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The Uptake and Intracellular Fate of PLGA Nanoparticles in Epithelial Cells

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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 hr), but are also found in other compartments within the cytoplasm, notably Golgi and, possibly, ER, as time progressed over the period of 4 to 24 hrs. 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.

Keywords

Nanoparticle; Epithelial cell; Intracellular; Organelle; Confocal microscopy

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

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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 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, $M_w = 30\text{--}70$ kDa with an inherent viscosity of 0.59 dL/g, was purchased from Birmingham Polymers Inc. (Birmingham, AL). Polyvinyl alcohol (PVA, $M_w = 12\text{--}23$ 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 seconds at a high setting. The tube contents were then sonicated for 3×10 seconds 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 hours 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°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=560nm/584nm), 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 1ml of dimethyl sulfoxide (DMSO) overnight. The sample was centrifuged at 10,000 rpm for 5 minutes 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 $\mu\text{g}/\text{ml}$ streptomycin, and 1mM 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 cm² 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% CO₂.

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 (~6×10⁵ NPs) in medium was added to the top of the filter and cells were incubated at 37°C, 5% CO₂ for defined time intervals. Controls included cells incubated at 4°C, as well as a ‘no treatment’ group.

For each timepoint (1, 2, 4, 6 and 24 hours), 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 minutes 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 minutes and then incubated with the primary α5-antibody (1:100) for 1 hour 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 minutes 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 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 $\Omega \cdot \text{cm}^2$.

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 timepoints. 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 minutes 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 minutes and then incubated with the primary antibody (1:100 for OK and Caco2; 1:50 for HBE) for 1 hour at room temperature. Cells were washed 3 times with permeabilization buffer and then incubated with the secondary antibody (1:100 for OK and Caco2; 1:50 for HBE) for 45 minutes at room temperature. (Antibodies: 1° EEA1 goat polyclonal or TGN38 goat polyclonal with 2° Donkey anti-goat IgG Cy5; 1° Rab7 rabbit polyclonal or Calnexin rabbit polyclonal with 2° Goat anti-rabbit IgG Cy5; 1° LAMP1 mouse monoclonal with 2° Rabbit anti-mouse IgG Cy5.) After secondary antibody incubation, cells were washed twice with permeabilization buffer, once with PBS, and mounted onto slides with Vectashield mounting media (Vector Labs, Burlingame CA).

2.3.6 Confocal Imaging—Filters/cells from the completed uptake and fate determination experiments were visualized by confocal laser scanning microscopy (LSM 510meta, Carl Zeiss Inc., Thornwood, NY). Samples were excited with 488 nm (green), 543 nm (red) and 633 nm (blue) laser lines. Images were captured with a C-Apochromat 40X/1.2 NA water-immersion lens in multi-tracking mode at an acquisition resolution of 1024×1024. Pinhole diameters were set to less than 1 airy unit where signal/noise ratio is optimal and optical slice sections of approximately 1µm were taken. Image stacks were analyzed using IMARIS software (Bitplane AG, Switzerland) on a per pixel basis.

Quantification of NPs throughout each image stack was completed using the SPOTS option of the IMARIS software package, which permits visualization of spherical objects throughout an image stack and designates a spatial position along x, y and z directions, as well as the intensity of the point it represents [20]. Summation of the number of spots is provided in the standard statistics package of SPOTS.

NP-organelle colocalization evaluation involved analysis of confocal images stacks with the IMARIS Coloc feature. This segment of the software implements the Costes & Lockett method (NIH, NCI/SAIC) which automates selection of colocalized voxels, thereby eliminating operator-related bias while providing analysis and visualization of colocalization in multi-channel data sets [20]. Colocalization of the rhodamine NPs with a specific organelle was determined on a per pixel basis.

Because it is difficult to distinguish between individual NPs and small clusters of NPs using our confocal imaging, the data are presented on a 'nanoparticle event' basis.

3. Results

3.1 Rhodamine NPS

Representative scanning electron micrographs (Figure 1) illustrate that NPs have a characteristic round shape and are monodisperse; quantitative analysis of particle size revealed an average diameter of 95 nm with a standard deviation of 20 nm. Rhodamine was released slowly from the NPs with a biphasic profile where an initial burst release is seen in the first several hours followed by a more sustained, uniform release (Figure 2). The release profile for a 20 day period is shown for rhodamine particles with a loading of 0.06% w/w (encapsulation efficiency was 62%).

3.2 In Vitro Experiments

Analysis of images acquired using confocal microscopy, along with TEER data, offered insight into the uptake and intracellular fate of NPs in cells across multiple epithelial cell lines.

