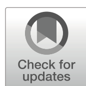

Chapter 6

Conjugation of Nucleic Acids and Drugs to Gold Nanoparticles

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Abstract

Gold nanoparticles (AuNPs) can be used as carriers for biomolecules or drugs in cell culture and animal models. Particularly, AuNPs ease their internalization into the cell and prevent their degradation. In addition, engineered AuNPs can be employed as sensors of a variety of biomarkers, where the electronic and optical properties of the AuNPs are exploited for a convenient, easy, and fast read out. However, in all these applications, a key step requires the conjugation of the different molecules to the nanoparticles. The most common approach exploits the great affinity of sulfur for gold. Herein, we summarize the methods used by our group for the conjugation of different molecules with AuNPs. The procedure is easy and takes around 2 days, where the reagents are slowly added, following an incubation at room temperature to ensure the complete conjugation. Finally, the unbound material is removed by centrifugation.

Key words Gold nanoparticles, Spherical nucleic acid, Functionalization, Oligonucleotides, Nanomedicine, Metal nanoparticles, Conjugation, Drug delivery, Sensors

1 Introduction

Oligonucleotides and drugs face some challenges for their optimal delivery in cells and animal models. Particularly, oligonucleotides (e.g., antisense, gapmers, and siRNAs) usually present low stability and suffer from reduced cell internalization and selectivity [1, 2] and, for these reasons, transfection reagents such as lipofectamine are usually employed to improve delivery. On the other hand, drugs can be too hydrophobic and require solubilizing molecules (e.g., dimethylsulfoxide [DMSO], ethanol). However, these kinds of chemicals present critical restrictions such as cytotoxicity or limited loading. To overcome these drawbacks, delivery systems based on nanoparticles can be employed [3]. There are different types of nanoparticles such as liposomes, micelles, dendrimers, inorganic

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particles, carbon-based nanostructures, viral nanocarriers, polymeric, peptide or metallic nanoparticles, etc. [4–12]. Each vehicle presents different characteristics that can be exploited to address specific challenges related to the delivery of bioactive molecules.

Among the different systems, gold nanoparticles (AuNPs) present excellent properties for the delivery of oligonucleotides because of their low toxicity, cost, and particularly their ease of preparation and functionalization [13]. AuNPs can be synthesized in the laboratory through simple methods, such as the one described by Turkevich [14] and detailed in Subheading 3.1.

The properties of AuNPs can be tuned through their modification with oligonucleotides. When the nanoparticles are densely loaded with oligonucleotides, the resulting nanostructures are known as spherical nucleic acids (SNA) [15]. This kind of nanostructure presents interesting features, such as high internalization in a wide variety of cells and low toxicity. Therefore, these derivatives can be employed for multiple applications, such as drug delivery systems, gene therapy and regulation, or molecular diagnosis [16, 17].

Regarding the vehiculization of therapeutics, AuNPs can be used for the delivery of hydrophobic drugs such as paclitaxel, doxorubicin, or AZD8055 without affecting their effectiveness [18, 19]. On the other hand, AuNPs functionalized with oligonucleotides (e.g., siRNAs, gapmers) could be used as a substitute for transfection reagents in different applications involving gene regulation, or even immunomodulatory processes, for the treatment of diseases such as cancer, sepsis, skin disorders, diabetes, etc. [16, 20–22].

In the case of diagnostics, it is worth mentioning that fast and accurate point-of-care diagnostic systems are critical in personalized medicine. In particular, nucleic acid detection is of great importance for the diagnosis and treatment of many diseases caused by genetic mutations, infectious agents, or other physiologically abnormal circumstances. Conventional methods such as RT-PCR offer high accuracy and sensitivity; however, these methods are not suitable for routine diagnosis because they are time-consuming and need highly trained personnel and expensive equipment. One development that seems to simplify the nucleic acid detection and we study in the lab is the use of SNA based on a single-stranded oligonucleotide with a unique stem-loop structure (Molecular Beacon, MB) [23, 24].

This chapter describes how to conjugate drugs or oligonucleotides to AuNPs, which can be further used as delivery systems of therapeutics and sensors.

To attach any compound to AuNPs, the high affinity of thiol groups to gold could be exploited. Thus, the molecules (e.g., oligonucleotides, drugs) should be functionalized with linkers

containing sulfur-based moieties, such as thiols or dithiolanes [25], which are commented in this chapter.

