Binding Enhancment of Antigen-Functionalized PEGylated Gold Nanoparticles onto Antibody-Immobilized Surface by Increasing the Functionalized Antigen using α-sulfanyl-ω-amino-PEG

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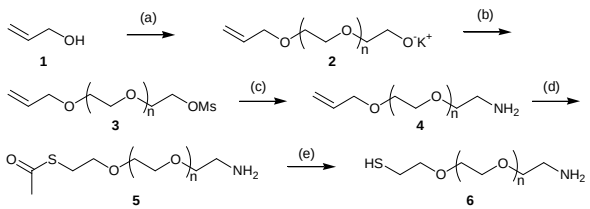
We established a technique for constructing PEGylated gold nanoparticles (GNP) that have small compounds with almost complete functionalities on their surfaces using a newly synthesized hetero-telechelic poly(ethylene glycol) (PEG), and their association/dissociation behavior on an antibody-immobilized surface was evaluated using a surface plasmon resonance (SPR) sensor.

GNPs have received widespread interest, particularly with regard to their uses in biotechnological systems for diagnostic applications,[1,2] and biological imaging,[3] due to the ease with which they can be prepared and bioconjugated, and their highly controlled optical properties. Notably, GNPs have recently attracted much attention in the medical field, since GNPs act not only as excellent agents for optical imaging, but also for photothermal therapy (PTT), which allows the selective killing of cancer cells, leaving healthy cells unaffected.[4] Since GNPs aggregate spontaneously under physiological salt concentrations due to surface charge shielding, several attempts have been made to stabilize GNPs in aqueous media.[5] Wuelfing et al. reported the reliable stabilization of GNPs in an aqueous milieu using a semi-telechelic PEG, α-methoxy-ω-mercapto-PEG (CH_3O-PEG-SH).[6] This PEGylation strategy confers appreciable stability and biocompatibility upon GNPs under physiological conditions owing to the steric repulsion effects of the tethered PEG strands, although the GNPs prepared by this method lack reactive groups for ligand immobilization onto the PEG-tethered chains, which enable the selective targeting and delivery of PEGylated GNPs.



Scheme 1 Synthetic route to HS-PEG-NH_2 (**6**): (a) potassium naphthalene, EO; (b) methane sulfanyl chloride; (c) ammonia; (d) thioacetic acid, azoisobutyronitrile, UV irradiation; (e) sodium methoxide-methanol, HCl. More detailed synthetic procedures were described in ESI.

We have been studying the synthesis of various types of hetero-telechelic PEGs based on the ring-opening polymerization of ethylene oxide (EO) using a metal alkoxide initiator with a protected functional group.[7] Using α-acetal-ω-sulfanyl-PEG (acetal-PEG-SH), an acetal group-terminated GNP can be prepared, which reacts with a small compound that carries an amino group, *p*-aminophenyl β-D-lactopyranoside, with low-level functionalities (ca. 65 %).[1b,8] Since this lactose-functionalized PEGylated GNP has a unique binding property, i.e., dissociation of the GNP from target proteins is easily accomplished by the addition of competitor molecules, the PEGylated GNP can be used in colorimetric assays for a lectin[1b] and as a signal-enhancement agent for the detection of galactose in the SPR sensor system[2]. In contrast, the development of a high-performance PTT agent requires both strong association and nondissociative properties with the target protein. Thus, modification of the PEG surface with ligands having high-level functionalities is crucial, and novel methods for improving functional efficiency at the PEG end are needed. However, to the best of our knowledge, a technique for constructing a ligand-functionalized PEGylated GNP with high-level functionalities and binding performance has not been reported to date.

We present herein a new technique for constructing a ligand-functionalized PEGylated GNP with almost complete functionalities, and show the effect of the immobilized amount of ligand in changing the binding property of ligand-terminated PEGylated GNPs. In the present study, a new heterotelechelic PEG, α-sulfanyl-ω-amino-PEG (HS-PEG-NH_2; M_n = 5k, M_w/M_n = 1.1), was synthesized by anionic ring-

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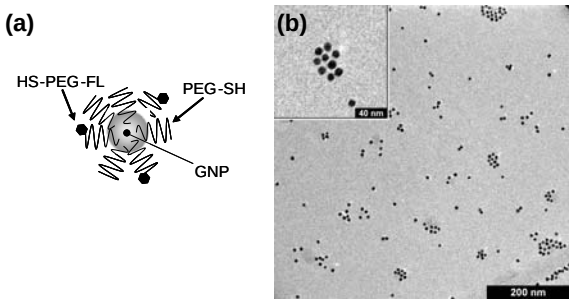

Figure 1 Schematic illustration (a) and TEM image of FL-terminated PEGylated GNP constructed using a combination of HS-PEG-NH_2 and PEG-SH (b).

opening polymerization of EO using the allyl alcohol/potassium naphthalene initiator system[9] (Scheme 1). Furthermore, a technique for constructing ligand-functionalized PEGylated GNP with almost complete functionalities was established, and its association/dissociation behavior on an antibody-immobilized gold surface was evaluated by SPR. As controls, PEGylated GNPs with various densities of surface-immobilized ligands were constructed using a combination of HS-PEG-NH_2 and α-mercapto-semi-telechelic PEG, and their binding properties were assessed.

