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Adsorption and Elution of Glucuronic Acid and Chondroitin Sulfate Using Amino-Group-Containing Spherical Gel

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Izumi Nakashima,[1] Akari Kishida,[1] Yuji Takaoka,[1] Shintaro Morisada,[1] Keisuke Ohto,[1] Hidetaka Kawakita,[1,†] Wataru Iwasaki,[2] Ramachandra Rao Sathuluri,[2] and Masaya Miyazaki[2]

[1]*Department of Chemistry and Applied Chemistry, Saga University*
(*1 Honjo-machi, Saga 840–8502, Japan*)
[2]*Advanced Manufacturing Research Institute, National Institute of Advanced Industrial Science and Technology*
(*807–1 Shuku, Tosu, Saga 841–0052, Japan*)

Abstract: A spherical gel containing amino groups was prepared using monomers of *N*,*N*-dimethylacrylamide and *N*,*N*-dimethylaminoethyl methacrylate, with a cross-linker composed of *N*,*N*′-methylenebisacrylamide prepared by suspension polymerization for the adsorption of glucuronic acid and chondroitin sulfate. The prepared gel was immersed in glucose, glucuronic acid, and chondroitin sulfate solutions to determine the adsorption performance in batch mode, which demonstrated that 20 % of the chondroitin sulfate was adsorbed to the amino-group-containing gel. The amino-group-containing gel was packed into a column to permeate the chondroitin sulfate-containing solution (0.40 g/L) at pH 2.0, and it adsorbed chondroitin sulfate to the gel at a space velocity of 4.5 h^{-1}. When the space velocity was changed to 1.5 h^{-1}, the amount of chondroitin sulfate increased. When 0.50 M NaCl solution was permeated through the chondroitin-sulfate-adsorbed gel in column mode, 70 % of the chondroitin sulfate was eluted. This spherical gel may be applicable for acidic glycan recovery using batch and permeation modes.

Key words: adsorption, gel, saccharides, chondroitin sulfate, elution

INTRODUCTION

Glycans play a central role in fundamental biological processes, including cell–cell recognition, detection, cell attachment, and detachment.[1)] Glycan structures containing relatively few components have the capacity to confer information. Various sugar compositions and modifications to those sugars, as well as different linkages and branch patterns, have been used to demonstrate that even short oligosaccharides confer a large amount of information. In addition to glycans with hydroxyl and amino groups, those with sialic acid with carboxyl groups and heparin sulfate with sulfonic acid exist. Sulfate glycan contributes to the formation of the minus domain among cells to transfer information by recognizing lectin and antibodies as well as control protein adhesion and enzymatic activity.[2)] Hyaluronic acid, composed of glucuronic acid and acetylglucosamine, forms the cell matrix and plays a role in the interaction between cells.[3)] Systems for recovering glycans are important for the purification of novel pharmaceuticals and glycan research.

A commonly used method for the recovery of glycans is adsorption. A solid material is immersed in glycan solution to concentrate glycan onto the materials. The non-selective adsorption of glycans results from similarities in glycan structures. Glycans possess multiple hydroxyl groups that are crucial to cellular functions. Moreover, glycans with amino groups and sulfonic groups exhibit complicated interactions with the solid material. Solid materials can be prepared by introducing functional groups with affinity to glycans. Boronic acid, amino groups, and biomolecules have been used with solid materials for the adsorption of glycans. Shinkai *et al*. prepared molecules complexed to glycans by using boronic acids.[4)5)] Matsumoto *et al.* used a chitosan-phenylborate polymer to adsorb modified glucose to determine the site of diol complexation in glucose.[6)] Polymeric resins can immobilize calcium or potassium ions adsorbed to glucose, fructose, and some oligosaccharides.[7)8)9)] Biomacromolecules of lectin immobilized on particles are typically used to separate glycans in a continuous mode.[10)]

The gel has a three-dimensional cross-linked structure including a solvent with a high volume fraction. After the gel chromatography column is filled with gel, the difference in uptake into the gel can be used to measure the polymer molecular weight. By introducing functional groups onto the gel, glycans can be adsorbed and concentrated in the gel interior via diffusion based on the concentration gradient. After adsorption, the gel contacts a reagent that cleaves the interaction between the adsorbed glycans and adsorption site to recover the glycans.

