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Cell shape imaging analysis: a fast and reliable technique for the investigation of internalised carbon nanotubes in flat macrophages

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Abstract: The aim of this work is to elucidate the mechanisms involved in the morphological adaptation and regulation of macrophages in the presence of internalised materials. This development will accelerate the toxicology assessment of novel nanomaterials and subsequently reduce their environmental and health exposure. For this purpose, we adapted our established in vitro culture system to investigate and measure cell shape changes with and without functionalized carbon nanotubes (CNTs). Two nanomaterials, such as fluorescent polystyrene (PS) beads and functionalized CNTs, were employed to track the material location under confocal microscopy, light microscopy and Transmission Electron Microscopy (TEM). It was found that particles equally spread throughout the entire cytoplasm in spherical macrophage; whereas when macrophages were forced to adhere to the substrate, via fibronectin coating, the accumulation of particles and tubes was limited to the vicinity of the nucleus due to the modified cellular micro architecture. TEM analysis also confirmed these findings and demonstrated that CNTs of about 5 μm laid at the bottom of adherent cells. Therefore, this cell shape analysis and manipulation may result very important for the quantification of internalised novel materials with high aspect ratio like nanotubes, nanorods and nanowires.

1. Introduction

Carbon nanotubes (CNTs) are one of the most well-studied nanomaterials in the literature since they were first reported back in 1991 [1]. The development of carbon nanotubes (CNTs) as delivery systems for nucleic acids, proteins, and drug molecules into mammalian cells and living tissues has attracted much attention [2]. Surface functionalization of carbon nanotubes is an effective way of improving the solubility and dispersion of the nanotube. Functionalization of carbon nanotubes has been proposed in various biological applications, such as DNA and protein biosensors, biocatalysts, and bioseparators. Nanotubes also find potential applications in the fields of cancer diagnosis and therapy. Pantarotto and coworkers reported on the internalisation of fluorescently labelled CNTs into

human T cells [3]. Recently, Kam et al. reported single-wall carbon nanotube (SWCNT)-streptavidin conjugates into human promyelocytic leukaemia (Hela or HL60), Chinese hamster ovary (CHO), and MC3T3 osteoblast cell line [4]. Getting the number of fluorescent SWCNT has been described in lung epithelium cell in the work of Lacerda et al [5]. However, it is necessary to calibrate a number of factors to get the right number of internalised nanomaterials.

It is well known that phagocytes, and in particular macrophages, play a key role in the cellular uptake and response to foreign bodies, such as particles, fibres or tubes. Alveolar macrophages in lungs represent the first defence against various hazardous environmental exposures such as particles and fibres when inhaled. To better describe *in vivo* environmental interactions and *in vitro* models, we should go beyond simple membrane filopodia to full actin-filaments cross-link which provides sufficient substrate for macrophage spreading.

Over the past decade, several methods have described how to enhance the macrophage adhesion by modifying the substrate surface-chemistry with immobilized ligands or proteins [6,7]. Current *in vitro* models use the interactions of macrophages with extra-cellular matrix such as fibronectin (Fn). This interaction is known to occur mainly via the amino acid sequence RGD [6]. Fn has been largely used to enhance actin polymerization rapidly in neutrophils, monocytes and macrophages [8]. Therefore an accurate concentration of Fn is essential for the formation of receptor clusters and enhancement of cell adhesion on substrates. In this paper, different concentrations of Fn were used to coat glass substrates to maximize alveolar macrophage adhesion and subsequently control cell, shape such as cell height and width.

To validate this shape control, well-defined commercially available 1 μm polystyrene particles were used as reference material. Polystyrene (PS) beads are regarded as reference material when it comes to their geometrical regularity, shape and low toxicity. It is known that due to their tube-shape geometry, in the nanoscale domain, nanotubes are not easy to focus and be detected under fluorescent microscopy. In this work, fluorescently conjugated labels onto CNTs were used for their visualization by confocal microscopy of the CNT post-internalisation and cell interaction. We speculate on the mechanisms that regulate the interaction between the macrophage cytoskeleton and the internalised CNTs. This may lead to develop a quantitative assay to measure the internalised mass of the nano-structured materials into macrophages.

