlcNAc conjugate-coated

wells compared to SF-coated wells (Fig. 5), indicating the existence of β-GlcNAc residues in SF-GlcNAc. Moreover, the interaction of SF-GlcNAc with lectins at a liquid/solid interface suggested that hydrophilic β-GlcNAc residues of solid SF-GlcNAc were exposed and recognized by lectins in an aqueous medium.

Since the existence of β-GlcNAc residues in SF-GlcNAc was established, the interaction of SF-GlcNAc conjugates with cytoskeletal protein, desmin was examined. As shown in Fig. 6, strong interaction with desmin was observed for the conjugate-coated wells while weak interaction was observed for SF-coated wells. From these results, it was considered that desmin specifically bound to SF-GlcNAc through the recognition of multiple β-GlcNAc residues while desmin non-specifically bound to SF. These results further denoted that β-GlcNAc residues in SF-GlcNAc were valid for recognition by desmin expressed on the cell surface.

Following the assessment of the interaction with desmin, the interaction with HCM was investigated using SF-GlcNAc conjugate-coated wells base on the fact that desmin is expressed on the surfaces of cardiomyocytes [20,27]. Since no difference in morphology was observed for the cells cultured on the wells coated with SF-GlcNAc conjugates or SF (Fig. 7), it was thought that the immobilization of the β-GlcNAc residue did not affect cell morphology. However, the numbers of attached cells and proliferating cells on the conjugate-coated wells were greater than those on SF-coated wells (Figs. 8a and 8b). The enhanced attachment of HCM on the conjugate-coated wells is consistent with the previous finding that the adhesion of vimentin- or desmin-expressing cells was promoted on the wells coated with GlcNAc-bearing polystyrene [27]. Furthermore, it was reported that the adhesion of cardiomyocytes to the wells coated with GlcNAc-bearing polystyrene was inhibited when the cells were transfected with anti-desmin siRNA, that reduced desmin-expressing levels [27]. Therefore, these results suggest that HCM adhered to SF-GlcNAc through desmin expressed on the cell surface. As

shown in Fig. 8b, cell proliferation was hardly observed on uncoated wells. Slow cell proliferation was observed on SF-coated wells as well as SF-GlcNAc conjugate-coated wells, although cell attachment on SF-coated wells was lower than that on uncoated wells. Thus, with respect to HCM, SF-coating onto plastic culture wells inhibited cell attachment but promoted cell growth. Since the speed of cell proliferation on SF-GlcNAc conjugate-coated wells was lower than that on SF-coated wells, the immobilization of β-GlcNAc residues was effective chiefly for promoting the attachment of HCM to SF. Based on the above findings, it is considered that the highest numbers of cells on the conjugate-coated wells at day 3 was due not only to the promotion of cell attachment by β-GlcNAc residues but also the promotion of cell proliferation by SF substrates.

Our results support the hypothesis that the immobilization of β-GlcNAc residues is a useful method for versatile functionalization of SF. Moreover, the present study indicates the potential of moldable SF-GlcNAc as a substrate for the culture of cytoskeletal protein-expressing cells such as HCM. Cardiomyocytes are used for cardiotoxicity testing, drug screening and drug validation as well as metabolism studies. A three-dimensional (3D) culture method is currently being developed to optimize use with cardiomyocytes [43,44]. Aqueous SF-GlcNAc solution can be reformed into 3D sponge substrates. Therefore, 3D culture of HCM using SF-GlcNAc sponges may be applied to the field of drug screening.

