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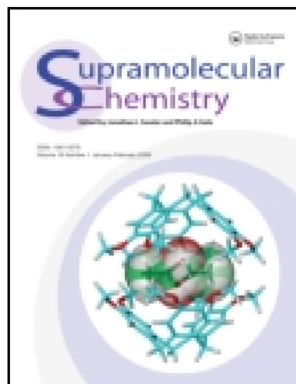

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Glycoconjugated gold nanoparticles (AuNPs) are of great interest as probes for detecting carbohydrate–protein interactions. Three sensing methods – absorption, light scattering and wavelength-ratiometric scattering – were used to detect polyvalent interactions between mannose-conjugated AuNPs and Concanavalin A (Con A). Nanoparticle aggregation was induced by protein–carbohydrate interaction, which shifted the plasmon absorption to longer wavelengths and increased the intensity of plasmon light scattering. The plasmon absorption wavelength shift and scattering light intensity enhancement were proportional to the concentration of Con A in the range 5.7–20.2 nM and 3.8–36.7 nM, respectively, and the corresponding limits of detection were 3.4 and 2.3 nM, respectively. The wavelength-ratiometric scattering method showed the lowest detection limits (1.9 nM) for Con A, at 5–40 times lower than those obtained using existing glycoconjugated AuNPs.

Keywords: gold nanoparticle; lectin; carbohydrate; thioglucose; surface plasmon resonance

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Introduction

Carbohydrates play an important role in numerous biological processes (*1*, *2*). Specific interactions between carbohydrates and proteins are essential in cell development and differentiation, viral and bacterial infection, immune response and metastasis of tumours (*3–5*). Therefore, an understanding of carbohydrate–protein interactions at the molecular level should lead to better insights into the biological processes of living systems and the development of novel therapies and diagnostics. However, carbohydrate–protein interactions are usually of low affinity, with dissociation constants typically in the range 10^{-3}–10^{-6} compared to dissociation constants of 10^{-8}–10^{-12} for the antigen–antibody interaction (*6*, *7*). Nature overcomes this low affinity through clustering of ligands or receptors on the cell surface. Polyvalent interactions between assembled ligands and proteins can be much stronger than the corresponding monovalent interactions (*8*, *9*). One of the advantages of glycoconjugated nanoparticles is that a nanoparticle with a large surface-area-to-volume ratio can present carbohydrates in a globular and polyvalent configuration on its surface, which provides a useful method for enhancing carbohydrate–protein interactions. Thus, glycoconjugated nanoparticles constitute an excellent biomimetic model for carbohydrate recognition and are a powerful and versatile tool in glycobiology.

Gold nanoparticles (AuNPs) are ideal for many of these studies due to their inherent advantages, including the possibility of preparation with well-defined sizes and shapes, easy surface modification, robustness and good biocompatibility (*10–15*). In addition, AuNPs exhibit strong optical absorption and scattering at visible and near infrared wavelengths due to a local surface plasmon resonance (LSPR) which involves collective oscillation of the conduction band electrons of the gold core (*16*). The maximum wavelength and shape of LSPR absorption and scattering are determined by the particle size and shape (*17–19*) and the local dielectric environment (*20*, *21*). Furthermore, aggregation of AuNPs leads to a dramatic colour change from red to blue or purple and a significant increase in light scattering. LSPR absorption and scattering can be measured using common absorption and fluorescence spectrometers, respectively. Due to the simplicity of these techniques, AuNPs have been extensively explored as probes for sensing a wide variety of analytes with high sensitivity and specificity (*22–42*). Here, we used LSPR absorption and scattering spectroscopy to probe the carbohydrate–protein interaction using glycoconjugated AuNPs.

