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Single Nanoparticle Tracking-Based Detection of Membrane Receptor−Ligand Interactions

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We developed a single nanoparticle tracking-based detection method for membrane-associated molecules using a paucivalent gold nanoparticle (AuNP)-modified supported lipid bilayer (SLB) platform. Here, the binding activity of membrane-associated molecules (cholera toxin binding to ganglioside GM_1 in this case) was determined by calculating the diffusion coefficients of membrane-tethered AuNPs. This nonbleaching nanoparticle-based method provides >100-fold improvement in sensitivity for the same target without optimization over the fluorophore-based method that also has photobleaching and photoblinking problems. This new detection platform and analysis method could be used for membrane-associated molecule biosensor and screening assay development.

A myriad of important biological events such as cell signaling are related with cell membrane, and over 50% of drug targets are involved with membrane proteins (e.g., G-protein coupled receptors).[1,2] It would be beneficial to develop a highly sensitive and selective biosensing method that detects membrane protein−ligand interactions for cell membrane study or screening membrane-associated protein-targeted drugs.[3] The supported lipid bilayer (SLB) system is a cell membrane-mimicking platform and has been of great interest. This ∼5 nm thick phospholipid bilayer membrane consists of two layers of amphiphilic lipid molecules and forms on a very thin water layer at a solid surface (e.g., glass).[4] In labeling and imaging lipids in SLB, conventional fluorescence-based methods, e.g., the fluorescence recovery after photobleaching (FRAP) method,[4,5] fluorescence cross-correlation spectroscopy (FCS),[4] and tethered fluorescent vesicle tracking,[5,6] have been widely used, but these fluorescent labels have problems such as photobleaching, photoblinking, limited multiplexing, low sensitivity, and narrow dynamic range. The single-particle tracking method is often used in live-cell imaging studies of cellular membrane dynamics.[7,8] Here, we developed a single nanoparticle tracking (SNT)-based detection method for membrane receptor-binding molecules. This method uses an SLB-tethered gold nanoparticles (AuNPs) as detection labels and a dark-field microscopy as a nanoparticle-tracking tool (Figure 1). In our scheme, ligand binding to membrane protein induces decrease in the lipid mobility around the gel−fluid transition temperature of the lipid bilayer. When multivalent ligands bind to membrane receptor molecules in fluid lipid membrane, ligand-bound receptors cluster to form rigid lipid domains (gel phase) on the SLB (fluid phase). These gel domains could act as obstacles that hinder free movement of neighboring lipids and result in decrease in whole lipid fluidity.[9] This fluidity change is monitored by tracking lipid-tethered AuNPs using dark-field microscopy herein. AuNP-based dark-field microscopy measures resonantly scattered light from metal NPs due to the intense localized surface plasmon resonance (LSPR) of metal NPs.[10] These metal NP labels, unlike chemical fluorophores, do not blink or photobleach and are suitable for real and long-term observation.[11] Moreover, the spatial resolution of SNT is approximately 2 orders of magnitude higher than fluorephore-based FRAP. Whereas FRAP averages over hundreds to thousands of diffusing molecules, SNT measures individual particles' trajectories.[12] Further, AuNP labels can be readily modified to thiolated or amine-modified lipids via straightforward conjugation chemistry. Finally, we prepared and compared multivalent and paucivalent AuNP probes as detection probes (Figure 3A). In the case of paucivalent AuNP probes, AuNPs were heavily covered by bovine serum albumins (BSAs) (detailed characterization of these probes is shown later in this article). This BSA-modified particle has little open surface for thiolated lipid binding, and the chance of multiple lipid-anchoring to a AuNP is very low. This results in more freely moving, paucivalent AuNP probes with high mobility, whereas multiple lipid-anchored, multivalent AuNP probes have more restriction in movement and lower mobility. Our detection assay results indicate that paucivalent probes indeed have better mobility on SLB and generate better particle mobility-based detection assay results with higher sensitivity than multivalent probes.