5. Conclusion

We prepared glycoconjugates, SF-GlcNAc composed of SF and monosaccharide GlcNAc as β-GlcNAc residue-bearing biopolymer. Analyses of ^{1}H-NMR spectrum of SF-GlcNAc clarified that monosaccharide GlcNAc was covalently immobilized into SF via CY and that the GlcNAc content in the conjugates was 9 wt%. The existence of β-GlcNAc residues in SF-GlcNAc was confirmed by the criteria using WGA and biotin-sWGA lectins. Cytoskeletal

protein, desmin bound more strongly to SF-GlcNAc conjugate-coated wells than SF-coated wells. As for desmin-expressing cells, HCM cultured on the conjugate-coated wells exhibited an increase in the numbers of attached cells and proliferating cells compared to the cells cultured on SF-coated wells. These results suggest that the immobilization of monosaccharide GlcNAc is a useful method for the versatile functionalization of SF as an application in tissue engineering.

Acknowledgements

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Figure legends

Fig. 1. Preparation of conjugates (SF-GlcNAc) composed of silk fibroin (SF) and

monosaccharide *N*-acetyl-D-glucosamine (GlcNAc) using cyanuric chloride (CY).

Fig. 2. Schematic representation of the procedure for detection of binding of biotin-sWGA onto SF-GlcNAc conjugate-coated wells by means of HRP-conjugated avidin.

Fig. 3. ^{1}H-NMR spectra of SF (a), a mixture of SF and GlcNAc (b) and SF-GlcNAc conjugates (c) in D_2O.

Fig. 4. Time course of absorbance change of SF-GlcNAc and SF solutions at 350 nm after addition of WGA.

Fig. 5. Comparison of the binding of biotin-sWGA to SF-GlcNAc conjugate-coated wells and SF-coated wells. *$p < 0.05$.

Fig. 6. Comparison of the binding of desmin to SF-GlcNAc conjugate-coated wells and SF-coated wells by ELISA. *$p < 0.05$.

Fig. 7. Phase-contrast micrographs of HCM on SF-GlcNAc conjugate-coated wells, SF-coated wells and uncoated wells after cultivation for 2 h, 1 day and 3 days. Scale bar indicates 100 μm.

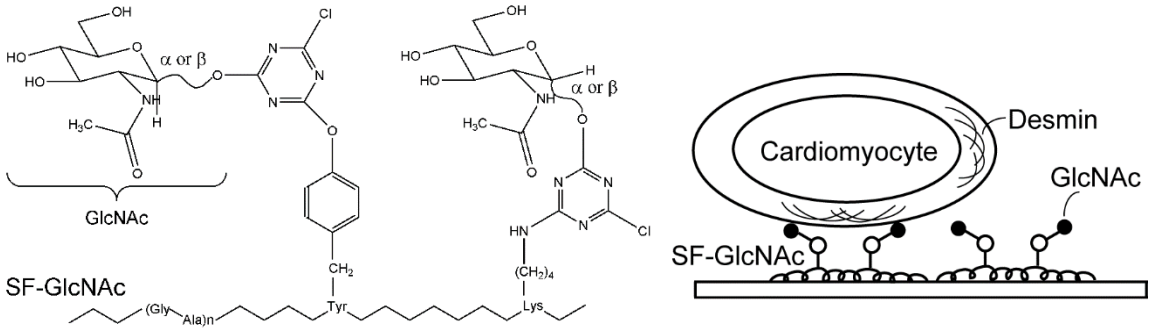
Fig. 8. (a) Attachment of HCM onto SF-GlcNAc conjugate-coated wells, SF-coated wells and uncoated wells, as assayed using WST-8. *$p < 0.05$. (b) Proliferation of HCM on SF-GlcNAc conjugate-coated wells, SF-coated wells and uncoated wells, as assayed using WST-8. *$p < 0.05$.

Highlights

• From silk fibroin (SF), *N*-acetyl-D-glucosamine-conjugated SF (SF-GlcNAc) was prepared.

