

Synthesis and Evaluation of Airway Targeted PLGA Nanoparticles for Drug Delivery in Obstructive Lung Diseases

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Abstract

Chronic airway inflammation is a hallmark of chronic obstructive airway diseases, including asthma, COPD (chronic obstructive pulmonary disease), and CF (cystic fibrosis). It is also a major challenge in delivery and therapeutic efficacy of nano-based delivery systems in these chronic airway conditions as nanoparticle (NP) need to bypass airways defense mechanisms as we recently discussed. NPs which are capable of overcoming airways defense mechanisms should allow targeted drug delivery to disease cells. Over the last decade there has been increasing interest in development of targeted NPs for cancer but relatively little effort on designing novel systems for treating chronic inflammatory and obstructive airway conditions. Here we describe methods for preparing drug loaded multifunctional nanoparticles for targeted delivery to specific cell types in airways. The formulations and methods for selective drug delivery, discussed here are currently under preclinical development in our laboratory for treating chronic airway conditions such as COPD, CF, and asthma.

Key words: PLGA nanoparticles, Therapeutics, CF, COPD, Asthma, Inflammation and lung disease

1. Introduction

We recently evaluated and discussed the efficacy of *poly-lactic-co-glycolic acid* (PLGA) based nano-systems for selective drug delivery to treat chronic obstructive lung diseases (1, 2). We have also recently described the diagnostic and therapeutic (theranostic) potential of these nanosystems in treating COPD (chronic obstructive pulmonary disease) and CF (cystic fibrosis) lung diseases (3).

A major drawback of PLGA nanoparticles (NPs) is that when formulated with the commonly used emulsifier polyvinyl alcohol (PVA), they have a high negative charge on their surface (3). As a result, such a system, when administered in experimental animals, is

rapidly cleared by airway defense system (1, 2, 4). The best way to overcome this challenge is coating of the drug delivery system with the outer layer of *poly-ethylene-glycol* (PEG) that endow these NPs with airway defense evading properties. PEGylation also increases the retention time of the NPs, thereby enhancing their propensity of accumulation in target diseased cells (1). NP mediated drug delivery presents with the added advantage of targeting the drug to specific diseased cells in the body, for example by conjugating it with an antibody that will target the system to the specific airway cells which over express the complementary antigen (our ongoing studies, as we recently discussed) (3).

In this chapter we describe methods and formulations of NPs as well as in vitro models to evaluate and standardize the targeted drug delivery to diseased cells. The nanosystem discussed here can be used to overcome airway defense mechanisms (i.e., macrophage, neutrophils, and mucus etc.) in chronic obstructive lung diseases and in variety of other pulmonary conditions.

2. Materials

2.1. Polymer Nanoparticle Synthesis and Conjugation

All chemical reagents used should be cell culture or pharmacological grade.

1. PLGA with L:G ratio 50:50 and MW: 5,000–15,000.
2. PEGylated phospholipids: Distereolylphos-phatidylethanolamine-mPEG²⁰⁰⁰ (DSPE-mPEG²⁰⁰⁰) (Avanti Polar Lipids).
3. Antibody: immunoglobulin A (IgA) (1 µg/mL) in PBS.
4. 7.5 mM 2-mercaptoethylamine (2-MEA), store in a desiccator at 4 °C.
5. 25 % HEPES in 0.9 % NaCl (pH 7.2).
6. Acetone.
7. Amicon Centrifugal Filter Device (Ultracel YM-100, Regenerated Cellulose 100 kDa MWCO, Millipore, YM-100).
8. Sonicator—32 W.
9. Refrigerated Rotary Evaporator (Buchi R220 Rotavap).
10. Antibiotics: ibuprofen or other suitable antibiotics.
11. CF corrector drug: VX-809/770 etc.
12. Nile RED (NR) dye (Invitrogen).
13. Rhodamine 6G (R6G) dye (Invitrogen).

2.2. Polymer Nanoparticle Characterization

1. 1 mM HEPES buffer for zeta potential measurements.
2. Formvar-coated copper grids for transmission electron microscopy (TEM) (Electron Microscopy Sciences Inc).
3. ZETAPLUS particle electrophoresis system or NanoDLS (Brookhaven Instruments Inc).
4. Polystyrene standards (Brookhaven Instruments Inc) for calibrating particle electrophoresis system.
5. PHILIPS EM 420 TEM.
6. 2 % (w/v) uranyl acetate solution in ethanol.
7. FACsCalibur flow cytometer (BD Biosciences).
8. CellQuest software (BD Biosciences).