Our research hypothesis is that cell shape can orientate CNTs when the cell height is less than the CNT length. This paper will show how the cytoskeletal reorganisation of geometrically-tubular internalised CNTs can be seen by light microscopy, confocal microscopy and TEM. Here, the macrophage cell shape is used as a tool to orientate the carbon nanotube and make them easier to detect under any of the microscopy techniques employed. From the results presented in this work we can confirm that this technique can be very useful for the toxicology assessment of tube shaped nano materials.

2. Materials and Methods

2.1. Particles and Nanotubes

Fluosphere particles of diameter 1.0 μm (Molecular probes F-8823) were purchased from Invitrogen (Invitrogen Ltd, UK). Particles were supplied at a concentration of 2.0 %_w suspended in 50 mM Na₃PO₄, 50 mM NaCl, pH 7.5. The number for particles, N, per ml of suspension can be determined from the following equation: $N = C (6 \times 10^{12}) / (\rho\pi\phi^3)$, where C is the concentration of suspended beads in g/ml (0.02 g/ml for 2 % suspension); diameter ϕ of particles in micrometres (1 μm), and density ρ of polystyrene in g/mL (1.05 g/cm³).

Multiwalled carbon nanotubes (MWCNTs) of 50 nm wide and 5 μm long were purchased from IJIN (Diamond Co. Ltd, Korea). CNTs were functionalized with amino acids, according to the work of Pantarotto and co-workers [3].

2.2. Substrate preparations with Fn

Fn coating of substrates for cell counting were achieved on purposely prepared glass cover slips ($\phi = 10$ mm, MatTek Corporation, MA., USA) by using 20 μl coating of 2.5×10^{-3} mg/ml of fibronectin III (Sigma Chemical Co. St. Louis, MO.).

2.3. Culture

A murine macrophage cell line RAW264.7 was grown in Dulbecco's minimal Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), 1% L-glutamine and 1% penicillin–streptomycin at 37°C, 5% CO₂ and 95% relative humidity (Incubator model: MCO-20AIC, SANYO, Japan). Macrophages and PS beads/CNTs are incubated in the plastic tubes. The tubes were then rotated at 38°C (rotator model ST, New Brunswick Scientific Company, New Brunswick, New Jersey) for 2 hours. Under these conditions more than 90 per cent of nanoparticles were ingested after 30 minutes of rotation.

2.4. Cell staining and confocal laser microscopy

First, cells were seeded onto coverslips and incubated for 2 hours in complete DMEM then were fixed with 4% paraformaldehyde in PBS at room temperature for 20 minutes. Cells were then washed again with PBS and permeated with 0.2% Triton X-100 for 5 minutes at room temperature. The samples were subsequently stained with mouse anti-actin (A4700, Sigma, Taufkirchen, Germany) and anti- α -tubulin (T4026, Sigma) antibodies by a FITC conjugated secondary anti-mouse antibody (F0257, Sigma) and Chromeo™ 642 Goat anti-Mouse IgG (ACTIVE MOTIF, Germany). Then the cell nucleus was stained by 10 mM Hoechst for 1 minute. Finally the stained coverslips were mounted for fluorescence microscopic examination. Data were obtained using a Zeiss Laser Scanning Microscope (LSM 510 META, Zeiss, Germany). The LSM was based on the Axiovert 100M inverted research microscope equipped with 4 laser sources (Argon, Helium-neon 1, Helium-neon 2 and Titanium : Sapphire).

2.5. Transmission Electron Microscopy (TEM) sample preparation

For TEM analysis the cell-CNT samples were prepared following the same protocol used for the fluorescent investigation. Macrophages with CNT at 0.05 μM concentration were incubated for 2 hours. After the incubation, cells were seeded onto fibronectin coated (2.5×10^{-3} mg/ml) glass slides for 2 hours. Afterwards, the cells were washed with 0.01M phosphate buffer (PBS) and fixed for 2h by 2.5% glutaraldehyde, which was previously dissolved in PBS (pH 7.4). The cells were embedded into 0.1% agar at 37 °C for 5min. This agar was fixed by 2.5% glutaraldehyde in PBS at 4 °C, for at least 2h. The samples were washed with PBS, and then fixed by 1% osmium tetroxide at 4 °C for 2h. Cells were dehydrated in graded series of ethanol and later embedded in epoxy resin, as described elsewhere [9]. The cells were cut by vertical and horizontal section. Ultra-thin cross sections (~50nm) of cells were observed under a Transmission Electron Microscope, Philips CM10.

