Chapter 11

Polymeric Nanoparticles for Drug Delivery

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Abstract

The use of biodegradable polymeric nanoparticles (NPs) for controlled drug delivery has shown significant therapeutic potential. Concurrently, targeted delivery technologies are becoming increasingly important as a scientific area of investigation. In cancer, targeted polymeric NPs can be used to deliver chemotherapies to tumor cells with greater efficacy and reduced cytotoxicity on peripheral healthy tissues. In this chapter, we describe the methods of (1) preparation and characterization of drug-encapsulated polymeric NPs formulated with biocompatible and biodegradable poly(D,L-lactic-*co*-glycolic acid)-poly(ethylene glycol) (PLGA-*b*-PEG) copolymers; (2) surface functionalization of the polymeric NPs with the A10 2′-fluoropyrimidine ribonucleic acid (RNA) aptamers that recognize the prostate-specific membrane antigen (PSMA) on prostate cancer cells; and (3) evaluation of the binding properties of these targeted polymeric NPs to PSMA-expressing prostate cancer cells in vitro and in vivo. These methods may contribute to the development of other useful polymeric NPs to deliver a spectrum of chemotherapeutic, diagnostic, and imaging agents for various applications.

Key words: Polymeric nanoparticles, polymer conjugation chemistry, targeted drug delivery, surface functionalization, aptamers, chemotherapy, microfluidics.

1. Introduction

Biodegradable polymers such as poly(D,L-lactic acid) (PLA), poly(D,L-lactic-*co*-glycolic acid) (PLGA), and poly (ε-caprolactone) (PCL) and their copolymers diblocked or multiblocked with poly(ethylene glycol) (PEG) have been commonly used to form polymeric nanoparticles (NPs) to encapsulate a variety of therapeutic compounds. These include polymeric micelles, capsules, colloids, dendrimers, etc. One such

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polymeric NP is Genexol-PM^{TM}, a PLGA-*b*-methoxyPEG NP encapsulating paclitaxel, which has received regulatory approval in South Korea for clinical use and is currently undergoing phase II clinical trials for a number of cancer indications in the United States. Polymeric NPs can be formulated by self-assembly of block copolymers consisting of two or more polymer chains with different hydrophobicity. These copolymers spontaneously assemble into a core-shell structure in an aqueous environment to minimize the system's free energy. Specifically, the hydrophobic blocks form the core to minimize their exposure to aqueous surroundings, whereas the hydrophilic blocks form the corona-like shell to stabilize the core through direct contact with water. Drug release rates from the polymeric NPs can be controlled by modifying polymer chemical and physical properties.

In this chapter, we describe the preparation and characterization of prostate cancer-targeted PLGA-*b*-PEG NPs as a model controlled-release drug delivery platform. We functionalized the NPs using the A10 2′-fluoropyrimidine ribonucleic acid (RNA) aptamers as a model targeting moiety, which binds to the prostate-specific membrane antigen (PSMA), a well-known prostate tumor marker, and characterized these targeted polymeric NPs for targeting to human prostate cancer cell lines in vitro and tumors in xenograft mouse models of prostate cancer in vivo.

2. Materials

2.1. PLGA-b-PEG Polymer Conjugation

1. Heterobifunctional PEG (amine-PEG-carboxylate) at molecular weight of 3,400 g/mol (NOF Corporation, Tokyo, Japan) is stored in dark at –20°C.
2. Poly(D,L-lactide-*co*-glycolide) (PLGA) with terminal carboxylate groups (PLGA-carboxylate) is stored at –20°C (Lactel Absorbable Polymers, Cupertino, CA).
3. Conjugation crosslinkers: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is stored in dark at –20°C; *N*-hydroxysuccinimide (NHS) is stored at 4°C (both from Pierce, Rockford, IL).
4. *N*,*N*-diisopropylethylamine (DIEA) is stored in dark at room temperature.
5. Solvents: dichloromethane (DCM), ethyl ether, acetonitrile, methanol are molecular biology grade (> 99% in purity).
6. Washing solution: anhydrous ethyl ether and methanol (50/50).