2.3. Nanoparticle Mediated Drug Delivery In Vitro

1. Secondary antibody to IgA (0.4 $\mu\text{g}/\text{mL}$ in PBS) for flow cytometry.
2. The cell permeabilization and fixation Cytofix/Cyterm Kit (BD Biosciences) for intracellular staining and flow cytometry.
3. Strain controlled rheometer (ARES 100, Rheometrics Scientific or TA Instruments).
4. Laser scanning confocal microscope (Zeiss LSM 510 or Nikon A1RMP).
5. Confocal microscope coupled to multiple particle tracking (MPT, SIT Camera VE-1000 Dage-MTI) device.
6. Metamorph software (Universal Imaging Corp) for time-lapsed 3-D imaging for quantifying NP mucus transport rates.
7. Epithelial cells: CF bronchial epithelial cells (CFBE41o-) and human bronchial epithelial cells (HBEo-).
8. Cell culture media, DMEM/F12 (Invitrogen).
9. 10 % Fetal bovine serum (FBS, Invitrogen).
10. 1 % Penicillin, streptomycin, and amphotericin (PSA, Invitrogen).
11. Cell culture lysis reagent, MPER (Pierce).
12. NF κ B reporter plasmid and renilla luciferase internal control.
13. Lipofecatmine 2000 (Invitrogen).
14. Dual Luciferase Assay kit (Promega).
15. Standard phosphate buffer saline (PBS): dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ in 800 mL of distilled H₂O, pH 7.4.
16. Spectrophotometer, e.g., VersMax (Molecular Devices) and SoftMaxPro Software (Molecular Devices) to quantify uptake of dye/drug loaded NPs by epithelial cells.

3. Methods

3.1. Polymer Nanoparticles Synthesis and Conjugation

1. Dissolve 30 mg of PLGA in 0.5 mL of acetone (see Note 1) while stirring.
2. Dissolve 0.5 mg of antibiotic or CF corrector drug in 0.5 mL of acetone (see Note 1) in a glass vial.
3. Dissolve 0.5 mg of dye (NR or R6G) in 0.5 mL of acetone (see Note 1) in a glass vial.
4. Add 0.5 mL of PLGA solution (step 1) drop wise to the DRUG (step 2) or dye (step 3) solution and mix well.
5. Dissolve 25 mg of DSPE-mPEG²⁰⁰⁰ in 0.5 mL of water (see Note 1) in a glass vial with gentle stirring.
6. Add 1 mL of PLGA^{DRUG/DYE} solution (step 4) drop wise to the DSPE-mPEG²⁰⁰⁰ solution (step 5) with vigorous stirring. A two-layered solution should form.
7. Sonicate the two-layered solution in an ice bath for 1–2 min at ~20 W until emulsification. Gradually increase the sonicating power/pulses from 0 to 30 W (see Notes 2 and 3).
8. Leave the emulsified solution overnight in cold room (4 °C) with gentle stirring to evaporate the organic solvent(s).
9. Centrifuge the resulting block copolymer (~5,000 × g, 20 min), wash in deionized water and dry for 30 min using refrigerated rotary evaporator to completely remove organic solvents.
10. Store PEGylated NPs (PNPs) at 4 °C. For the longer term storage freeze-dry the NPs and store at –20 °C.
11. Concentrate the PNPs (step 9) by filtration, using Amicon Centrifugal Filter Device (Ultracel YM-100; see Notes 4 and 5) at ~5,000 × g in 4 °C for 20 min. Equilibrate the Microcon columns with HEPES buffer prior to filtration.
12. Reduce 50 µL of 1 µg/mL IgA antibody by adding β-MEA (7.5 mM final concentration) for 30 min at 37 °C in HEPES buffer (see Note 6).
13. To conjugate the antibody, incubate the reduced antibody (step 12) with the PNP containing DSPE-mPEG (NP:ab = 5:1, v/v). Incubate at RT for 3–5 h (Fig. 1, see Note 6). Store PEGylated immune-conjugated NPs (PINPs) at 4 °C. For the longer term storage freeze-dry the NPs and store at –20 °C.