AuNPs conjugation requires a few simple steps of addition, incubation, and washes. The method might change slightly depending on the linker employed for the conjugation, which can be designed to control the release or stability of the cargo. In general, the use of dithiolane provides more robust structures and can be achieved in few hours, whereas the use of thiols implies more than 1 day. For the reader's convenience, we have included the preparation of the dithiolane linkers used in our group. The approach can be used for the conjugation of drugs, polymers (e.g., polyethylene glycol [PEG]), or the preparation of oligonucleotides in a DNA synthesizer using a tailored solid support, usually based on controlled pore glass (CPG).

2 Materials

2.1 AuNP Synthesis

2.1.1 Materials

- 250 mL round-bottom glass flask.
- Septum for a 250 mL round-bottom flask.
- 3.5 cm long magnet.
- 0.3 μm fritted filter for vacuum filtration.
- 250 mL Erlenmeyer flask with an output for vacuum.
- Plastic material such as conical centrifuge tubes and microcentrifuge tubes.
- 1 mL quartz cuvette.

2.1.2 Reagents

- Gold solution: 945.2 μM Hydrogen tetrachloroaurate (III) hydrate ($AuCl_4H_3O$) in 100 mL autoclaved Milli-Q grade water.
- Ultrapure reagent-grade water.
- Sodium citrate solution: 40 mM sodium citrate tribasic dihydrate (118 mg) in 10 mL autoclaved Milli-Q grade water.

2.1.3 Equipment

- Hot plate (7 cm radius) with magnetic stirring.
- Reflux column.
- Vacuum pump.
- UV-Vis spectrophotometer.

2.2 Dithiolane-Based Linkers Synthesis

2.2.1 Materials

- 50 mL round-bottom flask.
- 2-cm long magnet.
- 1 septum.
- Thin-layer chromatography (TLC) (sheets of silica gel 60F254).
- Filter paper.
- 2000 KDa tubing membrane.

2.2.2 Reagents

- Lipoic acid.
- *N*-hydroxysuccinimide (NHS).
- Tetrahydrofuran (THF).
- *N*,*N*′-dicyclohexylcarbodiimide (DCC).
- Ethyl acetate (AcOEt).
- Methoxypolyethylene glycol amine (PEG-NH_2).
- Drug with a primary amine (e.g., Gemcitabine).
- Dimethylformamide (DMF).
- Dichloromethane (CH_2Cl_2).
- Methanol (MeOH).
- Threoninol.
- 4,4′-Dimethoxytrityl chloride (DMTrCl).
- Dry pyridine (Py).
- Hexane.
- Succinic anhydride.
- Dry CH_2Cl_2.
- 4-(Dimethylamino)pyridine (DMAP).
- *N*,*N*-Diisopropylethylamine (DIPEA).
- Distilled water.
- Magnesium sulfate anhydrous ($MgSO_4$).
- 1-Hydroxybenzotriazole (HBO^t).
- Acetonitrile (MeCN).
- CPG: Aminopropyl-CPG, 1000 Å.
- Caping reagent A (CAP A): THF/pyridine/acetic anhydride (8:1:1).
- Caping reagent B (CAP B): 10% Methylimidazole in THF.

2.2.3 Equipment

- Flash column chromatography using silica gel (60 Å, 230 × 400 mesh).
- Rotavapor.

2.3 Functionalization of AuNPs

2.3.1 Materials

- Plastic material: one microcentrifuge tube per condition.
- A 96-well plate for absorbance measurements in a plate reader.

2.3.2 Reagents

- Oligonucleotides with sulfide-based modifications at micromolar concentration.
- Annealing buffer 3×: 30 mM Tris-HCl, 3 mM EDTA, 150 mM NaCl.
- Tris (2-carboxyethyl) phosphine hydrochloride solution (TCEP).
- Gold nanoparticles (AuNP) with a diameter of 13 ± 2 nm (*see* Subheading 3.1).
- Sodium chloride solution: 5 M NaCl.
- Oligonucleotide quantification kit (e.g., Quant-iT™ OliGreen™ ssDNA Assay Kit, Qubit™ ssDNA Assay Kit), including the Quant-iT OliGreen® ss DNA Reagent, TE 20× buffer, and oligonucleotide standard.
- Autoclaved Milli-Q grade water.
- PEG modified with a dithiolane group (*see* Subheading 3.2.2).
- Chemotherapeutic drugs with dithiolane-based linker (e.g., gemcitabine) (*see* Subheading 3.2.3).