2.2. PLGA-b-PEG NP Preparation

1. Solvents: acetonitrile (> 99% in purity), ultrapure water.
2. Docetaxel (> 97% in purity, Fluka).
3. Doxorubicin (> 98% in purity, Sigma).
4. Poly(vinyl alcohol) (PVA) (88% hydrolyzed, ~22 kDa, Fisher).
5. Amicon ultracentrifugation tubes with molecular weight cut-off (MWCO) of 10,000 Da (Millipore).
6. PDMS T-shaped microchannel (20 × 60 μm) mounted on a glass slide. Channels are made using PDMS Sylgard 184 (Dow Corning, Midland, MI).
7. Standard microfluidics equipment: syringe pumps, syringes, plastic tubing, and bright-field microscope for visualization.

2.3. Polymer and NP Characterization

2.3.1. Polymer Characterization

1. Bruker AVANCE 400 nuclear magnetic resonance (NMR, 400 MHz) (Bruker, Billerica, USA). Solvent: $CDCl_3$.
2. Gas permeation chromatography (GPC) (Waters Corporation). Solvent: tetrahydrofuran. Standards: polystyrene.

2.3.2. NP Characterization

1. Brookhaven 90 Plus particle sizer (676 nm laser) and ZetaPALS (Brookhaven Instruments Corporation). Standard-size disposable cuvettes.
2. Transmission electron microscope (TEM). Formvar-carbon-coated grids. Negative stain: 3% uranyl acetate solution stored at 4°C in dark (both from Electron Microscopy Sciences, Hatfield, PA).
3. Slide-A-Lyzer MINI dialysis unit, 10 K MWCO and floats (Pierce, Rockford, IL).
4. Phenomenex Curosil-PFP column (250 × 4.6 mm; 5 μm) (Phenomenex, Torrance, CA).
5. High-performance liquid chromatography (HPLC, Agilent 1100 series, Palo Alto, CA).

2.4. Targeted NP Preparation

1. Targeting aptamer: PSMA A10 2′-fluoropyrimidine RNA aptamer (sequence: 5′-NH_2-spacer GGG/AGG/ACG/AUG/CGG/AUC/AGC/CAU/GUU/UAC/GUC/ACU/CCU/U GU/CAA/UCC/UCA/UCG/GCiT-3′ with 2′-fluoropyrimidines, a 5′-amino group attached by a hexaethyleneglycol spacer, and a 3′-inverted T cap) custom synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium). Aptamers are stored as lyophilized powder at –80°C.
2. DNase- and RNase-free water (Invitrogen, Carlsbad, CA).

3. Dimethylsulfoxide (DMSO) (> 99% for molecular biology, Sigma).
4. 10% TBE-urea polyacrylamide gel (Invitrogen).

2.5. Reagents for In Vitro Cell Culture and Immunohistochemistry

1. LNCaP prostate epithelial cell line (Cat #: CRL-1740) cultured in RPMI-1640 medium and PC3 prostate epithelial cell line (Cat #: CRL-1435) cultured in F-12 K medium. All materials are from ATCC (Manassas, VA).
2. Both LNCaP and PC3 culture media are supplemented with 10% fetal bovine serum (FBS) and 1× (100 units/mL penicillin, 100 μg/mL streptomycin) of 100× penicillin–streptomycin (Invitrogen).
3. Opti-MEM I Reduced-Serum Media (Invitrogen).
4. CellBIND 75 cm^2 flask with rectangular, canted neck, vented cap; CellBIND 48-well plates (Corning, Lowell, MA) (*see* **Note 1**).
5. NBD cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol) (Molecular Probes, Invitrogen).
6. NBD-encapsulated targeted NPs (preparation described in **Section 3.4**) (*see* **Note 2**).
7. Freshly prepared 4% paraformaldehyde in PBS.
8. 0.1% Triton X-100 (Sigma) in PBS buffer.
9. Mouse monoclonal EEA-1 antibodies, mouse monoclonal mannose-6-phosphate receptor antibodies, Cy5 goat anti-mouse antibodies (Abcam, Cambridge, MA).
10. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).
11. Glass cover slips (Size: No. 1) (VWR, Batavia, IL).
12. Delta Vision Deconvolution Microscope (Applied Precision, Issaquah, DC).
13. Cell Proliferation Assay kit (ATCC).

