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Identification of multiple cellular uptake pathways of polystyrene nanoparticles and factors affecting the uptake: Relevance for drug delivery systems

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ABSTRACT

Nanoparticles may address challenges by human diseases through improving diagnosis, vaccination and treatment. The uptake mechanism regulates the type of threat a particle poses on the host cells and how a cell responds to it. Hence, understanding the uptake mechanisms and cellular interactions of nanoparticles at the cellular and subcellular level is a prerequisite for their effective biomedical applications. The present study shows the uptake mechanisms of polystyrene nanoparticles and factors affecting their uptake in bone marrow-derived macrophages, 293T kidney epithelial cells and L929 fibroblasts. Labeling with the endocytic marker FM4-64 and transmission electron microscopy studies show that the nanoparticles were internalized rapidly via endocytosis and accumulated in intracellular vesicles. Soon after their internalizations, nanoparticles trafficked to organelles with acidic pH. Analysis of the ultrastructural morphology of the plasma membrane invaginations or extravasations provides clear evidence for the involvement of several uptake routes in parallel to internalize a given type of nanoparticles by mammalian cells, highlighting the complexity of the nanoparticle–cell interactions. Blocking the specific endocytic pathways by different pharmacological inhibitors shows similar outcomes. The potential to take up nanoparticles varies highly among different cell types in a particle sizes-, time- and energy-dependent manner. Furthermore, infection and the activation status of bone marrow-derived macrophages significantly affect the uptake potential of the cells, indicating the need to understand the diseases' pathogenesis to establish effective and rational drug-delivery systems. This study enhances our understanding of the application of nanotechnology in biomedical sciences.

Introduction

Many diseases originate from alterations in biological processes that result from mutated genes, misfolded proteins, and infections caused by pathogens at the molecular or nanoscale level (1–100 nm) (Kim et al., 2010). These molecules and infectious agents are nanometers in size and their chemical properties, size and shape appear to dictate their transport to distinct cellular compartments and the interactions between the molecules (Kim et al., 2010). Nanoparticles (NPs) are similar in scale to these biological molecules or agents and can be engineered due to their unique physical and chemical properties to be used for diagnosis, vaccination and treatment of diseases at the molecular level. This can be achieved through encapsulating, covalently attaching or adsorbing molecules on such NPs to overcome biomedical pitfalls including sensitivity of diagnostic tools, therapeutic effectiveness, toxicity and side effects of drugs, and immunogenicity of vaccines (Briones et al., 2008; Karve et al., 2012; Li et al., 2011). Among these various applications, the development of medicines containing NP suspensions has made it possible to increase the therapeutic index of many components by selectively directing them toward the diseased tissues and cells, leading to medical breakthroughs (Couvreur, 2013; Karve et al., 2012). So far, NP-based chemotherapeutics for six cancer and more than 11 other diseases have been approved for clinical use and many more are being studied in clinical trials (Wang et al., 2012). For such a wide range of NP applications in medicine, understanding their uptake mechanisms and interactions at the cellular

Abbreviations: BMDM, bone marrow-derived macrophages; CpG ODN, oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanine; DMEM, Dulbecco's Modified Eagle Medium; RPMI, Roswell Park Memorial Institute; FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; NP, nanoparticle; PBS, phosphate-buffered saline; RT, room temperature; TEM, transmission electron microscopy.

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and subcellular level is a prerequisite and currently an active area of research because the route of uptake is critical for the intracellular fate of the particles and the induction of biological responses (Kumari et al., 2010; Scita and Di Fiore, 2010). However, the mechanism(s) of NP-cell interactions are still not fully understood and it has been suggested that the accurate knowledge of NP uptake mechanisms is an important criterion to progress in the field of nanomedicine (Chou et al., 2011; Iversen et al., 2011; Yan et al., 2012).