The amino group at the ω-end of HS-PEG-NH_2 can be used for the q uantita tive immobilization of amino-reactive compounds (ARC) that have an amino-reactive group, such as isothiocyanate and activated ester. Our strategy for constructing ligand-functionalized GNPs was based on the immobilization and co-immobilization through thiol-gold linkages of ARC-PEG-SH and ARC-PEG-SH/PEG-SH, respectively, onto a commercially available GNP (9.5 nm) (Figure 1a). Fluorescein isothiocyanate was used as a model compound, and fluorescein (FL)-functionalized PEGylated GNPs (FL-GNPs) with various densities of surface-immobilized FL were constructed. Using ion exchange chromatography and ^{1}H-NMR measurements, it was confirmed that FL was almost fully introduced at the ω-end of HS-PEG-NH_2 (see ESI). Figure 1b shows a typical transmission electron microscopy (TEM) image of the FL-GNP, which was constructed using a combination of HS-PEG-FL and PEG-SH, clearly indicating that the diameter of the constructed GNPs were about 10 nm. The PEGylated GNP sample showed no significant aggregation in solution under physiological conditions (150 mM NaCl, 50 mM phosphate buffer, pH 7.4).

FL-GNPs with various densities of surface-immobilized FL were prepared using mixtures of several molar ratios of HS-PEG-FL and acetal-PEG-SH (M_w = 5k) (Table 1). The FL content of FL-GNPs can be regulated by changing the molar ratio s of the HS-PEG-FL/acetal-PEG-SH mixture. The number of FL groups on the FL-GNP, as assessed by the fluorescence-based determination of HS-PEG-FL that leached from the GNP surfaces (see ESI), was increased as the HS-PEG-FL molar ratio in the mixed solution. The maximum number of FL groups that could be functionalized onto the PEGylated GNP was found to be 168, and all of the FL-PEG-immobilized GNPs prepared in this manner showed stable dispersibility in aqueous solutions with elevated ionic strength, which is an environment that immediately induced the aggregation of unmodified gold particles.[10]

Table 1 Number of FL-PEG immobilized on GNP surface.[a]

Sample	Molar ratio of HS-PEG-FL: acetal-PEG-SH mixed solution	Number of FL per single GNP surface
FL-GNP(0)	0 : 10	0
FL-GNP(6)	1 : 9	6
FL-GNP(22)	5 : 5	22
FL-GNP(49)	9 : 1	49
FL-GNP(168)	10 : 0	168

[a] Experimental conditions were described in ESI.

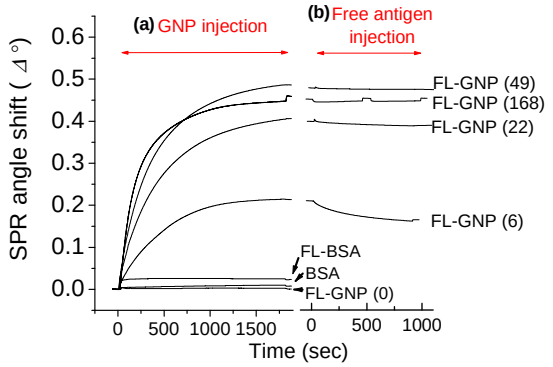

Figure 2 SPR sensorgrams for the association/dissociation behavior of 5 nM FL-GNPs(0)-(168) onto the Fab'/PEG-SH(2k) co-immobilized sensor surface. All samples and running buffer solutions were prepared with 50 mM sodium phosphate containing 150 mM NaCl at pH 7.4. Sample injections were carried out at a constant flow rate of 5 μL/min at 25 °C. Replacement reaction of FL-GNP(n) with FL was carried out by injecting of 1 μM FL solution.

The PEG properties of water-solubility, flexibility[11] and electroneutrality[12] confer upon FL-GNP the characteristics of stable dispersibility and nonfouling in physiological salt concentrations. However, the properties of nonfouling and molecular recognition on the PEGylated GNP surface were in tradeoff relation. To evaluate the binding performance of FL-GNP(0)-(168), SPR analysis was carried out on an antibody-immobilized gold sensor surface, whereby the fragment of antigen-binding (Fab') of the anti-fluorescein antibody (FL-antibody) and CH_3O-PEG-SH (M_w = 2k) were co-immobilized directly onto a bare gold surface *via* a thiol-gold linkage. Figure 2a shows the SPR sensorgrams for the adsorption behavior of the FL-GNPs on the constructed sensor surface. The extremely large binding responses of 0.2-0.5° were observed when FL-GNP(6), (22), (49), and (169) were injected into the flow path on the surface. The binding response tended to increase depending upon the number of functionalized FLs onto the FL-GNP surface, such that FL-GNP(49) showed the strongest SPR angle shift. In contrast, there was essentially no adsorption when bovine serum albumin (BSA) and FL-GNP(0) were flowed onto the same surface (Δ° = 0.001 and 0.008, respectively) (Fig. 2a). These results indicate that the FLs functionalized onto the PEGylated GNP surface can react selectively with the Fab' on the sensor surface, retaining the low nonspecific interaction of environmental materials. Interestingly, FL-conjugated BSA