Results and discussion

Preparation and characteristics of thioglucose-stabilised AuNPs

We recently developed thioglucose-stabilised AuNPs (TGlu-AuNPs) as a novel platform for constructing nanobiosensors (*43*). TGlu-AuNPs, which have free carboxyl groups on the particle surface, can be tailored to bind to target proteins selectively. Carbohydrate ligands

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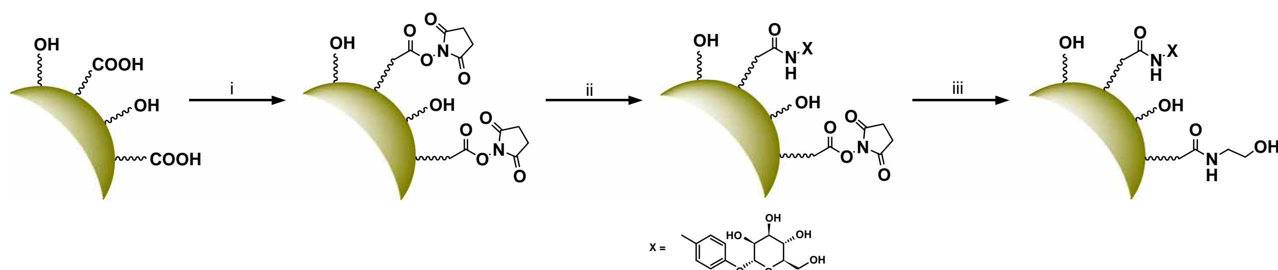

Scheme 1. Reagents and conditions. (i) NHS, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 25°C, pH 6.0, 1 h; (ii) X-NH_2, 25°C, pH 7.5, 2 h and (iii) 2-ethanolamine, 25°C, pH 7.5, 2 h.

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were covalently immobilised on the particle surface by coupling with activated *N*-hydroxy succinimide (NHS) esters with amino derivatives of carbohydrate (X-NH_2), as shown in Scheme 1. Figure 1 shows the plasmon resonance absorption and light scattering of TGlu-AuNPs in 10 mM {2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid} (HEPES) buffer. TGlu-AuNPs with a mean diameter of 45.4 nm exhibit characteristic plasmon resonance absorption at 528 nm, while showing complicated light scattering in the UV–vis region. The light scattering peaks at 361 and 438 nm are due to Rayleigh scattering of the aqueous solution. According to resonance light scattering theory, scattering is observed at or very near the wavelength of light absorption (*44*). Consequently, the light scattering peaks at 549 and 624 nm may be ascribed to plasmon resonance light scattering of TGlu-AuNPs.

Plasmon resonance absorption sensing of carbohydrate–protein interaction

Concanavalin A (Con A), a lectin obtained from jack beans, was chosen as a target protein. It exists as a dimer at low pH (<5.5) and a tetramer at high pH (>7), with each subunit containing a mannose- and glucose-specific binding site (*45*). When Con A was added to a solution of TGlu-AuNPs in 10 mM HEPES (pH 7.5), there was no change in the plasmon resonance absorption spectrum (data not shown), indicating that Con A cannot recognise a monolayer of thioglucose assembled on the particle surface. In contrast, the addition of Con A to mannose-immobilised AuNPs (Man-AuNPs) induced a significant red shift in LSPR, as shown in Figure 2(a). Dynamic light scattering (DLS) showed an increase in the mean diameter of Man-AuNPs from 45.8 to 444.3 nm, clearly demonstrating rapid aggregation of the nanoparticles. The dramatic colour change is due to electric dipole–dipole interactions and coupling between the plasmons of neighbouring particles, which depend on interparticle distance (*46–48*) and particle volume (*49*). Furthermore, the red shift of the plasmon absorption wavelength was sensitive to and correlated with the change in Con A concentration. These results indicate that, on addition of Con A to Man-AuNPs, multiple binding events occur between Con A and the mannoses immobilised on the particle surface, leading to cross-linking of the AuNPs, as shown schematically in Figure 3.

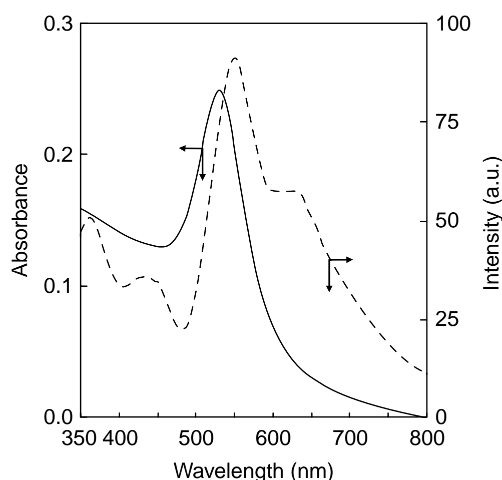

Figure 1. Plasmon resonance absorption and scattering spectra of TGlu-AuNPs ($d = 45.4$ nm) in 10 mM HEPES buffer (pH 7.5). [TGlu-AuNP] = 31 pM.

