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ORIGINAL ARTICLE

Biological specific recognition of glycopolymer-modified interfaces by RAFT living radical polymerization

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Glycopolymers with α-galactose (α-Gal) and α-mannose (α-Man) were synthesized by means of living radical polymerization with a reversible addition-fragment chain transfer reagent, and the thin-layer formation of glycopolymers was investigated in terms of protein recognition abilities. Thiol-terminated glycopolymers formed a thin layer of about 2.5 nm in thickness on a gold substrate, and the glycopolymer thin layer showed specific interaction with sugar recognition proteins (lectins and Shiga toxins (Stxs)). The interactions were highly specific, and the signal-to-noise ratio of protein recognition was greater than 16. Glycopolymer-substituted gold nanoparticles (GNPs) also showed biorecognition abilities and protein-specific aggregation. The protein recognition abilities of the GNPs were also analyzed. The glycopolymer-substituted GNPs were utilized for signal amplification of surface plasmon resonance (SPR) to detect protein-saccharide recognition. The glycopolymer with α-Gal showed a strong interaction with Stxs according to SPR measurements, suggesting a possible application of α-Gal-substituted GNPs in Stx-1 biosensing.
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Keywords: biointerface; glycopolymer; RAFT living radical polymerization; saccharide

INTRODUCTION

Saccharides on cell surfaces have important roles in life systems, such as cell–cell communication, immune response, pathogen invasion and cancer metastasis.[1] Because saccharide–protein interactions regulate life phenomena, a better understanding of saccharide functions would greatly aid elucidation of such phenomena and, hence, the diagnosis and detection of disease and pathogens.[2]

Saccharide–protein interactions are generally too weak to be used in biomaterials and devices, but interestingly, the interactions can be amplified by multivalency. Multivalent compounds of glycopolymers have been reported to exhibit strong saccharide–protein interactions.[3–5] We prepared various glycopolymers that can be used for cell cultivation,[6] toxin neutralization[7] and Aβ-inhibition.[8] In addition, saccharide–protein interactions are difficult to detect because of their lack of fluorescence or conductivity. Thus, conjugate materials with nanoparticles or substrates provide suitable materials for biosensing. We previously reported the preparation of glycopolymer-substituted gold nanoparticles (GNPs) and demonstrated the detection of protein–saccharide interactions by colorimetric changes.[9]

In this study, we investigated glycopolymer-substituted substrates and nanoparticles. We prepared glycopolymer-substituted substrates by reversible addition-fragment chain transfer (RAFT) living radical polymerization,[9–11] in which the terminal group of dithioester was converted to thiol to form a polymer layer through Au-S bonding on GNPs[12,13] and substrates by the 'grafting to' method.[14] Glycopolymers of polyacrylamide derivatives with α-galactose (α-Gal) and α-mannose (α-Man) and the corresponding thin layers were prepared. Precise control of the polymer resulted in a uniform biological interface, which led to a sensitive biosensing substrate.

The gold substrates and nanoparticles can be applied to various sensing methods such as surface plasmon resonance (SPR), quartz crystal microbalance, electrochemistry and colorimetric changes in nanoparticles. We measured the affinities and specificities of the glycopolymer thin layer to proteins, using SPR for the gold substrates and using colorimetric changes for the GNPs. In addition to the model interactions between lectin and saccharide, practical biosensing for Shiga toxins (Stxs) was also studied in this investigation using an α-Gal-substituted glycopolymer.[15]

EXPERIMENTAL PROCEDURE

Materials

The following reagents were used as received: D-mannose (Man) (Kishida Chemical, Osaka, Japan), *p*-nitrophenyl α-D-galactoside, acryloyl chloride (TCI, Tokyo, Japan), *p*-nitrophenol, 2.2′-azobis(2-amidinopropane)dihydrochloride (Kanto Chemical, Tokyo, Japan), bovine serum albumin (BSA), fibrinogen, lysozyme, (thiobenzoyl)thioglycolic acid (Sigma-Aldrich, Louisiana, MO, USA), peanut agglutinin (PNA), concanavalin A (ConA) (J-oil Mills, Tokyo, Japan), Stx-1 (Vero toxin-1 (Stx-1, from *Escherichia coli* O-157)) (Nacalai Tesque, Kyoto, Japan), high-flow nitrocellulose membrane (FH135) absorption pads (Millipore, Billerica, MA, USA), GNPs (40 nm) (Tanaka Kikinzoku, Tokyo, Japan) and a silver enhancer kit for membrane application (Funakoshi, Tokyo, Japan).

