Genotoxic effects of exposure to carbon nanotubes in human lung cells

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Oslo, June 2014

Eskild Hagenes
Abstract

Nanotechnology is a growing industry bringing with the increasing utilization of nanoparticles (NP). NPs are defined as particles with at least one dimension being smaller than 100 nm. They are an increasing concern because of their size and unique physico-chemical properties, but the knowledge of impact on human health is limited so far.

One class of NPs that has raised special concern is carbon nanotubes (CNTs). CNTs are high aspect ratio allotropes of carbon that are used to produce several manufactured nanomaterials (MNM). They possess many attractive features and potential for widespread applications with workplace and subsequent public exposure. CNTs structural similarities to asbestos fibers have raised concern that they may pose a comparable health hazard. Chronic inflammation following inhalation of fiber-like compounds like asbestos are known to lead to several adverse effects such as fibrosis and cancer and the same may hold true for CNTs.

This study was carried out to evaluate the genotoxic and inflammatory potential of CNTs on human lung cells. Crocidolite asbestos was included as a reference material to be able to directly compare effects between CNTs and asbestos. Endpoints evaluated includes cytotoxicity, and expression of the pro-inflammatory mediators Interleukin-1 alpha (IL-1α), Interleukin-1 beta (IL-1β), Tumor necrosis factor-alpha (TNF-α) and Tumor protein 53 (TP53). Protein levels of Extracellular signal-regulated kinase (ERK) were also investigated. In addition we investigated the effects of CNTs on intercellular communication.

The cytotoxicity results showed a clear dose dependent toxicity from both CNTs, and both were more toxic than asbestos. TNF-α was the only cytokine examined that showed upregulation of its mRNA levels after CNT exposure. Small changes were seen in ERK expression. Regarding effects of CNTs on cell to cell communication, levels of the gap junction gene Connexin 43 (Cx43) were downregulated at both mRNA and protein level. The intercellular communication was also shown inhibited by the use of the scrape loading assay. These are novel findings that point towards possible down regulation of gap junctions via Cx43 after CNT exposure.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCK-8</td>
<td>Cell Counting kit-8</td>
</tr>
<tr>
<td>Cdk4</td>
<td>Cyclin-dependent kinase 4</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
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<tr>
<td>CNT-1</td>
<td>Carbon nanotube 1</td>
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<tr>
<td>CNT-2</td>
<td>Carbon nanotube 2</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase type 2</td>
</tr>
<tr>
<td>Croc</td>
<td>Crocidolite asbestos</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>CTB</td>
<td>CellTiter-Blue</td>
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<tr>
<td>Cx</td>
<td>Connexin</td>
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<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-deoxy 5'-triphosphatase</td>
</tr>
<tr>
<td>DM</td>
<td>Dispersion medium</td>
</tr>
<tr>
<td>DPCC</td>
<td>1m2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap junction intercellular communication</td>
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<tr>
<td>HARN</td>
<td>High aspect ratio nanoparticles</td>
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<tr>
<td>HBEC-3KT</td>
<td>Human bronchial epithelial cells 3KT</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
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<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinases</td>
</tr>
<tr>
<td>MNM</td>
<td>Manufactured nanomaterial</td>
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<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial cells</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin and streptomycin</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>TPA</td>
<td>Skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>t-test</td>
<td>Student T-test</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
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</table>
1 Introduction

1.1 Nanomaterials

The word “nano” originates from the Greek word “nanos”, meaning dwarf. Scientifically speaking, nano is equivalent to 1 billionth of a unit, thus 1 nanometer (nm) is $1 \times 10^{-9}$ of a meter. Nanomaterials are defined as materials with at least one dimension being smaller than 100 nm (Warheit et al., 2008).

There have always been naturally occurring nanosized materials (e.g. volcanic ash, fine sand and dust), and the industrial revolution introduced sources of “incidental” nanoparticles from combustion engines, power plants and other processes of thermodegradation. The newest group of nanomaterials are manufactured nanomaterials (MNM) consisting of synthetic nanoparticles (NPs) which are deliberately made, with controllable size, shape and composition (Oberdorster et al., 2005).

The main classes of MNMs are made from metals, carbon and composites. They are also produced in many different forms such as tubes, rods, cones, wires and spheres (Singh et al., 2009). Materials in this size range will have different properties than bulk materials of the same composition (Dresselhaus et al., 2004). Their changed physico-chemical properties offer new and improved features such as better thermal or electrical conductivity, stronger and harder materials and improved catalytic and optical properties (Singh et al., 2009). This offers many new applications within industry, cosmetics, electronics and medicine (Oberstorster et al., 2005). Consumer products made up of MNMs are increasingly being advertised by the nanotechnology industry, and already now influence the daily life of households around the world.

The same properties that make NPs desirable for use in multiple consumer and industrial applications also raise concerns about their possible hazardous toxicological endpoints. In one study surveying problematic characteristics of NPs, the top ranking properties of concern were reactivity, size, composition, aspect ratio and surface charge (Berube et al., 2011). Problematic NPs in the same survey were in ranking order; carbon nanotubes (CNTs), quantum dots, metal/metal oxides and fullerenes. Regarding effects on human health, fibrous MNMs and particularly CNTs have been of special interest due to their structural similarity to
asbestos fibers. There is therefore concern that CNTs may also show similar adverse health effects.

1.2 Carbon nanotubes

CNTs are made entirely of carbon which exist as many different allotropes including graphite, amorphous carbon (soot) and diamond. These are all composed of the same material but have large differences as a result of contrasting bonding and structure. CNTs are planar sheets of graphite (called graphene) rolled up into seamless cylinders of one atom thickness. There are two types of nanotubes, single-walled carbon nanotubes (SWCNT) which consist of a single cylinder, and multi-walled carbon nanotubes (MWCNT) having multiple walls (Figure 1.1).

![Graphical representation from left to right of: graphene sheet, SWCNT and MWCNT. From (Graham et al., 2005).](image)

The first industrial synthesis of CNTs was in the 1980s and widespread research on CNTs began in the 1990s. The commercial activity has grown during the last decade, and from 2006 to 2012 worldwide CNT production increased by ten-fold (De Volder et al., 2013). Currently the largest applications of CNTs are in nanocomposite materials which are polymers containing CNT in varying amounts. Nanocomposites offer improved strength and lighter weight suited for sporting equipment, car parts and aviation industry (Hussain et al., 2006). CNTs are also promising for applications in medicine as diagnostic and therapeutic devices.

There are three methods used to synthesize CNTs including arc discharge, chemical vapor deposition (CVD) and laser ablation (Donaldson et al., 2006). All methods generate particles
by adding energy to a carbon source producing fragments consisting of groups or single carbon atoms, but the methods differ in the energy source utilized. There are a few companies producing CNTs in Scandinavia including production of MWCNTs by the arc discharge method (Schneider et al., 2007; Hedmer et al., 2014).

Although exposure to the general public is possible due to release of CNTs following wear and tear of consumer MNM products, the highest exposure scenarios may occur at occupational settings. Occupational exposure can occur in processes such as design, research, production, incorporation into secondary products, disposal and recycling (Bello et al., 2008). Airborne exposure to CNTs is seen to occur in research facilities. A study measuring airborne levels of MWCNT in a research laboratory found up to 0.43 mg/m³ (Han et al., 2008) and another study found that handling of dry CNT powders presumably results in the highest exposures (Dahn et al., 2013).

### 1.2.1 Physico-chemical properties

CNTs have many desirable qualities for industrial purposes, but these properties are also of toxicological concern. For industrial purposes the focus has been on their mechanical strength, electrical properties and optical and thermal properties. In experiments CNTs are found to be the strongest material yet discovered, with a tensile strength up to 200 Gigapascals (GPa), ten-fold stronger than steel (Yu et al., 2000). Physico-chemical properties that may influence toxicity are size and shape, purity, tendency to agglomerate and surface charge (Murdock et al., 2008). Lengths of CNTs are typically tens of microns while the diameter is in the nanometer range. The diameter depends on how many graphene layers it contains, with SWCNTs ranging from 1-3 nm and MWCNTs up to 100 nm (Hou et al., 2003). Aspect ratio is the length : width ratio of a fiber and CNTs have very high aspect ratio, typically over 3 and are therefore classified as high aspect ratio nanoparticles (HARN) (Tran et al., 2011).

Pure CNTs consist only of one or several graphite sheets of carbon rolled into tubes and are fairly non-reactive, and will only burn in air if heated over 500°C (Zhang et al., 2002). During manufacturing several residual impurities may stick to the surface of the fibers. Impurities that may remain from the synthesis process are metals (Co, Fe, Ni), organics (e.g. amorphous
soot) and support material (aluminates, silicates) (Donaldson et al., 2006). Especially important is metal contaminants like iron, which can increase toxicity by undergoing redox cycling and generate reactive oxygen species (ROS) (Kagan et al., 2006). The amount of metal contamination has been seen as a factor for the degree of *in vitro* cytotoxicity. In BEAS-2B lung cells exposed to CNTs with a content of 30% iron and 20% nickel, a significant decrease in viability and an increase in hydroxyl radicals was observed (Shvedova et al., 2009) while CNTs with a low iron contamination were much less toxic (Kagan et al., 2006).

