Toxicology in Vitro 31 (2016) 126–136

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Polystyrene nanoparticles internalization in human gastric adenocarcinoma cells

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ARTICLE INFO

Article history:
Received 14 July 2015
Received in revised form 14 October 2015
Accepted 9 November 2015
Available online 14 November 2015

Keywords:
Nanoparticles uptake
Gastric cells
Endocytosis
Interleukins

ABSTRACT

The increase in the use of nanoparticles, as a promising tool for drug delivery or as a food additive, raises questions about their interaction with biological systems, especially in terms of evoked responses. In this work, we evaluated the kinetics of uptake of 44 nm (NP44) and 100 nm (NP100) unmodified polystyrene nanoparticles (PS-NPs) in gastric adenocarcinoma (AGS) cells, as well as the endocytic mechanism involved, and the effect on cell viability and gene expression of genes involved in cell cycle regulation and inflammation processes. We showed that NP44 accumulate rapidly and more efficiently in the cytoplasm of AGS compared to NP100; both PS-NPs showed an energy dependent mechanism of internalization and a clathrin-mediated endocytosis pathway. Dose response treatments revealed a non-linear curve. PS-NPs also affected cell viability, inflammatory gene expression and cell morphology. NP44 strongly induced an up-regulation of IL-6 and IL-8 genes, two of the most important cytokines involved in gastric pathologies. Our study suggests that parameters such as time, size and concentration of NPs must be taken carefully into consideration during the development of drug delivery systems based on NPs and for the management of nanoparticles associated risk factors.

1. Introduction

Nanoparticles (NPs) are defined as materials with dimension range between 1 and 100 nm (Beer et al., 2012). They are being used in several application fields, such as electronic, cosmetic, food industry and medicine (Dekkers et al., 2011; Osmond and McCall, 2010). In medicine, in particular, they are a promising tool both in diagnosis and therapy (Cuenca et al., 2006; Schlorf et al., 2011; Soriano et al., 2013; Wickline and Lanza, 2003). NPs can be engineered with proteins, gene segments or siRNA encapsulated inside them or attached to their surface (Cai and Xu, 2011; Ferrari, 2005; Mora-Huertas et al., 2010; Panyam and Labhasetwara, 2003). On the other side, NPs can be accumulated in the environment and may be responsible for human pathologies when inhaled or ingested (Boczkowski and Hoet, 2010; Boraschi et al., 2012; Shang et al., 2014). Thus, it's clear that the first step for the use of NPs based systems is to characterize their interaction with cells, especially in terms of toxicity and internalization pathways.

Molecules enter in cells through different mechanisms of internalization: the most characterized are clathrin-dependent and clathrin independent pathways, such as macropinocytosis and phagocytosis (Bareford and Swaan, 2007). Briefly, in clathrin-mediated endocytosis, cells internalize molecules by the invagination of plasma membrane under the control of the small GTP-ase dynamin that is required for the budding of vesicles inside the cell. Typical size range of clathrin coated-pits is reported to be 60–200 nm (Rejman et al., 2004; Kirchhausen et al., 2008). Phagocytosis is mediated by Rho family GTP-ases that trigger the polymerization of actin at the site of ingestion to form membrane invagination that then culminate in the formation of phagolysosome. Although this process is typical of some cells, it is well known that also non-specialized cells in rare situations can activate phagocytosis (Gagnon et al., 2002). Macropinocytosis involves the internalization of various area of plasma membrane that lead to the formation of vesicle of approximately 150 nm of size (dos Santos et al., 2011). This invagination depends, in the same way of phagocytosis, by actin rearrangements, triggered by Rho-family GTP-ase (Mercer and Helenius, 2009).

In our study we used polystyrene nanoparticles (PS-NPs), that represent an interesting model to study interactions between NPs and cells for some practical reasons: first, they are commercially available and can be obtained in core-labeled fluorescent form allowing localization and tracking in living cells (Varela et al., 2012); second, they can be synthesized in a wide range of sizes or easily modified on their surface

Abbreviations: NPs, nanoparticles; PS-NPs, polystyrene nanoparticles; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; NP100, 100 nm rhodamine polystyrene nanoparticles (PS-NPs); NP44, 44 nm fluorescein isothiocyanate polystyrene nanoparticles (PS-NPs); MTT, 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyltetrazolium bromide test; IL, interleukins.

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http://dx.doi.org/10.1016/j.tiv.2015.11.006
0887-2333/

(Lunov et al., 2011); third, they have a high drug loading capacity and a colloidal stability in biological media (Holzapfel et al., 2005; Musyanovych et al., 2007). For these characteristics, there are many studies on the interactions between PS-NPs and cell lines; these studies were performed in the presence or in the absence of specific pharmacological inhibitors of endocytosis pathways. PS-NPs have been reported to enter in different cell types such as hepatocytes (Johnston et al., 2010), macrophages (Xia et al., 2008), lung cells (Geys et al., 2006) and glial astrocytoma cells (dos Santos et al., 2011). Results from these studies showed that parameters that govern the *in vitro* mechanism of internalization and the uptake rates of PS-NPs are the cell type, NPs shape and size, as well as the presence or absence of serum in the cell medium (Guarnieri et al., 2011; Smith et al., 2012). In this paper, we investigated the toxicity and the cellular uptake of fluorescent labeled PS-NPs of two representative sizes (44 nm and 100 nm) in human gastric adenocarcinoma (AGS) cell line: we chose human gastric cells since it has been accepted that for humans one of the primary route of contact with NPs is the ingestion of contaminated food, such as plant derivates (Nowack and Bucheli, 2007) and that huge amounts of PS-NPs reach, through the food chain, fish (Cedervall et al., 2012) with severe consequences on their metabolism and behavior (Mattsson et al., 2014), thus making conceivable the potential contact with human tissues through the alimentation. Both clathrin-mediated and caveolin-mediated endocytosis were studied using the dynamin inhibitor dynasore (Macia et al., 2006) while for clathrin independent pathways the selective Na^+/H^+ antiporter inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was used (Lagana et al., 2000). We demonstrated the efficiency of the internalization process, the amount of PS-NP uptake, internal cluster arrangement, the effect on cell viability and changing of gene expression of some genes involved in cell cycle and inflammation responses.