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Acrylamide (AAm) (Kanto Chemical) was used after recrystallization from chloroform and methanol. Acetylene-terminated disulfide and azide-terminated α-Man were synthesized according to the literature.[16,17]

Characterization

^{1}H (300 MHz) and ^{13}C (75 MHz) nuclear magnetic resonance spectra were recorded in $CDCl_3$ and D_2O using a Varian Gemini 300 spectrometer (Varian, Palo Alto, CA, USA) equipped with a Sun workstation. Gel permeation chromatography was carried out with a JASCO 800 high-performance liquid chromatographer (JASCO, Tokyo, Japan) with Shodex SB803 HQ columns (Showa Denko K. K., Tokyo, Japan) and phosphate-buffered saline as the eluent. Molecular weights were evaluated relative to a pullulan standard. The contact angle was determined using a Drop Master 300 (Kyowa Interface Science, Saitama, Japan). Infrared spectra were obtained at room temperature using an FTIR Spectrum 100 (PerkinElmer, Waltham, MA, USA). Fourier transform infrared-reflection absorption spectroscopy was carried out using a Reflector 2 instrument (Harrick Scientific, Pleasantville, NY, USA). The bonding on the gold substrates was measured by quartz crystal microbalance using a Q-SENSE D300 (QSENSE, Frölunda, Sweden) with a gold substrate of QSX301. SPR spectra were recorded using a BIACORE 1000 (GE Healthcare UK Ltd, Little Chalfont, UK) with phosphate-buffered saline. Atomic force microscopy images were recorded with a VN-8000 instrument equipped with an op-75042 cantilever (Keyence, Kyoto, Japan). Transmission electron microscopy images were recorded at 100 kV using a Hitachi HF-2000 (Hitachi, Tokyo, Japan) instrument equipped with an AMT-CCD camera (Hitachi). Gold nanoparticles (GNPs) for transmission electron microscopy were prepared by the deposition of buffer-dispersed particles onto a carbon film supported by a copper grid. Immunochromatography was conducted with high-flow nitrocellulose membrane (FH135) absorption pads (Millipore).

General procedure for polymerization

p-(*N*-Acrylamidophenyl)pyranosides (**1-1** and **1-2**) were synthesized according to previous procedures (Scheme 1).[18] A mixture of the corresponding sugar monomer, AAm, an initiator (2.2′-azobis(2-amidinopropane)dihydrochloride), RAFT reagent and solvent in a Pyrex tube was degassed by three freeze-thaw cycles. The tube was sealed under vacuum and heated at 60 °C with occasional agitation. Polymerization was terminated by cooling the tube, and the product was precipitated in acetone and collected by centrifugal sedimentation. Polymers were hydrogenated with $NaBH_4$ to obtain a thiol terminal. Further details are given in Supplementary information.

Preparation of glycopolymer-substituted gold substrates and nanoparticles

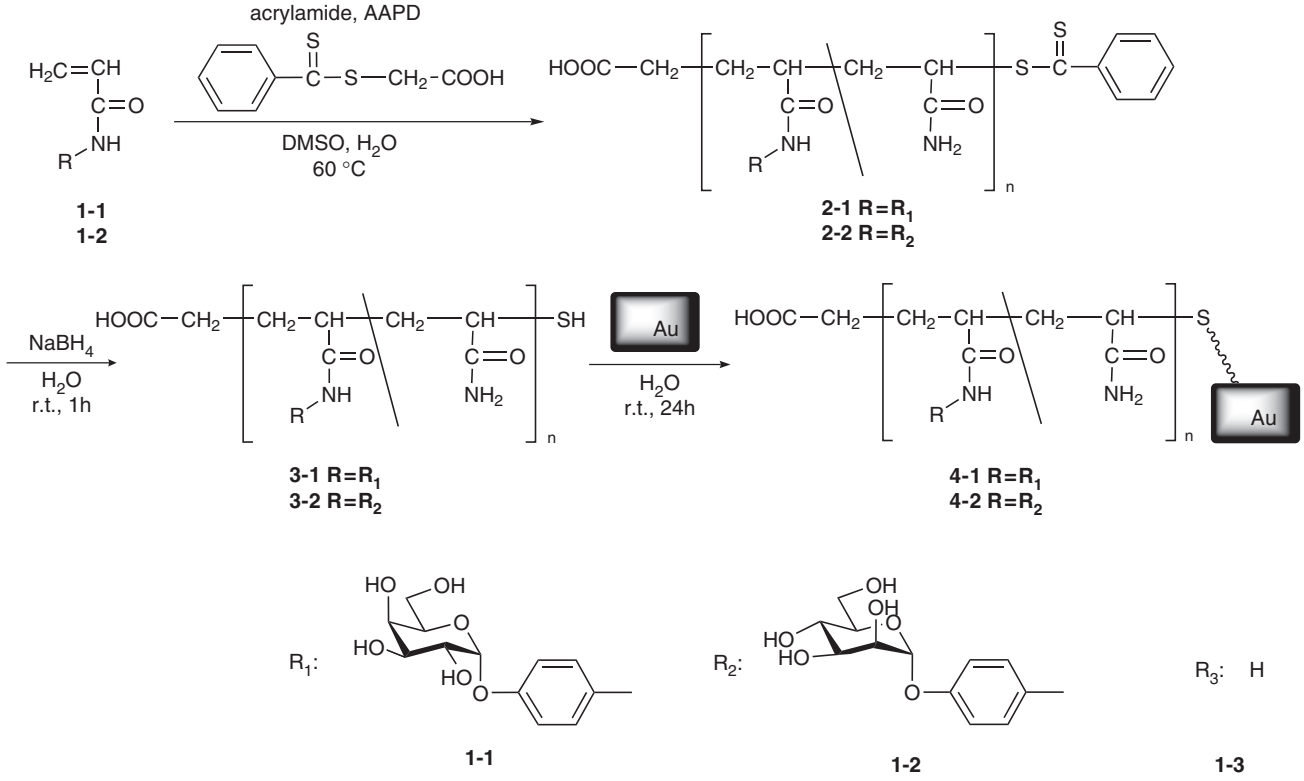
Gold substrates from GE Healthcare (SPR sensor chip Au) were used for SPR measurements, and gold substrates from Moritex Co. (Tokyo, Japan) were used for infrared, X-ray photoelectron spectroscopy and ellipsometry measurements.[9] The substrates were cleaned by ultraviolet/ozone irradiation (Bioforce Nanosciences, Ames, IA, USA) for 30 min before use. The thiol-terminated polymer was dissolved in MilliQ water at a concentration of 1.0 mg ml^{-1}. The substrate was immersed in the solution for 24 h, then rinsed with MilliQ water and dried under N_2.

