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Biocompatibility, uptake and endocytosis pathways of polystyrene nanoparticles in primary human renal epithelial cells

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ABSTRACT

Recent years have witnessed an unprecedented growth in the number of applications – such as drug delivery, nutraceuticals and production of improved biocompatible materials – in the areas of nanoscience and nanotechnology. Engineered nanoparticles (NPs) are an important tool for the development of quite a few of these applications. Despite intense research activity, mechanisms regulating the uptake of NPs into cells are not completely defined, being the phenomenon dramatically influenced by physico-chemical properties of NPs and cell-specific differences. Since the cellular uptake of NPs is a prerequisite for their use in nanomedicine, the definition of their internalization pathway is crucial. For this reason, we used 44 nm polystyrene NPs as a model to analyze the uptake and endocytosis pathways in primary human renal cortical epithelial (HRCE) cells, which play a key role in the clearance of drugs. NPs were found not to affect the viability and cell cycle progression of HRCE cells. Distinct internalization pathways were analyzed by the use of drugs known to inhibit specific endocytosis routes. Analyses, performed by confocal microscopy in combination with quantitative spectrofluorimetric assays, indicated that NPs enter HRCE cells through multiple mechanisms, either energy-dependent (endocytosis) or energy-independent.

1. Introduction

Nanoparticles (NPs) can be applied in the medical sector as sensors, in cell and organ imaging, implant coatings and drug delivery. Understanding the distinct endocytic mechanism(s) used by NPs to enter the target cell is essential for drug delivery. A thorough and consistent profile of NP uptake is difficult to describe, since literature offers contrasting opinions on this; it has been reported that NPs can enter cells both via passive and active transport and no general rules have been identified so far on size- and surface-dependent particle uptake. Moreover, several endocytic mechanisms often take place simultaneously and the mechanism of internalization may be dramatically influenced by physico-chemical properties (Hillaireau and Couvreur, 2009) of NPs. From a general point of view, a simplified classification of endocytosis into four routes, i.e. clathrin-mediated, caveolae-mediated, macropinocytosis, and caveolae-independent pathways, is generally taken into account (Doherty and McMahon, 2009; Howes et al., 2010; Sahay et al., 2010). Polystyrene (PS) NPs have been widely used as models because they can be easily synthesized in a wide range of sizes, which facilitates their application as biosensors (Velev and Kaler, 1999) in photonics (Rogach et al., 2000) and in self-assembling nanostructures (Boal et al., 2000). It has been reported that positively charged NPs are taken up via clathrin-coated vesicles (Jiang et al., 2010), while plain PS NPs mostly use macropinocytosis to enter cells (Jiang et al., 2011). It is noteworthy that differences in the internalization pathways may be due to cell-specific expression of endocytic routes. It is, for instance, known that smooth-muscle cells, fibroblasts, adipocytes and endothelial cells are rich in caveolae (Parton and Simons, 2007) and, therefore, preferentially use the caveolae-dependent route. The purpose of the present work is to analyze uptake, toxicity and stability of 44 nm PS NPs in renal cells. In our study, primary human renal

Abbreviations: EIPA, 5-(N-ethyl-N-isopropyl)amiloride; FITC, fluorescein isothiocyanate; HRCEC, human renal cortical epithelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPs, nanoparticles; PDI, polydispersity index; PS, polystyrene.

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cortical epithelial (HRCE) cells were used, as they play an important role in the clearance of drugs in vivo by reabsorbing glucose and amino acids in the glomerular filtrate, while allowing other substances of no nutritional value to be excreted in the urine. For biomedical applications, nanomaterials need to enter the body and directly contact tissues and cells without any toxic effects; therefore, it is crucial to explore PS NP biocompatibility. We found that 44 nm PS NPs do not affect viability of HRCE cells and do not interfere with cell cycle progression independently from NP concentration and exposure time. Furthermore, we analyzed the internalization pathway(s) of NPs by using different drugs known to inhibit different endocytic pathways. Confocal microscopy analyses, in combination with quantitative spectrofluorimetric assays, allowed us to draw a picture of the uptake mechanisms involved. We demonstrated that 44 nm PS NPs enter HRCE cells through different coexisting mechanisms, either energy-dependent or energy-independent. Our study represents a contribution in defining the internalization pathway of NPs to be used for drug delivery.

2. Materials and methods

2.1. Reagents

Green fluorescent PS NPs with a diameter of 44 nm were purchased from Duke Scientific Corporation (Palo Alto, CA, USA). All reagents and FITC-insulin were from Sigma–Aldrich (St. Louis, MO, USA). Mouse anti-human clathrin monoclonal antibody was purchased from ABR Affinity BioReagents (Golden, CO, USA); rabbit anti-human caveolin-1 polyclonal antibodies were from Abcam (Cambridge, UK); fluorescent goat anti-rabbit and anti-mouse IgGs were from Invitrogen (Carlsbad, CA, USA).

2.2. Size and ζ-potential measurements

The particle size and ζ-potential were determined by using a ZetaSizer Nano ZS, Malvern Instruments, Worcestershire, UK. Analyses were performed on NPs diluted in distilled water, serum free cell culture medium and complete cell culture medium. NPs were tested at a concentration of 0.1 mg/mL, following an incubation of 1 h at 37 °C. All samples were subjected to three measurements and data were reported as mean ± standard deviation (SD).

