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Nanoparticles Comprising a Mixed Monolayer for Specific Bindings with Biomolecules†

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Abstract: This work presents a strategy of using mixed monolayer protected nanoparticles for specific interactions with target biological molecules. The mixed monolayer is composed of a shielding component and a capture component. The shielding component utilizes ethylene glycol oligomers to prevent nonspecific binding with biomolecules. The capture component is chosen to specifically interact with the target of interest, such as a protein molecule. Such a concept was demonstrated by two synthetic systems. The first one is gold nanoparticles protected by a mixed monolayer of tri(ethylene glycol) thiol (EG_3-SH) and tiopronin (Tp), which was prepared by a one-step synthesis. Surface chemical composition studies using ^1H NMR spectroscopy revealed that the reactivity of EG_3-SH is 3 times as high as that of Tp in the nanoparticle formation. Gel electrophoresis analysis identified a critical ratio of (EG_3-S-)/Tp on the nanoparticle surface above which no nonspecific binding occurred. By further derivatizing Tp into a biotin group, we synthesized $Au(-S-EG_3)_n$/Tp-biotin particles that bind specifically to streptavidin with negligible nonspecific binding. The second system is gold nanoparticles protected by a mixed monolayer of EG_3-SH and glutathione (GSH). By controlling the feeding ratio of EG_3-SH and GSH, we made $Au(-S-EG_3)_n$/GSH particles that bind specifically to gultathione-*S*-transferase (GST) with negligible nonspecific binding.

Introduction

Nanoparticles are nanometer-size materials with unique physical and chemical properties and have been widely used for many years.[1] Macrobiological molecules, also in the nanometer-size range, possess functionalities that enable recognition and self-assembly. The combination of nanoparticles and biological molecules is very attractive and has gained tremendous attention from academics and industry, because such a combination could create new materials for electronics and optics and lead to new applications in genomics, proteomics, and biomedical and bioanalytical areas.[2−7]

A variety of studies have been done on DNA-functionalized nanoparticles. These include nanoparticle assemblies through either specific hybridization of a single strand DNA attached on the nanoparticle[8,9] or electrostatic interaction of positively charged nanoparticles with DNA molecules.[10,11] DNA-functionalized nanoparticles have been used successfully for DNA detection and as biosensors.[12−15] For DNA-related applications, nonspecific binding between DNA and nanoparticles is not a serious problem, because DNA is very hydrophilic and negatively charged. Unless the nanoparticles are positively charged, there is not much nonspecific binding between DNA and nanoparticles.

However, for proteins, the nonspecific binding could be a serious issue. Proteins are much more complicated than DNA. They can be either hydrophobic or hydrophilic, with either positive or negative charge, making it extremely challenging to avoid nonspecific binding with nanoparticles. Even though a number of articles have been published dealing with the interactions between nanoparticles and proteins and their potential applications in areas such as bioseparation,[16] biosensors,[17] immunoassays,[18,19] and enzyme inhibition assays,[20,21] the issue of protein nonspecific binding with nanoparticles has

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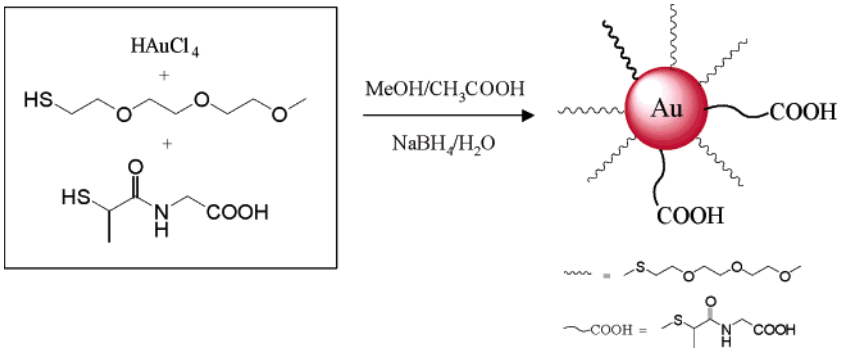
Scheme 1. One-Pot Synthesis of a Mixed Monolayer of Tri(ethylene Glycol) and Tiopronin Protected Gold Nanoparticle.



generally not been addressed.[22] To fully utilize the potential of protein−nanoparticle hybrids, specific interaction between proteins and nanoparticles are highly desirable. To the best of our knowledge, practical solutions for this nonspecific binding problem have not been developed.

In our previous studies,[23,24] we synthesized ethylene glycol monolayer protected gold nanoparticles, which are very stable in aqueous media and have complete resistance to nonspecific bindings with proteins, DNA, and RNA. However, these biologically inert nanoparticles do not provide functionality. To allow specific interactions or bindings with biological entities, a specific binding functionality has to be introduced onto the nanoparticles. For this purpose, we designed nanoparticles protected with a mixed monolayer of an ethylene glycol molecule and a ligand. The ethylene glycol short chains with well-defined lengths function as the shielding component to minimize the nonspecific interaction between nanoparticles and biological molecules, whereas the ligand acts as a capture agent to engage biological molecules specifically.

A number of issues need to be addressed in the mixed monolayer approach. Synthesis is the first one. Murray et al. pioneered ligand exchange reaction,[25] a general route for preparation of mixed monolayer protected nanoparticles. Since then, ligand exchange or S_N2 reaction has been widely used for preparing nanoparticles for applications in electrochemistry,[26,27] conductivity,[28,29] fluorescence,[30,31] biological bindings,[32,33] and coatings.[34] From the synthetic point of view, a direct synthesis has advantages over the replacement reaction for preparing a mixed monolayer protected nanoparticle, because it is simpler and better controlled. In this article, we report a one-step direct synthesis of water-soluble, mixed monolayer protected nanoparticles. The second issue is whether the number of ligands on a nanoparticle could be controlled. In a mixed monolayer protected nanoparticle system, a ligand molecule enables specific binding to a target. However, the ligand molecule itself might contribute to the nonspecific binding. Thus, a quantitative control of the ligand number or percentage is crucial to achieve the specific interaction. In the direct synthesis approach, it is easy to control the feeding ratio of the molecules. However, the essential information is the composition of the mixed monolayer on the nanoparticle surface, which is directly responsible for the surface properties and interactions with the target molecules. We therefore studied the relative reactivities of the shielding component and the capture component and used the results from the feeding ratio to determine the surface composition on a mixed monolayer protected nanoparticle. Thus, the ligand percentage on the nanoparticle could be controlled. The third issue is to determine what surface composition would eliminate the nonspecific interactions and yet provide maximum specific binding to the target molecules. Utilizing gel electrophoresis, we have developed a simple method to determine the critical ratio of the capture component to the shielding component that makes the nanoparticle inert to nonspecific binding with biological molecules while capable of maximum specific binding to biological molecules.

