Biomaterials 30 (2009) 2790–2798

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The uptake and intracellular fate of PLGA nanoparticles in epithelial cells

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ARTICLE INFO

Article history:
Received 14 January 2009
Accepted 29 January 2009
Available online 20 February 2009

Keywords:
Nanoparticle
Epithelial cell
Intracellular
Organelle
Confocal microscopy
PLGA

ABSTRACT

Biodegradable polymer nanoparticles (NPs) are a promising approach for intracellular delivery of drugs, proteins, and nucleic acids, but little is known about their intracellular fate, particularly in epithelial cells, which represent a major target. Rhodamine-loaded PLGA (polylactic-co-glycolic acid) NPs were used to explore particle uptake and intracellular fate in three different epithelial cell lines modeling the respiratory airway (HBE), gut (Caco-2), and renal proximal tubule (OK). To track intracellular fate, immunofluorescence techniques and confocal microscopy were used to demonstrate colocalization of NPs with specific organelles: early endosomes, late endosomes, lysosomes, endoplasmic reticulum (ER), and Golgi apparatus. Confocal analysis demonstrated that NPs are capable of entering cells of all three types of epithelium. NPs appear to colocalize with the early endosomes at short times after exposure (~2 h), but are also found in other compartments within the cytoplasm, notably Golgi and, possibly, ER, as time progressed over the period of 4–24 h. The rate and extent of uptake differed among these cell lines: at a fixed particle/cell ratio, cellular uptake was most abundant in OK cells and least abundant in Caco-2 cells. We present a model for the intracellular fate of particles that is consistent with our experimental data.

1. Introduction

Epithelial cells serve as the chief barrier that separates the internal contents of the body from the outside environment. The skin and mucosal surfaces of the gut, respiratory and reproductive tracts are also frequently the key obstacle to the administration of drugs due to their unique structure and barrier characteristics [1]. These cells are highly polarized, with apical surfaces exposed to the outside environment and basolateral surfaces connecting them to an underlying basal lamina. Epithelial cells impart strength and rigidity to tissue by forming a monolayer through cell–cell tight junctions [2,3]. Epithelia regulate selective transport of substances (e.g., ions, fluids, metabolic substrates and byproducts, macromolecules, and microparticulates) across the monolayer and alterations in the trafficking of apical membrane receptors, transporters and channels are often associated with disease [2].

Nanoparticulate delivery systems and their use *in vivo* is becoming increasingly popular, as they promise to overcome many of the obstacles inherently associated with the administration of certain drugs, vaccines, plasmid DNA, and RNAi material [4–8]. PLGA nanoparticles (NPs) for delivery of therapeutics are of particular interest due to their biocompatibility, biodegradability and ability to maintain therapeutic drug levels for sustained periods of time. The polymer matrix prevents the degradation of the drug and the duration and levels of drug released from the NPs can be easily modulated by altering the formulation.

Their successful application, however, is highly dependent on their interaction with epithelial cells, which serve the crucial barrier role in the body. Previous work demonstrates that most NPs, including those formed from biodegradable polymers such as poly(lactic-co-glycolic acid) or PLGA, are taken up by an endocytic process and that their uptake is concentration- and time-dependent [3,9–11]. Furthermore, it has been shown that nanoparticles gain access to the intracellular environment of epithelial cells [3,12–15]; however, little is known about their fate once inside the cells or whether NP uptake varies from one epithelial cell type to the next.

This report attempts to provide insight into these relatively unexplored areas by systematically analyzing the uptake pattern of biodegradable PLGA NPs *in vitro* in three different epithelial cell lines, representing distinct epithelial tissues: opossum kidney (OK) renal tubule cells, Caco-2 human intestinal cells, and human bronchial epithelial (HBE) cells. Experiments evaluating NP colocalization with specific intracellular organelles were conducted in an

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doi:10.1016/j.biomaterials.2009.01.057

effort to characterize the intracellular fate of these NPs. The various compartments, early endosomes, late endosomes, lysosomes, endoplasmic reticulum (ER), and the Golgi complex, were chosen for investigation as they are typically involved in biosynthesis, processing, transport, storage, release, and degradation of soluble and membrane-bound macromolecules [16].

2. Materials and methods

2.1. NP preparation

50:50 PLGA, $M_W = 30$–70 kDa with an inherent viscosity of 0.59 dl/g was purchased from Birmingham Polymers Inc. (Birmingham, AL). Polyvinyl alcohol (PVA, $M_W = 12$–23 kDa; 87–89% hydrolyzed) and Rhodamine B, a commonly used marker reagent [17], were obtained from Sigma–Aldrich (St. Louis, MO). Ethyl acetate and all other reagents used were supplied by Fisher Scientific (Fairlawn, NJ).

