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Bio-functionalized Gold Nanoparticles for Surface-Plasmon-Absorption-Based Protein Detection

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Bio-functionalized gold nanoparticles (AuNPs), which bio-specifically interact with biotin-(strept)avidin, were investigated in this study. AuNPs were functionalized with a synthetically-provided biotin-linked thiol (BLT), which was synthesized by amidation of the active ester of biotin with 2-mercaptoethylamine. The BLT-attached AuNP was bio-specific for streptavidin, making it potentially useful for biosensor applications. To test the bio-specific interactions, the colors, absorption spectra and TEM images were investigated for proteins such as streptavidin, cytochrome C, myoglobin and hemoglobin. The colors and absorption spectra changed when streptavidin was added to the BLT-attached AuNP solution. However, the color and spectra did not change when the other proteins were added to the same solution. These results show that the AuNPs provided a colloidal solution with excellent stability and highly selective absorption characteristics for streptavidin as a target molecule. Proteins were also screened in order to identify a general strategy for the use of optical biosensing proteins based on AuNPs. In addition, TEM images confirmed that streptavidin led the BLT-attached AuNPs to aggregate or precipitate.

Key Words : Gold nanoparticles, Biotin, Avidin, Self-assembly, Biomolecules

Introduction

Control over the assembly/disassembly of nanoparticles in solution and as solid substrates has great potential in many technologically important areas, such as nanobiosensors, electronic and optical devices, and magnetic data storage systems.[1] Among the techniques used to fabricate two- and three-dimensional hierarchical nanostructures, the coupling of nanoparticles and biomolecules has been investigated due to the possible utilization for biospecific interactions and the feasibility of controlling the assembly/disassembly of nanoparticles.[2] The first step toward the development of nanoparticle-based nanobiosensors is to attach appropriate biomolecules (e.g., ligands, DNA, carbohydrates and proteins) to nanoparticles and study the assembly of the nanoparticles through designed biospecific interactions ("bio-inspired self-assembly of nanoparticles"). Examples of biospecific interactions for the bio-inspired self-assembly of nanoparticles include DNA hybridization,[1b] antigen-antibody interactions,[3] biotin-(strept)avidin interactions,[4] carbohydrate-lectin interactions, and more.[5,6] (Strept)avidin interacts stoichiometrically with biotin, binding one biotin per subunit. Streptavidin is a biotin-binding protein that was originally isolated from *Streptomyces avidinii*. In contrast to avidin, streptavidin has no carbohydrate. The interaction between biotin and (strept)avidin has been used as a model system of biospecific interactions because the interaction is highly biospecific and strong ($K_D = 10^{-15}$ M). Also, the association between biotin and (strept)avidin is rapid and unaffected by extremes of pH, organic solvents, or other denaturing agents.

In this study, we synthesized a biotin-linked thiol (BLT) as a novel biospecific material that has a simple assembly process. To investigate biospecific characteristics of the assembly of biotin-attached gold nanoparticles (AuNPs), we measured the absorption spectra of the BLT-attached AuNP solution with the addition of proteins such as streptavidin, cytochrome C, myoglobin, and hemoglobin. The colors and absorption spectra changed when streptavidin was aggregated with the BLT-attached AuNP. However, they did not change when the other proteins were added to the solution. The BLT-attached AuNPs were highly selective for the target protein (streptavidin). Proteins (cytochrome C, myoglobin and hemoglobin) were also screened in order to identify a general strategy for the use of optical biosensing proteins based on AuNPs. Aggregation and precipitation were investigated *via* TEM images, which confirmed that streptavidin caused the BLT-attached AuNPs to precipitate. BLT was simply synthesized and biospecifically interacted with streptavidin, thus it would be useful for biosensors based on AuNPs.

Experimental Section

Materials, Reagents and Measurements. All reagents containing tetrachloroaurate ($HAuCl_4 \cdot H_2O$), bovine serum

albumin (BSA), biotin, streptavidin (from *Steptomyces avidinii*, MW ~ 60 kDa), trisodium citrate, 2-mercaptoethylamine, tetrafluorophenol, dipotassium bis(*p*-sulfonatophenyl)phenylphosphane dihydrate (BSPP), phosphate-buffered saline (PBS, pH 7.4), cytochrome C, myoglobin, hemoglobin, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), triethylamine (TEA) and *N,N*-dimethylformamide (DMF) were obtained from Sigma-Aldrich Co. and used as received. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM2000 TEM instrument operating at an accelerating voltage of 200 kV. An Eppendorf centrifuge 5417C was used to centrifuge the gold nanoparticle solution. UV-visible absorption spectra were recorded on a SHIMADZU UV-2501PC spectrophotometer. All glassware was cleaned with aqua regia and rinsed with deionized water prior to experiments.