Experimental Section

Materials and Methods. Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI) and used without further purification. Tri(ethylene glycol) thiol molecule (EG_3−SH) and tri(ethylene glycol) monolayer protected gold nanoparticles (Au−S−EG_3) were synthesized in our lab by following the procedures described in the previous publications.[23,24] Tiopronin-protected gold nanoparticles were synthesized by following the published procedures.[35]

Synthesis of Tri(ethylene Glycol) and Tiopronin Mixed Monolayer Protected Gold Nanoparticles [Au(−S−EG_3)$_n$/Tp]. The reaction is shown in Scheme 1. In a typical reaction with molar feeding ratio of EG_3−SH and Tp at 1:1, 45 mL of MeOH (HPLC grade from EM Science) and 7.5 mL of acetic acid (GR, min 99.7% from EM Science) were mixed in a 150-mL Erlenmeyer flask by stirring for 5 min. Then, 0.236 g (0.6 mmol) of tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$) (99.99%), 27.0 mg (0.15 mmol) EG_3−SH, and 24.5 mg of Tp (0.15 mmol) were added to the above mixed solvents and dissolved by stirring for 5 min, which gave a clear yellow solution. Next, 0.225 g (6.0 mmol) of sodium borohydride ($NaBH_4$, 99%) was dissolved in 7.5 mL Nanopure water. The $NaBH_4$ solution was added dropwise into the above solution with rapid stirring. When the first drop of $NaBH_4$ solution was added, the $HAuCl_4$ solution immediately turned to dark

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Table 1. Synthesis of Au(−S−EG_3)$_n$/Tp Nanoparticles[a]

capping agents	feeding molar ratio [EG_3–SH]/[Tp] 0.5	4	9	14	19
EG_3−SH	18.0 mg (0.1 mmol)	43.2 mg (0.24 mmol)	48.6 mg (0.27 mmol)	50.4 mg (0.28 mmol)	51.3 mg (0.285 mmol)
Tp	32.6 mg (0.2 mmol)	9.78 mg (0.06 mmol)	4.89 mg (0.03 mmol)	3.26 mg (0.02 mmol)	2.45 mg (0.015 mmol)

[a] For all reactions, 45 mL of methanol, 7.5 mL of acetic acid, and 0.236 g of $HAuCl_4 \cdot 3H_2O$ were used. A mixture of 0.225 g of $NaBH_4$ and 7.5 mL of Nanopure water was used for reduction.

Table 2. Synthesis of Au(−S−EG_3)$_n$/GSH Nanoparticles[a]

capping agents	feeding molar ratio [EG_3–SH]/[GSH] 4	12.3	14	19
EG_3−SH	43.2 mg (0.24 mmol)	50.0 mg (0.278 mmol)	50.4 mg (0.28 mmol)	51.3 mg (0.285 mmol)
GSH	18.4 mg (0.06 mmol)	6.9 mg (0.023 mmol)	6.2 mg (0.02 mmol)	4.6 mg (0.015 mmol)

[a] For all reactions, 45 mL of methanol, 7.5 mL of acetic acid, and 0.236 g of $HAuCl_4 \cdot 3H_2O$ were used. A mixture of 0.225 g of $NaBH_4$ and 7.5 mL of Nanopure water was used for reduction.

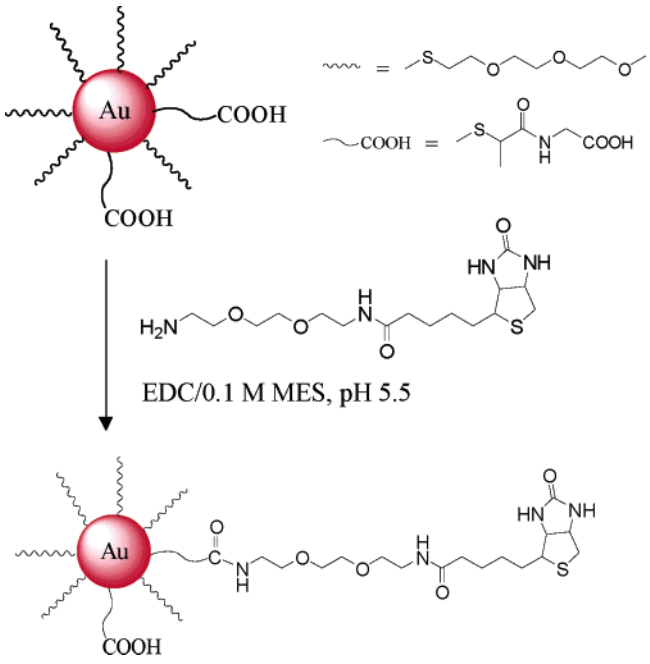
brown from yellow. It was noticed that this reaction was exothermic. The heat generated in the reaction made the solution warm for ∼15 min. Rapid stirring was continued for 2 h. The (EG_3−S−)/Tp mixed monolayer protected gold nanoparticles were soluble in water. When diluted, it became red purple and clear. The particle solution was transferred into a filter tube (50K MW cutoff, Millipore) and purified by centrifuging at 3500 rpm and washing with Nanopure water 4 times, and then dried in a lyophilizer for 3 days. The synthesis of different molar ratio of EG_3−S−/Tp mixed monolayer protected gold nanoparticles followed the same protocol as the above with varying weights of each reagent, as listed in Table 1.

Preparation of Glutathione Monolayer Protected Gold Nanoparticles. In a typical reaction, 60 mL of methanol (HPLC grade from EM Science) and 10 mL of acetic acid (GR, min 99.7% from EM Science) were mixed in an Erlenmeyer flask by stirring for 2−5 min. A quantity of 0.394 g of tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$, 99.99%) and 0.154 g of glutathione (GSH) (99% min) were added to the above mixed solvents and dissolved by stirring for 5 min, resulting in a clear yellow solution. A sodium borohydride solution was prepared by dissolving 0.6 g of $NaBH_4$ (99%) in 30 g of Nanopure water. The $NaBH_4$ solution was added dropwise into the above solution with rapid stirring. The rest of the procedure was the same as above. The glutathione monolayer protected gold nanoparticles were soluble in water, and when diluted the solution became clear purple.