NPs were prepared using a single-emulsion technique [7,18]. Two hundred milligrams of PLGA polymer was dissolved in 2 ml of ethyl acetate solvent in a glass tube. Twenty microliters of a 1 mg/ml solution of Rhodamine B was added to the polymer/solvent mixture. Subsequently, four milliliters of an aqueous 5% w/v solution of PVA was poured into a separate glass tube. While vortexing the surfactant solution at a high setting, the solvent mixture was added dropwise. Once all of the Rhodamine/polymer mixture was added to the PVA solution, the contents were vortexed for an additional 10 s at a high setting. The tube contents were then sonicated for 3×10 s at 38% amplitude with a TMX 400 sonic disruptor (Tekmar, Cincinnati, OH) to create an oil-in-water emulsion. Immediately after sonication, the emulsion was poured into 100 ml of an aqueous 0.3% w/v PVA solution, under rapid stirring with a magnetic stirrer. The resulting nano-sized particles were stirred in solution for 3 h to allow for ethyl acetate evaporation. The NPs were then collected by centrifugation, washed 3 times with Milli-Q treated water, and finally resuspended in 4 ml of Milli-Q treated water and dried on a lyophilizer. Long-term storage was in an airtight container at $-20\,^{\circ}C$.

2.2. NP characterization

The morphology, percent loading, and controlled-release profile were evaluated following synthesis of the particles.

NPs were fixed to aluminum sample stubs with double-sided carbon tape and sputter coated with gold for viewing by scanning electron microscopy. A scanning electron microscope (SEM; XL30 ESEM, FEI Company) was used to assess the particle surface properties. Micrographs were analyzed with NIH SCION imaging software (Scion Corporation, Frederick, MD) to determine particle size distribution.

Fluorescence techniques were used to evaluate the actual amount of Rhodamine dye encapsulated in the particles and its release profile. Because Rhodamine has inherent fluorescent properties (excitation/emission = 560 nm/584 nm), spectroscopy (SpectraMax, Molecular Devices) was used to generate a calibration curve of fluorescence values at known concentrations of the dye that allowed quantification of the percent loading of the dye and the controlled-release profile for the NPs.

In characterizing the release profile of Rhodamine NPs, a known quantity of the particles was suspended in Dulbecco's Phosphate Buffered Saline (PBS) in a glass container and incubated at 37 °C, 100 rpm in a rotary shaker. Samples of the supernatant were collected at designated time points and analyzed. To determine the amount of compound encapsulated in the particles, a known quantity of Rhodamine NPs was dissolved in 1 ml of dimethyl sulfoxide (DMSO) overnight. The sample was centrifuged at 10,000 rpm for 5 min followed by collection and spectroscopic analysis of the supernatant.

2.3. In vitro experiments

Three different cell lines were used to compare uptake and fate of NPs *in vitro*: (1) OK cells – model of renal proximal tubule, (2) Caco-2 cells – model of the gut epithelium, and (3) HBE cells – model of the respiratory airway. Studies exploring uptake and pathway/fate determination were conducted.

2.3.1. Antibodies and reagents

Primary antibodies (EEA1, LAMP1, Rab7, Calnexin, TGN38) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies were supplied by Sigma–Aldrich (St. Louis, MO) and Jackson ImmunoResearch Laboratories (West Grove, PA). Tissue culture reagents and all other reagents were from Invitrogen Corp. (Carlsbad, CA).

2.3.2. Culture conditions

OK cells were propagated in 75 cm^2 flasks in DMEM high glucose medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. Medium was changed every 2–3 days and cells were split at a ratio of 1:10 when 80% confluence was reached.

Caco-2 cells were propagated in 75 cm^2 flasks in DMEM high glucose medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Medium was changed every 2 days and cells were split at a ratio of 1:10 when 80% confluence was attained.

HBE cells were propagated in collagen-coated 75 cm^2 flasks in LHC-8 medium supplemented with 10% fetal bovine serum, 80 μg/ml tobramycin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone. Medium was changed every 2 days and cells were split at a ratio of 1:5 when 80% confluence was reached.

All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

2.3.3. NP uptake

Experiments that evaluated the method, rate, and extent of particle uptake used fluorescence techniques. These studies utilized NPs encapsulating Rhodamine and immunofluorescent labeling of cells with α5 antibody. The α5 antibody tags the Na^+/K^+ pump along the cell membrane thereby detailing the perimeter of each cell.

