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Drug Delivery Nanoparticles: Toxicity Comparison in Retinal Pigment Epithelium and Retinal Vascular Endothelial Cells

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Abstract

Multiple synthetic polymer nanoparticles (NPs) have been widely used as drug delivery systems. However, their toxicity to the retinal pigment epithelium and retinal endothelium remains unclear. In this study, we analyze the cytotoxic effects of three different kinds of NPs, made of poly lactic-co-glycolic acid (PLGA), polycaprolactone (PCL), and PEGylated PLGA (PEG-PLGA), in a retinal pigment epithelium cell line (ARPE-19) and in primary human retinal vascular endothelial cells (RVEC). PEG-PLGA NPs presented the lowest cytotoxicity on ARPE-19 cells and RVEC as assessed by MTT viability assay. While PLGA and PCL exhibited variable amount of toxicity, no significant toxicity was observed when incubating cells with high PEG-PLGA concentrations (100ug/ml), for up to 6 days. On both transmission electron microscopy and confocal microscopy, Rhodamine 6G-loaded PEG-PLGA NPs were observed intracellularly in multiple subcellular organelles. PEG-PLGA NPs are a potentially viable option for the treatment of eye diseases.

Keywords

Retina; nanoparticle; PEG-PLGA; pegylation; cellular uptake; subcellular localization

Introduction

The advent of nanotechnology has expanded the therapeutic potential allowing the delivery of drugs to cell-specific, subcellular organelles, using biodegradable materials in the nano-scale as carriers.¹ This nanotechnology-based targeted delivery is further facilitated by soluble polymer carriers, which, when combined with a drug, can advantageously modify its native pharmacological properties. These modifications include, among others, a prolonged half-life with controlled biodegradation rate, improved solubility of hydrophobic molecules, reduced immunogenicity, sustained or triggered-release of drugs, and selective accumulation in specific tissues.^{2,3} These changes are crucial to developing efficient and tailored nanoparticles (NPs), which could subsequently be used to improve the pharmacological

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efficiency and specificity of drugs. These drug-delivery NPs have already been implemented in the medical field, with encouraging results in several clinical trials.⁴

Recently, the use of engineered NPs in the ophthalmology arena has yielded positive results in animal eye models. The usage of NPs as a novel drug delivery system in eye disease models has gained much attention partly because of their particular ability to permeate eye barriers; Kim and colleagues showed that gold NPs administered intravenously could pass through the blood-retina barrier and be distributed in all retinal layers without cytotoxicity.⁵ Other studies have examined useful persistence in ocular tissues, thus contributing to the sustained release of a drug and its prolonged duration of action.⁵⁻⁸ Lipid-NPs loaded with myriocin, a powerful inhibitor of the rate-limiting enzyme of ceramide biosynthesis, can increase photoreceptor survival in the rd10 retinal degeneration mouse model of retinitis pigmentosa when administered by eye drops.⁹ Intravitreal injection of NPs loaded with a connexin43 mimetic peptide led to increased retinal ganglion cell survival in an acute retinal ischemia-reperfusion rat model.¹⁰ Finally, intravitreal and intravenous administration of expression plasmids-NPs have been shown to have an inhibitory effect on choroidal neovascularization (CNV) in several animal models.¹¹⁻¹³ Despite the promising results of these drug administration approaches, concerns on retinal toxicity are slowly being raised and the assessment of retinal pigment epithelium NP-related cytotoxicity remains to be addressed

The purpose of this in vitro study was to identify toxicity profile and potential of utilizing NPs to deliver drugs into retinal pigment epithelium (RPE) cells and retina vascular cells. The cytotoxic effects of three different kinds of NPs, made of either poly lactic-co-glycolic acid (PLGA), polycaprolactone (PCL), or PEGylated PLGA (PEG-PLGA), were analyzed using a human retinal pigment epithelial cell line (ARPE-19) and primary human retinal vascular endothelial cells (RVEC). We found that PLGA, PCL, and PEG-PLGA NPs have different toxicity profiles; PEG-PLGA NPs, in particular, presented the lowest cytotoxicity in both cell types, and a high incorporation rate by the ARPE-19 cells. In addition, these NPs are ubiquitously distributed in cell organelles.

