# IN-VITRO TESTING FOR IDENTIFICATION OF LONG-TERM EFFECTS OF NANOPARTICLES

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**Abstract**

Human exposure to nanoparticles increases because nano-sized materials are included in food, consumer and medicinal products, as well as being present in the environment. Routine cytotoxicity screening evaluates effects only upon acute exposure, usually 24h, but prolonged contact with nanoparticles might result in intracellular accumulation and disturbance of cell function. We studied effects of polystyrene particles, carbon nanotubes, and silica nanoparticles on endothelial cells and on monocytes using various culturing systems (microcarrier, CELLine CL350, and sub-culturing) for up to 28d. Particles were physicochemically characterized, cellular uptake studied, and cellular localization determined. Nanoparticles formed aggregates to different extent and accumulated in lysosomes to different degree. Exposure systems were suitable to different extent for the evaluation of chronic cytotoxicity. CELLine CL350 and sub-culturing of an aliquot of the exposed cells was less suitable than culture on microcarriers and sub-culturing by transfer of all exposed cells to a larger culture vessel. Cytotoxicity after long-term exposure was higher than after acute exposure. Differences between particles and cell lines were observed and adaptation to nanoparticle damage was also seen.

**Keywords:** Cellular effects, chronic effects, carbon nanotubes, silica particles

## 1. INTRODUCTION

Human exposure to nanoparticles (NPs) increases because nano-sized materials are being used in food, consumer, and medicinal products, as well as being present in the environment. NPs, which are most likely to accumulate in cells, organs, body, and the environment, are non-biodegradable NPs, such as metal and metal oxide and carbon-based NPs. Organ accumulation for instance of $SiO_2$ NPs has been demonstrated after intravenous and oral application to animals [1, 2]. The consequences of NP exposure for human health are difficult to assess because exact exposure doses, rate of uptake into the body, and chronic cellular effects are unknown. Main problems include inter-individual differences in the intake/use of these products, change of size and surface properties of particles when in contact with biological media, and difficulties in detection of NPs in complex matrices. Expected doses are rather low but exposure times long. This exposure scenario differs from the one used for routine cytotoxicity testing, where short-term effects (routinely 24h) at rather high doses of a given compound are tested. NPs, in contrast to conventional compounds, do not dissolve uniformly in the exposure medium and aggregates of different size are formed. As a consequence, contact of NPs with cells is determined by sedimentation and diffusion and lack of biological effect could be due to lack of contact between particles and cells. Specific software programs can calculate particle deposition on cells but due to uncertainties in medium-specific aggregate formation experimental determination of cellular doses is recommended.

In order to assess potential long-term effects on cells and organelles, 20 nm plain polystyrene particles (PPS20), 1-2 nm carboxylated short single-walled carbon nanotubes (SCNTc), and Aerosil OX50 silica (Aero50) nanoparticles, showing cytotoxicity in short-term exposure [3], were tested in adherent cells and monocytes using different culture systems. Endothelial cells were cultured on microcarriers in a benchtop

bioreactor BioLevitator and in sub-culturing. Monocytes were cultured in the bioreactor flask CELLine CL350 and by sub-culturing. Cellular uptake as well as short-term and long-term cytotoxicity was determined.

## 2. MAIN TEXT

### 2.1 Experimental section

#### 2.1.1 Particle characterization

Plain polystyrene particles (20 nm, Thermo Scientific), Aerosil OX50 (40 nm, Degussa), and single-walled carboxylated short carbon nanotubes (1-2 nm x1-2 µm, Cheap tubes) were used for the studies. Particle size and surface charge in the exposure medium were characterized by photon correlation spectroscopy and Laser Doppler Velocity. Size and metal contamination of carbon nanotubes were studied by transmission electron microscopy and energy dispersive X-ray spectroscopy. Biological contamination (endotoxin) was detected using PYROGENT Ultra clotting assay (Lonza).

