Quantitative and Reversible Lectin-Induced Association of Gold Nanoparticles Modified with α-Lactosyl-ω-mercapto-poly(ethylene glycol)

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Abstract: Gold nanoparticles (1−10 nm size range) were prepared with an appreciably narrow size distribution by in situ reduction of $HAuCl_4$ in the presence of heterobifunctional poly(ethylene glycol) (PEG) derivatives containing both mercapto and acetal groups (α-acetal-ω-mercapto-PEG). The α-acetal-PEG layers formed on gold nanoparticles impart appreciable stability to the nanoparticles in aqueous solutions with elevated ionic strength and also in serum-containing medium. The PEG acetal terminal group was converted to aldehyde by gentle acid treatment, followed by the reaction with *p*-aminophenyl-β-D- lactopyranoside (Lac) in the presence of $(CH_3)_2NHBH_3$. Lac-conjugated gold nanoparticles exhibited selective aggregation when exposed to *Recinus communis* agglutinin (RCA_{120}), a bivalent lectin specifically recognizing the β-D-galactose residue, inducing significant changes in the absorption spectrum with concomitant visible color change from pinkish-red to purple. Aggregation of the Lac-functionalized gold nanoparticles by the RCA_{120} lectin was reversible, recovering the original dispersed phase and color by addition of excess galactose. Further, the degree of aggregation was proportional to lectin concentration, allowing the system to be utilized to quantitate lectin concentration with nearly the same sensitivity as ELISA. This simple, yet highly effective, derivatization of gold nanoparticles with heterobifunctional PEG provides a convenient method to construct various colloidal sensor systems currently applied in bioassays and biorecognition.

Introduction

Nanometer-scaled semiconductor and metal particles are theoretically predicted to exhibit quantized properties and have become a focus for new applications in optoelectronics, thin film growth, catalysis, and most recently, medical diagnostics.[1−3] A simple and facile means of anchoring different ligand molecules onto particle surfaces allows their utilization as colloidal sensors.[4−6] Particularly, color changes induced by association of nanometer-sized gold particles provide a basis of a simple, yet highly selective, method for detecting specific biological reactions between anchored ligand molecules and receptor molecules in the milieu.[4−8] Mirkin and co-workers have shown that gold colloidal particles modified with oligonucleotides form large assemblies through the hybridization with complementary oligonucleotide strands, providing a new method for colorimetric detection of targeted DNA sequences.[7,8] With decreasing gold colloidal particle size, however, colloidal stability decreases significantly due to increased particle surface energy. Such gold nanoparticles aggregate in high ionic strength milieu as well as adsorb biomolecules such as proteins and DNA nonspecifically, resulting in reduced sensitivity and selectivity when used as colloidal sensor systems in biological fluids.

Several methods have attempted to stabilize nanoscale gold particles in aqueous milieu. Most of them have utilized surfactant and polymer stabilizers yet were not effective in preventing aggregation of nanoparticles particularly under physiological conditions (concentrated salt medium).[9] Recently, Wuelfing et al. reported that surface PEGylation of gold particles by CH_3O-PEG-SH significantly improved their dispersion stability in aqueous milieu due to steric repulsion effects of tethered PEG strands.[10] Yet, the CH_3O-PEGylated nanoparticles possess no reactive groups to further immobilize ligand molecules. Thus, a system possessing both sufficient colloidal stability and biofunctionality is strongly desired[11−13] to construct quantitative bioassays using gold nanoparticles. Here, we wish