Materials and Methods

Synthesis of nanoparticles

Three different kinds of polymer NPs, Poly D, L lactic-co-glycolic acid (PLGA), Polycaprolactone (PCL), and Poly D,L-lactic-co-glycolic acid-poly ethylene glycol (PEG-PLGA) were prepared by an oil-in-water (O/W) emulsion/solvent evaporation technique, as previously described.¹⁴ Briefly, 30 mg of each polymer were dissolved in an organic mixture of dichloromethane (DCM) and acetone, with a volume ratio of 8:2 and a final polymer concentration of 2.5%. The mixture was added drop-wise into an aqueous solution containing 2.5% polyvinyl alcohol (PVA). An O/W-type emulsion was achieved by sonication in an ice bath with a probe-type sonicator (Q500 Sonicator, Qsonica, Newtown, CT) at 200 W ultrasound power for 2 minutes. The organic solvent was allowed to evaporate by overnight stirring, at atmospheric pressure. The NPs were separated by ultracentrifugation at $133,000 \times g$ for 15 minutes (Sorvall Legend Micro 17R, Thermo Scientific, Wilmington, DE). After discarding the supernatant, NPs were washed 3 times

with deionized water to remove any residues. Finally, the product was dried by lyophilization and stored in -20 °C.

Synthesis of Rhodamine 6G-loaded nanoparticles

For the synthesis of Rhodamine 6G-loaded NPs, 30 mg of PEG-PLGA NPs were dissolved in 0.8 ml of DCM and mixed with a saturated Rhodamine 6G solution, including 30 mg of Acetone, 0.8 mL of DCM, and 0.2 mL of Rhodamine. The mixture was added drop-wise into a 2.5% PVA aqueous solution and the procedure described above was performed to synthesize the NPs.

Characterization of polymer nanoparticles

The size and shape of NPs were measured by transmission electron microscopy (TEM) and their hydrodynamic diameter was determined by light scattering, as previously described.¹⁵

Treatment of cells with different nanoparticles

The ARPE-19 cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium containing nutrient mixture F12 (1:1) (Gibco/BRL Life Technologies, Grand Island, NY), and supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Primary retinal microvascular endothelial cells were obtained from Cell Systems (Kirkland, WA). Cells were grown in conditioned media, which includes 10% serum (4Z0-500), attachment factor (4Z0-210), and supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin.

Cells were seeded into a 96-well plate, at a density of 1×10^4 cells per well, with 100 μ L of medium per well. The cells were allowed to grow for 24 hours at 37 °C in an incubator with 5% CO₂. Medium was replaced before treatment.

NPs were sterilized by UV light for 30 minutes and diluted into three different concentrations, i.e. 25, 100, and 200 μ g/mL. At 100% confluence, a volume of 100 μ L of the NP solution was added into each well. The cells were incubated at 37°C with 5% CO₂ for different periods of time before performing cytotoxicity assays.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To assess the *in vitro* cytotoxicity induced by NPs, an MTT assay (Invitrogen, CA) was performed according to the manufacturer's instructions. Briefly, media was aspirated from 96-well plates and cells were washed twice with sterile phosphate-buffered saline (PBS). MTT was added into each well at a concentration of 0.5 mg/mL. The final reading was measured by spectrometer (Spectra Max 190, Molecular Devices, Sunnyvale, CA) at a wavelength of 590 nm.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

In order to assess the DNA fragmentation induced by NPs, a TUNEL assay (Promega, WI) was performed using ARPE-19 cells, according to the manufacturer's protocol. Cells were treated with one of the three NPs evaluated, at a concentration of 100 μ g/mL, for 4 days. After TUNEL staining was completed, DAPI was used to stain for cell nuclei. Images were

taken by fluorescence microscopy (AXIO Imager 2, Carl Zeiss, NY). The ratio of TUNEL-positive cells per 10^4 total cells, was measured by manual counting of 144 images for each sample.