#### 2.1.2 Cells

EAhy 926 endothelial cells are adherent growing cells of human origin. THP-1 monocytes are derived from human acute monocytic leukemia and grow in suspension. Cell numbers were determined on an automated cell counter based on electrochemical sensing Casy TT.

#### 2.1.3 Cellular uptake

For uptake studies commercial red fluorescently labeled 20 nm plain polystyrene particles (Thermo Scientific) and 30 nm plain silica particles (micromod Partikeltechnologie) were used. Fluorescent CNTs were generated from SCNTc by adsorption of red fluorescently labeled bovine serum albumin. Cells were exposed to particles in a concentration of 20 µg/ml for 24h and washed. Fluorescence of cells and of serial dilutions of the exposure solution (standard curve) was read in a fluorescence plate reader and reported as % of applied amount of particles.

#### 2.1.4 Exposure

Cytotoxicity of particles in short-term exposure (24h) was determined by formazan bioreduction using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Human EAhy936 endothelial cells were exposed cultured on microbeads (Fig. 1A) or by subculturing of cells (Fig. 1C). Human THP-1 monocytes were exposed in the bioreactor CELLine CL350 (Fig. 1B) and by subculturing (Fig. 1C). Exposure concentrations of 12.5-25 - 50 µg/ml for polystyrene particles, 25-50-100 µg/ml for silica particles, and of 5-10-20 µg/ml for SCNTs were used. Maximal evaluation time was 28d for EAhy926 cells cultured on microbeads in the BioLevitator, 16d for THP-1 cells in CELLine CL350 and 16d for EAhy926 cells and THP-1 cells by sub-culturing.