3. Results

3.1. Measurements of cell geometry and particle location

After 2h exposure to polystyrene particles, RAW264.7 macrophages were successfully plated for 2 hours on fibronectin-coated glasses and untreated borosilicate glasses. This resulted in intrinsically induced morphological cell-shape changes of the plated macrophages, both at the cellular and subcellular level. In particular, the subcellular particle distributions varied a lot within the two different cell shapes, here call “spherical” and “flat”, as shown from the confocal fluorescence image (Figure 1). It can be seen that for so-call spherical cells, plated on untreated substrates, the particles are equally distributed through the cytoplasm (Figure 1A). Where as for the fibronectin-coated substrate the flat cells (cell height = 1.5 ± 0.2 μm) showed a preferential accumulation of the particles near the nucleus (Figure 1B). This is attributed to the modified cytoskeletal micro architecture network induced

by the fibronectin coating. Thus, the extracellular matrix coating has a direct impact on the cell focal adhesion and cell geometrical aspect ratio (diameter/height = 20). This high aspect ratio implies that spherical macrophages can be rearranged in their cytoskeletal scaffold into a nearly flat, 2-dimensional (2D) shape (Figure 1B). We thus call this cell shape ‘flat’. Enhanced imaging resolution can be immediately gained from the comparison of the two confocal images (Figure 1A and Figure 1B). Figure 1C illustrates parameters, such as height and diameter of each nucleus, heights and diameter of all the accumulated particles. All the geometrical information, quantification and localisation of accumulated particles are necessary for the 3-dimensional rendering in confocal microscopy.