***In vitro* cellular uptake and subcellular localization of nanoparticles in ARPE-19 cells**

To evaluate the *in vitro* subcellular distribution, ARPE-19 cells were treated for 24 hours with Rhodamine 6G-loaded NP-PLGA NPs, previously sterilized by UV light. Cells were harvested and stained with lysosomal (LysoTracker Green, Life Technologies), mitochondrial (Rhodamine 123, Life Technologies), and endoplasmic reticulum (ER) (ER-Tracker Green, Life Technologies) organelles dyes, and examined by confocal microscopy (Leica SP2, Bannockburn, IL).

Statistical Analysis

Statistical analysis was performed on JMP Pro software version 11.2.0 from SAS (Cary, NC). Statistical significance for differences between treatment groups was determined with one-way ANOVA with Tukey post-hoc correction. A p-value of < 0.05 was considered statistically significant.

Results

PEG-PLGA nanoparticles present the lowest cytotoxicity in ARPE-19 cells

To determine the cytotoxicity of different NPs, ARPE-19 cells were incubated with different concentrations (25-200 $\mu\text{g/ml}$) of PLGA, PCL and PEG-PLGA NPs. Cytotoxicity was determined by MTT assay at different time points. As seen in Figure 1, three different concentrations of PCL and PEG-PLGA NPs were tested. PLGA displayed a dose and time dependent toxicity, whereas PCL showed mostly time dependent toxicity at all dosages. PEG-PLGA was the most well tolerated NP exhibiting minimal reduction in MTT viability at the highest dose (200 $\mu\text{g/ml}$) only after 6 days of incubation. Overall, these data suggest that the PEG-PLGA NPs present the least toxicity on ARPE-19 cells.

To further confirm this finding, we performed a TUNEL assay in ARPE-19 cells treated with PLGA, PCL, and PEG-PLGA NPs for 4 days. As seen in Figure 2, PLGA and PCL NPs presented a higher ratio of TUNEL-positive cells compared to PEG-PLGA ($p < 0.01$). Cells treated with PEG-PLGA NPs showed no significant difference in the ratio of TUNEL-positive cells from the control. The TUNEL assay results correlate with the results from the MTT cell viability assay, confirming that PEG-PLGA has the lowest cytotoxicity on ARPE-19.

PLGA and PEG-PLGA nanoparticles present the lowest cytotoxicity in RVEC

Since intraocular drug administration has the potential to purposefully or inadvertently affect vascular cells we further assessed any possible cytotoxicity to human primary RVEC. Similar to ARPE19, PEG-PLGA exhibited no significant toxicity as assessed by MTT even at the highest dose (200 $\mu\text{g/ml}$) for up to 6 days in culture. In contrast both PCL and PLGA exhibited mostly a time dependent toxicity. Overall, we can conclude from this data that PEG-PLGA NPs have the lowest toxicity on human primary RVEC.

PEG-PLGA nanoparticles are round, smooth, with a diameter of 50 nm, and can be localized inside ARPE19 cell

Since PEG-PLGA NPs have the lowest toxicity in ARPE-19 and RVEC, we then proceeded to further characterize this particular type of NP. Transmission Electron microscopy showed them to be broadly round-shaped and their surface was without sharp edges (Fig. 4). The hydrodynamic diameter was 50 nm on average, as measured by light scattering. To assess cellular uptake, ARPE-19 cells were incubated with PEG-PLGA for 24 hours, and TEM images were obtained. As seen in Figure 4, PEG-PLGA NPs can be observed inside the cell, surrounded by membranous subcellular structures. We speculate that this can represent PEG-PLGA inside the endosomal system. The intracellular presence of these NPs was evidenced in all assessed cells. In conclusion, these results suggest that RPE cells can incorporate PEG-PLGA NPs.

PEG-PLGA nanoparticles are present in lysosomes, mitochondria and endoplasmic reticulum of treated ARPE-19 cells

