Aggregability of β(1→4)-linked glucosaminoglucan originating from a sulfur-oxidizing bacterium *Thiothrix nivea*

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β-1,4-glucosaminoglucan (GG) was prepared from the sheath of a sulfur-oxidizing bacterium *Thiothrix nivea*. Recently, GG was found to be adsorbed by cellulose (paper) and is therefore potentially applicable as an aminating agent for cellulose. We attempted to increase the yield of GG using a fed-batch cultivation method. Furthermore, behavior of GG molecules in water was theoretically and experimentally investigated. NMR analysis in combination with molecular dynamics calculation suggested that GG molecules tend to form soluble aggregates in water. It was experimentally revealed that the self-aggregation is enhanced by the addition of NaCl and reduced temperature. Adsorption of GG onto cellulose via hydrogen bonding was confirmed by molecular dynamics simulation. Adsorption was also promoted in the presence of NaCl but was inhibited by reduction in temperature. Only 11% of the amino groups in the GG-treated paper was reactive, suggesting that GG molecules adsorbed by the paper were forming aggregates.

Keywords: glucosaminoglucan; cellulose; aggregation; adsorption; *Thiothrix nivea*

It is well known that filamentous bulking (poor settling) of activated sludge is caused by the overabundance of bacteria possessing filamentous morphology [1]. *Thiothrix nivea* is a sulfur-oxidizing habitant of activated sludge and is regarded as a filamentous bacterium responsible for bulking [1]. Since *T. nivea* is a mixotrophic sulfur oxidizer, it requires both carbon and sulfur sources for its growth. *T. nivea* prefers simple organic acids as carbon sources and hydrogen sulphide or thiosulfate as sulfur sources [2]. Filamentous growth of *T. nivea* is attributed to longitudinal clumping of cylindrical cells as well as other species of the genus *Thiothrix*. Distinguishing morphological characteristics of *T. nivea* are formations of sheath [2,3] and S-layer [4] surrounding a line of cells. The sheath layer is located outside the cell wall and is an assemblage of a β-1,4-linked copolymer of glucose and glucosamine (GG) modified with unidentified deoxy sugar [3]. The S-layer, which has a lattice structure assembled from a hemolysin-like acidic protein, covers the sheath layer [4]. Since the S-layer protein tends to aggregate at pH 3-4, the filaments of *T. nivea* can be aggregated and precipitated by acidification, allowing easy recovery of the filaments after cultivation [4]. GG can be released from the sheath of *T. nivea* by partial hydrolysis (selective removal of the deoxy sugar residue) with dilute HCl [3]. *T. fructosivorans* is another representative sheath-forming species of the genus *Thiothrix* [5,6]. The major component of its sheath is GG similar to *T. nivea*, but the glucosamine residues are highly *N*-acetylated, which is not the case with *T. nivea* [3,6]. Therefore, completely deacetylated GG is not present in *T. fructosivorans*. It has been reported that the β-1,4-linked copolymer of glucose and *N*-acetyl glucosamine (*N*-acetylated GG) can be prepared as a semisynthetic polymer using reverse reaction (polymerization reaction) of chitinase or cellulase from an oxazoline repeating unit of β-Glc(1→4)GalNAc or a fluoride repeating unit of β-GalNAc(1→4)Glc, respectively [7]. Semisynthetic or synthetic preparation of GG

(copolymer of Glc and GlcN) has not been developed yet. Preparation of glucosamine-containing cellulose is possible by feeding a cellulose-producing bacterium with glucosamine, but the molar percentage of glucosamine in the heteropolysaccharide should not exceed 20% [8]. Consequently, GG (alternating copolymer of Glc and GlcN) can currently only be synthesized by *T. nivea*. Functionalization of cellulose by introducing reactive chemical species including amino groups is desirable in order to meet the increasing demands for environmentally friendly materials and technologies. GG was found to be irreversible entrapped by cellulose (paper) and could serve as a natural aminating reagent for it without any chemical reactions, by simply immersing paper into diluted hydrochloric acid containing GG [9]. This simple amination technique may be suitable for functionalization of cellulose nanofibers. The technique is attributed to the strong interaction between GG and cellulose. However, the yield of GG from *T. nivea* is too low for practical applications [3]. Moreover, the interaction mechanisms between GG and cellulose remain unknown. This study attempted to improve the GG yield through a fed-batch cultivation method. Furthermore, behavior of GG molecules in water was theoretically and experimentally elucidated by employing nuclear magnetic resonance (NMR) spectroscopy and molecular dynamics simulations, to find suitable conditions for amination of cellulose using GG.