3.2. Polymer Nanoparticles Characterization

3.2.1. Zeta Potential Measurement and Dynamic Laser Scattering

1. Reconstitute the powdered NPs in 250 µL of 1 mM HEPES buffer, pH 7.4.
2. The ZETAPLUS Instrument (Brookhaven Instruments Inc) is used to calculate the dynamic laser scattering (DLS) as per instruction manual (see Notes 7 and 8). DLS data indicates the hydrodynamic size distribution and colloidal stability of NPs (Fig. 2a).

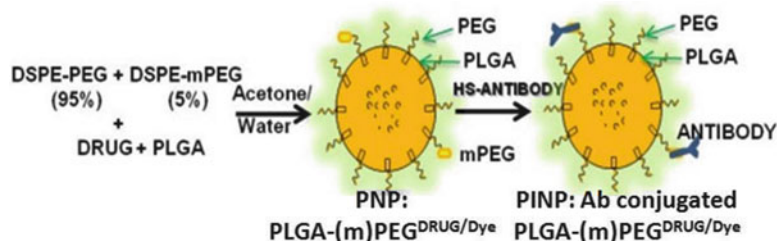


Fig. 1. Schematic of targeted nanoparticle synthesis.

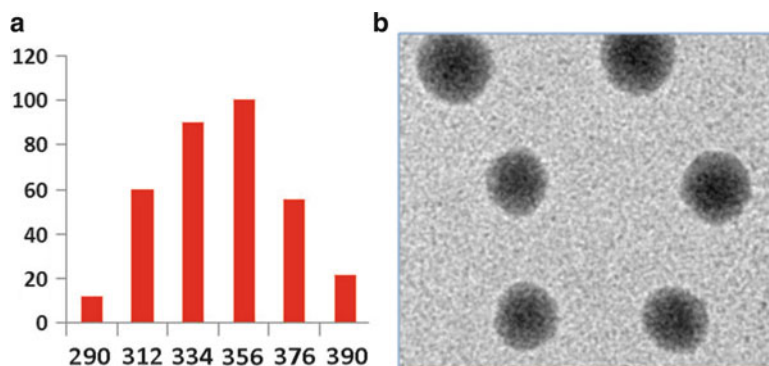


Fig. 2. Characterization of targeted NPs. Dynamic laser scattering (DLS) is employed to measure the hydrodynamic size distribution and colloidal stability of the targeted NPs while transmission electron microscopy (TEM) is used to characterize the size and shape of the NPs. (a) The DLS results verifies the hydrodynamic size of NPs. The hydrodynamic size of NPs varies by less than 15 %, suggesting that their colloidal stability is not affected under physiological pH. (b) TEM shows that targeted NPs are mono-dispersed and spherical. Moreover, it determines the NP size.

3.2.2. Transmission Electron Microscopy

TEM is used to determine the particle size, distribution, and surface morphology.

1. Dry the aqueous dispersion of the NPs on formvar-coated copper grids.
2. Stain the nanoparticles with 2 % uranyl acetate solution (negative staining) (see Note 9).
3. Visualize the nanoparticles using a PHILIPS EM 420 TEM.

3.2.3. Flow Cytometry, Protein Assay, and Fluorescence Microscopy

Flow cytometry is used to analyze the dispersion of marker dye (NR/R6G) loaded targeted NPs as compared to non-targeted NPs using BD FACSCalibur and CellQuest software.

1. Analyze the data using the FSC vs. SSC (Forward Scatter vs. Side Scatter) plots of PNPs (negative control obtained in Subheading 3.1, step 10) and antibody immunoconjugated PNPs obtained in Subheading 3.1, step 13) by FACS Caliber flow cytometer to determine the shift in FSC/SSC for PINPs (over PNPs) due to change in particle size (see Note 10).

2. Evaluate NP delivery (0.1 and 1 $\mu\text{g}/\text{mL}$) to bronchial epithelial cells cultured in DMEM-F12 media for 24–48 h, by analyzing dye (NR/R6G) loaded PINP (FL1) using flow cytometer as compared to the dye (NR/R6G) loaded PNP as a negative control (see Note 11).