2.6. Reagents for In Vivo Experiments

1. Male, 8-week-old, BALB/c nude mice (Charles River Laboratories, Wilmington, MA) (*see* **Note 3**).
2. 3×10^6 LNCaP cells/mouse in 200 μL RPMI-1640 medium with 10% FBS and 1× penicillin–streptomycin.
3. BD Matrigel matrix phenol-red free (BD Biosciences, San Jose, CA) (*see* **Note 4**).
4. 2.5% Avertin. To make 100% Avertin, mix 10 g of 2,2,2-tribromoethanol (99%) with 10 mL of tertiary amyl alcohol (Sigma). To make 2.5% Avertin, add 100% Avertin dropwise with constant stirring into PBS at 37°C (*see* **Note 5**).

5. ^{14}C-paclitaxel (Sigma)-encapsulated targeted NPs (preparation described in **Section 3.4**).
6. Solvable tissue solubilizer (Perkin-Elmer, Waltham, MA).
7. Hionic-Fluor scintillation cocktail (Perkin-Elmer).
8. 0.5 M EDTA, pH 8.0 (Invitrogen).
9. 30% H_2O_2.
10. Packard TriCarb Scintillation Analyser (Downers Grove, IL).

3. Methods

3.1. Synthesis of PLGA-b-PEG Polymer

Two of the most common methods to synthesize PLGA-*b*-PEG diblock copolymers are (i) conjugation of PLGA homopolymer with a carboxylate end group to a heterobifunctional amine-PEG-carboxylate using EDC and NHS as conjugation crosslinkers (1), and (ii) melt or solution copolymerization of lactide and glycolide in the presence of monomethoxy-PEG using stannous octoate as a catalyst (2). Here, we describe polymer conjugation using EDC/NHS chemistry (*see* **Note 6**).

1. Dissolve 250 mg of PLGA-carboxylate (0.005 mmol) in 1–2 mL DCM (*see* **Note 7**).
2. Dissolve NHS (3.0 mg, 0.025 mmol) and EDC (4.8 mg, 0.025 mmol) in 1 mL DCM (*see* **Note 8**).
3. PLGA-carboxylate is converted into PLGA-NHS by adding the EDC/NHS solution to PLGA-carboxylate solution with gentle stirring.
4. PLGA-NHS is precipitated with 20 mL ethyl ether/methanol washing solvent by centrifugation at 2,700×*g* for 10 min to remove residual EDC/NHS.
5. Repeat washing and centrifugation two times (*see* **Note 9**).
6. The PLGA-NHS pellet is dried under vacuum for 30 min to remove residual ether and methanol.
7. After drying under vacuum, PLGA-NHS (200 mg, 0.004 mmol) is dissolved in DCM (4 mL) followed by addition of amine-PEG-carboxylate (20.4 mg, 0.006 mmol) and DIEA (7.5 mg, 0.06 mmol). The mixture solution is incubated for 24 h at room temperature under gentle stirring.
8. Precipitate the resulting PLGA-*b*-PEG block copolymer with ether/methanol washing solvent and centrifuge to remove unreacted PEG.
9. Dry the purified PLGA-*b*-PEG polymer under vacuum (*see* **Note 10**).

3.2. PLGA-b-PEG NP Preparation

There are several methods available to prepare PLGA-*b*-PEG NPs including the (i) emulsification-solvent evaporation method (this method itself includes single emulsion and double emulsion) (3); (ii) nanoprecipitation (also known as solvent displacement method) (4); and the (iii) salting-out method (5). The choice of method depends on the nature of the drug to be entrapped within the NPs. For the encapsulation of hydrophilic drugs, double emulsion is preferred. For hydrophobic drugs, nanoprecipitation, single emulsion, and salting-out methods can be used. Concomitantly, recent interest exists in the use of microfluidic devices for the formation of polymeric particles because of the fast mixing and homogeneous reaction conditions in the microscale (6). Our group prepared homogeneous NPs through nanoprecipitation using microfluidics devices (7). Here, we describe in detail the preparation of NPs that encapsulate hydrophobic compounds using nanoprecipitation "in bulk" and in microfluidic channels, and the preparation of NPs that encapsulate hydrophilic compounds using double emulsion.