Preparation of Gold Nanoparticles. AuNPs are particularly easy to modify because they are often stabilized with a weakly binding layer of charged ligands (e.g., citrate) that can be replaced with molecules with chemical functionalities that bind more strongly (e.g., thiols, amines and disulfides)[7] to their surfaces than those ligands. AuNPs were synthesized by reducing sodium citrate with $HAuCl_4$.[8] An aqueous solution of $HAuCl_4$ (1×10^{-3} M, 500 mL) was brought to reflux while stirring, and then 50 mL of a 3×10^{-2} M trisodium citrate solution was added quickly, which resulted in a change in solution color from pale yellow to deep red. After the color change, the solution continued to reflux for an additional 10 min. The heating mantle was removed, and stirring was continued for an additional 15 min. The AuNP solution was cooled to room temperature and filtered through a 0.45 μm nylon filter. The AuNPs had an average diameter of 13 nm as measured by TEM.

Synthesis of Biotin-linked Thiol (BLT). BLT was synthesized in two steps by activation of the biotin carboxylic acid with tetrafluorophenol, followed by coupling of the active biotin ester with 2-mercaptoethylamine (Scheme 1). For the synthesis of biotin tetrafluorophenyl ester, biotin (0.20 g, 0.81 mmol) and 2,3,5,6-tetrafluorophenol (0.17 g, 1.06 mmol) were dissolved in 3 mL of anhydrous DMF at 80 °C under nitrogen. After cooling to room temperature, EDC (0.18 g, 0.98 mmol) dissolved in 3 mL of dry DMF was added to the reaction mixture and the reaction was reheated to 80 °C for 4 hours. The solvent was removed under reduced pressure and the crude product was purified by flash column chromatography on silica-gel using methylene chloride/methanol (8:1, v/v) to yield the biotin tetrafluorophenyl ester (white powder, 0.12 g, 38%). Biotin tetrafluorophenyl ester: FT-IR (KBr) 3256, 2941, 2873, 1795, 1710, 1525, 1487, 1179, 1096, 956 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.9 (m, 1H, Ph-H), 6.5 (s, 1H, NH), 6.4 (s, 1H, NH), 4.3 (dd, 1H), 4.1 (dd, 1H), 3.1 (m, 1H), 2.8 (dd, 1H), 2.7 (m, 2H), 1.4-1.7 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.4, 162.7, 146.7, 144.3, 141.3, 138.8, 128.6, 104.3, 61.0, 59.2, 55.2, 39.6, 32.3, 27.9, 24.3.

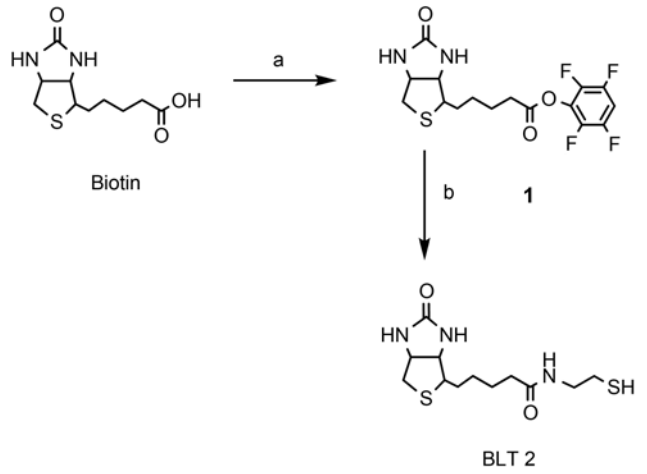
For the synthesis of biotinylated ethylthiol, 2-aminoethanethiol hydrochloride (0.29 g, 2.63 mmol) and freshly distilled TEA (0.12 mL, 0.87 mmol) were dissolved in 3 mL anhydrous DMF. The reaction was cooled on an ice bath and biotin tetrafluorophenyl ester (0.12 g, 0.29 mmol) was added over a period of 45 min under nitrogen. The reaction was stirred for 6 hours at room temperature and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica-gel using methylene chloride/methanol (7:1, v/v) to yield the BLT (white powder, 0.08 g, 86%). Biotinylated ethylthiol: FT-IR (KBr) 3296, 2933, 2870, 1710, 1639, 1551, 1459, 659 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 7.9 (t, 1H, CON-H), 6.4 (s, 1H, NH), 6.3 (s, 1H, NH), 4.3 (dd, 1H), 4.1 (dd, 1H), 3.2 (m, 2H, CH_2), 3.1 (m, 1H), 2.8 (dd, 1H), 2.5 (m, 2H), 2.1 (t, 2H, CH_2), 1.3-1.5 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.1, 162.7, 61.0, 59.2, 55.4, 42.0, 39.9, 35.1, 28.1, 25.2, 23.5.