2.3.3 Equipment

- Benchtop centrifuge.
- Vortex mixer.
- Orbital shaker.
- Plate reader suitable for absorbance and fluorescence determinations using 96-well plates.
- Evaporating centrifuge.

3 Methods

3.1 AuNP Synthesis

For the preparation of AuNP (13 ± 2 nm) the Turkevich method is used [14] as follows:

1. Turn on the heating plate to 140 °C.
2. Add 100 μL of $HAuCl_4$ solution (94.52 μmol) in a 250 mL round bottom flask containing 100 mL of sterile water.
3. Add the magnet to the round bottom flask.
4. Place the round bottom flask in the heating plate while stirring at 700 rpm approximately, with a reflux system, and heat it to reflux.

5. Prepare the sodium citrate tribasic dihydrate solution in a 50 mL conical centrifuge tube.
6. When the mixture boils, add the citrate solution quickly while stirring at 700 rpm.
7. Wait for 15 min and then remove the round bottom flask from the heating plate. During this period, the color of the solution turns from yellow to red (*see* **Note 1**).
8. Leave the mixture stirring at 300 rpm at room temperature and protect from the light for 16 h.
9. Filter the solution using a 0.3 μm fritted filter with the help of vacuum.
10. Determine nanoparticles' size: for proper characterization of gold nanoparticles, the size should be measured by TEM and the concentration determined by UV-Vis spectrophotometry using the Beer-Lambert law. It requires measuring the UV-Vis absorbance at 520 nm and using the corresponding extinction coefficient (ε) for its size [26]. For instance, to determine the concentration of 13 ± 1 nm AuNPs, which have an extinction coefficient (ε) of 2.7×10^8 M^{-1} cm^{-1}, you should use the following equation (Eq. 1):

$$\text{concentration} = \frac{A}{\varepsilon \times l} \tag{1}$$

where A is the absorbance at 520 nm, l is the optical path in cm, and ε extinction coefficient in M^{-11} cm^{-1}.

3.2 Dithiolane-Based Linkers Synthesis

The preparation of the dithiolane-based derivatives of drugs (**3**) and PEG (**2**) is summarized in Fig. 1 and described in the following instructions. In the case of oligonucleotides, the required solid support (CPG) containing a dithiolane moiety for the preparation of oligonucleotides is also described (**7**).

3.2.1 Compound 1 [2,5-Dioxopyrrolidin-1-yl(R)-5-(1,2-Dithiolan-3-yl) Pentanoate]

1. Dissolve lipoic acid (1 equiv.) and *N*-hydroxysuccinimide (1.2 equiv.) in tetrahydrofuran (0.5 M).
2. Stir the solution at 0 °C for 10 min.
3. Dissolve *N*,*N*′-dicyclohexylcarbodiimide (1.2 equiv.) in tetrahydrofuran (3.5 M) and add it slowly to the lipoic acid and *N*-hydroxysuccinimide solution obtained in **step 1**.
4. Stir the reaction at room temperature for 5 h.
5. Filter the mixture using a filter paper and wash the solid with cold ethyl acetate. Evaporate the solvent under vacuum to obtain compound **1** as a yellow oil.