To further confirm *in vitro* cell targeting efficiency and subcellular localization, ARPE-19 cells were treated with Rhodamine 6G-loaded PEG-PLGA NPs for 24 hours, and evaluated by confocal microscopy. As seen in Figure 5, NPs were incorporated by the cell and ubiquitously distributed, as the red fluorescence emitted from the Rhodamine 6G-loaded NPs was observed in all z-planes. This finding confirms that RPE cells can incorporate PEG-PLGA NPs. In regard to the subcellular localization of NPs, the fluorescence emitted by lysosomal stain LysoTracker Green presented a moderate and heterogeneous spatial overlapping with the red fluorescence emitted by the Rhodamine 6G-loaded NP. This signal suggests partial co-localization of PEG-PLGA NPs to the lysosomes consistent with the TEM results. Mitochondrial marker (rhodamine 123) and endoplasmic reticulum (ER) marker (ER-Tracker Green) suggested that PEG-PLGA NPs can also be target to the mitochondria and ER (Fig 5). In summary, these data indicate that PEG-PLGA is uptaken by ARPE-19 cells, and ubiquitously distributed in subcellular organelles, targeting lysosomes, mitochondria, and the endoplasmic reticulum.

Discussion

The results from this study showed that treatment with NPs can have a significant effect on ARPE-19 and RVEC viability. After comparing the cytotoxicity of three different drug delivery NPs, we found that PEG-PLGA NPs presented the lowest cytotoxicity on both RPE and retinal vascular endothelium cell lines. In addition, PEG-PLGA NPs can be localized in several organelles within the ARPE-19 cell, such as lysosomes, mitochondria, and endoplasmic reticulum. We speculate that the low cytotoxicity of PEG-PLGA NPs can be attributed to the PEGylation process, as compared to non-PEGylated PLGA NPs. Additionally, the morphologic characteristics of these particles, such as their size and shape, appear to influence the uptake pathway and specific organelles targeted.

PEGylation process is the covalent attachment of polyethylene glycol polymer (PEG) to PLGA NPs and has several potential benefits for drug delivery. PEGylation enhances the pharmacokinetics and tissue distribution of NPs¹⁶, prevents particle agglomeration¹⁷, and

enhances biocompatibility.^{18,19} In addition, PEG-coating increases the *in vivo* circulation time of NPs, most likely by increasing their hydrodynamic size and water solubility, therefore reducing their renal clearance.^{20,21} Recently, Moret and coworkers also demonstrated that non-PEGylated NPs can cause a dose-dependent reduction of cell viability in human lung cells.²² Peracchia and coworkers increased the body of evidence by showing that PEGylation reduced the *in vitro* cytotoxicity of NPs in a macrophage-monocyte cell line.²³ These findings correlate with our results in different cell lines, as PEG-PLGA NPs exhibit the lowest cytotoxicity in both ARPE-19 cells and RVEC, when compared to non-PEGylated NPs.

The diameter of NPs can condition the cellular uptake and organelle targeting. Sahu and coworkers demonstrated size-dependent cytotoxicity, showing that carbon black NPs with a diameter of approximately 50 nm can exhibit higher cytotoxicity and inflammation in human monocytes, in comparison to particles of 500 nm.²⁴ In our study, the hydrodynamic diameter of PEG-PLGA was 50 nm. In contrast to the study by Sahu et al, the toxicity of these NPs was insignificant and comparable to control treatment even after 6 days of exposure. This finding is in agreement with several works that suggest that epithelial cells can take up NPs of smaller size in a more efficient manner than larger ones, especially when compared to monocytes and macrophages.²⁵⁻²⁷ In summary, these reports substantiate that NP size-dependent cytotoxicity can differ among cell lines.

It has been suggested that the shape and potential sharpness of NP edges can affect its cellular uptake and localization within organelles. Chu and coworkers recently demonstrated that the morphology of NPs can independently determine their subcellular localization, regardless of size, chemical composition, or surface characteristics.²⁸ More specifically, NPs with sharp shapes can enter the cell via endocytosis, and later pierce the endosomal membrane prior to lysosome formation, avoiding excretion by exocytosis. Hence, they can escape to the cytoplasm and accumulate in the cytosol. From our results, PEG-PLGA NPs presented a smooth surface, without sharp edges. The presence of red fluorescence from Rhodamine 6G-loaded NPs inside ARPE-19 cells indicates that the PEG-PLGA NPs can persist inside this cell type.

The ubiquitous localization of PEG-PLGA NPs in lysosomes, mitochondria, and the endoplasmic reticulum, can be advantageous for targeting several diseases related to organelle dysfunction. Specifically, many retinal degenerative diseases are associated with mitochondria dysfunction. The availability of a NP with low cytotoxicity, cell-specificity, and somewhat preferential mitochondrial localization, may allow tailoring of NP drug-delivery systems suitable for the treatment of mitochondrial eye and/or systemic diseases.