Glycopolymer-substituted GNPs were prepared according to our previous report. Glycopolymers were mixed with an aqueous dispersion of GNPs for 12 h, and the resulting glycopolymer-substituted GNPs were purified by centrifugation (540 000 *g*, 2 h).

SPR measurements

Saccharide–protein interactions were analyzed according to SPR measurements obtained with a BIACORE 1000 (GE Healthcare UK Ltd) on glycopolymer-immobilized gold substrates. The flow rate of the buffer was 30 μl min^{-1}, and the sample solution was injected for 20 s. The substrate was regenerated twice by treatment with 10 mM glycine at pH 1.5 (GE Healthcare Bioscience, Little Chalfont, UK) as the dissociate solution for 2 min. Lectin binding to the corresponding saccharides (ConA to α-Man and PNA to Gal) was calculated from sensorgrams with the assumption of Langmuir binding (1:1) using BIAevaluation software (GE Healthcare UK Ltd). In the case of Stx-1, binding was calculated from Scatchard plots.

In the case of SPR signal amplification with GNPs, the flow rate of the buffer was 10 μl min^{-1}; the sample solution was injected for 60 s and then



Scheme 1 Syntheses and preparation of glycopolymer-substituted gold.

glycopolymer-substituted GNPs (OD_{529}=0.45) were injected for 60 s. The substrate was regenerated twice by treatment with 10 mM glycine at pH 1.5 (GE Healthcare Bioscience) as the dissociate solution for 5 min. Binding with PNA (a Gal-binding protein) and the nonspecific adsorption of fibrinogen were analyzed.

Immunochromatography

A solution of Stx-1 (2.4 μM, 10 μl) was applied to a nitrocellulose membrane as a test spot.[19] After drying for 30 min at room temperature, the membrane was blocked against nonspecific protein adsorption by immersion in 50 mM boric acid buffer containing 0.5% casein (pH 8.5) and incubated for 30 min at room temperature. The blocked membrane was then washed for 30 min at room temperature by immersion in 5.0 mM phosphate buffer (pH 7.5) containing 0.01% sodium dodecyl sulfate. After drying for 3 h, the membrane was prepared on a backing sheet, and an absorbent pad was attached. The solution of glycopolymer-substituted GNPs (OD_{529}=4.5) was placed on the membrane for 30 min and washed with MilliQ water (18.3 MΩ-cm). The membrane was incubated with the prepared silver enhancer (200 μl each of solutions A and B) for 30 min. After a suitable color intensity was attained, the membrane was rinsed for 3 min in a continuous stream of MilliQ water.

RESULTS AND DISCUSSION

Polymerization of glycopolymer

Polymerization of **1-1** and **1-2** (Scheme 1) was conducted with the RAFT reagent (thiobenzoyl)thioglycolic acid in a mixture of water and DMSO; polymers were obtained with low dispersities (<1.5) (Table 1). The yields of polymers increased with polymerization time, but the yield did not exceed 30% after 24 h. The yield of the polymer was low because of amine impurities from AAm, which induce degradation of the RAFT reagent to amine.[20] Amine was generated because of the aqueous solvent; thus, we are investigating RAFT living radical polymerization of glycopolymers with more appropriate conditions and RAFT reagents.