CNT aggregates will often associate with each other through van der Waal forces between the fibers. These so-called agglomerates are often larger than 100 nm in all dimensions and will therefore no longer behave as NPs which can alter their pathological potential. However, the forces holding them together are weak and they can be dispersed by lung fluid surfactants when deposited in the lungs (Wang et al., 2010). The van der Waal forces affecting MWCNTs with its multiple graphene sheets are weaker than for SWCNTs and therefore MWCNTs more commonly exist as individual fibers (Yu et al., 2000).

The harmful effect of particles depends on the combination of their surface area and their reactivity or intrinsic toxicity (Tran et al., 2000). When particle size decreases, more atoms become exposed on the particle surface. Therefore the ratio of surface to total volume increases exponentially with decreasing particle size, giving NPs a higher surface area to volume ratio than bulk material (Tran et al., 2000). As a consequence a greater amount of the material can interact with its surroundings, thus affecting reactivity.

### 1.2.2 Routes of exposure

The three major exposure routes for MNMs are inhalation, ingestion and dermal. CNTs are very light and can easily become airborne and potentially reach the lungs. Therefore inhalation exposure to CNTs has long been considered to be of major concern, especially in an occupational setting (Maynard and Kuempel, 2005).

The deposition and clearance from the respiratory tract varies significantly between NPs and larger particles (Kreyling et al., 2013). NPs can target all parts of the respiratory tract including, nasal, tracheobronchial and alveolar regions. Particles deposit at these different
regions according to their size, where smaller particles have greater potential of reaching further down the airways (Braakhuis et al., 2014). Once deposited NPs also have a greater risk of translocating across epithelial cells and enter the blood circulation (Choi et al., 2010). An in vivo study with TiO$_2$ NPs showed how particles were taken up in the lungs and passed through the air-blood barrier (Geiser et al., 2005) and similar pathways may apply to CNTs (Mercer et al., 2013a).

Epithelial cells are the first barrier in the lungs against the uptake of inhaled particles and fibers. Damage to the epithelial cells may give fibers free route into the pleura of the lung, where interaction with resident mesothelial cells may lead to diseases such as mesothelioma (Mercer et al., 2013b). Interaction between fibers and the cell surface or actively uptake of fibers by the endothelial cells may also be a cause of concern in regard to oxidative stress and inflammation.

CNTs may actively be taken up by clathrin- and caveolae- mediated endocytosis or they can enter the cells by passive diffusion or by piercing the cell membrane (Firme and Bandaru, 2010; Nel et al., 2006). Uptake of fibers may lead to inflammation, and epithelial cells are seen capable of engaging in an inflammatory response. Crocidolite asbestos strongly induces several pro-inflammatory cytokines via transcription factor Nuclear factor kappa B (NF-κB) in human alveolar epithelial cells (A549 cells) (Luster and Simenova, 1998). There is therefore concern that also CNTs through similar mechanisms may lead to serious pathologies (Sargent et al., 2014).

Exposure through ingestion and skin are considered minor routes for NPs. However dermal exposure may occur as MNMs such as TiO$_2$ and ZnO are frequently used in sunscreens and cosmetics. In healthy skin, the keratinized stratum corneum layer of the epidermis provides an excellent barrier and the use of nanoparticles in sunscreens has not led to reports of clinical toxicity in humans (Nel et al., 2006). Regarding CNTs there are no data on dermal or gastrointestinal toxicity.

1.2.3 Asbestos-like effects of CNTs

The experience with asbestos proved that fibers with a high aspect ratio can cause additional injury to the lung compared to compact particles (Donaldson et al., 2010). Asbestos has long
been known to cause serious injuries such as lung cancer, mesothelioma and asbestosis. Mesothelioma is almost exclusively linked to asbestos exposure and is a response confined to fibrous particles (Murphy et al., 2011). Therefore it is a major concern that a new fiber-shaped particle like CNT may show similar responses.

Several decades of research on asbestos led to the fiber pathogenicity paradigm (Figure 1.2), which one thinks also applies to other HARNs like CNT. The Fiber pathogenicity paradigm states that the toxicity behind fibers is a result of their length, diameter and biopersistence (Murphy et al., 2011). To be hazardous according to the Fiber pathogenicity paradigm a fiber must be thinner than 3 µm, longer than 10 to 20 µm and biopersistent enough in the lungs so that it does not break into shorter fibers (Murphy et al., 2011). Fibers with these characteristics can accumulate in the target tissue and induce inflammation and oxidative stress leading to pathogenicity.

![Figure 1.2: Model for the role of length, and biopersistence on clearance and pathogenicity according to the fiber pathogenicity paradigm. From (Donaldson, 2009).](image)
The width of the fiber determines its aerodynamic capabilities and where in the respiratory tract it will deposit. Mesothelioma arises in the parietal pleura on the inside of the thoracic cavities (Figure 1.3). Fibers that reach the alveolar and bronchiolar compartments are also potentially hazardous as this region is close to the blood for gas exchange (Donaldson et al., 2011).

![Diagram of the lung and types of injury caused by fibers at different sites.](image)

**Figure 1.3:** Anatomy of the lung and types of injury caused by fibers at different sites. From (Donaldson et al., 2011).

Mesothelioma is a tumor arising on the parietal pleura of the chest wall. The parietal pleura consist of connective tissue covered by flat mesothelial cells (Mutsaers et al., 2004). One *in vivo* study investigated the inflammatory effects of MWCNTs on the lungs and pleural cavity of mice following aspiration into the airspace of the lungs (Murphy et al., 2013). The MWCNTs reached the lungs where they were retained and caused substantial inflammation and lesion development in the pleural space. This finding supports the suspicion off asbestos-like mesothelioma hazard inflicted by CNTs.

When short fibers are inhaled and enter the parietal pleura of the lung, they will be translocated to the pleural space and later pass through the stomata and into the lymph. Long but weak fibers can break up into shorter fibers and will thus follow the same route. However, long and biopersistent fibers will be retained in the parietal pleura and accumulate and thereby induce inflammation that leads to pleural damage and later mesothelioma (Donaldson et al., 2012).
Length also determines if a fiber can be cleared by macrophages which are the key players in removing particles deep inside the lungs. Macrophages have a diameter of 10-20 µm and their phagocytic capabilities will be impaired if they attempt to engulf fibers beyond this size range (Donaldson et al., 2010). Long fibers (>15 µm) will not be completely engulfed and effectively phagocytosed and may lead to a process known as frustrated phagocytosis (Donaldson et al., 2013). Macrophages undergoing frustrated phagocytosis after deposition of long fibers release factors that promote a pro-inflammatory cytokine response in adjacent mesothelial cells (Murphy et al., 2012). This process therefore plays an important role in developing an inflammatory microenvironment following fiber exposure (Brown et al., 2007).

In a study with glass fibers of different length, long fibers (17 µm) were found to cause incomplete phagocytosis and activate production of the pro-inflammatory mediators NF-κB and tumor necrosis factor alpha (TNF-α) in mouse macrophages, while short (7 µm) fibers showed both complete phagocytosis and less expression of inflammatory mediators (Ye et al., 1999; Manke et al., 2014). This is also likely the situation in vivo as an experiment using scanning electron microscopy (SEM) to visualize macrophages after direct pleural installation of fibers in mice showed incomplete uptake with lengths exceeding 10 µm (Schinwald and Donaldson, 2012).

Biopersistent fibers will retain their fiber shape and can thus accumulate within the target tissue such as the lungs. CNTs are made from graphene, a very strong material that in several experiments have shown good biopersistence. In one study unmodified SWCNTs were exposed for 90 days in a phagolysosomal simulant milieu with pH 4.5 without showing any breakage (Liu et al., 2010). An in vivo study performing pulmonary exposure of MWCNTs to rodents found substantial amounts of CNTs in the lungs many weeks after exposure (Pauluhn et al., 2010; Mercer et al., 2013a). On the other hand, the lungs have defense mechanisms against particles, such as the mucociliary escalator in the airways that sweeps particles out of the lungs and alveolar macrophages that phagocytose them (Donaldson and Tran, 2002). Particles will first be problematic when these defenses are broken down which can occur when macrophages are damaged, inflammatory mediators are released or when particles are in high enough numbers to overload the defenses.
1.3 Mechanisms of toxicity by CNTs

1.3.1 Oxidative stress

Generation of ROS and the induction of oxidative stress are considered as the most plausible explanation of the toxic effects of inhaled NPs and fibers like CNTs (Garza et al., 2008). The term ROS applies to reactants such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH), superoxide anion (O$_2^{-}$) and singlet oxygen (‘O2) (Knaapen et al., 2004). Under normal conditions ROS are steadily generated at low levels, but are neutralized by antioxidants such as glutathione (GSH). Oxidative stress is the state that occurs when the antioxidant defense is overwhelmed by excess ROS production (by e.g. NPs) so GSH depletes while reduced glutathione (GSSG) increases (Xia et al., 2006). ROS secreted by phagocytes normally have a protective function by killing bacteria and parasites. However, when produced and accumulated in high numbers during oxidative stress they can have detrimental effects (Aggraval et al., 2006). ROS is known to cause oxidative DNA damage including base pair mutations, attack lipids and proteins (Shi et al., 1994) and activate signaling pathways controlling inflammatory responses. MWCNTs are found to be genotoxic to human lung cells in vitro at occupationally relevant doses (Siegrist et al., 2014).

Oxidative stress will trigger inflammation through activation of protein signaling pathways that could lead to activation of general transcription factors that regulate expression of multiple pro-inflammatory genes. For instance, at high levels of oxidative stress, inflammation is initiated by activation of mitogen-activated protein kinases (MAPKs) and NF-κB signaling cascades (Baldwin, 1996; Hayden and Gosh, 2004). This will induce downstream inflammatory mediators like cytokines in the Interleukin-1 (IL-1) and TNF-family.