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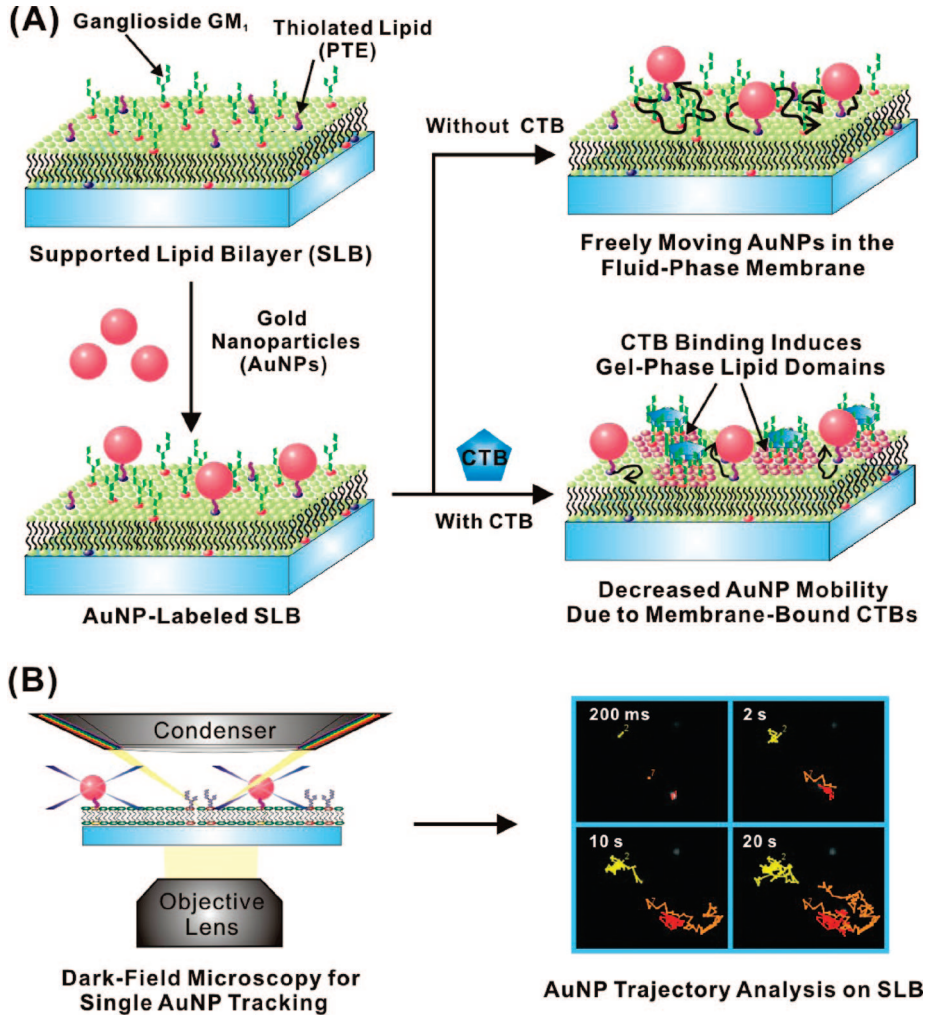

Figure 1. Single nanoparticle tracking-based detection on supported lipid bilayer platform (A) and time-lapse dark-field images of lipid-tethered AuNPs on supported lipid bilayer (B). The movements of AuNPs are changed by molecular binding events on the supported lipid bilayer.

