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Ligand Synthesis and Passivation for Silver and Large Gold Nanoparticles for Single-Particle-Based Sensing and Spectroscopy

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Summary

Silver and large gold nanoparticles are more efficient scatterers than smaller particles, which can be advantageous for a variety of single-particle-based sensing and spectroscopic applications. The increased susceptibility to surface oxidation and the larger surface area of these particles, however, presents challenges to colloid stability and controllable bio-conjugation strategies. In this chapter, ligand syntheses and particle passivation procedures for yielding stable and bio-conjugatable colloids of silver and large gold nanoparticles are described.

Key words: gold nanoparticles; silver nanoparticles; bio-conjugation; nanoparticle passivation

1 Introduction

Materials on the nanometer scale can exhibit optical properties different than the corresponding bulk materials. For noble metal nanoparticles, the source of the size-dependent optical response is the collective oscillation of conduction-band electrons in response to an applied electromagnetic field, *i.e.*, the particle's plasmon resonance (1–3). In the near-field region (distance to particle surface $\ll$ wavelength), nanoparticles can enhance the local field leading to emission enhancement for emitters present in the field. The plasmon resonance and near-field distributions can be tuned using differently shaped nanoparticles or hierarchical structures composed of multiple particles.

Noble metal nanoparticles, with plasmon resonances in the visible spectrum, have attracted a lot of attention in the literature for a variety of applications. The synthesis of these particles typically involves the reduction of the metal ions out of solution leading to Ostwald ripening of seed particles (4). Particles are typically ripened to a desired size and then stabilized with a surfactant.

The advantageous optical properties and the known surface chemistry of these nanoparticles have led experimenters to conjugate them to biological materials for a variety of applications. One can attach small molecules to the nanoparticle surface to prevent non-specific adsorption of biomolecules or attach molecules with

an orthogonal chemistry for hierarchical construction. Applications of these hybrid particles range from analyte detection (5), electron microscopy contrast agents (6), photo-thermal therapies (7, 8), drug delivery systems (9), bio-barcoding (10, 11), among others (12–16).

For certain applications, *e.g.* single-particle-based sensing and spectroscopy, using particles with increased scattering cross-sections such as larger-diameter gold nanoparticles may be desirable. Utility of any hybrid nano-material relies on the ability to produce stable colloids resistant to aggregation and non-specific adsorption of biomolecules under physiologically relevant conditions. As the particle size becomes greater, however, the increased susceptibility to surface oxidation and the larger surface area per particle present challenges to robust incorporation of these particles into the optical toolbox of nano-materials (17–20). The passivation of bare metal nanoparticle surfaces with organic ligands is important for colloid stability and minimizing non-specific adsorption of biomolecules. The attachment chemistry to the metal nanoparticle (21), coverage efficiency (22, 23), ligand length (24), and terminal group are all important control parameters (25).

Thiol groups are commonly used for attachment of small molecules to noble metal nanoparticles because chemical bonds are formed with the metal surface (26). To reduce ligand lability and reduce susceptibility to oxidative cleavage, dithiol groups have been used to increase coordination to the gold surface (21, 27, 28).

The ligand's terminal group can serve multiple roles. One of these is to increase steric and electrostatic repulsion between particles to increase colloid stability. Zwitterionic ligands, although uncharged, form a charged double layer around the nanoparticle and have been shown to yield stable colloids (29–33) under physiologically relevant conditions.

Another role for the terminal moeity is to provide an orthogonal chemistry for rational construction of biological or inorganic nanoparticles. Streptavidin, a 53 kDa tetrameric protein, binds strongly to biotin (34) and has been used to construct bio-inorganic composites for various applications (35–37).

For self-assembled monolayers on flat surfaces there exist techniques such as ellipsometry (45) and quartz crystal microbalance (46) measurements to quantify surface coverage. Nanoparticles typically display particle-to-particle variation; the extent of surface coverage (23, 47) and non-specific adsorption (40) need to be characterized to understand the scope of a new particle system. Ideally, one would desire the ligands to be resistant to non-specific adsorption and dissociation from the particle surface while also being bio-conjugatable.

Methods for the attachment of biological materials to noble metal nanoparticles are mature enough to allow the synthesis and purification of discrete 1-1 bio-conjugated nanoparticles (38). The more general topic of interfacing biological molecules with nanoparticle surfaces has been reviewed previously (39, 40). The majority of these bio-conjugation methods, however, have been applied to smaller-diameter gold nanoparticles (diameter < 40 nm). The increased surface area per particle for larger diameter noble metal nanoparticles presents challenges for non-specific absorption and colloid stability (19, 20).

