bio-protocol

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Preparation, Characterization, and Cell Uptake of PLGA/PLA-PEG-FA Nanoparticles

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Abstract

Oral administration of colon-targeting nanoformulations holds many advantages over the systemic delivery of free drugs, or traditional nontargeting formulations in the treatment of ulcerative colitis (UC). Currently, the most conventional method for constructing colon-targeting drug delivery systems (DDS) is by integrating the biocompatible materials poly(lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) into a copolymer. This PLGA/PLA-polyethylene glycol-folic acid (PEG-FA) copolymeric nanoformulation effectively delivers the drugs for uptake by various human colon cancer cells (*e.g.*, HT-29 and HCT-116) and mouse colon cancer cells (CT-26). There is, however, a distinct lack of comprehensive protocols for the construction of such copolymer. This protocol details an easy-to-follow single-step method for the construction of a colon-targeting PLGA/PLA-PEG-FA nanoformulation, which encapsulates a fluorescent dye and demonstrates the visualization of its cell uptake *in vitro*.

Keywords: Anti-inflammatory, Colon, Drug delivery, Inflammatory bowel disease, Nanoparticles, PLGA/PLA-PEG-FA, Ulcerative colitis

This protocol was validated in: J Crohns Colitis (2021), DOI: 10.1093/ecco-jcc/jjx115

Background

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with a mounting incidence in the United States (Yang *et al.*, 2020). Traditional anti-inflammatory drugs, including 5-aminosalicylic acid (5-ASA), corticosteroids, and thiopurines, are frequently used to treat mild-to-moderate UC (Tripathi and Feuerstein, 2019). However, these treatments yield inevitable side effects. Contrarily, UC-targeting formulations, especially colon-targeting nano-drug delivery systems, are designed to enhance colonic drug concentration, while reducing systemic drug distribution, effectively enhancing the drug's efficacy and reducing side effects.

Compared with other nanomaterials, FDA-approved poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), and Polycaprolactone (PCL) polymers are low-toxic, biodegradable, and can load both hydrophobic and hydrophilic drugs (Sindeeva *et al.*, 2021). These materials have also proven easy to co-polymerize and form complex nanoparticles. Recently, many laboratories, ours included, have shown that PLGA/PLA copolymer composed nanoparticles are colon-targeting and biocompatible (Zhao *et al.*, 2013; Zhang *et al.*, 2018). Additionally, we co-polymerized PLGA nanoparticles with PLA-polyethylene glycol-folic acid (PEG-FA) (a PLA-PEG polymer with folic acid modification), enhancing the targeting ability towards folic acid receptors on intestinal epithelial cells. Subsequent reassembling of PLGA/PLA-PEG-FA nanoparticles into a hydrogel further improves their colon-targeting efficiency (Zhang *et al.*, 2018). In this protocol, we will demonstrate the use of PLGA and PLA-PEG-FA as starting materials to make PLGA/PLA-PEG-FA copolymer nanoparticles. The uptake of these nanoparticles into epithelial cells would then be visualized by the incorporation of the fluorescent dye, Dil (dioctadecyl-tetramethylindocarbocyanine perchlorate). The nanoparticles prepared in this protocol have demonstrated their effectiveness in treating UC in recent research (Zhang *et al*., 2018).