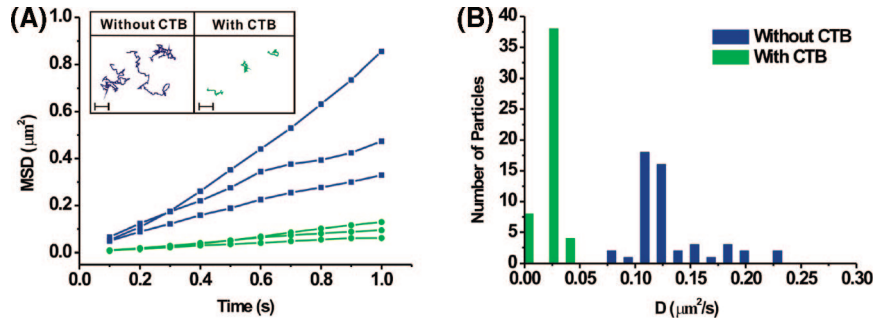

Figure 2. (A) The trajectories of lipid-tethered AuNPs (inset, the scale bars are 5 μm) and mean square displacement plots as a function of time. The diffusion coefficient (D) values are 0.12, 0.21, and 0.08 $\mu m^2/s$, respectively (from left to right in the inset), without CTB treatment. When 100 nM CTB was treated at room temperature, the D values are 0.03, 0.023, and 0.015 $\mu m^2/s$ (from left to right in the inset). (B) Histogram plot of diffusion coefficients for 50 AuNP labels. The average diffusion coefficients are 0.13 $\mu m^2/s$ without CTB and 0.02 $\mu m^2/s$ with CTB, respectively.

EXPERIMENTAL SECTION

Paucivalent Gold Nanoparticle Probes. The 50-nm gold particles (Ted Pella, Inc., Redding, CA) were centrifuged at 5000 rpm, 4 °C for 15 min. The resulting pellet was dispersed in the coupling buffer (glycine/KOH 2 mM, pH 8.5). The coupling buffer was chosen with consideration of the pI of BSA (pI = 5.6, purchased from Sigma-Aldrich, Milwaukee, WI). AuNPs were added to the tubes containing BSA with serial increase in BSA concentration and kept at least 1 h at 4 °C on an incubating shaker. The amount of needed BSA was determined by adding NaCl (final concentration of 1%) to each tube. Subsequent visual inspection, absorption measurement over the range of 320−800 nm at room temperature, and electrophoretic light scattering spectrophotometer (ELS) measurement were done subsequently. Color change of the AuNP solution from red to blue occurs when the particle aggregation occurs. To obtain paucivalent gold nanoprobes, the

lowest required amount of BSA to avoid nanoparticle aggregation was added (1.2 nM BSA/9 × 10^{10} AuNPs). The lowest protein concentration necessary to avoid the particle aggregation can be considered as the particle-stabilizing concentration.[13] Non-coupled BSA was titrated using Bradford assay, and assay result indicates that ~146 BSA molecules were fixed per 50 nm gold particle. The calculated number of BSA molecules to form a single shell around a AuNP is ~200 BSA molecules for a 50 nm AuNP. Finally, the coupling buffer containing the particles was replaced by centrifugation (5000 rpm, 4 °C, 15 min) by deionized water.

Lipid Vesicle Preparation. Small unilamellar vesicles (SUV, 100 nm diameter) of 95.25 mol % DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), 0.25 mol % GM_1 ganglioside, and 4 mol % PTE (1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol) were prepared by a vesicle extrusion method. In short, lipids (Avanti, Alabaster, AL) were dissolved and mixed in chloroform, and the solvent was removed by ~1 h of evaporation using a rotary evaporator. The dried thin lipid film on the side of a round-bottom flask was then dispersed in deionized water (1 mL) and incubated overnight at 4 °C. The resulted lipid concentration was 2 mg/mL. This lipid suspension was then passed through 100 nm polycarbonate membrane filters (Avanti, Alabaster, AL) 11 times using a miniextruder (Avanti, Alabaster, AL). The resulting SUV solution was stored at 4 °C prior to use.