Modification of Gold Nanoparticles. AuNPs (13 nm in diameter) were first stabilized by complexation with BSPP (Figure 1).[9] An aqueous solution of 0.06 mL BSPP (1.12×10^{-3} M) was added to a 2 mL AuNP solution (1.4×10^{-8} M) and shaken gently for 14 hours at room temperature. Upon completion of the complexation, the solution was centrifuged for 10 min and then dissolved in 2 mL deionized water. BLT-attached AuNPs were formed by adding 0.2 mL BLT (5×10^{-4} M) to a buffer solution (PBS, pH 7.4) of the BSPP-capped AuNPs and incubating the mixture at room temperature for 24 hours. Before assembling the BLT-attached AuNPs with avidin, BSA (0.05% w/v) was added to the BLT-attached AuNP solution to minimize non-specific adsorption of proteins.[10] Functionalization of the BLT-attached AuNPs was monitored by UV-visible absorption spectra.

Assembly of Au Nanoparticles. A stock solution of streptavidin (1×10^{-5} M) was prepared in deionized water and stored at 0 °C. After the 0.1 mL streptavidin (1×10^{-5} M) solution equilibrated to room temperature, it was added to a solution of 1 mL BLT-attached AuNPs. The resulting solution was shaken for 10 min and stored at room temperature until analysis. UV-visible absorption spectra were acquired in order to determine the functional characteristics of the BLT-attached AuNPs. Images of the the BLT-attached AuNPs surfaces and the assembly of AuNPs with avidin were obtained by TEM.

Results and Discussion

In this study, we synthesized AuNPs by reducing citrate with $HAuCl_4$. The average diameter of the AuNPs was 13 nm, as measured by TEM. AuNPs were complexed with BLT, which was synthesized by activating the biotin carboxylic acid group with tetrafluorophenol followed by coupling the active ester of biotin with 2-mercaptoethylamine as depicted in Scheme 1. The BLT-attached AuNPs were stabilized by complexation with BSPP. The BLT-attached AuNPs were formed by adding BLT to an aqueous solution of BSPP-capped Au NPs and incubating the mixture at room



Scheme 1. A schematic illustration of the preparation of BLT through amidation methods: (a) 2,3,5,6-tetrafluorophenol, EDC, DMF, 80 °C, 4 h and (b) 2-aminoethanethiol hydrochloride, TEA, DMF, RT, 6 h.

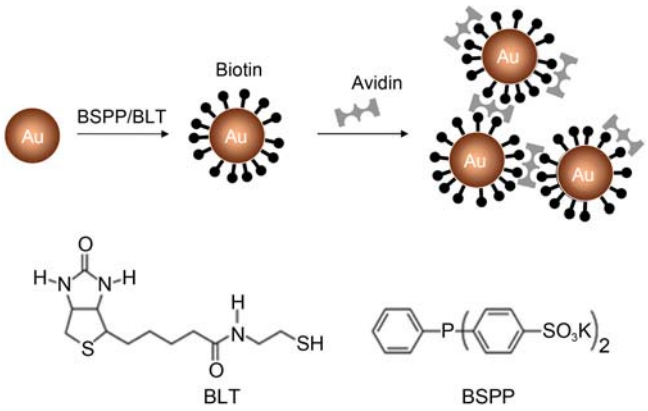


Figure 1. Schematic representation of the avidin-mediated assembly of biotin-attached gold nanoparticles. Chemical structures of the BLT and BSPP are also shown; homemade (left) and commercial BSPP (right).

temperature. Then BSA was added to the BLT-attached AuNP solution to minimize non-specific protein adsorption.