Materials and Reagents

1. Pipettes, 0.5–10 µL, 10–100 µL and 100–1,000 µL (Eppendorf, catalog number: 13-684-251)
2. Pipette tips, 0.1–10 µL, 1–300 µL, and 200–1,000 µL (Fisher Scientific, SureOne^{TM} micropoint pipette tips, catalog numbers: 02-707-441, 02-707-410, 02-707-407)
3. Eppendorf Safe-Lock Tubes, 1.5 mL (Eppendorf, catalog number: 022363204)
4. Falcom 15 mL and 50 mL conical centrifuge tubes (Fisher Scientific, catalog numbers: 05-527-90, 14-959-49A)
5. KimTech Science KimWipes (Kimberly-Clark, catalog number: 06-666)
6. Vacuum Filter (Millipore, catalog number: SCGVU01RE)
7. Mica sheet (Electron Microscopy Sciences, catalog number: 71856-01-10)
8. Malvern zeta potential capillary cell & plastic cap (Malvern, catalog number: DTS 1070)
9. Falcon 8-well cell culture slides (Corning, catalog number: 354108, 354118)
10. VWR micro cover glass (VWR, catalog number: 48382-138)
11. Nail polish (Electron Microscopy Sciences, catalog number: 72180)
12. MEDI-FIRST lens cleaner towelettes (Safetec of America, catalog number: 81586)
13. Countess cell counting chamber slides (Thermo Fisher Scientific, catalog number: C10283)
14. Polyvinyl alcohol (PVA, 87–90% hydrolyzed; Sigma-Aldrich, catalog number: P8136-250G)
15. Lactide:glycolide (75:25) (PLGA), molecular weight (MW) 66000–107000 (Sigma-Aldrich, catalog number: P1941-5g)
16. PLA-PEG (PEG MW 5000 and PLA MW 10000) (NSP-Functional polymers and copolymers, catalog number: DL-5k-10k)
17. PLA-PEG-FA (PEG MW 5000 and PLA MW 10000) (NSP-Functional polymers and copolymers, catalog number: 11440-10k-5000)
18. Phosphate-buffered Saline (PBS, Corning, catalog number: 21-040-CV)
19. 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (Dil, Thermofisher, catalog number: D282)
20. Methyl alcohol (Sigma-Aldrich, catalog number: 67-56-1)
21. Acetone (EMD, catalog number: AX0115P-1)
22. Dichloromethane (Sigma-Aldrich, catalog number: 32222-1L)

23. HEPES buffer pH 7.4 (Sigma-Aldrich, catalog number: F7876)
24. Spectrophotometer cuvettes, polystyrene (Sigma-Aldrich, catalog number: C5677-100EA)
25. RPMI 1640 cell culture medium (Corning, catalog number: 10-040-CV)
26. Fetal Bovine Serum (Atlanta Biologics, catalog number: S11150H)
27. StemPro Accutase Cell dissociation reagent (Life Technologies, catalog number: A11105-01)
28. Penicillin Streptomycin solution (Life Technologies, catalog number: 15070-063)
29. Trypan Blue stain 0.4% (Life Technology, catalog number: T10282)
30. Phalloidin, Fluorescein Isothiocyanate (FITC) Labeled (Sigma-Aldrich, P5282-.1MG)
31. DAPI (SouthernBiotech, catalog number: 0100-20)
32. Complete medium (see Recipes)
33. FITC staining solution (see Recipes)

Equipment

1. Milli-Q water purification system (Millipore-Sigma, model: advantage A10)
2. Electronic balance (OHAUS, model: explorer125)
3. Vortexer (Scientific Industries, model: 200-SI0236)
4. Digital Probe Sonifier (Branson, model: 450 Digital Sonifier)
5. Benchmark Hotplate and Magnetic Stirrer (Corning, model: PC-6200)
6. Rotary evaporator (Buchi, model: R-210)
7. Vacuum pump (Buchi, model: V-700)
8. Centrifuge (Thermo Fisher Scientific, model: Sorvalis ST16R)
9. Ultracentrifuge (Beckman Coulter, model: Optima L-90K)
10. Ultrasonicate cleaner (Branson, model: 3510R-MTH)
11. Zetasizer (Malvern, model: Nano-ZS90)
12. Atomic force microscopy (Nanosurf, model: CoreAFM)
13. Cell culture incubator (Thermofisher Scientific, model: Series II water jacket)
14. Cell counter (Life Technology, model: Countess 3)
15. Olympus fluorescence microscope (Olympus, model: BX63 with DP-25 camera)
16. Keyence microscope (Keyence, model: BZ-X700 Series)

Procedure

A. Preparation of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles

Note: This procedure should avoid intense lighting.