AuNP-Modified Supported Lipid Bilayer Formation. An SLB was formed on a piranha-etched glass coverslip by a vesicle fusion and rupture method. Briefly, microscopic coverslips (Fisher Scientific, Pittsburgh, PA) have been cleaned with piranha solution (3:1 = concentrated sulfuric acid/30% hydrogen peroxide) for 10 min, thoroughly rinsed with deionized water, and then dried with a stream of nitrogen. The SUV suspension was mixed 3:1 (v/v) with phosphate-buffered saline (PBS, pH 7.4; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl), and 100 μL of the resulting solution was placed onto a plastic Petri dish, after which the coverslip was placed on the droplet for 30 min at room temperature. The Petri dish was submerged in a 15 mM NaCl solution to remove excess vesicles. The SLB was then assembled in the well-slide (slide glass chamber, Live Cell Instrument, South Korea) as a sandwich configuration with another coverslip. Finally, the gold colloids were added to the newly formed SLB. After overnight incubation, the unbound AuNPs were rinsed off with deionized water.

Equipment and Settings for Imaging. A Carl Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Germany) with a dark-field condenser (NA = 1.4, oil-immersion) was used for imaging lipid-tethered AuNPs. The scattering images of AuNPs were taken using a 100× objective lens (NA = 0.8) and with a white light illumination from a 100 W halogen lamp. The experimental temperature was maintained at 24 °C. The temperature was slowly adjusted by controlling heating plate (slide glass chamber, Live Cell Instrument, South Korea), and the SLB chamber was allowed to equilibrate for another 10 min once the intended temperature was reached before single-particle tracking measurements were performed. For the cholera toxin B subunit (CTB) binding experiments, the appropriate amount of the CTB in 20 mM PBS for paucivalent AuNPs-labeled SLB or in deionized water for multivalent AuNPs-labeled SLB was injected into the chamber and incubated for 30 min before the sample was placed on the heating plate a microscope stage. The microscopy system was completely covered by a dark shield, which prevents ambient light interference.

Analysis of Lipid-Tethered Gold Nanoparticle Trajectories. Time-lapse dark-field images were taken every 200 ms for 100 frames, and this enabled 100−200 individual mobile particles to be collected, at least, from 10 different parts of the SLB. The two-dimensional (2D) lateral diffusive motion of individual particles could be analyzed by the single-particle tracking and analysis method. The trajectory of gold particles in each image was tracked by using the ImageJ program and particle tracker plug-in software. This plug-in implements a computationally efficient, 2D feature point tracking algorithm for the automated detection and quantitative analysis of particle trajectories as recorded by video imaging.[14]

For each particle, the tracking program can compute the mean square displacement (MSD) for every time interval by using the formula, $\mathrm{MSD}(t) = \langle (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \rangle$. The MSD was calculated from where a particle at one position (x_i, y_i) moved to other position (x_{i+n}, y_{i+n}) after a time interval, Δt, given by $n \times$ video frame time. This calculation covers the particle position ranged from 1 to $N - n$, where N is the total number of particle positions recorded and n takes on values 1, 2, 3,..., $N - 1$. Least-squares fits of the MSD versus time interval plots were calculated up to the 10th time interval. The diffusion coefficient D was calculated from the slope of the first four points (200−800 ms) in each MSD versus time interval plot according to $\mathrm{MSD} = \langle r^2 \rangle = 4D\Delta t$. Here D is the lateral diffusion coefficient. Particles that showed a nonlinear relationship ($R^2 < 0.97$) between MSD and time were rejected from further analysis. Here, the trajectories of nanoparticles with simple diffusion mode were used for the calculation of the MSDs and diffusion coefficients for the quantification of membrane receptor-binding molecules. Within the 2 s time frame that we used, the MSD−Δt plots of these particles (Figure 2A) showed a linear relationship, indicative of simple diffusion mode.

Stationary nanoparticles were not included for the calculation of the MSDs and diffusion coefficients. Our results indicate that stationary nanoparticles do not have a linear relationship between MSD and Δt. Importantly, when multivalent CTB molecule is bound to five units of GM_1 moieties, the AuNP-labeled lipids could be trapped within a gel-like domain that gives rise to stationary nanoparticles, and these particles should not be included for calculation. For all these reasons, the stationary nanoparticles were excluded in our calculation.

