Electrophoretic Deposition (EPD) of Lectin in the Presence of New Glycopolymers Aiming at Facile Detection of Carbohydrate-Protein Interactions

<table>
<thead>
<tr>
<th>Title</th>
<th>Macromolecular Chemistry and Physics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>218</td>
</tr>
<tr>
<td>Number</td>
<td>23</td>
</tr>
<tr>
<td>Page</td>
<td>1700351</td>
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<td>Year</td>
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Electrophoretic Deposition (EPD) of Lectin in the Presence of New Glycopolymers Aiming at Facile Detection of Carbohydrate-Protein Interactions

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Abstract: We performed atom transfer radical polymerization (ATRP) of allyl methacrylate (AMA) followed by a thiol-ene click reaction with 1-thio-β-D-glucose tetraacetate to afford a new type of glycopolymers via deacetylation. Their interactions with a sugar-binding protein were evaluated by electrophoretic deposition (EPD) procedures as well as fluorescence measurements. The deposition amount of concanavalin A (conA) in the presence of glucose or galactose measured by EPD was remarkably different from that in the presence of the synthesized glycopolymer, even though the concentration of sugar-residue was the same. The deposition amount, as well as the electric current that flowed during the EPD, was higher in the presence of the polymer, which confirmed that interaction of con A with the glycopolymer was detected by the EPD procedure.
INTRODUCTION

Recent progress in glycobiology has showed that cell surface oligosaccharides play essential roles in various biological recognition process, including intercellular recognition, adhesion, cell growth, and differentiation. Although the recognition process is essentially based on carbohydrate-protein interactions, individual interactions are generally weak. Some glycopolymers in which saccharide residues are incorporated in polymer backbones induce enhancement of binding affinity toward proteins which is ascribed to multivalent recognition, i.e., a “cluster effect” when the density and relative spatial arrangements of the carbohydrate residues are appropriate. Therefore, if the three-dimensional arrangement, i.e. the interval and direction of the pendant carbohydrate could be regulated, a remarkable enhancement of the binding ability might be possible. Aoi et al. used a dendrimer skeleton for the three-dimensional arrangement of a saccharide as a “sugar ball”. Matsuura et al. later proposed another strategy to prepare periodic glycosylated oligonucleotides (20-mers). In view of the improved binding ability of glycopolymers towards sugar-binding proteins, it is still necessary to explore facile and safe detection methods for the carbohydrate-protein interaction, because some biological infections that proceed via cell surface oligosaccharides lead to serious diseases, including influenza, Ebola hemorrhagic fever, as well as food poisoning caused by the O-157 strain of the E. coli bacteria.

On the other hand, design of new materials that can effectively coat a metal substrate at a nano- to micrometer scale is a fascinating subject from the viewpoints of biorelated, energetic, and environmental fields. Electrophoretic deposition (EPD) is a promising and facile technique for the fabrication of inorganic or organic or inorganic / organic composite films onto electrodes. EPD is based on the controlled electric-field-induced deposition of charged particles or molecules in an orderly manner onto an electrode. The characteristics of EPD include the capability of film formation on a complex surface shape, easy control of thickness, and formation of a homogeneous layer.
During the past five years, our group has focused on sulfone-containing polymers for the investigation of the electrophoretic behavior of interesting non-ionic polymers. Recently, our group found that an aliphatic poly(ester-sulfone) prepared by thiol-ene click polymerization and subsequent oxidation showed anode-selective electrophoresis under EPD conditions. Anode-selective EPD coatings of bioactive glass (45S5Bioglass) and titanium dioxide (TiO₂) were also demonstrated. Furthermore, these observations and the interesting electrophoretic behavior of the sulfone-containing polyester prompted us to explore why the poly(ester-sulfone) is deposited onto the anode, and what part of its structure is responsible for this unusual electrophoretic behavior. As far as we know, there are few reports dealing with application of EPD procedure for biological applications, even though sugar-binding proteins, e.g. lectins, act as electrolytes in aqueous media. This research background and the recent pandemic of infectious diseases based on carbohydrate-protein interactions prompted us to investigate the EPD behavior of lectins in the presence of new glycopolymers synthesized in our laboratory.