MWCNTS showed a dose-dependent toxicity in an in vitro experiment on human lung cells (BEAS-2B) and induced substantial ROS production and mitochondrial damage, indicating oxidative stress (He et al., 2011). NPs can generate ROS by three different mechanisms: Intrinsic generation of oxidants from the particles in absence of cells, from interaction of particles with target cells or during inflammation by particle driven activation of inflammatory cells like macrophages and neutrophils (Unfried et al., 2007). ROS can be
generated through redox cycling (fenton reaction) via NP-coatings such as various metals (Nel et al., 2006). Metals can be washed away with acid treatment but purification of the nanotubes may weaken them and create carboxylic acid (-COOH) residues. This creates areas in the CNT structure that are more reactive than others, like defects with missing carbon atoms (Lin et al., 2003). Defects in material composition can generate electron-donor or acceptor active groups that can react with O$_2$ and create O$_2^-$ (Fenoglio et al., 2008). Thus, several characteristics of CNTs can contribute to ROS generation. An in vitro study using human lung A549 cells showed a significant increase in intracellular ROS production to untreated CNTs, but no ROS generation was reported using acid treated CNTs (Pulskamp et al., 2007), pointing towards metal contaminants as the causative agent of ROS production seen in these cells.

1.3.2 Inflammation

Inflammation is a host response to tissue injury, pathogens, or irritants designed to heal the afflicted tissue. Changes in blood flow, tissue permeability and release of cytokines recruits leukocyte effectors to the target tissue (Coussens and Werb, 2002). Of these effectors are macrophages specially important and closely linked to chronic inflammation due to their secretion of TNF-$\alpha$ and IL-1 that can drive the acute inflammation onward into a chronic state. Acute inflammation is a short lived response where effectors eliminate the hazardous agents and damaged tissue may be repaired. After an acute response macrophages need to switch from producing pro-inflammatory mediators to anti-inflammatory mediators thereby suppressing the inflammatory response. Acute inflammation must be terminated when no longer needed and is usually self-limiting, but dysregulation by the controlling factors can lead to a long lasting chronic inflammation, followed by cellular destruction and pathogenesis (Coussens and Werb, 2002).

Many of the same inflammatory mediators such as cytokines, chemokines and ROS are generated in both acute and chronic inflammation. Chronic exposure to these mediators is a risk factor and linked to many diseases (Schachter and Weitzman, 2002), including cancer, cardiovascular diseases, arthritis, pulmonary and autoimmune diseases (Aggraval et al., 2006). Cytokines comprise small proteins that are active in cell signaling and are released in response to external stimuli and exert their effect by binding to their receptors on cell membranes. Cytokines are mainly produced by immune cells like macrophages, T cells and B
cells, but also many other cell types including endothelial and epithelial cells can produce and secrete these mediators (Takizawa, 1998).

1.4 Activation of inflammatory transcription factors

MWCNT can activate the transcription of IL-1 and TNF-α through generation of ROS and the subsequent activation of pro-inflammatory transcription factors like NF-κB and AP-1 (Figure 1.4).

![Diagram of activation of inflammation induced by MWCNTs via the IL-receptor pathway. MWCNTs can generate ROS which activates NF-κB and AP-1 via MAPK phosphorylation cascades. Activation causes translocation of NF-κB to the nucleus where it functions as a transcription factor on pro-inflammatory genes such as IL-1, TNF-α, Cyclooxygenase 2 (COX-2), IL-6 and others. IL-1 can bind to the IL-1 receptor causing a positive feedback loop and amplification of the initiated inflammation. From (Arnoldussen et al., 2014)]

NF-κB is the transcription factor central to inflammatory and innate immune responses. It is ubiquitously expressed and can be detected in its inactive form in almost all cell types (Gloire et al., 2006). Activation of NF-κB is mediated through phosphorylation of Inhibitor of κB (IκB) by MAPK cascades, followed by the subsequent degradation of IκB at the proteasome (Hayden and Gosh, 2004). Known inducers of NF-κB activity are ROS, TNF-α receptor, IL-1
receptor, as well as the Toll-like receptors that recognize pathogens. CNTs are known to cause both release of ROS and induction of IL-1 and TNF-α and can thus activate NF-κB by several different pathways. Induction of IL-1 or TNF-α receptors or sufficient ROS production causes activation and translocation of NF-κB to the nucleus where it switches on genes that drive the inflammation (Han et al., 2010). CNTs are also seen to activate NF-κB in vitro. MWCNTs activated the NF-κB pathway in RAW264.7 macrophages, following secretion of several cytokines (TNF-α, IL-1β, IL-6), thereby promoting inflammation (He et al., 2011).

The pro-inflammatory cytokine TNF-α controls inflammatory cell populations as well as mediating many other aspects of inflammatory processes (Coussens and Werb, 2002). Binding of TNF-α to the TNF receptor causes activation of NF-kb or AP-1 leading to increased transcription of genes regulating inflammation, cell proliferation and apoptosis. Monocytes exposed to MWCNTs in vitro had a significantly elevated release of TNF-α, as well as raised ROS production (Brown et al., 2007). Chronically produced TNF-α can act as a promoter contributing to the enhancement and progression of the inflammatory process (Balkwill and Mantovani, 2001).

The IL-1 family is intimately linked to the innate immune response and binds to the IL-1 receptor. The two best studied members are IL-1α and IL-1β, both secreted by many cell types but mainly by macrophages. IL-β promotes the immune response indirectly by inducing gene expression of other inflammatory mediators like cyclooxygenase type 2 (COX-2) and inducible nitric oxide synthase (iNOS) (Dinarello, 2009). The latter activates nitric oxide (NO) production and thus elevated levels of reactive nitrogen species (RNS). IL-1β also increases the expression of adhesion molecules such as Intercellular adhesion molecule 1 (ICAM-1) and Vascular cell adhesion molecule 1 (VCAM-1) on endothelial cell walls which leads to tissue remodeling (Dinarello, 2009). In an in vitro study long CNTs resulted in significant IL-β release from THP-1 macrophages (Murphy et al., 2012). Further, conditioned media from the same CNT-activated macrophages resulted in an extensive increase in IL-1β release from mesothelial cells.

CNTs can as shown in Figure 1.4 induce MAPK cascades through generation of ROS which in turn may lead to induction of transcription factors AP-1 and NF-kB and regulation of downstream pro-inflammatory genes. MAPKs can be triggered by tyrosine kinase membrane receptors like the epidermal growth factor receptor (EGFR). EGFR can again be activated by
direct ligand binding or by oxidative stress (Abdelmoisen et al., 2003). The MAPKs Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK thereby control cell fate decisions like proliferation, differentiation and apoptosis (Fey et al., 2012). Dysregulation of such important cell fate decisions which can lead to aberrant proliferation and reduced apoptosis is a hallmark of many diseases including cancer (Hanahan and Weinberg, 2011). Activation of JNK is also found to be essential for the induction of apoptosis following NP exposure (Unfried et al., 2007).

Another important protein in apoptosis is the tumor protein 53 (TP53) that regulates the cell cycle. It can arrest the cell cycle at the G1/S checkpoint until DNA repair proteins have repaired the damage or it can initiate apoptosis if the damage is irreversible (De Zio et al., 2013). In normal healthy cells TP53 exists at low concentrations and in an inactive state. Under cellular stress it is activated and can in such circumstances block the progression of the cell cycle, inducing apoptosis through activation of Bax and caspase-3 (Srivastava et al., 2011). Caspase-3 plays a vital role in caspase cascades that is characteristic for apoptosis (Perry et al., 1997).

1.5 Intercellular communication

Cell-cell communication facilitates the coordination of activities between cells. A mechanism for direct cell-cell communication is gap junction intercellular communication (GJIC). Gap junctions are found in the plasma membranes of most cell types and form channels interconnecting the cytoplasm of adjacent cells (Evans and Martin, 2002). They permit the passage between cells of small (<1 kD) soluble molecules and ions (Veenstra et al., 1995). Gap junctions consist of two connexon hemichannels from adjacent membranes that join to form one channel and each connexon consists of six connexin (Cx) molecules (Evans and Martin, 2002). Twenty one different connexin genes have been identified in humans where Cx43 is the most common and is expressed in most tissues (Evans and Martin, 2002).

In response to cellular injury downregulation of GJIC is a possible mechanism to protect adjacent healthy cells from injured cells and prevent the spread of toxic molecules such as excess of Ca^{2+} ions (Saez et al., 1987). Gap junctions help maintain tissue homeostasis via intercellular exchange of molecules associated with cell growth and apoptosis (Trosko and Chang, 2001). Communications through gap junctions are therefore important in regulation of
normal cell growth and function (Opsahl and Rivedal, 1999). Several carcinogens have been shown to inhibit GJIC (Budunova and Williams, 1994), and were in one study found to be significantly inhibited by cigarette smoke condensate (McKarns et al., 2000). GJIC are inhibited through hyperphosphorylation of Cx43 chemically by the skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and by EGF, both via the action of MAPKs (Rivedal and Opsahl, 2001). Another study also investigated the effects of TPA, but also included the chlorinated insecticide endosulfan (Kenne et al., 1994). Long term exposure to endosulfan resulted in hypophosphorylation of Cx43, indicating that phosphorylation as well as dephosphorylation are important factors in regulation of Cx43. The effect of CNTs on GJIC has not yet been investigated.