Colloidal silver nanoparticles have an exceptionally strong and sharp plasmon resonance useful for different applications. Due to their utility, there have been numerous synthetic procedures using different reducing agents, such as citric acid (41), ascorbic acid (42), sodium borohydride (43), and hydrogen gas (44). On the other hand, silver nanoparticles are even more susceptible to surface oxidation than gold nanoparticles, thereby impacting on the proper preparation for biological applications. This can be overcome by synthesizing the desired silver nanoparticles in house (rather than purchasing them from commercial sources) and passivating the freshly synthesized particles immediately. Once the silver nanoparticles are properly passivated, they can be stably stored for longer period of time and still retain the reactivity for further bioconjugation.

This chapter details the practical procedures that have been routinely used in our laboratory for the preparation of nanoparticles for biological applications. The methods section is conceptually divided into three parts. The first part describes describes the synthesis of three ligands for particle passivation. The first is a compact zwitterionic ligand containing a sulfobetaine moiety derived from lipoic acid (LA). The second is a dithiol ligand with a biotin terminal group, enabling the bio-conjugation to streptavidin modified species. The third procedure describes the modification of single stranded DNA with LA, for direct bio-conjugation of DNA onto larger gold nanoparticles using EDC chemistry. The relatively low cost of aminated primers allows for the increased preparative scale necessary to passivate large gold nanoparticles with DNA. Here, amine 5'-modified ssDNA is coupled to LA and then a complementary strand, 5'-modified with biotin, is annealed to the LA-modified strand. The second part describes a silver nanoparticle synthesis using a modified ascorbic acid reduction method and nanoparticle passivation with dithiol ligands. The last part describes methods to characterize the effectiveness of the passivation. Particles are characterized by absorption spectroscopy, gel electrophoresis, and column chromatography to ensure the robustness of the passivation. It should be pointed out that the latter two characterization methods are routinely used in preparing proteins and nucleic acids; ensuring that the passivated gold and silver nanoparticles are compatible with these standard biochemical methods is a necessary step to enable subsequent manipulation of the desired hybrid nano-materials.

2 Materials

2.1 Chemicals

1. 18.2 MΩ-cm Water.
2. Room temperature is 20 °C.
3. $(\pm)\alpha$-Lipoic acid , $\geq$ 99%.
4. Thionyl chloride ($SOCl_2$), $99+\%$.
5. 3-Dimethylamino-1-propanol, 99%.

6. 1,3-Propanesultone, 99%.
7. Sodium borohydride ($NaBH_4$).
8. Chloroform ($CHCl_3$), ACS grade.
9. Acetone, HPLC grade.
10. Sodium bicarbonate.
11. Ethyl acetate (EtOAc), ACS grade.
12. Agarose, bioreagent grade.
13. New England Biolabs Tridye 1kb DNA ladder.
14. Ethanol, 200 proof.
15. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), stored in a desiccator at -20 °C.
16. Sulfo-NHS, stored in a dessicator at 4 °C.
17. Ethylenediaminetetraacetic acid (EDTA), bioreagent grade.
18. 0.1 M MES (2-(N-Morpholino)ethanesulfonic acid sodium salt) pH 6, bioreagent grade.
19. Sephacryl S-300, size exclusion resin (GE Lifesciences).
20. Chromatography media sampler pack (Bio-Rad).
21. 80 nm Gold nanoparticles (British BioCell International).
22. Silver nitrate, 99.9999%.
23. Sodium acetate, bioreagent grade.
24. Sodium citrate dihydrate, granular/certified.
25. L-Ascorbic acid, ACS grade.
26. Sodium hydroxide, ACS grade.
27. DithiolalkanearomaticPEG6-NHS (CAS# 936115-55-8 , SensoPath Technologies) (see Note 1).
28. EZ-Link Amine-PEG2-Biotin (Pierce).
29. 10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) pH 8, bioreagent grade.

2.2 Equipment

1. Aluminum foil.
2. Pasteur pipette.
3. Cotton balls.
4. Mini-tube rotator.
5. Rotary evaporator.
6. Speed-Vac, *i.e.,* Savant SVC-100 or equivalent.
7. Schlenk line.
8. Temperature-controlled water bath.
9. Three-neck jacketed round bottom flask.
10. Syringe pumps.
11. Agarose gel electrophoresis apparatus.
12. UV-Vis absorption spectrophotometer.