**Experimental Section**

**Materials and Methods.** Chemicals were obtained from commercial sources and used without further purification. Allyl methacrylate (AMA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 1,1,4,7,10,10-Hexamethyltriethylenetetramine (HMTETA) and ethyl 2-bromoisobutyrate were purchased from Aldrich Chemical Co. (St. Louis, MO, U.S.A.). 1H-NMR spectra were acquired at 27 °C using a Bruker Analytik DPX400 spectrometer (400 MHz). Tetramethylsilane was used as the internal standard (0 ppm). The number-average molecular weight (Mₙ) and the polydispersity index (Mₘ/Mₙ) of each polymer were determined by size exclusion chromatography (SEC) using a JASCO PU-2080 plus pump system, a refractive index (RI) detector (JASCO RI-2031 plus), and either a TSK gel Super Multipore HZ-M column (eluent, chloroform; flow rate, 0.35 mL/min; temperature, 40 °C; Tosoh Corp.) or using a size exclusion chromatography
system consisting of a Tosoh DP8020 pump system, a JASCO RI-2031T plus differential refractometer, and Shodex OH pak SB-803HQ columns [eluent, NaCl aq. (0.2 M); flow rate, 0.5 mL/min; temperature, 40 °C; Tosoh Corp.], in which pullulans are used as the calibration standard.

**Synthesis of Poly(AMA) by Atom Transfer Radical Polymerization (ATRP).**
Polymerization conditions were surveyed, an example follows. AMA 0.67 mL (5 mmol), ethyl 2-bromoisobutyrate 15 μL (0.1 mmol), 1,1,4,7,10,10-hexamethyltriethylene-tetramine (HMTETA) 27 μL (0.1 mmol), CuBr 14 mg (0.1 mmol), and 5 mL of toluene were added to a 10 mL round-bottomed flask with a magnetic stir bar under a nitrogen atmosphere. The mixture was stirred at 50 °C for 6 h, the ATRP proceeded smoothly to give poly(AMA) (51% conversion, $M_n = 6.0 \times 10^3$, $M_w/M_n = 1.2$). $^1$H-NMR (400 MHz, CDCl$_3$, δ): 5.97-5.82 (brm, CH$_2$CHCH$_2$OC=O, 1H), 5.39-5.29 and 5.29-5.21 (brm, CH$_2$CHCH$_2$OC=O, 2H), 4.65 (brs, CH$_2$CHCH$_2$OC=O, 2H), 2.05-1.89 and 1.89-1.77 [brm, O=CC(CH$_3$)$_2$, 2H], 1.16-1.00 and 0.93-0.82 [brm, C(C$\cdot$H$_3$)$_3$, 3H]. IR (KBr) cm$^{-1}$: 3086 (νC=C–H), 2934 (νC–H), 1730 [νC=O(ester)], 1649(νC=C), 1449 (δC–H), 1267 and 1147 [νC=O(ester)] (see also Figure S1).

**Thiol-ene Functionalization of Poly(AMA) with 1-Thio-β-D-glucose Tetraacetate.** Poly(AMA) (141 mg, 1.12 mmol repeating unit, 1 equiv) was dissolved in 2.5 mL of THF. 1-Thio-β-D-glucose tetraacetate (500 mg, 3.16 mmol, 2.8 equiv) and 1-hydroxycyclohexyl phenyl ketone (Irgacure 184; 114 mg, 0.5 equiv) were added, the mixture was degassed, the ambient atmosphere replaced with nitrogen, and exposed to UV light using a ASAHI SPECTRA MAX-303 Xenon light resorce (300 W 250 nm-385 nm) for 2h. The solvent was concentrated in vacuo. The polymer was dissolved in CHCl$_3$ and was purified by precipitation into an excess amount of MeOH (87.9 % yield, $M_n = 1.0\times10^4$, $M_w/M_n =2.1$). $^1$H-NMR (400 MHz, CDCl$_3$, δ): 5.25 (brt, sugar H-2, 1H, 8.2 Hz), 5.10 (brt, sugar H-3, 1H, 9.7 Hz), 5.03 (brt, sugar H-4, 1H, 9.2 Hz), 4.61 (brs, sugar H-1, 1H), 4.32-4.23 and 4.18-4.10 (brm, sugar H-6, 2H), 4.03 (brs, SCH$_2$CH$_2$CH$_2$OC=O, 2H),
3.84-3.75 (brm, sugar H-5, 1H), 2.84-2.75 and 2.75-2.66 (brm, SCH₂CH₂CH₂OC=C, 2H), 2.10, 2.08, 2.03, and 2.02 (4s, acetyl group, 3Hx4), 1.99-1.89 (brm, SCH₂CH₂CH₂OC=O, 2H), 1.59-1.46 [brm, CH₂CH(CH₃), 2H], 2.10, 2.08, 2.03, and 2.02 (4s, acetyl group, 3Hx4), 1.99-1.89 (brm, SCH₂CH₂CH₂OC=O, 2H), 1.59-1.46 [brm, CH₂CH(CH₃), 2H], 2.10, 2.08, 2.03, and 2.02 (4s, acetyl group, 3Hx4), 1.99-1.89 (brm, SCH₂CH₂CH₂OC=O, 2H), 1.59-1.46 [brm, CH₂CH(CH₃), 2H], 1.00, 0.92, 0.81 [3brs, C(CH₃), 3Hx3].

