

Selected Papers

Preparation of Glycopolymer-Modified Gold Nanoparticles and a New Approach for a Lateral Flow Assay

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A novel recognition element has been synthesized using glycopolymer-modified gold nanoparticles (GNPs) in sugar ratios of 0, 6, 12, and 50%. The as-synthesized glycopolymer-modified GNPs have been employed in a novel lateral flow assay for the detection of proteins. A test solution of concanavalin A (ConA) at 0.01–100 $\mu g\,mL^{-1}$ was readily detectable with the glycopolymer-modified GNPs with a sugar ratio of 6%. The results were clear enough to be visible with the naked eye, which demonstrates the convenience of this lateral flow assay for performing the detection of Shiga toxins and influenza viruses. Therefore, this lateral flow assay provides an attractive biosensor for the detection of proteins without the handling of toxic reagents, while allowing an easy and rapid procedure.

Saccharides on cell surfaces play important roles in living systems and interact with proteins, cells, and viruses.[1] Because the saccharide–protein interaction is weak generically, the detection of proteins is difficult to achieve.[2] As such, there is a need to improve the biosensing capabilities for proteins.[3] However, the saccharide–protein interaction can be amplified by the multivalent effect of densely packed saccharides, which is called the glyco-cluster effect. In natural systems, glycolipids form densely packed "raft" and "caveola" structures,[1] which increase the efficacy of the protein–saccharide interaction. Since the glyco-cluster effect amplifies the interaction, utilizing a densely packed saccharide structure in artificial glycopolymers represents an important strategy for increasing the effectiveness of biosensing devices for the detection of proteins. We have reported toxin detection using a self-assembled monolayer (SAM) of a saccharide with a quartz crystal microbalance (QCM)[4] and amyloid β peptide detection using an electrochemical chip.[5] The protein–saccharide interaction is a prominent tool for biosensing devices and proteome analysis.

In relation to novel biosensing tools, the conjugation of biomolecules with metal nanoparticles has received much interest due to the unique physical and chemical characteristics of the conjugates. For example, DNA-modified gold nanoparticles (GNPs) show a color change by hybridization.[6,7] Antibody-modified GNPs have been utilized in immunochromato test strips,[8–11] and in electrochemical immunoassay sensor chips.[12] Metal nanoparticles are desirable as a signal transducer based on their optical and electrical properties, and are generally more stable than other kinds of labeling reagents such as fluorescent dyes and enzymes.

The development of biosensing devices is increasing rapidly and such devices have been widely applied in various fields including medical, environmental, and forensic applications.[13] The development of the lateral flow assay has been under intense investigation because of its desired properties, such as user friendly operation and short analysis time. The lateral flow assay, using a membrane strip as the immunosorbent, provides a unique analytical platform that permits a one-step, rapid, and low-cost analysis.[14] The lateral flow assay has high sensitivity, which is comparable to enzyme-linked immunosorbent assay (ELISA). Furthermore, the concentration levels of analyte can be observed directly with the naked eye. Because of these advantages, the lateral flow assay has been widely used as a convenient assay system.

In the lateral flow assay, ligands such as antibodies are immobilized on a nitrocellulose membrane. The analytical signals are observed after the specific interaction between the ligands and the analyte, such as antigen–antibody reactions. These specific interactions take place in the membrane by capillary action. In the sandwich assay, detection is achieved as a result of the reactions between the analyte, nanoparticle modified secondary antibody and antibodies that are immobilized on the membrane. After these reactions, the color caused by the accumulation of nanoparticle modified secondary antibody appears on the membrane. In these applications, the nanoparticles usually employed are either colored lateral beads or gold. Monitoring the strength of the color provides the basis for the quantitative determination of the target protein.

In the present study, we synthesized glycopolymer-modified GNPs as protein recognition ligands, and proposed a new approach of a lateral flow assay system for the simple, rapid,