3 Methods

3.1 Protocol for Dihydrolipoic Acid - Sulfobetaine (DHLA-SBE) Synthesis

1. All reactions performed under N_2 with a Schlenk line, unless otherwise stated.
2. Dilute lipoic acid (1000 mg, 4.85 mmol) in 10 mL $CHCl_3$ and cool in an ice bath.
3. Dissolve $SOCl_2$ (5.34 mmol) in 2-3 mL $CHCl_3$ and add drop-wise.
4. After addition of $SOCl_2$, stir 30 minutes at room temperature.
5. Remove the solvent by rotary evaporation.
6. Re-dissolve the product in 10 mL $CHCl_3$, cool in an ice bath and purge with N_2.
7. Dilute 4.85 mmol 3-dimethylamino-1-propanol with 2-3 mL $CHCl_3$ and add drop-wise.
8. Stir the reaction vigorously overnight at room temperature.
9. Quench the reaction with $NaHCO_3$ (9.7 mmol) in 20 mL water.
10. Extract the quenched reaction with EtOAc (2x) and wash the combined organic extract with saturated aqueous $NaHCO_3$ (2x).
11. Combine organic layers, dry over $MgSO_4$ and filter.
12. Remove the solvent by rotary evaporation.
13. Dissolve the product in 10 mL acetone.
14. Dissolve 4.85 mmol 1,3-propanesultone in 2-3 mL acetone and add drop-wise at room temperature.
15. Reflux the reaction at 75 °C overnight under N_2. The final product will precipitate out of solution.
16. Filter the precipitate and wash with $CHCl_3$. Dissolve the product in water and extract the aqueous layer with $CHCl_3$. The desired product will be in the aqueous layer.
17. Filter through a pasteur pipette with a cotton plug and evaporate under reduced pressure.
18. Characterize the product. ^{1}H NMR (500 MHz, DMSO-d6): δ [ppm]: 3.46-3.50 (t, 2H), 3.38-3.43 (m, 2H), 3.28-3.35 (m, 2H), 2.98-3.06 (s, 6H), 2.77-2.85 (m, 2H), 3.46-3.50 (m, 4H), 2.19-2.23 (t, 1H), 1.87-2.08 (m, 4H), 1.79-1.86 (m, 2H), 1.27-1.63 (m, 6H). ESI MS m/z: 414.14451 (M+H)+.
19. Reduce dithiol bond with an equimolar amount of $NaBH_4$ just before use.
20. Store unreduced product as a solid in -20 °C freezer until needed. The structure shown in Figure 1 is of the product after reduction of the disulphide bond with $NaBH_4$.

3.2 Protocol for Dithiol-biotin Synthesis

1. Store 10 mg (12.6 μmol) dithiolalkanearomatic PEG6-NHS ligand at -20 °C, unopened in a dessicator until required.
2. Dissolve 6 mg (16 μmol) EZ-Link Amine-PEG2-Biotin in 500 μL 10 mM Hepes buffer at pH 8 .
3. Add solution *immediately* to freshly opened bottle of dithiolalkanearomatic PEG6-NHS (see Note 1)
4. Let mixture react for 2 hours at room temperature.
5. Store the final solution (dithiol-biotin) at -20 °C.

3.3 Preparation of Lipoic Acid Functionalized Single Stranded DNA

1. Prepare 10 mg/mL solution of lipoic acid in DMSO.
2. Equilibrate EDC and sulfo-NHS to room temperature.
3. Add 360 μL of 10 mg/mL lipoic acid (0.0174 mmol) solution to 640 μL 0.1 M MES at pH 6.
4. Add aqueous lipoic acid solution to an Eppendorf tube containing 14 mg (0.073 mmol) EDC and 22 mg (0.1 mmol) sulfo-NHS.
5. Let mixture react 5 minutes at room temperature to activate carboxylic acid.
6. Prepare 500 μL 0.5 mM aminated DNA in water and add to activated lipoic acid mixture.
7. Rotate on a mini-tube rotator at room temperature for 2 hours.
8. Ethanol precipitate DNA using 1 mL 0.3 M sodium acetate in ethanol.
9. Incubate at 4 °C for 1 hour.
10. Spin at 13,000 rpm for 1 hour at 4 °C in a microcentrifuge.
11. Carefully discard the supernatant.
12. Add 500 μL 70% ethanol, cooled to 4 °C.
13. Centrifuge the solution at 13,000 rpm for 20 minutes at 4 °C.
14. Carefully discard the supernatant.
15. Dry the DNA pellet with a Speed-Vac.
16. Dissolve the pellet in 50 μL 10 mM Hepes buffer at pH 8.
17. Quantitate the DNA by absorbance at 260 nm.
18. Dilute complementary ssDNA, 5'-modified with a biotin, in 10 mM Hepes buffer at pH 8.
19. Add an equimolar amount of complementary ssDNA to lipoic acid-functionalized single-stranded DNA.
20. Add sodium chloride to a concentration of 100 mM.
21. Heat DNA to 95 °C for 2 minutes.
22. Cool DNA on bench top at room temperature.
23. Store the lipoic acid-DNA at -20 °C until needed.