In order to confirm that the aggregation was induced by mannose-selective recognition of Con A, control experiments were carried out with another lectin, Lotus tetragonolobus lectin (Lotus) or peanut agglutinin lectin (PNA), which are specific for fucose (Fuc) and galactose (Gal), respectively (*50*). When these lectins were mixed with Man-AuNPs, no change in the UV–vis extinction spectrum was observed, as shown in Figure 2(b), indicating that no interaction between the Man-AuNPs and the Fuc- and Gal-specific lectins had occurred. It was concluded, therefore, that the aggregation induced by Con A was specific to the mannose binding sites located around the particle surface. When the experimental data were plotted as the normalised maximum wavelength shift ($\Delta\lambda/\Delta\lambda_{max}$) vs. the concentration of Con A, a sigmoidal form was obtained (Figure 4). This indicates that the binding of Con A to mannose on the AuNPs can be reliably detected only when the concentration of Con A is in the range 5.7–20.2 nM. The limit of detection for Con A at a signal-to-noise ratio of 3 was 3.4 nM.

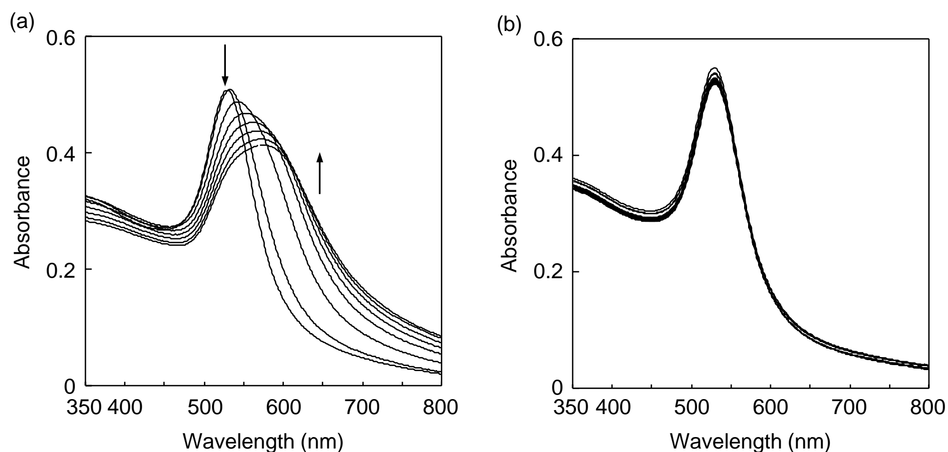

Figure 2. Change in the LSPR absorption spectrum of Man-AuNPs upon addition of 0–25 nM of (a) Con A and (b) Lotus in 10 mM HEPES buffer (pH 7.5) at 25°C. [Man-AuNP] = 60 pM.

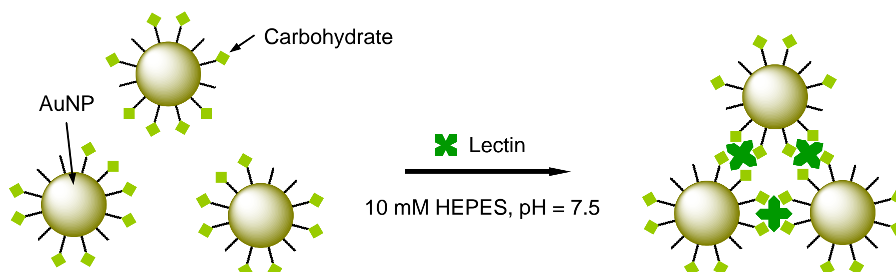

Figure 3. Schematic illustration of lectin-induced AuNP aggregation.