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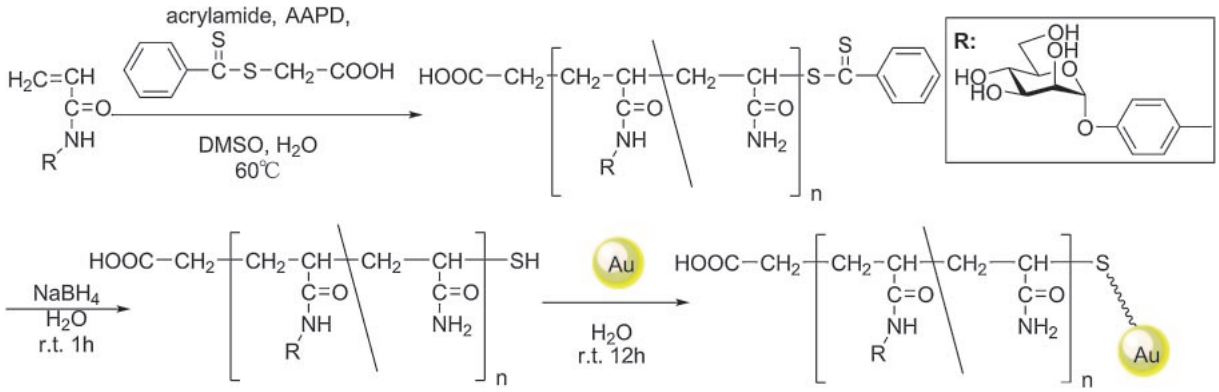

Scheme 1. Preparation of glycopolymer-modified GNPs via RAFT living radical polymerization.

and quantitative detection of proteins, where the glycopolymer-modified GNPs were synthesized via reversible addition–fragmentation chain transfer (RAFT) living radical polymerization. We have reported the detection of sugar–protein interaction of the GNPs with UV spectra.[15,16] However, the sensitivity was at most 10^{-6} M, and not comparable to the sensitivity ELISA of 10^{-12} M. In addition, the assay procedure was complicated. The lateral flow assay with the glycopolymer-modified GNPs improved the sensitivity and the tedious experimental procedure. We measured the sugar–protein interaction with the combination of concanvalin A (ConA) and mannose as model, and showed the possibility of the glycopolymer-modified GNPs for the biosensing applications.

Experimental

Materials. The following reagents were used as received; 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPD), 2-propanol, $NaBH_4$, DMSO (Kanto Chemical, Tokyo, Japan), bovine serum albumin (BSA), (thiobenzoyl)thioglycolic acid, ConA (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-ConA antibodies (EY Laboratories, Inc., CA, USA), wheat germ agglutinin (WGA) (J-oil Mills, Tokyo, Japan), and gold nanoparticles of diameter 40 nm (Tanaka Kikinzoku, Co., Ltd., Tokyo, Japan), casein, boric acid, sodium dodecyl sulfate, sucrose, K_2HPO_4, Na_2HPO_4, NaH_2PO_4, NaCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and nitrocellulose membrane with packing sheet (Nihon Millipore, Tokyo, Japan). Acrylamide (Kanto Chemical, Tokyo, Japan) was used after recrystallization from chloroform and methanol.

The monomer of *p*-acrylamidophenyl-α-D-mannoside (α-Man) was synthesized according to a literature method.[15,16]

Characterization. ^{1}H NMR (400 MHz) spectra (Bruker Biospin, Avance III, Rheinstetten, Germany) was used for determination of sugar ratio. Absorbance of gold nanoparticles was measured by UV–vis spectrometer (JASCO, Tokyo, Japan). Gel permeation chromatography (GPC) was carried out with a JASCO 800 high-performance liquid chromatography instrument equipped with SB803 HQ columns (Shodex, Tokyo, Japan) using phosphate buffered saline (PBS) as eluent. Molecular weights were evaluated using pullulan standard. The immunochromatography strips were prepared with an XYZ work station (XYZ 3200, BioDot Inc., Irvine, CA, USA). The color intensity of the detection line of the membrane was measured using an immunochromato-reader (ICA-1000 Immunochromato reader, Hamamatsu Photonics K. K., Hamamatsu, Japan).

Polymerization of Glycopolymer. The preparation of the glycopolymer used in this study is shown in Scheme 1. Polymerization of *p*-acrylamidophenyl-α-D-Man and acrylamide to afford the glycopolymer was conducted in the presence of a RAFT reagent of (thiobenzoyl)thioglycolic acid in a solvent mixture of water and DMSO. Briefly, a mixture of the monomer, an initiator (AAPD), the RAFT reagent, and the solvent mixture was placed in a Pyrex tube which was then degassed by three freeze–thaw cycles. The tube was sealed under vacuum and heated at 60 °C for 4 h with occasional agitation.

Preparation Glycopolymer-Modified GNPs. Preparation of glycopolymer-modified GNPs is also shown in Scheme 1. The synthesized glycopolymers were purified by dialysis in cellulose tubes with a molecular weight cutoff of 3500. Each polymer was then dissolved in water, and finally reduced using $NaBH_4$ to yield the thiol-terminated glycopolymer. The thiol-terminated glycopolymer was mixed with an aqueous dispersion of GNPs for 12 h, and the resulting glycopolymer-modified GNPs were stored at 4 °C.