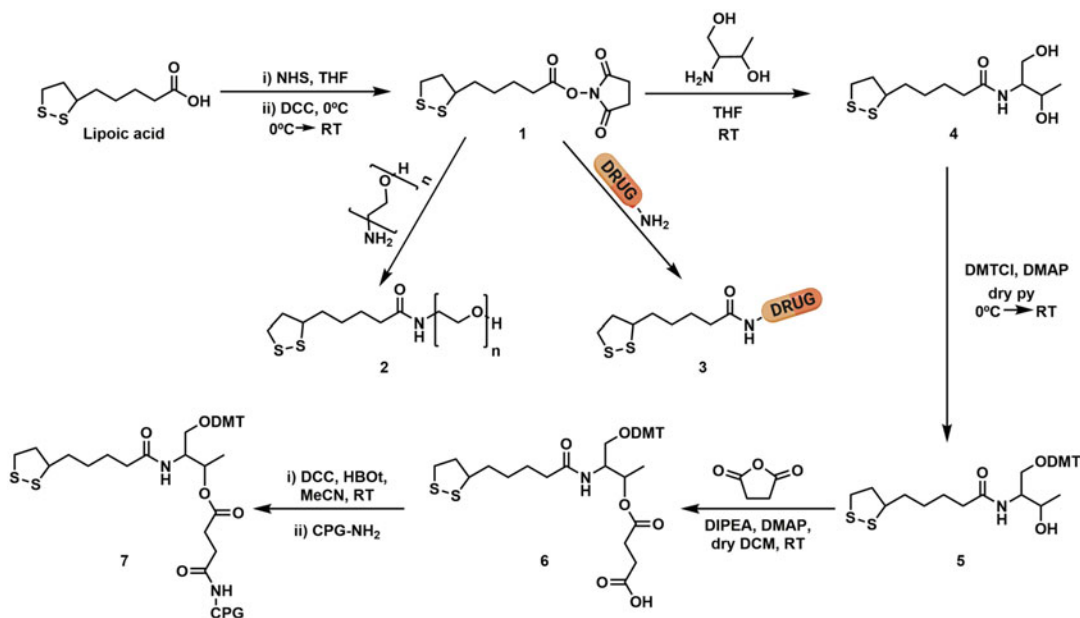

Fig. 1 Schematic representation of the synthesis of dithiolane-modified products: PEG, drug, and CPG

*3.2.2 PEG (**2**)*

1. Dissolve compound **1** (2 equiv.) and PEG-NH_2 (1 equiv.) in tetrahydrofuran.
2. Stir the reaction at room temperature for 18 h.
3. Purify the crude by dialysis with a 2000 KDa tubing membrane.
4. Stir the solution for 18 h to obtain compound **2**.

*3.2.3 Drug-Modified Linker (**3**)*

1. Dissolve compound **1** (2 equiv.) and a drug containing a primary amine (e.g., Gemcitabine) (1 equiv.) in dimethylformamide (0.1 M).
2. Stir the reaction at room temperature for 24 h.
3. Eliminate the solvent in vacuum.
4. Purify the crude by flash chromatography (CH_2Cl_2:MeOH/ 25:1) to obtain compound **3.**

*3.2.4 Compound **4**: N-(1,3-Dihydroxybutan-2-yl)-5-(1,2-Dithiolan-3-yl) Pentanamide*

1. Dissolve compound **1** (1 equiv.) and threoninol (1.1 equiv.) in THF (0.15 M).
2. Stir the solution at room temperature for 18 h.
3. Eliminate the solvent in vacuum.
4. Purify the crude by flash chromatography (CH_2Cl_2:MeOH/ 25:1) to obtain compound **4** as a yellow oil.

3.2.5 Compound **5**: N-(1-(bis(4-Methoxyphenyl)(Phenyl)Methoxy)-3-Hydroxybutan-2-yl)-5-(1,2-Dithiolan-3-yl) Pentanamide

1. Dissolve compound **4** (1 equiv.) in dry pyridine (0.3 M).
2. Dissolve DMTrCl (1.2 equiv.) in dry pyridine (1 M) and add to the compound **4** solution in dry pyridine at 0 °C.
3. Stir the reaction at 0 °C for 30 min, then at room temperature for 18 h.
4. Eliminate the solvent in vacuum.
5. Purify the crude by flash chromatography (Hexane:AcOEt/1:1) to obtain compound **5** as a beige foam.

3.2.6 Compound **6**: 4-((3-(5-(1,2-Dithiolan-3-yl) Pentanamido)-4-(bis(4-Methoxyphenyl)(Phenyl) Methoxy)Butan-2-yl)oxy)-4-Oxobutanoic Acid

1. Dissolve compound **5** (1 equiv.), DMAP (0.1 equiv.) and DIPEA (1.4 equiv.) in dry CH_2Cl_2 (0.13 M).
2. Dissolve succinic anhydride (1.3 equiv.) in dry CH_2Cl_2 (0.3 M) and add the solution slowly to the mixture prepared in the previous step at 0 °C.
3. Stir the reaction at room temperature for 18 h.
4. Wash the solution with water 3 times.
5. Dry the organic layer with $MgSO_4$.
6. Eliminate the solvent in vacuum to obtain compound **6**.