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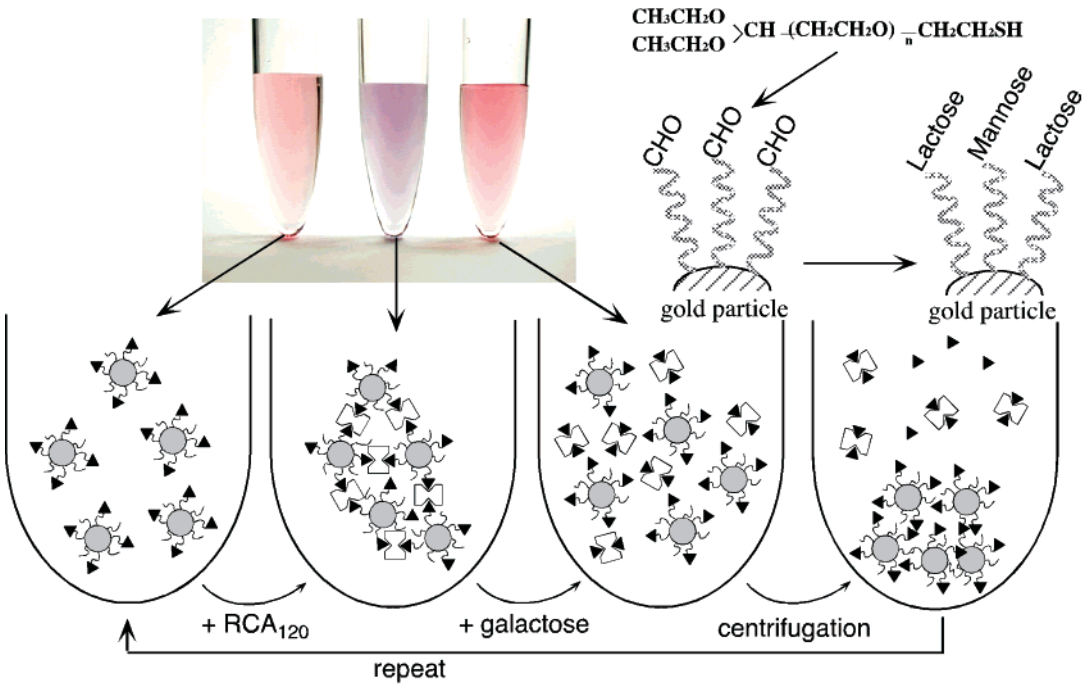
Scheme 1. Schematic Representation of the Reversible Aggregation−Dispersion Behavior of Lac-PEGylated Gold Nanoparticles by Sequential Addition of RCA_{120} Lectin and Galactose with Actual Concommitant Change in Color from Pinkish-Red → Purple → Pinkish-Red



to report a facile route to functionalize PEGylated gold nanoparticles, having a potential utility for colloidal sensor systems, by the use of newly designed heterobifunctional PEG derivatives.

Our strategy to synthesize various types of heterobifunctional PEGs is based on the ring-opening polymerization of ethylene oxide using a metal alkoxide initiator with a protected functional group.[14−18] Specifically, synthesis of heterobifunctional PEG containing both mercapto and acetal terminal groups (α-acetal-ω-mercapto-PEG, acetal-PEG-SH) was recently reported.[14] The acetal moiety can readily be transforned into a reactive aldehyde group by simple treatment with dilute acid. An aldehyde-functionalized PEGylation of gold nanoparticles using this acetal-PEG-SH (M_n = 3090) was achieved in this study, obtaining gold nanoparticles with high dispersion stability, particularly in physiological milieu, and appreciable aldehyde reactivity to immobilize ligand molecules on the PEG coronas which additionally impart sensitivity and selectivity due to their nonfouling properties. As a model ligand, lactose was successfully introduced in the distal end of PEG chain to induce lectin-mediated quantitative and reversible association of gold nanoparticles under physiologically relevant conditions, producing a concomitant color change (red → purple → red) as described in Scheme 1.