Materials and methods

Preparation of glucosaminoglucan (GG) and GG-treated cellulose

T. nivea ATCC35100 was cultured using the following fed-batch method. The strain was statically grown at 30 °C for 3 days in a 500 mL flask containing 200 mL of a medium composed of 1.5 g sodium acetate, 0.3 g $Na_2S \cdot 9H_2O$, 0.2 g NH_4Cl, 0.01 g K_2HPO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 20 mL $CaSO_4$ saturated solution, and 5 mL trace

element solution (DSMZ155) per liter of water. Subsequently, 0.4 mL $Na_2S_2O_3 \cdot 5H_2O$ solution (0.1g/mL) was aseptically added to each flask followed by shaking for 1 day. The same amount of the $Na_2S_2O_3 \cdot 5H_2O$ solution was added to each flask followed by shaking another 2 days. The culture was then acidified (to about pH 3) to flocculate the bacterial filaments by the addition of 0.6 mL 0.5 M HCl. The flocs were harvested by centrifugation and subjected to GG preparation via sheath isolation according to a previously described method [3]. To prepare HCl salt of GG (GG-HCl), purified GG (0.1 g) was dissolved in 10 mM HCl (50 mL) followed by lyophilization. GG-treated cellulose was prepared as follows: GG (1 mg) was dissolved in 1 mL of 10 mM HCl containing 1 M NaCl. A disk (φ8 mm) or strip (2 × 7 mm) of filter paper (Whatman #1; GE Healthcare, Little Chalfont, UK) was soaked in 0.5 mL of the GG solution and then incubated at 37 °C. After incubation for 18 h, the disk or strip was recovered, rinsed with 20 mM HCl (3 times), and then rinsed with water (3 times). GG-treated cellulose was obtained by drying the washed disk or strip *in vacuo*. The standard conditions for preparation of a GG-treated paper was defined as soaking paper in 1 mg/mL GG solution containing 1 M NaCl, at 37 °C for 18 h.

Inter-proton (H-H) distance estimation

GG-HCl (5 mg) was dissolved in D_2O (0.75 mL) followed by lyophilization. The lyophilization was repeated three times and thereafter, the sample was dissolved in 0.75 mL of D_2O. The pH of the solution was adjusted to 3 with dilute DCl in the sensor chamber of the Horiba pH-11B pH meter. The above-mentioned operations were performed under an Ar atmosphere. The solution was subjected to NMR spectroscopy using Bruker AVANCE III HD 600 spectrophotometer at 35 °C, to acquire the ^{1}H- and ^{13}C-NMR spectra. Trimethylsilyl propanoic acid (δH 0.00 ppm) and deuterated acetone

(δH 31.45 ppm) were used as internal standards. The Bruker standard pulse sequences for DQF-COSY, TOCSY (mixing time 40, 80, 200 ms), NOESY (mixing time 80 ms), HSQC, HMBC (optimized for 5 Hz coupling constant), and ^{1}H non-decoupled HSQC were used in 2D-NMR analyses. NOESY correlations were integrated on both sides of the diagonal and averaged. The Nuclear Overhauser effect (NOE) signals between the protons of position 6 (H6,6') of D-Glc were also integrated and averaged for internal reference. The intensity was set to 1.8 Å based on the crystal structure of cellobiose [10]. H-H distances were calculated by applying the usual r^{-6} NOE-distance relationship.

Molecular weight determination

The number average molecular weight of GG was determined by NMR spectroscopy after derivatization. Prior to NMR analysis, the reducing end of GG was modified with 4-aminobenzoic acid ethyl ester (ABEE) as follows. GG-HCl (10 mg) was dissolved in 0.3 mL of water and 0.3 mL of the ABEE labelling mixture (GlyScope ABEE Labelling Kit; J-CHEMICAL, Tokyo, Japan) was added for reductive amination of the reducing terminal residue of GG. After heating at 80 °C for 2 h, water (1 mL) and chloroform (2 mL) were added to the reaction mixture, followed by vortexing. The upper aqueous phase was recovered after centrifugation, and 3 volumes of ethanol added to it to precipitate the derivative (GG-ABEE). GG-ABEE recovered by centrifugation was washed with 90 vol% ethanol, and then dried *in vacuo*. To completely remove residual ABEE, GG-ABEE was dissolved with 5 mM of phosphoric acid (1 mL), and the solution was passed through a C_{18} silica gel cartridge (DISPO COLUMN; Advantec, Tokyo, Japan) followed by lyophilization. The lyophilizate was dissolved in D_2O containing trimethylsilyl propanoic acid (δH 0.00 ppm) as an internal standard and

subjected to NMR spectroscopy to acquire the ^{1}H-NMR spectrum at 40 °C using JEOL ECA-500 spectrophotometer. The molecular weight of GG was calculated based on the relative intensity of the ^{1}H signals of the GG moiety and ABEE residue.