In summary, our results suggest that PEG-PLGA can be a suitable molecule for drug delivery systems in eye diseases. The low cytotoxicity and preferential cellular localization of PEG-PLGA NPs in the ocular cells examined *in vitro* is the first essential step in the developmental process of a safe drug delivery modality aimed at treating intricate diseases of the posterior segment of the eye. Given that certain NPs have already been shown to be able to overcome the blood-ocular barriers, as well as help maximize ocular drug absorption, we believe a NP drug delivery system utilizing PEG-PLGA could be a viable new option for

the treatment of retinal diseases. This prospect should be further investigated *in vivo*, in animal models.

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Figure 1. PEG-PLGA nanoparticles present the lowest cytotoxicity in ARPE-19 cells
Cytotoxicity assessment by MTT assay on ARPE-19 cells treated with PLGA, PCL, and PEG-PLGA NPs at three different concentrations, 25, 100, and 200 µg/mL, at 2, 4 and 6 days (A, B, C). Y-axis in each figure represents the MTT absorbance normalized to the control. PEG-PLGA NPs show the lowest toxicity of the three types of NPs, while PLGA NPs present the highest toxicity through day 6. * $p < 0.05$.

Figure 2. PEG-PLGA nanoparticles present the lowest ratio of DNA fragmentation in ARPE-19 cells

DNA fragmentation or nicking, a characteristic hallmark of apoptosis, was assessed by TUNEL assay of ARPE-19 cells treated with PLGA, PCL, and PEG-PLGA NPs. (A) Fluorescence microscope images of TUNEL assay. Cells were incubated with 100 μ g/ml of PLGA, PCL, and PEG-PLGA NPs or medium only (control). Blue channel represents cell nuclei stained by DAPI, and the green channel represents the DNA-nicked TUNEL positive apoptotic cells. (B) Quantitation of TUNEL positive cells. A total of 144 images per group were counted manually, and results are expressed as the ratio of TUNEL positive cells per 10,000 normal cells. Compared with the control, the PLGA NPs showed the highest and statistically significant toxicity on ARPE-19 cells, approximately 8-fold. Similarly, PCL toxicity also presented a significant increase in toxicity, in this case, 2-fold higher than control. In contrast, PEG-PLGA NPs presented a similar ratio of apoptotic cells to the control, without statistical significance. * $p < 0.01$.

Figure 3. PEG-PLGA nanoparticles present the lowest cytotoxicity in retinal microvascular endothelial cells

Cytotoxicity assessment by MTT assay on RVEC treated with PLGA, PCL, and PEG-PLGA NPs at three different concentrations, 25, 100, and 200 µg/mL, at 2, 4 and 6 days (A, B, C). Y-axis in each figure represents the MTT absorbance normalized to the control. PEG-PLGA NPs show the lowest toxicity of the three types of NPs, while PCL NPs present the highest toxicity through day 6. * $p < 0.05$.

Figure 4. PEG-PLGA nanoparticles present a diameter of 50 nm, with blunt edges, and can be localized inside the cell

TEM images from ARPE-19 cells treated with PEG-PLGA NPs. (A) Characterization of size and shape of PEG-PLGA NPs. The hydrodynamic diameter was 50 nm, as measured by light scattering. Edges were blunt, without sharp spicules. (B) ARPE-19 cells treated with PEG-PLGA evidenced presence of the NPs within the cellular compartment, most likely in the endosomal system (arrows).

Figure 5. PEG-PLGA nanoparticles are internalized and can be localized in the lysosomes, mitochondria, and endoplasmic reticulum

Confocal microscopy images of ARPE-19 cells treated with Rhodamine 6G-loaded PEG-PLGA NPs and stained with different organelle stains. (A-C) Rhodamine 6G-loaded PEG-PLGA NPs can be observed at all z-planes inside the cells. These NPs can also be co-localized in the lysosomes (D-F), mitochondria (G-I), and endoplasmic reticulum (J-L), as seen in the merged signal with their respective trackers.