3.2.1. Nanoprecipitation for Encapsulating Hydrophobic Compounds

3.2.1.1. "Bulk" Nanoprecipitation

1. Dissolve PLGA-*b*-PEG (10 mg/mL) and docetaxel (0.1 mg/mL) in acetonitrile.
2. Add the polymer/drug mixture dropwise to 3–5 volumes of stirring water giving a final polymer concentration of 3.3 mg/mL (*see* **Note 11**).
3. Stir NPs for 2 h, and remove remaining organic solvent in a rotary evaporator at reduced pressure.
4. The NPs are concentrated by centrifugation at 2,700×*g* for 15 min using an Amicon filter, washed with deionized water and reconstituted in PBS.

3.2.1.2. Microfluidic-Based Nanoprecipitation

1. Dissolve PLGA-*b*-PEG (20 mg/mL) and docetaxel (0.2 mg/mL) in acetonitrile (*see* **Fig. 11.1**).
2. Fill one syringe with water and place it in a syringe pump. Fill the other syringe with polymer/drug solution and place it in another syringe pump.
3. Set water and acetonitrile flow rate at 10 μL/min and 1 μL/min, respectively (*see* **Note 12**).
4. NPs are collected at the outlet stream (*see* **Note 13**).
5. NPs are washed and recovered as described in Step 4 of **Section 3.2.1.1**.

3.2.2. Double Emulsion (w/o/w) for Encapsulating Hydrophilic Compounds

1. An aqueous solution of doxorubicin (2.5 mg/mL, 0.4 mL) is emulsified in 2 mL PLGA-*b*-PEG dissolved in DCM (50 mg/mL) using a probe sonicator (Fisher Scientific) at 20 W for 45 s.

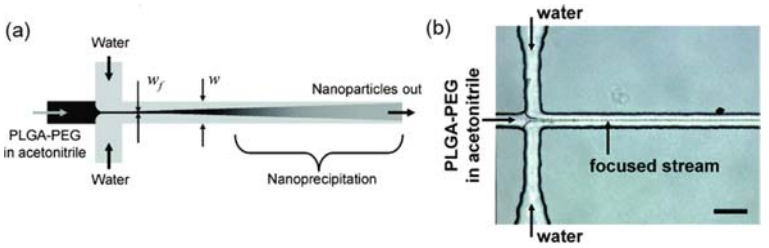

Fig. 11.1. (**a**) Schematic of synthesis of NPs by nanoprecipitation in microfluidic channels. The polymer stream is focused into a thin stream between two faster flowing water streams, in a process commonly called hydrodynamic flow focusing. In the channel, rapid mixing and solvent displacement occur by the diffusion of the organic solvent out of the focused stream and diffusion of water into the focused stream. (**b**) Micrograph of T-shaped device in operation. Scale bar = 50 μm. Reproduced with permission from *Nano Lett* 2008, 8, 2906–2912 (8). Copyright 2008 American Chemical Society.

2. Transfer the emulsion to a PVA aqueous solution (0.1% w/v, 50 mL) and sonicate at 20 W for 1 min.
3. Gently stir the w/o/w emulsion formed at room temperature for 2 h.
4. NPs are washed and recovered as described in Step 4 of **Section 3.2.1.1**.

3.3. Characterization of PLGA-b-PEG Polymer and NPs

3.3.1. Characterization of PLGA-b-PEG Block Copolymer

The composition of PLGA-*b*-PEG is characterized using a 400 MHz ^{1}H nuclear magnetic resonance (NMR). Prepare samples by dissolving 5 mg of the PLGA-*b*-PEG diblock copolymer in 1 mL of deuterated chloroform ($CDCl_3$). An example of a PLGA-*b*-PEG NMR spectrum is shown in **Fig. 11.2**. Conjugation of PLGA-PEG is confirmed using GPC. Compare the PLGA-*b*-PEG molecular weight distribution curve and elution time to PLGA and PEG alone. The GPC sample is prepared by dissolving 4–6 mg of diblock copolymer in 1 mL of tetrahydrofuran.