Preparation of Lateral Flow Assay Strip. The newly developed lateral flow assay strip is shown in Figure 1. A rabbit anti-ConA antibody solution with concentration of 1 mg mL^{-1} was prepared by diluting with PBS (pH 7.4). For immobilization at the detection line on the nitrocellulose membrane of the assay, 650 µL of an antibody solution was mixed with 20 wt % sucrose solution diluted with 300 µL of 50 mM K_2HPO_4 buffer (pH 7.5) and 100 µL of 2-propanol. The antibody solution was applied to the nitrocellulose membrane by using the XYZ dispense platform dispensing system. After drying for 1 h at room temperature, the membrane was protected from undesirable nonspecific protein adsorption by immersing in 50 mM boric acid solution containing 0.5 wt % casein (pH 8.5) and incubating for 30 min at room temperature. Then, the protected membrane was washed by immersion in PBS (pH 7.5) containing 0.01 wt % sodium dodecyl sulfate for 30 min at room temperature. After drying the membrane overnight, an absorption pad was pasted onto the membrane and the membrane was cut into individual strips using a guillotine cutting system.

Measurement of Lateral Flow Assay. A sample solution (40 µL) and glycopolymer-modified GNPs were mixed in a 96

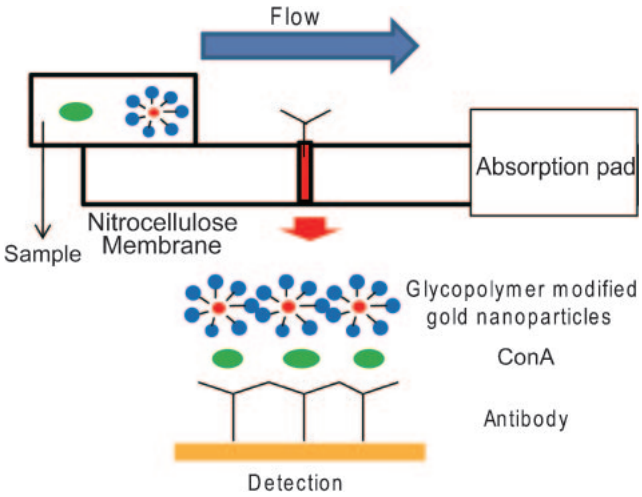

Figure 1. The schematic illustration of the procedure for the lateral flow assay with anti-ConA antibodies.

Table 1. Polymerization of *p*-Acrylamidophenyl-α-Man and Acrylamide in the Presence of a RAFT Reagent[a)]

Run No.	Monomer (0.30 mmol)		M_w[c)]	M_w/M_n	Sugar ratio in polymer /%[d)]
	Saccharide monomer[b)] /mmol	Acrylamide /mmol			
1	0	0.30	8.6×10^3	1.3	0
2	0.010	0.29	2.0×10^4	1.2	6.0
3	0.030	0.27	2.1×10^4	1.3	12
4	0.15	0.15	2.1×10^4	1.3	50

a) RAFT 0.5 mol %, AAPD 0.2 mol %, Solvent: H_2O:DMSO = 1:1. b) *p*-Amidophenyl-α-Man. c) By the Pullulan standard with Shodex SB803 HQ columns and PBS as eluent. d) By ^{1}H NMR.

well microtiter plate. The mixed solution was then absorbed into the lateral flow strip using capillary force. The intensity of red color afforded by the accumulation of glycopolymer-modified GNPs on the detection line, was measured as the reflective absorbance using the ICA-1000 Immunochromato reader. The measurement time of lateral flow assay was 10 min.