Molecular dynamics (MD) simulation of molecular aggregation

MD simulations were carried out for 100-300 ns using the GROMACS 2019 package [11]. A PDB (Protein Data Bank) file for the dodecamer chain of β-1,4-kinked D-glucose was generated using the GLYCAM06 software [12]. The PDB file was edited using the Avogadro package [13] to prepare PDB files for dodecamer chains of cellulose, fully protonated chitosan, and fully protonated GG. A GROMACS format topology file for each dodecamer chain was then generated using ACPYPE [14]. The initial configuration of a system for monomolecular simulation was constructed by inserting a dodecamer chain into the water of a TIP3P model (10 × 10 × 10 nm box). For charge neutralization of the system inserted with GG or chitosan, Cl^- of the AMBER99SB [15] force field was inserted. Simulation (300 ns) was performed keeping a constant pressure (1 atm) and temperature (300 K), after energy minimization and subsequent equilibration (NVT followed by NPT). For examination of the self-assembly process of fully protonated chains of GG (and chitosan for comparison), an initial system was constructed by randomly inserting 4 dodecamer chains into water (10 × 10 × 10 nm box) followed by the insertion of Cl^- for charge neutralization, and simulation was carried out for 300 ns. The interaction between cellulose and the fully protonated chain of GG was investigated by constructing an initial system by inserting a cellulose nanocrystal and 10 dodecamer chains of GG or chitosan randomly into water (12 × 12 × 12 nm box) followed by the insertion of Cl^- for charge neutralization. A cellulose nanocrystal composed of 13 tetradecamer chains was constructed by using the

Avogadro package. The PDB file of the cellulose I_β crystal model (JINROO01) was obtained from the Cambridge Structural Database (CSD).

Adsorption test

A paper disk (φ8 mm) of known weight was soaked in 0.5 mL of 10 mM HCl containing 1 mg/mL of GG and 0-1 M NaCl. A GG solution without a paper disk was also prepared as a control. After maintaining the mixtures at different temperatures in the range of 4-37 °C for 18 h, the liquid phase was recovered for GG quantification (ninhydrin reaction). The amount of GG adsorbed on the paper disk (cellulose) was calculated from the difference in GG concentrations in both mixtures. Adsorption tests for regenerated cellulose (Whatman RC55; GE Healthcare), absorbent cotton, and bacterial cellulose (BC) were performed under standard conditions (1 mg/mL GG, 1 M NaCl, 37 °C, 18 h). BC was prepared as follows: *Komagataeibacter xylinus* ATCC10245 was statically grown at 30 °C for 7 days in 100 mL of a medium (5 g polypeptone, 5 g yeast extract, 5 g mannitol, 5 g glucose, and 1 g $MgSO_4 \cdot 7H_2O$ in 1 L of water). A biofilm formed at the gas-liquid interface after cultivation which was then recovered. The biofilm was washed with water followed by heating in 50 mL of 0.25 M NaOH solution at 100 °C for 1 h. The NaOH-treated biofilm was washed with water and then dried *in vacuo* to obtain BC.

Aggregation test

GG (1 mg) was dissolved in 0.5 mL of 10 mM HCl. To this solution, 0.5 mL of 10 mM HCl containing 0-4 M NaCl was added. The mixture was then split into two equal aliquots and each aliquot was settled at 4-37 °C for 72 h. One aliquot was centrifuged at 12,000 rpm for 5 min to recover the supernatant for quantification of soluble GG

(insoluble GG was removed as a precipitate). The other aliquot was subjected to GG quantification without centrifugation as a control (total GG). The quantification of GG was performed colorimetrically using a ninhydrin reaction. The amount of insoluble GG was estimated from the difference between soluble and total amounts of GG.