2. Materials and methods

2.1. Cell culture

Human gastric adenocarcinoma epithelial cells (AGS, American Type Culture Collection CRL1739, Manassan, VA) were grown in Dulbecco's Modified Eagle's medium (DMEM) (LONZA), supplemented with 10% fetal bovine serum (FBS), 2 mm L-glutamine and antibiotics (100 U/ml penicillin/streptomycin, 10 μg/ml gentamicin) in a humidified incubator at 37 °C and 5% CO_2. When confluent, the cell line was detached enzymatically with trypsin-ethylenediamine tetra-acetic acid (Trypsin–EDTA) and subcultured into a new cell culture flask. The medium was replaced every 2 days. Cells were used for experiments between passages 15–25.

2.2. Nanoparticle characterization and chemicals

100 nm rhodamine PS-NPs (NP100) and 44 nm fluorescein isothiocyanate PS-NPs (NP44) unmodified polystyrene nanoparticles (PS-NPs) were purchased from Duke scientific corporation (Palo Alto, CA, USA) and used without further modifications (Table 1). Dynamic light scattering (DLS), made with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK), was performed to measure PS-NPs size distribution, z-potential and polydispersity index (PdI) which is the value that indicates whether the distribution in the size of NP is more or less homogeneous. PdI values less than 0.2 indicate homogeneous distribution of PS-NPs in the solution. Measures were conducted at 25 °C and 37 °C, without sonication, using 10 μg/ml PS-NPs in DMEM or in H_2O. The nanoparticles were suspended in DMEM at concentration of 5 mg/ml and after 1 h were diluted to 10 μg/ml for measurement in the ZetaSizer (Fröhlichet al., 2012).

Determination of size and z-potential was performed in triplicate and every value represents the mean of five measures. NP dispersions were prepared by diluting the concentrated stock solutions into the serum-free medium used for cell culture, at room temperature, with or without inhibitor drugs, immediately prior to the experiments on cells, with an identical time delay between diluting and introducing to the cells for all experiments. The medium was kept at room temperature and not pre-warmed to 37 °C to ensure better NP dispersions. Before sampling, NPs were vigorously mixed by vortexing. For inhibition studies two different drugs were used in the following final concentration: dynasore (25.8 μg/ml) for clathrin-dependent pathways and 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (30 μg/ml) for macropinocytosis/phagocytosis (both from Sigma). Concentration used for inhibitor drugs refers to the work of Daunsend et al. (2008) for HeLa cells. All experiments were performed in the dark. To assess the efficacy of inhibitor drugs, transferrin and dextran were used as positive control, for clathrin dependent and independent pathways, respectively (data not showed).

2.3. Fluorescence microscopy

For fluorescence microscopy 5×10^4 AGS cells were allowed to attach in 4-well chamber slide overnight. The following day, cells were incubated with 10 μg/ml nanoparticle dispersions in serum-free medium (1 h at 37 °C). After the incubation time, cells were rinsed twice with PBS, in order to eliminate non-internalized PS-NPs and then fixed for 5 min with 4% paraformaldehyde in PBS. Cell nuclei were counterstained with Höechst 33258 (1 μg/ml). Inhibition studies were assessed for 1 h after pre-treatment for 30 min with dynasore and EIPA. Fluorescent images were taken on an Axioskop (Carl Zeiss) epi-fluorescence microscope using a 40× (NA = 0.75) and 100× oil immersed (NA = 1.3) objectives. Axiocam MRc5 and the acquisition software Axiovision 4.7 (Carl Zeiss) were used to capture the images in three different channels: Rhodamine for NP100 (ex: 580 nm; em: 605 nm), FITC for NP44 (ex: 488 nm; em: 535 nm) and Höechst 33258 for nuclei (ex: 350 nm; em: 461 nm). Three independent experiments were performed for each experimental condition and different fields were randomly chosen for data analysis.

2.4. Spectrofluorimetric assays

To evaluate the ability to internalize PS-NPs both dose and time course were evaluated. The time course was performed using 1×10^5 cells/well seeded in white bottom 96 multiwell. At 24 h post-seeding, cells were incubated with PS-NPs at the final concentration of 10 μg/ml in DMEM serum free for different times (1 min, 10 min,

Table 1
Chemical features of polystyrene nanoparticles (PS-NPs).

NPs	Composition	Concentration	Contents	Diameter (metric) mean	Diameter (metric) nominal	Dye type	Particle density	particle size	Uniformity	Refractive index
PS-NPs44 nm	Polystyrene	1% solids	Dyed polystyrene microspheres in water	0.04 μm	0.04 μm	Firefli™ fluorescent green (468/508 nm)	1.05 g/cm^3	0.04 μm	<15%	1.59 at 589 nm (25 °C)
PS-NPs 100 nm	Polystyrene	1% solids	Dyed polystyrene microspheres in water	0.10 μm	0.10 μm	Firefli™ fluorescent red (542/612 nm)	1.05 g/cm^3	0.10 μm	<10%	1.59 at 589 nm (25 °C)