Synthesis of Tri(ethylene Glycol) and Glutathione Mixed Monolayer Protected Gold Nanoparticles [Au(−S−EG_3)$_n$/GSH]. In a typical reaction with molar feeding ratio of tri(ethylene glycol) thiol and glutathione at 1:1, 45 mL of MeOH (HPLC grade from EM Science) and 7.5 mL acetic acid (GR, min 99.7% from EM Science) were mixed in a 150-mL Erlenmeyer flask by stirring for 2−5 min. Then, 0.236 g (0.6 mmol) of tetrachloroauric acid ($HAuCl_4 \cdot 3H_2O$) (99.99%), 27.0 mg (0.15 mmol) of EG_3−SH, and 46.1 mg of glutathione (0.15 mmol) were added to the above mixed solvents and dissolved by stirring for 5 min, which gave a clear yellow solution. Next, 0.225 g (6.0 mmol) sodium borohydride ($NaBH_4$, 99%) was dissolved in 5.0 mL of Nanopure water. The $NaBH_4$ solution was added dropwise into the above solution with rapid stirring. The rest of the procedure was the same as described above. The (EG_3−S−)/GSH protected gold nanoparticles were soluble in water. When diluted, the solution became red, purple, and clear. The synthesis of different molar ratios of EG_3−SH and GSH mixed monolayer protected gold nanoparticles followed the same protocol as above with varying weights of each reagent, as listed in Table 2.

Synthesis of Biotinylated Tri(ethylene Glycol)/Tiopronin Mixed Monolayer Protected Gold Nanoparticles [Au(−S−EG_3)$_n$/Tp-Biotin]. A typical reaction is shown in Scheme 2. A quantity of 15.0 mg of (EG_3−S−)/Tp mixed monolayer protected gold nanoparticle, [Au−(S−EG_3)$_{4fr}$Tp] (prepared with the feeding ratio of EG_3−SH and Tp at 4:1) was mixed with 1.0 mL of 0.1 M *N*-morpholinoethane sulfonic acid (MES) at pH 5.5 in a 5-mL round-bottom flask. Then, 22.0 mg of EZ-Link biotin-PEO-amine ((+)-biotinyl-3,6-dioxaoctanediamine, MW 374.5, from Pierce, Rockford, IL) and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) (from Pierce, Rockford, IL) were added. The mixture was stirred overnight at room temperature. The reaction mixture was then transferred into 1-mL filter tubes (50K MW cutoff, Millipore) and purified by centrifuging at 3500 rpm and washing with Nanopure water 4 times.

Scheme 2. Covalent Attachment of the Biotin Molecules to a (EG_3−S−)/Tp Mixed Monolayer Protected Nanoparticle.



Synthesis of Biotinylated Tri(ethylene Glycol)/Glutathione Mixed Monolayer Protected Gold Nanoparticles [Au(−S−EG_3)$_n$/GSH-Biotin]. In this example, a biotin ligand was introduced to the nanoparticle by reaction with the surface carboxylic acid group of GSH. In a typical synthesis, 7.7 mg of (EG_3−S−)/GSH mixed monolayer protected gold nanoparticle, Au(−S−EG_3)$_{4fr}$GSH (with the feeding ratio of EG_3−SH and GSH at 4:1), was mixed with 1.5 mL of 0.1 M MES at pH 5.5 in a 5-mL round-bottom flask. Then 7.3 mg of EZ-Link 5-(biotinamido)pentylamine (MW 328.48 from Pierce, Rockford, IL) and 50 mg of EDC (from Pierce, Rockford, IL) were added. The mixture was stirred overnight at room temperature. The reaction mixture was then transferred into a filter tube (50K MW cutoff, Millipore) and purified by centrifuging at 3500 rpm and washing with Nanopure water 4 times.

^{1}H NMR Spectroscopy of the Mixed Monolayer Protected Gold Nanoparticles. Nanoparticle samples were prepared by dissolving 15.0 mg of the dry nanoparticle in 2 mL of DMSO-d_6 solvent. ^{1}H NMR spectra of the nanoparticles were recorded with a Varian Inova 400

MHz spectrometer at room temperature in DMSO-d_6. The baseline was flattened by using a spline baseline corrector. The peak assignments can be found in previous publications.[23,24]

Gel Electrophoresis Analysis of Nanoparticle−Protein Binding Reactions. Nanoparticle−protein binding reactions were carried out in 1.5-mL eppendorf tubes by directly mixing appropriate amount of aqueous nanoparticle solution with 5−10 μg of protein. After incubation at room temperature for 10 min, 1/10 volume of 20% glycerol aqueous solution was added to the reaction tube, and the entire mixture was loaded on the gel. To optimize resolution, agarose gels with 0.8, 1, and 4% cross-linking were used for different experiments as described in the figure legends. Gel electrophoresis was typically run in 1X TBE buffer (Tris-borate-EDTA) at 90 V constant voltage for 20−40 min. Gel pictures were taken by directly scanning the gel on a HP ScanJet 7400C.

Results

1. Synthesis of a Mixed Monolayer of EG_3−SH and Tp Protected Gold Nanoparticle and Relative Reactivities of EG_3−SH and Tp. Synthesis. To get better control of the surface composition, we developed a one-pot synthesis instead of the two-step ligand replacement reaction for preparing gold nanoparticles protected with a mixed monolayer of an ethylene glycol and a ligand. The first example was a mixed monolayer of a (EG_3−S−)/Tp protected gold nanoparticle, which was synthesized by adding a reducing agent ($NaBH_4$) to a mixture of $HAuCl_4$, EG_3−SH, Tp, methanol, and acetic acid, as shown in Scheme 1. In our previous studies,[23,24] we discovered that the water content in the reaction mixture was critical for synthesizing ethylene glycol molecule protected gold nanoparticles with high yield. The optimum water concentration is 9−18%. This method also applies to the synthesis of gold nanoparticles protected with a mixed monolayer in which ethylene glycol is the major component. The synthesis utilized the optimum condition that water for dissolving $NaBH_4$ was added into the mixture of reagents to a final concentration of 12.5% (v/v). Under this condition, the reaction went well, and no precipitation was seen. The yield was about 40%. The purified nanoparticles are very stable in pure water as well as concentrated electrolyte solutions, such as 1.0 M aqueous NaCl. These nanoparticles did not degrade after one-year storage in water. The nanoparticles are also soluble in common organic solvents, such as methanol, ethanol, acetone, DMF, DMSO, THF, and chloroform. By controlling the ratio of [$HAuCl_4$]/([EG_3−SH] + [Tp]) at 2.0, we observed that the synthesized gold particles have an average of approximately 3-nm diameter when the molar feeding ratio of [EG_3−SH]/[Tp] is 1:1, as determined by TEM measurement. With increasing the molar feeding ratio of [EG_3−SH]/[Tp] to 4:1 and higher, the average particle size is approximately 3.5 nm. The impact of different ligand reactivities on the nanoparticle growth kinetics and the final particle size was expected but not investigated in this study.