Quantification of reactive amino groups

Reactive amino group was quantified using a modified 2,4,6-trinitrobenzene sulfonic acid (TNBS) reaction [16]. In this procedure, a GG-treated paper disk was soaked in 0.5 mL of sodium tetraborate solution (50% saturation) containing 0.15 mg/mL TNBS, followed by incubation at 37 °C for 18 h, to initiate a reaction between TNBS and amino groups in the paper disk. An untreated paper disk was treated in the same as control. The solution was diluted with 3 mL water and 1 mL of the diluted solution was recovered, to which 80 μL of adipic dihydrazide solution containing 50% saturated sodium tetraborate was added. After incubation at 37 °C for 1 h, absorbance of the mixture at 500 nm was measured to determine the amount of residual TNBS. The amount of reactive amino groups in the GG-treated paper disk was calculated by comparing the amount of residual TNBS in the test and reference systems.

Enzymatic degradation test

The GG-treated and untreated paper strips prepared under standard conditions were soaked in 1 mL of 0.1 M sodium acetate buffer (pH 4) containing 0.5 mg/mL cellulase. The reaction mixture was incubated at 45 °C observing the shape of the strips. In order to quantitate enzymatic degradation, the GG-treated or untreated paper disks were soaked in 1.5 mL of the cellulase solution, and the reaction mixture was gently shaken at 30 °C. The reaction mixture (50 μL) was recovered at specific time intervals to

measure the cellobiose concentration. The cellobiose concentration was colorimetrically determined by measuring the reducing power using dinitrosalicylic acid reagent [17].

Results

Cultivation of T. nivea

T. nivea was statically batch cultured in previous studies [3,4] using Erlenmeyer flasks, in a medium supplemented with Na_2S and sodium acetate since the bacterium requires both sulfur and carbon sources to grow [2]. Following this protocol in this study initially yielded poor quantity of GG not exceeding 10 mg/L-medium. To improve the yield, 3-folds of the original amount of sodium acetate was added to the medium and $Na_2S_2O_3$ was fed during cultivation. By introducing the fed-batch cultivation, the yield of GG was increased to about 40 mg/L-medium. In contrast to $Na_2S_2O_3$, feeding with Na_2S, a basic salt, proved to be unsuccessful, probably because of drastic pH increase in culture medium. Though $Na_2S_2O_3$ is a suitable energy source for feeding, it is inferior to Na_2S as an energy source especially in the initial stage of cultivation. Poor and slow bacterial growth in a medium supplemented with $Na_2S_2O_3$ instead of Na_2S implies that Na_2S (H_2S) activates the sulfur-oxidizing pathway of *T. nivea*. It was also found that bacterial growth was depressed by shaking, especially in the early stage of cultivation, suggesting sensitivity to mechanical impact of *T. nivea* at this stage.

Dynamic behavior of GG molecules in water

Monomolecular MD simulations of GG, cellulose, and chitosan chains (12 mer) were performed, in order to identify distinguishing features of GG molecules in comparison with cellulose and chitosan molecules. As shown in Fig. 1, GG mainly exhibits linear conformation in simulations. Fig. S1 shows the torsional angle distribution pattern of

240 the glycoside bonds (Glc-Glc, GlcN-GlcN, Glc-GlcN, GlcN-Glc) in the models during
241 the stable period (200-300 ns) of each simulation. The torsional angle distribution
242 patterns of Glc-Glc (Fig. S1a) in cellulose, Glc-GlcN (Fig. S1b) in GG, GlcN-GlcN
243 (Fig. S1c), and GlcN-Glc (Fig. S1d) in GG during this period resembled that of each
244 other. In all simulations during this period, the inter-residual hydrogen bonds between
245 positions 3 and 5 of the sugar residues shared majority (>75%) of the total inner
246 molecular hydrogen bonds (Table S1). It was assumed, therefore, that the dynamic
247 configuration of GG molecules in water is similar to that of cellulose and chitosan
248 molecules due to the stable inter-residual (Glc-GlcN, GlcN-Glc) hydrogen bonds
249 between positions 3 and 5. To confirm the linear conformation of GG indicated by the
250 simulations, the theoretical inter-proton (H-H) distances were compared with the
251 experimental distances. The average H-H distances in a single GG chain were
252 calculated from the last half of the monomolecular MD simulation as theoretical
253 distances. Experimental H-H distances were obtained by NMR analysis (NOESY
254 experiment) of a GG solution (pH 3). As the amino groups of chitosan have a pKa of
255 6.4 [18], the amino groups in the GG molecules in solution were also expected to be
256 completely ionized (positively charged). As shown in Fig. S2, all NOE signals in the
257 NOESY spectrum were successfully assigned by consulting the former assignment of
258 ^{1}H-MNR signals [3] and the experimental H-H distances were estimated based on the
259 intensities of the NOE signals. The theoretical and experimental H-H distances of each
260 H-H pair are listed in Table 1. Most of the experimental H-H distances were shorter
261 than the theoretical H-H distances. Notable difference of 2.5 Å were observed in the
262 distance between position 4 of GlcN (GlcN4) and position 4 of Glc (Glc4). Since
263 position 4 of each sugar residue is involved in glycosidic linkage, the protons at position
264 4 must be distantly located as estimated by the MD calculations. The unexpected