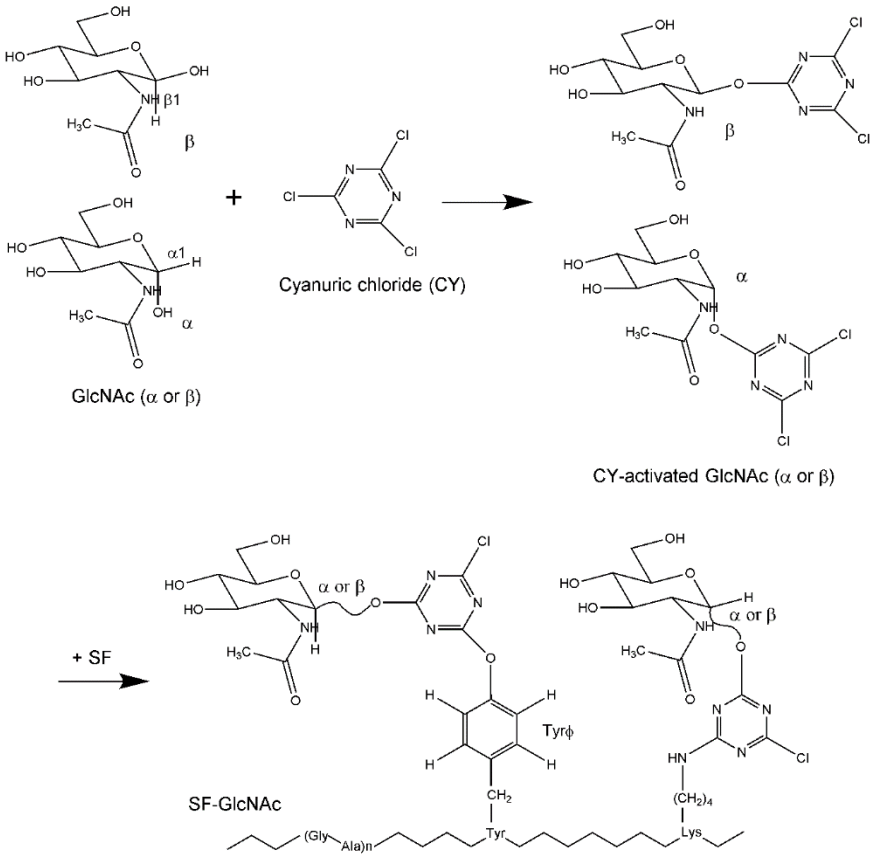
• WGA lectins and desmin bound more strongly onto SF-GlcNAc conjugates than SF.

• Attachment and proliferation of cardiomyocytes were improved on SF-GlcNAc compared to SF.



N-Acetyl-D-glucosamine-conjugated silk fibroin (SF-GlcNAc)

Graphical abstract (It has no figure legend)



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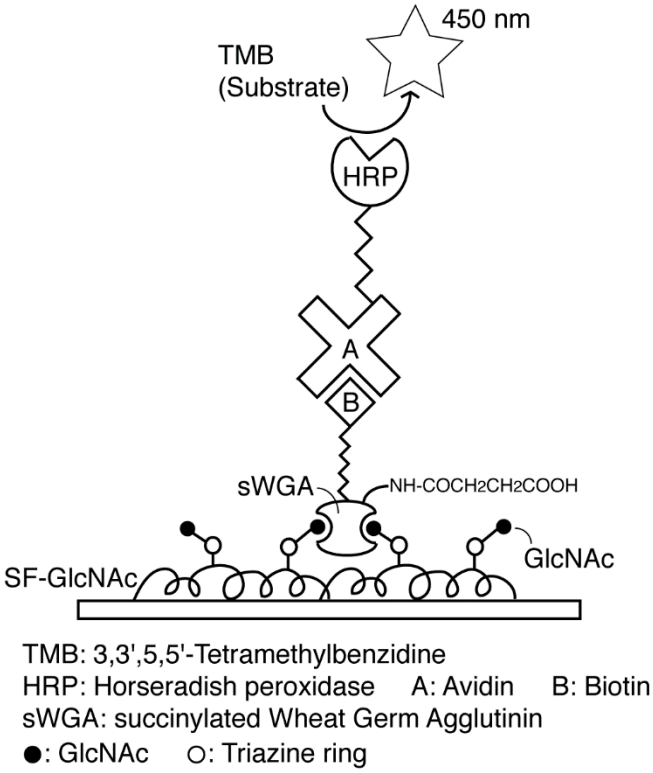