Preparation of glycopolymer on the gold substrate

The glycopolymers of Run no. 3 (**P3**) and 6 (**P6**) were hydrogenated to result in a terminal thiol group, and the gold substrates were modified by the addition of 1 wt% glycopolymer solution, which we called **P3-gold** and **P6-gold**, respectively. The glycopolymers used were selected by following the previous report.[9] The contact angle of the substrate obtained was rapidly reduced by the addition of glycopolymers, from 82° to 24° after 0.5 h, and stabilized at 14.5° after 12 h.[21] Formation of the polymer layer was also confirmed by Fourier transform infrared-reflection absorption spectroscopy; bands for the hydroxyl groups (δOH about 3300 cm^{-1}), amide I (νC=O at 1680 cm^{-1}) and amide II (δNH at 1518 cm^{-1}) (Figure 1), were observed. The thicknesses of **P3**- and **P6-gold** were measured using ellipsometry and atomic force microscopy as 2.5 ± 0.2 nm (Supplementary information). The area per molecule of the glycopolymer as determined by quartz crystal microbalance was 1.6 nm^2, following the Sauerbrey equation in air (Supplementary Information). These values for thickness and bonding indicate a polymer layer with random orientation and a non-dense structure, indicating that a pancake layer formation occurred because of the 'grafting to' method.[22]

SPR analyses of glycopolymer–protein interactions

Substrates with glycopolymers were subjected to interaction evaluation with proteins (Con A, PNA, BSA, lysozyme and fibrinogen) by SPR measurements. The time course of the resonance unit (RU) change was monitored for the glycopolymer-substituted substrates: a representative time-course curve of the RU with a 500-nM protein injection is shown in Figure 2. The glycopolymer-substituted substrate showed strong interaction with the corresponding lectins because of the multivalent effect of the glycopolymer.[3] The K_A values of **P3-gold** to PNA and **P6-gold** to ConA were 2.0×10^7 and 1.6×10^7 (M^{-1}), respectively (Table 2).

Protein adsorption was compared in terms of specific recognition (Figure 2 and Table 3). The RU_{eq} change of **P3-gold** for PNA (500 nM) was 8.1×10^2, whereas the change for other proteins was at the most 31 (S/N > 26). The RU_{eq} change of **P6-gold** with ConA was 2.4×10^3, and the change for those with noncorresponding proteins were at the most 1.5×10^2 (S/N > 16). These SPR results indicate the formation of a highly bio-specific poly(sugar-co-AAm) surface because of the hydrophilic polymer layer. We also prepared an α-Gal monolayer without a polymer backbone and measured the interaction with proteins by SPR (Figure 3). The α-Gal monolayer showed an interaction with the corresponding lectin of PNA, but the specificity was less than that for glycopolymer substrates. For example, the amount of fibrinogen bound to the substrate was

Table 1 Polymerization of *p*-acrylamidophenyl α-Gal (1-1) and *p*-acrylamidophenyl α-Man (1–2) with acrylamide (1–3) in the presence of RAFT reagent[a]

	Monomer						
Run no.	*Saccharide monomer (mmol)*	*Acrylamide (mmol)*	*Time (h)*	*Yield (%)*	*10^{-3}Mn (g mol^{-1})[b]*	*Mw/Mn*	*Sugar ratio in polymer[c]*
1	**1-1**	**1-3**	2	7.7	5.3	1.2	0.53
	0.030	0.27					
2	**1-1**	**1-3**	3	13	6.6	1.3	0.38
	0.030	0.27					
3	**1-1**	**1-3**	4	17	7.0	1.4	0.24
	0.030	0.27					
4	**1-1**	**1-3**	5	19	10	1.4	0.27
	0.030	0.27					
5	**1-1**	**1-3**	24	34	12	1.4	0.20
	0.030	0.27					
6	**1-2**	**1-3**	4	20	8.1	1.3	0.25
	0.030	0.27					

[a]Monomer 0.30 mmol, AAPD 0.20 mol%, (thiobenzoyl)thioglycolic acid 0.50 mol%, 60 °C.
[b]By pullulan standard.
[c]Determined using 300 MHz ^{1}H NMR.

