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Intracellular dynamics and fate of polystyrene nanoparticles in A549 Lung epithelial cells monitored by image (cross-) correlation spectroscopy and single particle tracking

Sarah Deville [a,b], Rozhin Penjweini [a,c], Nick Smisdom [a,b], Kristof Notelaers [a], Inge Nelissen [b], Jef Hooyberghs [b,d], Marcel Ameloot [a,*]

[a] Biomedical Research Unit, Hasselt University, Agoralaan Building C, 3590 Diepenbeek, Belgium
[b] Flemish Institute For Technological Research, Environmental Risk and Health Unit, Boeretang 200, 2400 Mol, Belgium
[c] Department of Radiation Oncology, School of Medicine, University of Pennsylvania, 3400 Civic Boulevard, Philadelphia, PA 19104, USA
[d] Theoretical Physics, Hasselt University, Agoralaan Building D, 3590 Diepenbeek, Belgium

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ABSTRACT

Novel insights in nanoparticle (NP) uptake routes of cells, their intracellular trafficking and subcellular targeting can be obtained through the investigation of their temporal and spatial behavior. In this work, we present the application of image (cross-) correlation spectroscopy (IC(C)S) and single particle tracking (SPT) to monitor the intracellular dynamics of polystyrene (PS) NPs in the human lung carcinoma A549 cell line. The ensemble kinetic behavior of NPs inside the cell was characterized by temporal and spatiotemporal image correlation spectroscopy (TICS and STICS). Moreover, a more direct interpretation of the diffusion and flow detected in the NP motion was obtained by SPT by monitoring individual NPs. Both techniques demonstrate that the PS NP transport in A549 cells is mainly dependent on microtubule-assisted transport. By applying spatiotemporal image cross-correlation spectroscopy (STICCS), the correlated motions of NPs with the early endosomes, late endosomes and lysosomes are identified. PS NPs were equally distributed among the endolysosomal compartment during the time interval of the experiments. The cotransport of the NPs with the lysosomes is significantly larger compared to the other cell organelles. In the present study we show that the complementarity of ICS-based techniques and SPT enables a consistent elaborate model of the complex behavior of NPs inside biological systems.

1. Introduction

Nanoparticles (NPs) are defined as particles with at least 50% in the number size distribution having one or more external dimensions in the size range between 1 nm and 100 nm [1]. The study of the interaction of NPs with living systems is of steadily growing interest. Since NPs are in the same order of magnitude as biomolecules and viruses, they can easily enter cells and interact with the cellular machinery [2–5]. Although NPs hold great promise for new nanoscaled diagnostics and targeted drug delivery in biological systems [6], they may also provoke toxic side effects [7]. NPs enter cells predominantly through a complex interplay of endocytic pathways and thereafter travel throughout the cell by using its endocytic machinery [8,9]. Primary endocytic vesicles can fuse together with the early endosomes to deliver the cargo for further cellular processing [10]. Early endosomes move towards the perinuclear space, where the transition towards the late endosomes takes place. Finally, the late endosomes fuse with the lysosomes [11]. The intracellular transport of vesicles and cell organelles is mediated through both the microtubules and the actin microfilaments [12]. By studying the trafficking of NPs within living cells and cell organelles, novel insights in NP uptake processes, intracellular transport and their multiple outcomes can be obtained [13].

In the present study, the application of image correlation spectroscopy (ICS) for the determination of interactions of fluorescently stained carboxylated polystyrene (PS) NPs within the cell is presented. PS NPs are commonly used as model NPs to study interaction with biological systems due to their commercial availability, high quality and wide variety of sizes and surface chemistries [8]. ICS is a fluorescence-based microscopic technique suitable for defining the diffusion and directed motion on time scales ranging from microseconds to milliseconds [14–16]. This information is obtained from the fluctuations of the fluorescence intensity within a region of interest (ROI) of a time-lapse image series. Various ICS variants exist which differ in the way how the fluorescence fluctuations in the image series are analyzed. Where in temporal ICS (TICS), fluorescence fluctuations in time for the recorded pixels of an image series are exploited for the correlation analysis [15,17], spatiotemporal ICS (STICS) correlates fluorescence fluctuations

* Corresponding author.
E-mail address: marcel.ameloot@uhasselt.be (M. Ameloot).

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both in space and time [15,18,19]. With these techniques the diffusion and/or flow (magnitude and direction) of molecular complexes can be monitored. They have been used in previous studies to quantify the intracellular transport of the photosensitizer PVP-hypericin, polyplexes, lipoplexes and silica NPs [20–23]. Interaction between molecular complexes is investigated by spatiotemporal image cross-correlation spectroscopy (STICCS) in which space-time correlation functions from the fluorescence intensity fluctuations within a two-channel fluorescence image series are correlated [24]. This approach is used here to study the association of NP complexes with cell organelles. Besides these ensemble averaging techniques, single particle tracking (SPT) can be used to quantify the individual NP motions, which registers and tracks the NP frame by frame [25], as demonstrated for gene nanocarriers and polymeric NPs [26,27].

Here we report on the intracellular dynamics of fluorescently stained carboxylated PS NPs in human alveolar epithelial A549 cells, mimicking the biological responses of lung cells to NP exposure, which is highly relevant for both nanomedicine and nanotoxicology as inhalation is one the major entry routes of NPs to the human body. TICS and STICS were used to characterize the diffusion and map the flow magnitude and direction of PS NPs inside the cell. PS NP motions were also analyzed by SPT, which facilitated in the translation of these general motion models to effective NP dynamics created by biological processes. This was corroborated by dynamic interactions of NPs with the early endosomes, late endosomes and lysosomes established and quantified with STICCS. In addition, the role of cytoskeleton in the NP transport was explored by means of ICS and SPT.