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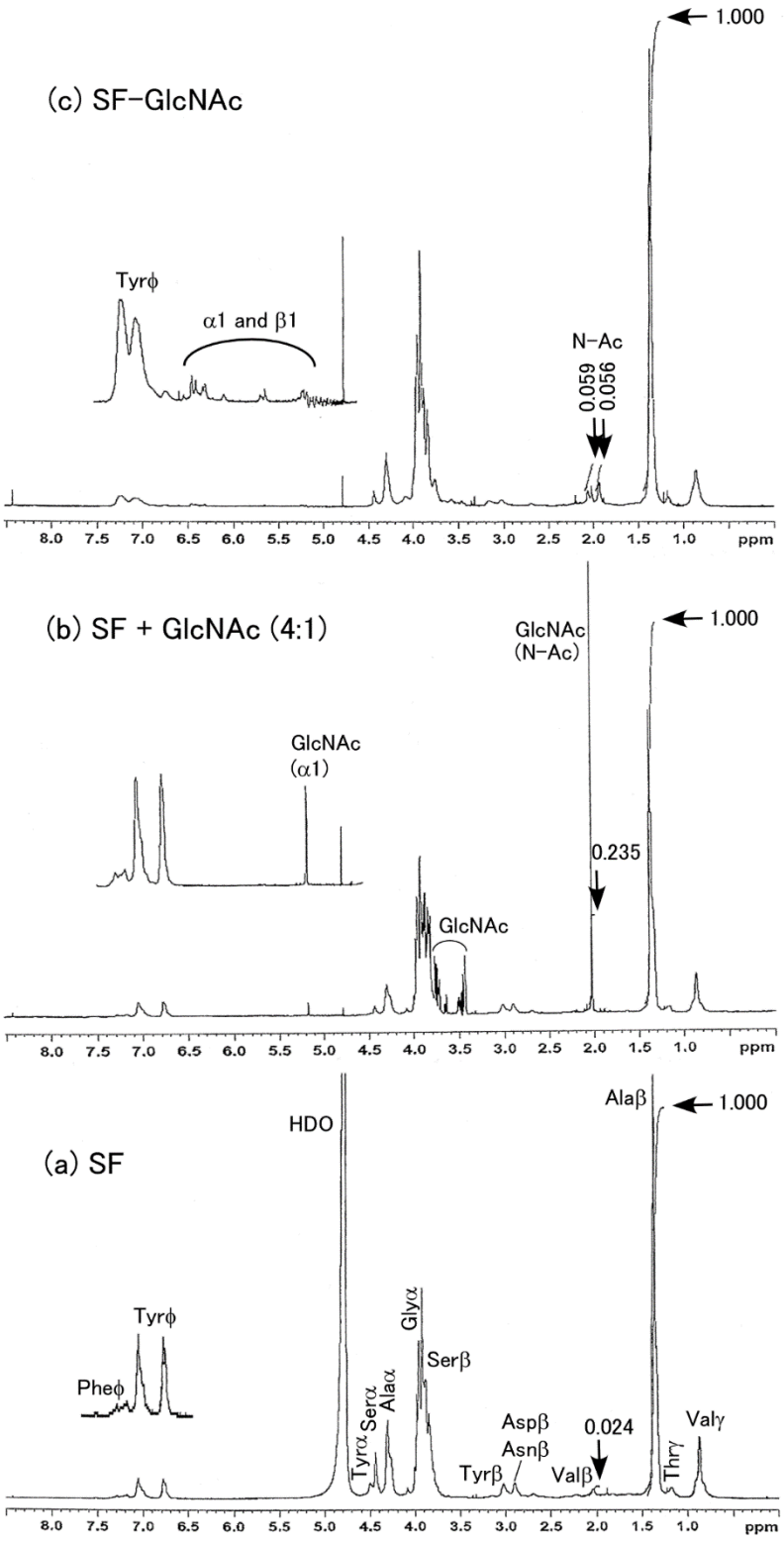