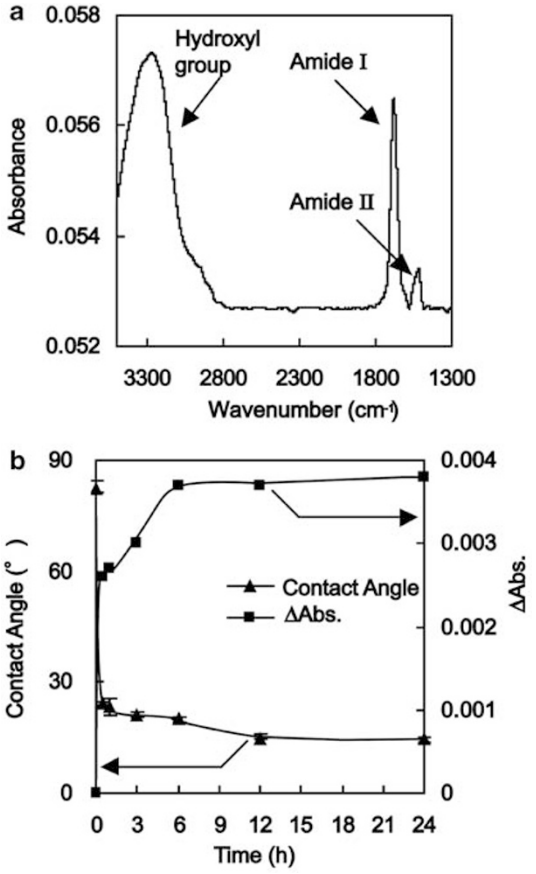


Figure 1 The characterization of a thin layer of glycopolymer (P3). (**a**) Fourier transform infrared-reflection absorption spectroscopy spectrum of P3-gold. (**b**) Time-course changes of the contact angle and the absorbance of amide I.

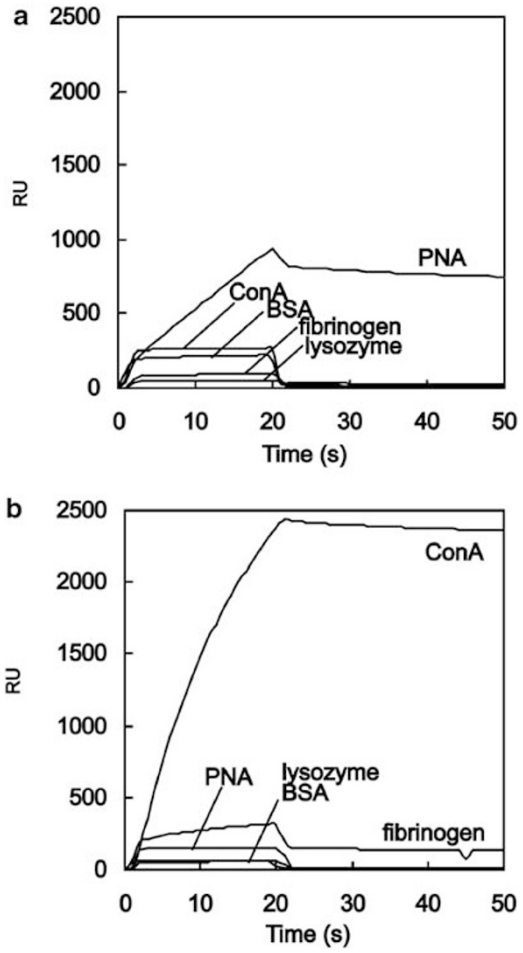


Figure 2 Surface plasmon resonance analyses of (**a**) P3-gold and (**b**) P6-gold with various proteins (500 nM) in phosphate-buffered saline buffer at 25 °C.

more than that of PNA, and the RU_{max} values of ConA, lysozyme and BSA were only 40% of that of PNA. In addition, the amount of PNA bound to the monolayer was only 20% of that of glycopolymer **P3-gold**.

Basically, the sugar-immobilized substrates show strong and specific recognition abilities to the corresponding lectins, because of hydrophilicity. The interactions of glycopolymer layers with lectins were more specific and stronger than those of the saccharide monolayer, because the glycopolymer formed a thicker uniform hydrophilic layer. The thicker hydrophilic polymer layer provides 'free water'[23] at the interface to prevent nonspecific protein adsorption.[24–28] However, a small amount of nonspecific protein adsorption was still observed on the glycopolymer layer. Glycopolymers have multiple OH groups, which could disturb the water structure by hydrogen bonding and induce nonspecific adsorption.[29] The hydrogen-bond formation of glycopolymers with proteins also induces nonspecific adsorption. In the case of the sugar monolayer, the surface was more hydrophobic, with a contact angle of 37°, and not only the hydrogen bonding of sugar but also hydrophobic interactions contributed to nonspecific adsorption.

Overall, glycopolymers exhibit strong affinity on the basis of multivalency and allosteric effects.[30] On the whole, the glycopolymer-substituted substrate showed suitable properties for biosensing applications. Biosensing with a glycopolymer thin layer will be investigated in a later section.

