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Acta Biomaterialia 2 (2006) 409–419
Acta BIOMATERIALIA
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Uptake of C_{60} by human monocyte macrophages, its localization and implications for toxicity: Studied by high resolution electron microscopy and electron tomography

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Received 21 December 2005; received in revised form 9 February 2006; accepted 22 February 2006

Abstract

Despite great interest in the engineering applications of carbon-based nanoparticles, recent studies have raised concerns about their potential toxicity and safety. The release of C_{60} into the environment has been suggested to be a potential risk with possible ecological implications. Here we evaluate energy-filtered transmission electron microscopy (EFTEM) and scanning transmission electron microscopy (STEM)-based electron tomography as techniques for imaging the three-dimensional (3-D) distribution of nanoparticles within cells. Our aim was to establish if human monocyte macrophages internalise nanoparticles and to assess whether nanoparticles are modified by cells following uptake. Using these techniques we were able to show a marked increased in the amount of information gained from 3-D imaging. 3-D electron tomography revealed several sub-cellular compartments containing C_{60} within the cell: secondary lysosomes, along the outer and nuclear membrane and most notably inside the nucleus of the cell. Using EFTEM and STEM-based techniques we were able to visualize cell structures such as membranes, the mitochondria, ribosomes and the nucleus, without the need for traditional staining techniques. In particular we demonstrate the potential of electron tomography for whole cell studies to enable 3-D distributions of particles within cells. The concentrations of C_{60} used in this study were not toxic and were chosen to study which sub-cellular compartments accumulated C_{60}. Knowledge of the sites of accumulation of nanoparticles will allow us to predict vulnerability if the nanoparticles can generate free radicals.
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Keywords: 3-D electron tomography; Buckminster fullerene; Toxicity; Intracellular distributions

1. Introduction

Carbon-based nanoparticles, such as buckminster fullerene (C_{60}) [1–3], have attracted a great deal of interest due to their unique chemical and physical properties. Although applications of fullerenes in their native form have been somewhat limited, unique properties may be achieved by chemically tailoring the outer fullerene surface [2,4] or its inner "cage" (endohedral fullerenes) [5]. Potential applications for carbon-based nanoparticles include optical and electronic materials, superconductors and drugs or drug delivery vehicles in the treatment of debilitating diseases such as HIV [4]. However, recent studies have also raised concerns about their potential toxicity [6–8], with the release of C_{60} into the environment suggested as having possible ecological implications [6–9].

It has been hypothesised that the toxicity of C_{60} is related to its ability to cause oxidative stress [6,7]. In water, uncoated C_{60} forms crystalline colloids, described as nano-C_{60} [7]. These colloids have a high electron affinity and a propensity for generating superoxide anions [7]. In vivo

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doi:10.1016/j.actbio.2006.02.006

tests have shown that nano-C_{60} is both redox-active and lipophilic, and produces oxidative damage in the brain of largemouth bass fish [6]. Similarly, exposure of human fibroblasts to nano-C_{60} causes decreased cell viability arising from lipid peroxidation of cell membranes [7]. Peroxidation in the lipid bilayer causes membranes to become "leaky", compromising ion homeostasis [7]. Interestingly, neither study reported any change in DNA concentration, protein oxidation, mitochondrial activity or monitored particle uptake [8]. An understanding of C_{60} particle uptake and intracellular distribution in three dimensions is critical to unravel the putative mechanisms of toxicity.

Electron tomography is a well established technique in the biological sciences for determining three-dimensional (3-D) components and structure of macromolecular assemblies and sub-cellular organisation, see, e.g., Refs. [10,11]. The technique is based on recording a series of electron micrographs over a range of tilt angles from a specimen of interest. A back-projection algorithm is then used to compute the 3-D reconstruction of the sample. Images may be acquired using conventional energy-filtered transmission electron microscopy (EFTEM) [12,13] or by high angular annular dark field (HAADF) scanning TEM (STEM) [14]. EFTEM is often used to form "zero-loss" loss images in which electrons that have undergone inelastic scattering are removed to improve the contrast of bright-field TEM images. Alternatively, elemental maps can be acquired by using characteristic ionisation edges. However, both EFTEM techniques have limited value when investigating sections thicker than ~100 nm, or whole cells, as multiple scattering events dominate, greatly reducing the usable signal. We will show that EFTEM tomography can be used to reveal useful information from non-stained sections of moderate thickness. HAADF STEM uses an annular detector to collect electrons which have been scattered to high angles. As there is no post-specimen imaging lens, the image quality does not suffer from the effect of chromatic aberration, which compromises images from thick sections. Beam broadening will occur in STEM imaging of thick sections, but its effects can be minimised by using small focussed beams with smaller convergence angles.

