Time-dependent appearance of nanometer-sized particles in living cells

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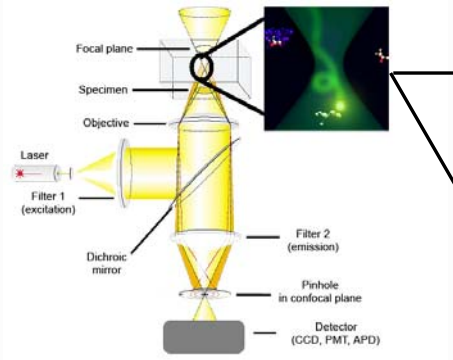
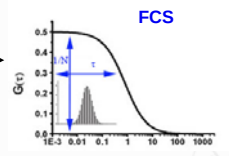
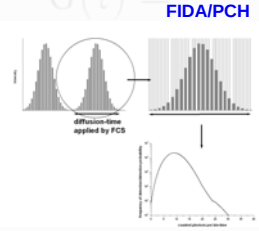
Abstract

Possible negative health effects became an issue in environmental sciences as nanoparticulate matter appears also in ambient air as a result of a variety of technological processes. Especially the PM2.5 fraction of these emissions, which consist mainly of carbon associated with metals, oxygen, and organic matter, is assumed to threaten public health and even heritable effects are possible. Nanoparticles and ultrafine particles are also an issue in job safety and therefore global research activities are focused on different kinds of aerosol sources, such as coal fly ash and diesel exhaust particles from modern combustion and fabrication processes. Exposed workers (e.g., road- or tunnel construction-sites) carry an incalculable risk to contract physiological sequels due to the exposure. A detailed understanding of their interaction with cells is a prerequisite for the appraisal of hazardous effects. The general and unspecific uptake was shown with conventionally used fluorescence detection methods like Laser Scanning Microscopy (LSM). Fluorescence fluctuation methods were applied to follow the time course of the translocation and distribution of fluorescent 20nm polystyrene nanoparticles in HeLa cells under physiological conditions. The experimental results demonstrate that singular particles enter the cell without significant contribution by endocytotic mechanisms and are distributed within the cytoplasm. Subsequently aggregation is observed, which can be blocked by cytotoxins, like Genistein and Cytochalasin B, interfering with cellular uptake processes. The observed non-active uptake is due to non-specific interactions with the cell surface and could be responsible for distribution of nanometer-sized materials in tissue.



Schematic illustration of the Confocor setup. A laser is fiber coupled with the system. The beam passes a collimator and an excitation filter. The beam is reflected by dichroic mirror and focused into the sample by the tubus and objective lense. the emission beam passes again the dichroic mirror and a excitation filter. The pinhole blocks "out of focus" light and the emitted photons are detected by a single--photon--detecetor (avalanche photon detector --APD)





Materials and Methods

- LSM:

Live cell confocal images were performed using a confocal laser scanning microscope (Leica SP1, Germany), a CW Ar^+ laser and an oil immersion objective (PL APO 100x1.4 OIL UV). Images for the fluorescent particles were performed using the laser line at λ = 488nm. Images for the stained cells were performed using the laser line at λ =568nm. Imaging started 20 minutes after adding the particles and all images were averages of 3 individual scans. Images were extracted and overlays were performed using the Leica Simulator Software (Leica, Germany) or Adobe Photoshop 6.0.

- FFS (FCS and PCH)

For FCS the APD is coupled to a fast digital ALV5000E correlator card that calculates the real--time autocorrelation function. Fluorescence signals were detected for 42 seconds with 3 seconds interval and 12 seconds correlator scaling. The auto correlation was fitted using FCS Access version 1.0.12 (Evotec, Germany) applying a structural parameter of 5.6.

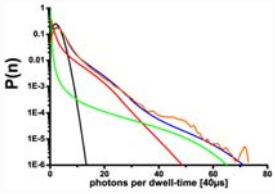
To obtain raw data for PCH, fluorescence signals were detected using an APD coupled with a Time Measurement Histogram Accumulating Real-Time Processor (PC Board for Time Correlated Single Photon Counting, Time Harp 200, PicoQuant, Software version 3.0) triggered with 7.4MHz. The histogram of counted photons is acquired by monitoring the signal intensity during 30 seconds and plotting the numbers of photons detected in a time window of 40µs.