Reactivities of EG_3−SH and Tp in the Formation of the Mixed Monolayer Protected Nanoparticles. To understand the relative reactivity of EG_3−SH and Tp in the formation of the gold nanoparticles, various ratios of EG_3−SH and Tp were fed into the HAuCl solution, while keeping all other reaction conditions constant. A number of nanoparticles were synthesized with [EG_3−SH]/[Tp] feeding ratio controlled at 0.5, 1, 4, 9, 14, and 19, as listed in Table 1. After trying various techniques for quantitative analysis of surface composition, such as microanalysis, electron spectroscopy for chemical analysis (ESCA), FT-IR, and thermogravimetric analysis (TGA), we found that the best way to characterize the mixed monolayer was by ^{1}H NMR spectroscopy, for it gave the most accurate and reproducible data. NMR spectroscopy has been widely used to characterize nanoparticle surface structure and composition. However, it usually results in very broad peaks, as reported in numerous articles.[36−40] For ethylene glycol protected nanoparticles, we were able to get high-resolution ^{1}H NMR spectra that are comparable to those of free small molecules.[23,24] We were initially very surprised by such high-resolution peaks. We conducted a thorough study to prove that these sharp peaks are not from the free EG_n−SH ligands. (1) We used filters (MWCO 10, 30, or 50 kD) to purify Au−S−EG_n nanoparticles by centrifuging and washing with water at least 5 times. The final filtrate was concentrated and tested by ^{1}H NMR and LC−MS, and no free EG_n−SH molecules were found. (2) We also deliberately added free EG_3−SH to Au−S−EG_3 nanoparticles and ran ^{1}H NMR spectroscopy. The spectra clearly showed two different −SCH_2− peaks. One was from Au−S−CH_2−, a single triplet with the chemical shift at 2.88 ppm. The other one was from HS−CH_2−, a double triplet peak with the chemical shift at 2.66 ppm. Both experimental results demonstrated that the observed sharp NMR peaks from the nanoparticles were not from free ligands. In addition, we performed many ^{1}H NMR measurements for other types of nanoparticles, such as alkyl chain, tiopronin, and GSH monolayer protected gold nanoparticles. All these spectra showed broad peaks. We were also able to obtain high-resolution ^{1}H NMR spectra for gold nanoparticles protected with a mixed monolayer of EG_3−S− and a functional ligand. It appears that if the particles have an ethylene glycol component, the ^{1}H NMR peaks are always sharp. The detailed mechanism for the unusual sharp peaks associated with the ethylene glycol bonded nanoparticles was not examined in this study. We speculate that the −CH_2− or CH_3− groups on the ethylene glycol chain could rotate more freely because of the higher degree of rotational freedom around a C−O bond. The motion of the ethylene glycol molecules could also lead to higher degree of motion of the minority component and, therefore, sharper NMR peaks for the mixed monolayer. For alkyl molecules, the hydrocarbon chains are densely packed and intermolecular hydrophobic interactions make the −CH_2− or CH_3− groups very difficult to rotate, resulting in broad peaks. For Tp or GSH alone on the nanoparticle, the intermolecular H-bonding makes these molecules have little freedom to rotate, also resulting in broad peaks.

Figure 1 shows the ^{1}H NMR spectrum of the Au(−S−EG_3)$_{4fr}$/Tp nanoparticle (with the feeding ratio of [EG_3−SH]/[Tp] at 4:1). The triplet peak at 2.87−2.92 (δ) is attributed to the protons of −SCH_2− group from EG_3−S−, and the doublet peak at 1.32−1.36 (δ) is characteristic of −CH_3 group from Tp. The ratio of these two peaks' integrals is converted to the ratio of [EG_3−S−]/[Tp] on the gold nanoparticle, when multiplied with a factor of 3/2. When the feeding ratio of [EG_3−SH]/[Tp] was

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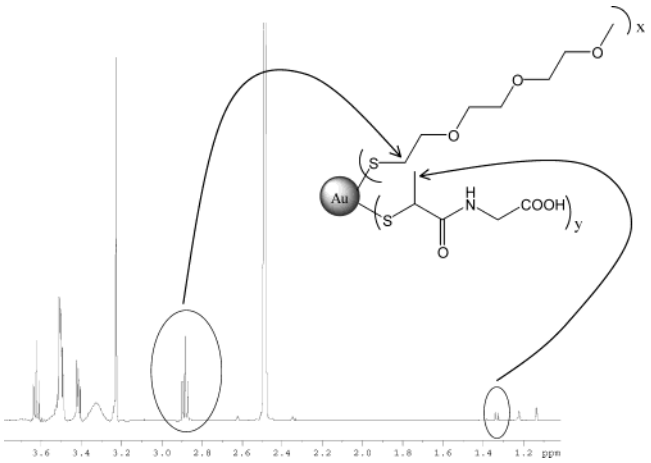


Figure 1. ^{1}H NMR spectrum of a mixed monolayer of tri(ethylene glycol) and tiopronin protected gold nanoparticle in DMSO-d_6. The molar feeding ratio of EG_3-SH and Tp for the synthesis of the nanoparticle is 4/1.

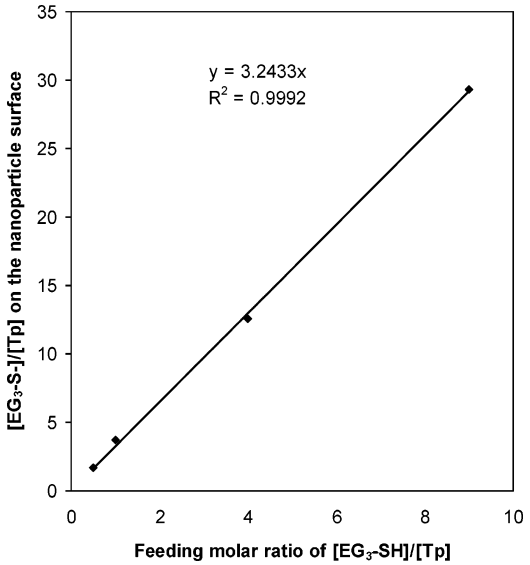


Figure 2. Relative reactivity of EG_3-SH vs Tp in the formation of a mixed monolayer protected gold nanoparticle.