**Deprotection of the Acetyl Groups of Glyco Poly(AMA).** The product was dissolved in 2 mL of MeOH and 1 mL of CH₂Cl₂ containing hydrazine monohydrate ([hydrazine]₀/[acetyl group]₀ = 5/1) and stirred at room temperature for 16 h. After the reaction, acetone was added to the solution and the mixture was stirred for 4 h to quench the reaction. The reaction mixture was evaporated in vacuo, and the remaining residue was dissolved in H₂O, and it was then purified by precipitation into an excess amount of MeOH. After dialysis for 2 days (cut off MW = 100-500), a white powder was obtained using a freeze-dryer (44.0 % yield, Mₐ = 5.6×10³, Mₖ/Mₐ = 1.4). H-NMR (400 MHz, CDCl₃, δ): 4.51 (brd, sugar H-1, 1H, 7.1 Hz), 4.13 (brs, SCH₂CH₂CH₂CO=O, 2H), 3.90-3.81 and 3.76-3.63 (m, sugar H-6, 2H), 3.52-3.36 and 3.36-3.24 (brm, sugar H-2,3,4 and 5, 4H), 2.97-2.68 (brm, SCH₂CH₂CH₂CO=O, 2H), 2.21-1.95 and 1.95-1.74 [brm, SCH₂CH₂CH₂OC=O and CCH₂(CH₃), 2H], 1.26, 1.04, 0.85 [3brs, C(CH₃), 3Hx3]. IR (KBr) cm⁻¹: 3406 (νOH), 2925 (νC-H), 1721 [νC=O(ester)], 1449 (δC-H), 1247 and 1159 [νC=O(ester)] (see also Figure S2).

**EPD of Con A in the Presence of Glycopolymer**. Concanavalin A (con A) was dissolved in water or HEPES buffer in the absence and presence of glycopolymer. For the deposition of con A onto a stainless steel electrode (0.2 × 15 × 40 mm stainless steel SUS 301 electrodes, the Japan Metal Service Corporation, Saitama, Japan) by EPD, the distance between the stainless steel electrodes was 6.5 mm, and the deposition time was 90 s at 50 V. EPD of con A was also performed in the presence of glucose, galactose, or mannose.

**Recognition of Glycopolymer by Lectin (con A) Detected by Fluorescence Spectra.** Fluorescence spectra (at 518 nm) of FITC-labeled con A lectin were recorded
on a JASCO FP-8500 spectrofluorometer with excitation at 490 nm in the range of 1-6 mM sugar residue concentration. The solutions were contained in 10 mm quartz cells maintained at r.t. The concentration of con A in 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) buffer (10 mM, pH 7.2) was 15 µg/mL.

Results and Discussion

Preparation of Poly(AMA) by Atom Transfer Radical Polymerization (ATRP). ATRPs of AMA were performed in toluene (1.0 M) at 50 °C for 6 h, using ethyl 2-bromoisobutyrate, 1,1,4,7,10,10-hexamethyltriethylene-tetramine (HMTETA) and CuBr as the initiator and catalyst, respectively (Scheme 1). The ATRP proceeded smoothly to give poly(AMA) (51% conversion, $M_n = 6.0 \times 10^3$, $M_w/M_n = 1.2$). The results of polymerizations at 50 °C and 60 °C are summarized in Figure 2. In all runs, the structure was confirmed by IR and $^1$H NMR spectra (Figure 1) and the expected polymethacrylate having pendent carbon-carbon double bonds was obtained. As shown in Figure 2, at 60 °C the $M_w/M_n$ ratio increased with the monomer consumption, for which at 60% conversion the value of $M_w/M_n$ was 2.7. The broadening of the molecular weight distribution was ascribed to termination of the propagating radical at a pendent double bond to produce the corresponding branched polymer. At 50 °C, the increase of $M_w/M_n$ was suppressed even with an increase of monomer consumption, for which at 40% of conversion, the poly(AMA) with $M_n=3.5 \times 10^3$ ($M_w/M_n=1.7$) was formed.

Scheme 1. Atom Transfer Radical Polymerization (ATRP) of AMA.
Figure 1. $^1$H NMR spectrum of poly(AMA) (in CDCl$_3$, 25 °C, 400 MHz).
Figure 2. Conv. vs. $M_n$ plots (blue symbols) and conv. vs. polydispersity index (PDI) = $M_w / M_n$ plots (orange symbols) for ATRP of AMA in toluene ([M]₀: 1 M) at (a) 50 °C and (b) 60 °C (initiator: ethyl 2-bromoisobutyrate).