Experimental Section

Materials. Commercial tetrahydrofuran (THF) and 3,3-diethoxy-1-propanol (Aldrich Chemical Co., Inc., Milwaukee, WI) were purified by conventional methods.[19] Ethylene oxide (EO) (3M, Japan) was dried over calcium hydride and distilled under an argon atmosphere. Potassium naphthalene was used as a THF solution, whose concentration was determined by titration. Hydrogen tetrachloroaurate(III) tetrahydrate ($HAuCl_4 4H_2O$) (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), *p*-aminophenyl-β-D-lactopyranoside (*p*-aminophenyl-Lac, Sigma Chemical Company, St. Louis, MO), *p*-aminophenyl-β-D-mannopyranoside (*p*-aminophenyl-Man, Sigma Chemical Company, St. Louis, MO) and *R. communis* agglutinin (RCA_{120}, Vector Lab., Burlingame, CA) were used as received. Water used in this study was purified by a Milli-Q System (Nihon Millipore Co., Tokyo, Japan) to have a specific conductivity of less than 0.1 μS cm^{-1}.

Synthesis of α-Acetal-ω-mercapto-PEG. The heterobifunctional poly(ethylene glycol) (PEG) with a mercapto group at one end and an acetal group at the other end was synthesized by a one-pot anionic ring-opening polymerization of ethylene oxide (EO) initiated with potassium 3,3-diethoxypropanolate (PDP) as an initiator at room temperature under argon. Because the detailed procedure was described in the previous paper,[14] only a brief description is presented here; To prepare α-acetal-ω-mercapto-PEG, the two-step synthesis method shown in Scheme 2 was employed. After the PEG possessing an acetal group at the α-chain end was prepared using PDP as an initiator, the alkolate group at the ω-chain end was converted to the methansulfonyl group by the addition of an excess amount of methanesulfonyl chloride. Furthermore, the acetal-PEG-SO_2CH_3 was reacted with potassium-*O*-ethyldithiocarbonate to convert the methane sulfonyl end group to the *O*-ethyldithiocarbonate group, which can be easily cleaved by *n*-propylamine in THF to generate a mercapto group. The end mercapto group was analyzed by UV spectroscopy using 2-pyridyl disulfide (2-PDS) where the SH group reacts with 2-PDS, resulting in a release of 2-thiopyridon, which can be quantified by UV absorbance at 360 nm. The functionality of the SH-end-group per prepared acetal-PEG-SH was calculated to be 0.85.[14]

From the size-exclusion chromatography (SEC) analysis, the number-average molecular weight (M_n) and the molecular weight distribution (MWD) were determined to be 3090 and 1.05, respectively.[14]

Synthesis of Au Nanoparticles. Acetal-PEG-SH obtained from the above synthesis and $HAuCl_4$ (0.2 mM L^{-1}) were dissolved in deionized water, separately. Then, both solutions were mixed in a vessel to make

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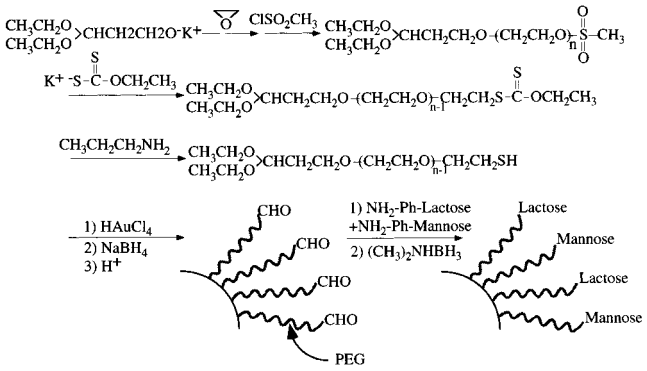
Scheme 2. Preparation of Functionalized Gold Nanoparticles Covered with Heterobifunctional PEG



desired concentrations of acetal-PEG-SH and $HAuCl_4$ at the acetal-PEG-SH:$HAuCl_4$ molar ratio of 1/6:1. The solutions were vigorously stirred during the addition of an excess amount of ice-chilled aqueous $NaBH_4$ (10 times the molar concentration of $HAuCl_4$) at room temperature for 0.5 h. The aqueous medium was removed after the spinning-down of gold nanoparticles at the centrifugation force of 360.6K × *g* (corresponding to 70K rpm) for 3 h at 4 °C. The precipitate was washed twice with purified water through an ultrasonic redispersion−centrifugation process and was finally suspended in phosphate buffer solution (PBS) with varying concentrations.

