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# Effects of carbon nanotubes on primary neurons and glial cells

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ABSTRACT

Carbon nanotubes (CNTs) are among the most promising novel nanomaterials and their unique chemical and physical properties suggest an enormous potential for many areas of research and applications. As a consequence, the production of CNT-based material and thus the occupational and public exposure to CNTs will increase steadily. Although there is evidence that nanoparticles (NPs) can enter the nervous system via the blood stream, olfactory nerves or sensory nerves in the skin, there is still only little knowledge about possible toxic effects of CNTs on cells of the nervous system.

The goal of the present study was to analyse the influences of single-walled CNTs (SWCNTs) with different degrees of agglomeration on primary cultures derived from chicken embryonic spinal cord (SPC) or dorsal root ganglia (DRG). As measured by the Hoechst assay treatment of mixed neuro-glial cultures with up to 30 μg/mL SWCNTs significantly decreased the overall DNA content. This effect was more pronounced if cells were exposed to highly agglomerated SWCNTs as compared to better dispersed SWCNT-bundles. Using a cell-based ELISA we found that SWCNTs reduce the amount of glial cells in both peripheral nervous system (PNS) and central nervous system (CNS) derived cultures. Neurons were only affected in DRG derived cultures, where SWCNT treatment resulted in a decreased number of sensory neurons, as measured by ELISA. Additionally, whole-cell patch recordings revealed a diminished inward conductivity and a more positive resting membrane potential of SWCNT treated DRG derived neurons compared to control samples.

The SWCNT suspensions used in this study induced acute toxic effects in primary cultures from both, the central and peripheral nervous system of chicken embryos. The level of toxicity is at least partially dependent on the agglomeration state of the tubes. Thus if SWCNTs can enter the nervous system at sufficiently high concentrations, it is likely that adverse effects on glial cells and neurons might occur.


## 1. Introduction

Carbon nanotubes are hollow cylinders made of graphene sheets rolled in on themselves to form a tube. They are produced as SWCNTs or multi-walled CNTs (MWCNTs) consisting of one or multiple concentrically rolled layers of graphene, respectively (Baughman et al., 2002). The unique structural, mechanical, electrical and optical properties of CNTs are currently exploited in many areas of research and application, including biosensors (Chen et al., 2003), field emission (Minoux et al., 2005), energy storage (Patchkovskii et al., 2005), molecular transporters for drug delivery (Kam et al., 2005) and neural prosthetics (Lovat et al., 2005; Wei et al., 2007). However, despite their increasing use, the effects of CNTs on human health and on the environment are largely unknown. Most of the recent research dealing with potential health hazards of CNTs or other nanoparticles has focused on cells and tissues that are likely to get immediately into contact with airborne particles. Initial toxicological studies demonstrated that intratracheal or pharyngeal instillation of SWCNT suspensions in mice caused a persistent accumulation of carbon nanotube aggregates in the lung, followed by the rapid formation of pulmonary granulomatous and fibrotic tissues at the site (Lam et al., 2004; Shvedova et al., 2005; Warheit et al., 2004). Respiratory exposure to high concentrations of mostly agglomerated SWCNTs was shown to provoke not only pulmonary toxicity but also caused vascular effects related to mitochondrial oxidative modifications as well as accelerated atheroma formation (Li et al., 2007). Exposure of human keratinocytes to CNTs increased oxidative stress, led to the accumulation of peroxidative products, induced a cytokine-mediated inflammatory response, inhibited cell proliferation, and provoked ultrastructural and morphological changes in the cells (Manna et al., 2005; Monteiro-Riviere et al., 2005; Shvedova et al., 2003). On the other hand, CNTs are extensively explored for their beneficial use in nervous system tissue engineering, including CNT-based nerve scaffolds to drive nerve regeneration across a lesion site (Wei et al., 2007). However,

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there is only sparse knowledge about potential neurotoxic effects of CNTs. Recent experiments with rats and fish showed that nanosized carbon particles can be taken up by olfactory neurons in the nose and are translocated to the brain, making neurotoxicity of CNTs an important issue (Elder et al., 2006; Oberdorster, 2004; Oberdorster et al., 2004).

The goal of the present study was to elucidate the effects of well-characterised SWCNTs with different degrees of agglomeration on primary cells of the nervous system. Mixed neuro-glial cultures from the spinal cord (SPC) of the central nervous system (CNS) or the dorsal root ganglia (DRG) of the peripheral nervous system (PNS) were exposed to SWCNT-agglomerates of submicron sizes (SWCNT-a) as well as SWCNT-bundles (SWCNT-b) consisting of 10–20 parallel aligned tubes. We found that SWCNT-a and SWCNT-b affect glial cells from both tissue types. Concerning the overall DNA content of primary cultures, higher concentrations of SWCNT-a were more toxic than the same amounts of SWCNT-b. These observations are consistent with our previous results (Wick et al., 2007) indicating that the aggregation state of SWCNTs might be an essential factor in determining their toxic effects. Furthermore the ionic conductance, the resting membrane potential and the capacity of PNS derived neurons were affected by SWCNTs, while neurite outgrowth and electrophysiological properties of SPC derived neurons were not. Taken together, these findings demonstrate that SWCNTs adversely influence mixed neuro-glial cultures from both CNS and PNS.