**1.6 Project objectives**

The aim of this study was to investigate cellular responses and molecular mechanisms following exposure of two CNTs. CNTs are of toxicological concern because of the structural similarities to asbestos. Due to this similarity, inhalation of CNT fibers are suspected to induce inflammatory and oxidative stress responses promoting chronic inflammation, leading to adverse effects such as fibrosis and cancer.

The CNTs used were two MWCNTs with different characteristics regarding fiber length, diameter, shape, structure and number of isolated fibers. The toxicological effects and molecular mechanisms were investigated in a human cell line established from normal human lung tissue. This cell model is relevant as the main exposure route of exposure to the highest doses of CNTs is through inhalation where lung cells are the main target of toxicity. To compare the effects with asbestos fibers, the UICC crocidolite fibers were included as a reference material.

A number of toxicological endpoints were studied including cell viability using two different assays, gene expression of inflammatory genes, phosphorylation of ERK as the representative MAPK protein and effects of CNTs on intercellular communication.
2 Materials and Methods

2.1 Cell lines

The human bronchial epithelial cells 3 KT (HBEC-3KT) were kindly provided by Dr. J.D Minna at Hamon Center for Therapeutic Oncology Research, Texas, USA. The cells were isolated from non-cancerous lung tissue from a 65- year old female and are immortalized with cyclin-dependent kinase 4 (Cdk4) and human telomerase reverse transcriptase (hTERT) as described in (Ramirez et al., 2004).

For the scrape loading experiments the Hela cells stably transfected with a vector containing Cx43 and therefore overexpressing Cx43 were used (kindly provided by Dr Edward Leithe, at Department of Cancer Prevention, Institute for Cancer Research, the Norwegian Radium Hospital, Oslo).

2.2 Nanomaterials and asbestos

The carbon nanotube 1 (CNT-1) has been produced by the CVD method whereas the carbon nanotube 2 (CNT-2) is produced by the Arc discharge method. Both are MWCNTs containing multiple layers of nanotubes. UICC crocidolite asbestos was prepared in Johannesburg under the joint supervision of the MRC, Pneumoconiosis unit, Penarth, Wales and the National Research Institute for Occupational Diseases in South Africa and was a gift from the MRC Pneumoconiosis Unit in Penarth.

2.3 General cell culture work

HBEC-3KT cells were grown in LHC-9 medium (Gibco), supplemented with 5% penicillin and streptomycin (PS). HBEC-3KT cells were grown on collagen-coated dishes. Hela cells were grown in DMEM medium (Thermo Scientific), supplemented with 10 % Fetal bovine serum (FBS) (PAA cell culture company) and 1 % PS. Cell culture work was performed under sterile conditions in a OAS LAF bench. Cell cultures were incubated at 37°C at 95%
humidity and 5% CO\textsubscript{2} (Thermo Forma incubator). Cells were passaged when reaching about 70% confluency and medium was changed every second day.

### 2.3.1 Seeding of cells

For experiments cells were seeded in petri dishes (NUNC) of various sized depending on the type of experiment. Growth medium and volumes may differ according to each experiment. Protocol for seeding of cells starting with 70% confluent cells in 100 mm petri dishes.

1. Petri dishes with cells were washed twice with 8 ml 1x Phosphate buffered saline (PBS) (Lonza).
2. 1 ml of trypsin (Biochrom) was added to each dish.
3. The petri dish was placed in 37°C for 10 minutes until the cells detached from the dish.
4. 4 ml of medium was added and the cell suspension was collected.
5. Cells were transferred to a falcon tube and centrifuged (Eppendorf centrifuge 5707) at 1000 rpm for 4 minutes.
6. The supernatant was discarded and the pellet resuspended in 4 ml of medium.
7. 10μl of cell suspension was mixed with 10 μl of tryphan blue stain (Invitrogen). 10 μl of this mixture was transferred to a counting slide and cells were counted using Countess Automated Cell Counter (Invitrogen).
8. The right amount of medium was added to the cell suspension to reach the desired density of cells.
9. Cells were seeded out on appropoiate dishes.

### 2.3.2 Preparation and sonication of particles for experiments

In all experiments cells were exposed to CNT-1 and CNT2 in addition to crocidolite asbestos. Before exposure the particles were weighed, suspended in dispersion medium (DM) and sonicated. DM contains PBS supplemented with 0.6 mg/ml Bovine Serum Albumin (BSA), 0.01 mg/ml 1m2-dipalmitoyl-sn-glycero-3-phosphocholine (DPCC) and 5.5 mM d-Glucose as described in (Porter et al., 2008). This DM provides a lower degree of agglomeration for the
test materials and is meant to mimic the environment of the lungs. Sonication uses ultrasound to evenly disperse the particles in the DM solution.

Between 1 and 1.5 mg of the CNTs and asbestos were weighed up in eppendorf vials and the correct amount of DM was added to reach a concentration of 1 mg/ml. One drop of Tween 20 (Sigma) detergent was added to avoid aggregation and increase solubility. Vials were vortexed for 15 seconds before they were placed on ice and sonicated (Branson SLPe) at 30% amplitude for 3 x 5 minutes. The 1mg/ml stock solution of particles was newly prepared before each experiment and diluted into cell growth media at the indicated concentrations.

**2.3.3 Exposure of particles for each experiment**

Cells were seeded out as explained in section 2.3.1 and exposed to the particles after 24 hours. The 1mg/ml stock solution of CNTs and asbestos (2.3.2) were diluted in cell culture media to obtain the concentrations used in each experiment (1-50µg/ml). Cells were then incubated at 37°C for the specific time points used in the different experiments.

**2.4 Cytotoxicity Assays**

**2.4.1 Experimental setup for Cytotoxicity assays**

<table>
<thead>
<tr>
<th>Type of exposure</th>
<th>Exposure concentration (µg/ml)</th>
<th>Time points (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-1:</td>
<td>5, 10, 20, 50</td>
<td>6 and 24</td>
</tr>
<tr>
<td>CNT-2:</td>
<td>5, 10, 20, 50</td>
<td>6 and 24</td>
</tr>
<tr>
<td>Asbestos:</td>
<td>5, 10, 20, 50</td>
<td>6 and 24</td>
</tr>
<tr>
<td>Control (DM):</td>
<td>50</td>
<td>6 and 24</td>
</tr>
</tbody>
</table>
2.4.2 CellTiter-Blue and Cell Counting kit-8 assay

Two cytotoxicity assays were used in this study, CellTiter-Blue (CTB) (Promega) and Cell Counting kit-8 (CCK-8) (Sigma). CTB is based on the ability of living cells to reduce the redox dye resazurin into the end product resorufin, which is highly fluorescent at 590 nm. The fluorescence produced is proportional to the number of viable cells, as nonviable cells lose their metabolic capacity. CCK-8 uses the dye WST-8 which is reduced by cellular dehydrogenases to an orange formazan product which is proportional to the number of living cells. Bioreduction requires the presence of an electron carrier and thus is a measure for cell viability and mitochondrial integrity. The protocol for CTB and CCK-8 is the same except for the reagent added (CTB or CCK-8). In addition, CTB measures fluorescence while CCK-8 measures absorbance.

For both assays 5000 cells per well were seeded in a black 96 well plate (Thermo Scientific). The cells were incubated for 24 hours before 5 different concentrations of CNT-1, CNT-2 and asbestos were added in triplicate to each plate. The different concentrations were 5, 10, 20 and 50 µg/ml. Each 96 well plate also included wells with cells seeded out in different densities (500, 1000, 2000, 3000, 4000, 5000 and 10 000 cells/well) to generate a standard curve. These wells were only exposed to DM alone and were used as a control, set to 100% cell viability.

After exposure for 6 or 24 hours the media from all wells was removed and washed with 100 µl of PBS. Then 100 µl of media including 10 µl of CTB or CCK-8 was added to each well and the plates were incubated at 37°C for 4 hours. For the plates with CTB, fluorescence was measured using a Modulus plate reader (Turner Biosystems). For the plates with CCK-8, absorbance was measured at 450 nm and 750 nm, with the Modulus plate reader. For CCK-8 the measurements at 750nm wavelength were used for background detection and were subtracted from the sample wavelength of 450nm. Three experiments where performed for both assays, with three replicates of each exposure and time points in each experiment. Both assays were performed simultaneously.
2.4.3 Calculations of LC$_{50}$ values

![LC$_{50}$ Croc CellTiter-Blue 6 hours](image)

**Figure 2.1:** LC$_{50}$ calculations for asbestos after 6 hours measured with CTB. Y-axis is % of dead cells, X-axis is concentration of asbestos in µg/ml.

LC$_{50}$ values were calculated based on the data obtained from the cytotoxicity assays in section 2.4.2. Percentage of live cells for the different doses were recalculated to percentage of dead cells and placed in a scatter plot (Figure 2.1). A logarithmic curve was fitted to the data points and the resulting equation was solved for $y=50$, giving the concentration on the x-axis corresponding to 50% dead cells.

2.4.4 Calculations of Cytotoxicity according to total number of fibers

The results from the cytotoxicity assays were recalculated from expressing exposure concentration as mass (µg/ml) to total number of fibers (fibers/ml). The number of viable cells from the cytotoxicity assays in section 2.4.2 were plotted against the number of fibers present per mass of each fiber type. For each fiber type the total number was obtained from the characterization of the fibers (Table 3.1) whereas the number of UICC asbestos fibers (2.93 x $10^6$ fiber/ug) were obtained from (Goodglick and Kane, 1990; Moalli *et al.*, 1987).
2.5 Gene expression

qPCR was performed for analysis of gene expression. Before qPCR was run (2.4.6), RNA was isolated from the exposed cells (2.4.2), cDNA synthesis was performed (2.4.4) and primers were designed (2.4.5).