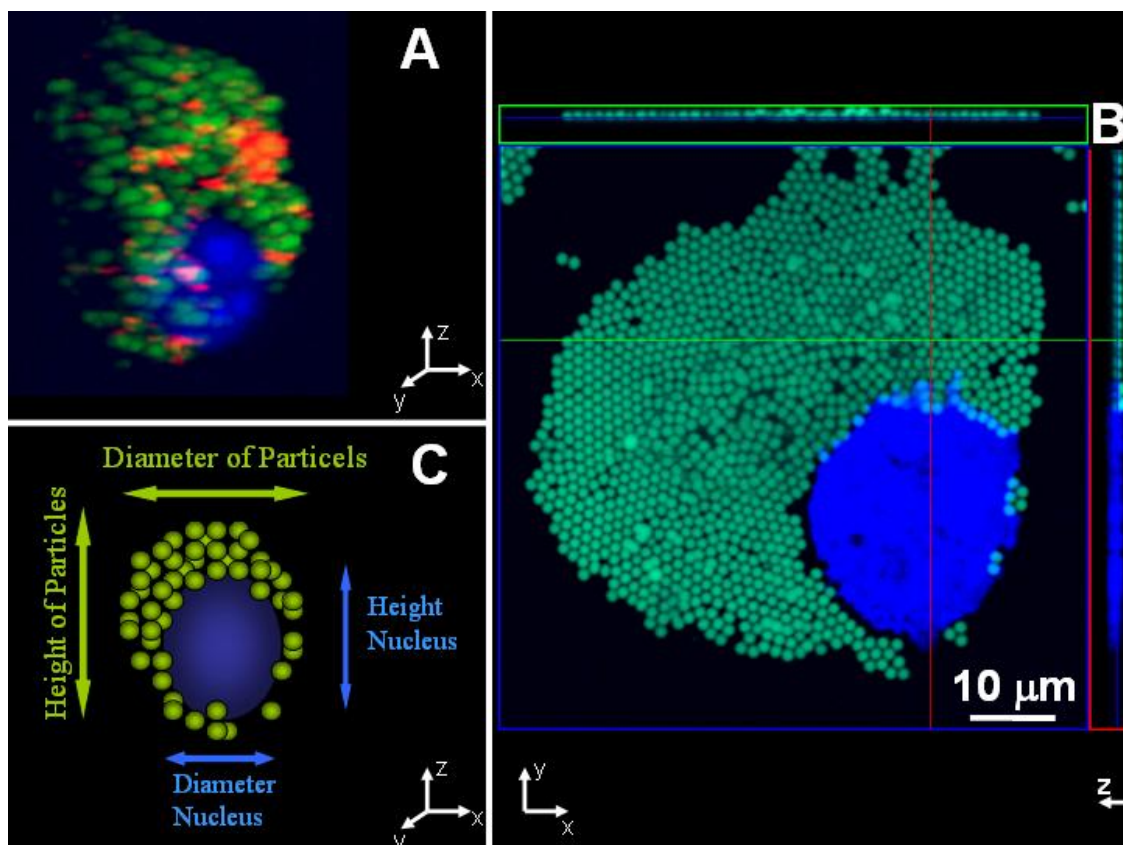


Figure 1. Morphology of a ‘spherical’ and ‘flat’ macrophages with internalised 1 µm fluospheres. (A) Particle distribution in a spherical macrophage. Uniform distribution throughout the cytoplasm of a spherical cell; 2D image. (B) X-Y and Z-plane section of a macrophage adherent to fibronectin-coated substrate. The Z-plane section (right side of B image) showed a flat cell with a height of 1.5 µm. (C) 3D schematic image illustrating a macrophage with internalised 1 µm fluorescent beads (in green) and nucleus (in blue).

3.2. Cell adaptation to particles: cytoskeletal reorganisation

From our observations it was found that spherical macrophages do not show any stretched cytoskeletal structural organisation. Instead, a rather diffuse organisation of the microtubule- and actin filaments was observed, as shown by Figure 2A. This is highlighted by the recurrent colour merging between the green fluorescent polystyrene beads and the yellow fluorescent stained microtubules, as it is shown in Figure 2A.

On the other hand, when the flattened macrophages were analysed and imaged, it was clearly distinguishable that microtubules and actin filaments were spreading throughout the cytoplasm. This cytoskeletal organisation identifies a filamentous intracellular network. Actin-bundle structures appeared in straight filaments; whereas microtubules occurred as subtle structural networks, radiating throughout the cytoplasm. In this study the simplified structural scaffold is only limited to actin filaments and microtubules since they are the main active filaments involved during cell active cytoskeletal adaption to the ingested particles. They filamentous scaffold results so organised that the green fluorescence of the polystyrene particles can be seen entangled with the yellow microtubules (Figure 2B). A schematic representation of what we believe is intervening during this structural cell-scaffolding rearrangement on flattened macrophages is shown in Figure 2C. Our hypothesis is that the cell membrane (in violet) and the actin filaments (in red) are constricting the cytoplasm structure and moved the rearrangement of the cytoskeletal components (i.e., actin and microtubules) which subsequently force the particles to redistribute in organised layers underneath the filamentous structures. This constrains an effective accumulation of particles to the immediate vicinity of the nucleus. By our microscopic analysis we also observed that when high concentration of particles were forced towards the nucleus, they rearranged into a dense 2-dimensional packed clusters, as shown in Figure 1B. These combined evidences lead us to assume that the relatively “bulky” 1 μm polystyrene particles were simply accumulated around the perinuclear region of the cytoplasm due to the cell lack of space. Speculations on a tighter packing order can be drawn by the presence of cytoskeletal forces rearranging the cell shape.

