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Comparison of fluorescence-based methods to determine nanoparticle uptake by phagocytes and non-phagocytic cells in vitro

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ABSTRACT

At many portals of entry the relative uptake by phagocytes and non-phagocytic cells has a prominent effect on availability and biological action of nanoparticles (NPs). Cellular uptake can be determined for fluorescence-labeled NPs. The present study compares three methods (plate reader, flow cytometry and image analysis) in order to investigate the influence of particle size and functionalization and medium content on cellular uptake of fluorescence–labeled polystyrene particles and to study the respective method's suitability for uptake studies. For comparison between the techniques, ratios of macrophage to alveolar epithelial cell uptakes were used. Presence of serum protein in the exposure solution decreased uptake of carboxyl-functionalized and non-functionalized particles; there was no clear effect for the amine-functionalized particles. The 200 nm non- or carboxyl-functionalized NPs were taken up preferentially by phagocytes while for amine-functionalized particles preference was lowest. The presence of the serum slightly increased the preference for these particles.

In conclusion, due to the possibility of calibration, plate reader measurements might present a better option than the other techniques to (semi)quantify differences between phagocytes and non-phagocytic cells for particles with different fluorescence. In order to obtain unbiased data the fluorescent labeling has to fulfill certain requirements.

1. Introduction

In human tissues, phagocytes and non-professional phagocytes are located in close vicinity. Phagocytes are part of the mononuclear phagocyte system (MPS), also called reticuloendothelial system. These cells play an important role in the uptake of foreign materials, pathogens and damaged cells. The extent of their phagocytic activity can influence concentrations of particles, including nanoparticles (NPs), at epithelial barriers and in tissues. Cellular uptake is linked to biological effects as well as to cytotoxicity (Sabella et al., 2014) and influenced by several parameters. Binding of macromolecules to the particle surface ('protein corona') has been identified as main determinant of biological effects (Rahman et al., 2013) and many studies were dedicated to the characterization of medium-specific composition, time dependence, and dynamic structure of the protein corona. These studies, however, were not able to predict qualitative and quantitative differences in the cellular uptake, making uptake measurements in the given exposure condition necessary.

Size, shape and surface properties are the most decisive particle-related parameters for cellular uptake and might influence the relation of phagocyte to non-phagocte uptake (Li, 2014). Cellular uptake in vitro is influenced by medium composition, particularly by the protein content, and by exposure conditions, e.g. particle concentrations, height of the medium on top of the cell layer, and perfusion with medium. Biological parameters that

Abbreviations: A549, human lung adenocarcinoma epithelial cells; DMBM-2, murine macrophages; DMEM, Dulbecco minimal essential medium; FBS, fetal bovine serum; NPs, nanoparticles; CPS20 and CPS200, carboxyl-functionalized polystyrene, sizes 20 nm and 200 nm; AMI20 and AMI200, amine-functionalized polystyrene, sizes 20 nm and 200 nm; PPS20 and PPS200, plain polystyrene, sizes 20 nm and 200 nm.

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influence particle uptake include cell size, proliferation rate, growth pattern and expression of uptake routes (Fröhlich et al., 2012; Rees 2013).

An important area for both therapeutic interventions and environmental exposure is the alveolar region of the lung where alveolar epithelial cells and macrophages are localized. Particle uptake by alveolar macrophages together with removal by mucus through mucociliary clearance represents the main clearance mechanism in the deep lung (Tomashefski and Farver, 2008). Phagocytes, such as alveolar or peritoneal macrophages, and non-phagocytic cells, such as epithelial cells, differ in their ability to ingest particles. Alveolar macrophages preferentially phagocytize particles of 3–6 μm size (Hirota et al., 2007) but may also ingest NPs to higher rate than non-phagocytes. The pinocytosis rate of murine fibroblasts is 18.7 μm^3/h/cell compared to 46.5 μm^3/h/cell in murine peritoneal macrophages (Edelson et al., 1975; Steinman et al., 1974). NPs of various materials have been localized inside cells (polystyrene: (Kuhn et al., 2014; Schimpel et al., 2015); silica: (Kasper et al., 2013); quantum dots: (Chakraborty and Jana, 2015)) but quantitative data as well as systematic comparisons of particle uptake between different cell types are scarce. This is mainly due to lack of appropriate quantification methods or problems in the differentiation between intact particles and dissolved particles in the case of several metal and metal oxide NPs. Furthermore, the differentiation between plasma membrane-bound and ingested particles poses problems. The contribution of extracellular fluorescence to the signal obtained by flow cytometry was estimated as 30% in one study (Vranic et al., 2013). As for many other biological readout parameters of NP action there are no standardized protocols for the measurement of uptake. Therefore, the researchers are using different methods and it is not clear if the different techniques provide the same information. The establishment of Standard Operation Procedures (SOPs) for NP testing is the aim of several European projects, for instance of NanoDiaR (Roubert et al., 2016). In order to evaluate existing protocols for their suitability as SOP several parameters, such as requirements from the particle side, influencing factors, and obligatory controls have to be known.