Results and Discussion

Preparation of Glycopolymer-Modified GNPs. Copolymers of *p*-acrylamidophenyl-α-Man with acrylamide were synthesized in sugar ratios of 0, 6, 12, and 50% (Table 1), and these glycopolymers were obtained with low dispersities (<1.3). The corresponding four kinds of glycopolymer were converted to thiol-terminated polymers by reaction with the RAFT reagent (thiobenzoyl)thioglycolic acid, which were then finally reduced using $NaBH_4$ and then dispersed in GNPs of diameter 40 nm to afford glycopolymer-modified GNPs, hereafter referred to as GP-0%-GNPs, GP-6%-GNPs, GP-12%-GNPs, and GP-50%-GNPs, respectively. The particle size of each glycopolymer-modified GNP was measured using dynamic light scattering (DLS) to give estimated diameters of around 110 nm. Each glycopolymer-modified GNP showed a red color with a λ_{max} of around 520 nm. A glycopolymer-modified GNP solution with an absorbance of $OD_{520} = 1.7$ was

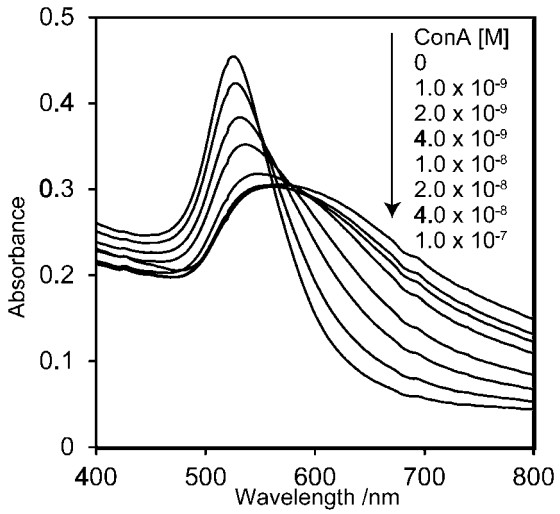

Figure 2. The UV–vis spectra of the GP-6%-GNPs with the addition of ConA in varying concentrations.

adopted as a stock solution and was used in the following experiments.

Protein Recognition of Glycopolymer-Modified GNPs in Aqueous Solution. Recognition between glycopolymer-modified GNPs and ConA was investigated using UV–vis spectra (Figure 2). It is known that addition of the corresponding protein to the GNPs induced aggregation, resulting in a change in the color of the dispersion.[15] When ConA was added into the GP-6%-GNP solution, the peak at 520 nm showed the red shift and the absorbance was decreased due to the aggregation. In contrast, other proteins such as WGA and BSA as negative controls did not interact with the glycopolymer-modified GNPs (Supporting Information, Figure S1). The specific recognition of ConA with glycopolymer-modified GNPs was confirmed in the aqueous solution.

The glycopolymer-modified GNPs showed a change in their respective UV spectra with addition of ConA due to the specific interaction with α-Man. The strong molecular recognition was achieved by the multivalent effect of the glycopolymers. The protein recognition of the glycopolymer-modified GNPs was found to be highly specific due to the hydrated properties of the polymer. The GNPs recognized the corresponding lectin of ConA, but did not recognize the non-corresponding lectin of WGA or the non-sugar recognizing protein of BSA.

Protein Recognition of Glycopolymer-Modified GNPs on Nitrocellulose Membrane. The interaction between the glycopolymer-modified GNPs and ConA was conducted on the nitrocellulose membrane (Figure 3). First, ConA (0.6 mg mL^{-1}) was immobilized on the nitrocellulose membrane like an antibody, in order to monitor the protein recognition abilities of the glycopolymer-modified GNPs in the lateral flow assay, comparing to the color change in the solution state (Supporting Information, Figures S2 and S3). The results of the study are shown in Figure 3.

Efficacious absorbance was observed in the case of GP-6%-GNPs and GP-12%-GNPs. The GP-6%-GNPs, compared with the GP-12%-GNPs, showed a large change in the reflective absorbance values. For GP-50%-GNPs, the glycopolymer-