Figure 5 shows the time-dependent absorbance at 590 nm for Con A-induced aggregation of Man-AuNPs. The absorbance increased with the addition of Con A titrant solution, eventually reaching a maximum. Once the maximum was reached, the aggregate was stable for several hours. The change in absorbance was modelled as an exponential function of time:

$$\Delta A = A_t - A_0 = \Delta A_{t\to\infty}[1 - \exp(-k_{\mathrm{app}}t)],$$

where ΔA is the increase in absorbance, A_0 and A_t are the absorbance at time equal to 0 and t, respectively, $\Delta A_{t\to\infty}$ is

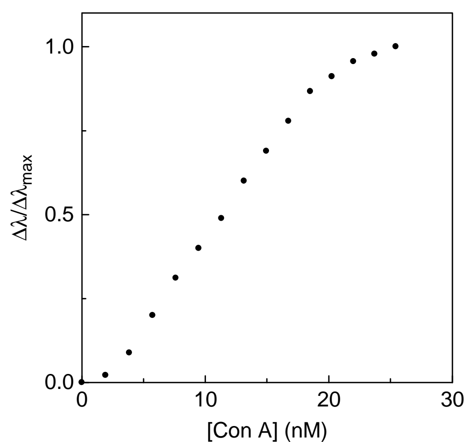

Figure 4. LSPR absorption calibration curve for Con A (0–37 nM) using Man-AuNP in 10 mM HEPES buffer (pH 7.5) at 25°C. [Man-AuNP] = 60 pM, [Con A] = 0–25 nM.

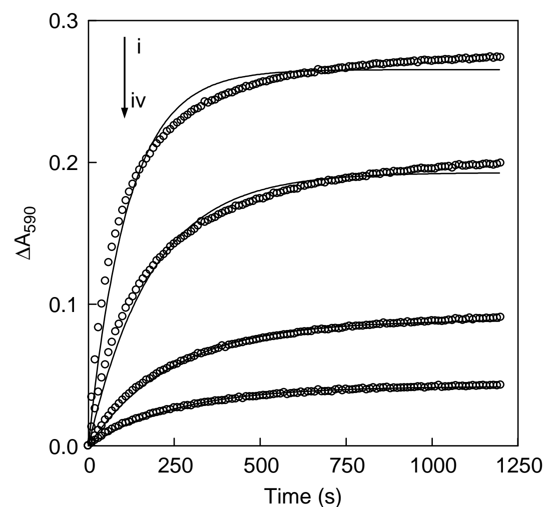

Figure 5. Time-dependent change in the LSPR intensity at 590 nm with varying concentrations of Con A. (i) 25.2 nM, (ii) 22.1 nM, (iii) 18.9 nM and (iv) 12.6 nM.

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the ΔA at time equal to infinity and k_{app} is the rate constant for absorption change due to aggregation. The k_{app} values for aggregation induced by Con A solutions of 12.6, 18.9, 22.1 and 25.2 nM were calculated at 3.90×10^{-3}, 4.06×10^{-3}, 5.47×10^{-3} and $6.67 \times 10^{-3}\,s^{-1}$, respectively. The response time t_{90} for reaching 90% of the final value is less than 10 min. Aslan et al. (*42*) reported that 500 kDa dextran-coated AuNPs gave the t_{90} of 20–100 min for aggregation induced by Con A. Thus, monosaccharide-functionalised AuNPs in the system under discussion have the advantage of a shorter response time than polysaccharide-functionalised AuNPs.