1. Prepare a 2.5% polyvinyl alcohol (PVA) solution, by adding 2.5 g PVA to 100 mL of deionized water. Stir the solution using a magnetic stirring stick at room temperature (25°C) overnight, and filter it through a 0.2 µm vacuum filter (Figure 1a).
2. Weight 75 mg PLGA and add it to a 10 mL glass bottle.
3. Add 5 mL of dichloromethane (DCM) to dissolve the PLGA.
4. Spike 100 µL of Dil (1 mg/mL in acetone) to the above solution and mix. Cover the bottle with aluminum foil to avoid direct lighting.
5. Transfer 25 mL of 2.5% w/v aqueous PVA solution to a 250 mL flask.
6. Place the flask containing the aqueous PVA solution in an ice bath.

7. Insert the sonication probe (4 mm diameter) into the flask, until it is immersed by the aqueous PVA solution (Figure 1b–1c).
8. Initiate the probe sonifier at 50% amplitude for 30 s (three times), to sonicate the PVA solution over an ice bath. Meanwhile, using a glass pipette, add the DCM solution (contains Dil and PLGA) dropwise to the PVA solution, to form an oil-in-water emulsion during the sonication process.
9. Weigh 15 mg PLA-PEG and 10 mg PLA-PEG-FA and place them into a 10 mL glass bottle.
10. Add 200 µL of DCM to the above bottle to dissolve PLA-PEG/PLA-PEG-FA mix.
11. Under magnetic stirring, add the PLA-PEG/PLA-PEG-FA solution dropwise to the emulsion (prepared in step 8).
12. Transfer the solution to a 100 mL glass flask; add a clean stir bar, place the glass flask on the stir plate, adjust the speed and temperature (25°C), and stir (at 500 rpm) overnight to reduce the DCM solvent (Figure 1d). Cover the bottle with aluminum foil to avoid direct lighting, and use needle punch holes on the foil to allow the DCM to evaporate.

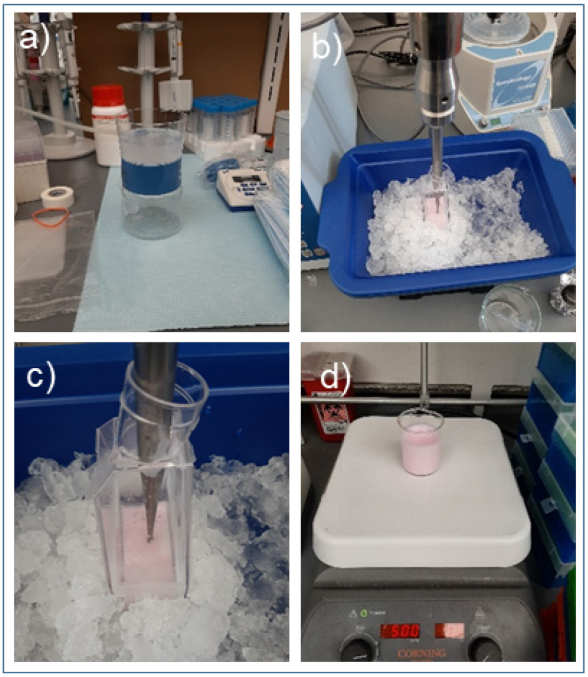

Figure 1. Preparation of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles.
a) Filtering the PVA solution; b) Immersing the sonifier probe; c) Adjusting the probe position; d) Stirring the emulsified nanoparticle suspension.

