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Synthetic Glycogels for Affinity Electrophoresis: A Facile and Efficient Method for Investigating Sugar-Lectin Interaction[1]

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A novel and efficient method for analyzing sugar-lectin interaction using affinity electrophoresis (AEP) is described. Polyacrylamide gels covalently conjugated with 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) residues were successfully prepared by radical copolymerization of highly reactive 3-(*N*-acryloylamino)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside with acrylamide in the presence of *N,N'*-methylenebisacrylamide (BIS). When the glycogels carrying various densities of GlcNAc branches were employed for polyacrylamide gel electrophoresis (PAGE) of lectins, the mobilities of wheat germ agglutinin (WGA) were specifically reduced by increasing the concentrations of the GlcNAc residues in gels, although concanavalin A (Con A) showed no significant change in the mobility. It was also demonstrated that the association constant of WGA with immobilized GlcNAc residue can be determined by combined use of this stable glycogel and an automated gel-scanning system associated with fluorometric spectroscopy. The association constant of WGA with the GlcNAc moiety was estimated to be 1.24×10^4 M^{-1}.

Key words: affinity electrophoresis, fluorometric detection, glycogel, glycomonomer, lectin-sugar interaction.

Oligosaccharide components of glycoconjugates play increasingly important roles in cellular recognition (*1*). The recognition processes are mostly dependent on the specific interaction of sugars with a variety of carbohydrate-binding proteins, including lectins (*2*). Although many methods of sugar-protein interaction such as ultraviolet or fluorescent spectroscopies (*3-5*), equilibrium dialysis (*6*), nuclear magnetic resonance (NMR) (*7*), differential scanning calorimetry (*8*), frontal affinity chromatography (*9*), and capillary zone electrophoresis (*10, 11*), have been reported for the characterization, these methods usually require large amount of samples or predominant labeling with suitable chromogenic probes. It is, therefore, of great importance to develop easier and more efficient analytical methods that can be performed with highly diluted concentrations of each ligand.

Affinity electrophoresis (AEP) is a useful method for analyzing biomacromolecular interactions such as those between enzyme and substrate (*12*) or antigen and antibody (*13*). AEP on macromolecule-incorporated polyacrylamide disk gels has also been used for the determination of dissociation constants of lectin-sugar complexes (*14, 15*). Allyl glycosides were also examined as possible polymerizable ligands for direct preparation of homogeneous glycopolymer gels, but were found unsuitable due to their lower polymerizability than that of acrylamide: large amounts of unpolymerized glycomonomers remained in the gel and had to be removed by washing. Therefore, methods that can be used to immobilize ligands for AEP seem to be severely limited, because it is preferable to utilize conjugating methods and gel media that do not cause high electroendoosmic flow and do not disturb the subsequent quantitative detections of separated proteins. Thus, we considered that copolymerization of glycomonomers showing similar polymerizability to acrylamide would proceed smoothly and afford the desired homogeneous glycogels in which the ligand molecules are distributed quite randomly.

In our studies on synthetic glycoconjugates and their biochemical functions, we found that the polymerizability of glycomonomers is remarkably enhanced by introducing aglycones having a terminal acrylamide-structure through an appropriate spacer moiety (*16*). We also demonstrated that these water-soluble glycopolymers can be used as highly sensitive and specific ligands of lectins (*17-21*). We describe herein a facile and efficient method for the preparation of novel and stable glycogels and their evaluation in AEP analysis of sugar-lectin interaction.

MATERIALS AND METHODS

Materials—Unless otherwise stated, all commercially

[1] Synthetic Glycoconjugates. 9: For Synthetic Glycoconjugates. 8., see Furuike, T., Nishi, N., Tokura, S., and Nishimura, S.-I. *Chem. Lett.*, 823-824 (1995).