The objectives of this paper were: (i) to evaluate EFTEM and STEM-based electron tomography as techniques for imaging the 3-D distribution of nanoparticles within thick sections, and indeed within whole freeze-dried cells, (ii) to confirm uptake to identify where the nanoparticles accumulate, and (iii) to assess whether nanoparticles are modified by the cells following uptake. Macrophages form the first line of defence in the immune response to foreign materials in many tissues, including the lung, and have a large pro-inflammatory potential. A significant proportion of nanoparticles entering the pulmonary airways are likely to be scavenged by macrophages before they transcytose across epithelial and endothelial cells into the blood and lymph circulation. Therefore, we chose human monocyte macrophages (HMMs) as an in vitro model for the exposure of nanoparticles to cells or tissues.

2. Methods

2.1. Preparation of nanoparticles

2.1.1. C_{60} preparation

0.01 g C_{60} (99.9% pure, Aldrich, Dorset, UK) was dispersed in 10 ml of freshly distilled, filtered and de-gassed tetrahydrofuran (THF) (1 g/l). The solution was left in a vial with a screw cap for 24 h stirring at ambient temperature, allowing it to become saturated with soluble C_{60}.

2.1.2. Characterization of C_{60}

For TEM observations of C_{60} in THF, lacy carbon 300 mesh copper grids (Agar Scientific, Dorset, UK) were immersed in stirred solutions of C_{60} in THF. The size distribution of the fullerene clusters was studied by dynamic light scattering (DLS), which was performed at 24 °C on a DLS-system (Malvern Zetasizer) equipped with an He–Ne laser.

2.2. In vitro cell culture studies

2.2.1. Isolation and culture of human monocyte macrophages

Mature human macrophages were obtained by in vitro culture of human monocytes isolated from human buffy coat residues (National Blood Transfusion Service, Brentwood, UK). Buffy coat residue was washed once with phosphate-buffered saline (PBS), and the resulting cell sediment was mixed with an equal volume of fresh PBS. Thirty milliliter of diluted buffy coat residue was layered onto 15 ml of LymphoPrep (Axis-Shields, Oslo, Norway) and, after centrifugation at 20 °C for 30 min at 600*g*, the opaque interphase of mononuclear cells was removed and washed three times with PBS containing 4 mg/ml bovine serum albumin (BSA) to remove platelets. Monocytes were then enriched by an additional centrifugation step in a Percoll gradient [15]. Mononuclear cells were re-suspended in 4 ml of PBS and mixed with 8 ml of Percoll: Hanks' balanced salt solution (10× concentrate) (6:1, at pH 7.0). After centrifugation at 20 °C for 30 min at 450*g*, the monocytes were collected from the top of the gradient, washed in PBS/BSA and seeded in 24 and 48-well tissue culture plates at $1–2 \times 10^6$ cells/well and $0.5–1 \times 10^6$ cells/well respectively using macrophage serum-free medium (Mϕ-SFM, Invitrogen) unless otherwise stated. For microscopy, cells were seeded on formvar coated gold TEM grids in 50 μl culture medium at a density of $0.25–0.5 \times 10^6$ cells/grid. After incubation for 1 h at 37 °C, any remaining non-adherent cells were removed by washing twice with PBS. Adherent monocytes were cultured at 37 °C in humidified air/5% CO_2 using Mϕ-SFM for at least 6–7 days prior to experiments unless stated otherwise, renewing the culture medium twice a week.

2.2.2. Neutral red assay

Cell viability was assessed using the neutral red assay. Neutral red is a dye, which accumulates in lysosomes of live cells only. HMMs were incubated with various

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