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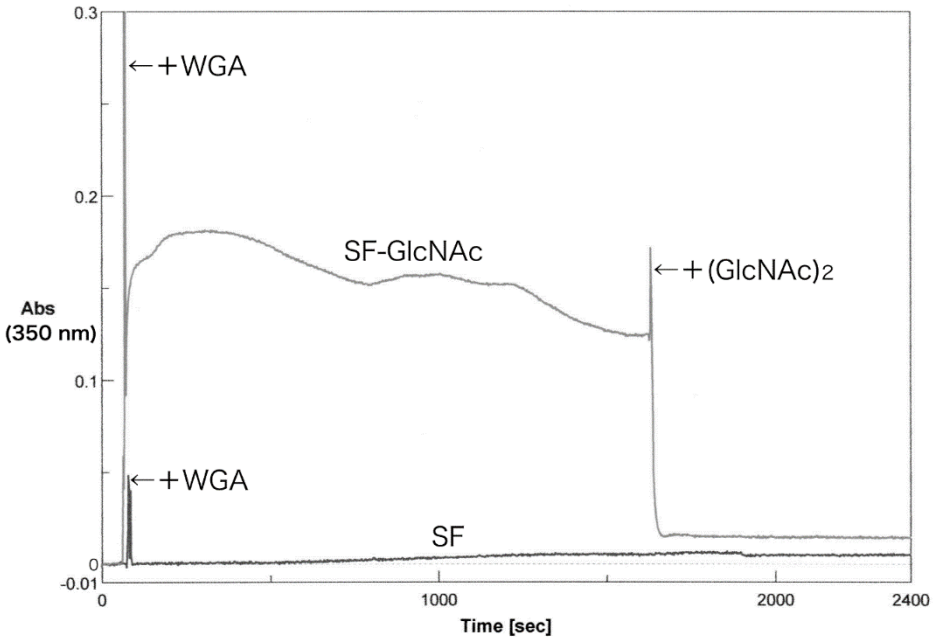

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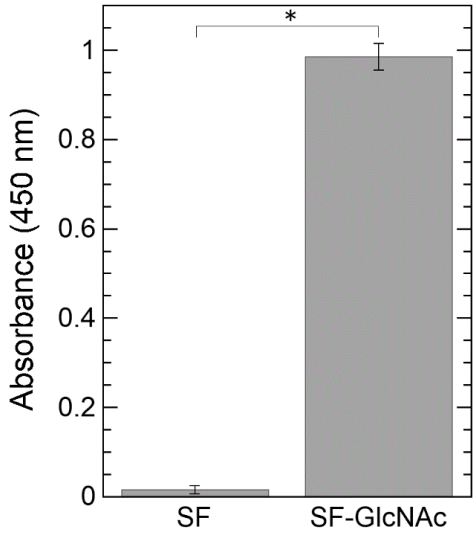

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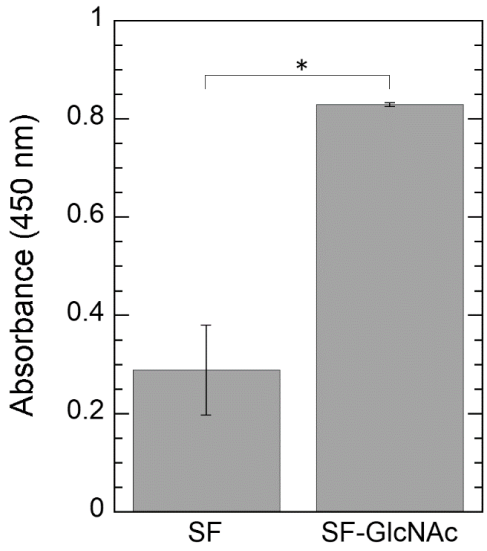