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Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; AP-GlcNAc, 3-(*N*-acryloylamino)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside; BIS, *N,N'*-methylenebisacrylamide; TEMED, *N,N,N',N'*-tetramethylethylene-diamine; APS, ammonium persulfate; PAGE, polyacrylamide gel electrophoresis; AEP, affinity electrophoresis; Con A, concanavalin A; WGA, wheat germ agglutinin; NMR, nuclear magnetic resonance.

available solvents and reagents were used without further purification. WGA (Lot No. 62404) and Con A (Lot No. G9Q80141) were purchased from Seikagaku Corporation, Tokyo and used without further purification. 3-(*N*-Acryloylamino)propyl 2-acetamido-2-deoxy-β-D-glucopyranoside (AP-GlcNAc) was prepared by the method reported previously (*16*). Acrylamide was purchased from LKB-Produkter AB, Bromma, Sweden, and recrystallized from benzene before use. BIS, APS, and TEMED were purchased from Wako Pure Chemical Industries, Tokyo.

Characterization of Glycogel Formation by NMR Spectroscopy—^{1}H NMR spectra were recorded at 400 MHz with a JEOL EX-400 spectrometer in deuterium oxide using 3-(trimethylsilyl)propionic acid sodium salt (TSP) as internal standard. Polymerizability of AP-GlcNAc was evaluated as follows: AP-GlcNAc (10.0 mg, 30 mmol), acrylamide (10.6 mg, 0.15 mmol), and BIS (300 μg, 2.0 μmol) were dissolved in deuterium oxide (0.5 ml) and the solution was transferred into an NMR tube. To the solution were added TEMED (0.15 μl) and APS (0.35 mg), and the tube was immediately placed in the magnet system of the NMR spectrometer for further continuous measurement of the copolymerization profiles of each monomer.

Preparation of Glycogels and Affinity Electrophoresis—AEP analyses were essentially performed in 5% disk gels (0.38 M acetate buffer, pH 4.5) containing various concentrations of immobilized GlcNAc branches ranging from 0 to 1.5×10^{-3} M and 2.5% of BIS. The disk gels were prepared in glass tubes (5.0 mm $\times$ 115 mm) under the general conditions for radical copolymerization at room temperature for several hours. Electrophoresis was carried out using Model 3000-150 power supply (Wakamori, Tokyo) at 3 mA per tube and 20.0$\pm$0.1°C for 3 h using 0.35 M acetic acid and 0.35 M β-alanine as cathodic and anodic solutions, respectively. Lectins were dissolved in 56 mM potassium acetate solution (pH 6.8) and the concentrations of WGA and Con A were estimated to be 6.5 and 4.3 μM, respectively. About 10-20 μg of protein was applied on each electrophoretic tube. Mobilities of lectins were recorded by use of a Perkin Elmer LS50B luminescence spectrometer equipped with a plate reader system through fiber-optics for easy and speedy analysis, although Coomassie Brilliant Blue staining and silver staining were also applicable. Figure 1 shows a schematic drawing of the gel-scanning system used. A typical procedure for the determination of the protein-mobility is as follows: (A) After electrophoresis, the glass tube containing gel was directly placed on the plate reader system, which was originally designed for automated fluorometric analysis of 96-well microplates or thin layer chromatographic plates. (B) Relative fluorescence intensity of the gel at 370 nm (excitation at 285 nm) was continuously recorded from one end of the tube to the other by the LS50B spectrometer. (C) Difference spectra were obtained by using a fluorescence data manager for LS50B (FLDM, Perkin Elmer).