To analyze the precipitation or bio-specific binding of the AuNPs with avidin in detail, absorption spectra of the solutions containing the BLT-attached AuNPs were acquired by UV-visible absorption spectra. Figure 2 shows the absorption spectra of the assembly of AuNPs, the BLT attached AuNPs, and the proteins added BLT-attached to the AuNPs. The peak wavelength (λ_{max}) of the absorption spectrum of the solution of the assembled AuNPs was 519 nm. Following stabilization with BSPP, the absorption peak of the BLT-attached AuNPs minimized and red-shifted from 519 nm to 526 nm when BSA was added to the solution. Upon addition of 0.1 mL (strept)avidin solution at a concentration of 1×10^{-5} M to the BLT attached AuNPs solution, the absorption spectrum shifted and the peak absorption wavelength was 550 nm. This process was accomplished within 10 min at room temperature. These results indicate that the BLT-attached AuNPs were aggregated and precipitated by (strept)avidin.

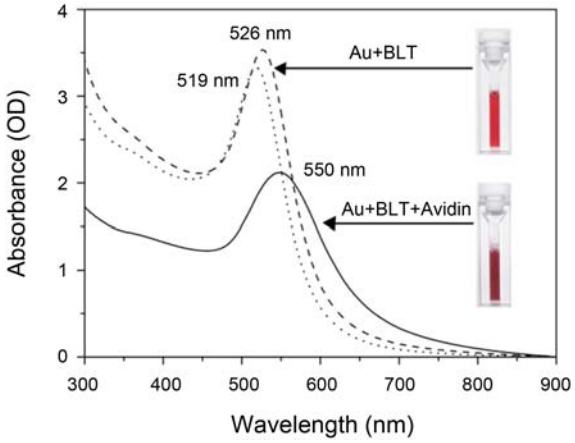


Figure 2. UV-visible absorption spectra of bare AuNPs (519 nm), (a) biotin attached AuNPs (526 nm) and (b) after addition of 1×10^{-5} M (strept)avidin solution (550 nm).

To test of the nonspecific binding characteristics of the BLT-attached AuNPs, absorption spectra were measured for the BLT-attached AuNP solution with additions of other proteins (cytochrome C, myoglobin and hemoglobin). The resulting spectra were different from the spectrum of the solution containing streptavidin shown in Figure 3. While the addition of (strept)avidin to the BSPP-capped AuNP solution led to a shift of λ_{max} to 550 nm, the absorption spectra for the other proteins did not change λ_{max} and were almost the same as the spectrum of the BLT-attached AuNPs solution, except at ~400 nm. The colors of the solution containing BLT-attached AuNPs stabilized in BSPP were observed in UV-cell (model: Hellma, 10 mm) in order to assess bio-specific interactions. Here, the small absorption peak around 400 nm are unique peak of cytochrome C, myoglobin and hemoglobin. Figure 4 shows that the color changed when 0.7 mL of the BLT attached Au-NPs solution interacted with 0.1 mL protein (1×10^{-5} M) such as avidin, cytochrome C, hemoglobin or myoglobin. The red color deepened when (strept)avidin was added to the BLT-attach-

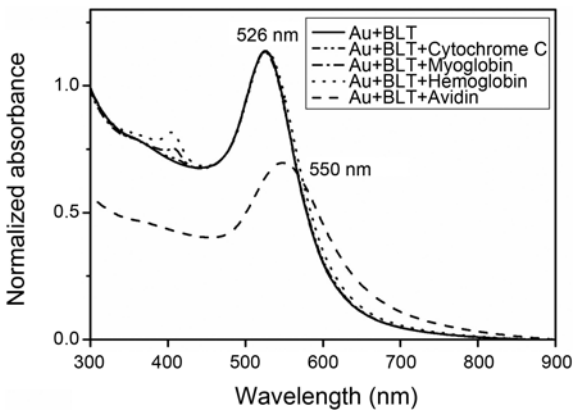


Figure 3. Comparison of UV-visible absorption spectra for cytochrome C, myoglobin, hemoglobin (526 nm) and (strept)avidin (550 nm).