Glycopolymer-substituted gold nanoparticles and lectin recognition

Gold nanoparticles with glycopolymer substitution were prepared using the same polymers of **P3** and **P6**, which are abbreviated as **P3-GNP** and **P6-GNP**, respectively. Nanoparticles were stably dispersed in an aqueous solution, and **P3-GNP** and **P6-GNP** showed a red color with λ_{max} at 529 and 524 nm, respectively. Bonding of the glycopolymer on GNPs was calculated by weight change, comparing the weight measured before and after preparation. As a result, the area per molecule was 1.7 nm^2 (Supplementary information). This result is consistent with that of gold substrates. Addition of the corresponding lectin (Gal to PNA and Man to ConA) induced aggregation, resulting in a change in the color of the dispersion. In the case of **P3-GNP**, the addition of PNA induced aggregation and a red shift in λ_{max} from 529 to 531 nm (Figure 4a). In contrast, λ_{max} did not shift with the addition of the non-corresponding proteins wheat germ agglutinin and BSA. Aggregation was confirmed by transmission electron microscopy observation. **P3-GNP** was placed on a grid, and the addition of PNA induced the formation of a $220 \times 280\ nm^2$ aggregate of about 40 nanoparticles (Figures 4b and c). Aggregates were not observed by UV–vis observation with the addition of ConA or BSA. In the case of **P6-GNP**, aggregation of nanoparticles and color changes were also observed with the addition of the corresponding lectin.

Table 2 Kinetic constants for lectins with glycopolymer-modified substrate

Lectin	*Substrate*	k_a *(1/Ms)*	k_d *(1/s)*	K_A *(1/M)*	RU_{max}	RU_{eq}
PNA	**P3-Gold**	2.13×10^{3}	1.09×10^{-4}	1.99×10^{7}	9.43×10^{2}	8.07×10^{2}
ConA	**P3-Gold**	—	—	—	1.77×10^{2}	3.10×10
PNA	**P6-Gold**	—	—	—	5.90×10	1.10×10
ConA	**P6-Gold**	2.50×10^{4}	2.13×10^{-3}	1.64×10^{7}	2.38×10^{3}	2.41×10^{3}

Abbreviations: ConA, concanavalin A; PNA, peanut agglutinin.

Table 3 SPR response of glycopolymer-modified substrate with proteins

	Substrate	RU_{max}	RU_{eq}
PNA	**P3-Gold**	9.43×10^{2}	8.07×10^{2}
ConA	**P3-Gold**	1.77×10^{2}	3.10×10
BSA	**P3-Gold**	2.65×10^{2}	1.20×10
Lysozyme	**P3-Gold**	4.80×10	1.20
Fibrinogen	**P3-Gold**	9.70×10	1.90×10
PNA	**P6-Gold**	5.90×10	1.10×10
ConA	**P6-Gold**	2.38×10^{3}	2.41×10^{3}
BSA	**P6-Gold**	3.00×10	7.00
Lysozyme	**P6-Gold**	1.53×10^{2}	9.00
Fibrinogen	**P6-Gold**	3.17×10^{2}	1.46×10^{2}

Abbreviations: BSA, bovine serum albumin; ConA, concanavalin A; PNA, peanut agglutinin.

SPR signal amplification with glycopolymer-modified gold nanoparticles

Surface plasmon resonance signal amplification was investigated with **P3-GNP** on the basis of local SPR.[31,32] Figure 5 shows an SPR profile for a diluted protein concentration on an α-Gal monolayer. The protein was first injected at a diluted concentration of 10 nM, and the RU_{eq} was difficult to observe because of the small amount of adsorption of the protein. The SPR response returned with the injection of **P3-GNP.** The SPR signal was not observed without the injection of proteins in advance. By contrast, this signal amplification was not observed with the **P3-gold** substrate.

The glycopolymer induced multiple binding to the sugar recognition sites, and PNA on **P3-gold** attached to **P3-GNP**, which induced an local SPR signal between **P3-gold** and **P3-GNP**. The binding of the α-Gal monolayer to proteins was nonspecific (Figure 3b).