0.5, 1, 4, and 9, the surface ratio of $[EG_3-S-]/[Tp]$ on the nanoparticle was 1.7, 3.7, 12.6, and 29.3, respectively, as plotted in Figure 2. The linear relationship between the two ratios with a slope of 3.2 means the reactivity of EG_3-SH is roughly 3 times as high as that of Tp in the formation of the mixed monolayer of $(EG_3-S-)/Tp$ protected gold nanoparticles. To study the reproducibility of the data, we chose the feeding ratio of $[EG_3-SH]/[Tp]$ at 4:1 and repeated the synthesis 5 times. These five experiments gave an average surface ratio of $[EG_3-S-]/[Tp]$ 12.3 ± 0.9. This result proved that the data had good reliability.

The aforementioned results demonstrated that different thiol molecules have different reactivity for bonding with gold in the formation of the nanoparticles. Possible causes for the reactivity difference include (1) difference in the electron density of sulfur atom (−S−) and (2) presence or absence of a metal chelation effect. The electron-withdrawing carbonyl group (−C═O) in tiopronin makes its sulfur atom less electron dense compared with the sulfur in EG_3-SH. According to the nanoparticle formation mechanism proposed by Shiffrin et al.,[36,41,42] as shown in eqs 1 and 2, the more electron-rich thiol

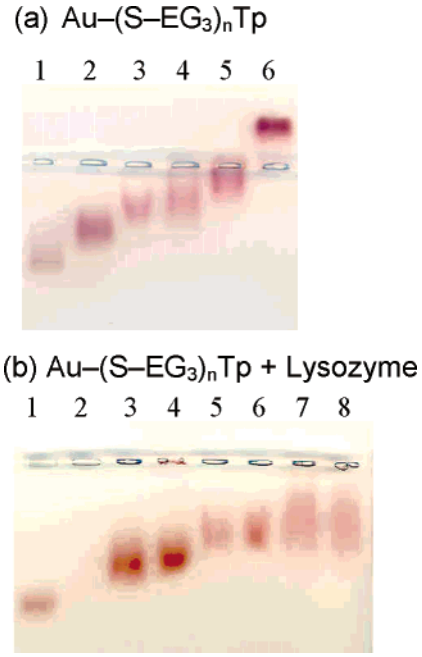


Figure 3. (a) Gel electrophoresis image illustrating the migration of $(EG_3-S-)/Tp$ mixed monolayer protected nanoparticles as a function of the percentage of Tp on the nanoparticle surface. A 4% agarose gel was used and run at 90 V for 40 min. Lanes 1, 2, 3, 4, and 5 are $Au(-S-EG_3)_nTp$ with $n = 3$, 12, 27, 42, and 57, respectively. Lane 6 is the pure $Au-S-EG_3$ nanoparticle. In all cases, 10 μL of gold particles at a concentration of 50 μM were loaded onto the gel. (b) Gel electrophoresis image of the nanoparticles from Figure 3a bonded with lysozyme. A 1% agarose gel was used and run at 90 V for 20 min. Lanes 1, 3, 5, and 7 are $Au(-S-EG_3)_nTp$ with $n = 3$, 12, 27, and 42, respectively. Lanes 2, 4, 6, and 8 are the mixtures of lysozyme and $Au(-S-EG_3)_nTp$ with $n = 3$, 12, 27, and 42, respectively. Protein binding reactions were done by mixing 7 μL of 50 μM gold particles with 7 μL of 1 mg/mL lysozyme solution, with 10 min incubation time at room temperature.

group (HS−) in EG_3-SH reacts faster with $AuCl_4^-$ in the polymer formation step. After the polymer was reduced by BH_4^-, the nanoparticle surface is bound more favorably to EG_3-SH. In addition, the Tp molecule has an acid group (−COOH) that could chelate to Au^{3+} metal salt to prevent the formation of Au^I-S-R, resulting in low reactivity.

$$AuCl_4^- + HSR \rightarrow (-Au^ISR-)_n(\text{Polymer}) \quad (1)$$

$$(-Au^ISR-)_n + BH_4^- \rightarrow Au_p(SR)_q \quad (2)$$

2. $Au(-S-EG_3)_nTp$ Nanoparticles and Their Interactions with Proteins. Qualitative Characterization of the Surface Ligand Density. The nanoparticles in our studies are a few nanometers in size and water soluble. Gel electrophoresis is a perfect tool for characterizing some of their surface properties. Tiopronin has a −COOH functional group at the end that makes the nanoparticle negatively charged at pH 8 (TBE gel running buffer). A qualitative understanding of the percentage of tiopronin on the gold nanoparticle could be easily monitored by gel electrophoresis. Figure 3a showed the migration of the gold nanoparticles bonded with a mixed monolayer of (EG_3-S-) and Tp at various surface ratios from 3 to 57.

(41) Brust, M.; Walker, M.; Bethell, D.; Schiffrin, D. J.; Whyman, R. *J. Chem. Soc., Chem. Commun.* **1994**, 801−802.
(42) Schaaff, T. G.; Shafigullin, M. N.; Khoury, J. T.; Vezmar, I.; Whetten, R. L.; Cullen, W.; First, P. N.; Gutierrez-Wing, C.; Ascensio, J.; Jose-Yacaman, M. *J. Phys. Chem. B* **1997**, *101*, 7885−7891.

Lane 1 is Au−(S−EG_3)$_3$Tp (representing the surface ratio of (EG_3−S−)/Tp at 3:1 on the gold nanoparticle). Lanes 2−6 are the same amount of gold particles with the surface ratio of (EG_3−S−)/Tp at 12:1, 27:1, 42:1, 57:1, and pure Au−S−EG_3, respectively. The migration speed of Au−(S−EG_3)$_n$Tp nanoparticles decreased with decreasing percentage of tiopronin on the nanoparticle surface. This is due to the decrease of the negative charge (−COOH) on the nanoparticle surface. This agrees with the results from ^{1}H NMR measurements of these nanoparticles.