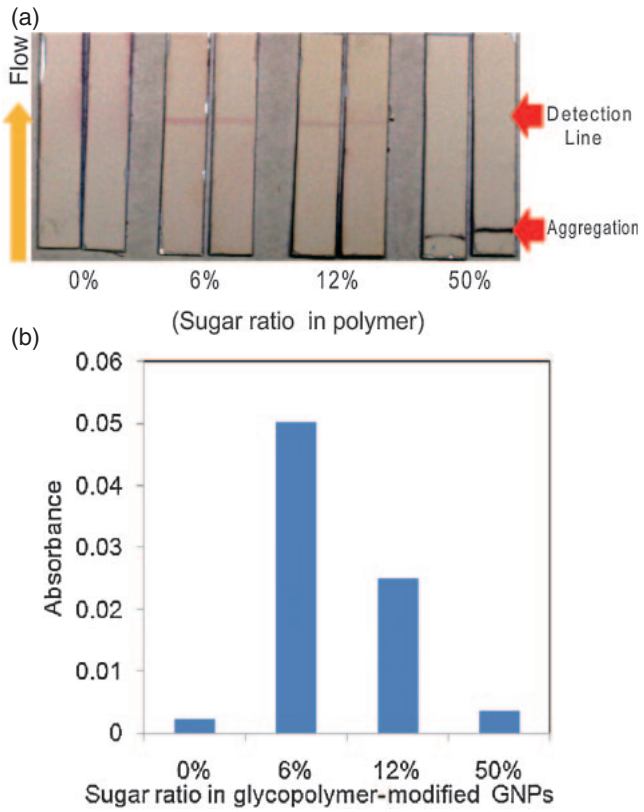

Figure 3. Relationship between the absorbance and the sugar ratio of the glycopolymer-modified GNPs for the lateral flow assay: (a) the image of test strips with various sugar ratios of 0, 6, 12, and 50%, and (b) the reflective absorbance with the various sugar ratios of the glycopolymer-modified GNPs.

modified GNPs aggregated with the ConA sample and could not move through the nitrocellulose membrane by capillary action. In the case of GP-0%-GNPs, no red line was observed. These glycopolymer–protein interactions demonstrate the ability of the Man part of the polymer to recognize the lectin part of ConA. From these observations, the ConA recognition of the glycopolymer-modified GNPs was confirmed by the appearance of the expected red line on the nitrocellulose membrane. In contrast, other proteins such as a solution of 1% WGA and BSA did not induce the appearance of the red line due to the lack of recognition capabilities.

It was found that the sugar ratio of the glycopolymers much affected the lateral flow assay, because the physical properties of GNPs such as fluidity and solubility changed the detection efficacy. The glycopolymer was composed of the acrylamide derivative of bulky side chain (*p*-nitrophenyl Man), and so the glycopolymer with higher sugar ratio might have the high isotacticity.[17] The stereoregular polymerization of glycopolymer with high sugar ratio formed a stiff conformation, which results in the low fluidity and solubility. Therefore the glycopolymer with modest sugar content (GP-6%-GNPs) had better fluidity than that with high sugar content (GP-12%-GNPs), and showed better sensitivity. The glycopolymer-modified GNPs with higher sugar ratios were found to be inappropriate for the lateral flow assay, suggesting that the

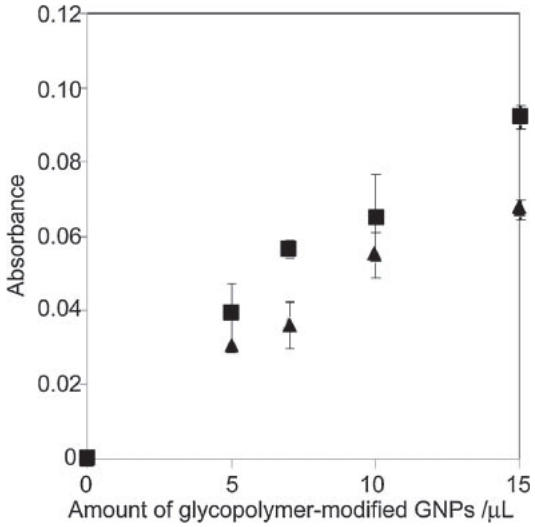

Figure 4. Relationships between the absorbance and the volume of the glycopolymer-modified GNPs for the lateral flow assay with sugar ratios of (■) 6% and (▲) 12%.

polymer-coated GNPs need to have adequate design for each biosensing technique such as immunoprecipitation,[18] localized surface plasmon resonance (LSPR),[19] and the lateral flow assay. The detailed results of glycopolymers with modest sugar ratios are described in the following section.

Optimization of Lateral Flow Assay Strip. To optimize the conditions of the lateral flow assay, we changed the amount of GNPs, and investigated the absorbance in the detection line in the presence of $1\,\mu g\,mL^{-1}$ of ConA. The reflective absorbance values increased gradually and reached maximum values at 15 μL for both GP-6%-GNPs and GP-12%-GNPs (Figure 4). However, the aggregation was observed in the case of 12% at 15 μL of GP-12%-GNPs ($OD_{520} = 1.7$), and the aggregation properties were diminished by decrease of GNPs solution until 5 μL (Supporting Information, Figure S5). Optimal volumes of GP-6%-GNPs (15 μL) and GP-12%-GNPs (5 μL) were required for $1\,\mu g\,mL^{-1}$ of ConA sample (40 μL), respectively.