2.5.1. Experimental setup for gene expression experiments

Table 2.2: Exposure types, concentrations and time points used in all gene expression experiments.

<table>
<thead>
<tr>
<th>Type of exposure</th>
<th>Exposure concentration (µg/ml)</th>
<th>Time points(h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-1:</td>
<td>5 and 10</td>
<td>3 and 6</td>
</tr>
<tr>
<td>CNT-2:</td>
<td>5 and 10</td>
<td>3 and 6</td>
</tr>
<tr>
<td>Asbestos:</td>
<td>10</td>
<td>3 and 6</td>
</tr>
<tr>
<td>Control (DM):</td>
<td>10</td>
<td>3 and 6</td>
</tr>
</tbody>
</table>

2.5.2 RNA isolation

Cells were seeded in 6-well plates with a density of 3*10^5 cells per well. After 24 hours of incubation, CNT-1 (5 and 10 µg/ml), CNT-2 (5 and 10 µg/ml) and asbestos (10 µg/ml) were added to the cells. DM alone was added to the cells that were used as a negative control. After 3 and 6 hours of exposure the wells were washed three times with PBS and stored at -80°C.

The following procedure was used for RNA extraction.

1. 1ml Isol (5`Prime) was added to each well. The cell lysate was pipetted up and down several times, transferred to an eppendorf vial and incubated at room temperature for 5 minutes.
2. 0,2 ml of chloroform (AnalaR NORMAPUR) was added to each vial and the vials were shaken for 15 seconds before incubation for 2-3 minutes at room temperature.
3. Vials were centrifuged (Eppendorf centrifuge 5417R) at 12 000g for 15 minutes at 4°C
4. The upper (water soluble) phase was transferred into new vials. 0.5 ml of iso-propanol (Merck) was added to each vial and mixed together before incubation in 10 minutes.

5. Samples were centrifuged at 12,000 g for 15 minutes at 4°C.

6. The supernatant was removed and the cell pellet was washed with 75% ethanol (in DEPC-H2O). Samples were mixed and vortexed quickly.

7. Samples were centrifuged at 12,000 g for 5 minutes at 4°C.

8. The supernatant was removed and the pellet let to dry for 30 minutes. The pellet was then dissolved in 15 µl of DEPC-H2O and the vials were put on ice.

9. Samples were incubated at 65°C for 2 minutes before mixing the content. Samples were then stored at -80°C.

2.5.3 Measurements of RNA concentration and quality

RNA concentration of each sample was used to obtain a dilution of 1 µg/µl RNA used in cDNA synthesis. RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific). Nucleic acids have an absorption maximum at 260 nm and the RNA concentrations were determined by measuring optical density (OD) at 260 nm.

NanoDrop can also give a measure of RNA purity. The ratio OD_{260}/OD_{280} is a measure of contamination by proteins or phenols which have an absorbance maximum at 280 nm. Pure RNA has an OD_{260}/OD_{280} ratio of 2.0. The OD_{260}/OD_{230} ratio is a measure of organic contamination as carbohydrates have an absorbance maximum at 230 nm. This ratio should also be close to 2.0.

2.5.4 cDNA synthesis

Before gene expression was analyzed using qPCR the RNA was converted into complementary DNA (cDNA). cDNA is double-stranded DNA derived from RNA and is much more stable than RNA which is easily degradable by RNases. cDNA synthesis was performed on the isolated RNA using the qScript cDNA synthesis kit (Quanta BioSciences). The kit consists of qScript reaction mix which includes buffer, magnesium, oligo dT primers, random primers, and dNTPs for synthesis of the first-strand. The kit also included reverse transcriptase (RT) for the second strand synthesis.
First all RNA samples were diluted with dH₂O to a final concentration of 0.5 µg RNA/ml. Then 4 µl Reaction Mix, 1 µl RT and 5 µl of N-H₂O (Nuclease-free H₂O) were added to each well of a 96-well PCR reaction plate (Applied biosystems). RNA was added to each well except for the negative controls, where similar amounts of N-H₂O were added instead. RT-negative controls were also made that included Reaction mix and RNA, but no RT. The plate was gently vortexed and centrifuged for 10 sec at 1000 g. Plates were placed in a thermal cycler (Eppendorf Mastercycler nexus) and run by the following program: 22°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and put on hold at 4°C. The cDNA was then stored at -20°C.

### 2.5.5 Primer design

Primers listed in Table 2.3 were designed using PrimerBLAST (NCBI), and specificity and unwanted self complementarity was checked using the beacon designer (Premier Biosoft). PCR primers were designed to span introns to avoid amplification of genomic DNA. Primers should optimally have a length of 18-22 bp, GC content between 40-60% and a melting temperature TM below 60°C. Primers were made for all the tested genes in addition to β-actin that was used as a reference gene. All primers were ordered by Thermo Fisher Scientific and tested before gene expression analysis was performed.

**Table 2.3: Primer sequences used in qPCR in this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Bp</th>
<th>% GC</th>
<th>TM°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb forward</td>
<td>GCGAGAAGATGACCCAGATCA</td>
<td>21</td>
<td>52,4</td>
<td>54,0</td>
</tr>
<tr>
<td>Actb reverse</td>
<td>GATAGCAGACGCTGGATAGCAA</td>
<td>22</td>
<td>50,0</td>
<td>53,7</td>
</tr>
<tr>
<td>IL-1α forward</td>
<td>TCACGGCTGCTGCATTACAT</td>
<td>20</td>
<td>50,0</td>
<td>54,1</td>
</tr>
<tr>
<td>IL-1α reverse</td>
<td>GGGTATCTCAGGGCATCTCCTTC</td>
<td>22</td>
<td>54,5</td>
<td>53,4</td>
</tr>
<tr>
<td>IL-1β forward</td>
<td>TGAGCTCGCCAGTGAATGA</td>
<td>20</td>
<td>50,0</td>
<td>54,1</td>
</tr>
<tr>
<td>IL-1β reverse</td>
<td>GGTGGTCCGGAGATTTCGTAGC</td>
<td>20</td>
<td>50,0</td>
<td>54,1</td>
</tr>
<tr>
<td>TP53 forward</td>
<td>CTATGAGCGCCGCTGGGTGG</td>
<td>20</td>
<td>60,0</td>
<td>53,5</td>
</tr>
<tr>
<td>TP53 reverse</td>
<td>GCACAAACACGCACCTCATAA</td>
<td>20</td>
<td>50,0</td>
<td>54,4</td>
</tr>
<tr>
<td>TNF-α forward</td>
<td>ACCACGCTCTTCTGCCTGCTT</td>
<td>20</td>
<td>50,0</td>
<td>56,4</td>
</tr>
<tr>
<td>TNF-α reverse</td>
<td>TGAGGGTTTGCATCAACATGGGCT</td>
<td>24</td>
<td>50,0</td>
<td>60,5</td>
</tr>
</tbody>
</table>
2.5.6 qPCR

Real-time polymerase chain reaction (qPCR) is a quantitative method for measuring relative gene expression of specific genes. qPCR will amplify the DNA sequences flanked by the forward and reverse primers. Differences in gene expression will be reported through the detection and quantification of the fluorescent reporter SYBR green. This reporter binds to double stranded cDNA and emits fluorescence at 520 nm upon excitation so the level of fluorescence will increase proportionally to the amount of PCR product.

One qPCR cycle consists of three steps: denaturation, annealing and extension. Under denaturation the temperature is raised to 95°C and DNA strands are separated. Annealing occurs when the temperature is lowered and the two primers bind the complementary sequences in the template. Finally during the extension step DNA polymerase binds to the 3'end of the primers and extends the DNA sequence by incorporating 2'-deoxy 5'-triphosphatases (dNTPs) so the complementary cDNA strand is produced. These three steps are run for 40 cycles.

Primer stock solutions were first diluted with n-H$_2$O to yield a concentration of 10 pmol/µl. cDNA samples were diluted in various concentrations depending on each gene expression level. Mastermix was made for each gene and includes Perfecta SYBR Green Fast mix (Quantas), forward and reverse primers and n-H$_2$O in concentrations shown for one reaction in Table 2.4.

Table 2.4: Mastermix solution for 1 reaction.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfecta SYBR Green FastMix</td>
<td>10</td>
</tr>
<tr>
<td>Forward primer (10 pmol/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>Reverse primer (10 pmol/µl)</td>
<td>0.4</td>
</tr>
<tr>
<td>n-H$_2$O</td>
<td>7.2</td>
</tr>
</tbody>
</table>
18 µl of the reaction mastermix specific for each gene and 2 µl of cDNA samples were added in parallels to a 96-well plate. For each gene negative controls (n-H_2O), RT-negative controls and a standard curve were included. The standard curve was made from a 2x dilution series of cDNA comprised of three samples. The 96-well plates were covered by a plastic film and centrifuged at 2200 rpm for 20 seconds.

The StepOnePlus qPCR machine (Applied Biosystems) was used and run on the following program: 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds followed by a melt curve stage. Results were then analyzed with the StepOne Software (Applied Biosystems). The software generates an amplification plot (Figure 2.2) that shows the fluorescent signal from each sample plotted against cycle number, and represents the accumulation of product (fluorescence) during the qPCR experiment.