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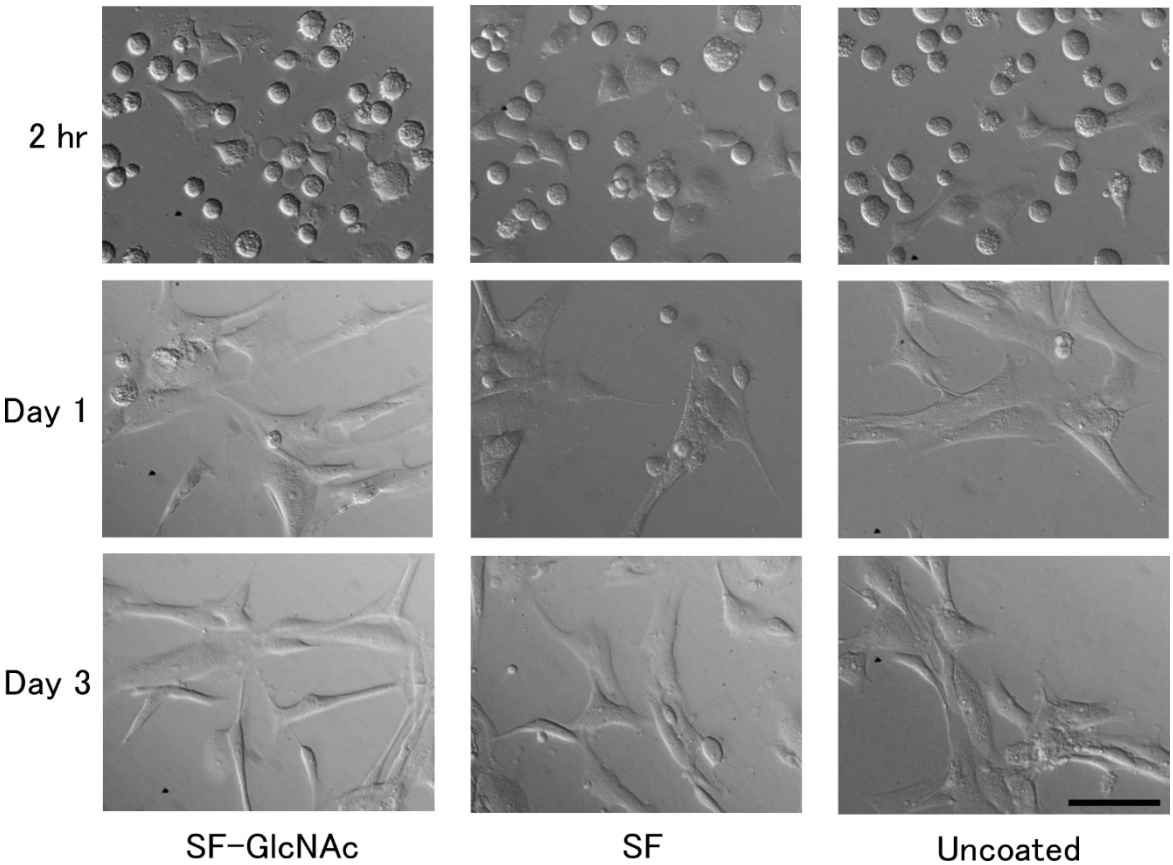