UV−vis spectra of solutions before and after the reduction of $HAuCl_4$ were measured with a UV spectrophotometer (V-550 UV/VIS spectrophotometer, JASCO, Japan) at 1-cm path length. Average sizes of obtained gold nanoparticles were determined from a transmission electron microscope (TEM) images of approximately 300 particles. The TEM samples were prepared by mounting a drop of the solution on carbon-coated Cu grids and allowing them to dry in air. TEM observation was carried out with a Hitachi H800 (Hitachi, Tokyo, Japan) operating at 200 kV. The size distributions were obtained by treating the degitized photo image with a Scion image software package (Scion Co., Frederick, MD).

Conversion of the Acetal Group into an Aldehyde Group. The acetal-PEG-SH-coated gold nanoparticle was immersed into aqueous media adjusted to pH 2 using hydrochloric acid for 5 h to transform an acetal group at the PEG chain end into an aldehyde end group. The resulting particle was immersed into the mixture solution of 0.5 mM of *p*-aminophenyl-Lac and *p*-aminophenyl-Man, which was carefully adjusted to pH 7 with PBS, and then subjected to 0.5 mM of borane dimethylamine complex ($(CH_3)_2NHBH_3$). The reacted gold nanoparticles was purified by the centrifugation with a force of 360.6K × *g*, followed by the suspending in purified water. This procedure was repeated three times to completely remove the unreacted labeling reagent.

Compositional Imaging Using Energy-Filtered TEM (EFTEM). The compositional imaging mode of TEM is used to directly image the distribution of thiolate molecules on the surface of gold nanoparticles. EFTEM and electron energy loss spectroscopy (EELS) were acquired using a LE0912 electron microscope (LEO Electron Microscopy, Oberkochen, Germany). The acceleration voltage was $E = 120$ keV. Element distribution images were acquired via the three-window method. To determine the sulfur distribution, the $L_{2,3}$ edge at $\Delta E = -200$ eV was used. The background (preedge) images were taken at $\Delta E_{1,S} = -137$ eV and $\Delta E_{2,s} = -153$ eV, respectively, with an energy width (δE) of 15 eV.

Results and Discussion

Gold nanoparticles were prepared by reduction of metal salt (0.2 mM of $HAuCl_4$ solution) with $NaBH_4$ in the presence of acetal-PEG-SH at acetal-PEG-SH:$HAuCl_4$ molar ratio of 1/6:1. The UV−visible spectrum of the pinkish-red solution produced after chemical reduction with $NaBH_4$ indicated formation of gold nanoparticles with an absorption band near

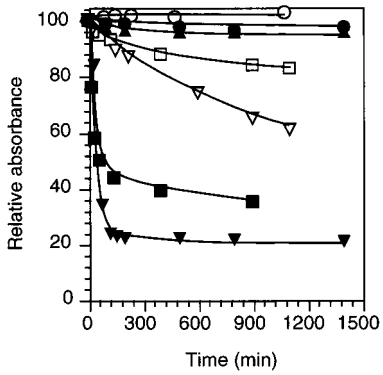


Figure 1. Dispersion stability of gold nanoparticles with time in various environments. Acetal-PEG-SH-protected gold nanoparticles in 0.15 M PBS (●), 0.3 M PBS (▲), and 0.15 M PBS with 2% serum (○); acetal-PEG-OH-adsorbed gold nanoparticles in 0.03 M PBS (■), 0.15 M PBS (▼), 0.03 M PBS with 2% serum (□), and 0.15 M PBS with 2% serum (▽).

$\lambda = 520$ nm, assigned to a gold nanoparticle plasmon band. The gold nanoparticles thus prepared were modestly polydisperse (typically <22% standard deviation) exhibiting an average diameter of 8.9 nm as measured by TEM (see Supporting Information a)).