3.4 Large Gold Nanoparticle Passivation

1. Thaw DHLA-SBE from section Section 3.1, dithiol-biotin from Section 3.2, and lipoic acid-DNA from Section 3.3 to room temperature.
2. Add 10 μL of 50 mM DHLA-SBE to either 1 μL of 10 mM dithiol-biotin or 10 μL of 500 μM lipoic acid-DNA (see Note 2 and 3).
3. Add an equimolar amount of $NaBH_4$ to the mixture and incubate 5 minutes.
4. Add reduced thiols to 500 μL of 80-nm gold nanoparticle(10^{10} particles/mL).
5. Rotate the mixture on mini-tube rotator overnight at room temperature.
6. Save an aliquot of mixture for characterization by gel electrophoresis and uv-vis absorption spectroscopy.
7. Dialyze gold nanoparticles in a 300-kDa membrane for 2 hours at room temperature in 4 L water (see Note 4).
8. Exchange the 4 L water every two hours for a total of 3 exchanges.

3.5 Silver Nanoparticle Synthesis and Passivation

1. Prepare 20 mg/mL silver nitrate stock solution fresh for each synthesis.
2. Prepare 70 mg/mL trisodium citrate, 2 mg/mL ascorbic acid, and 0.1 N NaOH stock solutions.
3. Connect a 500 mL three-neck jacketed round bottom flask to a circulating water bath set to 75 °C.
4. Wrap flask with aluminum foil. An example synthesis apparatus is depicted in Figure 2.
5. Add 100 mL of ultra-pure 18.2 MΩ-cm water along with a stir bar.
6. Center the flask on a stir plate set to 750 rpm.
7. Cap the open necks of the flask with rubber stoppers.
8. Load two syringe pumps with NaOH and ascorbic acid stock solutions. Set syringe pumps to 250 μl/min for two minutes of NaOH addition, and 2 mL/min for five minutes of ascorbic acid addition.
9. Once the water reaches 75 °C, equilibrate for five minutes before adding reactants.
10. Quickly add 1 mL of silver nitrate. Wait 5-10 seconds, then quickly add 1 mL of trisodium citrate stock solution by syringe.
11. Add 500 μL of NaOH at 250 μL/min for 2 minutes and 10 mL of ascorbic acid at a rate of 2 mL/min for a total of five minutes.
12. Remove any injection needles and let react at the set temperature for 30 minutes.
13. Store product at 4 °C.
14. Spin 10 mL of 0.5 nM silver nanoparticles at 12,125 rpm for 30 minutes.
15. Remove the supernatant and re-suspend the pallet in methanol.
16. Add DHLA-SBE to a final concentration of 500 mM.
17. Rotate on a mini-tube rotator for 24 hours at room temperature.

3.6 Absorption Spectra of Large Gold Nanoparticles

1. Record uv-vis absorption spectrum for each particle sample before and after dialysis. If the particles are stable and monodisperse, they should still retain their characteristic plasmon band. If the particles are incompletely passivated, then the absorption spectra may display a large shift of the plasmon resonance and characteristic increase in near infra-red absorption (48). A representative figure comparing particle aggregation after dialysis is shown in Figure 3.

3.7 Gel Electrophoresis of Large Gold Nanoparticles

1. Cast a 0.5% 0.5X TBE agarose gel. Use a comb with smallest teeth available. The teeth of the comb used in this protocol are 3 mm x 1.5 mm x 10 mm (see Note 5).
2. Mix 15 μL gold nanoparticle sample with 5 μL 15% Ficoll solution as the loading buffer.
3. Load samples into the gel with 0.5X TBE as the running buffer. Reserve a lane in the gel for a DNA marker such as New England Biolab's Tridye 1 kb DNA marker. The Tridye marker contains three organic dyes which are a good visualization tool to monitor the progress of the gel (see Note 6).
4. Run samples at a field strength of 6.5 V/cm (see Note 7).
5. Record a brightfield image of the particle on a lightbox. The large gold nanoparticles, if properly passivated, should run as a tight pink band. A representative gel is seen in Figure 4.