For each of the epithelial cell lines, cells were grown on permeable filter supports (Costar Transwell® filter plate, 0.4 μm pore size; collagen-coated for HBE cell line) until confluence. Caco-2 cells were given an additional 14–21 days of growth post-confluence to allow for enterocytic differentiation into "gut-like" cells that possess microvilli, junctional complexes, a tall columnar appearance and a basophilic nucleus [19].

Immediately before co-incubation with Rhodamine NPs, medium at both the top and bottom of the filter was changed. Two hundred microliters of a 0.5 mg/ml suspension of NPs ($\sim 6 \times 10^5$ NPs) in medium was added to the top of the filter and cells were incubated at 37 °C, 5% CO_2 for defined time intervals. Controls included cells incubated at 4 °C, as well as a 'no treatment' group.

For each time point (1, 2, 4, 6 and 24 h), filters were removed from the incubator, placed in a clean plate and placed on ice. Cells were washed twice with PBS to remove excess NPs, fixed with cold methanol and then washed an additional 3 times with PBS. Filters were carefully cut away from their supports and cells were permeabilized (PBS^{++} containing 0.3% Triton X-100 (v/v), 0.1% BSA (w/v)) for 15 min at room temperature. The samples were blocked with dilution buffer (PBS containing 10% goat serum (v/v), 2% saponin (w/v), 10 mM glycine) for 30 min and then incubated with the primary α5-antibody (1:100) for 1 h at room temperature. Cells were washed 3 times with permeabilization buffer and then incubated with fluorescein isothiocyanate-conjugated goat antimouse IgG antibody (1:100) for 45 min at room temperature. Following secondary antibody incubation, cells were washed 3 times with PBS and mounted onto slides with Vectashield mounting medium.

2.3.4. NP pathway

To determine the effect NPs have on the bioelectric parameters of epithelial cells, transepithelial electrical resistance (TEER) measurements were taken.

Cells were grown on permeable filter supports (Costar Transwell® filter plate, 0.4 μm pore size; collagen-coated for HBE cell line) until confluence. Caco-2 cells were given an additional 14–21 days of growth post-confluence to allow for enterocytic differentiation. Prior to the addition of NPs, the medium in both the top and bottom of the filter was changed and samples were returned to the incubator and allowed to equilibrate.

An EVOM Epithelial Voltmeter (World Precision Instruments, New Haven, CT) was used to measure the 'filter + cells' resistance prior to adding NPs. After the addition of NPs (300 μl or 500 μl of a 0.5 mg/ml suspension of NPs in media), resistance measurements were taken at defined intervals. A 'control filter + cells only' sample was measured for the full time course of the study. TEER values were obtained by subtracting the resistance value of the 'filter + cells alone' from the 'filter + cells + NPs' resistance value. The differences were then multiplied by the surface area of the filter and expressed as Ω cm^2.

2.3.5. NP intracellular fate

To examine the position of particles relative to different intracellular compartments, antibodies directed against organelle specific indicators were used: EEA1 as a marker of early endosomes, Rab7 as a marker of late endosomes, LAMP1 as a marker for lysosomes, TGN38 as a marker for the Golgi complex, and Calnexin as a marker for the ER. Fluorescent labeling of a compartment, together with the use of Rhodamine NPs, allowed microscopic analysis of NP-organelle colocalization.

This set of experiments followed the general design of the uptake experiments detailed previously, in which cells were co-incubated with NPs for a set time period, fixed, and immunofluorescently labeled for areas of interest. Briefly, following incubation of NPs on cell monolayers grown on Transwell® filters, samples were collected at specified time points. Samples were removed from the incubator, placed in a clean collection plate and placed on ice. Cells were washed twice with PBS, fixed in 4% paraformaldehyde and then washed an additional 3 times with PBS. Filters were carefully cut away from their supports and cells were permeabilized (PBS^{++} containing 0.3% Triton X-100 (v/v), 0.1% BSA (w/v)) for 15 min at room temperature. The samples were blocked with dilution buffer (PBS containing 5% donkey serum (v/v), 2% saponin (w/v), 10 mM glycine) for 30 min and then incubated with the primary antibody (1:100 for OK and Caco-2; 1:50 for HBE) for 1 h at room temperature. Cells were washed 3 times with permeabilization buffer and then incubated with the secondary antibody (1:100 for OK and Caco-2; 1:50 for HBE) for 45 min at room temperature. (Antibodies: 1° EEA1 goat polyclonal or TGN38 goat