Acetal-PEG-SH-coated gold nanoparticles centrifuged at 360.6K × *g* (corresponding to 70K rpm) for 3 h at 4 °C can be readily resuspended in deionized water, various buffers, serum-containing medium, and organic solvents. This cycle of spinning-down and resuspension can be repeated several times without any observed loss of colloidal stability. They were stable even in buffers of elevated salt concentrations (0.15−0.3 M phosphate buffered solutions; PBS, pH 7.4), an environment which immediately induces aggregation of unmodified gold particles.[20] As shown in Figure 1, the decay of the optical absorbance of the particle suspension at $\lambda = 520$ nm was negligible for acetal-PEG-SH-coated gold nanoparticles (●, ▲) even in 0.3 M PBS, while gold nanoparticles physically stabilized through the adsorption of 2 mM acetal-PEG-OH (Mn = 3000) (■, ▼) aggregated immediately even in 0.03M PBS. Notably, acetal-PEG-SH-coated gold nanoparticles are stable even in 0.15 M PBS including 2% serum (○) in a sharp contrast with a gradual aggregation behavior of gold nanoparticles physically stabilized by acetal-PEG-OH (□, ▽). Highly enhanced stabilization of gold nanoparticles bearing acetal-PEG-SH compared to adsorbed acetal-PEG-OH appears to be a consequence of the formation of the PEG brush layer on gold tethered by the gold−thiol linkage. The well-established nonfouling properties of PEG chemistry, highly effective in reducing nonspecific and uncontrolled interactions with biological components including cells and proteins,[21,23] imparts the observed high stability as well as selectivity of PEGylated gold particles in the presence of serum, allowing this system to be developed for diagnostics in blood serum-containing samples.

Further, the conversion of the acetal group into an aldehyde group was conducted smoothly by immersing the PEGylated gold nanoparticles into aqueous media adjusted to pH 2 using hydrochloric acid for 5 h.[21,23] The presence as well as the

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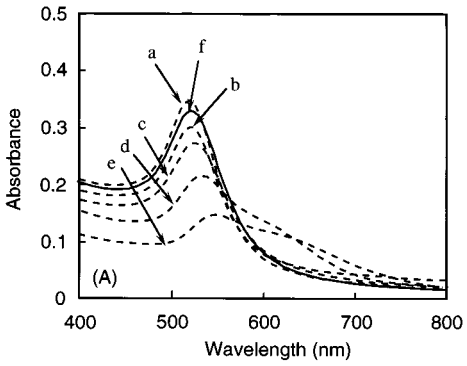

Figure 2. Optical spectra of the Au−Lac(0.5) nanoparticle samples recorded 8 h after the addition of different concentrations of RCA_{120}: (a) 0 μg/mL; (b) 5 μg/mL; (c) 10 μg/mL; (d) 20 μg/mL; (e) 50 μg/mL; and (f) (solid line) the optical spectrum of redispersed aggregates, corresponding to condition (e), after the addition of excess D-galactose.

reactivity of the aldehyde group at the PEG-chain end was experimentally confirmed through a model reaction of an aldehyde group with an amino-functionalized ESR probe, 4-amino-2,2,6,6-tetramethylpiperidinyloxy (4-amino-TEMPO).[21,23] Lactose (Lac) and mannose (Man) groups were successfully reacted in this study with the distal PEG-chain ends on the gold particles through reductive amination reaction of PEG terminal aldehyde and corresponding sugar derivatives having *p*-aminophenyl moieties at the C-1 position (*p*-aminophenyl-β-D-lactopyranoside or mannopyranoside) as shown in Scheme 2. Terminal Lac content could be regulated by varying the molar ratio of *p*-aminophenyl-Lac with *p*-aminophenyl-Man in reaction mixtures [sample 1, *p*-aminophenyl-Lac:*p*-aminophenyl-Man = 5:5 (abbreviated as Au−Lac(0.5)), and sample 2, *p*-aminophenyl-Lac:*p*-aminophenyl-Man = 2:8 (abbreviated as Au−Lac(0.2))], respectively. Since the reactivity of aminophenyl group with aldehyde is likely to be equal between *p*-aminophenyl-Lac and *p*-aminophenyl-Man, the ratio of the two sugar groups on the gold surface is assumed to be equal to the molar ratio of the reaction mixture.