Determination of the Critical Ratio of (EG_3−S−)/Tp on the Nanoparticle Surface for Elimination of the Nonspecific Binding with Biological Molecules. Protein binding to gold nanoparticles can be conveniently monitored by gel electrophoresis, since protein−nanoparticle complexes are expected to migrate differently than the free gold particles. In our previous studies,[23,24] we conducted a thorough investigation of the nonspecific binding between the nanoparticles and various biomolecules. It was found that Au−S−EG_3 did not have any nonspecific bindings with positively charged proteins, negatively charged proteins, DNA, or RNA. Au−Tp nanoparticles had variable degrees of binding with positively charged proteins, such as lysozyme, cytochrome C, and ribonuclease A, but did not show nonspecific bindings to negatively charged DNA, RNA, or proteins, such as BSA. These results are presumably caused by negatively charged Tp. In the mixed monolayer of (EG_3−S−)/Tp protected gold nanoparticles, the major contribution for nonspecific binding is from the electrostatic interaction introduced by Tp molecules. Therefore, lysozyme is a very good indicator for testing the nonspecific binding. The binding results for Au−(S−EG_3)$_n$Tp nanoparticles are shown in Figure 3b. Lanes 1, 3, 5, and 7 are the nanoparticles with the surface ratio of (EG_3−S−)/Tp at 3, 12, 27, and 42, respectively. Lanes 2, 4, 6, and 8 are identical to lanes 1, 3, 5, and 7, respectively, except that particles were mixed with lysozyme. When the molar percentage of Tp on the nanoparticle surface is less than 7.7%, the nanoparticles have negligible binding with lysozyme. This simple gel electrophoresis analysis established a method to determine the critical ratio of a ligand to ethylene glycol molecule on the nanoparticle surface for elimination of the nonspecific interaction.

Biotinylated Nanoparticle Having Specific Binding with Streptavidin. The biotin−streptavidin binding reaction has been widely used for studying binding specificity and strength. Here we used this well-known binding pair to demonstrate that Au(−S−EG_3)$_n$Tp nanoparticles could be further functionalized to provide a biotin moiety for specific interaction with streptavidin protein. First we needed to determine which ratio of (EG_3−S−)/Tp on the gold nanoparticle surface would provide optimum binding properties. The optimum surface ratio should give a nanoparticle with (1) negligible nonspecific interactions with biological molecules and (2) maximum functional groups on the nanoparticle surface for specific bindings. From the gel electrophoresis results of Au(−S−EG_3)$_n$Tp nanoparticles with proteins as shown in Figure 3a, we could determine that the nanoparticle has negligible nonspecific interactions with proteins when the surface ratio of (EG_3−S−)/Tp is larger than 12. Therefore, we chose Au(−S−EG_3)$_{12}$Tp as the starting material for preparing biotinylated nanoparticles. The reaction for attaching the biotin molecule to Au(−S−EG_3)$_{12}$Tp nanoparticles

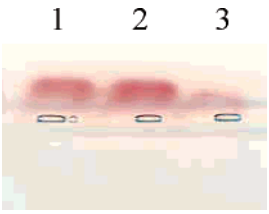


Figure 4. Gel electrophoresis image illustrating specific binding of Au(−S−EG_3)$_{12}$Tp-biotin nanoparticle with streptavidin protein. A 1% agarose gel was used and run at 90 V for 40 min. Lane 1 is 5 μL of the nanoparticle at a concentration of ~100 μM aqueous solution, and lanes 2 and 3 are the same amount of the nanoparticle incubated with 5 μL of lysozyme and streptavidin, respectively. All the protein concentrations are 1 mg/mL in Nanopure water.

is shown in Scheme 2. Before the reaction, the Au(−S−EG_3)$_{12}$-Tp nanoparticle has 7.7% of Tp molecules on its surface. This negatively charged nanoparticle moved rapidly toward the positive electrode in gel electrophoresis, as shown in Figure 3a. After reaction with EZ-Link biotin-PEO-amine, the nanoparticle did not move toward the positive electrode in gel electrophoresis, as shown in Figure 4. This is due to the reactions of the acid groups (−COOH) from Tp with amine groups of EZ-Link biotin-PEO-amine, which made the nanoparticles neutral and move slightly toward the negative electrode. The purified biotinylated gold nanoparticle, Au(−S−EG_3)$_{12}$Tp-PEO-biotin was used to test specific binding with streptavidin by gel electrophoresis, as shown in Figure 4. Lane 1 is 5 μL of the biotinylated nanoparticles at ~0.1 mM; lanes 2 and 3 are the same amount of nanoparticles as lane 1, but 5 μg of lysozyme and 5 μg of streptavidin were added, respectively. The gel experiment showed that the biotinylated gold nanoparticle did not undergo nonspecific binding with lysozyme, a positively charged protein, but did bind strongly to the target protein, streptavidin. Because of the four binding sites of streptavidin, most of the mixture of Au(−S−EG_3)$_{12}$Tp-PEO-biotin nanoparticle and streptavidin became precipitated and did not move out of the well. Only a small fraction of the mixture moved slightly out of the sample well. The protein binding experiments by gel electrophoresis demonstrated that the biotinylated gold nanoparticle has two functions: eliminating the nonspecific binding with proteins such as lysozyme and enabling the specific binding with streptavidin protein through biotin−streptavidin interaction.

3. Au(−S−EG_3)$_n$GSH Nanoparticles and Their Interactions with Proteins. For a mixed monolayer protected nanoparticle to be applied in the biological environment, the critical issue is how to design a surface that allows the nanoparticle to avoid nonspecific interactions and in the meantime provides maximum binding to a specific target. In the above example of Au(−S−EG_3)$_n$Tp nanoparticles, we quantitatively characterized the surface composition by using ^{1}H NMR spectroscopy and then determined the relationship between the surface composition and the binding properties by gel electrophoresis. For most applications, accurate understanding of the surface composition might not be necessary. If we could correlate the feeding ratio of EG_3−SH and the ligand with the nanoparticle binding performance, that should be sufficient information to design the synthesis of nanoparticle with only specific interactions. Here we used Au(−S−EG_3)$_n$GSH as another example to demonstrate that a simple method using gel electrophoresis analysis can identify the critical feeding ratio for the nanoparticle to perform specific binding with the targets.