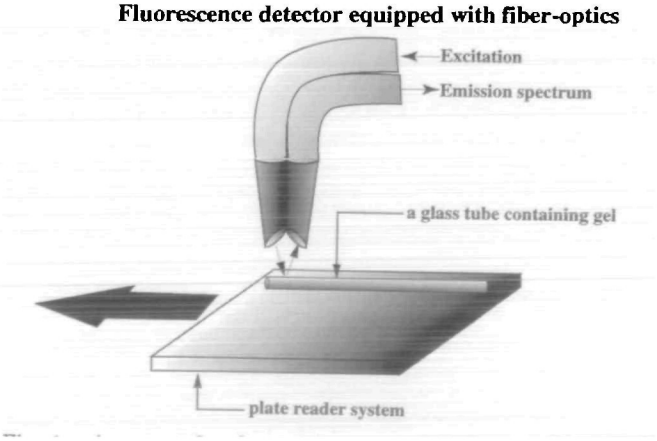

Fig 1. **Automated gel-scanning system using luminescence spectrometer LS50B.** Operations were carried out by fluorescence data manager (FLDM) in TLC mode. A gel-containing glass tube was placed on the plate and analyzed as described in "MATERIALS AND METHODS."

Theory and Data Analysis—In general, the complexation of a lectin and a ligand is an equilibrium reaction and the association constant (K_a) can be determined experimentally based on the following theoretical derivation. Assuming that the binding sites are independent of each other and non-interacting, the dissociation-association reaction at equilibrium is described as:

$$L+S \longleftrightarrow LS$$
$$K_a=[LS]/[L][S] \quad (1)$$

where [L], [S], and [LS] are the concentrations of free lectin, free sugar, and the resulting complex, respectively. Since the electrophoretic mobility of lectin is directly proportional to the concentration of the free lectin ([L]), the mobility of the lectin in the presence (l) or absence (l_0) of immobilized sugar can be expressed as:

$$l_0/l=[L]_t/[L] \quad (2)$$

where $[L]_t$ is the total concentration of the lectin which is initially charged on the top of the disk gel. Therefore, $[L]_t$ is given by

$$[L]_t=[L]+[LS] \quad (3)$$

where $[L]_t$ is considerably lower than the concentration of free sugar immobilized on the gels ([S]). Therefore, [S] is given by

$$[S]=[S]_0-[LS]\cong[S]_0 \quad (4)$$

where $[S]_0$ is the initial concentration of the sugar moiety in the gel, which can be easily calculated from the feeding ratio of the glycomonomer, acrylamide, and BIS. Rearranging Eqs. 1-4 leads to the final equation:

$$1/l=K_a[S]_0/l_0+1/l_0 \quad (5)$$

By plotting $1/l$ *versus* $[S]_0$ should give a straight line, from which the association constant K_a or $1/K_a$ can be calculated from the slope or the x-intercept.

RESULTS AND DISCUSSION

Characterization of Glycogel Formation by NMR Spectroscopy—Polymerizability of AP-GlcNAc as a glycomonomer was directly monitored and evaluated by ^{1}H NMR spectroscopy and the spectra are shown in Fig. 2. To investigate the solubility of AP-GlcNAc in the buffer solution and compatibility with acrylamide, in addition to its polymerizability, copolymerization was examined with much higher concentration of the AP-GlcNAc monomer

($[S_0] = 6.0 \times 10^{-2}$ M) than those employed for AEP analyses described below. Thus, the copolymerization of AP-GlcNAc, acrylamide, and BIS (molar ratio: 1.0 : 5.0 : 0.067) was initiated with APS and TEMED at 23.5°C. After 20 min (Fig. 2B), the signals due to double bonds at 5.75-5.85 ppm (-CH=) and 6.20-6.35 ppm ($=CH_2$) apparently became smaller than those of the original spectrum (Fig. 2A). The progress of polymerization was also supported by the appearance of new signals due to polymer backbone structures at 1.50-1.85 ($-CH_2-$) and 2.10-2.40 (-CH-) and significant broadening of the sugar ring protons at 3.20-4.50 ppm. Finally, the signals due to double bonds disappeared nearly quantitatively after 50 min (Fig. 2D). Figure 3 shows the time course of the polymerization reaction estimated by the integration data of the signals due to the double bonds. It was clearly observed that more than 98% of monomers including AP-GlcNAc were efficiently converted to the stable copolymer gel within 50 min, and the trace amount of unpolymerized monomers might not cause serious trouble for further AEP analysis. This suggests that polymerizability and compatibility of AP-GlcNAc were satisfactory for the facile preparation of homogeneous polyacrylamide gels bearing immobilized sugar residues in which the sugar contents could be easily controlled by the feed ratio of carbohydrate monomers and acrylamide.