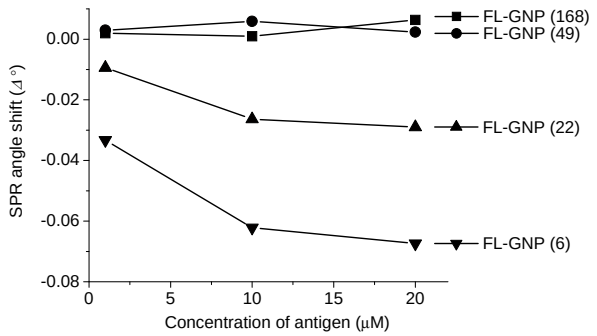

Figure 3 SPR angle shift changes in the competitive reaction between FL-GNP(n) and free FL on the Fab'/PEG co-immobilized gold surface. 5 nM GNP was pre-modified to the surface and FL solutions were injected at 20 μL/min flow rate for 15 min at 25 °C. All sample and running buffer solution were prepared with 50 mM sodium phosphate containing 150 mM NaCl at pH 7.4.

(FL-BSA), the volume of which is significantly larger than that of GNP, also showed slight changes in the SPR angle shift ($\Delta° = 0.022$). This means that the response signals of FL-GNP(n)s bound to the antibody-immobilized sensor surface are strongly enhanced by the coupling of the localized surface plasmon of GNP to the propagating plasmon on the gold sensor surface.

To evaluate the dissociation of FL-GNPs from the antibody-immobilized gold sensor surface, a competitive reaction with a 1 μM solution of free FL was carried out (Figure 2b). The largest decrease in the SPR sensorgram was observed at the FL-GNP(6)-modified sensor surface, whereas negligible changes were observed when 1 μM of free FL was injected into the flow path on the FL-GNP(22)-, FL-GNP(49)-, and FL-GNP(168)-modified sensor surfaces. To evaluate the dissociation behavior in more detail, the SPR angle shift changes for the dissociation of FL-GNPs from the sensor surface were monitored by increasing the free FL concentration (Figure 3). In the case of FL-GNP(22)- and FL-GNP(6)-modified sensor surfaces, decreases in the SPR angle shift change were observed with increases in the free FL concentration; and eventually 5% and 20% of the respective GNPs were dissociated from the surfaces. In contrast, negligible SPR angle shift changes were observed for the FL-GNP(49)- and FL-GNP(168)-modified sensor surfaces. These results give a new insight into the binding performance of ligand-functionalized PEGylated GNPs, in that the association abilities and nondissociative properties of the ligand-functionalized PEGylated GNPs with antibodies were increased by increasing the number of antigens immobilized on the PEGylated GNP surface. The high-level binding accompanying the nondissociative properties of FL-GNP(49) and FL-GNP(168) are interesting, and this binding property is suitable for the selective and effective staining of targets by PEGylated GNPs. Multipoint interactions might play an important role for the present binding behavior.

In summary, we synthesized a hetero-bifunctional PEG, HS-PEG-NH_2, which reacted with ARC with almost full functionality through the amino group at the ω-end. This new hetero-telechelic PEG enables the construction of FL-functionalized PEGylated GNPs with high-level functionality on its surface. Interestingly, SPR analysis revealed that FL-GNP(49) and FL-GNP(168)) had high binding affinities accompanying the nondissociative property for the FL-antibody Fab'-immobilized sensor surface. These results give a new insight into the construction of high-performance PTT agents, since the number of ligand immobilized on the PEG surface affects not only the association properties but also the dissociation properties of PEGylated GNPs. Thus, PEGylated GNPs with high ligand functionalities could be used as high-performance photothermal agents in PTT. On the other hand, PEGylated GNPs with various ligand functionalities can be constructed using a combination of HS-PEG-FL and acetal-PEG-SH, and the association/dissociation behavior of the PEGylated GNPs with low functionalities make them suitable for the GNP replacement assay for the detection of small molecules in the SPR instrument[1b]. The established technique for preparing a surface-functionalized PEGylated GNP using HS-PEG-NH_2 should be useful for the derivation of PEGylated GNPs with high densities of surface-immobilized molecules.

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