In this study, a continuous adsorption and elution system

†Corresponding author (Tel. +81-952-28-8670, Fax. +81-952-28-8548, E-mail: kawakita@cc.saga-u.ac.jp).
Abbreviations: DMAA, *N*,*N*-dimethylacrylamide; DMAEMA, *N*,*N*-dimethylaminoethyl methacrylate; SV, Space velocity.

of acidic glycans was developed using a spherical amino-group-containing gel. The gel was packed into a circular column to permeate the saccharide solution for continuous adsorption. The gel was polymerized using the monomers dimethylacrylate and *N*,*N*-dimethylaminoethyl methacrylate in suspension polymerization. Glycans generally contain a carboxylic acid or sulfonic group in their structure, in addition to a hydroxyl group. Gels prepared with an amino group formed a complex with the carboxylic acid or sulfonic group of glycans via ion-exchange interactions. The glycans used in this study were glucose, glucuronic acid, and chondroitin sulfate. Glucuronic acid and chondroitin sulfate have a carboxyl group and sulfonic group, respectively. To separate glycans using the gel-packed column, glycans flowed by convection would move through the gaps in the gels, interact with the functional groups on the surface of the gel, and diffuse towards the functional groups in the gel. The interactions between the gel and glycans depend on the functional groups present and the diffusion of glycans into the gel depends on the molecular weight and size of glycans. The gylcans glucuronic acid and chondroitin sulfate were used to evaluate the effect of different functional groups and molecular weights of glycans. The aims of this study were as follows: 1) preparation of a spherical gel with amino groups, 2) adsorption of glycans in batch mode, and 3) continuous adsorption and elution of glycans using a gel-packed column to recover the glycans.

MATERIALS AND METHODS

Materials. Monomers, *N*,*N*-dimethylacrylamide (DMAA) (049-19185), *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) (044-16276), and the cross-linker *N*,*N*′-methylenebisacrylamide (M0506) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. The stabilizers Span 80 and Tween 80 used during polymerization were purchased from Tokyo Chemical Industry Co., Ltd. and Sigma-Aldrich Co. (St. Louis, USA), respectively. The initiator ammonium persulfate (018-03282) was obtained from Wako Pure Chemical Industries, Ltd. The column (I.D. 5.0 mm, length 10 cm) used for gel packing was obtained from Bio-Rad (Hercules, USA). Sodium glucuronic acid monohydrate and chondroitin sulfate C sodium salt used for adsorption were purchased from Wako Pure Chemical Industries, Ltd. Other chemicals were of analytical grade or higher.

Spherical gel preparation by water-in-oil suspension. The water phase including monomer, cross-linker, and initiator was dropped onto the organic phase of hexane for suspension polymerization to obtain the spherical gel.[11] The polymerization scheme is shown in Fig. 1. The polymerization conditions of monomers, cross-linker, and initiators are summarized in Table 1. Span 80 and Tween 80 were dissolved in hexane in a three-necked flask. The flask was placed in a water bath (Tokyo Rikakikai Co., Ltd., Tokyo, Japan, SB350) at 348 K with stirring (Tokyo Rikakikai Co., Ltd., NZ-1100) at 360 rpm. To prepare the amino-group-containing gel, monomers of DMAA, DMAEMA, the cross-linker *N*,*N*′-methylenebisacrylamide, and water were mixed and then ammonium persulfate was added and dissolved. The water phase was dropped onto the organic phase using a dropping funnel. The temperature during polymerization was maintained at 348 K with stirring at 360 rpm for 1 h. After polymerization, radicals were scavenged by air bubbling and the reactor was cooled at room temperature for 20 min. After filtering the polymers by vacuum, the gels were washed with 250 mL hexane, ethanol in sonification. The gels were recovered by centrifugation at 6,000 rpm for 15 min. The gels were washed three times each with ethanol and water. The obtained gel was stored in water at a concentration of 5.0 (wt/wt)%. DMAA gel was prepared without DMAEMA monomer. The prepared gels using DMAA and using DMAA and DMAEMA were referred to as DMAA gel and DMAA-DMAEMA gel, respectively. The gel was observed by optical microscopy (VH-1000, Keyence, Osaka, Japan) to determine the size distribution from more than 200 images.

