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Synthesis, Purification, Characterization, and Imaging of Cy3-Functionalized Fluorescent Silver Nanoparticles in 2D and 3D Tumor Models

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Abstract

Silver nanoparticles (AgNPs) have a high affinity for sulfhydryl (thiol) groups, which can be exploited for functionalization with various tracking and targeting moieties. Here, we describe how to reliably and reproducibly functionalize AgNPs with the fluorescent moiety cyanine3-polyethylene glycol (5000 molecular weight)-thiol (Cy3-PEG₅₀₀₀-SH). We also demonstrate how to purify and characterize Cy3-functionalized AgNPs (Cy3-AgNPs). Additionally, we describe how these Cy3-AgNPs can be imaged in 2D and 3D tumor models, providing insight into cellular localization and diffusion through a tumor spheroid, respectively.

Keywords

Silver; Nanomaterials; Fluorescence; Imaging; 3D tumor models

1 Introduction

Silver nanoparticles (AgNPs) are the most widely applied nanomaterial for both commercial and clinical biomedical applications. Due to their anti-bacterial properties, AgNPs have been adapted for use in disinfectants for aseptic environments, as surface coatings for neurosurgical shunts and venous catheters, and in bone cement. They have also been shown to enhance wound healing and improve skin regeneration [1]. Preclinical studies of AgNPs show that they possess cytotoxic activity toward a variety of cancer cell lines including breast [2–5], glioblastoma [6–8], cervical [9], liver [10], lung [11], and leukemia [12, 13].

AgNPs possess a strong affinity for sulfhydryl (thiol) groups [14, 15], and their physicochemical attributes can be easily tailored to produce different surface characteristics that are important for stability in physiological conditions and to aid in targeting [16, 17]. This unique property can be exploited to functionalize AgNPs with various targeting and tracking moieties that contain a thiol group. It is well documented that the cytotoxic

properties of nanomaterials are dependent upon characteristics including size, charge, and coatings, all of which affect the uptake [18]. Therefore after functionalization, it is imperative to characterize particles based on hydrodynamic diameter and ζ -potential because these properties may affect stability and biodistribution of nanoparticles in addition to cytotoxicity [18].

Here, we describe how to functionalize AgNPs with the fluorescent tracking moiety Cyanine3, which is stabilized by a polyethylene glycol (PEG) group. PEG has been added to nanomaterials and small-molecule drugs to increase stability and bioavailability in physiological conditions [19, 20]. This fluorescent tracking moiety also contains a terminal thiol group that can readily interact with the AgNP. We demonstrate how ultraviolet/visible (UV/Vis) spectrophotometry can be used to monitor the reaction of the thiol group binding with the AgNP. Additionally, we describe how to purify the Cy3-PEG₅₀₀₀-SH functionalized AgNPs (Cy3-AgNPs) from the starting materials (25 nm AgNPs and Cy3-PEG₅₀₀₀-SH), and how to identify each using UV/Vis spectrophotometry. We demonstrate how dynamic light scattering (DLS) can further validate that the particles are functionalized based upon changes in hydrodynamic diameter and ζ -potential. Lastly, we describe how to visualize the Cy3-AgNPs in 2D cell monolayers and 3D tumor spheroids using confocal microscopy and the EVOS FL Auto Cell Imaging System, respectively.

2 Materials

Prepare all AgNPs and Cy3-PEG₅₀₀₀-SH dispersions in deionized water at room temperature and store at 4 °C light protected.

2.1 Cy3-AgNP Synthesis and Purification Components

1. 25 nm PVP Redispersible Silver Nanoparticles (nanoComposix Inc.): dissolve 25 mg of AgNPs in 2.5 mL deionized water.
2. Cy3-PEG₅₀₀₀-SH (Nanocs, Inc): dissolve in warm deionized water to make a 200 mM solution.
3. TCEP Bond Breaker solution (Thermo Scientific).
4. 100,000 MWCO column.
5. PD-10 Desalting Column 1.7 mL microcentrifuge tubes.

2.2 Characterization Components

1. UV/Vis Spectrophotometer.
2. Quartz cuvette.
3. Malvern Zetasizer Nano ZS90 (Malvern Panalytical).
4. Folded capillary Zeta cells.
5. Disposable, clear plastic cuvette.

2.3 2D and 3D cell culture imaging components

1. MDA-MB-231: were purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA): Cells were grown in DMEM supplemented with 10% FBS (vol:vol), 2 mM L-glutamine, penicillin (250 U/mL), and streptomycin (250 µg/mL).
2. 1 × Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium.
3. 16% Formaldehyde solution (w/v), Methanol-free: dilute 16% formaldehyde 1:4 (vol:vol) with 1 × DPBS to make 4% formaldehyde solution.
4. Four well glass slide chamber slides.
5. Vectashield Hard Set Mounting Medium with DAPI.
6. Microscope Cover Glass.
7. Clear fingernail polish.
8. Confocal Microscope.
9. Round bottom 96-well plates.
10. Six-well tissue culture-treated plates.
11. Matrigel Matrix (Corning): dilute to 2% in growth medium for MDA-MB-231 cells outlined above.
12. EVOS FL Auto Cell Imaging System (Invitrogen) or similar equipment.