AEP Analysis of Sugar-Lectin Interaction—Disk gel electrophoresis of lectins was performed under the conditions indicated in Table I and the results of the scanning fluorometric titrations by fluorescence spectrometer equipped with fiberoptics are shown in Fig. 4. The concentration of AP-GlcNAc ($[S]_0$) was successively increased from 0 to 1.5 mM. As seen in Fig. 4, it was found that the electrophoretic mobility of WGA (l) was markedly decreased from 0.412 (l_0) to 0.0438 by increasing the ligand

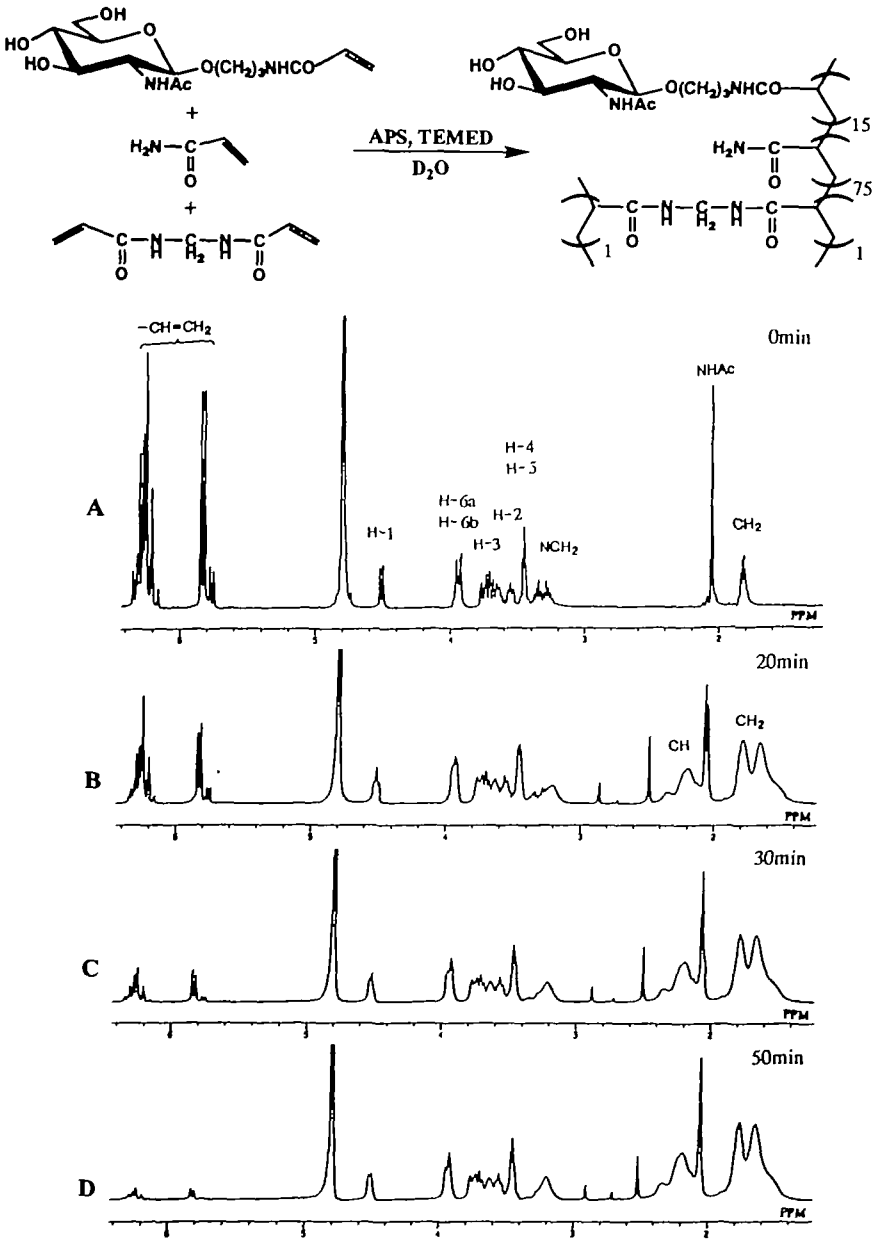

Fig. 2. **Glycogel formation observed by ^{1}H NMR spectroscopy.** The copolymerization was initiated under the conditions described in "MATERIALS AND METHODS." (A) 0 min, (B) 20 min, (C) 30 min, and (D) 50 min.

TABLE I. **Conditions and results of affinity electrophoresis.**

Disk No.	GlcNAc monomer conc. ($\times 10^{-4}$ M)	WGA			Con A	
		Distance[a] (mm)	Mobility (l)	$1/l$	Distance[a] (mm)	Mobility (l)
1	0.00	54.0	0.412	2.43	28.2	0.722
2	1.88	67.8	0.247	4.05	28.3	0.728
3	3.76	72.1	0.196	5.10	30.0	0.706
4	5.64	77.8	0.121	8.23	29.0	0.720