The water volume in the DMAA gel and DMAA-DMAEMA gel was determined based on the weight change before and after drying, and revealed that the water percentages were 85 and 86 %, respectively. If all of the monomer was polymerized, the amino group density in 1 g of dry DMAA-DMAEMA gel was calculated to be 0.86 mmol.

Glycans adsorption to spherical gel in batch mode. The molecular weight of chondroitin sulfate was determined by gel permeation chromatography. The analysis conditions were as follows: elution: 0.10 M phosphate buffer (pH 7.0), column: G3000PW$_{XL}$ (Tosoh Corporation, Tokyo, Japan), pump: Waters 515, detector (Milford, USA): Waters Refractive index 2414, recorder: PC recorder (Run Time Corporation, Tokyo, Japan).

For batchwise adsorption, glucuronic acid monohydrate (0.40 g/L), glucose (0.20 g/L), and chondroitin sulfate (0.80 g/L) were dissolved in water. Next, 0.10 wet gel was added to 15 mL of glycan solution. The pH was adjusted using 0.1 M HCl and 0.1 M NaOH solutions. Adsorption was performed at 308 K. After adsorption, the solution was filtered (Advantec, Taipei, Taiwan, 5C, pore: less than 5.0 μm). The glycan concentration remaining in the solution was determined using the phenol-sulfuric acid method after each adsorption. The adsorption percentage of glycans was determined as follows:

$$\begin{aligned}&\text{Adsorption percentage (\%)}\\&= 100\ (\text{initial concentration of glycan}) -\\&\quad (\text{concentration of glycan in the sample solution})/\\&\quad (\text{initial concentration of glycan})\end{aligned} \tag{1}$$

Adsorption and elution of chondroitin sulfate through gel-packed column in permeation mode. The obtained wet gel was packed in a column for the permeation of saccharide solution. Cotton (0.0037 g) was packed into the bottom of the column to a height of 1.0 cm. On the packed cotton, wet gel (volume 0.29 or 0.88 cm^3) was packed into the column to a height of 1.5 or 4.5 cm. Using a syringe pump (S-1235, Atom Medical International Inc., Tokyo, Japan), a solution of chondroitin sulfate (0.40 g/L, pH 2 or 7) was flowed from the top of the column. The effluent vol-