Plasmon resonance scattering sensing of carbohydrate–protein interaction

It has been reported that the aggregation process induces enhanced light scattering (*40–42*). We applied LSPR scattering spectroscopy to detect Con A using Man-AuNPs. First, the initial concentration of Man-AuNPs was optimised because the LSPR scattering band of Man-AuNPs is sensitive to the AuNP concentration. Figure 6(a) shows the concentration-dependent scattering of Man-AuNPs. The scattering intensity at 548 nm increased and shifted to 577 nm with increasing AuNP concentration, while the shoulder peak around 628 nm became much more distinctive and intense. A plot of the ratio of the scattering light intensity at 628 nm to that at 577 nm (I_{628}/I_{577}) vs. the concentration of Man-AuNPs over the concentration range 70–150 pM exhibits an inflection point at 60 pM. Although DLS showed no detectable increase in the mean diameter of the Man-AuNPs at a concentration range of 30–150 pM, the Man-AuNPs might be partially aggregated at concentrations of greater than 60 pM. Thus, in order to optimise the LSPR scattering measurements, the initial concentration of Man-AuNPs was set at less than 60 pM. The addition of Con A to a 6 pM solution of Man-AuNPs in 10 mM HEPES (pH 7.5) resulted in a dramatic enhancement of the resonance light scattering intensity at around 624 nm (Figure 7(a)). DLS was used to measure the particle sizes before and after the addition of Con A; an increase in the size of the nanoparticles from 45.8 to 195.3 nm was observed. Control experiments were carried out using the Fuc-specific lectin (Lotus) and the Gal-specific lectin (PNA). When these lectins were mixed with Man-AuNPs, no change in the LSPR scattering spectrum was observed, as shown in Figure 7(b), indicating that no interaction between the Man-AuNPs and the Fuc- and Gal-specific lectins had occurred. Thus, the Con A-induced enhancement in the light scattering intensity is mediated exclusively by means of specific mannose–Con A interaction. A calibration plot of the normalised light scattering intensity change ($\Delta I/\Delta I_{max}$) vs. the concentration of Con A yielded a detection limit of 2.2 nM. The dynamic range of the nanoparticle sensor was 2.3–36.7 nM, as shown in Figure 8(a). The detection limit for the LSPR scattering method was somewhat lower than that for the LSPR absorption method.

Wavelength-ratiometric scattering sensing of carbohydrate–protein interaction

The plasmon resonance light scattering method allowed us to use wavelength-ratiometric measurement, which has several advantages over plasmon-resonance-absorption-based sensing (*51*). In order to choose appropriate wavelengths for the wavelength-ratiometric measurements, the scattering light intensity of aggregated AuNPs can be divided by the scattering light intensity of monomer AuNPs at each corresponding wavelength, and the resulting ratio was plotted vs. wavelength (data not shown). To obtain the optimum sensitivity for ratiometric determination, we chose wavelengths at the maximum (598 nm) and minimum (529 nm) of the spectrum ratio. Figure 8(b) shows a plot

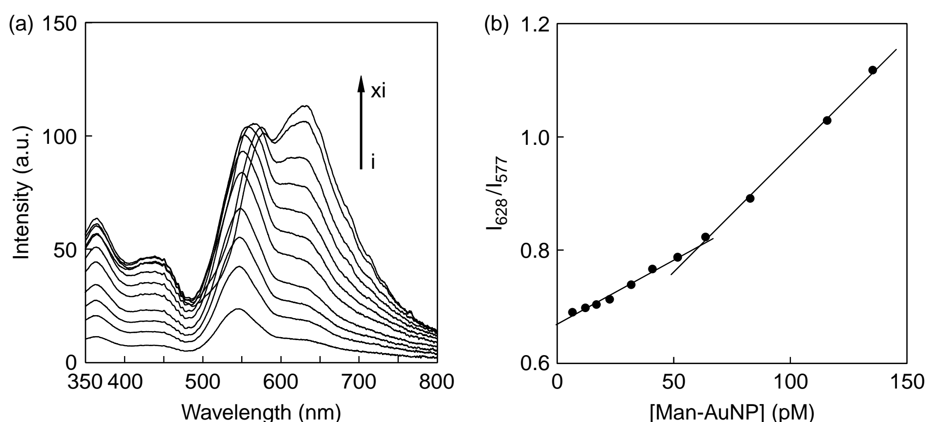

Figure 6. (a) Plasmon resonance scattering spectra of Man-AuNPs in aqueous solution at various Man-AuNP concentrations: (i) 6.56 pM, (ii) 12.2 pM, (iii) 17.0 pM, (iv) 22.5 pM, (v) 32.0 pM, (vi) 41.0 pM, (vii) 51.8 pM, (viii) 63.9 pM, (ix) 82.8 pM, (x) 115.9 pM and (xi) 135.5 pM and (b) Relative intensity (I_{628}/I_{577}) plotted as a function of the corresponding concentration.