5	7.52	80.9	0.0895	11.2	27.8	0.722
6	9.40	82.7	0.0658	15.2	30.0	0.707
7	11.3	83.4	0.0595	16.8	29.6	0.710
8	15.0	84.7	0.0438	22.8	29.6	0.709

[a]Distance from the starting point of the gel measured by LS50B according to the method described in the experimental section.

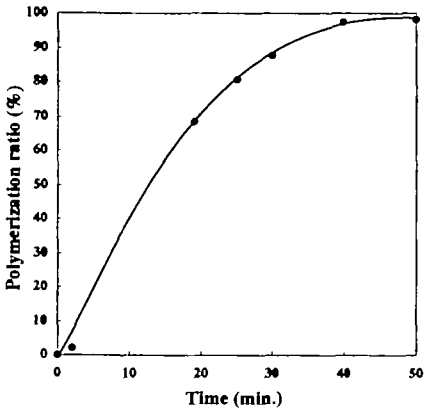

Fig. 3. **Time course of the copolymerization reaction.** The degree of the polymerization was calculated by integration data at 6.20-6.35 ppm due to the signals of double bonds (=CH_2).

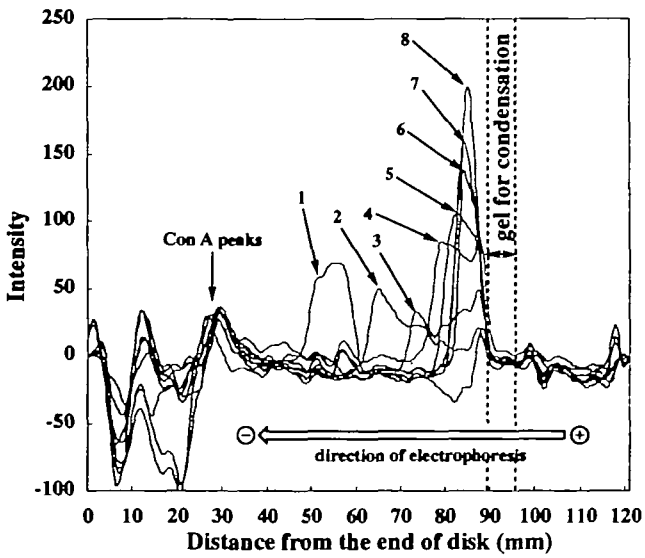

Fig. 4. **Affinity electrophoresis of WGA and Con A.** Fluorescence difference spectra were obtained on FLDM by subtracting the base line fluorescence intensity of the standard polyacrylamide gel from the original scanning data. The vertical and horizontal axes show the relative fluorescence intensity of the difference spectra and the distance from the bottom to top of the gel disk, respectively. The electrophoretic mobilities of WGA are indicated by numbers with arrows and correspond to the disk numbers listed in Table I.