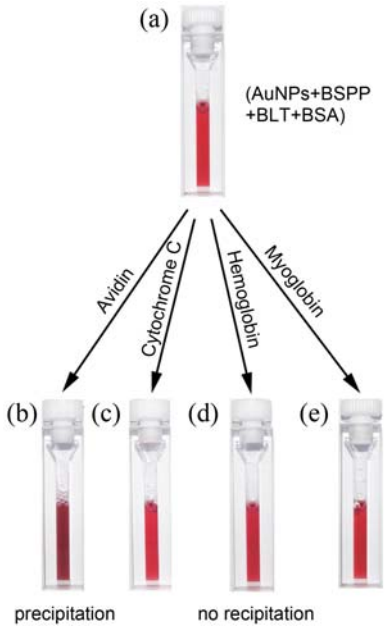


Figure 4. Addition of proteins (cytochrome C, myoglobin, hemoglobin and (strept)avidin) to the BLT. (a) Complexation of Au-NPs, BSPP, BLT and BSA. (b) after the addition of 0.1 mL streptavidin (1×10^{-5} M). (c) after the addition of 0.1 mL cytochrome C (1×10^{-5} M). (d) after the addition of 0.1 mL myoglobin (1×10^{-5} M). and (e) after the addition of 0.1 mL hemoglobin (1×10^{-5} M).

ed AuNPs solution in the UV-cell (Figure 2(b)), indicating that the AuNPs were aggregated and precipitated. However, the of cytochrome C, hemoglobin and myoglobin solutions in Figure 4 are almost the same color as the original solution containing no protein (Figure 4(a)), indicating no precipitation.

The shift in absorption was visually evident, and this has been the basis of some biological assays. Assembly was biospecific; the addition of streptavidin to the BSPP-capped AuNP solution did not change λ_{max}. The lack of change in λ_{max} indicates that (1) the observed aggregation was mediated by the biospecific biotin-avidin interaction and (2) the non-specific adsorption of (strept)avidin to the AuNPs was negligible. The (strept)avidin-mediated assembly of BLT-attached AuNPs was further confirmed by TEM (Figure 5). While BLT-attached AuNPs were well-dispersed in the BSA solution (Figure 5(a)), the addition of (strept)avidin to the BLT-attached AuNPs solution resulted in aggregates (Figure 5(b)). Nanoparticle-based biological sensing is based on biospecific interactions between a target and a probe attached to the nanoparticle. In this study, as a proof-of-concept, we used (strept)avidin as a target and biotin as a probe molecule, and screened several proteins. The addition of proteins, such as cytochrome C, myoglobin, and hemoglobin, neither changed λ_{max} in the UV-visible absorption spectra (Figure 3) nor led to any precipitation. This study provides a very simple method, using biotin-(strept)avidin interactions, for the optical biosensing of proteins based on AuNPs.

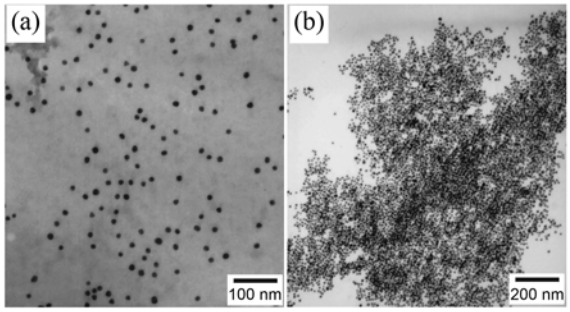


Figure 5. TEM micrographs (a) before assembly and (b) after assembly.

Conclusions

In summary, we synthesized a BLT by activating the carboxylic acid group of biotin with tetrafluorophenol, followed by coupling the active biotin ester with 2-mercaptoethylamine. The BLT and AuNPs were stabilized by complexation with BSPP and BLT-attached were formed AuNPs by adding BLT to phosphate-fabricated biotin-attached gold nanoparticles. We showed the bio-specific interactions by observing colors, absorption spectra and TEM microscopic images. (Strept)avidin changed the color of the spectrum, but the other proteins did not. Our findings suggest that these biomolecule-attached nanoparticles with biospecific features could be used as an easy and reliable optical biosensing method.

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