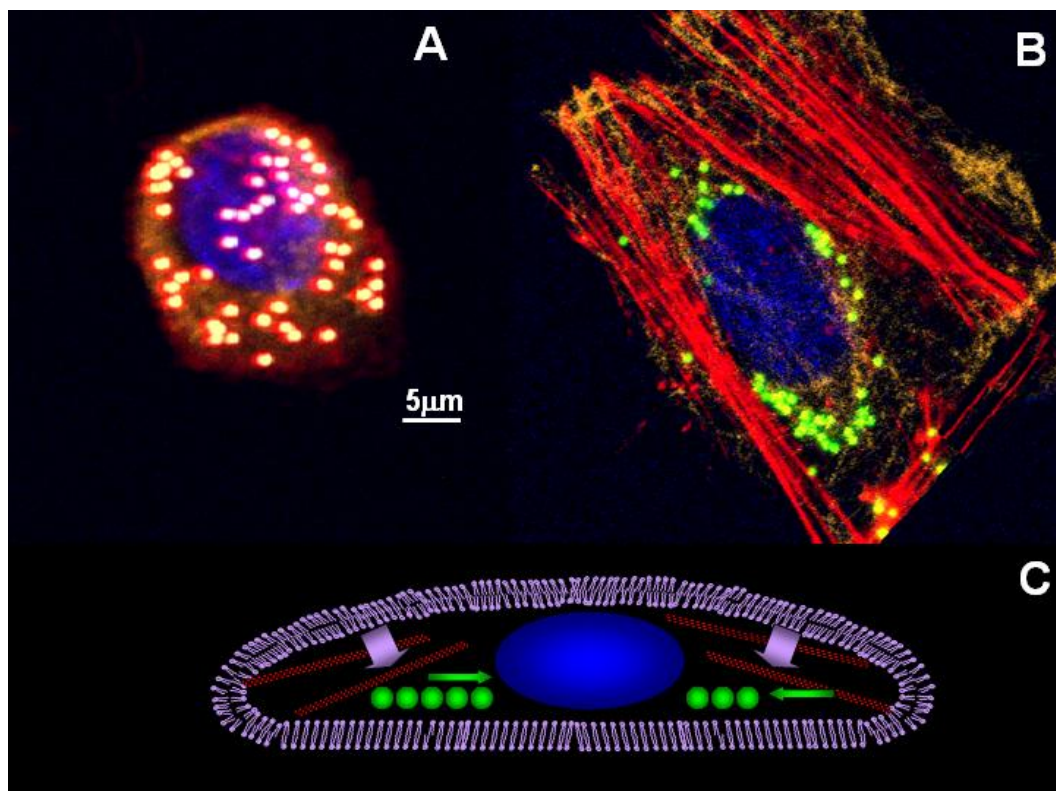


Figure 2. Particle distribution and macrophage morphological geometry: images and schematic representation; (A) Top view of spherically rounded macrophage (adhesion on untreated glass substrate); (B) Top view of flattened macrophage (adhesion on fibronectin coated glass substrate); (C) Schematic illustration of X-Z plane section of flattened macrophage (from Figure B); The nucleus is stained blue, microtubules are stained yellow, and actin is stained red. Green fluorescent particles are moved by the microtubule and actin cytoskeletal reorganisation.