Reaction of these sugar-derivatized gold nanoparticles with bivalent galactose-binding lectin (*R. communis* agglutinin, RCA_{120})[24,25] was followed as a function of time through optical changes in the surface plasmon band in the UV−vis spectrum. Figure 2 shows representative UV−vis spectra recorded 8 h after addition of RCA_{120} lectin with varying concentrations to colloidal suspensions of Au−Lac(0.5). These solutions were initially a pinkish-red color due to the well-dispersed nature of the particles (Scheme 1). After introduction of the RCA_{120} lectin in 0.15 M PBS, the color gradually changes from red to purple (Scheme 1). In line with this directly observable change in appearance, significant differences in the optical spectra over time were observed, specifically a broadening and red-shift in the particle surface plasmon resonance from 523 nm to longer wavelength.[26] This is attributed to distance-dependent changes in the optical properties of three-dimensionally aggregated gold nanoparticles[4−8] cross-linked by RCA_{120} lectin which recognizes lactose residues on the PEGylated gold surface. TEM images of the lectin-particle system depicted an extended aggregation of many gold nanoparticles (Figure 3b). Further, to directly

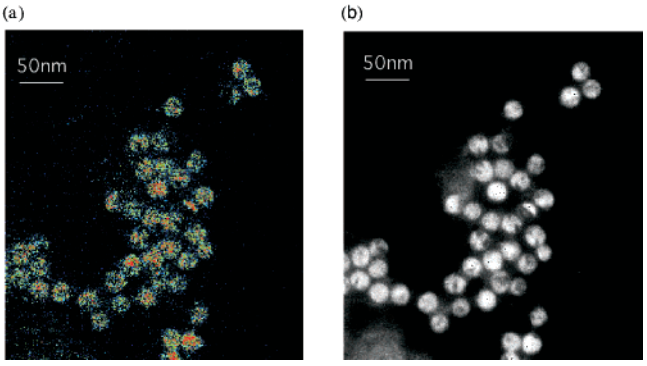

Figure 3. (a) Energy-filtered TEM image to determine the sulfur distribution on the Lac-PEG-SH-immobilized aggregated gold nanoparticles and (b) the electron energy loss image of the same region.

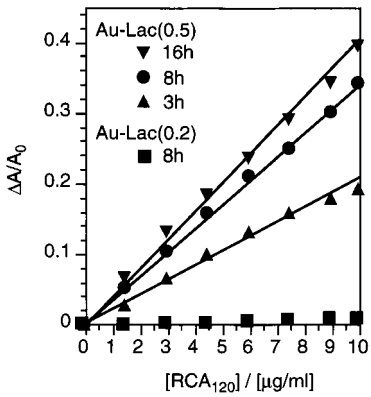

Figure 4. Change in the surface plasmon band intensity (ΔA (=$A_0 - A$)/A_0) with RCA_{120} concentration ([RCA_{120}]) for nanoparticle Au−Lac(0.5) (▲, ●, ▼) and Au−Lac(0.2) (■) at different times after onset of the aggregation reaction. A_0 is the surface plasmon band intensity in the absence of RCA_{120}.

image the thiolate molecules distributed on the gold nanoparticles, the element spectroscopic imaging technique (ESI)[27] (or energy-filtered TEM; EFTEM) was applied as shown in Figure 3a. The image contrast of ESI is directly proportional to the thickness-projected density of the sulfur atom. The sulfur distribution in ESI image (Figure 3a) nicely corresponds to the high contrast image of gold nanoparticles (Figure 3b) obtained with the electron energy loss at 250 eV, indicating that the thiolate end groups (S-atoms) of Lac-PEG are certainly anchored on the gold surfaces.

