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Preparation of 2 nm gold nanoparticles for *in vitro* and *in vivo* applications

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Summary

Gold nanoparticles have been a versatile tool in recent years for the exploration of biological systems. However, challenges with purification and adequate surface coverage limit the biocompatibility of gold nanoparticles. Here, we describe a detailed procedure for the synthesis, purification, and functionalization of biologically compatible gold nanoparticles for *in vitro* and *in vivo* studies.

Keywords

Gold nanoparticle; biocompatibility; purification

1. Introduction

Gold nanoparticle (AuNP) properties and applications continue to be the focus of variety of fields since their discovery in 1857 by Michael Faraday (1). Among these areas, avid research has been pointed towards the creation of biocompatible nanoparticles (2). The precise control introduced by the two-phase Brust-Schiffrin method (3,4) allowed more systematic studies of nanoparticle properties to be performed. However, the use of tetra-n-octylammonium bromide as the phase transfer catalyst limits biological applications due to the difficulty in separating this high cytotoxic chemical from the nanoparticles (5,6). Additionally, the functionality of nanoparticle surfaces has been a prominent concern due to the direct correlation of cytotoxicity with the functionality of the surface monolayer (7). To further improve nanoparticle utility, a fine balance between hydrophobic functionalization near the metallic core to confer monolayer stability (8) and non-fouling character provided by poly(ethylene glycol) moieties must be achieved (9). An excellent ligand base for biological applications is 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol (Figure 1) as its components confer the desired stability and non-fouling properties (10). This structure can be easily modified to provide *selective* binding making these nanoparticles a promising platform for systematic biological studies (11). Employing these concepts, this protocol elaborates on the synthesis, purification and functionalization of gold nanoparticles with the biocompatibility required for *in vivo* and *in vitro* studies.

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2. Materials

1. Hydrogen tetrachloroaurate (III) hydrate.
2. Tetra-n-octylammonium bromide (TOAB).
3. 1-Pentanethiol.
4. Sodium borohydride.
5. 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol, synthesized according to the reported procedure (12).
6. 200 proof ethanol, acetone, hexanes and acetonitrile.
7. 10,000 MWCO snakeskin pleated dialysis tubing membrane.
8. 3.0 cm Grade 1 filter paper circles.

3. Methods

All procedures are done at room temperature unless otherwise specified.

3.1. Synthesis of gold nanoparticles (from the Brust-Schiffrin method) (3)

1. Pour 1.0 g (2.5 mmol, 1 eq) of hydrogen tetrachloroaurate (III) hydrate and 150 mL of Type I ultrapure water into a 1000 mL round bottom flask. Stir slowly for 5 minutes using a 2 inch egg-shaped magnetic stir bar until complete dissolution. Solution should be clear yellow (*see Note 1*).
2. Premix 2.1 g (3.8 mmol, 1.5 eq) of tetra-n-octylammonium bromide in 150 mL of toluene and add it to the gold solution in the round bottom flask. Stir at maximum speed for 15 minutes until solution is a cloudy dark orange color (*see Note 2*).
3. While continuing to rapidly stir, cover the round bottom flask with a septum stopper and using a syringe add dropwise 0.7 mL (5 mmol, 2 eq) of 1-pentanethiol over the course of 15 minutes. Stir until the solution presents a cloudy deep white color (*see Note 3*).
4. Remove the stopper and quickly add a freshly prepared solution of 2.0 g (50 mmol, 20 eq) of sodium borohydride in 10 mL of Type I ultrapure water. Stir the solution for 5 hours (*see Note 4*).

¹Weigh the gold salt using a plastic spatula, not a metallic one that can potentially reduce gold salts. Make sure all the gold salt is dissolved.

²Stirring is one of the key components of the process. Maintain fast stirring for the rest of the synthesis, using the recommended stir bar.

³Do not add more 1-pentanethiol to achieve the white color. Wait a prudent amount of time (20 minutes) and the color should appear, otherwise some of the components are impure and the process should be restarted with new materials.

⁴A black color should appear as soon as the sodium borohydride is added. If it takes more than 3 seconds to appear, the process should be started again and the solution discarded as slow appearance of the black color will indicate polydisperse product.