proximity of the protons at position 4 in the NMR spectroscopy suggested that GG molecules are forming soluble (small) aggregates in water despite the intermolecular electrostatic repulsion. To theoretically verify the assumption of molecular association, a self-assembly simulation was attempted by randomly placing 4 chains in a cubic box of water (Fig. 2a). As shown in Fig. 2b, assemblages composed of 2 GG chains were formed during the simulation. In contrast, self-assembly of chitosan chains was not observed (Fig. 2c,d). The molecular association characteristic of GG was supported by the increase in the total number of interchain hydrogen bonds (Fig. S3a) and the decrease in the total solvent-accessible surface area (Fig. S3b) in the course of the simulation. In contrast, there are no time-dependent change in the total number of interchain hydrogen bonds (Fig. S3a) and the total solvent-accessible surface area (Fig. S3b) in the system inserted with chitosan chains. Based on these results, the molecular association (aggregation) of GG molecules in acidic solutions was demonstrated as a distinguishing feature of GG.

Molecular weight of GG

It has been previously reported that the molecular weight (peak top molecular weight) of GG estimated by size exclusion chromatography (using 10 mM phosphoric acid as mobile phase) is 8.2×10^4 Da [3]. Taking into account the aggregability of GG in acidic solutions, the accurate molecular weight is expected to be below 8.2×10^4 Da. To accurately determine the molecular weight of GG, the aldehyde group (reducing end) of GG was modified with ABEE and the 1D-^{1}H-NMR data of the derivative was acquired (Fig. 3). The signals detected in the range of 3.0-5.5 ppm are typical for GG [3]. The nonoverlapping proton signals (Glc1, GlcN1, Glc2, and GlcN2) were successfully assigned (Fig. 3) according to former assignment [3]. Among these signals,

GlcN2 was selected as the reference for molecular weight estimation. Out of the GG signal region (< 1.8 ppm, > 5.5 ppm), minor doublet (6.82 and 7.87 ppm) and triplet signals (1.35 ppm) were observed. Using the ChemDraw program, the doublet signals were assigned to the aryl protons in the benzene ring of ABEE residue and were used as another standard, while the triplet signal was assigned to the methyl protons, as shown in Fig. 4. Based on the intensity of GlcN2 and the total intensity of the aryl protons, the number of the disaccharide repeating units (GlcN-Glc) of GG was estimated to be 30. Accordingly, the number average molecular weight of GG was calculated to be about 1.0×10^4 Da. Since this value is smaller than the chromatographically estimated peak top molecular weight, the formation of soluble aggregate of GG molecules in acidic solutions was strongly suggested.

Insolubilization of GG

Since it has been reported that chitosan molecules tend to be assemble in the presence of salt in a solution [19], the effect of NaCl on the aggregation of GG was examined (Fig. S4). As shown in Fig. S4a, a GG solution supplemented with 1 M NaCl was transparent at 25 °C. The solution became opaque when it was refrigerated at 4 °C (Fig. S4b), indicating that aggregation (insolubilization) of GG is accelerated by the addition of salt and refrigeration. In contrast, no visible change was observed in a refrigerated chitosan solution containing 1 M NaCl (Fig. S4b), demonstrating that GG is more sensitively affected by NaCl than chitosan. The refrigerated GG solution stayed opaque even when it was kept at 25 or 37 °C. Heating at 50 °C for 2 min was required for clarification of the opaque solution (Fig. S4c). These results suggested that the insoluble aggregates of GG formed by refrigeration in the presence of NaCl are metastable. For a more detailed analysis of salt- and temperature-dependent