2. Materials and methods

2.1. Cell culture

The human alveolar epithelial A549 cell line was routinely maintained in modified eagle's medium with glutamax (MEM, Gibco, Paisley, United Kingdom) supplemented with 10% non-heat inactivated fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (P/S, Gibco) at 37 °C under 5% CO_2. Before reaching confluence, cells were washed with versene (Gibco) and detached using 0.05% trypsin containing 0.02% EDTA (Sigma-Aldrich, Ayrshire, United Kingdom).

2.2. Nanoparticles

Dark red (λ^{Ex} 660, λ^{Em} 680) fluorescent carboxylated polystyrene nanoparticles (PS NPs) were purchased from Molecular Probes (Invitrogen, Merelbeke, Belgium). PS NPs were characterized by dynamic light scattering (ZetaPALS, Brookhaven Instruments Corporation, Holtsville, USA), yielding a mean hydrodynamic diameter of 116 ± 1 nm (polydispersity index (PDI) of 0.19) in water and 152 ± 2 nm (PDI of 0.18) in complete cell culture medium.

2.3. Cell exposure

The cells used for microscopic observation were plated one day before the experiment in 8 well μ-Slide (Ibidi GmbH, Martinsried, Germany) at a density of 30×10^3 cells/cm^2. Cells were exposed for 30 min to 50 μg/ml of PS NPs diluted in MEM containing 10% FBS, but without phenol red and without P/S at 37 °C under 5% CO_2. After exposure, cells were washed 3 times to remove the PS NPs that were not taken up by the cells and STICS/TICS measurements were conducted. In order to disturb the cytoskeleton mediated transport within the cell, 1 μM latrunculin A (Merck Millipore, Overijse, Belgium) and 20 μM nocodazole (Sigma-Aldrich) were added to the culture medium after NP treatment and washing. After incubation for 30 min, STICS/TICS measurements were performed. For the STICCS measurements, the cells were labeled prior to PS NP exposure with organelle specific dyes. 1 μM LysoTracker® Green DND-26 (Molecular Probes) was added for 30 min. CellLight® Early Endosomes-GFP (Molecular Probes) or CellLight® Late Endosomes-GFP (Molecular Probes) were added to the cultures in a final concentration of 25 particles per cell 24 h prior to the experiments, according to the manufacturer's protocol.

2.4. Immunofluorescence

For visualization of the tubulin and F-actin cytoskeleton, cells exposed to PS NPs, latrunculin A and/or nocodazole were fixed with 4% formalin (Sigma-Aldrich). Cells were permeabilized with 0.5% Tween®20 (Sigma-Aldrich) in PBS for 10 min, and blocked with 0.1% Tween®20 and 2% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min. Subsequently, cells were incubated with mouse anti-tubulin antibodies (T9026, Sigma-Aldrich) for 30 min. After washing the unbound fraction, donkey anti-mouse antibodies conjugated with Alexa Fluor® 488 and CytoPainter Phalloidin-iFluor 555 Reagent (Abcam, Cambridge, UK) were applied for 30 min. Finally, after rinsing the sections extensively, Vectashield® mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, United Kingdom) was added.

2.5. Confocal microscopy

Image time-series were acquired using an epifluorescence Axiovert 200 M equipped with a Zeiss LSM 510 Meta confocal laser scanning unit (CLSM, Zeiss, Jena, Germany) and a LD C-Apochromat 40×/1.1 W Korr UV–VIS-IR water immersion objective (Zeiss), placed on a vibration isolation table in an air-conditioned room kept at constant temperature. Cells were kept at 37 °C by the means of a stage incubator (Tempcontrol 37–2 digital, PeCon, Erbach, Germany). Alexa Fluor® 488, CellLight® Early Endosomes-GFP, CellLight® Late Endosomes-GFP and LysoTracker® Green DND-26 were excited with the 488 nm line of an argon ion laser. CytoPainter Phalloidin-iFluor 555 was excited with a 543 nm helium–neon laser. Dark red PS NPs were excited a 633 nm helium–neon laser. DAPI was excited using a 150 fs pulsed laser light of a Ti:Sapphire laser (MaiTai DeepSee, Spectra-Physics, California, USA) tuned at an output wavelength of 730 nm. The excitation light was directed to the sample by a dichroic beam splitter (HFT UV/488/543/633). For the detection and separation of the different emitted fluorescence signals, a secondary dichroic beam splitter NFT 545, as well as four band-pass filters BP 390-465 (DAPI), BP500-550 (Alexa Fluor® 488, CellLight® Early Endosomes-GFP, CellLight® Late Endosomes-GFP, LysoTracker® Green DND-26), BP565-615 (CytoPainter Phalloidin-iFluor 555) or BP650-710 (dark red PS NPs) were used. Signals were directed towards an internal analog photomultiplier tube of the confocal unit. Confocal pinhole was set to a maximum of 1.5 Airy units to provide sufficient z-sectioning. Each image time-series comprised typically 100 frames with a 512 by 512 resolution, a pixel size between 40 and 120 nm, a frame rate of 0.63 Hz without extra time delay between subsequent frames, and a pixel dwell time of 2.51 μs.

2.6. Image (cross-) correlation spectroscopy and colocalization analyses

A brief review of image correlation spectroscopy is given in the Supporting Information. Image (cross-) correlation spectroscopy analysis was performed using custom written MATLAB routines (The MathWorks, Eindhoven, The Netherlands), based on the original work of the Wiseman Research Group (McGill University, Canada). The original routines and details of analysis have been published before [18,19,24,28–31]. Immobile populations within the image series were removed by the means of Fourier-filtering [18]. Due to the flat morphology of the A549 cells, only two-dimensional dynamics were considered.