3.2.7 Compound **7**: 4-((3-(5-(1,2-Dithiolan-3-yl) Pentanamido)-4-(bis(4-Methoxyphenyl)(Phenyl) Methoxy)Butan-2-yl)oxy)-4-Oxobutanamide CPG

1. Dissolve compound **6** (1 equiv.) in MeCN (0.05 M).
2. Dissolve DCC (1 equiv.) and HBO^t (1 equiv.) in MeCN (0.2 M) and add to compound **6** solution at room temperature for 3 h.
3. Filter the solution with filter paper and add to the CPG (5 equiv. in mg).
4. Stir the mixture for 3 h at room temperature.
5. Remove the solvent and wash the CPG with MeOH three times and with CH_2Cl_2 three times.
6. Dry the CPG.
7. Add a mixture of capping reagents [CAP A:CAP B (1:1)] (1 mL per 175 mg of CPG).
8. Stir the solution for 1 h at RT.
9. Remove the solvent and wash the CPG with MeOH three times and with MeCN three times.

3.3 AuNP Functionalization with Thiol-Modified Oligonucleotides

Oligonucleotides can be easily attached to AuNPs using a thiol-based linker, which is commercially available, and most oligonucleotide providers offer this modification. However, the thiol group should be deprotected, as detailed below, before incubating the oligonucleotides with AuNPs (Fig. 2).

1. Incubate the oligonucleotide with TCEP (*see* **Note 2**) using a 100× excess relative to the oligonucleotide's thiol (*see* **Note 3**)

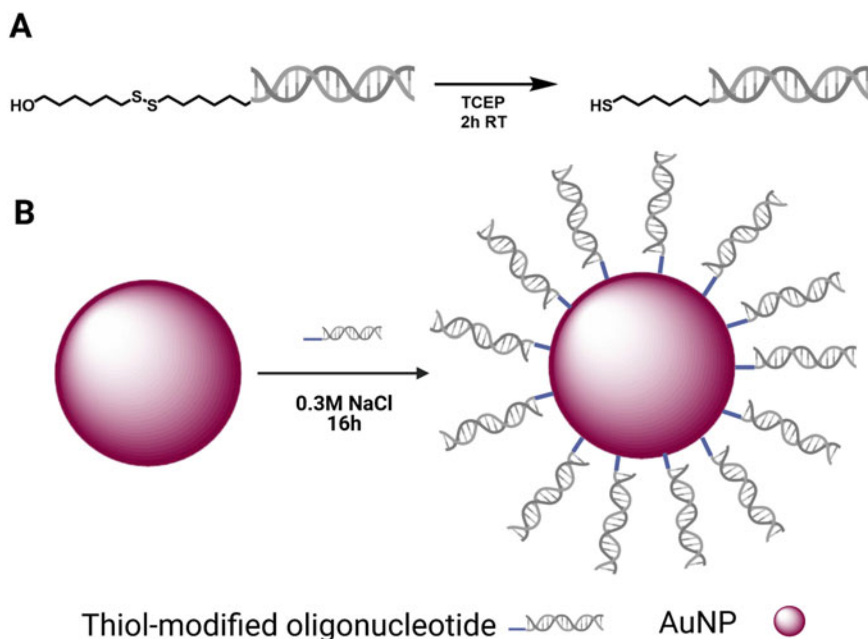

Fig. 2 Schematic representation of: (**a**) Deprotection of oligonucleotides bearing a thiol moiety. (**b**) Functionalization of AuNPs with thiol-modified oligonucleotides

for 2 h at room temperature and moderate agitation on a mini-shaker (e.g., for deprotect 500 μL of an oligonucleotide solution at 20 μM (i.e., 20 pmol/μL) use 2 μL of TCEP at 0.5 M) (*see* **Note 4**).

2. Add the deprotected oligonucleotide slowly to the AuNP solution prepared at 12 nM.
3. Incubate the mixture for 45 min at room temperature and moderate agitation.
4. Add 60 μL NaCl solution to a final concentration of 0.3 M through the addition of small volumes (e.g., 5–10 μL) (*see* **Note 5**). Vortex the solution quickly after each addition of NaCl solution and incubate the sample for at least 10 min on a mini-shaker between each addition.
5. Incubate the sample for 16 h at room temperature on a mini-shaker with moderate agitation.
6. Remove any unbound material by centrifugation at 13.2 rpm and 4 °C for 30 min. After the centrifugation, remove the supernatant and save it for later use. The pellet should be resuspended by vortexing in sterile water using the same volume of water removed to keep the concentration constant. Repeat the cleaning **step 3** times (*see* **Note 6**).
7. Evaporate the collected supernatants and use the Quant-iT™ OliGreen™ ssDNA Assay Kit protocol to determine the

unbound oligonucleotide from the solution (*see* Subheading 3.4) (*see* **Note 7**).