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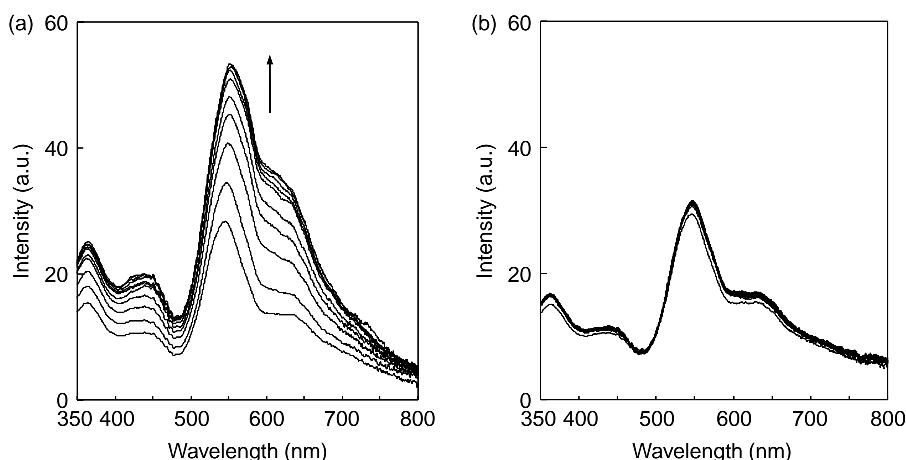

Figure 7. Change in the LSPR scattering spectrum of Man-AuNPs (6 pM) upon addition of 0–37 nM of (a) Con A and (b) Lotus in 10 mM HEPES buffer (pH 7.4) at 25°C.

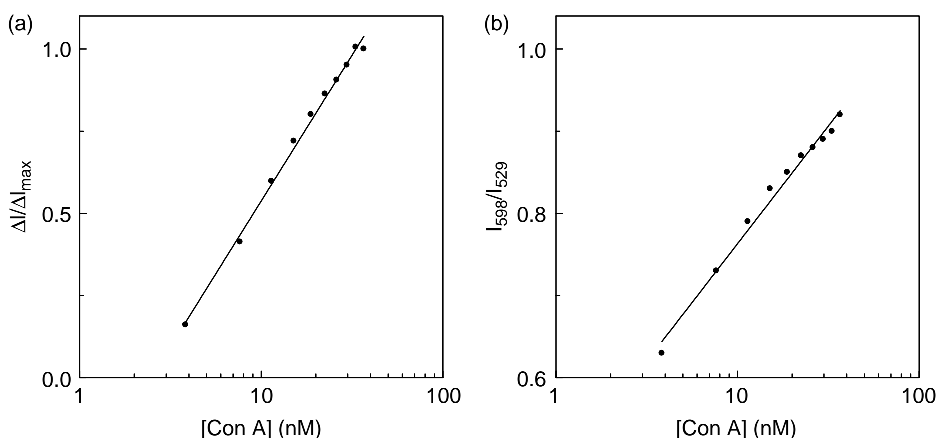

Figure 8. Calibration curve for Con A using Man-AuNP (6 pM) in 10 mM HEPES buffer (pH 7.5) at 25°C. (a) LSPR scattering method and (b) wavelength-ratiometric scattering method.

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of the LSPR scattering intensity ratio (I_{598}/I_{529}) as a function of Con A concentration. A good linear relationship was obtained in the concentration range 3.8–36.7 nM ($R^2 = 0.998$). The limit of detection for Con A was 1.9 nM in aqueous buffer, which is 5–40 times lower than those obtained for existing glycoconjugated AuNPs (*33–39*).

Conclusions

We have demonstrated that highly sensitive and selective sensing of protein–carbohydrate interactions can be achieved using glycoconjugated AuNP-based LSPR absorption and scattering methods, which allow the detection of Con A over the range 5.7–20.2 and 3.8–36.7 nM, respectively. By taking advantage of the high sensitivity of the LSPR scattering method, quantitative determination of Con A with a detection limit of 1.9 nM was achieved. The system exhibited shorter response times and higher sensitivity than existing LSPR absorption and scattering methods involving glycoconjugated AuNPs. These methods do not require protein labelling and can be performed using common absorption and fluorescence spectrometers. Furthermore, they are easily generalised for the study of other carbohydrate–protein interactions by varying the carbohydrate on the particle surface.

Experimental

Chemicals and materials

Hydrogen tetrachloroaurate trihydrate and trisodium citrate dehydrate were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 1-Thio-β-D-glucose sodium salt and 4-aminophenyl α-D-mannose (Sigma-Aldrich, St Louis, MO, USA) were used as received. NHS, *N*-3-(dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), 2-aminoethanol and HEPES were obtained from Nacalai Tesque (Kyoto, Japan). *Concanavalia ensifomis* (Con A),

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Lotus tetragonolobus (Lotus) and *Arachis hypogaea* (PNA) lectins were obtained from J-OIL Mills, Inc. (Tokyo, Japan). All chemicals were used as received.