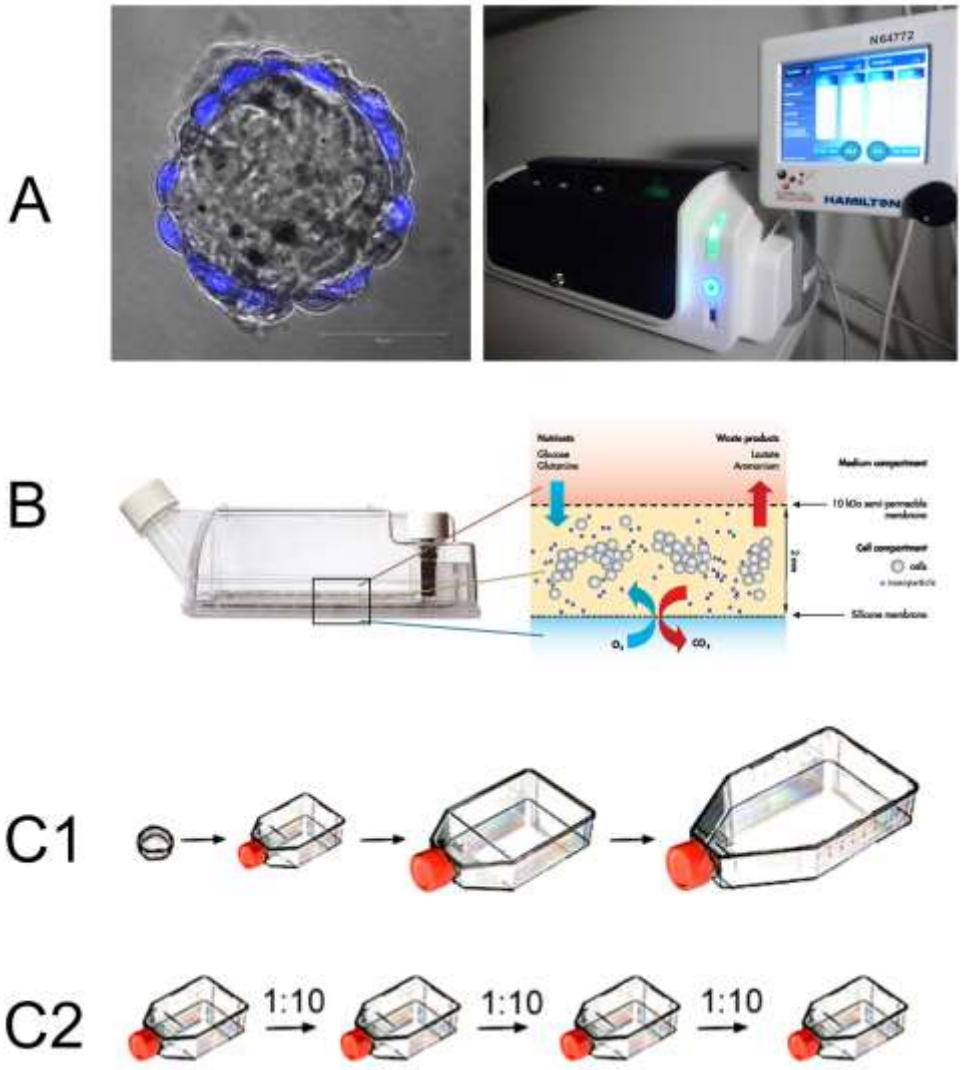


**Fig. 1**: Exposure protocols: A: Culture of cells on microbeads in the bench top bioreactor BioLevitator. B: Culture of cells in the flask bioreactor CELLine 350. C: sub-culturing of cells in the way that all cells are transferred into a larger culture vessel when control cells reach confluency (C1) or cells are harvested and sub-cultured 1:10 in a new culture vessel of the same size (C2).

### 2.1.5 Results and discussion

Sizes of all particles were greater in cell culture medium than reported for primary particles suggesting agglomeration; carboxylated single-walled carbon nanotubes immediately aggregated to bundles with a negligible fraction of single tubes, the mean peak of the polystyrene particle increased from 46-62 nm at day1 to 101-140 nm at ≥day2, and the fraction of silica aggregates (522-731 nm) increased from 60% at day1 to 90% at day10. CNTs did not contain heavy metal contamination, which had often been reported to be involved in the cytotoxic action of these particles [4]. There was also no obvious contamination with endotoxin.

After ingestion by the cells, particles accumulated partly in lysosomes (Fig. 2). Cellular particle uptake in EAhy926 cells was around 2% for all particles and around 7% in THP-1 cells. Particle uptake rates by THP-1 cells was higher than that reported for carboxyl polystyrene particles of similar size [5]. The higher degree of particle uptake of professional phagocytes was not related to increased cytotoxicity.