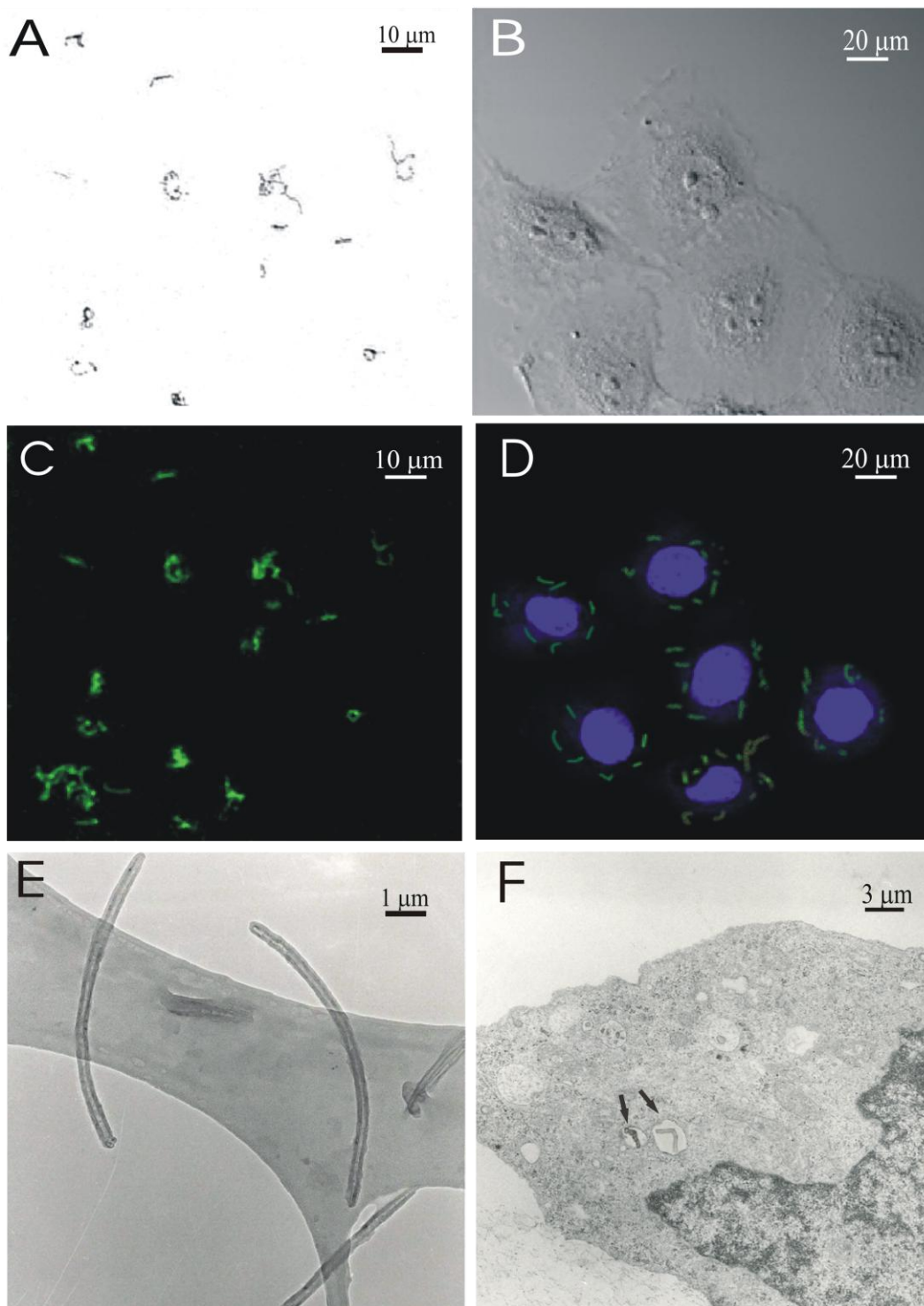


Figure 3. Micrographs of carbon nanotubes (CNTs) and cells with internalised CNTs; (A) and (C) highlight a heterogeneous distribution of CNTs onto a glass substrate under phase contrast and dark-field fluorescent microscopy, respectively; (B) and (D) show cells with internalised CNTs under light (phase contrast) and fluorescent (dark-field) microscopy (CNTs: green, nucleus: blue); (E) shows a HRTEM image of entangled MWCNTs with a membrane; (F) shows TEM contrast details of the internalised CNTs inside a macrophage, their localization and storage inside vesicles.

3.3. Determination of ingested CNTs in flat macrophages

A direct consequence of flattening macrophages is the possibility to image individual CNTs with a standard fluorescent microscope. After ingesting functionalized carbon nanotubes (CNT), macrophages were plated on fibronectin-coated substrates and the internalised CNTs were observed using three different microscopy techniques: phase contrast (Figure 3A and B), fluorescence (Figure 3C and D) and high resolution transmission electron microscopy (HRTEM) (Figure 3E and F). The left column of Figure 3 (Figure 3A, 3C, and 3E) shows images of separated CNTs; whereas the right column of Figure 3 shows cultured macrophages with internalised CNTs (Figure 3B, 3D, and 3F). The phase contrast micrographs show CNTs separated and spread on the glass slides (Figure 3A). From the matching fluorescent microscopy image (Figure 3C) the green fluorescent tube-shape structures exactly matched the CNTs imaged by phase contrast technique. Therefore, we achieved individual carbon nanotube imaging by fluorescence microscopy.