LSPR absorption and scattering spectroscopy

LSPR absorption measurements were performed on a double-beam JascoV560 UV–vis spectrophotometer (JASCO Ltd, Tokyo, Japan) at 25°C, using 1 cm path length quartz cells. LSPR spectra were obtained using an F-5500 fluorescence spectrometer (JASCO Ltd) by simultaneous scanning of the extinction and emission monochromators at $\Delta\lambda = 0$ nm.

Dynamic light scattering

DLS experiments were conducted using a light scattering spectrometer DLS-6000 (Photal Otsuka Electronics Co., Ltd, Osaka, Japan). Incident light was provided by an argon ion laser ($\lambda_{ex} = 514.5$ nm) operating at 75 mW and by a He–Ne laser ($\lambda_{ex} = 514.5$ nm) operating at 10 mW. Scattering light was collected at a fixed angle of 90°.

Synthesis of 1-thio-β-D-glucose-stabilised AuNPs and AuNPs modified with saccharides

AuNPs were prepared according to the method previously reported (*43*), with slight modification. Briefly, an aqueous solution of $HAuCl_4$ (200 ml, 0.40 mM) was heated to boiling under reflux. To the vigorously stirred aq. $HAuCl_4$ was added, all at once, an aqueous solution of 1-thioglucose sodium salt (50 ml, 0.72 mM). The colour of the aqueous solution turned from pale yellow to ruby red within 10 s. The reaction mixture was further heated under reflux for 20 min and allowed to cool to room temperature.

The solution of prepared TGlu-AuNPs ($d = 50$ nm) was adjusted to pH 6.0 using aq. NaOH. An aqueous solution of NHS and EDC (2.0 ml, 80 μM NHS and EDC) was added to 20 ml of the pH-adjusted AuNPs with a concentration of 100 pM (determined by measuring the absorbance at 520 nm using an extinction coefficient of $1.20 \times 10^{10}\,M^{-1}\,cm^{-1}$). The resulting mixture was adjusted to pH 7.5 with aq. NaOH. A 5.7 mM solution of 4-aminophenyl α-D-mannose (1.0 ml) was added to the surface-activated AuNPs and incubated for 2 h at 25°C. The remaining NHS-activated carboxylate groups on the particle surface were blocked with a 5.8 mM solution of 2-ethanolamine (1.0 ml) for an additional 2 h. The resulting mixtures were dialysed overnight in 3 l of 10 mM HEPES buffer (pH 7.5) using a dialysis membrane (Spectra/Por® Biotech Regenerated Cellulose, molecular-weight cut-off 12,000–14,000). These particle solutions were stored in the dark and refrigerated at 4°C for the specific binding experiments with lectin.

LSPR absorption (colorimetric) method using AuNPs

A 2 ml aliquot from a stock solution of AuNPs in 10 mM HEPES buffer (pH 7.5) was transferred to a 1 cm UV cell. A 0.16 mg/ml solution of lectin in 10 mM HEPES buffer was prepared, and small aliquots (10 μl) were added to the solution of AuNPs in the UV cell, which was equipped with a polytetrafluoroethylene (PTFE) magnetic stirring bar. The solutions were equilibrated by stirring and standing for 5 min prior to the acquisition of UV–vis spectra. The wavelength shift in the LSPR was monitored as a function of lectin concentration.

LSPR scattering method using AuNPs

A 3 ml aliquot from a stock solution of AuNPs (6 pM) in 10 mM HEPES buffer (pH 7.5) was transferred to a 1 cm quartz cell. A 80 μg/ml solution of lectin in 10 mM HEPES buffer was prepared, and small aliquots (10 μl) were added to the solution of AuNPs in the quartz cell, which was equipped with a PTFE magnetic stirring bar. The solutions were equilibrated by stirring and standing for 5 min prior to the acquisition of light scattering spectra. Changes in the light scattering intensity were monitored as a function of lectin concentration.

Acknowledgements

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