3.3.2. Characterization of PLGA-PEG NPs

The particle size and size distribution are measured by dynamic light scattering at 25°C, scattering angle of 90°C, using a NP concentration of approximately 1 mg/mL (*see* **Note 14**). The NP surface zeta potential is measured by ZetaPALS and is recorded as the average of three measurements. Transition electron microscopy (TEM) is used to confirm the size and structure of the NPs. A solution of NPs in distilled water (0.5–2 mg/mL) is absorbed on grids and negatively stained for 1 min. For each sample, 5–6 grids are prepared and viewed with a JEOL 200 CX TEM equipped with an AMT 2 k CCD camera. Image at 27–41,000× magnification (*see* **Note 15**).

3.3.2.1. NP Drug Release Kinetics

1. Prepare drug-encapsulated NPs at 5–20% wt of the polymer (*see* **Note 2**).
2. Wash away free drug three times using an Amicon filter.

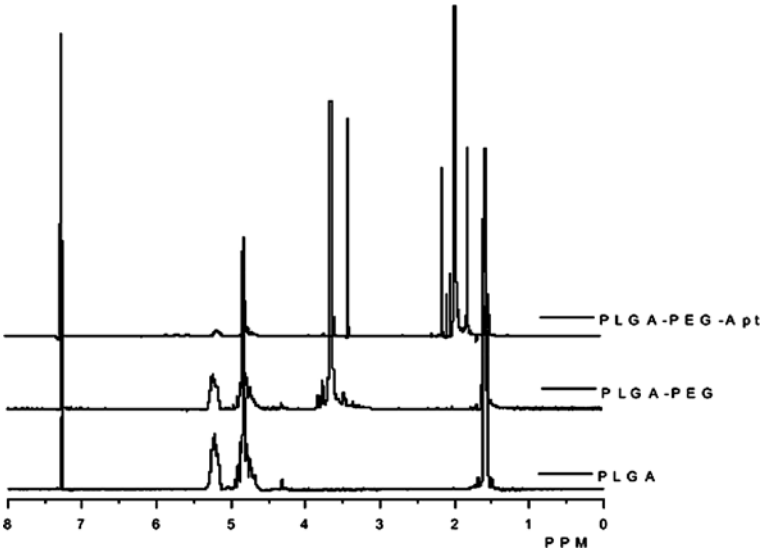

Fig. 11.2. ^{1}HNMR characterization of PLGA-*b*-PEG and PLGA-*b*-PEG-*b*-APT. PLGA: δ 5.2 (m, ((OC$\underline{H}$(CH$_3$)C(O)OCH$_2$ C(O))$_n$)); δ 4.8 (m, ((OCH(CH$_3$)C(O)OC$\underline{H}_2$C(O))$_n$)); δ 1.6 (d, ((OCH(C$\underline{H}_3$)C(O)OCH$_2$C(O))$_n$). PEG: δ 3.7 (s, (C$\underline{H}_2$C$\underline{H}_2$O)$_m$). APT: peaks between δ 1.8 and 2.2 ppm. Reproduced with permission from *Proc Natl Acad Sci USA* 2008**,** 105, 2586–2591 (2). Copyright 2008 National Academy of Sciences, USA.

3. Resuspend NPs in PBS buffer and split them equally into 30 Slide-A-Lyzer MINI Dialysis units (100 μL of sample per tube).
4. Dialyze these microtubes in 4 L of PBS buffer with gentle stirring at 37°C.
5. Change the PBS buffer every 24 h during the whole dialysis process.
6. Collect samples ($n = 3$) at specific time points, such as at 3, 6 or 12 h intervals.
7. Mix samples with an appropriate volume of organic solvent (as required for the mobile phase in Steps 9 and 10) and vortex overnight to dissolve the NPs.
8. Assay free drug content in each sample by measuring drug absorbance using a UV–Vis detector at the appropriate wavelength and mobile phase, against a standard curve of known drug content.
9. Measure docetaxel absorbance at a wavelength of 227 nm and a retention time of 12 min in 1 mL/min nongradient (50/50) acetonitrile/water mobile phase.
10. Measure doxorubicin absorbance at a wavelength of 490 nm and a retention time of 3 min in 1 mL/min nongradient (40/60) acetonitrile/water mobile phase.