## 2. Materials and methods

### 2.1. Carbon nanotube preparation and characterization

SWCNT raw material (SWCNTrm) produced by the conventional arc-discharge evaporation of graphite rods filled with Ni and Y powder as catalysts, was purchased from Yangtze Nanotechnology, Shanghai. To obtain SWCNT-agglomerates (SWCNT-a), the SWCNTrm was purified by heating at 320 °C for 30 min followed by treatment with 37% HCl in an ultrasonic bath for 15 min. SWCNT-a was suspended in 40 μg/mL polyoxyethylene sorbitan monooleate (PS80, Sigma P4780) to a final concentration of 5.4 mg/mL. To obtain SWCNT-bundles (SWCNT-b), the SWCNTrm was sterilised for 3 h at 160 °C and suspended in Millipore water containing 40 μg/mL PS80. The resulting suspension (250 μg/mL) was sonicated in an ultrasonic bath for 15 min, left overnight at room temperature (RT) and sonicated again for 15 min. A purified fraction of SWCNT-b was obtained in the supernatant after centrifugation at $1000 \times g$ for 10 min. The concentration of SWCNT-b was determined by measuring the optical density (OD) of the dispersion at 405 nm. Serial dilutions of SWCNTs ranging from 0.9 to 500 μg/mL were used for calibration. The standard curve was linear over a concentration range from 0.9 to 250 μg/mL ($r = 0.9969$)(Wick et al., 2007). SWCNT-a and SWCNT-b were characterised by scanning electron microscopy (SEM), transmission electron microscopy (TEM), Raman spectrometry, near infrared spectroscopy and inductively coupled plasma optical emission spectrometry (ICP OES) as described before (Wick et al., 2007).

### 2.2. Primary cell cultures and treatment with SWCNTs

Primary cultures containing a mixture of different types of neuronal and glial cells were prepared from Leghorn chicken embryos. To distinguish between effects of SWCNTs on cells derived from CNS or PNS, cultures were prepared from the ventral part of the SPC of E6 chicken embryos (Hamburger–Hamilton stage 28/29) or DRG of E10 chicken embryos (Hamburger–Hamilton stage 36), respectively. Both tissue types were carefully dissected, triturated and dissociated with 0.25% trypsin for 10 min at 37 °C. Cell suspensions were prepared in modified minimum essential medium (MMEM) (MEM supplemented with 2 mM glutamine, 5% heat-inactivated horse serum, 1% nutrient concentrate, 2% chicken embryo extract and 10% muscle cell conditioned medium) at a concentration of $1 \times 10^5$ cells/mL. Cells were seeded on poly-D-lysine and laminin coated plates or cover slips and maintained in 5% $CO_2$ at 37 °C.

Primary glial cultures were prepared from SPC or DRGs of E6 and E10 chicken embryos, respectively. Resulting cell suspensions were plated in tissue culture flasks at a density of $1 \times 10^5$ cells/mL in MMEM and maintained in 5% $CO_2$ at 37 °C for 30 min. Following shaking, floating cells were washed away and medium was changed. Cultures were trypsinised and re-plated after 6 days, expanded for additional 6 days and were finally trypsinised and re-plated in 96-well plates at a density of $6 \times 10^4$ cells/mL.

Twenty-four hours after plating (DIV1), cells were treated with PS80-suspended SWCNT-a, PS80-suspended SWCNT-b or PS80 alone for the duration and at the concentrations indicated. For electrophysiological experiments half of the medium volume was renewed on DIV4.

### 2.3. Reaggregates and neurite outgrowth analysis

After chicken eggs were incubated for 70 h at 37 °C SPC cells of chicken embryos were transfected *in ovo* with a modified RFP-plasmid vector pRFP-N1 (Clonetech, USA) as described previously (Pekarik et al., 2003). To visualise cells on non-transparent substrates an ubiquitously expressed variant kindly provided by Prof. J.-C. Perriard (Institute of Cell biology, ETH, Switzerland) was used. $2 \times 10^6$ cells were incubated in 4 mL MMEM in a 25 mL Erlenmeyer flask (Brand, D) on a gyratory shaker (HT, Infors AG, Bottmingen, CH) for 20 h at 37 °C. The resulting spherical aggregates with diameters in the range of 100–300 μm were transferred to poly-D-lysine/laminin coated cover slips and maintained in 5% $CO_2$ at 37 °C overnight. Neurite outgrowth was monitored using a Confocal Laser Scanning Microscope (CLSM, Zeiss Axioplan 2 with LSM510 scanning module) and analysed as described previously (Kaiser and Bruinink, 2004).

### 2.4. Characterization of primary mixed neuro-glial cultures by indirect immunofluorescence microscopy

Cells were grown on poly-D-lysin/laminin coated cover slips, fixed with 4% paraformaldehyde and 0.2% Triton X-100 in PBS for 10 min at RT, washed three times with PBS and processed for indirect immunofluorescence. After a blocking step with 5% goat serum in PBS for 30 min at RT cells were incubated with primary antibodies for 3 h at RT, or overnight at 4 °C. Antibodies used were glial-specific mouse anti-glial fibrillary acidic protein IgG (GFAP) (1:1000, Chemicon), neuron-specific mouse anti-neurofilament 68 kDa IgG (NF68) (1:1600, Sigma), glial-specific guinea pig anti-vimentin IgG (1:500, Progen) and oligodendrocyte-specific mouse anti-$O_4$ IgM (1:170, Chemicon) and mouse anti-Galactocerebrosid IgG (GalC) (1:700, Chemicon). After three washing steps in PBS cells were incubated with the appropriate secondary antibodies (goat anti-mouse IgG Alexa Fluor 546, goat anti-guinea pig IgG Alexa Fluor 488, goat anti-mouse IgM Alexa Fluor 546, all 1:400, Molecular Probes) in the presence of 100 ng/mL 4′,6-diamidine-2-phanylindole (DAPI) for 1–2 h at RT. All antibodies were diluted in PBS containing 1% bovine serum albumin (BSA). Cells were analysed by fluorescence microscopy using an Axio Imager.M1 epifluorescence microscope (Zeiss).