5. Using a separatory funnel, remove the water phase (colorless) from the toluene solution (black). Evaporate the majority of the solvent under reduced pressure at 40 °C (*see Note 5*).
6. Add 900 mL of 200 proof ethanol and mix until the solid is completely dispersed. Store the flask at –20 °C.

3.2. Purification of gold nanoparticles

1. Two days after the synthesis, the nanoparticles precipitate and a black solid is observed at the bottom of the flask. Carefully remove the ethanol solution (brown color) without dispersing the precipitate (*see Note 6*).
2. Add fresh 200 proof ethanol and mix the solution until the nanoparticles are dispersed. Leave the solution at –20 °C until precipitation is complete (*see Note 7*).
3. Repeat the first and second steps until the ethanol solution is colorless after precipitation (approximately 5 times) (*see Note 8*).
4. Remove the solvent and redisperse the precipitate in 10 mL of 200 proof ethanol. Sonicate the solution for 10 minutes.
5. Using a 3.0 cm grade 1 filter paper circle over a 25 mm filter holder (No. 5 stopper) attached to a vacuum line, wash the nanoparticles several times with 200 proof ethanol redispersing the precipitate in each wash (*see Note 8*).
6. Recover the solid from the filter paper.
7. Dissolve the nanoparticles in a minimal amount of toluene and analyze the pentanethiol-capped AuNPs using mass spectrometry (LDI) paying particular attention for the presence of a peak at 466 m/z (TOAB). If the peak is present repeat steps 5 and 6 until it is no longer observed (*see Notes 9 and 10*) (13).
8. Use transmission electron microscopy (TEM) to obtain the average particle diameter and use nuclear magnetic resonance (solvent - CDCl₃) to confirm purity.

3.3. Functionalization of gold nanoparticles (from the Murray place exchange) (14)

1. Dissolve 30 mg of the purified pentanethiol-capped AuNPs in 10 mL of dry dichloromethane and bubble the solution with nitrogen for 5 minutes.

⁵No more than 40 °C should be used, otherwise nanoparticles will grow larger and polydispersity will be observed.

⁶If there is no observable precipitation (solution too dark), check with a glass pipet to observe if the solution is clear or still cloudy. In case of a cloudy solution, let sit at –20 °C for another day.

⁷No sonication should be used in the first cleanings.

⁸Acetone can be used for faster precipitation/filtration.

⁹If TOAB is still present after several cleanings, pour the solid nanoparticles into a 1000 mL round bottom flask, add 5 mL of toluene to dissolve (brownish color appears), and add 900 mL of ethanol (5). Repeat the process from step 1 (this procedure can be repeated until TOAB is removed).

¹⁰If TOAB is still present, an alternative procedure for cleaning is the use of acetonitrile. Dissolve the AuNPs in hexanes and extract multiple times with acetonitrile (AuNPs remain in upper hexanes phase and the TOAB transfers to the lower acetonitrile layer). Collect the hexanes phase and evaporate the solvent at reduce pressure (<40°C).

2. Separately dissolve 150 mg of 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol (or the ligand of interest) in 10 mL of dichloromethane and bubble with nitrogen for 5 minutes.
3. Mix the two solutions in a 50 mL round bottom flask, cap with a plastic stopper, and stir (0.5" octagonal magnetic stir bar) for 2 days.
4. Remove the solvent under reduced pressure and redissolve the now functionalized nanoparticles in 10 mL Type I ultrapure water, obtaining a brownish colored solution.
5. Purify the solution in 10,000 MWCO snakeskin pleated dialysis tubing membrane by dialyzing the system for 5 days using deionized water in a 5000 mL polypropylene beaker (see **Note 11**).
6. Recover the AuNP solution from the dialysis bag and lyophilize the nanoparticles. A black solid is obtained.
7. Dissolve a small amount of the solid in D₂O (for NMR) and the rest in 2 mL Type I ultrapure water.
8. Calculate the concentration according to the reported extinction coefficient methodology using the size value obtained from TEM (15).
9. Store at 4 °C.

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¹¹During the first day water should be changed every two hours, the second and third day every 6 hours, and during the last three days every 12 hours.

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Fig. 1. Gold nanoparticle chemical structure. The ligand is composed of three parts namely the sulfur atom (binding with gold), alkyl chain (nanoparticle stability) and a tetraethylene glycol spacer (biocompatibility and solubility).