Chapter 4

Gold Nanoparticle as a Marker for Precise Localization of Nano-objects Within Intracellular Sub-domains

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Abstract

Delivery of nano-objects to certain intracellular sub-domains is crucial for nanomedicine. Therefore delivery of nano-object to desirable cellular compartment has to be confirmed. The most valuable confirmation of the delivery comes from direct visualization of the nano-object. This visualization usually requires use of microscope and corresponding probe which has to be conjugated with the nano-object. There are two most popular methods of the visualization: confocal and electron microscopy. The former has significant limitations due to diffraction limited resolution of confocal systems and three-dimensional convolution of fluorescence. The latter should be significantly modified for needs of the visualization. Here we describe the method for precise localization of nano-object within the cell using electron microscopy and 1–2 nm gold particles as a nanomarker.

Key words Gold nanoparticle, Delivery of nano-objects, Intracellular sub-domains, Electron microscopy

1 Introduction

Therapeutic delivery of genes and drugs to intracellular sub-domains with nanocarriers requires the creation of reliable delivery systems (1, 2). The methods available for monitoring the delivery of nano-objects could be divided into retrieval of the products of the nano-object delivery and visualization of nano-objects. The former is especially important in the case of gene delivery when the level of corresponding proteins clearly confirms the delivery. The latter, visualization, has to show the location of nano-objects directly.

The direct visualization of the nano-object requires use of microscope and corresponding probe which has to be conjugated with the nano-object. There are two most popular methods of the visualization: confocal and electron microscopy. They require correspondingly fluorescent or electron-dense probe to be tagged to nano-objects. Unfortunately, confocal microscopy could be used.
mostly for in vitro experiments. Also, the precise localization of nano-objects with confocal microscopy is significantly complicated due to diffraction limited resolution of confocal systems and threedimensional convolution of fluorescence (3). This along with folding of cellular membranes and clamping of nanoparticles makes practically impossible precise localization of nano-objects within structures smaller than 0.5 μm. For example, diameter of isolated mitochondria is about 1 μm, and mitochondrial inner membrane makes multiple invaginations. It is clear that confocal microscopy cannot resolve between fluorescent dot located inside mitochondrial matrix and another dot located in the mitochondrial intermembrane space.

Hence, for purposes of localization of nanoparticles more accurate confocal microscopy methods, such as Förster resonance energy transfer (FRET) or more accurate systems, as superresolution structured illumination microscopy (SSIM) or electron microscopy (EM), should be used. However, both FRET and SSIM has some limitations of confocal microscopy and cannot be used for localization of nanoparticles in tissue. The EM allows studying of tissue fragments after in vivo experiments and precise localization of electron-dense objects >10 nm in diameter within ultrathin sections of the tissue (50–90 nm).

To make this localization of nanogold particles more precise, we slightly modified the usual procedure of EM preparation (4, 5). Namely, we employed water-soluble resin for cell polymerization and silver enhancement within ultrathin sections (4, 5). Figure 1 shows the major steps of the method. The silver enhancement significantly increases size of gold nanoparticles and makes their distribution obvious. In addition, the silver enhancement is more effective closer to the surface of the slice; therefore, the size of silver grains allows deciding about location of the gold sol within the ultrathin section. Our approach allows precise localization of 1–2 nm gold particles that could be tagged to any nano-object.

Here we describe the method for precise localization of nano-object within the cell using the EM. The method could be useful for electronopaque nano-objects and nanoparticles tagged to electron-dense marker, such as gold sols.

## 2 Materials

1. LR White resin.
3. Gold nanoparticles. To prevent aggregation of the gold particles in experimental solution and their binding to cell proteins, the nanoparticles should be pretreated (coated) with polyvinylpyrrolidone (PVP). For that we incubated gold sols in 1% PVP (MW 10,000) for 10 min with gentle agitation (see Note 1).
4. Ultramicrotome.

5. Transmission electron microscope.

6. Cell culture. Freshly isolated cells should be plated on coverslips coated with laminin.

7. 0.1 M Na⁺-cacodylate buffer:
   (a) Prepare 0.2 M stock solution of sodium cacodylate in double distilled water (21.4 g/500 ml).
   (b) Add 27 ml of 0.2 M HCl per 500 ml cacodylate stock solution.
   (c) Add double distilled water to a final volume of 1 L.

8. Fixation buffer: 6 ml of 25% glutaraldehyde in 19 ml of 0.1 M Na⁺-cacodylate buffer.

9. Rinse buffer: Na⁺-cacodylate buffer supplemented with 0.2 M RNAase-free sucrose in 500 ml of 0.1 M Na⁺-cacodylate buffer.

10. Postfix buffer: 1% osmium tetroxide in the 0.1 M Na⁺-cacodylate buffer.

11. 2% aqueous uranyl acetate solution.

12. Lead citrate solution (Reynold’s lead citrate stain):
   (a) 50 ml lead solution: 0.19 M Pb(NO₃)₂ in double distilled boiled (30 min, CO₂-free) and filtered H₂O.
   (b) 50 ml of 0.28 M tribasic sodium citrate solution in double distilled boiled (30 min, CO₂-free) and filtered H₂O. Add one drop of the lead solution.