3 Methods

Perform all the steps at room temperature and protect from light unless otherwise specified.

3.1 Synthesis of Cy3-AgNPs

1. Disperse 25 nm AgNPs in deionized water via bath sonication for 5 min at 4 °C resulting in a brownish-gray opaque solution.
2. Dissolve Cy3-PEG₅₀₀₀-SH in 1 mL warm deionized water at a 200 mM concentration via vortexing resulting in a pink solution.
3. Add TCEP bond breaker solution to the Cy3-PEG₅₀₀₀-SH at a ratio of 50:1 (molarity) for 20 min (*see* Note 1).
4. Combine 2 mL of AgNPs with the Cy3-PEG₅₀₀₀-SH + TCEP solution and additional deionized water to bring the final volume to 4 mL with a final concentration of 5 mg/mL AgNPs.
5. Monitor the reaction every 15 min by UV/Vis spectrophotometry (*see* Note 2) (Fig. 1).

6. After 45 min, concentrate the Cy3-PEG₅₀₀₀-SH AgNPs dispersion using a 100,000 MWCO vivaspin column to approximately 1 mL volume via centrifugation at 3000.0 rcf for 10 min (*see* Note 3).

3.2 Purification of Cy3-AgNPs

1. Remove the cap of the PD-10 desalting column and remove the column storage solution.
2. Cut the tip of the column at the indicated notch.
3. Fill the column with deionized water and allow it to enter the column completely. Repeat this **step 4** times, discarding the flow-through (*see* Note 4).
4. Filter the concentrated volume from **step 6** of the synthesis of Cy3-AgNPs through the equilibrated PD-10 desalting column with deionized water to separate free Cy3-PEG₅₀₀₀-SH, unfunctionalized AgNPs, and Cy3-AgNPs (*see* Note 5).
5. Collect fractions in 1.7 mL microcentrifuge tubes in approximately 500–800 μ L volumes (approximately 10 drops).
6. Analyze the fractions collected from the PD-10 column by UV/Vis spectrophotometry to identify fractions containing Cy3-AgNPs (*see* Note 6) (Fig. 2).
7. Combine fractions containing Cy3-AgNPs, and store light-protected at 4 °C.

3.3 Characterization of Cy3-AgNPs

3.3.1 Hydrodynamic Diameter of Cy3-AgNPs

1. Dilute 10 μ L of Cy3-AgNPs in 1 mL of deionized water or 1 $\times$ DPBS in a plastic cuvette.
2. Read the sample in triplicate on the Zetasizer Nano ZS90 with the following settings: 25 °C, automatic settings, adjust for refractive index of the dispersant (*see* Note 7) (Fig. 3a).

3.3.2 Zeta Potential of Cy3-AgNPs

1. Transfer the sample diluted in deionized water from the plastic cuvette to a folded capillary cell.
2. Read the sample in triplicate on the Zetasizer Nano ZS90 with the settings outlined above for the hydrodynamic diameter measurements (*see* Note 8) (Fig. 3b).

3.4 Imaging of Cy3-AgNPs in 2D Culture

1. Plate 5×10^5 MDA-MB-231 cells per well in a four-well glass slide chamber slide, and let it adhere for 48 h at 37 °C.

2. Treat cells with Cy3-AgNPs or unfunctionalized 25 nm AgNPs diluted in MDA-MB-231 growth medium for 1 h at 37 °C.
3. Wash cells twice with 1 × DPBS.
4. Fix cells with 4% formaldehyde for 15 min at room temperature.
5. Remove chambers on the slide.
6. Add 2–3 drops of Vectashield Hard Set Mounting Medium with DAPI, and place a glass microscope cover on the slide.
7. Seal the edges with clear fingernail polish, and allow the mounting media to harden overnight, light-protected at 4 °C.
8. Image using a confocal microscope (*see* Note 9) (Fig. 4).

3.5 Imaging of Cy3-AgNPs in 3D Multicellular Spheroids (3D Culture)

1. Prepare MDA-MB-231 spheroids by plating 5000 MDA-MB-231 cells in a 96-well round bottom plate in 200 µL of 2% Matrigel matrix.
2. Allow spheroids to grow at 37 °C for 4 days.
3. Treat spheroids with 1 × DPBS, Cy3-AgNPs, or Cy3-AgNP filtrate (obtained from the concentration of the particles (*see* Subheading 3.1, **step 6**) diluted in growth medium for 6 or 24 h.
4. Using a P1,000 pipette, transfer spheroids to a six-well plate containing fresh growth medium (*see* Note 10).
5. Image using the transmitted light and red fluorescent protein (RFP) lenses on the EVOS FL Auto Cell Imaging System or similar machine. Merge transmitted light and RFP images (*see* Fig. 5).