**Thiol-ene Functionalization of Poly(AMA) with 1-Thio-β-D-glucose Tetraacetate.** The poly(AMA) obtained as described above was coupled with an excess amount (2.8 equiv) of 1-thio-β-D-glucose tetraacetate in THF for 2h using 1-hydroxycyclohexyl phenyl ketone (Irgacure 184; 0.5 equiv) as the photo-radical initiator (Scheme 2), where the reaction mixture was exposed to UV light (300 W 250 nm-385 nm) during reaction. After the reaction, the mixture was purified by precipitation into an excess amount of MeOH to give the expected acetylated glycopolymer (87.9 % yield $M_n = 1.0 \times 10^4$, $M_w/M_n =2.1$). The structure was confirmed by IR and $^1$H NMR (Figure 3) spectra and the expected polymethacrylate having pendent acetylated glucose was obtained. The functionality was calculated using $^1$H NMR intensity ratio of peaks at 2.84-
2.75 and 2.75-2.66 (SCH$_2$CH$_2$CH$_2$OC=C) ppm and at 1.00, 0.92, 0.81 [backbone $\alpha$-methyl; C(CH$_3$)].

Scheme 2. Synthesis of new glycopolymer prepared via ATRP of AMA and thiol-ene click reaction using Irgacure 184 as the photo radical inducer.

Figure 3. $^1$H NMR spectrum of glycopolymer prepared via ATRP of AMA and thiol-ene click reaction (in CDCl$_3$, 400 MHz, 25 °C).

Deprotection of the Acetyl groups of Glyco Poly(AMA). Tetracetylated Glc-
substituted poly(AMA) was deacetylated in MeOH/CH₂Cl₂ (2/1, v/v) containing hydrazine monohydrate ([hydrazine]₀/[acetyl group]₀ = 5/1) and stirred at room temperature for 16 h to give glucose-containing poly(AMA) in good yield (44-53 %) (Scheme 3). After the reaction, the reaction mixture was evaporated in vacuo, the residue was dissolved in H₂O, and it was then purified by dialysis for 2 days (cut off MW = 100-500), to produce a white powder using a freeze-dryer (44.0 % yield, \( M_n = 5.6 \times 10^3 \), \( M_w/M_n = 1.4 \)). Their structure was elucidated by IR, \(^1\)H (Figure 4) and \(^{13}\)C NMR spectra. The FT-IR spectrum of the product polymer showed disappearance of the absorption at 1748 cm\(^{-1}\) due to ester groups. The SEC, \(^1\)H and \(^{13}\)C NMR analyses of glucose-containing poly(AMA) shows that the deprotection proceeded without any side reaction.

**Scheme 3.** Deacetylation of new glycopolymer prepared via ATRP of AMA and thiol-ene click reaction.
**Figure 4.** $^1$H NMR spectrum of deacetylated glycopolymer prepared via ATRP of AMA and thiol-ene click reaction (in D$_2$O, 25 °C, 400 MHz).

**EPD of conA in the Presence of Glycopolymers.** First, the EPD behavior of concanavalin A (conA) was investigated in water. ConA is one of the well-studied plant lectins and specifically recognizes glucose. For the deposition of conA the distance between the stainless steel electrodes was 6.5 mm, and the deposition time was 90 s at 200 V. As shown in Figure 4 (left), the amount deposited on the stainless steel increased with an increase in the added conA concentration. Using water as the medium, the pH around the electrode was locally changed by electrolysis of water and polymeric films of conA formed on the anode as well as the cathode, as shown in Figure 5 (right).
Figure 5. Changes of deposited amount (mg/cm$^2$) as a function of concentration of conA (g/L) (left) and pictures of the anodic and cathodic electrodes (right).

Next, therefore, the EPD of con A was performed in 10 mM HEPES buffer. Using the HEPES buffer, the pH was kept at 7 and deposition on the anode was preferred (Figure 6). When we used 10mM HEPES buffer kept at pH 8, conA moved mainly to the anode under the EPD conditions and the EPD behavior described blow is discussed under same conditions.
Figure 6. Deposited amount (mg/cm²) as a function of pH of the conA aq (top) and pictures of the anodic and cathodic electrodes at pH 7 and 8 (bottom).