Glycopolymer-modified GNPs with higher sugar ratios exhibited poor fluidity on the nitrocellulose membrane. Even though GP-6%-GNP and GP-12%-GNP both showed responses to ConA in the lateral flow assay, the higher sugar ratio (12%) exhibited poorer results due to its rigid rod-like structure, which is inadequate for application in lateral flow assays. A small difference in the sugar ratio showed significant differences in the results of the lateral flow assay.

Lateral Flow Assay Using Glycopolymer. The detection efficacy of protein (ConA) was investigated by the lateral flow assay in detail. The calibration curves of each glycopolymer-modified GNP are shown in Figure 5, in which the absorbance of GP-6%-GNP was notably stronger and showed better response to ConA. The detection of ConA at concentrations of 100, 10, 1, 0.1, and $0.01\,\mu g\,mL^{-1}$ using GP-6%-GNPs was visible with the naked eye (Figure 6). Compared with the absorbance values obtained using GP-6%-GNPs and GP-12%-GNPs, GP-6%-GNPs was found to be most suitable for the lateral flow assay.

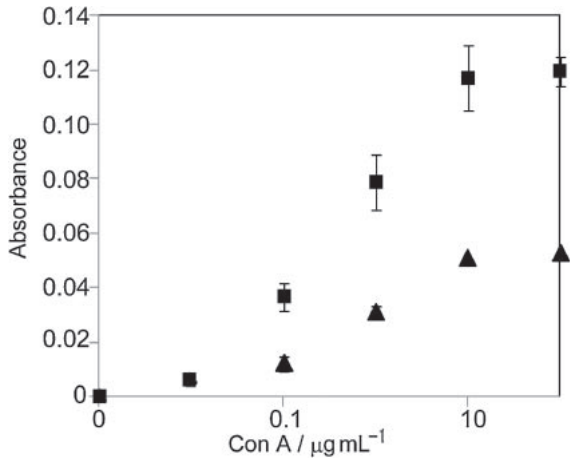

Figure 5. The calibration curves of ConA by the lateral flow assay with the GP-6%-GNPs (■) and GP-12%-GNPs (▲).

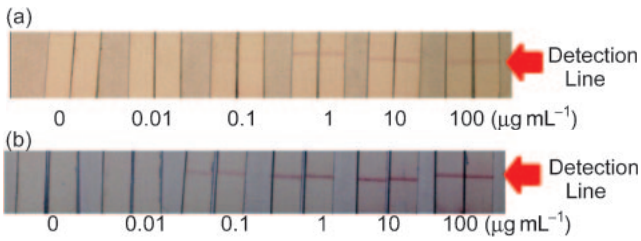

Figure 6. The image of strips for the lateral flow assay of (a) GP-12%-GNPs and (b) GP-6%-GNPs. The arrows point to the detection line.

On the other hand, other proteins such as BSA and WGA solution were also measured to investigate the detection specificity. When 1% BSA solution and 1% WGA solution in PBS (pH 7.4) were applied as negative controls, no red line could be observed for GP-6%-GNPs. Analytical ranges were estimated for ConA at concentrations of 10–0.01 $\mu g\,mL^{-1}$ (9.6×10^{-8}–9.6×10^{-11} M) for GP-6%-GNPs and 10–0.1 $\mu g\,mL^{-1}$ (9.6×10^{-8}–9.6×10^{-10} M) for GP-12%-GNPs (Figure 5). The glycopolymer-modified GNPs monitored using UV–vis spectra have been reported to show ConA recognition, but at μM level sensitivities with few exceptions.[15] As a result, it was suggested that this method was a more sensitive detection method than using UV–vis spectra.

The various pathogens of toxin proteins and viruses have been reported to have a high affinity to saccharides. For example, it has been reported that Shiga toxins and influenza viruses have a high affinity to globoside[4] and siallyllactose saccharides,[20] respectively. We envisage the lateral flow assay will be useful for specific pathogen detection.

Conclusion

A new recognition element has been synthesized using glycopolymer-modified GNPs. The as-synthesized glycopolymer-modified GNPs have been employed in a novel lateral flow assay for the detection of proteins. The rapid observation of clear results with the naked eye will ensure the convenience of performing the detection of Shiga toxins and influenza viruses. Therefore, our lateral flow assay provides an attractive biosensing device without the handling of toxic reagents, while allowing an easy and rapid procedure.

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Supporting Information

The UV–vis data, the schematic illustration of the assay and lateral flow assay are available free of charge on the internet at http://www.csj.jp/journals/bcsj/.

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