3.2.1 NP Uptake—NPs are capable of gaining intracellular access in all cell lines investigated. Uptake was quantified by acquiring and evaluating 1 μ m-thick image slices of the cell monolayer that were stacked and analyzed, together with cross-sectional slices perpendicular to the plane of the cell monolayer midpoint (z-axis). The z-slices showed that distinct NPs were present in different planes throughout the thickness of the monolayer. Cross-sectional slices confirmed that NPs were indeed inside the cells and not simply adsorbed to the outer surface.

The rate and the extent to which NPs were taken up differed, however, among the cell lines. OK cells took up the PLGA NPs quite readily. As early as 30 minutes post addition, NPs were found within the cells and it appears that a saturation limit was reached at about 4 hours (average of 91 NP events at 1 hour with 421 NP events present at 4 hours post-incubation - Figures 3 and 6). After 4 hours, the amount of NPs associated with the cells decreases. In both the intestinal (Caco-2) and bronchial epithelial (HBE) cells, the rate of uptake was considerably slower than in OK cells. In HBE cells, NPs also gain entry at early timepoints, but to a lesser extent than OK cells. At 1 hour, an average of 28 NP events was seen with a steady increase observed for each subsequent timepoint reaching 93 NP events (average) at 24 hours post NP co-incubation (Figures 4 and 6). It does not appear that a saturation limit was reached in HBE cells within the 24 hour period. A very different behavior was observed for Caco-2 cells, namely that these gut-like cells did not readily take up the NPs. The first sign of NP association with Caco-2 cells is at 24 hours (Figure 5) with only a very small number gaining access (average of 26 NP events per image stack).

To determine whether the uptake mechanism of NP entry into epithelial cells was mediated by endocytosis, controls were included in which cells were co-incubated with NPs and maintained at 4°C. Endocytosis, an energy dependent process, is blocked at low temperatures. We observed that NP uptake was significantly decreased at 4°C (Figure 6), suggesting that indeed NP uptake is mediated by endocytosis. Because a very low number of particles were taken up by Caco-2 cells, it was difficult to ascertain whether NP uptake was inhibited at 4°C in this cell line specifically; however previous work using NPs in Caco-2 cells demonstrates that endocytosis is the mechanism of NP uptake with these cells [15].

3.2.2 NP Pathway—Transcellular transport requires solutes to cross the plasma membranes of the epithelium, while paracellular passage involves movement between the cells through ‘leaky’ tight junctions. The formation and permeability of tight junctions between cells determine the resistance and integrity of a particular tissue and the electrical resistance properties of the epithelial barrier can be altered by physiological stimuli [21]. TEER can be measured to assess changes in the integrity or tightness of an epithelial monolayer. Our data showed that TEER measurements in OK, Caco-2 and HBE cells were not significantly affected by the addition of NPs when compared to control cell values. This result suggests that the particles and their uptake into cells did not interfere with the integrity of the monolayers.

3.2.3. NP Intracellular Fate—Analysis of immunofluorescently labeled compartments in OK and HBE cells showed that internalized NPs colocalized with markers of early endosomes and the Golgi complex (Figure 8). In OK cells, peak colocalization is seen at 4 hours. In HBE cells, peak colocalization with early endosomes is observed at 2 hours, while greatest colocalization with Golgi is detected at 24 hours post NP addition. Presence of NPs in lysosomes was observed, but to a far lesser extent than other compartments in both cell lines. One surprising finding was that NPs were found to colocalize with the ER in HBE cells, however, the convoluted structure of the ER and the limited resolution of our method of analysis does not permit us to conclude definitively that NPs are actually contained within that specific compartment. It is also worth noting in this context that NPs co-localizing with Golgi markers may not be contained within the saccules of the Golgi complex itself, but may rather be encapsulated by Golgi-associated late endosomal vesicular structures.

It was not possible to determine NP-organelle colocalization in Caco-2 cells due to the low number of NPs taken up by the cells; however, previous work using transmission electron microscopy (TEM) analysis with colloidal gold-labeled nanospheres *in vivo* showed particles to be present in the cytoplasm, inside the Golgi apparatus, and in secretory vesicles [22].

4. Discussion

Our aim was to quantify the rate and extent of uptake of PLGA NPs in different epithelial cell lines and learn more about the intracellular fate of NPs that gain entry into cells. An understanding of the dynamics of uptake and intracellular trafficking could provide insight into the mechanism by which drug-loaded NPs allow improved therapy when compared to free drug [4, 23, 24] and provide further information as to whether these vehicles are suitable for intracellular delivery of RNAi material [25–27]. Moreover, knowledge of the intracellular fate of unmodified PLGA NPs can serve as a baseline for design of surface-modified NPs intended for improved, targeted delivery [28, 29].