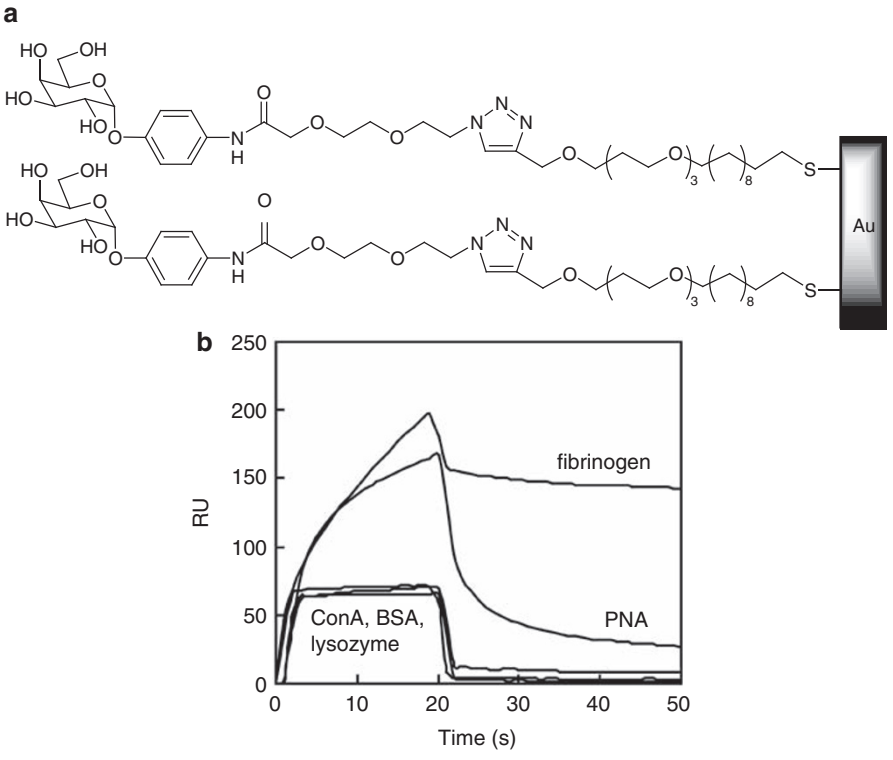


Figure 3 Binding analyses of α-Gal monolayers. (**a**) A schematic illustration of α-Gal on the gold substrate. (**b**) Surface plasmon resonance analyses of α-Gal monolayers with various proteins (500 nM) in phosphate-buffered saline buffer at 25 °C.

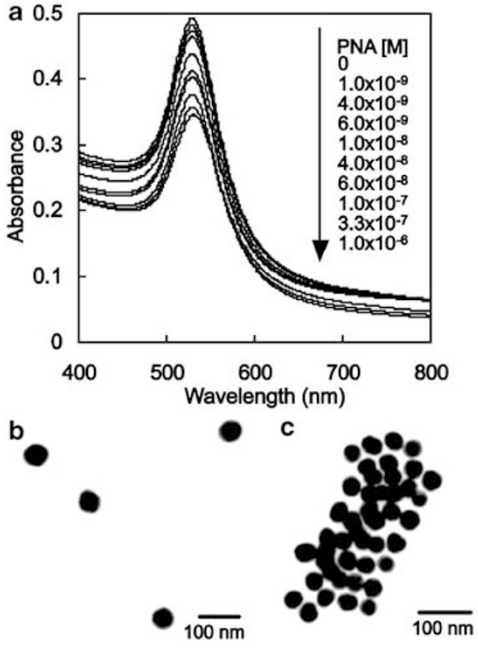


Figure 4 Observations of P3-GNP with the addition of proteins. (**a**) Ultravisible spectra of P3-GNP with the addition of peanut agglutinin. Transmission electron microscopy observations (**b**) without the addition of proteins and (**c**) with the addition of peanut agglutinin.

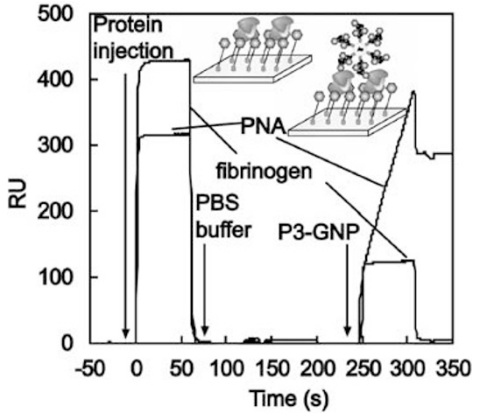


Figure 5 Signal amplification of surface plasmon resonance by the addition of P3-GNP (OD_{529}=0.45) and a schematic illustration for a protein concentration of 10 nM.

The injection of glycopolymer-substituted GNPs can be used to visualize the specific binding of PNA because of the binding specificity of **P3**, but the signal amplification can be observed only for a slow flow rate (10 μl min^{-1}) because of the weak interaction. The combination of a saccharide-modified substrate and nanoparticles enables the sensitive detection of proteins.

Biosensing of shiga toxin-1 using glycopolymer-modified substances

We investigated a more practical target and biosensing using glycopolymer-substituted materials. Considering that α-Gal-terminated saccharides bind to Stxs, we studied the interaction of Stx-1 with **P3-gold** and **P3-GNP**.

The **P3-gold** substrate was subject to measurements by SPR for determining the interaction with Stx-1. RU_{max} showed a clear dose dependency on the concentration of Stx-1, and the binding constant based on the Scatchard analysis was $4.1\times10^5\ M^{-1}$ (Figure 6). **P3-GNP** showed a slight increase in ultraviolet absorbance with the addition of Stx-1, and **P3-GNP** formed small aggregates in the solution (Figure 7). Finally, the immunochromatography of Stx-1 was investigated. A vague pink spot was observed with the addition of 2.4 μM Stx-1, although the spot was not observed with other proteins (Supplementary information).