13. Transfer the solution to a pear-shaped glass bottle, and evaporate the solution with rotary evaporation at 40°C for 2 h, to remove the DCM solvent residue (Figure 2a).
14. Transfer all the mixture to the centrifuge tube (Figure 2b), and centrifuge at 2,795 × *g* and 4°C for 15 min (Figure 2c). Discard the pallet (remove the micro-sized particles).
15. Transfer the supernatant to a 70 mL ultracentrifuge tube, to centrifugate the supernatant at 25,155 × *g* and 4°C for 45 min (Figure 2d). Discard the supernatant.
16. Slowly add deionized water to the tube, avoid disturbing the pellet, and then aspire the water. Repeat this step three times, to gently wash the pellet.
17. Add deionized water and pipette the suspension, to retrieve the nanoparticles.
18. Lyophilize the suspension to obtain a dry powder (~20 mg).

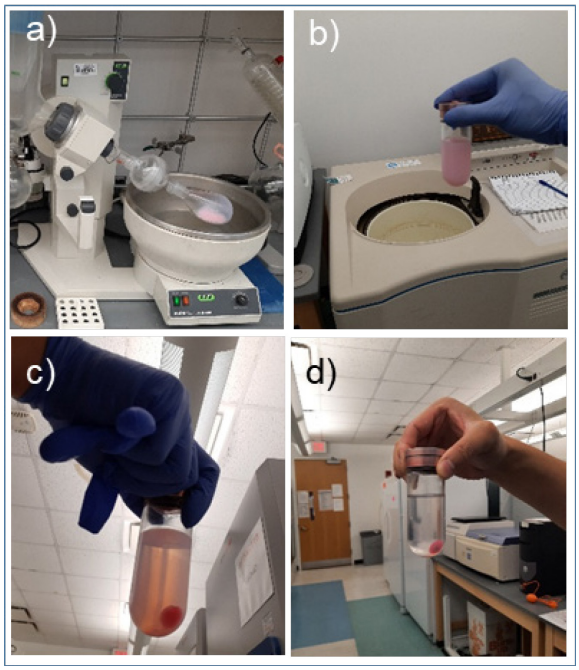

Figure 2. Preparation of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles.
a) Removing the DCM via rota evaporation; b) Submitting the suspension to the centrifugation; c) Visual of the sample after the 2,795 × *g* centrifugation for 15 min; d) Visual of the sample after 25,155 × *g* ultracentrifugation for 45 min.

B. Characterization of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles

Measure particle size and zeta potential

The particle size and zeta potential of NPs were determined using a Malvern Zetasizer Nano ZS90 Apparatus [Malvern Instruments, Malvern, UK] at room temperature.

1. Suspend the NPs in a PBS solution (Figure 3a); the approximate concentration of NPs is 10–100 μg/mL.
2. Add 1.5 mL of nanoparticle suspension to a cuvette.
3. Cover the cuvette with a plastic cap.
4. Insert the cuvette into the Malvern Zetasizer, and set the parameter to measure size.
5. Carefully add 0.8 mL of nanoparticle suspension to a capillary cell (Figure 3b).
6. Cover the capillary cell with a plastic cap.
7. Insert the cell into the chamber of the zeta potential analyzer (Figure 3c).
8. Set the parameter to measure the surface zeta potential.

Notes:
a. Use room temperature PBS for accurate size measurement.
b. After size measurement, the same solution can be used to measure the zeta potential.

Acquire atomic force microscopy (AFM) image

1. Prepare a freshly peeled mica sheet.
2. Deposit ~2.5 μL of PLGA/PLA-PEG-FA NP (suspended in water) to the mica sheet (Figure 3d).
3. Dry the sample at room temperature for 30 min (Figure 3e).
4. Gently rinse the mica sheet with 5 μL of distilled water.
5. Dry the sample at room temperature for 30 min again.

6. Place the mica sheet on the AFM stage (Figure 3f).
7. Scan the sample with an area of about 5 × 5 μm and 2–50 nm in height.
8. A typical AFM picture can be found in Figure 5b.