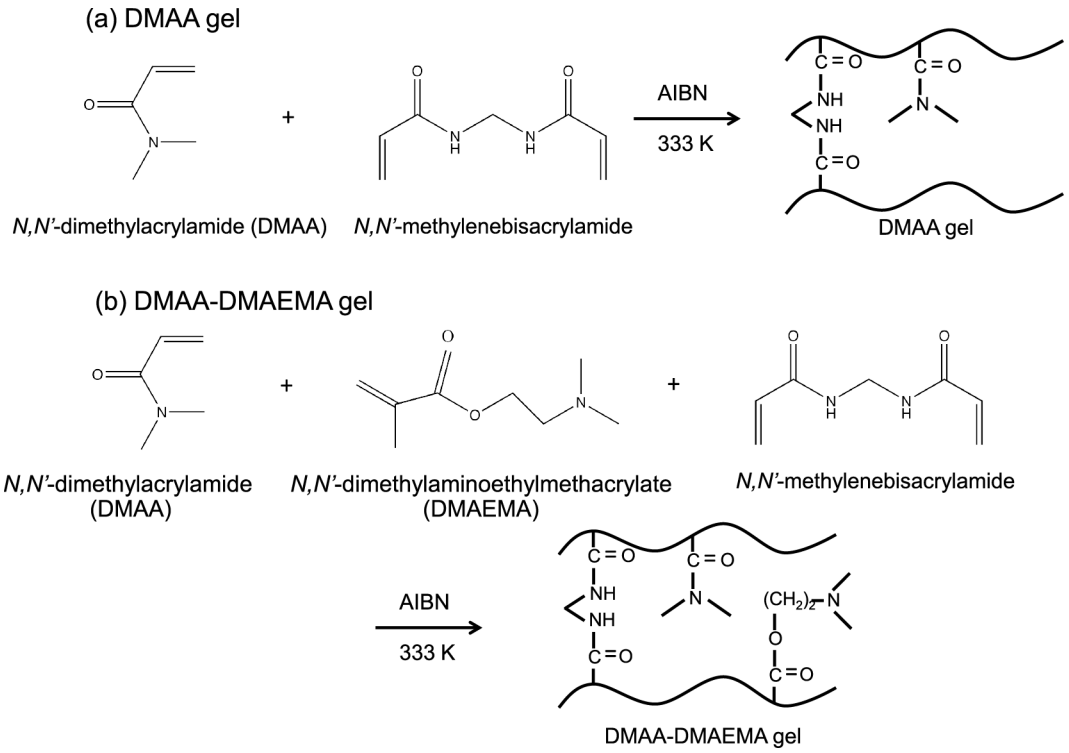

Fig. 1. Polymerization scheme of gels.
(a) DMAA gel and (b) DMAA-DMAEMA gel.

Table 1. Polymerization of spherical gel particle.

	Material	DMAA gel	DMAA-DMAEMA gel
Water phase	*N,N*-Dimethylacrylarnide (DMAA, monomer)	9.32	9.32
	N,N-Dimethylaminoethy1 methacrylate (DMAEMA, monomer)	-	1.48
	N,N'-Methylene bisacrylarnide (cross-linker)	0.16	0.16
	Ammonium persulfate (initiator)	0.33	0.33
	Distilled water	34.4	34.4
Organic phase	Span 80 / Tween 80 = 3 / 1 (stabilizer)	12	12
	Hexane	100	100

ume from the column was collected and the concentration in the effluent was determined using the phenol-sulfuric acid method. Space velocity (SV) was calculated as follows:

$$\text{Space velocity } (h^{-1}) = (\text{effluent volume})/(\text{gel volume}) \quad (2)$$

After the adsorption of chondroitin sulfate to the gel in the column, 0.50 M NaCl solution was flowed through the column to continuously elute adsorbed chondroitin sulfate.

RESULTS AND DISCUSSION

Preparation of spherical gel.

Glycans have hydroxyl, amino, carboxyl, and sulfonic functional groups in their structures. When a gel is present in glycan solution, molecules with affinity to these groups are adsorbed via diffusion into the gel. Using the suspension polymerization method, monomers dissolved in water were dropped into an organic solvent to form spherical droplets. Surfactants in the organic solvent were adsorbed to the liquid-liquid interface to stabilize the droplets. Initiator in the water media was cleaved to generate radicals to begin polymerization as temperature was increased.

Optical images and the size distribution of the obtained DMAA gel and DMAA-DMAEMA gel are shown in Fig. 2. The gels were spherical and contained a high percentage of water. The size of each gel was approximately 200 μm. Although the DMAEMA possessed amino groups to improve hydration, the size of each gel was similar.

Characteristics of spherical gels.

The gel was formed from the three-dimensional structure of the cross-linked polymer. The interior of the gel contained pores formed by the polymer matrix. When the obtained gel was used for glycan adsorption, the glycans reached the adsorption site by diffusing through the pores. Calculation of the gel pore size may be important for determining the kinetics of adsorption into the gel.