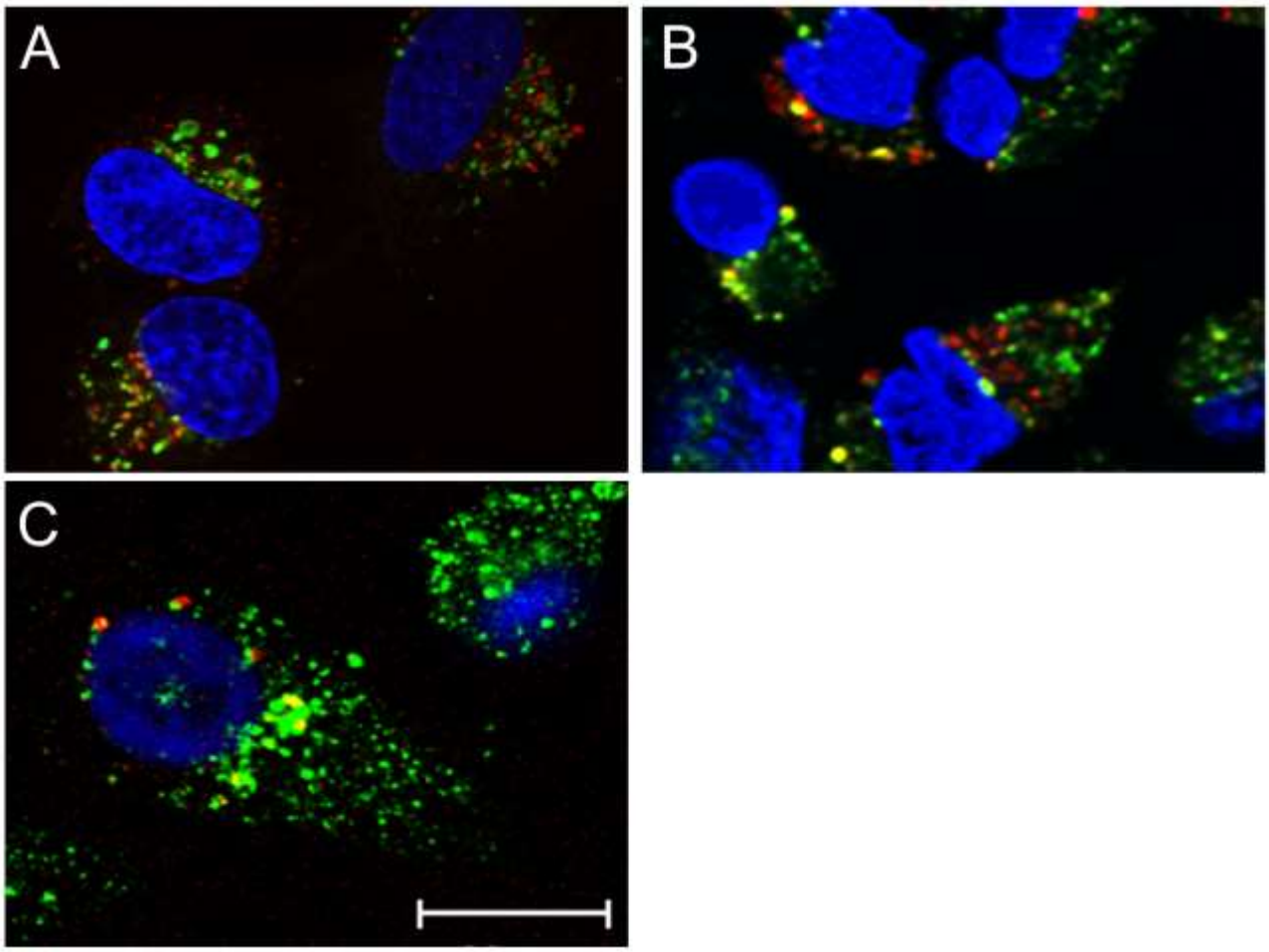


**Fig. 2**: Uptake of red fluorescently labeled NPs, PPS20 (A), SiO2 (B) and SCNTc (C), into EAhy926 cells after 24h of exposure. Particles (red) co-localize with lysosomes (identified by green fluorescent vital dye LysoTracker) to different extent. Co-localization is seen in yellow. Scale bar: 20 µm.

The choice of the exposure system had a great influence on the detected cytotoxicity and not all systems performed in an optimal way. The CELLine CL350 system evaluated for chronic cytotoxicity in cells growing in suspension (THP-1 cells) proved not to be suitable for this assessment due to great dependence on cell density. In the original protocol, which is being used for antibody production, high cell numbers are seeded and antibodies are harvested after short time [6]. For cytotoxicity testing high cell densities have to be avoided because control cells must grow without inhibition. Therefore, cells have to be seeded in a way that growth inhibition by high cell density, lack of nutrients, accumulation of waste products, changes in pH, etc. does not occur during the entire observation time [7]. In the CELLine CL350 system a cell density enabling reproducible growth in control cells enabled only an observation time of 5 days, whereas lower cell density allowing an observation time of 16 days did not produce reproducible cell growth. For the evaluation in sub-culturing experiments, the protocol, where all plated cells were transferred to the new culture flask (Fig. 1C1) was more reproducible than the protocol, where an aliquot of the exposed cells was sub-cultured 1:10 every week (Fig. 1C2). In the later protocol cells decreased to about 80% of control cells at mildly acute cytotoxic concentrations and this value was constant over the entire observation time. This exposure scenario resembles more acute exposure because cells, which might have accumulated NPs could have been removed during the manipulation (trypsinization and re-seeding).