![Amplification Plot](image)

**Figure 2.2:** A qPCR amplification plot from this study with indicated baseline, threshold and Ct-values.

The baseline of a qPCR reaction refers to the signal in the first initial cycles of PCR and is set as a background signal. The threshold is a statistical significant increase over the baseline signal. The cycle threshold (Ct) value is the cycle number where the fluorescent signal of each sample crosses the threshold. Ct values are inversely proportional to the amount of nucleic acid in the target i.e. the lower the Ct value, the higher amount of nucleic acid in the sample.

The genes of interest were normalized to the reference gene β-actin to account for variations in cDNA from different samples. β-actin is a housekeeping gene expressed at similar levels in cells and is expected not to be affected by exposure. The relative expression for each gene
was calculated using the Livak method. This method, also called DeltaDelta CT uses the following equation:

\[
\Delta\Delta CT = (CT(target,unexposed) - CT(ref,unexposed)) - (CT(target,exposed) - CT(ref,exposed))
\]

Three experiments were performed for all genes, with two replicates of each exposure and time point in each experiment.

### 2.6 Protein expression

#### 2.6.1 Experimental setup for protein expression experiments

Table 2.5: Exposure types, concentrations and time points used in all protein expression experiments.

<table>
<thead>
<tr>
<th>Type of exposure</th>
<th>Exposure concentration (µg/ml)</th>
<th>Time points (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-1:</td>
<td>5 and 10</td>
<td>24 and 48</td>
</tr>
<tr>
<td>CNT-2:</td>
<td>5 and 10</td>
<td>24 and 48</td>
</tr>
<tr>
<td>Asbestos:</td>
<td>10</td>
<td>24 and 48</td>
</tr>
<tr>
<td>Control (DM):</td>
<td>10</td>
<td>24 and 48</td>
</tr>
</tbody>
</table>

#### 2.6.2 Particle exposure and protein extraction

Cells were seeded out in 100 mm petri dishes at a concentration of 3*10^5 cells per dish. After 24 hours the cells were exposed to CNT-1, CNT-2, asbestos or DM at concentrations and incubation times listed in Table 2.5.

After 24 and 48 hours, respectively, the cells were harvested into eppendorf vials using a cell scraper. Vials containing cells suspended in 1.2 ml PBS were centrifuged (Sigma centrifuge 4k15) at 6000 rpm at 4°C for 10 minutes. Supernatant was removed and 100 µl lysis buffer (Table 2.6) was added to each cell pellet. Vials were incubated with lysis buffer for 2 hours before centrifugation at 13000 rpm at 4°C for 10 minutes. The supernatant was transferred to a new vial and protein concentration was measured using the Bradford assay (AppliChem) on the Modulus plate reader.
Table 2.6: Constituents of the lysis buffer used for protein extraction

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HePes pH 7.7 (Sigma)</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl (Merck)</td>
<td>300 mM</td>
</tr>
<tr>
<td>EDTA (Merck)</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Triton x100 (Sigma)</td>
<td>0.1%</td>
</tr>
<tr>
<td>MgCl$_2$ (Merck)</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>DTT (Sigma)</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>β-Glycerolphosphate (Sigma)</td>
<td>20 mM</td>
</tr>
<tr>
<td>Sodiumortovandate (Sigma)</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

2.6.3 Western blotting

SDS PAGE gels were prepared according to table 2.7.

Table 2.7: Separating and stacking gel constituents.

<table>
<thead>
<tr>
<th></th>
<th>10 ml Separating gel (12%)</th>
<th>10 ml Stacking gel (6%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x buffer</td>
<td>2.5 ml sep.buffer</td>
<td>2.5 ml stack. buffer</td>
</tr>
<tr>
<td>Acrylamide 40%</td>
<td>3 ml</td>
<td>1.125 ml</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>4.5 ml</td>
<td>6.375 ml</td>
</tr>
<tr>
<td>APS 10%</td>
<td>50 µl</td>
<td>60 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Equal amounts of protein samples containing dH$_2$O, 4x loadingbuffer and 10x DTT were denatured at 100°C and cooled down on ice, before loaded on to the gel. Page Ruler Prestained Protein ladder (Thermo Scientific) was added to the first well of the gel to easily identify specific bands.

Proteins on the gel were then transferred to a PVDF membrane (Bio-Rad) using Trans-Blot Turbo (Bio-Rad) for 30 minutes. After transfer the membrane was blocked in 5 % dry milk dissolved in Tris buffered saline (TBS) for 1 hour. After blocking, the membrane was incubated overnight at 4°C with primary antibody in 5 % BSA (AppliChem). Antibodies used in this study were against β-Tubulin, phJNK, totalJNK, phERK, totalERK, Cx43 (all from...
Cell Signaling Technology), Cx26 (Millipore) and Cx32 (Millipore). The following day membranes were incubated for 1 hour in horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody (Cell Signaling Technology) in 5% dry milk dissolved in TBST.

SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detection of bands. The bands were visualized using the LAS-4000 Luminescent Image Analyzer (FujiFilm) and were quantified using ImageJ software (NIH Image).

2.7 Scrape loading assay

This assay makes it possible to measure possible communication between cells. The fluorescent dye Lucifer Yellow is applied to the cells and a scalpel is used to make a cut in the cell monolayer. The dye will enter cut cells and diffuse into neighboring cells via gap junctions. Therefore the amount of cells that have taken up the dye represent the amount of communicating cells.

2.7.1 Experimental setup for scrape loading experiments

Table 2.8: Exposure types, concentrations and time points used in all cell-cell communication experiments.

<table>
<thead>
<tr>
<th>Type of exposure</th>
<th>Exposure concentration (µg/ml)</th>
<th>Time points (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-1:</td>
<td>1 and 2</td>
<td>6 and 24</td>
</tr>
<tr>
<td>CNT-2:</td>
<td>1 and 2</td>
<td>6 and 24</td>
</tr>
<tr>
<td>Asbestos:</td>
<td>2</td>
<td>6 and 24</td>
</tr>
<tr>
<td>Control (DM):</td>
<td>2</td>
<td>6 and 24</td>
</tr>
</tbody>
</table>
2.7.2 Preparation of a solution of Lucifer Yellow for use in experiments:

10 mg Lucifer Yellow Di-lithium salts (Sigma) were dissolved in 100 μl 0.33 M Lithium Chloride (LiCl) (Sigma). The solution was then diluted in 20 ml PBS (without Mg\textsuperscript{2+}/Ca\textsuperscript{2+}) and aliquoted in glass vials containing 1 ml each of Lucifer Yellow stock solution. One vial of stock solution Lucifer Yellow was dissolved in 20 ml PBS (without Mg\textsuperscript{2+}/Ca\textsuperscript{2+}).

2.7.3 Scrape loading

Scrape loading was performed with both HBEC-3KT and Hela cells. The same protocol was used for both cell lines. 12-well plates were coated after a cover slip was added to each well. Cells were then seeded out in a concentration of 1.5*10\textsuperscript{5} cells/well on top of each cover slip. The cells were grown for 24 hours until they were fully confluent. After 24 hours the cells were exposed to CNT-1 (1 and 2 μg/ml), CNT-2 (1 and 2 μg/ml) or crocidolite asbestos (2μg/ml) for 6 and 24 hours. After the indicated exposure times wells were washed twice with 5 ml PBS (including Ca\textsuperscript{2+}/Mg\textsuperscript{2+}). 2 ml of Lucifer Yellow solution was added to each well before the cell monolayer on each cover slip was cut three times with a sterilized scalpel. Then 3 minutes of incubation followed before all wells were rinsed four times with 2 ml of PBS (including Ca\textsuperscript{2+}/Mg\textsuperscript{2+}). Cells were then fixed in 3.7% of formaldehyde in PBS over night.

The following day wells were rinsed once with 2 ml of PBS (including Ca\textsuperscript{2+}/Mg\textsuperscript{2+}). Mowiol was used for mounting the coverslips which were then kept at 4°C under dark conditions before investigated using confocal microscopy.

2.7.4 Visualization and quantification of scrape loading samples

LSM 710 Confocal microscope (Zeiss) and the Zen software (Zeiss) were used to visualize and take images of the cells. All images were taken with the x10 lens under the same settings with respect to laser intensity and gain and offset settings. Pictures were taken in 1024x1024 resolution and a scanning speed of 15 sec.
Signals were quantified using ImageJ Software (NIH Image). The steps are pictured in Figure 2.3. The image to be quantified (Figure 2.3 A) is first analyzed using the phase contrast setting (Figure 2.3 B). This is done to easily visualize cells and verify that cells are evenly grown without confounding factors. By using the ImageJ software the threshold is set to distinguish between signal and background noise (Figure 2.3 C). The threshold was set according to the control (DM) sample and the same threshold was used on all other samples for direct comparison. The signal was converted into black and white (Figure 2.3 D) and the percentage of signal (black) to background (white) was quantified and is an indication of the amount of communicating cells.

Figure 2.3: Pictures taken with a LSM 710 Confocal Microscope and analyzed using ImageJ. A: Fluorescence picture of cells emitting Lucifer Yellow. B: Phase contrast picture. C: Threshold adjusted to match fluorescent signal. D: Picture converted to black and white.
2.8 Statistics

2.8.1 Standard deviations and standard errors

Standard deviation is an index of how closely individual data points cluster around the mean, i.e. variability of individual data points while Standard Error (SE) reflects the variability of the mean values and is therefore also called Standard Error of the mean. In this study we used SD of the results where only one experiment is shown and SE when using mean values of several experiments.