RESULTS AND DISCUSSION

We chose cholera toxin as a detection target for proof-of-concept experiments. Cholera toxin is a virulent factor secreted by *Vibrio cholerae* and has a hexameric protein structure containing two different types of subunits in an AB_5 configuration. The cholera toxin B subunit specifically binds to the ganglioside GM_1 pentasaccharide head-groups. These multivalent ligand−

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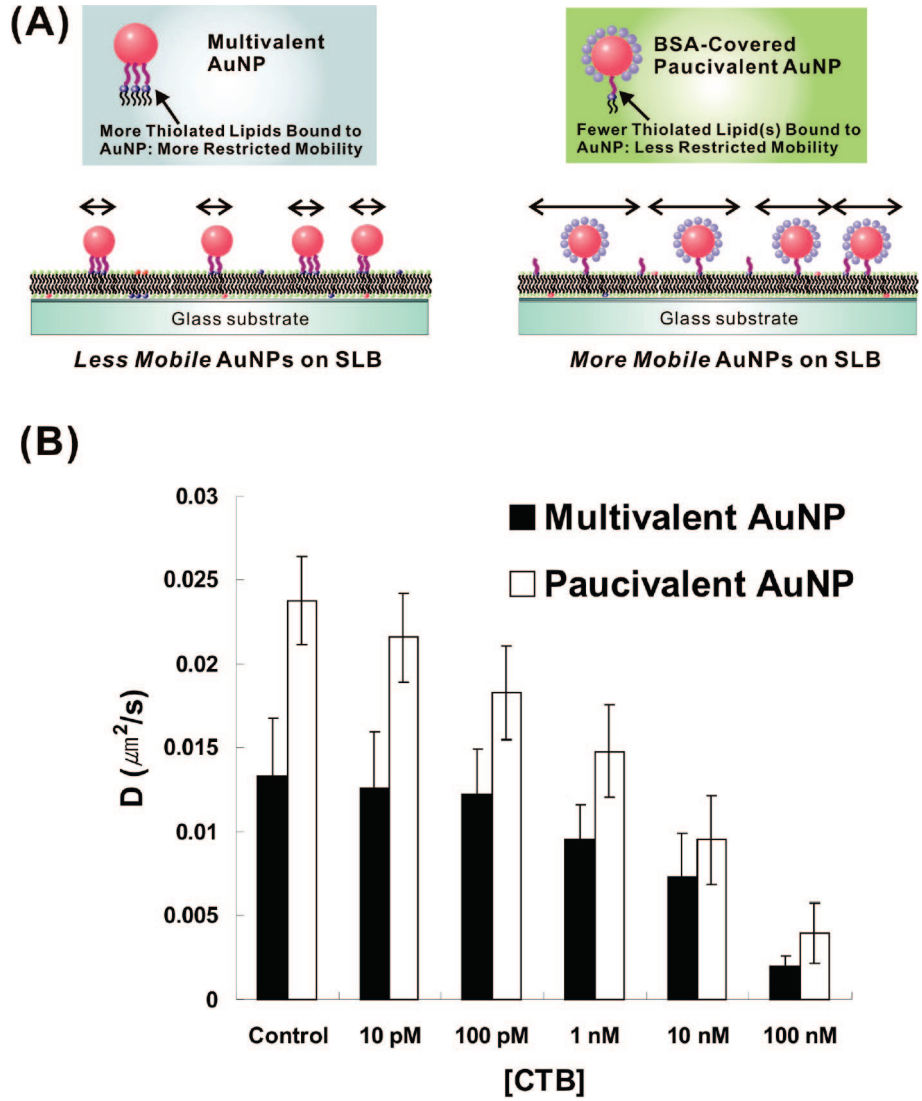