With the overall aim to suggest one strategy for the quantification of cellular uptake of fluorescence-labeled particles, this study investigated the influence of particle parameters (size, surface functionalization) and exposure conditions (medium, culture) on particle uptake by phagocytes and epithelial cells by different methods. Pulmonary exposure was mimicked and fluorescence-labeled 20 nm and 200 nm non-functionalized plain (PPS20 and PPS200), carboxyl-functionalized (CPS20 and CPS200) and amine-functionalized (AMI20 and AMI200) polystyrene particles were used as models. Exposure conditions were kept constant for all evaluation methods. Alveolar lining fluid and plasma contain serum albumin and immunoglobulin as major components (Kim and Malik, 2003). Fetal bovine serum (FBS) content of the exposure solution was chosen based on protein content between 1.3 mg/ml and 5.3–9 mg/ml in the alveolar lining fluid (Fick et al., 1984; Olsson et al., 2011) and 32–70 mg/ml protein in FBS preparations (Lindl, 2002). Human A549 alveolar epithelial cells were chosen as representatives for epithelial cells and DMBM-2 murine macrophages for phagocytes. A549 cells are frequently used in studies on pulmonary toxicity, while they are less suitable for studies on the alveolar barrier function (see for instance (Akhtar et al., 2012; Fröhlich et al., 2013; Lankoff et al., 2012; Stoehr et al., 2011)). Murine macrophages phagocytize particles similarly to human macrophages but behave more homogenous in culture (Gantt et al., 2001). In addition to monocultures also co-cultures of both cell types were studied. Co-culture of human and murine cells are used in several fields of research, particularly in the study of stem cell differentiation. The murine cells induce similar physiological reactions in human cells but are less efficient than the human counterparts (Matsuzaki et al., 1999; Reichert et al., 2015; Stecklum et al., 2015).

2. Methods

2.1. Particles

Red carboxyl-functionalized particles 20 nm and 200 nm (FluoSpheres® carboxylate-modified microspheres, Invitrogen), 20 nm amine-functionalized particles (green dyed Estapor F2-XC, Merck Millipore), 200 nm red amine-functionalized particles (FluoSpheres® amine-modified microspheres, Invitrogen) and non-functionalized 20 nm and 200 nm red dyed polystyrene particles (Fluoro-Max R25 and R200, ThermoScientific) were used.

2.2. Particle characterization

Hydrodynamic size and zeta potential were determined in particle suspensions with a concentration of less than 1 mg/ml. The particles were suspended in DMEM with 0% FBS, 2% FBS or 10% FBS and put into an Elmasonic S40 water bath (ultrasonic frequency: 37 kHz, Elma) for 10 min. Particles were measured via photon correlation spectroscopy (PCS, Malvern Zetasizer, Malvern Instruments) equipped with a 532 nm laser. The zeta potential was measured by Laser Doppler Velocimetry (scattering angle of 17°) coupled with Photon Correlation Spectroscopy (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) and calculated out of the electrophoretic mobility by applying the Henry equation. It has been reported that agglomerates of amine-functionalized in contrast to other polystyrene particles after 24 h at 37 °C differed from the initial size (Paget et al., 2015). Therefore, particle sizes were determined at the start of the incubation and after 24 h at 37 °C in the different exposure media.

To identify the presence of free dye in the solution, fluorescence of freshly prepared suspensions were measured. Particle suspensions (20 μg/ml) in medium + 0% FBS were used and fluorescence of serial dilutions of the suspensions measured (FLUOstar Optima, BMG Labortechnik) with the Ex/Em wavelengths (584/612 nm, CSP20, CPS200, AMI200; 544/612 nm, PPS20, PPS200; 485/520 nm, AMI20). After centrifugation at 220,000*g* in an OPTIMA L-90k ultracentrifuge (Beckman Coulter) for 60 min fluorescence of the supernatants were determined. Alternatively, particle suspensions were filtered through a 0.1 μm syringe filter (Minisart® 0.1 μm, Sartorius) and fluorescence in the filtrate compared to that of the non filtrated suspension.

2.3. Cell culture

DMBM-2 mouse macrophages and A549 cells (derived from a human lung adenocarcinoma) were obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH. DMBM-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% horse serum, 2 mM L-glutamine and 1% penicillin/streptomycin. A549 cells were cultured in DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin. Cells were sub-cultured at regular intervals.

Cells in monocultures ($2*10^5$ DMBM-2 and $1*10^5$ A549 per well) were seeded 24 h before treatment in 12-well plates in their cell-specific medium for plate reader and flow cytometry. Different cell densities had to be used to generate the sub-confluent exposure condition needed because DMBM-2 cells are markedly smaller than A549 cells.

For image analysis cells were seeded in chamber slides. $8*10^4$ A549 cells were seeded per chamber (Nunc® Lab-Tek® Chamber