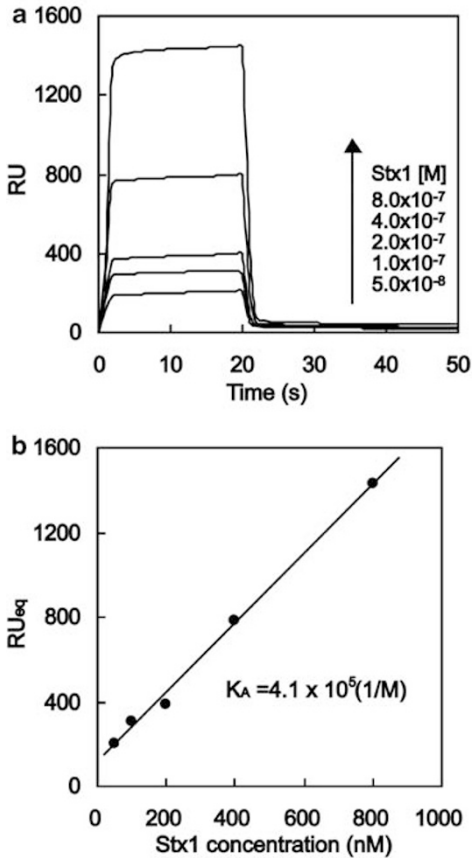


Figure 6 (**a**) Surface plasmon resonance analyses of P3-gold with Stx-1 and (**b**) Scatchard plot of P3-gold with Stx-1.

The affinity of **P3-gold** to Stx-1 was not sufficient for detecting Stx-1 because the pendant saccharide structure of α-Gal is different from the natural ligand structure of Gb3 (Galα1-4Galβ1-4GlcCer).[33] However, these results indicate that the glycopolymer thin layer can be applied for the biosensing of sugar recognition toxins. The immunochromatography of glycopolymer-substituted nanoparticles is still under investigation with glycopolymers carrying a natural ligand, in order to develop highly sensitive immunochromatography.

CONCLUSION

Gold surfaces modified with glycopolymers were prepared by living radical polymerization with RAFT. We prepared glycopolymers using a range of saccharides, molecular weights and sugar densities. Thiol-terminated glycopolymers were used for the modification of gold substrates and GNPs. The glycopolymer-substituted gold substrates showed strong, specific molecular recognition abilities with lectins, because of the hydrophilic polymer properties. Biosensing using glycopolymer-modified GNPs showed specific interactions with target proteins and toxins.

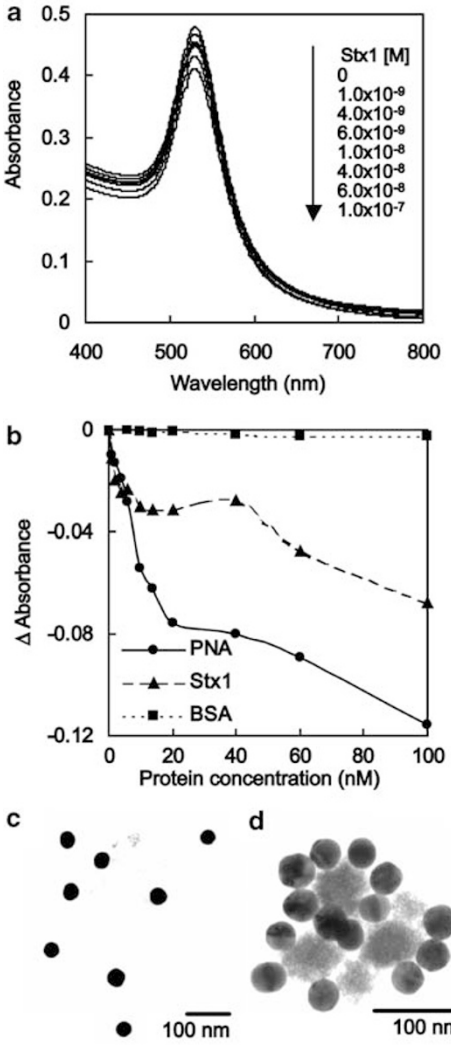


Figure 7 Ultra-visible spectra of (**a**) P3-GNP with the addition of Stx1 and (**b**) the intensity at 529 nm for varying protein concentrations ((●) peanut agglutinin, (▲) Stx1 and (■) bovine serum albumin). Transmission electron microscopy micrograph of Stx-1 stained with 1 wt% OsO_4 with P3-GNP. The entire image of Stx-1 (**c**) before and (**d**) after the addition of Stx1.

Electronic supporting information available

The syntheses of glycomonomers, atomic force microscopy imaging of the glycopolymer-modified layer, SPR analyses, evaluation of GNPs and immunochromatography are available through the Internet.

ACKNOWLEDGEMENTS

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