insolubilization, GG solutions containing different NaCl concentrations (0-2 M) were equilibrated at 4, 15, 25, or 37 °C followed by centrifugation, to remove the insoluble aggregates in the form of a precipitate. The GG concentration in the supernatant was determined to estimate the precipitation rate (precipitated amount/total amount × 100), as summarized in Fig. 4. No precipitate was detected up to 0.5 mM NaCl regardless of temperature. In the presence of 1.0 M NaCl, a precipitate was formed at 4, 15, and 25 °C. The precipitation rate decreased with rising temperature, and no precipitate was detected at 37 °C in the presence of 1.0 M NaCl. In the presence of 2.0 M NaCl, precipitate was formed even at 37 °C. From these results, it was concluded that the molecular aggregation of GG (formation of metastable aggregates) is facilitated by increasing salt concentration and decreasing temperature. More specifically, insolubilization of GG occurs below 37 °C in the presence of ≥ 1 M NaCl.

Adsorption of GG

An MD simulation was performed by randomly arranging 10 chains of GG (12 mer) around a cellulose assemblage (10 chains of 12 mer) in a water box to confirm the association between GG and cellulose. As the simulation proceeded, the GG chains and their self-aggregates became attached to the surface of the cellulose assemblage (Fig. S5a). As expected, no stable attachment was observed between the chitosan chains and the cellulose assemblage (Fig. S5b). Fig. S5c shows the time course of the interchain hydrogen bond number formed between cellulose and GG or chitosan during the simulation. The average number (during 200-300 ns) of the hydrogen bonds between GG and cellulose in the water box was calculated to be 31.0, while that between chitosan and cellulose was 7.9, demonstrating that GG is superior to chitosan in interacting with cellulose via hydrogen bonds. Considering the positive effect of NaCl

on the self-aggregation of GG, effect of NaCl on adsorption of GG to paper was experimentally investigated. The filter paper disks were soaked in GG solutions of varied NaCl concentrations at 37 °C. After equilibration for 18 h, the adsorption (mg-GG/mg-cellulose) was estimated by measuring the GG concentration in the aqueous phase (Fig. 5). The adsorption increased proportionally with NaCl concentration and the adsorption in the presence of 1 M NaCl was about 0.045 mg-GG/mg-cellulose, revealing that adsorption as well as self-aggregation of GG is accelerated by NaCl. For the next step, effect of temperature (4-37 °C) on adsorption of GG to paper was studied in the presence of 1 M NaCl with results being shown in Fig. 6. The adsorption increased according to the temperature and highest adsorption of 0.05 mg-GG/mg-cellulose was achieved at 37 °C, indicating that higher temperature is favorable for the adsorption of GG. Since self-aggregation of GG is induced by cooling in the presence of 1 M NaCl (Fig. 4), it was revealed that temperature change exerts opposite influences on self-aggregation and adsorption of GG. Based on these results, the standard condition for adsorption was defined to be 1 mg/mL GG, 1 M NaCl, and 37 °C. Besides filter paper, adsorption of GG to cotton (0.015 mg-GG/mg-cellulose), regenerated cellulose (0.034 mg-GG/mg-cellulose), and BC (0.114 mg-GG/mg-cellulose) was also confirmed under the standard conditions. It was revealed that adsorption of GG is possible regardless of the crystal structure of cellulose. The difference in adsorption amount might be attributed to the difference in surface area available for GG adsorption.

Properties of GG-treated paper

Quantification of the reactive amino groups in the GG-treated filter paper, which was prepared in the standard condition, was performed by the TNBS method.

The amount of reactive amino groups in the GG-treated paper was estimated to be 11 nM/mg-cellulose. Since the adsorption was about 0.045 mg-GG/mg-cellulose (Figs 5,6), the concentration of amino groups in the filter paper is expected to be about 100 nM/mg-cellulose. Consequently, only 11% of the total amino group in the GG-treated filter paper was reactive. The limited reactivity of amino group introduced to the paper with GG might be caused by steric hindrance due to self-aggregation of GG, suggesting that most GG molecules adsorbed on the GG-treated paper form aggregates. To determine whether GG-treated paper is completely covered with GG aggregates or not, its cellulose degradability was examined (Fig. S6). As shown in Fig. S5, cellulase tolerance of the GG-treated paper strip was revealed. The quantitative evaluation of the cellulase tolerance was attempted by comparing the reducing power (release of cellobiose) in the reaction mixtures containing the GG-treated or non-treated paper disk. The cellobiose concentration was found to increase over time, even in the GG-treated paper-containing mixture (Fig. 7). The degradation rate of the GG-treated paper was estimated to be about 14% of that of the non-treated paper, revealing that the GG-treated paper is not completely covered with GG. Thus, the GG-treated paper strip was found to be mostly covered with GG aggregates and exert certain degree of cellulase-tolerance.