3.8 Column Test for Large Gold Nanoparticles

1. Prepare 10 mL of a 50% slurry of Sephacryl S-300 size exclusion resin with 18.2 MΩ-cm water (see Note 8).
2. Pack a glass drip column (1.5 cm inner diameter) with 5 mL of slurry (see Notes 9 and 10).
3. Equilibrate the column with two column volumes of water.
4. Let the water drip through column until the meniscus of the mobile phase reaches the top of the packed resin bed.
5. Once the water has reached the top of the column, carefully load 500 μL of passivated particle sample from Section 3.4 to the top of the column.
6. Let gold sample move into the column.
7. After the gold has entered the top of the resin bed, carefully wash the column with enough water to wash the particles through the columnn. Incompletely passivated gold nanoparticles will remain at the top of the column, such as in Figure 5.

8. Repeat column test with other anion exchange, cation exchange, DEAE, and hydroxyapatite resins from Bio-Rad's chromatography media sampler pack to determine scope of usable resins with the particle system. The passivated particles should not aggregate at the tops of the columns.

4 Notes

1. The NHS ligand is very reactive and will hydrolyze quickly if not used immediately. Store at $-20\ ^{\circ}\mathrm{C}$ and use *immediately* after opening. Do not store unreacted stock solutions.
2. Nanoparticles passivated by lipoic acid derived ligands appear to be most stable when a mixture of at least two ligands are used. In the presented protocol, DHLA-SBE is mixed with a dithiol biotin ligand. Other ligands, such as lipoic acid modified peptides, can also be used.
3. Optimizing the ratio of the relative ligands can help with particle stability. For large gold nanoparticles, millimolar concentrations of ligands are necessary to ensure adequate passivation.
4. Excess DNA can be difficult to remove by dialysis. Using a Sephacryl S-300 size exclusion column described in Section 3.8 to separate large gold nanoparticles from unbound DNA. The large gold nanoparticles will be eluted in the excluded volume limit, whereas the DNA will enter the resin.
5. When casting a gel, combs with narrow teeth are useful for visualizing dilute samples. The path length of light passing through the sample increases with the total sample height in a transillumination configuration.
6. DNA can be visualized by ethidium bromide staining. Unlike small gold nanoparticles, large gold nanoparticles show some increased scattering by uv transillumination. Visualize the gel by uv transillumination before staining with ethidium bromide to account for the particle scattering signal.
7. Setting the voltage too high will cause particles to streak in the gel.
8. The pore size of the Sephacryl S-300 resin is such that nanoparticles greater than approximately 60 nm will run in the excluded volume limit. Particles smaller than this limit, such as quantum dots and DNA molecules $\leq$ 90bp can be resolved by the resin.
9. Use the minimal amount of resin necessary to achieve desired separation. Excessively large columns can dilute the recovered sample and make reconcentrating the sample difficult.
10. Acetone has a strong uv absorbance and can be used to determine the retention limit of a freshly packed column. One can use passivated large gold nanoparticles to determine the excluded volume limit.

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Fig. 1 The chemical structure of dihydrolipoic acid - sulfobetaine (DHLA-SBE).

Fig. 2 Setup of the apparatus used for silver nanoparticle synthesis. Two syringe pumps are connected to a 500-mL thermally jacketed three-neck round bottom flask. The flask is placed on top of a stir plate. The temperature of the water flowing through the water-jacket is 75 °C.

Fig. 3 The absorption spectrum of 80-nm gold nanoparticles passivated with a monothiol ligand (red squares) taken after dialysis shows increased near-infrared absorption with respect to the unpassivated particles before dialysis (black curve). The near-infrared absorption absorption band is indicative of aggregation. The spectrum taken after dialysis of particles passivated with DHLA-SBE (green circles) do not show the same increase. All spectra are normalized to the absorption maximum at the plasmon resonance wavelength.

Fig. 4 The brightfield illuminated (A), UV illuminated (B), and overlaid images (C) of a 0.5% 0.5X TBE agarose ethidium bromide stained gel. Lane 1 is New England Biolab's Tridye 1kb DNA ladder while lanes 2 and 3 are the passivated and unpassivated particles, respectively. The passivated particles (red arrow) move with quickly into the gel while the unpassivated particles (black arrow) remain near the load well after 35 minutes at 6.5 V/cm.

Fig. 5 Incompletely passivated large gold nanoparticles will become immobilized at the top of various liquid chromatography resins. Here, unpassivated 80 nm diameter gold nanoparticles have become immobile on a Sephacryl S-300 column.