3.4. Synthesis of Targeted NPs

3.4.1. Surface Functionalization of Polymeric NPs

1. Suspend 10 mg/mL PLGA-*b*-PEG NPs prepared as described in **Sections 3.1** and **3.2** in DNase- and RNase-free water, and incubate them with EDC (400 mM) and NHS (200 mM) for 20 min with gentle stirring.
2. Wash NPs three times in DNase- and RNase-free water using an Amicon filter.
3. Aptamers are dissolved in water and denatured for 5 min at 90°C followed by snap-cooling on ice, to allow the aptamers to assume binding conformation.
4. The resulting NHS-activated NPs are then reacted with aptamers (1 mg/mL) for 2 h at room temperature with gentle stirring.
5. Wash NP-Apt conjugates three times using an Amicon filter.
6. Keep the NP suspensions at 4°C until use.

3.4.2. Characterization of Targeted PLGA-b-PEG NPs

To verify the presence of the aptamer in the NPs, incubate PLGA-PEG NPs with (+EDC) and without (–EDC) the crosslinker to confirm covalent conjugation. Wash the targeted NPs and separate by polyacrylamide gel electrophoresis (PAGE) and visualize with ethidium bromide (8).

3.5. In Vitro Cell Culture Experiments

3.5.1. In Situ Hybridization to Visualize Endocytosis on Targeted NPs

1. LNCaP and PC3 cells were grown in 8-well microscope chamber slides at concentrations that allow 70% confluence in 24 h (i.e., LNCaP: 40,000 cells/cm^2).
2. On the day of the experiments, wash cells with pre-warmed PBS and incubate with pre-warmed Opti-MEM I Reduced Serum Media for 30 min at 37°C.
3. Incubate cells with ~50 μg of targeted NPs (with a final dye concentration of 1 μg/mL) for 30 min at 37°C in the Opti-MEM media.
4. Wash cells twice with pre-warmed PBS and fix them with 4% formaldehyde for 20 min.
5. Wash cells three times with PBS and permeabilize the cell membrane in PBS containing 0.1% Triton X-100 and 1% BSA and rinse again twice with PBS.
6. Incubate early endosomal marker (mouse monoclonal EEA-1) and late endosomal marker (mouse monoclonal mannose-6-phosphate receptor) antibodies with cells for 1 h at room temperature.
7. Wash cells twice with PBS.
8. Incubate cells with Cy5 goat anti-mouse antibodies for 1 h at room temperature and wash cells twice with PBS.
9. Mount the microscope chamber slides with Vectashield mounting medium with DAPI and glass coverslips. Observe slides by fluorescence microscopy (*see* **Note 16**).

3.5.2. In Vitro Cytotoxicity Assays with Drug-Encapsulated Targeted NP

1. LNCaP and PC3 cells were grown in CellBIND 48-well plates at concentrations that allow 70% confluence in 24 h (i.e., LNCaP: 40,000 cells/cm^2).
2. On the day of the experiments, wash cells with pre-warmed PBS.
3. Incubate cells with targeted NPs (final drug concentration of 1 μg/mL) for 1 h at 37°C.
4. Wash cells twice with pre-warmed PBS. Incubate in fresh complete media for 72 h at 37°C.
5. Assess cell viability colorimetrically with the MTT reagent following the standard protocol provided by ATCC.

3.6. In Vivo Experiments (see Note 17)

3.6.1. In Vivo Biodistribution Experiments

1. LNCaP cells were cultured as described in **Section 2.5**. They were grown to 90% confluency, counted, and resuspended to give 15 million cells/mL (*see* **Note 18**).
2. Weigh the mice and anesthesize intraperitoneally with 2.5% Avertin (200 mg/kg body weight) (*see* **Note 5**).
3. Resuspend 3×10^6 LNCaP cells in 200 μL media/Matrigel mixture suspensions with a media/Matrigel volume ratio of 1:1. Subcutaneously inject the suspensions into the left flank of each mouse. Between injections, gently invert the vials to mix suspensions for more regular-sized tumors. Allow LNCaP tumors to develop for ~3 weeks (*see* **Note 19**).
4. When tumors are sufficiently large (~250–300 mm^3), prepare NPs for administration. Tumor length and width can be measured by digital calipers using the formula: $(\text{width}^2 \times \text{length})/2$. Divide mice randomly into different groups to minimize tumor size variations between groups ($n = 4$–7).
5. Suspend ^{14}C paclitaxel-NP formulations in 200 μL PBS before administration sterile filter.
6. Anesthetize mice with 2.5% Avertin and dose with NPs via tail-vein intravenous injection.
7. After dosing, euthanize different groups at different time points such as ~3, 6, 12, or 24 h. Draw 200 μL of blood by cardiac puncture from each mouse. Harvest the tumor, heart, lungs, liver, spleen, and kidneys from each animal, weigh them, and place them directly in scintillation vials. The liver from each mouse has to be homogenized due to its large size and only ~100 mg of tissue is analyzed.
8. Solubilize each organ in 2 mL Solvable for ~12 h at 60°C. For the blood sample, add only 400 μL Solvable.