Fig. 1 Simplified schematic representation of a method for localization of PVP-coated gold nanoparticles within cells (reproduced from ref. 1 with permission from Future Medicine)
(c) 50 ml of freshly made 1 N sodium hydroxide solution in double distilled boiled (30 min, CO₂-free) and filtered H₂O.

(d) Lead citrate solution: mix 21 ml lead solution and 21 ml lead citrate solution and shake vigorously for 2 min (solution will be a milky white); then in 30 min of gentle shaking, add 8 ml 1 N NaOH (the solution should be clear).

(e) Store in syringes (needle down into the rubber cork, without air) at 4°C.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

In our experiments we used primary culture of rat ventricular myocytes (single freshly isolated cells); however, any monolayer cell culture of any confluency could be used.

1. Fix cells or small pieces of tissue (~1 mm³) in 2 ml (per sample unit) 6% glutaraldehyde in 0.1 M Na⁺-cadodylate buffer (pH = 7.4), for 20 min. Rinse two times with the Na⁺-cadodylate buffer supplemented with 0.1 M sucrose. Postfix the cells with 1% osmium in Na⁺-cadodylate buffer for 1 h.

2. Stain samples en bloc with 1% uranyl acetate in 25% ethanol for 1 h. Dehydrate cells in ethanol and acetone step by step as shown:

(a) Increase ethanol concentration by moving the cells from one solution to another. Amounts of ethanol in water solution: 30, 40, 50, 60, 70, 80, 90% (every step is 10 min, 1 time), and 100% (10 min, 3 times).

(b) Acetone: 100%—10 min, 3 times.

3. Embed the cells in increasing concentrations of LR White resin. Proportions of LR White to acetone (use 50 mm glass Petri dishes): (1) 1 to 3, (2) 2 to 3, (3) 3 to 1, and (4) fresh resin. Every step is 12 h.

To embed cultured cells (on coverslips) in LR White resin for the final step, use a 1.5-ml tube with cap cut off. Fill the tube with the resin; cover (seal) the tube with the cover slip, so that cells face the resin; and tightly bind the construction with parafilm, scotch tape, and foil to prevent the resin from exposure to air.

4. For resin polymerization, put the tubes upside down into thermostat (~60°C) for 24 h.

5. Remove the bandage from the tube. To separate embedded cells from coverslip, dip the coverslip into liquid nitrogen for
about 5 s. Now your cells are on the top of polymerized resin, and you can see them with binocular microscope.

6. Sharpen the block for ultramicrotome cutting.

7. Obtain ultrathin sections with ultramicrotome. Most valuable slices are 85–90 nm thick (they have a light gold color). Collect the slices on formvar-coated nickel grids.

8. Perform silver enhancement with Silver Enhancing Kit (follow instructions from Ted Pella, Inc.) (see Note 2).

9. After drying, stain slices for 15 min with 2% aqueous uranyl acetate and then for 2 min with lead citrate.

10. Dry them. Now the slices are ready for electron microscopy.

11. Store images in tiff format (see Notes 3 and 4). Representative micrographs (Fig. 2) show the typical distribution of nanoparticles within ventricular cardiomyocytes.

4 Notes

1. To prevent aggregation of gold nanoparticles and their binding to proteins, after conjugation, gold sols have to be coated with polyvinylpyrrolidone (PVP) or polyethylene glycol PEG (Fig. 3).
Fig. 3 Stabilizing the effect of polymer polyvinylpyrrolidone (PVP) on gold nanoparticles. Electron micrographs (without silver enhancement) show 10 nm gold sols (a) in water, (b) in 150 mM potassium aspartate solution (pH 7.2), and (c) in particles pretreated with 1% PVP and in aspartic acid solution. The suspensions were air-dried on formvar-coated grids. No staining. (d) The absorption spectra for colloidal gold (peak at 520 nm) and mixtures of gold and 150 mM potassium aspartate and/or 5% PVP. OD optical density (depends on gold particles concentration); dashed lines show the absorbance maximum for colloidal gold (523 nm). (e) The absorption spectra for 10 nm colloidal gold stabilized with 5% PVP before and after adding 1% bovine serum albumin (BSA) to the cuvette (reproduced from ref. 4 with permission from Cell press).

For that, coating the sols has to be pretreated for 10 min in 1% PVP10 (neutral, MW = 10,000). To measure the size of the PVP-coated nanoparticles, we employed dynamic light scattering (Protein Solutions Ltd., England). These measurements showed that 1% PVP increased the diameter of the gold particles, adding about 2 nm to their original size (4).

2. The size of the silver grains depends on the time of exposure and the accessibility (i.e., depth of position within the section) of the gold. We applied the solution for 8 min.

3. To find real silver grains on the EM micrograph, reduce contrast by 70–80% (until less dense cell structures vanish) with Adobe
Photoshop (Adobe Systems Incorporated, San Jose, CA). Then mark them and retune the image contrast to normal.

4. To calculate the number of particles, we recommend using Image J 1.31v (National Institutes of Health, Bethesda, USA).

References