The TEM image in Figure 3E shows a 10^4 -fold magnification on three individual CNTs. There, the CNTs appear well separated, as single structure on the carbon film substrate. The right column of the images shows cells grown on fibronectin-treated glass slides. According to the phase contrast image (Figure 3B), the cells appear as flat-shaped and well spread. The smooth surface of the cell indicates that there are not any CNTs on the outer side of the cell membrane. By using the fluorescence microscopy, the fluorescent CNTs are shown as green tubes arranged around the nucleus (Figure 3D). There, the arrangement of the CNTs appears in a 2-dimensional orientation. This allow for the geometrical sizing of the internalised CNT lengths at around 5 μm each. The TEM image in Figure 3F shows two internalised CNTs, engulfed and stored inside individual vesicles. Furthermore, the high staining contrast between cell membrane and cytoplasm made it also possible to visually quantify the number of CNTs internalised in each cell.

4. Discussion

The total account of “potentially hazardous” materials such as nanoparticles or CNTs requires the establishment of their potential bio-effect, uptake and subcellular localisation. Many studies have reported on the cell uptake of carbon nanotube by confocal microscopy, fluorescent microscopy and TEM [2, 3]. In those works, bulky CNTs resulted very difficult to observe because nanotubes were entangled into the cell-cytoplasm and overlapped with all the others internalised tubes. In our work, it was possible to described how the cytoskeleton would oriented the carbon nanotube inside the cytoplasm by using FN coating to flatten the cells.

From our results, we also found that in well-adherent flat-shaped macrophages, fluorescent particles were systematically accumulated around the perinuclear region in a geometrically regular packing order. Hence, we suggested that the cell membrane bending forces, exerted by the recruited actin filaments traction, may confine the large particles to the region where a maximal void space is available. A similar explanation has also been suggested in the study of Kodali and co-workers [10]. To further support of our findings, it has been suggested that, after their cellular uptake, these particles follow the dynamic instability of the microtubules and are directed towards the nucleus [11,12]. In our experimental settings, the preferential accumulation of particles, closer to the nucleus, was observed only when the cells reached their maximum flattened shape, which represents an almost 2-dimensional condition. There, we highlighted that the geometry of the cytoskeleton micro architecture limits the accumulation of particles near the nucleus. This cell response is most likely caused by the constricted cell height which directs particles toward an ordered arrangement. On the basis of this, the intracellular particle accumulation model could also be applied to explain the agglomeration of carbon particles and paclitaxel etanidazole nanoparticles closer to the cytoplasmic nucleus [13,14].

From the phase contrast images we also showed the flat shape of macrophages grown on fibronectin treated glass slides. There, the arrangement of the CNTs appears in a 2-dimensional orientation. The lack of cell membrane coarse rugosity indicates that the CNTs did not perforate the

cell membrane; this was also shown by fluorescence microscopy (Figure 3D). Where from the TEM image in Figure 3F it was reported that each CNT were neutralised and stored within the cell cytoplasm [3]. Furthermore, the high contrast made it possible to quantify and localize low density number of internalised CNTs per cell. Orientation of the nanotubes is possible in flat-shaped cells. However, it has been shown that there is a limit for localisation and orientation of CNTs. This has been reported in Lacerda's work, at a concentration of 500 µg/ml [5]. On the other hand, another work reported the imaging of individual SWCNTs, inside macrophage, by using EFTEM (Energy Filtered Transmission Electron Microscopy). In their work, Porter and co-workers reported a of 5 µg/ml which is far lower than the one described of Lacerda et al. [5, 14]

In our work, CNTs appeared well separated, as single tubes. This has been shown by the TEM image and suggested a high geometrical aspect ratio. However, the main limitation of TEM imaging technique is the applicability to very low density of CNTs. Therefore, the imaging technique here proposed has an advantage over the other techniques, by mapping the particles into a 2D plane. This also facilitates the use of light transmission microscopy (or similar techniques) for the quantification and localization of the internalised nanoparticles.

This implies that the detection and quantitative measurement of CNTs can be carried out from a small amount of cells (< 10,000 cells). This is commonly occurring in clinical medicine and occupational health when the cell samples are obtained from biopsies or bronchoalveolar lavages (BAL). Therefore, if the quantitative technique is not sensitive enough the toxicological analysis of the alveolar macrophages will be negatively biased. The technique here presented would facilitate and accelerate the experimental preparation for BAL investigations, and increase the laboratory resource capacities by simply reducing the cost per experiment due to the low sample volume employed. Finally, we can expect that this technique can be very useful for the toxicology assessment of any tube-like and spherically shaped nanomaterials.

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