3.3. Nanoparticles Mediated Drug Delivery In Vitro

3.3.1. In Vitro Efficacy of NP Mediated Targeted Cell Delivery

Determine the in vitro specificity and cellular uptake of the dye-loaded PINPs using spectrofluorimetry.

1. Seed bronchial epithelial cells at 25,000-cells/mL/well in DMEM-F12 media in a 24-well plate and allow them to attach for 24 h at 37 °C and 5% CO_2 .
2. Determine efficacy of NP uptake by incubating these cells (step 1) with suspension of dye loaded PNP/PINP in growth medium for 15 min, 1 and 4 h.
3. Wash cells three times with PBS.
4. Lyse cells using 100 μL of 1 $\times$ cell culture lysis reagent for 5 min at room temperature.
5. Analyze cell lysates for NP uptake by quantifying the amount of dye recovered from these lysates using the spectrophotometer. Record the changes in optical density at excitation/emission wavelengths of 525/555 to determine the uptake of dye loaded NPs. The amount of dye recovered from the lysates (the changes in optical density) directly correlate with the efficiency of PINP uptake by the target cells. The non-antibody conjugated PNPs should be used as a control to determine if antibody conjugation improves efficacy of PINP uptake by target cells.

3.3.2. Mucus Transport and In Vivo Kinetics

1. To measure mechanical properties of COPD and CF mucus use a strain-controlled rheometer. Measure elastic and loss moduli, $G'(\omega)$ and $G''(\omega)$, by applying 1 % amplitude oscillatory deformations of frequencies between 0.01 and 100 rad/s and quantify the in- and out-of-phase components of the resulting stress (see Note 12).
2. Load the layer (1–5 μm thick) of undiluted mucus on a 24 well plate followed by ~ 100 μL of dye loaded PINPs.
3. Use Laser scanning confocal microscopy and MPT Camera for time-lapsed 3-D imaging to determine NP transport rates through the mucus using the Metamorph software. The rates of PINP penetration through the mucus layer from various patients (at least $n = 15$) can be used to determine if these NPs can effectively penetrate mucus for drug delivery in chronic obstructive lung diseases.

3.3.3. NF κ B Reporter Assays

1. Seed and transfect bronchial epithelial cells with reporter plasmids (0.1 μg of NF κ B and 0.01 μg Renilla luciferase internal control).

2. Six hours post-transfection, treat the cells with PINP^{DRUG} (10 and 1 μ L drug, from Subheading 3.1, step 13) and incubate plates for 24 h. To determine the efficacy of PINP mediated drug delivery in controlling NF κ B mediated inflammation; step 2 is also done in the presence of 10 ng/mL IL1- β /TNF α .
3. Use standard Dual Luciferase Assay Promega kit and follow manufacturer's instructions to calculate the NF κ B reporter activity normalized to renilla luciferase internal control for step 2. The data will determine the efficacy of PINP mediated drug delivery in controlling NF κ B mediated inflammatory response.

4. Notes

1. The acetone is used as a solvent based on the solubility of selected DRUG/Dye. Accordingly, other solvents can be used based on the solubility of selected drug/dye. Single/Double emulsion technique can be used depending on the hydrophilic/phobic nature of the drug.
2. Stirring rod should be removed prior to sonication.
3. Keep your tubes on ice while sonicating.
4. Microcon filter device column only holds 500 μ L volume, use multiple spins of same sample for higher volumes. Make sure the column does not run dry.
5. Use low speed centrifugation (800 $\times g$, 30 s) or microcon filter to remove unbound target antibody.
6. IgA antibody is used to selectively bind to the polymeric immunoglobulin receptors (IgR) on the surface of epithelial cells in the lungs. Other target antibodies can be used based on the alveolar cell type.
7. The instrument should be calibrated using polystyrene standards (e.g., from Brookhaven Instruments Inc) prior to the measurements.
8. Serial dilution (10^{-1} – 10^{-4}) may be required.
9. Uranyl acetate stains PEG (negative staining) and enhances its electron density. This staining results in a dim ring surrounding the PLGA core (Fig. 2b).
10. We developed this approach to verify antibody conjugation to PINPs, in addition to classical ELISA's or protein quantification.
11. This is an important step to verify both NPs epithelial cell delivery and efficacy.

12. The mechanical properties of mucus are variable among patients and this will help correlate the efficacy of NP transport rates through mucus with varied consistency.

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