Photon Counting Histogramming (PCH)

$$G(\xi) = \exp\left[(\xi - 1)\lambda tw + \sum_s c_s \int_V \exp\{[(\xi - 1 q_s tw B(r)] - 1\} dV\right]$$

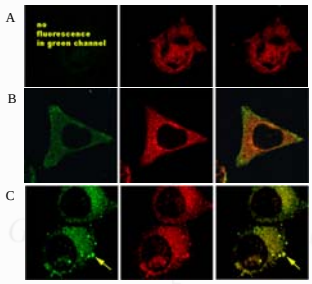
Fluorescence Correlation Spectroscopy (FCS)

$$G(\tau) = \frac{\langle \delta(N_i \times Q_i)(t)\,\delta(N_i \times Q_i)(t+\tau)\rangle}{\langle \delta(N_i \times Q_i)(t)\rangle^2} = 1 + \frac{1}{\sum_{i=1\ldots3}(N_i \times Q_i)} \sum_{i=1\ldots3} \frac{F_i}{\left(1+\frac{\tau}{\tau_i}\right)} \frac{1}{\sqrt{\left(1+\frac{\tau}{SP\tau_i}\right)}}$$

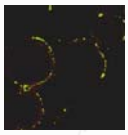


Simulation of the brightness distribution for three species with different concentration and brightness values. This theoretical and convoluted graph (blue) corresponds to three different species whereas the contribution of every single species is shown in black (Species 1),red (Species 2), and green (Species 3). For comparison to the convolution of these species an experimental curve (orange) is plotted. P(n) represents the probability of detected photon counts.

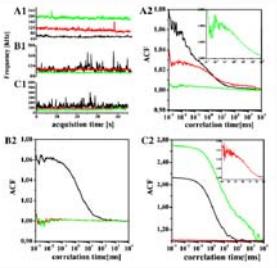
Laser Scanning Microscopy (LSM)



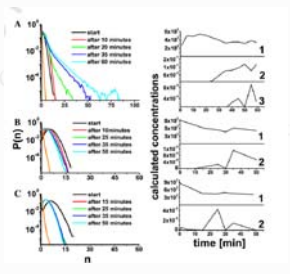
Confocal images of HeLa cells. (A) HeLa cells stained with Nile-Red and treated with Genistein. (B) Native HeLa cells stained with Nile-Red and incubated with green fluorescent particles. (C) HeLa cells stained with Nile-Red, treated with Genistein, and incubated with green fluorescent particles. Images represent sections of 500 nm thickness taken 3–4 µm above the glass surface.



Confocal images of dead HeLa cells. Particles are just aggregated at the outer side of the cellular membrane (no translocation is observed)



Fluorescence correlation measurements in HeLa cells. (A1–C1). Intensity scans measured at different time-points. (A2–C2) Auto correlation function (ACF) obtained at different time-points.Untreated cells (black), cells treated with Genistein (red) and CytochalasinB (green).



Normalized distribution of photon counts. Untreated samples (A), cells treated with Genistein (B) or Cytochalasin B (C). On the right side the temporal evolution of the particle-concentration is shown. The numbers in the graphs indicate the particular species. The legends show the different time-points of the measurement. Controls without particles are plotted in orange.

Table 1
Average size distribution and relations of detected species

	Species 1		Species 2		Species 3	
	r_{hydr} (nm)	Detected (%)	r_{hydr} (nm)	Detected (%)	r_{hydr}	Detected (%)
Native cells (min)						
After 2	10–19	100	ND*	ND*	ND*	ND*
After 15–20	13–25	97	88–117	3%	ND*	ND*
After 50–60	19–37	90	88–146	7	352–675	3
Cells treated with Genistein (min)						
After 2	17–20	100	ND*	ND*	ND*	ND*
After 15–20	No correlation found					
After 50–60	27–39	97	161–308	3	ND*	ND*
Cells treated with Cytochalasin B (min)						
After 2	12–17	100	ND*	ND*	ND*	ND*
After 15–20	No correlation found					
After 50–60	27–39	98	161–308	2	ND*	ND*

* ND, not detected.

Results - Conclusions

- LSM

LSM images showed that negatively charged polystyrene particles with a diameter of 20 nm are effectively internalized by human HeLa cells independent whether the cells were treated with Genistein or not. In native cells isolated particles and small aggregates were found throughout the cytoplasm. Additionally bright aggregates were found mainly at the periphery of the cell. Such aggregates of individual particles could result from incorporation into endosomes or similar structures (e.g. lysosomes, exosomes, multivesicular bodies). In cells treated with Genistein only small aggregates were found near to the cytoplasm membrane. One explanation could be that in that case particles are not efficiently packed into vesicles or that such structures are not actively transported within the cell. Translocation of particles needs a viable, fluid membrane as particles are not found within dead cells.

- FCS/PCH

FCS identified highly mobile particles with a hydrodynamic radius of about the size of the applied particles in living cells. Additionally larger species were found at later times with a broad distribution of radii (see Table 1). The appearance of such aggregated species was inhibited if the cells were treated with Genistein or Cytochalasin B.

The photon counting histograms were dominated by a species of rather low brightness, identified as individual 20nm particles, over the whole observation period independent of the treatment of the cells. In untreated samples a considerably brighter species accompanied the decrease of the number of individual particles with time. At longer observation times singular events of an even brighter species were observed. In cells treated with cytotoxins aggregation is only detected in minute amounts. The long term limit of these observations coincides with the results obtained by LSM.

Aggregation must not primarily occur on the surface of the cell but could be the result of active processes within the cell.

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For more informations see publication on: http://www.sciencedirect.com (Biochemical and Biophysical Research Communications 332 (2005) 109–116)