The direct and indirect determination of the gel pore has been previously examined. The pores were indirectly observed by X-ray and neutron scattering; Shibayama *et al*. studied gelatin gel to quantitatively examine the aggregated polymer structure.[12)]

Morisada *et al*. evaluated gel pores based on the amount

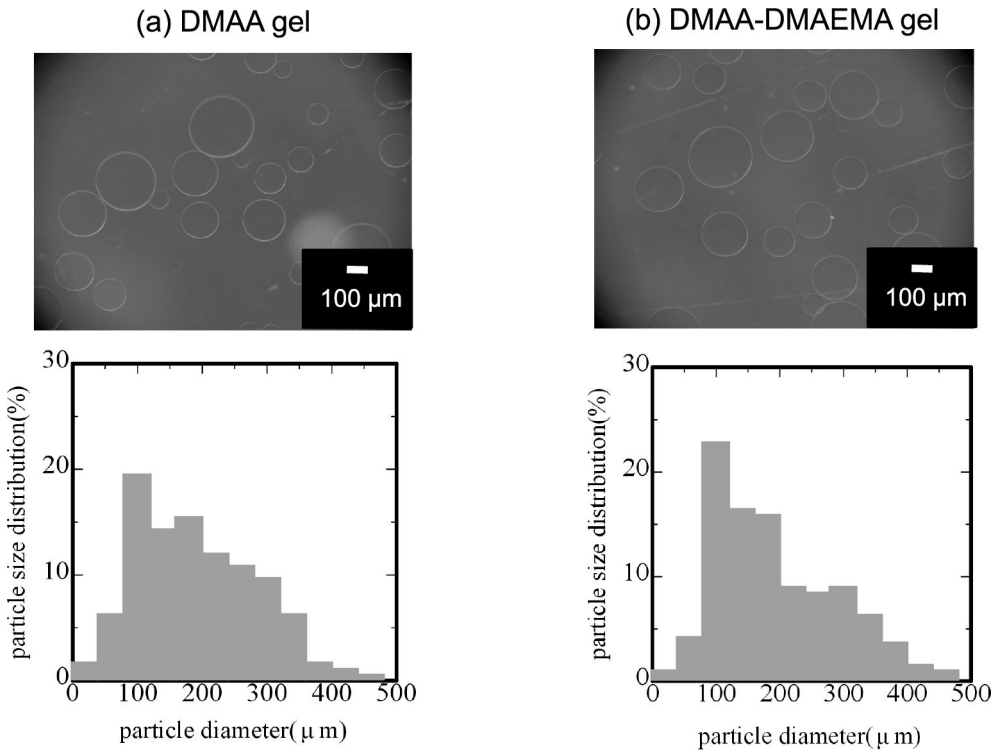

Fig. 2. Images and size distribution of (a) DMAA gel and (b) DMAA-DMAEMA gel.

of monomer and cross-linker as well as the volume of water media in polymerization.[13] The pore size in the DMAA-DMAEMA gel used for adsorption was calculated as follows. The gel volume was referred to as V_T. The distance between the cross-linkers, L_T, can be approximately described as follows:

$$L_T = \left(\frac{V_T}{N_c}\right)^{1/3} \quad (3)$$

where N_c is the number of cross-linkers in the gel. Setting the number of monomers in the polymer as N_m and number of monomers as n_m, the number of cross-linkers is represented as follows:

$$n_m = \frac{N_m}{N_c} \quad (4)$$

Assuming that the cross-linker connects two polymers, the number of the monomers, $n_m/2$, can be determined between the cross-linkers. The polymerized monomer had an all-trans structure, and binding angle was assumed to be θ = 109.5°. The length of the polymer, l, in the gel was calculated as follows:

$$l = \left(2 \cdot \frac{n_m}{2} - 1\right) l_b \sin\frac{\theta}{2} \quad (5)$$

where the bond length, l_b, was 0.153 nm. The size of the gel in this study was approximately 200 μm. Using the polymerization conditions shown in Table 1, L_T and l were calculated to be 17.9 and 12.8 nm, respectively.

The correlation length of acrylamide gel prepared by radical polymerization was directly determined by static light scattering, which showed that the length was 10 nm.[14] The length between monomers was calculated to be 18 nm and the calculated length was 2-fold longer than that the observed length.

Saccharides adsorption to spherical gel.