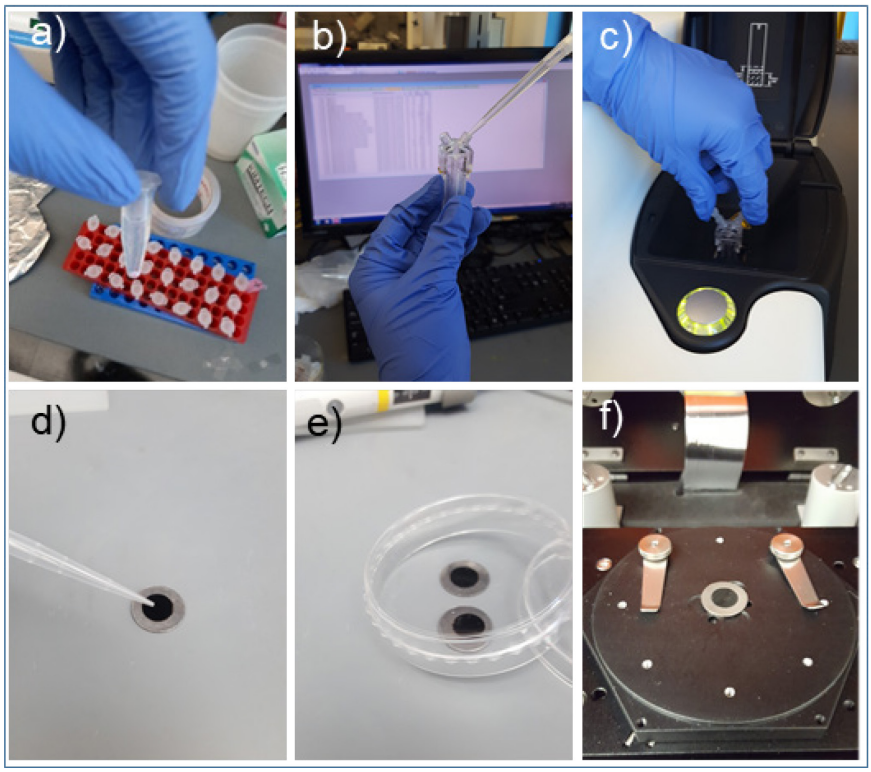

Figure 3. Characterization of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles.
a) Reconstituting the nanoparticle by adding water to the powder; b) Adding the nanoparticles suspension to the cuvette; c) Inserting the cuvette to the zetasizer to measure the size and zeta potential; d) Adding a tiny drop of nanoparticle suspension to the freshly peeled mica sheet; e) Covering the sheet and letting it dry at room temperature; f) Placing the sheet on the AFM stage.

C. CT-26 uptake of PLGA/PLA-PEG-FA Nanoparticles

Revive CT-26 Cells

1. Warm the medium to 37°C in a water bath; transfer the warmed medium (RPMI 1640, 15 mL) into a 75 cm^2 cell culture flask.
2. Revive the CT-26 cells from a liquid N_2 tank. Thaw the cells and spike the cell suspension into the culture flask.
3. Culture the flask at 37°C in a humidified atmosphere containing 5% CO_2.
4. After 24 h, replace the medium and wait until the colon-26 cells grow to 75% confluence in the 75-cm^2 flasks.
5. Subculture the cells in an 8-well plate. Add 1 mL of medium (containing ~75,000 cells) to each well.
6. Continue to culture the cells until they are 75% confluent (Figure 4b).
7. Prepare the NPs in a PBS suspension (0.1 mg/mL).
8. Add 20 μL of NP-PBS suspension to the 8-well plate (Figure 4a).
9. After 2 hrs, aspire the medium, wash the plate with 0.5 mL of PBS, and fix the cells with 0.25 mL of icy cold methanol for 15 min (at 4°C).
10. Aspire the methanol and wash with icy cold PBS three times (0.25 mL each time). Add 100 μL of FITC

solution, and incubate the plate at room temperature for 30 min.