3.4 Oligonucleotide Quantification

For the quantification of oligonucleotides in the supernatant, use an oligonucleotide quantification kit. In this case, the Quant-iT OliGreen kit is used. The general procedure is as follows.

3.4.1 Standard Curve Preparation

1. Prepare a standard curve for each oligonucleotide using at least 5 dilutions of the specific oligonucleotide in TE buffer (1×), e.g., 0, 1000, 750, 500, 250 ng/mL.
2. Prepare a solution of Quant-iT OliGreen® ss DNA Reagent (2 μg/mL) in TE (1×).
3. Mix each oligonucleotide dilution from **step 1** with 1 mL Oligreen solution prepared in **step 2**. Incubate the solution for 5 min at room temperature protected from light.
4. Take 200 μL of each solution prepared in **step 3** and measure it in a plate reader (excitation 480 nm, emission 520 nm).
5. Plot the data in a concentration (ng/mL, *X*-axis) vs. absorbance (a.u., *Y*-axis) graph. Fit the data to a simple linear regression model and use this equation to calculate future concentrations.

3.4.2 Oligonucleotide Quantification in AuNPs

1. Evaporate to dryness the supernatant collected during the cleaning of modified AuNPs (*see* **step 7** in Subheading 3.3).
2. Resuspend the pellet in 1 mL TE (1×).
3. Prepare a solution of Oligreen reagent (2 μg/mL) in TE (1×).
4. Mix the resuspended supernatant with 1 mL Oligreen solution (*see* **step 2** in Subheading 3.4.1). Incubate the solution for 5 min at room temperature protected from light.
5. Take 200 μL of each solution prepared in **step 4** and measure it in a plate reader (excitation 480 nm, emission 520 nm).
6. Interpolate the data obtained in the standard curve equation (*see* **step 5** in Subheading 3.4.1) to determine the amount of unbounded oligonucleotide.

3.5 AuNP Functionalization with Dithiolane-Modified Oligonucleotides or Drugs

Oligonucleotides could be attached to AuNPs in a faster way using a dithiolane-based linker, which does not require a deprotection step, as in the case of thiols (Fig. 3).

3.5.1 AuNP Functionalization with Dithiolane-Modified Oligonucleotides

1. Add the oligonucleotide to 1 mL 12 nM of 13 ± 2 nm gold nanoparticles (AuNP) (*see* **Note 3**).
2. Incubate the solution for 15 min at room temperature on a mini-shaker at a moderate speed.
3. Add 60 μL NaCl solution to a final concentration of 0.3 M through the addition of small volumes (e.g., 5–10 μL) (*see*

Note 5). Vortex the solution quickly after each addition of NaCl solution and incubate the sample for at least 10 min on a mini-shaker between each addition.

4. Incubate the sample for 4 h at room temperature on a mini-shaker at a moderate speed.
5. Continue with the washing steps, as described previously (*see* **step 6** in Subheading 3.3) and the quantification of the unbound material (*see* Subheading 3.4) (*see* **Notes 6** and 7).

3.5.2 AuNP Functionalization with Dithiolane-Modified Drugs

In this case, the drugs have to be modified with a dithiolane-based linker (Fig. 4), and the AuNPs should be stabilized with oligonucleotides or PEG containing a sulfide-based linker. In this case, for 1 mL of 13 ± 2 nm AuNP (12 nM) add at least 2000 pmol of stabilizing agent (e.g., PEG, oligonucleotide) and then the required amount of the modified drug for a total of 10,000 pmol (stabilizing agent + drug) in the solution.

1. Add the stabilizing agent and incubate it for 15 min at room temperature on a mini-shaker at a moderate speed.
2. Add the modified drug very slowly and incubate it for 16 h at room temperature on a mini-shaker at a moderate speed (*see* **Note 5**).
3. Wash the nanoparticles as described in **step 6** in Subheading 3.3 (*see* **Note 6**).
4. Evaporate the collected supernatant to the initial volume (1 mL) to quantify the drug and determine the nanoparticles' loading.
5. Measure the supernatant in a spectrophotometer (according to the specific absorbance of the drug) and calculate the unbound drug using the Beer-Lambert formula (*see* **Note 8**).