Data from our experiments in three distinct epithelial cell lines confirmed that NPs are taken up by means of an endocytic process. This finding is consistent with previous work that showed endocytosis or receptor-mediated endocytosis to be the pathway mitigating intracellular localization of PLGA NPs [3, 10, 30, 31]. The minimal level of uptake that was observed to persist at 4°C has also been seen by other groups and is thought to be most likely due to a passive process such as diffusion [3, 31]. We noted that the rate and extent of NP uptake, however, differs among cell lines. Cells that served as a model for the renal proximal tubule readily endocytose particles and reach a saturation point between 4 and 6 hours after exposure. Evidence of saturation of particle uptake has been previously documented in the literature in other cell types (e.g., vascular smooth muscle cells, Caco-2 cells) [15, 31]. After 4–6 hours, fewer particles are seen within the OK cells, suggesting that they are being lost to another process, most likely exocytosis. Consistent with this

hypothesis, spectroscopic analysis of medium collected during OK and HBE NP uptake experiments revealed increasing amounts of fluorescence (presumably associated with NPs) in the medium within the basolateral section of the well over time (data not shown).

Uptake of NPs was observed in HBE cells, but to a much lesser degree, and with slower uptake kinetics. At 2 hours post-incubation, an average of 58 NP events was observed across the HBE monolayer while at the same timepoint, OK cell monolayers contained an average of 247 NP events. This assessment held true when data were analyzed on a per cell basis (Figure 7). Caco-2 cells take up particles at the slowest rate with the first signs of NP events visible only after 6 and 24 hours. The evidence of intracellular uptake in OK, HBE and Caco-2 cells is consistent with the literature [12–15], however direct comparison of rates to previous work is not possible, as differences between NP formulation, dose, and cell culture conditions exist.

It is not surprising to see that the three cell lines compared possess different NP uptake rates, as it is well known that endocytosis rates are specific to cell type. For example, the rate at which plasma membrane is internalized by pinocytosis varies between cell types: a macrophage ingests 3% of its plasma membrane each minute, while fibroblasts endocytose at a somewhat lower rate of 1% per minute [32]. Moreover, if one considers the basic function of each of these cell lines, we see that the uptake pattern is consistent with the cells' intended role. Caco-2 cells are models of enterocytes within the intestinal epithelium whose main purpose is to control passage of macromolecules and pathogens, while at the same time allowing digestive absorption of dietary nutrients [4]. Hence, one would expect very selective transport of macromolecules and particles. The airway epithelia (HBE cell model) is similar, as its primary function is mucociliary clearance and protection against noxious agents [33]. Renal proximal tubule cells (OK cell model), on the other hand, are the major site for reabsorption of filtered proteins and consequently exhibit a high rate of apical endocytotic activity [34, 35].

Previous work with epithelial cells demonstrates that trafficking pathways and regulatory mechanisms vary between epithelial cell types and that transepithelial transport largely depends on the polarized expression of transporters and channels at either the apical or the basolateral membrane [2, 36]. In one study, comparison of different transporters present at the apical membrane of renal proximal tubules, intestinal and lung epithelia demonstrated that while intestinal and lung epithelia have the same type of cotransporters present at their apical surface, renal proximal tubules did not [36]. This work confirms the existence of specific differences in the cellular machinery of OK, Caco-2 and HBE cells and underscores the complexity involved even among cells of the same general structure.

We propose that NP uptake in certain cells may be the result of receptor-mediated endocytosis. Observation of increased NP uptake in OK cells could be directly related to two glycoproteins, cubulin and megalin, present at the surface of the cells. Cubulin and megalin serve as endocytic receptors in the kidney proximal tubule and facilitate the reabsorption of albumin from the glomerular filtrate [37, 38]. In this situation, it is possible that PLGA NPs bind albumin present in the culture medium, thereby causing the protein to function as a ligand at the surface of the NPs; the receptor interaction with 'albumin-coated' NPs would allow increased intracellular absorption. Previous work has shown that NPs, with their large surface area, bind or absorb proteins that function as receptor-agonists and permit selective access through receptor-mediated endocytosis [39]. Additional studies with PLGA NPs would need to be conducted to confirm this uptake mechanism in renal tubule cells.

Qualitative assessment of confocal images reveals NP distribution that is either punctate (suggesting localization of particles in specific intracellular compartments) or of a more

diffuse nature (suggesting that particles are spread throughout the cytoplasm). Similar distribution patterns were seen in NP work in rabbit conjunctival epithelial layers by Qaddoumi et al [3]. In our study, completely blurred areas detected in the red channel (predominantly with Caco-2 cells) are thought to be due to free rhodamine release in the medium and subsequent intracellular uptake of the dye. Experiments in which free rhodamine was applied to the cultured cells in medium confirmed this hypothesis (data not shown).