11. Aspire the FITC and dry the plate.
12. Add ~0.3 mL of DAPI and nail polish to seal the plate (Figure 4c). Allow the plate to dry in a dark and cold place for 2 h.
13. Place the slide on the microspore stage (Figure 4d), and use fluorescence microscopy to image the samples.

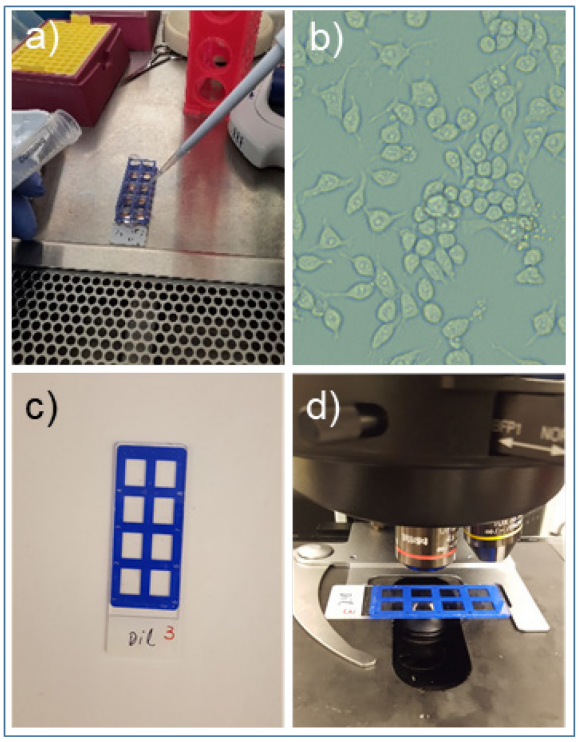

Figure 4. CT-26 uptake of Dil-labeled PLGA/PLA-PEG-FA Nanoparticles.
a) Adding nanoparticle PBS suspension to 8-well cell culture plate; b) Imaging of attached cells observed under a reverse light microscope (40×); c) A prepared slide for fluorescence microscope analysis; d) Placement of the slide on the microscope stage.

Data analysis

1. Dynamic light scattering analysis showed that the hydrated nanoparticle size is about 170.5 ± 52.87 nm in diameter with a polydispersity index (PDI) of 0.096 (Figure 5a).
2. The Zeta potential of nanoparticles varies from -18.1 to -18.6 mV (Figure 5c).
3. AFM images show that the Dil-labeled PLGA/PLA-PEG-FA nanoparticles are nano-sized particles with a spherical shape (Figure 5b).
4. Fluorescence microscopy photos demonstrate that the colon epithelial cells uptake the NPs after 2h of incubation (Figure 6).