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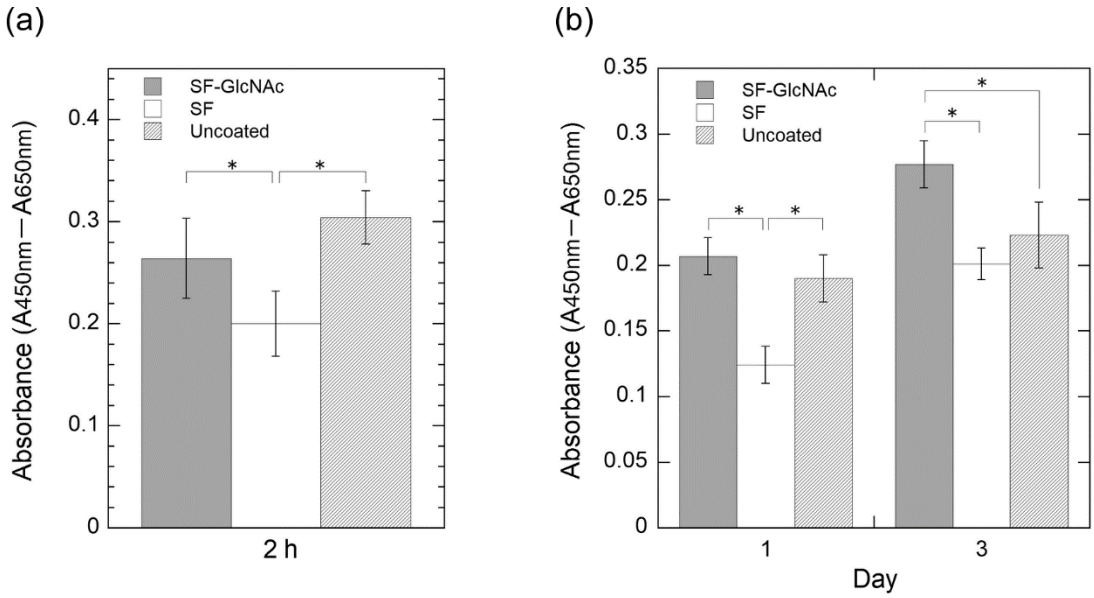

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Supplementary materials

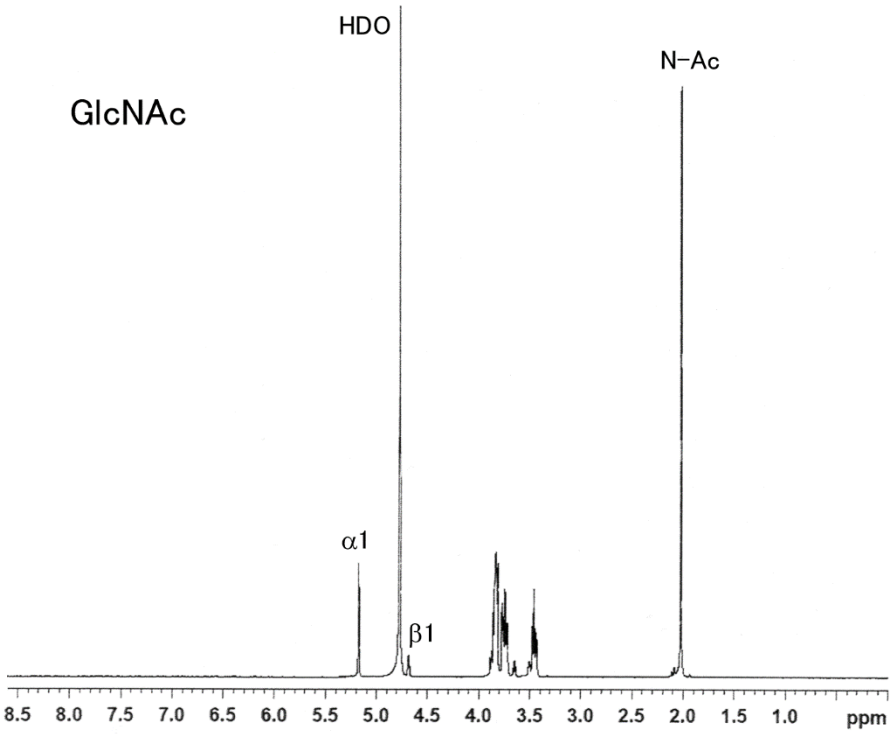

Fig. S1. ^{1}H-NMR spectrum of GlcNAc in D_2O.