9. Add 200 μL of 0.5 M EDTA to each vial to help reduce foaming that occurs upon the addition of H_2O_2.
10. Add 200 μL of 30% H_2O_2 to each vial for 1 h at 60°C to decolorize the sample.
11. Cool samples and add Hionic-Fluor scintillation cocktail to the top of the vial (Perkin-Elmer).
12. The ^{14}C content of tissues were counted using a scintillation counter. To determine the 100% dose, count vials of the formulated NPs with tissue samples. Data are presented as percent injected dose per gram of tissue (% i.d./g).

4. Notes

1. LNCaP cells are an adherent cell line that originated from human prostate carcinomas. They demonstrate slow growth and poor attachment to tissue culture plates. The use of CellBIND plates (Corning) increased cell attachment.
2. Dye-encapsulated NPs are prepared similar to drug-free and drug-encapsulated NPs by mixing the dye or drug with the polymer in the organic phase.
3. Since this was a prostate cancer study, only male mice were used.
4. Phenol-red free media should be used for experiments that require fluorescence microscopy to reduce background auto-fluorescence.
5. When making 2.5% Avertin, dropwise addition with constant stirring at 37°C avoids crystals in the final solution that will cause death in mice via intestinal necrosis. The 2.5% stock solution should be sterile filtered, aliquoted into a series of sterile microcentrifuge tubes, capped tightly, and stored wrapped in foil at 4°C (stable for at least 1 year).
6. The reagent amounts included in this protocol is adequate for in vitro experiments.
7. PLGA viscosity can influence the rate of PLGA-*b*-PEG conjugation. For high-viscosity PLGA, dilute PLGA in DCM to 0.1–0.25 g/mL before adding EDC/NHS.
8. For maximum conjugation efficiency, dissolve EDC/NHS in DCM immediately before adding to PLGA-carboxylate.
9. Supernatant from the washing steps should be clear, especially after the second wash.
10. To achieve a more efficient polymer conjugation, use a high-power vacuum pump to rapidly evaporate residual solvents. The reaction yield should be between 90 and 95%.

11. In order to avoid NP aggregation, the acetonitrile/water volume should be larger than 1:2.
12. The ratio of aqueous stream to organic stream is important. It has been found that as this ratio increases, mixing time decreases as well as particle size (7). As the ratio of water to organic solvent increases, the focused stream becomes thinner and mixing by diffusion occurs faster. A range of 10–30 yields more homogeneous particles and narrower size distributions.
13. The amount of NPs collected at the current flow rates (10 μL/min) is relatively low compared to the "bulk" method. Flow rates can be increased but always maintaining the flow ratio of aqueous to organic stream constant.
14. To maintain NP colloidal stability, always formulate NPs in pure water before reconstituting in PBS or other desired media.
15. For better results, try to use freshly made NPs and avoid waiting longer than 1 h between the time the stain is applied and imaging by TEM.
16. To preserve imaging quality, NPs should be freshly encapsulated with fluorescent dyes and slides should be imaged immediately. Slides can be stored at –20°C in the dark.
17. Animals care and experimental procedures were performed in accordance with the regulations of the Massachusetts Institute of Technology Division of Comparative Medicine and the Principles of Laboratory Animal Care of the National Institutes of Health.
18. To obtain fast-growing tumors, reconstitute LNCaP cells in complete growth medium before mixing with Matrigel.
19. Carefully monitor health status and blood vessel growth around the tumor in the mice. If tumors are too big, the central region would have necrosed and will not be accessible by blood vessels; conversely, smaller tumors would have insufficient neovasculature. Observe for blood vessels around the tumor area.

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