Figure 3. Single nanoparticle tracking-based CTB detection results on SLB for various concentrations of CTB using multivalent and paucivalent AuNP labels. (A) Multivalent AuNP probes vs paucivalent AuNP probes. (B) Measured average diffusion coefficients of tethered AuNPs on SLB as a function of CTB concentration from 10 pM to 100 nM.

carbohydrate interactions would easily change the physical condition of membrane,[15] and thus understanding membrane physical chemistry in this model system may provide insight into membrane receptor biosensing and strategies for inhibitory drug screening.[16] In a typical experiment, thiolated SLB, composed of 95.25% DMPC, 4% PTE, and 0.25% GM_1 ganglioside, was first prepared on a transparent glass slide. Next, 50 nm AuNPs were added to the SLB and incubated overnight. This AuNP-conjugated SLB was then washed with deionized water. To confirm the modification of AuNPs to SLB, the UV−vis spectrum at 520 nm was measured (the modification of AuNPs to SLB was monitored at 520 nm). The results indicate that AuNPs were successfully conjugated on PTE−SLB and PTE is necessary for this conjugation (Figure S1 in the Supporting Information). As Lee et al. observed a random Brownian motion of membrane-bound NPs,[17] the PTE-modified AuNPs (PTE−AuNPs) were freely moving on SLB. Although nonconjugated AuNPs were not completely washed away, the motion of lipid-conjugated AuNPs on SLB is easily distinguished because nonmodified AuNPs have three-dimensional movements with untraceable, rapid velocities. The trajectories of individual membrane-bound AuNPs were obtained by analyzing consecutive images recorded at a fixed time interval using the ImageJ program (http://rsb.info.nih.gov/ij/, Figures 1B and 2A), and the MSDs were calculated subsequently. Finally, diffusion coefficient (D) for tethered AuNP was calculated using these data. Even 1 week after the initial preparation, lipid-tethered AuNPs were stable and sustained their fluidity.

In a CTB detection experiment, CTB solution was added to the SLB, the resulting solution was incubated at room temperature for 30 min, and the trajectories of PTE−AuNPs were imaged and analyzed. The particles travel slower, and their travel trajectories get distinctly shorter, after the addition of CTB (see the inset in Figure 2A and MSD values). Figure 2B shows the histogram plots of 50 independent particles' diffusion coefficients. Analysis results indicate that smaller D values were obtained after CTB treatment to SLB. The average D value in the absence of CTB is 0.13 $\mu m^2/s$, and this

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D value is decreased to 0.02 $\mu m^2/s$ after CTB addition to SLB. The apparent decrease of D upon CTB binding to SLB suggests that SLB-bound CTB molecules indeed affect the lipid mobility and AuNP movement.