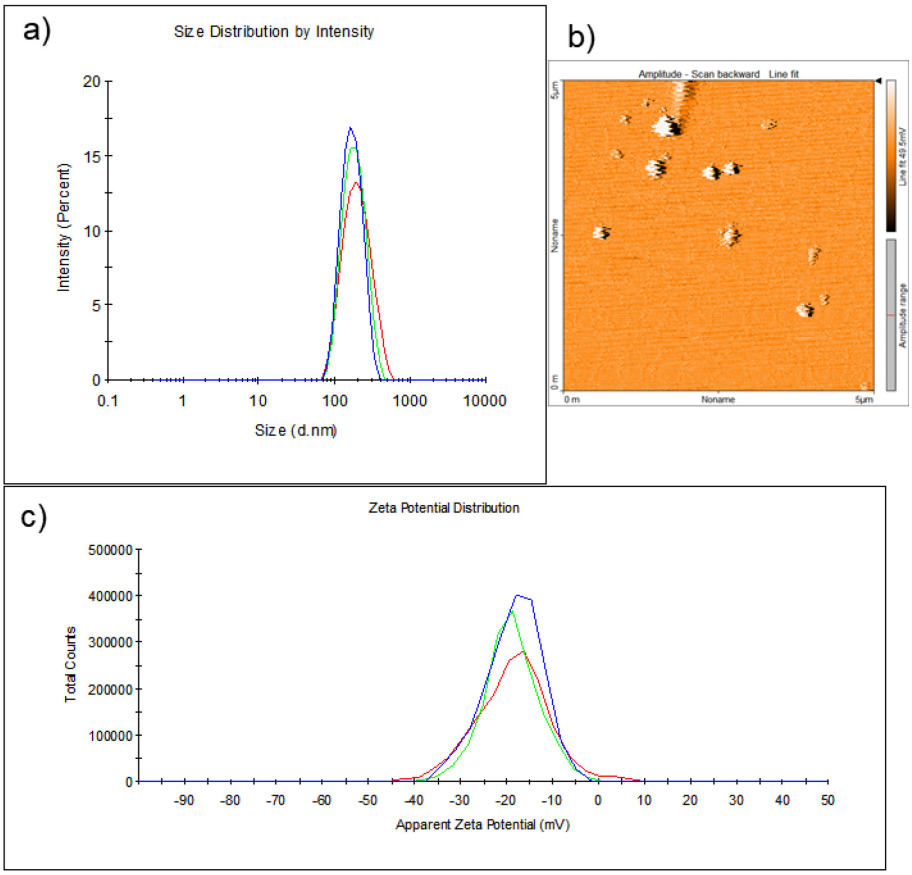

Figure 5. Size, zeta potential, and morphology analysis of the nanoparticles.
a) Size distribution of the nanoparticles; b) An AFM photo of the nanoparticle c) zeta potential of the nanoparticle. Different colored lines represent 3 readings of the same preparation.

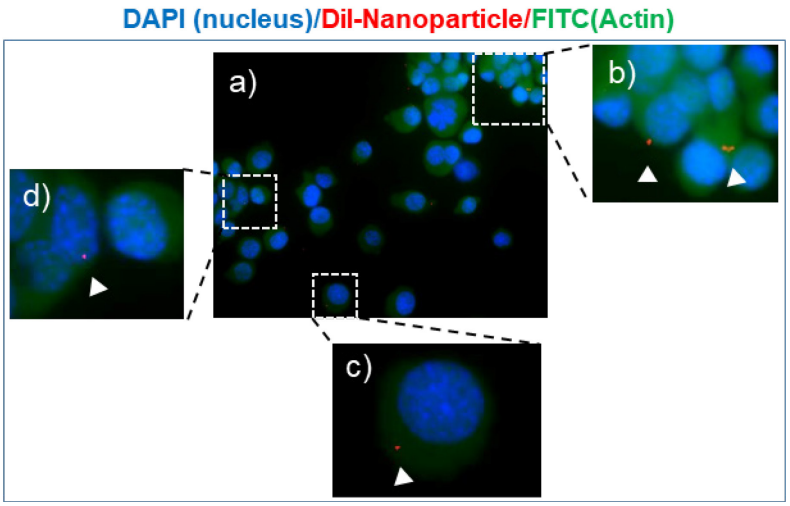

Figure 6. Fluorescence microscopy analysis of the cell uptake of the Dil-labeled nanoparticle.
a) A representative fluorescence microscopy photo. b–d) Enlarged photo represents the nanoparticles internalized in the cytoplasm.

Notes

1. When using the sonication probe, use a large flask and place it on the ice bath, to prevent local overheating.
2. Avoid touching the bottom of the bottle with the sonication probe, to avoid abrasions and potential breaking of the bottle.

Recipes

1. **Complete medium**
   Complete medium for Colon-26 is RPMI 1640, supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and heat-inactivated fetal bovine serum (10%).

2. **FITC staining solution**
   Dissolve 0.1 mg Phalloidin, Fluorescein Isothiocyanate with 10 mL Methanol.

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Competing interests

The authors declare no conflicts of interest within the work.

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