Concentrations causing long-term cytotoxicity in THP-1 cells cultured by transfer in larger flasks (protocol Fig. 1C) were half of the acutely cytotoxic concentration. This difference resembles the pattern of ZnO NP

cytotoxicity in keratinocytes, which were 20 µg/ml after short-term exposure and at 10 µg/ml ZnO NPs for long-term exposure [8]. For other NPs more pronounced differences in cytotoxic concentrations between 24h and 10d of exposure were seen. While a concentration of 50 µg/ml $CeO_2$ NPs caused significant reduction of HepG2 cell viability after 24h similar decreases were already detected at 0.5 µg/ml $CeO_2$ NPs after 10d of exposure [9].

Culturing on microcarriers in the BioLevitator™ was identified as the most physiological system for adherent cells and also the most sensitive system to identify long-term cytotoxicity. Similar to the situation in the human body cell grow on a collagen-containing matrix. In the microcarrier-based BioLevitator™ system HepG2 cells were reported to show increased survival and differentiation [10, 11]. Differences between cytotoxic concentrations in short-term and long-term exposure were high for polystyrene particles. While cell numbers decreased steadily upon exposure to the polystyrene particles, EAhy 926 cells exposed to SCNTs showed a prominent decrease in cell number to 45% after 21days and then increased after 28days of exposure (Fig. 3). This could be interpreted as adaptation mechanism similar to the reaction of cells exposed to $TiO_2$ and Au NPs, where decrease of cell viability of Au NPs-treated MG63 osteoblast-like cells was lower after 5d than after 21d [12]. One potential mechanism for this adaptation has been identified in CHO-K1 cells, which showed lower ROS levels after exposure to 10-40 µg/ml $TiO_2$ NPs for 60d than after 2d [13].

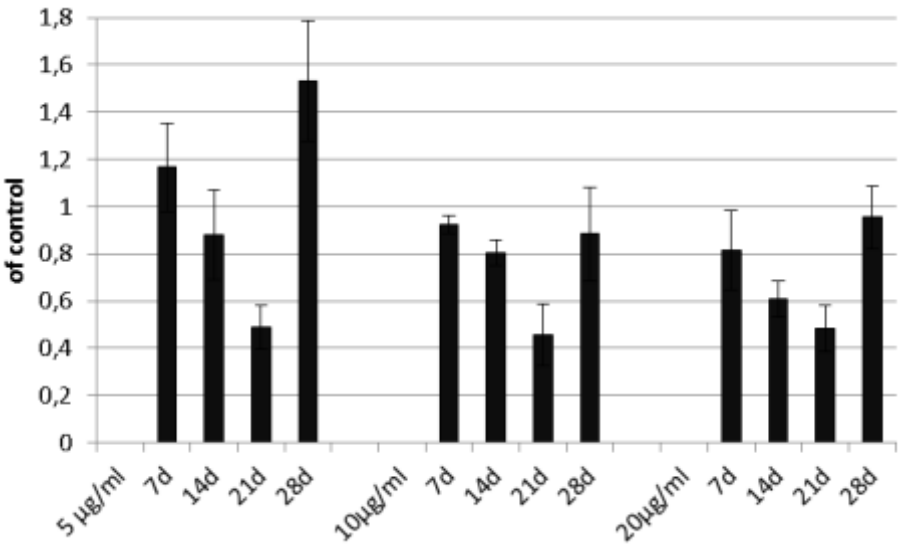


**Fig. 3:** Changes in cell numbers of EAhy926 cells cultured on microbeads in the BioLeviator after exposure to SCNTc particles in different concentrations. Cell numbers were normalized to untreated control.

## CONCLUSION

Long-term exposure to NPs results in cell damage at lower concentrations than short-term exposure. The concentration, at which cytotoxicity is detected, depends to a high degree on the exposure model. For cells growing in suspension conventional screening can give an approximate indication on cytotoxic levels upon longer exposure when all cells are harvested and transferred to a new culture vessel. For adherent cells routine cytotoxicity screening might not be ideal and culturing on scaffolds like microcarriers provide a more realistic screening system for long-term cytotoxicity.

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