4 Notes

1. AuNP solution should be kept in darkness.
2. Keep TCEP under an inert atmosphere to prevent its oxidation. Once opened, store the compound in 20 μL aliquots at −20 °C.
3. Duplexes should be annealed from their corresponding oligonucleotides before conjugation. In short, combine equal concentration and volume of each strand and add the same volume of annealing buffer (3×). Then, incubate the sample at 95 °C for 10 min and leave to cool slowly to room temperature.

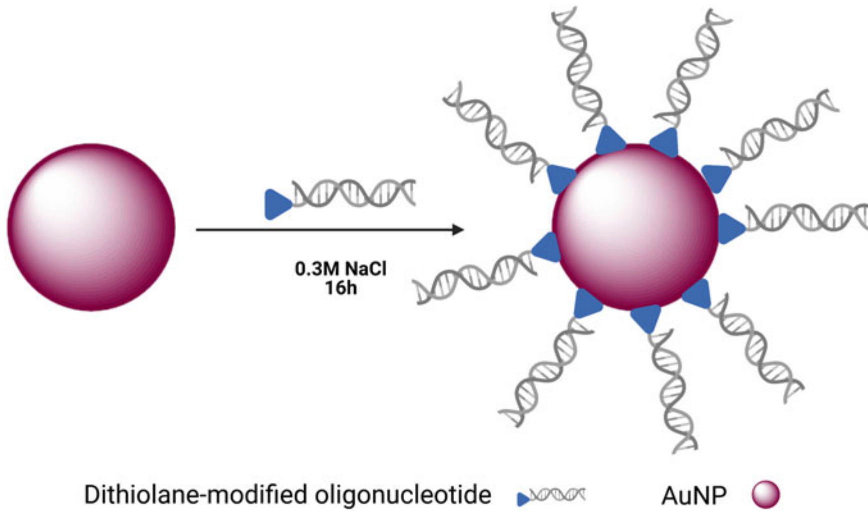

Fig. 3 Schematic representation of AuNPs functionalization with dithiolane-modified oligonucleotides

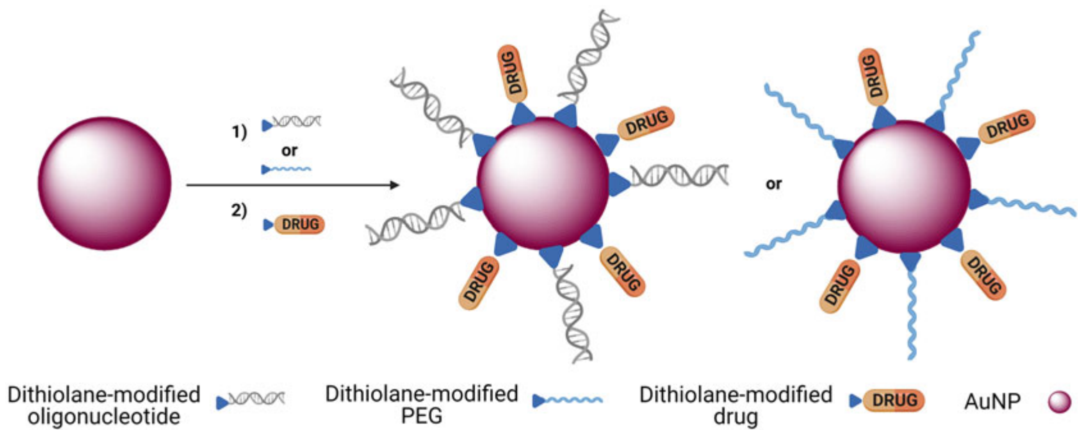

Fig. 4 Schematic representation of AuNPs functionalization with a dithiolane-modified drug using oligonucleotides or PEG as stabilizers

4. To get a complete functionalization of the nanoparticles, we recommend adding 10,000 pmol of the oligonucleotide to 1 mL AuNP (12 nM, 13 nm).
5. If you see that the AuNPs are being attached to the plastic tubes, move the solution to other plastic tubes immediately.
6. When AuNPs are changing their color to blue, it is due to aggregation. If vortexing the solution does not re-solubilize them, discard the preparation.
7. Oligonucleotide quantification could also be done by releasing the attached oligonucleotide [27]. To do so, treat the sample with 1 mM GSH for 8 h 37 °C. Then centrifuge the sample for

30 min at 13.2 rpm. Collect the supernatant and measure it as described in Subheading 3.3 and 3.4.

8. Drug quantification could also be done by comparing the drug supernatant absorbance or fluorescence with a proper standard linear calibration curve of the drug [19] or using analytical chromatography (HPLC).

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