concentration of the disk gels, while the mobility of Con A was independent of the concentration of AP-GlcNAc. The

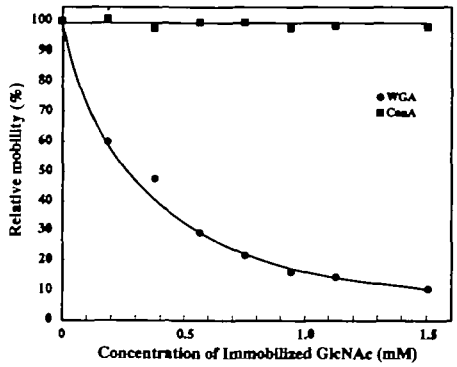

Fig. 5. **Influence of the concentration of immobilized GlcNAc on the electrophoretic mobility of WGA and Con A.** Symbols: square, Con A; circle, WGA.

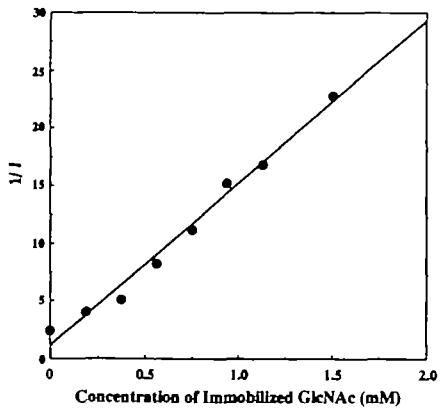

Fig. 6. **Plot of $1/l$ *versus* the concentration of immobilized GlcNAc for the calculation of the association constant according to Eq. 5.** Data are taken from Table I and Fig. 5.

relationship between the changes in electrophoretic mobility caused by GlcNAc residues immobilized in the affinity gel and ligand concentration is given in Fig. 5. And by applying Eq. 5, a straight line was obtained for WGA with a regression coefficient better than 0.985 (Fig. 6), suggesting that the assumption underlying this equation is correct. Consequently, the association constant calculated from the x-intercept was 1.24×10^4 M^{-1}. This agreed well with the association constant of WGA with the monomeric GlcNAc residue or its simple derivatives that was preliminarily reported from the fluorometric titration (*3*) and equilibrium dialysis (*6*) to be 1.98×10^4 M^{-1} (25°C) and 1.32×10^3 M^{-1} (4°C), respectively. Apparently, our method is supe-

rior to the conventional spectrometric methods since it requires only 10-20 μg of protein samples for each analysis. It also has the advantages of being are rapid, easy, and widely applicable to the neutral carbohydrates without negatively charged sugar derivatives required for the capillary zone electrophoresis. Moreover, the automated fluorescent gel-scanning system employed here facilitated the direct detection of the mobility of the proteins without the tedious and time-consuming procedures for staining with Coomassie Brilliant Blue.

In conclusion, we have demonstrated the feasibility of affinity electrophoresis by using a new type of polymerizable glycosides for the efficient analyses of the sugar-lectin interactions. The development of the acrylamido-type polymerizable glycomonomers was found to improve the previous method of preparation of homogeneous acrylamide gels for the affinity electrophoresis of sugar-binding proteins. The association constant of WGA with immobilized GlcNAc residues was determined by the combined use of the stable glycogels and an automated gel scanning system associated with fluorometric spectroscopy. The versatility of this method is now under investigation by using further complicated and longer oligosaccharide sequences containing sialic acid and sulfated sugars, which are known to be important ligands of selectins. The results will be reported in the near future.

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