Over the past years, considerable numbers of studies have been conducted to understand the route through which NPs are taken up by different cells. The results indicate that NP entry takes place through endocytosis mechanisms operating in mammalian cells (Chou et al., 2011; Iversen et al., 2011). These distinct endocytotic pathways have been characterized on the basis of their differences in ultrastructure, pharmacology, cargo (membrane protein, or receptor plus ligand) and coat protein composition (Hansen and Nichols, 2009; Sandvig et al., 2011; Xu et al., 2013). The ultrastructural morphology of nascent endocytic intermediates at the plasma membrane provides a crucial parameter for classifying endocytic pathways (Hansen and Nichols, 2009). Typically, endocytosis occurs by multiple mechanisms that fall into two broad categories: 'Phagocytosis' or cell eating by which cells internalize large solid particles, and 'pinocytosis' or cell drinking, where cells take up both fluid and solutes from their environment. Pinocytosis can be further sub-classified and at least four basic mechanisms can be distinguished: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent pathways (Conner and Schmid, 2003; Yan et al., 2012). The identification of several uptake routes and pathways complicates the study of the cellular uptake of NPs. Furthermore, these uptake mechanisms of NPs have been shown to be highly influenced by the physicochemical properties of NPs including size, surface functionalization, geometry and other factors like concentration, time or cell types (Albanese et al., 2012; Dos Santos et al., 2011; Herd et al., 2013; Saha et al., 2013) making the comparison of different findings even more difficult. In multicellular organisms, the distinct endocytic pathways are highly regulated to control all aspects of intercellular communications including hormone-mediated signal transduction, immune surveillance, antigen presentation, and cellular and organismal homeostasis (Conner and Schmid, 2003; Sanjuan et al., 2007; Underhill and Goodridge, 2012). As a result, one of the goals of designing NP-based delivery systems is to be able to correlate uptake routes with the physicochemical properties of the engineered NPs in order to guide the entry into the cell and, consequently, control the cellular responses. For example, Karlson et al. (2013) described that bone marrow-derived dendritic cells use caveolin-dependent pathways to take up 40–50 nm polystyrene NPs. Thus, unlike most other NPs, they do not induce extracellular signal-regulated kinases that mediate inflammatory pathways during their applications in vaccines. Furthermore, it has been suggested that alterations in the physicochemical properties of nanomaterials can regulate uptake mechanisms and the intracellular fate of NPs (Herd et al., 2013; Saha et al., 2013; Yan et al., 2012). However, there is little consensus in the literature regarding this issue. For instance, Rejman et al. (2004) studied size-dependent internalization of particles and concluded that microspheres of greater size (>500 nm) predominantly involve caveolae-dependent endocytosis. On the other hand, Yan et al. (2012) found that caveolae pathways are generally limited to smaller (<150 nm) materials. Moreover, Fernando et al. (2010) suggested that the cellular uptake of 18 (±5) nm size polymer NPs occurs via constitutive macropinocytosis rather than clathrin-dependent or caveolin-dependent mechanisms. Importantly, it has not yet been fully resolved whether a single cell or cell type uses several uptake pathways simultaneously for a given nanomaterial or NPs having the same physicochemical properties. In addition to the lack of strict specificity of some pharmacological inhibitors, this might have contributed to the inconsistent results obtained in different studies (Vercauteren et al., 2010). Furthermore, the effect of infection or the diseases' pathogenesis on the cellular uptake mechanisms of NPs has never been fully investigated.

Moreover, the type of laboratory techniques applied, the variability in fluorescence of some NPs used for tracking, the lack of uniformity in their physicochemical properties and the toxic nature of some NPs contribute to the complexity and inconsistent findings regarding the specific endocytic route involved in the cellular uptake of NPs (Chou et al., 2011). To minimize such limitations, we used non-cytotoxic and commercially available polystyrene NPs which are suitable for quantitative cellular uptake studies and were previously used as model NPs (Dos Santos et al., 2011). The aim of the present study was to systematically investigate the cellular uptake mechanisms and to explore the factors affecting cellular uptake of polystyrene NPs. In particular, we wanted to investigate whether a single cell type employs several uptake mechanisms simultaneously to internalize a given type of NPs.

Materials and methods

Polystyrene latex beads and molecular probes

Fluorescent polystyrene latex beads of different sizes (20 nm, 100 nm, 200 nm, 500 nm, 1 μm and 2 μm) were purchased from Life Technologies (F-8888, Darmstadt, Germany) at 2% solids and used without any further modification. The beads were sonicated (Bandelin Sonorex Super RK 106, Berlin, Germany) for 10 min immediately prior to every experiment and were used at a 1:1000 v/v dilution from the 2% solids stock for all experiments unless it was mentioned. FM4-64FX dye (F34653) which is a fixable analog of FM 4-64, Hoechst 33342 (H3570) and LysoSensor Blue DND-167 (L-7533) were similarly purchased from Life Technologies. AlamarBlue cell viability assay reagent was purchased from Trinova Biochem GmbH (Giessen, Germany) and all were used according to the manufacturer's instructions.

Drug treatment

For inhibition of distinct types of endocytosis pathways, cytochalasin D (C8273), wortmannin (W1628), chlorpromazine hydrochloride (C8138), ikarugamycin (SML0188) and dynasore (D7693) were purchased from Sigma–Aldrich (Deisenhofen, Germany) and dissolved in DMSO to make stock solutions that were further diluted in medium to make their final working concentrations. Oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanine (CpG ODN 1668) with the sequence TCCATGACGTTCCTGATGCT were purchased from Eurofins MWG Operon (Ebersberg, Germany), diluted in distilled water and used at 2.5 μg/ml concentrations to activate macrophages.

Cell culture

Primary bone marrow-derived macrophages (BMDM) were generated from bone marrow of BALB/c mice (Charles River Breeding Laboratories, Sulzfeld, Germany) according to the following protocol. The mouse was sacrificed by cervical dislocation. After disinfecting all external surfaces with 70% ethanol, tibia and femur were surgically removed without damaging the epiphysis and placed into a 50 ml polypropylene tube containing phosphate-buffered saline (PBS). The bone marrows were opened by a sharp scissor in conditioned Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (heat-inactivated), 5% horse serum (heat-inactivated), 50 μM β-mercaptoethanol, 10 mM

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