Confocal analysis demonstrates that NPs are capable of intracellular uptake in all three cell lines, that they can escape/avoid endo-lysosomal degradation, and that NPs are trafficked to relevant subcellular compartments after endocytosis. PLGA NPs appear to colocalize with the early endosomes, Golgi apparatus and in certain instances, the ER. Our findings are consistent with the previous studies in the literature, in which researchers evaluating NPs in other cell types have shown their particular NP preparation to be taken up via the transcellular and paracellular routes and NPs to be capable of endo-lysosomal escape [10, 40]. Additionally, TEM evaluation of NP-treated Caco-2 cells by other groups observed NPs present in the cytoplasm, within the Golgi apparatus, and in secretory vesicles of the cells [22].

We suggest one possible intracellular pathway that NPs follow is of a 'typical' endocytosis-exocytosis route (Figure 9); NPs first encounter endosomes (main site for sorting of endocytosed material and membrane-recycling in the cell), followed by retrieval or escape from the compartment and subsequent interaction with the exocytic organelles of the cell: the ER, the Golgi apparatus, and secretory vesicles [16, 32]. NPs largely avoid lysosomes and are thus able to bypass the main intracellular digestive compartment. Evaluation of colocalization trends in HBE cells illustrates the proposed pathway: NPs strongly colocalize with early endosomes at 2 hours post incubation, followed by a decrease in early endosome association and an increased association/colocalization with the ER and Golgi over time. Given that NPs are not highly colocalized with late endosomes or lysosomes relative to the other compartments, we infer that these NPs may largely escape endo-lysosomal degradation. Comparison of insulin processing at the cellular level with the proposed NP intracellular route reveals a similar pattern. Free aqueous insulin present in the lumen of the intestinal tract is absorbed by binding to specific receptors in intestinal enterocytes, enters the endosomal compartment of epithelial cells and is then transferred to the blood circulation via the Golgi apparatus and the basolateral plasma membrane interdigitations [41].

5. Conclusions

Data collected in our work demonstrates that PLGA NPs are taken up intracellularly in three distinct epithelial cells lines. The rate and extent of uptake among these cell lines differs, most likely due to regulatory mechanisms inherent to the particular cell type. We propose an intracellular route which describes the fate of particles following uptake. Our results suggest that when designing *in vitro* and *in vivo* NP experiments, researchers should include a preliminary study to assess the rate and extent of uptake by running a series of NP concentrations over a minimum time course of 0–24 hours to better ascertain appropriate dose, incubation time, and intracellular targets relative to the application/cell type of choice and to ensure successful study outcome. Finally, the findings of our study provide insight into NP trafficking that can be used to further efforts in targeted NP applications, where cell recognition and association with cell membranes can be enhanced by the use of surface ligands to bind specific receptors on cell membranes [42–44], and in the area of RNAi delivery, where efficient cellular uptake, rapid lysosomal escape and sustained intracellular drug-release characteristics make PLGA NPs an attractive vehicle for gene-silencing applications [8, 45, 46].

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Figure 1.
Representative scanning electron micrograph of rhodamine NPs at ~ 30,000X magnification.

Figure 2.
Controlled-release profile for NPs 0.06% loaded with Rhodamine.

Figure 3.

NP uptake in OK cells. Z-stack images for 1, 2, 4, 6, and 24-hour timepoints are shown. Green channel (row 1), red channel (row 2) and color overlay (row 3) images are provided. Green denotes the cell perimeter ($\alpha 5$ label for Na⁺/K⁺ pump), while red indicates Rhodamine NPs. Scale bar represents 50 μ m.

Figure 4.

NP uptake in HBE cells. Z-stack images for 1, 2, 4, 6, and 24-hour timepoints are shown. Green channel (row 1), red channel (row 2) and color overlay (row 3) images are provided. Green denotes the cell perimeter (α5 label for Na⁺/K⁺ pump), while red indicates Rhodamine NPs. Scale bar represents 50 μm.

Figure 5. NP uptake in Caco2 cells. Z-stack images for 1, 2, 4, 6, and 24-hour timepoints are shown. Green channel (row 1), red channel (row 2) and color overlay (row 3) images are provided. Green denotes the cell perimeter ($\alpha 5$ label for Na^+/K^+ pump), while red indicates Rhodamine NPs. Scale bar represents 50 μm .

6 (a)

6 (b)

Figure 6. Quantitative comparison of total NP-associated events for OK (a) and HBE (b) cells for the full 24-hour time course. Endocytosis control data is provided in the last column.

7 (a)

7 (b)

Figure 7. Quantitative comparison of NP-associated events per cell for OK (a) and HBE (b) cells. Endocytosis control data is provided in the last column.

8 (a)

8 (b)

Figure 8. Colocalization analysis for OK (a) and HBE (b) cells for each of the five intracellular compartments evaluated.

Endocytosis

Figure 9.

Proposed NP intracellular pathway. Black arrows denote typical endocytosis-exocytosis pathway; orange arrows indicate proposed NP path. Illustration from [16].