Discussion

Cellulose is a major constituent of the cell walls of plants and a highly important structural polysaccharide in living organisms. To expand the range of industrial and biomedical applications of cellulose, functionalization of cellulose is commonly achieved by introducing carboxy, aldehyde or amino groups. The most popular carboxylation method is TEMPO-catalyzed oxidation [20]. Aldehyde groups are readily

390 generated by periodate oxidation [21]. While amination has been attempted by using
391 highly reactive compounds, such as epichlorohydrin [22,23], *p*-toluenesulfonyl chloride
392 [24], silane coupling agents [25], and others [26-28]. However, these chemical
393 derivatization methods for functionalization do not make the best use of cellulose,
394 which is an environmentally-friendly material. In contrast the chemical derivatization
395 methods ever developed, amination with GG of bacterial origin is an environmentally-
396 friendly and low-emission technique for functionalization of cellulose. Not only for
397 functionalization in terms of chemical reactivity, GG is useful as a preservative for
398 paper because it confers biological stability (cellulase resistance) to cellulose.
399 During MD simulation for examination of the self-assembly of completely
400 protonated GG chains, both parallel and antiparallel double strands were commonly
401 observed while triple strands were only observed temporarily. The major intermolecular
402 hydrogen bonds (donor-acceptor) in these assemblages were, GlcN6-Glc5, Glc2-Glc6,
403 Glc6-Glc2, Glc6-GlcN6, and GlcN6-Glc6. It is likely that pairwise parallel and
404 antiparallel associations via diverse hydrogen bonds may be a characteristic of GG.
405 Complexed pairwise association among GG molecules may cause the development of
406 aggregation.
407 It was revealed that solutions with high salt concentration are favorable for
408 adsorption of GG to cellulose (amination of cellulose with GG). In general, both high
409 salinity and low temperature facilitate interactions between polymer molecules. It is
410 reasonable that self-aggregation (insolubilization) of GG was promoted under high
411 salinity and cool temperature conditions. Though high salinity was preferable for
412 adsorption of GG to cellulose, cool temperature depressed adsorption revealing that
413 adsorption should be performed under high salinity and high temperature conditions.
414 Opposite effect of temperature on aggregation and adsorption can be interpreted by

415 postulating the competition between aggregation and adsorption as follows. At low
416 temperature and in the presence of salt, most GG molecules associate to form insoluble
417 (large) aggregates before contact with cellulose. At high temperature in the presence of
418 salt, most GG molecules cannot form insoluble aggregates but remain in soluble (small)
419 aggregates which have potential to be irreversibly entrapped by cellulose. The
420 predominance (higher stability) of adsorption over self-aggregation at high temperature
421 can be attributed to electrostatic repulsion, only arise among GG molecules. As far as
422 we have tested, suitable salt concentration and temperature for amination using GG
423 solution are 1 M NaCl and 37 °C, respectively. It should be noted that only 11% of the
424 amino groups in the GG-treated paper are reactive probably because of steric hindrance
425 caused by aggregation of GG on the treated paper. For effective amination with GG, its
426 aggregation should be minimized. Preparation of GG of lower molecular weight might
427 be preferable for minimizing the aggregation. For optimization of aminating conditions,
428 equilibrium and kinetic analyses under varied GG molecular weight, GG concentration,
429 GG/cellulose ratio, and temperature will be necessary.
430 In this study, amination was only examined by dipping (soaking) a paper in a GG
431 solution but dropping of a GG solution on paper is another option for amination of a
432 paper. Since the amination using GG is possible only by making contact between GG
433 with cellulose in water, local amination of paper will proceed by applying GG solution
434 as droplets. Ink jet printing systems or sprayers will be suitable for dropping
435 application, while brushing will be a simpler method for amination of limited area. In
436 dropping or painting application of GG solution, drying of the solution will have a
437 significant influence on amination efficiency. Though the yield of GG was improved in
438 this study by introducing fed-batch cultivation, admittedly the productivity of GG is still
439 not enough for practical use. Considering the susceptibility of *T. nivea* to shaking

(mechanical impact), bubble tower type reactors might be more adequate than jar fermenters for high density cultivation.