The two types of gels, DMAA gel and DMAA-DMAEMA gel, were added to glycan solutions (glucuronate monohydrate, glucose, and chondroitin sulfate) at pH 6. The time course curves of the adsorption percentages of three glycans to DMAA gel and DMAA-DMAEMA gel are shown in Fig. 3. The adsorption percentages of glucose and glucuronic acid to DMAA gel at 120 h were 10 and 6 %, respectively. For the DMAA-DMAEMA gel, the adsorption percentages of glucuronic acid and chondroitin sulfate gradually increased, and at a reaction time of 140 h, 20 % of the chondroitin sulfate was adsorbed. The adsorption percentage of glucuronic acid was 14 % at 120 h, demonstrating that the amount of gluronic acid adsorbed was 0.27 mmol/g-dry gel. The density of amino groups in the DMAA-DMAEMA gel was calculated to be 0.86 mmol/g, and 31 % of amino groups adsorbed glucuronic acid. At pH 6, the amino groups in the DMAA-DMAEMA gel were positively charged. Glucuronic acid and chondroitin sulfate, which have a carboxyl group and sulfonic group, respectively, had negative charges for adsorption to the gel via ionic interactions.

The resins and gels for glycan adsorption prepared by polymerization were evaluated. Acrylamide and *N*,*N*–methylenebisacrylamide with methacryloyl-histidine-copper ion complex was copolymerized for the adsorption of glucuronic acid at 342 mg/g of gel.[15] Ethylene glycoldimethylacrylamide with methacryloylamidohistidine-copper

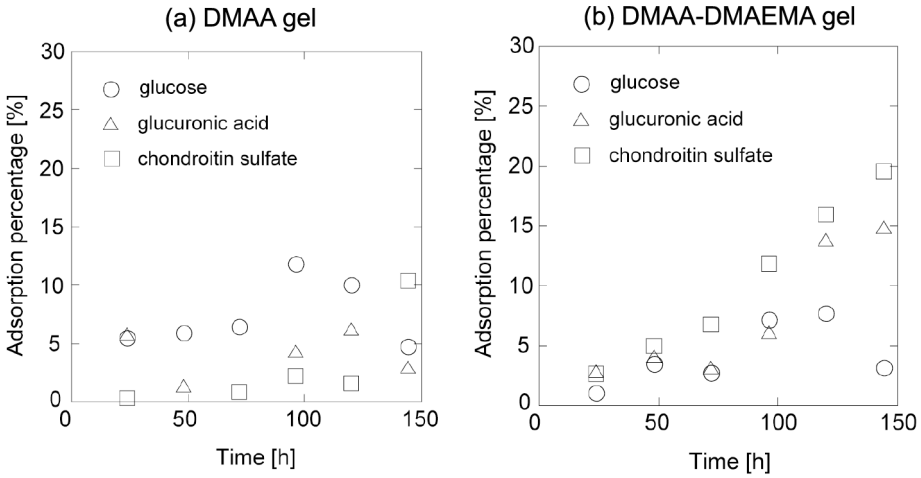

Fig. 3. Adsorption of saccharides at pH 6 to (a) DMAA gel and (b) DMAA-DMAEMA gel.

ion was copolymerized to prepare a gel. The gel adsorbed glucuronic acid at 233 mg/g.[16] The amount of glucuronic acid adsorbed to the gel in this study was 0.27 mmol/g (52 mg/g), which was 15−25 % higher than that in previous studies. However, as the amount of DMAEMA in the polymer increased, glycan adsorption increased.

Polymer size depends on environmental conditions such as pH and ionic strength. In this study, the size of chondroitin sulfate was not determined directly. Based on gel chromatography, the peak for chondroitin sulfate was observed at an earlier time than that of dextran at a molecular weight of 2,000 kDa. The size of dextran at 2,000 kDa was approximately 30 nm,[17] indicating that the size of chondroitin sulfate was a few tens of nanometers. The gel pore size, as described above, was calculated to be 20 nm, and chondroitin sulfate was adsorbed only to the surface of the spherical gel via ionic interactions. During adsorption, diffusion into the gel matrix is the rate-determining step. To achieve high-speed adsorption, the density of amino groups in the gel should be increased and pore formation in gel for diffusion is required. A method for forming pores has been proposed; silica particles were added to the water media in suspension polymerization and the silica particles were removed from the spherical gel.[18] The sites at which silica particles were removed from the gel would be the connected pore in gel. As the DMAA-DMAEMA gel effectively adsorbed acidic glycans in this study, more sophisticated gel preparation for continuous performance may be possible.