To increase the mobility of lipid-tethered AuNPs and improve assay sensitivity, we prepared paucivalent AuNPs to label lipids on SLB, and these paucivalent AuNPs were compared with multivalent AuNP labels (Figure 3A). In most cases, when colloidal gold particles are used as probes, they are conjugated with antibodies or DNA on the whole area of the particles.[18] For more effective diffusion of individual AuNPs on SLB, the number of AuNP-bound lipids should be minimized. If many lipids were bound to an AuNP, extra lipids would move in random directions and restrict a diffusion motion of a PTE−AuNP. For this reason, AuNP labels need to be designed and prepared with as a few extra binding sites as possible to obtain more reliable information on the dynamics of individual particles on SLB with larger diffusion coefficient values.[17] Moreover, in the case of multivalent AuNPs with more open surface area, there would be higher chance for nonspecific gold−gold interaction-based particle aggregation. To obtain such paucivalent AuNP labels, we coated AuNPs with BSA for minimal exposure of thiolated lipid binding AuNP surface prior to the addition of these particles to the SLB. BSA binding to the gold surface occurs via electrostatic interaction. The electrostatic interactions between citrate and BSA molecules consist of salt-bridges between the citrate and the lysine on the protein surface.[13] To synthesize paucivalent AuNP probes, AuNPs were incubated with various concentrations of BSA and then noncoupled BSA was quantified to calculate the number of bound BSA molecules per AuNP. Bradford titration showed the number of BSA molecules associated with a AuNP was gradually increased as the concentration of BSA in the coupling buffer was increased (Figure S2 in the Supporting Information). The fact that BSA can readily dimerize and even form multimers is consistent with these data. The minimal BSA concentration, approximately corresponding to the coating with one protein monolayer, can be readily determined by the NaCl resistance test to confirm the lowest protein concentration necessary to avoid the aggregation. Electrophoretic light scattering spectrophotometer results further confirm the formation of BSA-monolayered AuNP probes (~7 nm increase for paucivalent, BSA-modified AuNPs in particle diameter compared to multivalent, nonmodified AuNPs; Figure S2 in the Supporting Information). Under the minimal concentration of BSA (1.2 nM BSA/9 $\times$ 10^{10} AuNPs) for making paucivalent AuNPs, we found out that ~146 BSA molecules were modified to a single AuNP. Assuming BSA as a 5-nm spherical protein, it was estimated that the number of BSA molecules required to cover the whole AuNP surface is ~200 BSA molecules for 50-nm AuNP. This AuNP probe had less spaces for conjugation with thiolated lipid on SLB, and the number of AuNP-bound lipids will be lower than multivalent AuNPs with no BSA passivation (Figure 3). In Figure 3, the control experimental results in which CTB was not added show that the paucivalent particles have higher D values than the multivalent particles because paucivalent, BSA-modified particles have fewer gold−gold interactions and less restriction in AuNP movement. Next, we examined the relationship between target concentration and diffusion coefficient. Our results show that D values are dependent on the concentration of CTB (Figure 3B). The more CTBs were added and bound with GM_1 moieties, the more the fluidity decreased. This trend becomes more distinct with a wider dynamic range when paucivalent AuNPs were used as detection probes (Figure 3B). Target was detected from 10 pM to 100 nM, and the data suggest that current detection limit for CTB is 10−100 pM with paucivalent AuNP probes. When compared with fluorescence signal-based detection methods that have ~5 nM detection limit,[4] the detection limit for the same target with this SNT-based method has been improved >100-fold.

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CONCLUSION

In summary, we developed a single AuNP tracking-based method on an SLB platform that allows for detecting and quantifying molecular binding to membrane receptor-binding targets of interest. When ligand molecules (CTB in this case) are bound to ganglioside GM_1 on SLB, the gel-phase lipid domains are formed, and AuNP mobility was subsequently affected by bulky, multivalent CTB binding to ganglioside GM_1 on SLB. The magnitude of decreased mobility was quantified by calculating and comparing MSD and diffusion coefficient values of lipid-tethered AuNPs before and after CTB addition to ganglioside GM_1-modified SLB. Unlike conventional fluorophore-based methods that have photobleaching and photoblinking problems, this dark-field microscopy-based SNT-based sensor offers real and long-time monitoring capability of AuNP-modified lipids. Importantly, when paucivalent AuNPs that have much less thiolated lipid-binding area are used as detection labels, higher diffusion coefficient values, better sensitivity, and wider dynamic range were obtained than when multivalent AuNPs were used as detection labels. The detection limit using this paucivalent NPs for CTB targets was almost 10 pM without optimization. This is >100-fold sensitivity improvement over the fluorophore-based method for the same target. Moreover, the dynamic range for this method is from 10 pM to 100 nM. By optimizing modified nanoparticle concentration, lipid composition, binding kinetics, observation time, the number of observed particles, particle modification chemistry, buffer condition, etc., the assay sensitivity and reliability could be easily improved. Finally, this SNT-based method paved the way of using AuNP labels for the detection of ligand binding to membrane-associated molecules. This approach should be useful in studying any membrane-associated molecules and practical applications including membrane receptor-based drug screening assays that require high sensitivity, wide dynamic range, and long/real-time monitoring and study in the cell−SLB interface.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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