2.8.2 Statistical methods used

Statistical analysis was performed using SPSS version 20.0.0.1 (IBM). Student T-test (t-test) is used when comparing the same parameter in two different groups. In this study all statistics involved comparing an exposed sample to a control sample. The t-test requires normally distributed data. All results were therefore first checked for normal distribution with the Shapiro-Wilk normality test. The t-test was performed on the results that passed this test. Results that were not normally distributed were analyzed by the non parametric Mann-Whitney rank sum test.

From the statistics we obtained a P-value that is used to determine significantly different samples. The P-value is a measure of the probability to obtain the observed result if the null hypothesis was true. The null hypothesis states that there is no relationship between the samples investigated and is rejected when the P-value is less than the predetermined significance level. The significance level is in our study set to 0.05.
3 Results

3.1 Physico-chemical properties of the CNTs

Physico-chemical properties of CNT-1 and CNT-2 have previously been determined (Arnoldussen, et al., 2014). The CNTs were characterized and counted using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Energy-dispersive X-ray spectroscopy (EDX). As shown in Table 3.1 CNT-1 and CNT-2 differ in several important parameters such as length, diameter, aspect ratio and morphology. Chemical analysis using TEM and EDX showed that the two CNTs consisted of multiple layers of carbon tubes with no traces of metals (Arnoldussen et al., 2014).

Table 3.1 Length, diameter and aspect ratio for CNT-1 and CNT2

<table>
<thead>
<tr>
<th></th>
<th>CNT-1 (n=156)</th>
<th>CNT-2 (n=150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>129</td>
<td>15</td>
</tr>
<tr>
<td>Length (nm)</td>
<td>30990</td>
<td>214</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>620</td>
<td>62</td>
</tr>
</tbody>
</table>

*Number of fibers counted for each CNT
Figure 3.1: SEM image of CNT-1. Scale: 100µm. (C) SEM image of CNT-2. Scale: 300µm. Pictures from (Arnoldussen et al., 2014).

CNT-1 fibers showed varied morphology displaying straight, bent and curved shapes (Figure 3.1 A). CNT-1 was also often found aggregated in fiber bundles. The fibers in CNT-2 samples were morphologically different from CNT-1 being shorter, primarily single and straight (Figure 3.1 B). The number of fiber structures per mass unit was also different: CNT-1 contained $1.5 \times 10^6$ single fibers per µg, while the numbers for CNT-2 were $13 \times 10^6$ single fibers per µg.

### 3.2 Cytotoxicity of CNTs in human lung cells

#### 3.2.1 Cytotoxicity related to the mass of fibers

Cytotoxic effects of the CNTs on HBEC-3KT cells were investigated using the two commercial cytotoxicity assays CTB (Figure 3.2 A and B) and CCK-8 (Figure 3.2 C and D) after exposure for 6 or 24 hours. Both assays were conducted simultaneously using cells subcultured at the same time and same particle suspensions. Two references were also included: the UICC crocidolite asbestos (Croc) fibers as the positive reference for fibertoxicity and the dispersion medium (DM) as the zero toxicity reference (100% viability).
Cell viability decreased significantly in a time and concentration dependent manner for both CNTs. Both CNTs are also consistently more toxic than asbestos at all measured concentrations and time points. There is a slight variation between the two CNTs, where CNT-1 seems to be more toxic than CNT-2 at most time points. The two different assays show the same trends but CCK-8 showed more viable cells after exposure to all fibers in the two highest concentrations (20 µg/ml and 50 µg/ml).
3.2.2 Comparison of the Lethal Concentration 50 (LC$_{50}$) for various fibers

LC$_{50}$ is the concentration of a given agent that reduces cell viability to 50 %. LC$_{50}$ was calculated for asbestos, CNT-1 and CNT-2 (Table 3.2) using values obtained from the cytotoxicity experiments (Figure 3.2), to be able to compare the two cytotoxicity assays.

Table 3.2. LC$_{50}$ values calculated for crocidolite asbestos, CNT1 and CNT2 after 6 and 2 4hours measured with CTB and CCK-8 cytotoxicity assays.

<table>
<thead>
<tr>
<th></th>
<th>CTB 6 hours</th>
<th>CCK-8 6 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestos</td>
<td>17,5 µg/ml</td>
<td>14,5 µg/ml</td>
</tr>
<tr>
<td>CNT-1</td>
<td>4,8 µg/ml</td>
<td>3,7 µg/ml</td>
</tr>
<tr>
<td>CNT-2</td>
<td>6,1 µg/ml</td>
<td>0,85 µg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CTB 24 hours</th>
<th>CCK-8 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestos</td>
<td>6,84 µg/ml</td>
<td>3,48 µg/ml</td>
</tr>
<tr>
<td>CNT-1</td>
<td>0,24 µg/ml</td>
<td>0,21 µg/ml</td>
</tr>
<tr>
<td>CNT-2</td>
<td>0,25 µg/ml</td>
<td>0,05 µg/ml</td>
</tr>
</tbody>
</table>

In both assays CNT-1 and CNT-2 required a lower concentration than asbestos to reduce cellular viability to 50% of the cells, i.e. more toxic. Comparing the two assays CCK-8 is more sensitive and has higher levels of toxicity for all fibers than CTB. The highest variation in toxicity between the two assays is for CNT-2.

3.2.3 Cytotoxicity related to concentration (number) of fibers

To relate the number of fibers on cellular cytotoxicity, the cell viability fractions from the cytotoxicity assays in Figure 3.1 were plotted against the number of fibers present per mass of each fiber type. For each fiber type the total number was obtained from the characterization of the fibers (Table 3.3) whereas the number of UICC asbestos fibers (2.93 x 10$^6$ fiber/ug) were obtained from (Goodglick et al 1990; Moalli et al 1987). The cell viability related to fiber concentration is shown in Figure 3.3.
Figure 3.3: % Cell viability depicted as fibers/ml. % cell viability recalculated from µg/ml to fibers/ml using results obtained in figure 3.2. All treatments are shown as % viability of DM treated cells. CTB 6 hours (A), CTB 24 hours (B), CCK-8 6 hours (C) and CCK-8 24 hours (D) Statistical analysis performed with the Mann-Whitney rank sum test. Error bars = SE. *=p< 0.05 significantly different from DM.

CNT-2 showed considerably less toxicity than asbestos and CNT-1 when cytotoxicity is related to number of fibers. Both asbestos and CNT-1 showed significant cytotoxicity at all measured concentrations and time points. Comparing asbestos and CNT-1 they are more similar by this dose metric.

3.3 Effect of CNTs on inflammatory and apoptotic genes

There are indications that CNTs can have similar pathological effects as asbestos that is known to induce chronic inflammation. To investigate the inflammatory potential of CNTs the mRNA levels of three known pro-inflammatory mediators (*IL1-a, IL-1B, TNF-a*) in addition to the pro-apoptotic gene *TP53* were measured using qPCR. Protein expression and
phosphorylation of the MAPK ERK, involved in induction of pro-inflammatory transcription factors was also determined.

### 3.3.1 Gene expression of inflammatory and apoptotic genes.

In all experiments HBEC-3KT cells were exposed to CNT-1 and CNT-2 at the indicated concentrations (5µg/ml and 10µg/ml) and time points (3 and 6 hours). Dispersion medium alone (DM) was used as a negative control and crocidolite asbestos (10µg/ml) was used as the positive control. Changes in mRNA expression level for each gene are indicated as fold change compared to unexposed controls (DM).

![Figure 3.4: mRNA expression of IL-1α (A), IL-1β (B), TNF-α (C) and TP53 (D) in HBEC-3KT cells exposed to crocidolite asbestos (10 µg/ml), CNT-1 (5 and 10 µg/ml), CNT-2 (5 and 10 µg/ml) or left untreated (DM) after 3 and 6 hours. Fold change = $2^{\Delta\Delta CT}$. The results from three independent experiments in duplicate are shown. Statistical analysis was performed with the t-test. Error bars = SE. *=$p<0.05$ significantly different from DM.](image-url)
A slight increase in mRNA expression of IL-1α after exposure of 10 µg/ml CNT-1 for 3 hours (fold change=2.3±1) was observed (Figure 3.4 A). For CNT-2 a significant decrease in mRNA expression was observed after 6 hours exposure at both concentrations.

IL-1β (Figure 3.4 B) follows the same trends as IL-1α. In CNT-1 a significant decrease was seen after exposure of 5 µg/ml at both 3 and 6 hours. 10 µg/ml of CNT-1 gave an increase in mRNA expression after 3 hours, although nonsignificant. Exposure of CNT-2 gave reduction in IL-1β mRNA levels at both concentrations and time points, significantly so after 6 hours exposure.

The mRNA levels of TNF-α were significantly upregulated after exposure to both CNT-1 and CNT-2 for both concentrations (Figure 3.4 C), however, CNT-2 exposure gives a smaller upregulation compared to CNT-1.

In addition mRNA expression of the pro-apoptotic gene TP53 was investigated (Figure 3.4 D), and showed a slight upregulation at all exposures and a significant upregulation after 5 µg/ml of CNT-2 for 3 hours.
3.3.2 Phosphorylation of the ERK protein

Cellular extracts were isolated from the exposed and unexposed cells and western blotting was used to detect phosphorylation levels of ERK. To evaluate phosphorylation levels, membranes were incubated with phospho-ERK antibody and compared to levels of total-ERK. Figure 3.5 A and 3.5 B show the results for CNT-1 and asbestos. Figure 3.5 C and 3.5 D show the results for CNT-2.