The pH dependence of adsorption percentage of glucuronic acid and chondroitin sulfate to the DMAA-DMAEMA gel is shown in Fig. 4. The pH in the solution changes the dissociation and association state of functional groups in the gel and glycans. To determine the pH dependence of the adsorption rate, adsorption time was set to 70 h. The adsorption percentage of glucuronic acid and chondroitin sulfate reached a maximum at pH 4.5 and 2, respectively. In the adsorption of acidic glycans, amino groups derived from the DMAEMA in the gel were involved. The pK_a values of DMAEMA were 0.6–1. [9] The sulfonic acid group of chondroitin sulfate is negatively charged at pH 1−7. Because at lower pH values, the amino group in DMAA-DMAEMA gel had positive charge, the adsorption percentage of chondroitin sulfate increased, showing maximum adsorption of chondroitin sulfate at pH 2. In contrast, the pK_a of the carboxylic acid of glucuronic acid is approximately 4.5 and this group is negatively charged at pH 4.5 or higher. As pH increased, the positive charge of the amino group in the DMAA-DMAEMA gel reduced the adsorption percentage of glucuronic acid, which showed a maximum at pH 4.5. As pH was further increased, further decreasing the positive charge of the amino group of the DMAA-DMAEMA gel, the adsorption percentage of glucuronic acid was reduced.

Continuous glycan adsorption and elution using gel-packed column.

The gel was packed into the column (I.D. 5.0 mm) and the solution of chondroitin sulfate was added for continuous adsorption. As shown in Fig. 4, the pH of the solution was found to be important for controlling glycan adsorption. The flow rate of glycan solution through the gel-packed column is also critical because of changes in the residence time of glycans in the gel. A breakthrough curve shows the relationship between the concentration in the effluent and the effluent volume. In Fig. 5, the *y*-axis is glycan concentration in the effluent divided by that of feed solution, while the *x*-axis is the effluent volume per packed gel volume. In the DMAA-packed gel, the concentration in the effluent reached one quickly on the *y*-axis, demonstrating that no adsorption occurred. As the chondroitin sulfate at pH 7.0 was flowed through the DMAA-DMAEMA gel-packed column, the amount of chondroitin sulfate was decreased, while at pH 2.0, adsorption was higher. When SV was changed from 4.5 to 1.5 h^{-1}, the amount of chondroitin sulfate was higher because the residence time of chondroitin sulfate in the gel was longer.

The DMAA-DMAEMA gel adsorbed glucuronic acid and chondroitin sulfate via ionic interactions. As the acidic glycan-containing gel was immersed into high-concentrated NaCl solution, the adsorbed glycan was desorbed by exchange of the glycan with the Cl^- ion. After adsorption, the chondroitin sulfate in the gel-packed column was eluted by the flow of 0.50 M NaCl solution, as shown in Fig. 6. The concentration of chondroitin sulfate in the effluent from the first column was 4-fold higher than that in feed concentra-

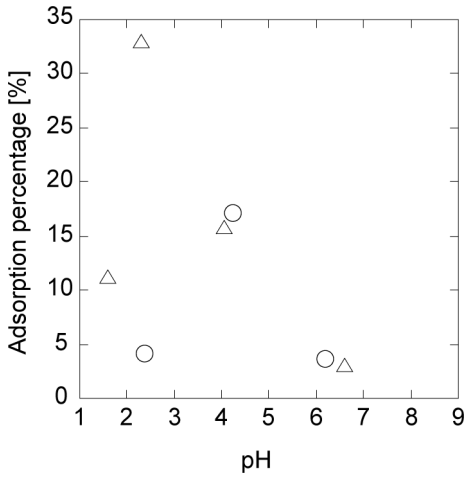

Fig. 4. Adsorption percentage to DMAAA-DMAEMA gel as a function of pH.
○, glucuronic acid; △, chondroitin sulfate. Reaction time, 70 h.