4 Notes

1. TCEP bond breaker solution is necessary to prevent disulfide bond formation among the Cy3-PEG₅₀₀₀-SH fluorescent moieties. Disulfide bond formation between the moieties would prevent thiol binding of the AgNPs, and thus prevent functionalization of the particles.
2. AgNPs have a strong absorbance peak at 405 nm, while Cy3-PEG₅₀₀₀-SH has a strong absorbance peak at 550 nm. Upon functionalization via thiol binding, a dampening of the AgNP peak at 405 will occur along with a red shift. Additionally, a small peak around 550 should be observed to indicate the presence of the Cy3-PEG₅₀₀₀-SH.
3. Additional centrifugation may be required if the volume is still above 1 mL. Although the PD-10 desalting column specifies that larger volumes (up to 2.5 mL) may be used, smaller volumes provide better separation of unfunctionalized AgNPs, Cy3-AgNPs, and free Cy3-PEG₅₀₀₀-SH. If the particles become

entrapped in the membrane, a P200 gel loading tip can be used to loosen particles from the membrane.

4. Equilibration of the PD-10 desalting column may be performed while the reaction is occurring or while the reaction mixture is being concentrated. Ensure that the column does not dry out after equilibration by filling the column with deionized water and recapping the bottom of the column with the caps provided. When ready to filter the AgNPs, remove the cap and allow the deionized water to move into the packed bed of the column before adding the AgNPs.
5. Because the Cy3-AgNPs increase in size following functionalization, they will elute in the early fractions. The unfunctionalized AgNPs will elute in the middle fractions. The free Cy3-PEG₅₀₀₀-SH will elute last because the small size will cause entrapment in the fenestrations of the column leading to a slow release from the column. Unbound Cy3-PEG₅₀₀₀-SH may remain trapped in the PD-10 column as indicated by a light pink discoloration of the column.
6. Fractions containing Cy3-AgNPs will have two absorbance peaks: one peak at 405 nm indicating the presence of the AgNPs and one at 550 nm to indicate the presence of the Cy3-PEG₅₀₀₀-SH fluorescent moiety. Fractions displaying only a peak at 405 nm contain unfunctionalized AgNPs, and fractions displaying only a peak at 550 nm contain free Cy3-PEG₅₀₀₀-SH.
7. An increase in hydrodynamic diameter will occur, which is indicative of functionalization of the particle.
8. The ζ -potential of the Cy3-AgNPs becomes more neutral compared to negative ζ -potential for unfunctionalized AgNPs. This change in ζ -potential is indicative of functionalization via thiol binding.
9. Free Cy3-PEG-SH displays a strong absorbance peak at 550 via UV/Vis spectrophotometry. However for visualization of the Cy3-AgNPs in vitro, the optimal wavelength is between 565 and 665 nm.
10. When removing spheroids from the 96-well plates to the six-well plates, place all spheroids receiving the same treatment in one well of the six-well plate for imaging.

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Fig. 1.

UV/Vis spectrophotometry absorbance readings for monitoring the reaction of Cy3-PEG₅₀₀₀-SH binding to AgNPs. Unfunctionalized AgNPs have a strong absorbance peak at 405 nm. When thiol binding occurs, a damping of the peak at 405 nm occurs along with the development of a peak at 550 nm where Cy3-PEG₅₀₀₀-SH absorbs

Fig. 2. UV/Vis spectrophotometry absorbance readings of various fractions obtained during the purification process utilizing the PD-10 desalting column. **(a)** Example of unfunctionalized particles: displays one absorbance peak at 405 nm. **(b)** Example of free (unbound) Cy3-PEG₅₀₀₀-SH: displays one absorbance peak at 550 nm. **(c)** Example of Cy3-AgNPs: displays a large peak at 405 nm indicating the presence of AgNPs and a smaller peak at 550 nm indicating successful binding of the Cy3-PEG₅₀₀₀-SH fluorescent moiety

Fig. 3. Dynamic light scattering (DLS) data comparing unfunctionalized 25 nm AgNPs (black) and Cy3-AgNPs (red). **(a)** An increase in hydrodynamic diameter indicates the formation of Cy3-AgNPs. **(b)** Positive zeta potential shift indicates functionalization of AgNPs to form Cy3-AgNPs

Fig. 4. Confocal images of MDA-MB-231 breast cancer cells after 1 h treatment with 25 nm AgNPs or Cy3-AgNPs. The first panel shows cells after 1 h treatment with 25 nm AgNPs at 20× magnification. The second and third panels show cells after a 1 h treatment with Cy3-AgNPs at 20× and 40× magnification, respectively

Fig. 5. EVOS images of MDA-MB-231 3D tumor spheroids treated for 6 or 24 h with $1 \times$ DPBS, Cy3-AgNPs, or filtrate collected from Cy3-AgNPs