Author contributions

MT is responsible for overall design of the research and supervised the experiments and analyses. MK, KU, KK, and IS assisted in designing the research and writing the paper. RT, HM, MK, RU, and TM performed cultivation and sample preparation. KK and KT performed NMR analysis. RT and KU performed computational analysis. RU and TM performed molecular weight determination and enzymatic degradation experiment. HM and MK performed adsorption and aggregation experiments. All authors read and approved this paper.

Disclosure statement

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536 Table 1. Comparison of theoretical and experimental H-H distances (Å) for GG

Proton pair for comparison[a]		Theoretical distance (MD calculation)	Experimental distance (NOESY experiment)	Difference[b]
GlcN1[c]	GlcN5	2.5	2.3	0.2
GlcN1[d]	**Glc3**	**4.4**	**3.1**	**1.3**
GlcN1	Glc4	2.9	2.0	0.9
GlcN1	Glc6	3.1	3.0	0.1
GlcN1	Glc6'	3.7	2.7	1.0
GlcN2	GlcN4	2.7	2.7	0
GlcN2	GlcN5	4.0	3.1	0.9
GlcN2	**Glc4**	**4.0**	**2.6**	**1.4**
GlcN4	Glc1	2.6	2.1	0.5
GlcN4	**Glc4**	**5.1**	**2.6**	**2.5**
GlcN5	GlcN6	2.6	2.7	-0.1
GlcN6	GlcN6'	1.8	2.2	-0.4
GlcN6	Glc1	3.2	3.0	0.2
GlcN6'	Glc1	3.9	2.9	1.0
Glc1	Glc3	2.8	2.2	0.6
Glc1	**Glc4**	**4.0**	**2.8**	**1.2**
Glc1	Glc5	2.5	2.2	0.3
Glc2	Glc4	2. 8	2.5	0.3
Glc2	**Glc5**	**4.0**	**2.8**	**1.2**
Glc3	Glc4	3.0	2.6	0.4
Glc4	Glc5	3.1	2.2	0.9
Glc5	Glc6	2.6	3.0	-0.4
Glc5	Glc6'	2.5	2.2	0.3
Glc6	Glc6'	1.8	1.8	0

537 [a] Proton pairs which exhibited NOE contacts in the NOESY experiment are listed.

538 [b] Difference = theoretical distance (Å) – experimental distance (Å).

539 [c] Hydrogen atoms are designated as Glc1 (position 1 of glucose residue), etc.

540 [d] The proton pairs which exhibited large difference (> ±1.2 Å) are indicated in bold.

541 Figure legends

542 Figure 1. Snapshots at 50 ns of GG (a), cellulose (b), and chitosan (c) chains (12 mer)
543 during MD simulations.

544

545 Figure 2. Self-assembly of fully protonated dodecasaccharide chains of GG in MD
546 simulation. Four chains of GG (a) were inserted in the water box and simulations were
547 performed for 300 ns (b). Simulations for chitosan chains were also performed for
548 comparison, (c) for 300 ns (d).

549

550 Figure 3. 1D-^{1}H NMR spectrum of GG-ABEE. The possible chemical structure of GG-
551 ABEE is illustrated above the spectrum. The nonoverlapped signals identified are
552 designated as GlcN1 (H1 of GlcN) and ABEE-CH_3 (methyl proton of ABEE), etc. The
553 signals used for the molecular weight estimation of GG are underlined.

554

555 Figure 4. Effects of NaCl concentration and temperature on precipitation of GG.
556 Symbols: ●, 4 °C; ■, 15 °C; ◆, 25 °C; ▲37 °C.

557

558 Figure 5. Effect of NaCl concentration on adsorption of GG to cellulose at 37 °C. The
559 unit (mg/mg) denotes the amount (mg) of GG adsorbed to 1 mg of cellulose (paper
560 disk).

561

562 Figure 6. Effect of temperature on adsorption of GG to cellulose in the presence of 1 M
563 NaCl. The unit (mg/mg) denotes the amount (mg) of GG adsorbed to 1 mg of cellulose
564 (paper disk).
565
566 Figure 7. Effect of cellulase on GG-treated (●) and non-treated (◆) paper disks. The
567 reaction mixtures were incubated at 30 °C. The degradation of the paper disks was
568 monitored by the increase in cellobiose concentration.
569