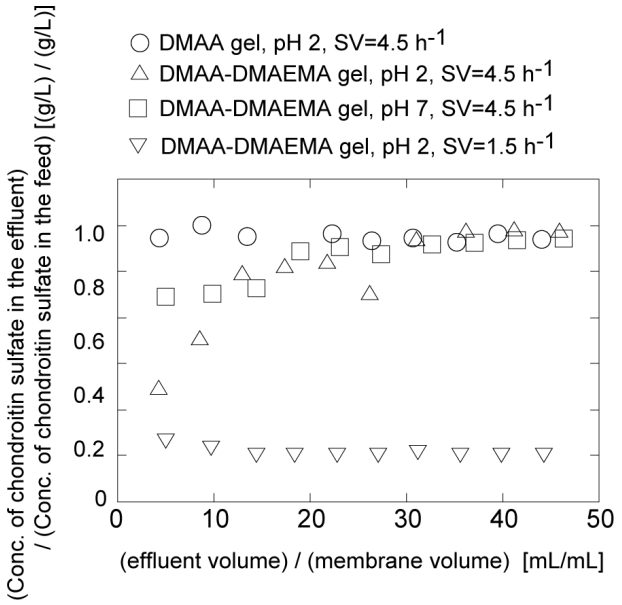

Fig. 5. Breakthrough curves of chondroitin sulfate to gel-packed column at pH 2 and 7, and SV = 1.5 and 4.5 h^{-1}.

tion, resulting in concentrated saccharide. Up to an effluent volume per gel volume of 45, 70 % of adsorbed chondroitin sulfate was eluted from the packed gel.

In the food and bioengineering industries, glycans play an important role. Glycan possesses a carboxyl group or sulfonic group, as well as hydroxyl groups or ether, with different molecular weights and branched structures. Our method can be carried out continuously in a column packed with DMAA-DMAEMA gel to recover glycans possessing various acidic functional groups.

CONCLUSIONS

In the food engineering and bioengineering industries, glycans and sugar chains plays are very important. In particular, in the analysis and medicinal use of glycans, it is important to establish a method for recovering the glycans. In this study, the amino group-containing gel was copolymerized with monomers possessing an amino group. The acidic glycans of glucuronic acid and chondroitin sulfate were concentrated by adsorption and elution in column mode. The size of the amino-group-containing-spherical gel prepared by suspension polymerization was approximately 200 μm. The adsorption percentage of chondroitin sulfate to the gel was 20%. This is because the sulfonic group of chondroitin sulfate formed a complex with the amino group in the gel via an ion-exchange interaction. When the solution of chondroitin sulfate was passed over the column filled with gel, the adsorption performance was dependent on pH and the flow rate of the feed solution. When 0.50 M sodium chloride solution was passed through glycan-adsorbed gel in the column, the adsorbed chondroitin sulfate was eluted, demonstrating that the gel-packed column can be applied for continuous glycan recovery.

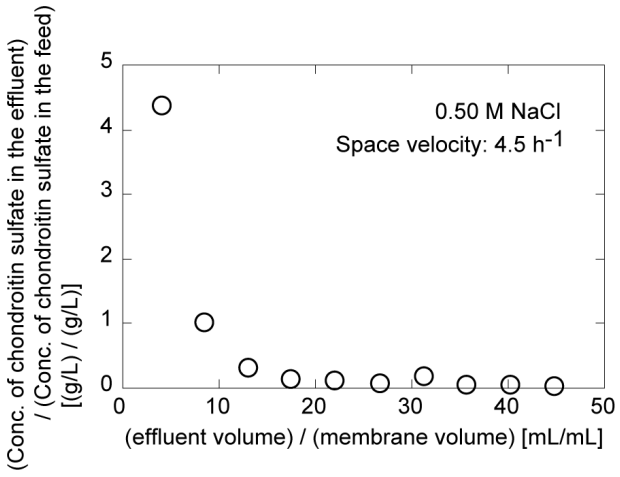

Fig. 6. Elution curves of adsorbed chondroitin sulfate from DMAA-DMAEMA gel-packed column.

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