2.3. Cell culture

Human renal cortical epithelial (HRCE) cells (Innoprot, Derio – Bizkaia, Spain) were cultured in basal medium, supplemented with 2% foetal bovine serum, epithelial cell growth supplement and antibiotics, all from Innoprot, in a 5% CO_2 humidified atmosphere at 37 °C.

2.4. Cell viability and cell cycle analyses

To test the effects of NPs on cell viability, HRCE cells were seeded in 96-well plates (100 μL/well) at a density of 5×10^3/well. In the case of long time exposure to NPs (168 h), cells were plated at a lower density (1×10^3/well). 24 h after seeding, PS NPs were added to the cells for time- and dose-dependent cytotoxic assays. At the end of incubation, cell viability was assessed by the MTT assay. MTT reagent, dissolved in DMEM in the absence of phenol red, was added to the cells (100 μL/well) to a final concentration of 0.5 mg/mL. Following 4 h incubation at 37 °C, the culture medium was removed and the resulting formazan salts were dissolved by adding isopropanol containing 0.01 N HCl (100 μL/well). Absorbance values of blue formazan were determined at 570 nm using an automatic plate reader (Microbeta Wallac 1420, Perkin Elmer, Waltham, MA, USA). Cell survival was expressed as a percentage of viable cells in the presence of NPs with respect to control cells grown in the absence of NPs. In all the experiments, controls were performed by supplementing the cell cultures with identical volumes of surfactant for the same lengths of time.

For cell cycle analyses, HRCE cells were seeded in 100 mm diameter cell culture dishes at a density of 3×10^4 cells/mL and incubated at 37 °C with PS NPs at a concentration of 40 μg/mL for 72 or 168 h. Following incubation, cells were detached with non-enzymatic cell dissociation solution, washed with PBS and incubated in ice cold methanol for 20 min at −20 °C. Following incubation, cells were hydrated in PBS for 30 min and centrifuged. Cell pellet was then dissolved in PBS containing 50 μg/mL RNase A. After 30 min at room temperature (RT), cells were centrifuged and dissolved in propidium iodide (PI) staining buffer (0.1% sodium citrate containing 50 μg/mL PI) at a density of 0.5×10^6 cells/mL. Cell cycle analysis was then performed using a FACS Calibur Flow Cytometer (Becton Dickinson, Oxford, UK). The percentage of cells in each phase of the cell cycle (G1, S and G2/M) was calculated by using Modfit software (Becton Dickinson), and compared with the results obtained with untreated cells as a control.

2.5. Fluorescence studies

HRCE cells were seeded on glass coverslips in 24-well plates and grown to semi-confluency. Cells were incubated for the indicated times in complete medium with green fluorescent PS NPs (10 μg/mL) or FITC-insulin (0.1 mg/mL). In order to inhibit specific internalization pathways, different experimental procedures were used. To inhibit energy-dependent endocytosis, HRCE cells were pre-incubated at 4 °C for 10 min and then incubated with NPs or with FITC-insulin for 1 h at 4 °C. Alternatively, cells were pretreated with sodium azide (200 μM) for 30 min at 37 °C and then incubated with NPs or with FITC-insulin for 1 h at 37 °C. To inhibit microfilament-mediated endocytosis, cells were pre-treated with lantrunculin A (1 μM) for 10 min at 37 °C and then incubated with NPs for 1 h at 37 °C. To inhibit clathrin-mediated endocytosis, cells were pre-treated with 900 μM sucrose for 30 min at 37 °C and then co-incubated with NPs or with FITC-insulin for 1 h at 37 °C. Endocytosis mediated by clathrin, caveolin and lipid rafts was inhibited by using dynasore. Cells were pre-treated with dynasore (80 μM) for 30 min at 37 °C and then incubated with NPs for 1 h in the presence of the inhibitor. To inhibit macropinocytosis, cells were pre-treated with ethyl-isopropyl amiloride (EIPA, 100 μM) for 30 min at 37 °C and then incubated with NPs for 1 h in the presence of the inhibitor. At the end of the incubations, cells were treated with 1 μg/mL Hoechst 33342 for 10 min at 37 °C and washed with PBS. Cells were then fixed for 10 min at RT with 2% paraformaldehyde in PBS. For immunofluorescence analyses, cells were permeabilized with 0.5% Triton X-100 in PBS (5 min) and then incubated for 30 min with 3% goat serum in PBS, in order to saturate nonspecific binding sites. Afterwards, cells were incubated overnight at 4 °C with mouse anti-clathrin monoclonal or rabbit anti-human caveolin-1 polyclonal antibodies, and then rinsed with 0.1% Triton X-100 in PBS. Finally, cells were incubated 1 h in the darkness with fluorescent goat anti-rabbit or anti-mouse secondary antibodies. Slides were then washed with 0.1% Triton X-100 in PBS and then with PBS, and mounted in 50% glycerol in PBS. Samples were examined using a Leica 6000 UV microscope and a Leica TCS SP5 confocal microscope, equipped with a Leica application suite software. All images were taken under identical conditions, unless specified differently.

2.6. Spectrofluorimetric analyses

HRCE cells were seeded in 12-well plates and grown to semi-confluency. Cells were then incubated for the indicated times in