In the presence of added sugar (glucose, galactose, or glycopolymer), the EPD behavior of conA aq. was monitored and the amount deposited on the stainless steel anodes was plotted as a function of sugar concentration, as shown in Figure 7, accompanied by a picture of the conA aqueous solution. Even though similar EPD behavior was observed in the presence of glucose or galactose, as compared with that in the absence of additives, the change in the deposited amount was negligible. In the presence of glycopolymer, the conA aq. looks turbid because of aggregation of conA induced by the glycopolymer having pendent glucose residues. In the plot of the deposited amount on the anode as a function of sugar concentration in the glycopolymer, the
deposited amounts were higher than those in the presence of glucose (Figure 6, bottom).

**Figure 7.** Changes of deposited amount (mg/cm²) on anode as a function of concentration of glucose or galactose (top) and glucose or glycopolymer (bottom) and in the presence of conA (1.5 g/L) accompanied by pictures of the conA aq. solution.

When we used a HEPES buffer solution of conA. kept at pH 8, a remarkable difference was observed (Figure 8), because the conA interacted with glucose and caused it to move to the anode selectively. It is apparent that the amount deposited onto a stainless steel electrode is higher with glycopolymer than in the presence of glucose. The results indicated that an EPD procedure in an HEPES medium can detect the improved sugar-
protein interaction resulting from aggregation of the sugar-binding lectin with glycopolymer, in which the new slip surface preferred for electrophoresis appeared in the EPD carried out in HEPES media.

The occurrence of an improved sugar-protein interaction was supported by fluorescence measurements of FITC-labeled lectin in the presence of glycopolymer (please see also supporting information available Figure S3), according to reported procedure. Changes of the fluorescence intensity of FITC-labeled conA lectin at 518 nm (Ex 490 nm) were plotted as a function of sugar concentration ([sugar]) using eq 1. The binding constant ($K_a$) of the glycopolymer ($M_n=5.6\times10^3$, $M_w/M_n=1.41$) with conA was calculated to be $5.4 \times 10^2$, which is significantly higher than that of glucose ($4.8\times10^2$) or mannose ($6.7\times10^2$), as well as galactose ($3.5\times10^2$) (please see also supporting information available Table S1).

$$F_0[sugar]/\Delta F = F_0[sugar]/\Delta F_{\text{max}} + F_0/\Delta F_{\text{max}} K_a \quad (1)^{11}$$

In order to check remarkable “cluster effect”, we synthesized a glycopolymer having pendent glucosides with higher molecular weight ($M_n=10.3\times10^3$, $M_w/M_n=2.55$). When we calculated the $K_a$ of the glycopolymer, obtained apparent $K_a$ ($M^{-1}$) with conA was $1.4\times10^3$ ($M^{-1}$), which is higher than that ($5.4\times10^2$ $M^{-1}$) of the glycopolymer ($M_n=5.6\times10^3$). Expectedly, the deposited amount of the former ($221 \mu g/cm^2$) was higher than that ($187 \mu g/cm^2$) of the latter at the concentration of sugar residue ([sugar]=4.7 mM) in the EPD conditions. The results supported our new aspect in EPD behavior of conA in the presence of glycopolymer.
Figure 8. Changes of deposited amount (mg/cm$^2$) on anode as a function of concentration of glucose, galactose, or mannose (top) and glycopolymer ($M_n=5.6\times10^3$, $M_w/M_n=1.41$) or glucose (bottom) in the presence of conA (1.5 g/L) accompanied with the pictures of the conA aq. solution [10mM HEPES buffer (pH 8)].

Conclusion
This article deals with a novel application of an EPD procedure for biological application to sugar-binding proteins, i.e. lectins, which act as electrolytes in aqueous media, employing newly-synthesized glycopolymers. The improved sugar-protein
interaction with the polymer was clearly detectable using the EPD procedure. The sugar-binding lectin aggregated with the glycopolymer and preferentially produced a new slip surface on stainless-steel electrodes preferred for electrophoresis carried out in the HEPES media. These fundamental results provide a new aspect in EPD with glycopolymers.

ASSOCIATED CONTENT
SUPPORTING INFORMATION Representative experimental procedures, spectral and biding constant data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Acknowledgement. The authors express their sincere gratitude to Professor M. Higuchi, Nagoya Institute of Technology for their help to use the UV apparatus. This work was funded by the Ministry of Education, Science, and Culture of Japan (Grant-in-Aid for Development Scientific Research, no. No. 15K04872).

References and Notes


For Table of Contents Use Only

Graphical Abstract

for *Macromolecular Chemistry and Physics*

<table>
<thead>
<tr>
<th>Electrophoretic Deposition (EPD) of Lectins in the Presence of New Glycopolymers Aiming at Facile Detection of Carbohydrate-Protein Interactions</th>
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