Changes in the surface plasmon band observed in Figure 2 (breadth and λ_{max}) became more significant with increasing concentrations of added lectin: higher concentrations of added RCA_{120} induce larger changes in the spectra. Additionally, no precipitation or aggregation of PEGylated gold nanoparticles was observed in the presence of free D-galactose (1 mg/mL), demonstrating that aggregation observed upon addition of RCA_{120} to Lac-functionalized PEGylated gold particles was indeed the result of specific interactions between the immobilized lactose moieties on gold surface and the RCA_{120} lectin.

Notably, relative changes in the optical absorbance of the Au−Lac(0.5) system at $\lambda = 523$ nm with increasing amount of added RCA_{120} produced the linear relationship at different times after onset of the binding experiment, as shown in Figure 4. Thus, the degree of colloidal association can be controlled by adjusting the concentration of RCA_{120} lectin in the medium.

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Importantly, lectin concentration can be quantitated in unknown samples from this calibration curve. Assay sensitivity is appreciably high enough to detect lectin concentration to ≅l μg/mL (1 ppm), comparable to that of immunological assay methods such as ELISA. Hence, with these properties, this type of colorimetric assay using functionalized PEGylated gold nanoparticles in physiological buffer salt concentrations exhibits practical utility for further exploitation due to a simple and handy assay system in such fields as medical diagnostics.

Influence of the degree of gold nanoparticle lactose derivatization on lectin detection was further studied by reducing the amount of PEG-immobilized Lac moiety (sample Au−Lac(0.2)). As shown in Figure 4 (closed square plots), no decrease in absorbance was induced for the Au−Lac(0.2) system with lectin concentrations up to 50 μg/mL. This indicates the requirement of a critical cross-linking density in the RCA_{120}/Au−Lac system to induce optically observable particle aggregation through specific multiple lactose−lectin interactions.

The reversibility of binding between RCA_{120} lectin and lactose-immobilized gold nanoparticles was confirmed by addition of 1 mg/mL D-galactose to a suspension of fully aggregated Au−Lac(0.5) nanoparticles with RCA_{120} (Au−Lac(0.5):0.l mg/mL, RCA_{120}:50 μg/mL). Complete recovery of the optical spectrum relative to the particle surface plasmon band prior to RCA_{120} addition is seen in Figure 2 (solid line f), indicating dissociation of gold nanoparticles cross-linked with RCA_{120} lectin. The TEM image of the galactose-treated sample further supported the complete redispersive property of RCA_{120}-cross-linked Au−Lac(0.5) nanoparticles by excess galactose (see Supporting Information c). This reversibility may be driven by the steric stabilization effect of PEG strands based on the entropic elasticity. Dissociated Au−Lac(0.5) nanoparticles were then separated from solution by centrifugation and redispersed in fresh buffer (0.15 M PBS) to demonstrate repeated aggregation through the re-addition of RCA_{120} lectin. This reversible process was confirmed to be repeatable through several cycles.

In conclusion, new stable and functionalized PEG−gold nanoparticles were prepared by in situ aqueous reduction of $HAuCl_4$ in the presence of newly developed α-acetal-ω-mercapto-PEG. The PEGylated gold nanoparticles thus prepared exhibited appreciable stability even in concentrated buffer salt solutions as well as in those including serum. Lactose immobilization to PEG distal ends on gold particles facilitates RCA_{120} lectin-induced aggregation in both quantitative and reversible manners with a concomitant change in optical color (red → purple → red). The degree of particle aggregation is proportional to added lectin concentration, allowing the system to be utilized for quantitative assay. A simple yet highly effective derivatization of gold nanoparticles with heterobifunctional PEG is expected to increase the utility of colloidal gold systems in diverse technologies including bioassays and biorecognition.

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Supporting Information Available: TEM images representing the reversible aggregation−dispersion behavior of Lac-PEGylated gold nanoparticles by sequential addition of RCA_{120} lectin and galactose (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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