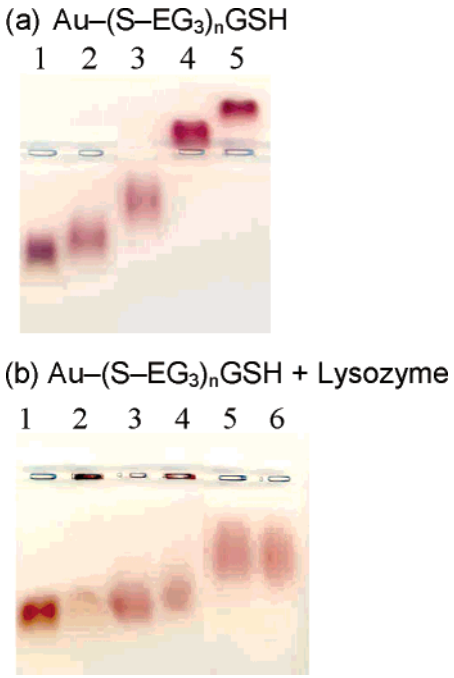
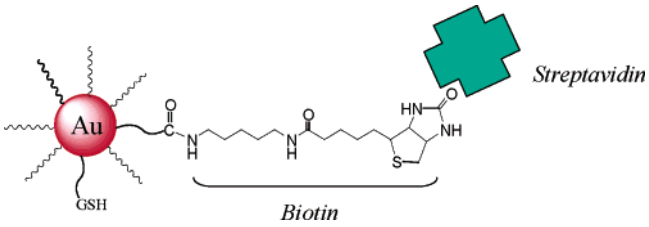


Figure 5. (a) Gel electrophoresis image illustrating the migration of (EG_3−S−)/GSH mixed monolayer protected nanoparticles as a function of feeding ratio of [EG_3−SH]/[GSH]. A 4% agarose gel was used and run at 90 V for 40 min. Lanes 1, 2, 3, and 4 are Au(−S−EG_3)$_{nfr}$GSH with n = 1, 4, 14, and 19, respectively. Lane 5 is the pure Au−S−EG_3 nanoparticle. In all cases, 10 μL of gold particles at concentration of 50 μM were loaded onto the gel. (b) Gel electrophoresis image of the nanoparticles from Figure 5a bonded with lysozyme. A 1% agarose gel was used and run at 90 V for 20 min. Lanes 1, 3, and 5 are Au(−S−EG_3)$_{nfr}$GSH with n = 1, 4, and 14, respectively. Lanes 2, 4, and 6 are the mixture of lysozyme and Au(−S−EG_3)$_{nfr}$GSH with n = 1, 4, and 14, respectively. Protein binding reactions were done by mixing 7 μL of 50 μM gold nanoparticles with 7 μL of 1 mg/mL lysozyme solution, with 10 min incubation time at room temperature.

Au(−S−EG_3)$_n$GSH Nanoparticle Synthesis and Its Nonspecific Bindings with Proteins. [Au(−S−EG_3)$_n$GSH] nanoparticles were synthesized by following the same protocol as that for [Au(−S−EG_3)$_n$Tp]. When the water content in the reaction mixture was controlled in the range of ∼9−18%, [Au(−S−EG_3)$_n$GSH] nanoparticles were readily prepared. The synthesis was very reproducible. The [Au(−S−EG_3)$_n$GSH] nanoparticles were also very stable over storage in dry format as well as in solvents such as water, methanol, ethanol, and other organic solvents. With a constant ratio of $HAuCl_4$ and capping agents (EG_3−SH and GSH) at 2.0, a series of nanoparticles were synthesized with the feeding ratio of [EG_3−SH]/[GSH] at 1, 4, 14, and 19, respectively. Au(−S−EG_3)$_{4fr}$GSH represents the nanoparticle synthesized with the feeding ratio of [EG_3−SH]/[GSH] at 4. The charge density of Au(−S−EG_3)$_{nfr}$GSH nanoparticles representing the surface density of GSH was qualitatively characterized with their migration speed in the gel electrophoresis, as shown in Figure 5a. Lane 1 is Au(−S−EG_3)$_{1fr}$GSH nanoparticles with EG_3−SH/GSH feeding ratio at 1:1. Lanes 2−5 are the same amount of Au(−S−EG_3)$_{nfr}$GSH nanoparticles with n = 4, 14, 19, and pure Au−S−EG_3, respectively. The migration speed of Au(−S−EG_3)$_{nfr}$GSH nanoparticles decreased with decreasing percentage of GSH on the nanoparticle surface. In Figure 5b, Lanes 1, 3, and 5 are Au(−S−EG_3)$_{nfr}$GSH nanoparticles with n = 1, 4, and 14, respectively. Lanes 2, 4, and 6 are the same amount of Au(−S−EG_3)$_{nfr}$GSH nanoparticles with n = 1, 4, and 14, respectively, mixed with lysozyme. When the molar feeding percentage of GSH is less than 20%, (EG_3−S−)/GSH mixed monolayer protected nanoparticles have negligible binding with lysozyme. In this example, the ratio of (EG_3−S−)/GSH on the nanoparticle surface was unmeasured and unknown.

Scheme 3. Schematic Illustration of the (EG_3−S−)/GSH Mixed Monolayer Protected Nanoparticle Chemically Bonded with a Biotin Molecule and Its Interaction with a Streptavidin Molecule.



Biotinylated Nanoparticle [Au(−S−EG_3)$_n$GSH-Biotin] Having Specific Binding with Streptavidin. The above simple gel electrophoresis analysis guided us to pick Au(−S−EG_3)$_{4fr}$GSH nanoparticles as the starting material for a specific binding to a target. A biotin molecule was chemically attached to GSH via EDC coupling reaction, as shown in Scheme 3. The biotinylated nanoparticle, Au(−S−EG_3)$_{4fr}$GSH-biotin indeed showed no binding with either BSA, a negatively charged protein, or lysozyme, a positively charged protein, but had strong binding with the target protein, streptavidin, as shown in Figure 6.

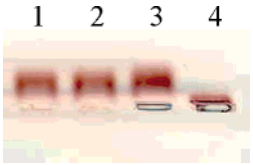


Figure 6. Gel electrophoresis image illustrating specific binding of Au(−S−EG_3)$_{4fr}$GSH-biotin with streptavidin protein. A 1% agarose was used and run at 90 V for 20 min. Lane 1 is 2 μL of the nanoparticle at concentration of ∼200 μM, lanes 2−4 are the same amount of nanoparticle incubated with 8 μL of BSA, lysozyme, and streptavidin, respectively. All the protein concentrations are 1 mg/mL in water.

Au(−S−EG_3)$_n$GSH Nanoparticle Having Specific Binding with GST Protein. The GSH molecule on the Au(−S−EG_3)$_n$GSH particle is a natural substrate for the enzyme glutathione-*S*-transferase (GST). In contrast to the biotin−streptavidin binding pair, the binding of GSH by GST is far weaker. The GST−GSH binding pair thus provides an opportunity to demonstrate the specific binding between nanoparticles and proteins under more common biological conditions. From the nonspecific tests of Au(−S−EG_3)$_{nfr}$GSH, as shown in Figure 5, we saw negligible binding with lysozyme for the nanoparticle at the [EG_3−SH]/[GSH] feeding ratio at 4. The nanoparticles with the feeding ratios at 9 and 14 were chosen for specific interaction with GST protein. Figure 7 demonstrates that neither Au(−S−EG_3)$_{9fr}$GSH nor Au(−S−EG_3)$_{14fr}$GSH bound to lysozyme (lanes 2 and 5, respectively), while both particles migrated faster (lanes 3 and 6) after incubation with GST protein, indicating their specific interaction with GST. The faster migration of the Au(−S−EG_3)$_{nfr}$GSH−GST complex is presumably due to the negative charge of the GST protein (pI = 6.2) in the pH 8 gel running buffer (TBE).

Discussion

Mixed Monolayers. In this study, we developed mixed monolayer protected nanoparticles for specific interactions with

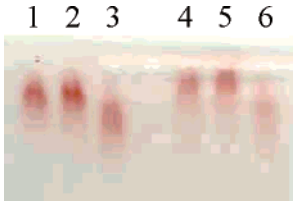


Figure 7. Gel electrophoresis image illustrating specific binding of Au(−S−EG_3)$_{nfr}$GSH (n = 9 and 14) with GST protein. A 0.8% agarose gel was used and run at 90 V for 20 min. Gold particles were suspended in H_2O with a concentration of ~50 μM. Lane 1 is 10 μL of Au(−S−EG_3)$_{9fr}$-GSH; lanes 2 and 3 are the same amount of gold particles incubated with 1 μL of lysozyme (10 mg/mL in water) and 5 μL of GST (0.5 mg/mL in water), respectively. Lanes 4 to 6 are identical to lanes 1−3, except that Au(−S−EG_3)$_{14fr}$GSH is used.

biological molecules. The principal requirement of the shielding component is that it does not have any binding with biological molecules, and typically it is an uncharged, water soluble molecule of well-defined length. Examples of suitable shielding components include short chain ethylene glycol oligomers, sugars, crown ethers, and polyacrylamide. Although oligomers and polymers are suitable for repelling biological binding, long chain major components may block the capture ligand binding functionality, preventing any binding from occurring. Polymers of excessive length may not be practical, and thus polymer chain length must be controlled.

The capture component is a ligand that can be recognized by biological targets, such as a protein, a nucleic acid, or even a cell. The capture agent itself could also cause the nonspecific binding with biological molecules. Therefore, control of the percentage of the capture agent on the nanoparticle is crucial for the nanoparticle to specifically bind the target of interest while excluding all other materials. The capture component can be attached to the nanoparticles either through direct synthesis, for example GSH, or through further functionalization of a mixed monolayer on the nanoparticle, for example, biotin attaching to tiopronin molecule. The capture component has a specific affinity for single or multiple targets. This article only demonstrated two examples: a very tight binding pair, biotin−streptavidin, and a weak binding pair, GSH−GST. Certainly the mixed monolayer protected nanoparticles could be applied to a variety of chemical and biological based binding pairs. Examples include the class of immune-type binding pairs, such as antigen/antibody or hapten/anti-hapten systems, and the class of nonimmune-type binding pairs, such as biotin/avidin, folic acid/folate binding protein, complementary nucleic acid segments, including peptide nucleic acid sequences, and protein A or G/immunoglobulins.

Coated Nanoparticles. Nanoparticles may be composed of a variety of metals, such as gold, silver, platinum, palladium, copper, cobalt, and alloys composed of these metals. The method of direct synthesis of the nanoparticles with a shielding component and a binding domain can be applied to the preparation of the nanoparticles with more than one binding domain, for example, two different binding domains for various targets. A pool of nanoparticles could be prepared with monolayers of different binding ligands. These nanoparticles with specific binding ligands may then be used in assays to detect or isolate targets of interest. Alternatively, the targets of interest may also be used to immobilize or assemble the nanoparticles into nanowires or other components of nanoelectric devices.

This article provided a very simple method, using gel electrophoresis, for optimizing nanoparticle binding to a target in a specific fashion. This method is so simple that it could be adopted for routine use in bioanalytical and biomedical applications. To make the gel electrophoresis assay more informative, a narrow bandwidth for nanoparticles is very much desired. For a mixed monolayer protected nanoparticle, its bandwidth is determined by two factors: the particle size distribution and the ligand number distribution on the same size particle. Controlling either of these distributions should make the bandwidth narrower.

Conclusions

Mixed monolayer EG_3−S−/capture agent protected gold nanoparticles were prepared by a one-step direct synthesis. For Au(−EG_3−S−)$_n$/Tp nanoparticles, ^{1}H NMR measurement revealed that the reactivity of EG_3−SH is 3 times as high as that of Tp in the formation of the nanoparticles. By controlling the feeding ratio of [EG_3−SH]/[Tp], the percentage of Tp on the gold nanoparticle surface could be quantitatively controlled. This approach could be easily applied to other nanoparticles protected with a mixed monolayer of an ethylene glycol molecule and a ligand. Gel electrophoresis of the nanoparticles was used to identify the critical ratio of a capture ligand at which the nanoparticle enables maximum specific binding with a biological target without the interference of nonspecific interactions. Such a quantitative control of the ligand in the mixed monolayer protected nanoparticles is useful for understanding the mechanism of the interaction of nanoparticles and biological entities and for better design of the new nanomaterials. However, in some cases, quantitative understanding of the surface ratio of the capture ligand to shielding component is not necessary. A correlation between the synthesis feeding ratio of the capture agent and EG_3−SH and the migration of the nanoparticles in gel electrophoresis when mixed with proteins is sufficient to select the optimum nanoparticles for applications in a specific binding fashion. The nanoparticles developed in this work have potential utilities in nanoscale electronic devices, multifunctional catalysts, biosensors, and biological assays.

Acknowledgment. This work comes from Du Pont CR&D Bioelectronics Project. We thank Dr. Fredric Davidson and Mr. Steven F. Krakowski for their support on ^{1}H NMR spectroscopy and Dr. Nicole Reinartz for reading our manuscript.

Supporting Information Available: TEM images of nanoparticles Au(−S−EG_3)$_{1fr}$GSH, Au(−S−EG_3)$_{4fr}$GSH, Au(−S−EG_3)$_{9fr}$GSH, Au(−S−EG_3)$_{14fr}$GSH, Au(−S−EG_3)$_{19fr}$GSH, Au(−S−EG_3)$_{1fr}$Tp, Au(−S−EG_3)$_{4fr}$Tp, and Au(−S−EG_3)$_{5.6fr}$Tp. This material is available free of charge via the Internet at http://pubs.acs.org.

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