![Western Blot Results](image)

Figure 3.5: Western blotting results showing phosphorylated and total protein levels of ERK in HBEC-3KT cells after exposure to CNT-1 and crocidolite asbestos (A) and (B) and after exposure to CNT-2 (C) and (D). (A) and (C) show representative western blots and (B) and (C) show quantification of normalized phospho ERK levels to total ERK. All treatments are set relative to DM 24h. Data are shown in arbitrary units. The result of one experiment is shown.

Exposure to CNT-1 resulted in a slight decrease in phosphorylation of ERK after 24 hours. Both concentrations of CNT-1 give at this point a lower degree of phosphorylation than asbestos. At 48 hours CNT-1 gives a rise in phosphorylation compared to DM and asbestos. After 24 hours CNT-2 gives a higher degree of phosphorylation with the highest dose (10 µg/ml). While after 48 hours CNT-2 only leads to a higher degree of phosphorylation at the lowest dose (5 µg/ml).
3.4 Effects on cell-cell communication

It was hypothesized that CNTs affect cell-cell communication via interaction with gap junctions. Gap junctional proteins were therefore investigated by gene expression via qPCR, protein expression via western blot and also visualized through the scrape loading method.

3.4.1 Gene expression of two Gap junction genes

The mRNA expression of the genes coding for gap junction proteins Cx43 and Cx26 were studied using qPCR. In all experiments HBEC-3KT cells were exposed to CNT-1 and CNT-2 at the indicated concentrations. Changes in mRNA expression are measured as fold change compared to unexposed control cells (DM).

![Figure 3.6: mRNA expression of Cx43 (A) and Cx26 (B) in HBEC 3kt cells exposed to crocidolite asbestos (10 µg/ml), CNT-1 (5 and 10 µg/ml), CNT-2 (5 and 10 µg/ml) or left untreated (DM) after 3 and 6 hours. Fold change = $2^{\Delta \Delta CT}$. The experiments were repeated three times and were performed in duplicate. Statistical analysis performed with the t-test. Error bars = SE. *p<0.05 significantly different from DM.]

A decrease in Cx43 mRNA expression was observed after CNT-1 exposure at both time points, significantly after 6 hours at a concentration of 5 µg/ml (Figure 3.6 A). Furthermore, there was an increase in Cx43 mRNA levels in response to CNT-2 after 3 hours (fold change =1.5 ± 0.5) followed by a decrease after 6 hours (fold change =0.5 ± 0.3) (Figure 3.6 A). For asbestos no changes were observed. The mRNA levels of Cx26 did not change considerably in response to CNT-1, CNT-2 or asbestos (Figure 3.6 B).
3.4.2 Protein expression of gap junction genes

To evaluate the protein expression of the Gap junction protein Cx43 membranes were stained with Cx43 antibody. Cx43 bands are normalized to the loading control Tubulin which is constitutively expressed. Figure 3.7 A and 3.7 B show the results for CNT-1 and asbestos. Figure 3.5 C and 3.5 D show the results for CNT-2.

![Western blotting results showing Cx43 levels in HBEC-3KT cells after exposure to CNT-1 (A) and (B) and after exposure to CNT-2 (C) and (D). (A) and (C) show representative western blots and (B) and (C) show quantification of normalized total Cx43 levels to Tubulin. All treatments are set relative to DM 24h. Data are shown in arbitrary units. The result of one experiment is shown.](image)

For CNT-1 5 µg/ml there is a 2-fold decrease in protein expression of Cx43 after 24 hours, while the higher concentration (10µg/ml) gives no change. Asbestos gives a 2-fold increase after 24hours. After 48 hours all exposures lead to a more than 10-fold decrease in Cx43 expression, with the higher effect by CNT-1 compared to asbestos. For CNT-2 there is a small decrease after 24 hours at both concentrations. After 48 hours CNT-2 gives a more than 20-fold decrease at both concentrations compared to DM.
3.4.3 Visualization of intercellular communication

To further investigate if exposure to CNTs may affect cell communication scrape loading was performed. Here, cells were grown on coverslips and incubated with the fluorescent dye Lucifer Yellow and then cut using a scalpel as described in the materials and methods section. The dye will enter cut cells and diffuse into neighboring cells via gap junctions therefore being indicative of communication between the cells. Compromised gap junctions will lead to lower rate of diffusion and the dye will enter fewer cells than in uninjured cells.

A representative selection of images for all treatments after 6 hours of exposure are shown in Figure 3.8.

<table>
<thead>
<tr>
<th></th>
<th>Hela (6 hours)</th>
<th>HBEC-3KT (6 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control DM:</strong></td>
<td>Avg. % area: 5.0</td>
<td>Avg. % area: 1.4</td>
</tr>
<tr>
<td><strong>Croc 2µg/ml:</strong></td>
<td>Avg. % area: 3.4</td>
<td>Avg. % area: 1.2</td>
</tr>
</tbody>
</table>
Figure 3.8: Visualized degree of cell communication for Hela and HBEC-3KT cells for indicated exposure and time points. Cells with a black color have taken up the dye. A total number of ten pictures were taken for all treatments and time points for each experiment. Degree of cell-cell communication is represented as % area of cells that have taken up the dye.
To give a better overview, the results presented in Figure 3.8 were quantified and are presented in Figure 3.9.

**Figure 3.9:** Degree of cell-cell communication as % area. Cells were exposed to DM alone, crocidolite asbestos (2µg/ml), CNT-1 (1 and 2µg/ml) and CNT-2 (1 and 2µg/ml). Pictures were analyzed using imageJ software and % area of communicating cells were calculated. (A): Cx43 transfected Hela cells. Experiments in (A) were performed one time with 10 replicates. Error bars = SD. *=p<0.05 significantly different from DM. (B): HBEC-3KT cells. Experiments in (B) were performed in triplicate and repeated three times. Statistical analysis performed with the Mann-Whitney rank sum test. Error bars = SE. *=p<0.05 significantly different from DM.

The Hela cells that constitutively express Cx43 show a significant reduction in cell-cell communication after exposure to both CNTs at all indicated concentrations and time points. Furthermore, asbestos exposure gave a similar reduction. The trend in HBEC-3KT was also towards a reduction in cell-cell communication and a significant reduction is seen for CNT-2 (1 µg/ml) at 6 hours.
4. Discussion

4.1 Choice of nanomaterials, cells and methods

The present study was undertaken to study the toxicity and molecular mechanisms of two different MWCNTs using a human lung epithelial cell line as an *in vitro* model system. The nanomaterials chosen are both multi-walled carbon nanotubes but differ in fiber length, diameter and aspect ratio. The aspect ratio of fibers is an important parameter of the fiber-pathogenicity. Carbon nanotubes are suspected to cause asbestos-like health effects and therefore the crocidolite asbestos was used as the reference fiber. Human lung epithelial cells were chosen because the lung is the first target organ to be exposed to CNTs through inhalation at work places. The lung epithelium is the first barrier met by inhaled particles and is vital for protecting the lungs against pathogenic effects. The HBEC-3KT cells used in this study are immortalized and can be grown continuously in culture and are well suited for *in vitro* experiments. The cells have a normal phenotype, thus displaying similar characteristics and cellular responses as primary lung cells (Ramirez et al., 2004). However, there are limitations in using an *in vitro* model. An *in vitro* model simplifies biological processes that take place in normal tissue and does not necessarily reflect the situation *in vivo*. However, an *in vitro* model provides a much more detailed and focused view on molecular mechanisms of cellular effects.

CNTs are suspected to interfere with dyes used in cytotoxicity assays. We therefore performed two different assays simultaneously with the same cell and particle suspensions to be able to compare the results. There have been conflicting reports regarding cytotoxicity of CNTs. Several studies using the dye based MTT assay have reported strong cytotoxicity (Jia et al., 2005; Murr et al., 2005), but it is speculated that CNTs attach to the insoluble MTT product and disturb the test (Worle-Knirsch et al., 2006). Prior to performing the cytotoxicity assays, we performed tests where the CNTs were incubated with CTB or CCK-8 in a cell-free medium and we found no elevated levels of absorbance/fluorescence indicating minimal interference of the CNTs with these assays.
4.2. Toxicity of CNTs in human lung cells

Exposure of epithelial cells to CNTs are suspected to lead to a variety of responses ranging from direct effects like cell damage and cell death (cytotoxicity) to indirect effects like the induction of pro-inflammatory mediators, disruption of intercellular communication and protein phosphorylation. In this study, we have studied possible effects of CNTs on all of these endpoints.

Cytotoxicity assays were performed to measure the toxicity on HBEC-3KT cells by the MWCNTs compared to asbestos fibers. All tested materials showed a clear dose-dependent toxicity. This is in concordance with other studies where cytotoxicity has been measured by LDH assay (Muller et al., 2005; Simon-Deckers et al., 2008), though some studies (Davoren et al., 2007) had to use very high concentrations (400 µg/ml) before any cytotoxicity was measured.

With regard to the LC₅₀ values calculated in our study both MWCNTs are also at least threefold more toxic than asbestos fibers. Comparing the two CNTs, CNT-1 and CNT-2 showed similar cytotoxicity measured by the CTB assay while CNT-2 was more toxic when measured by the CCK-8 assay indicating there are some properties of the fibers affecting either cytotoxicity or interfering with the assays. The physico-chemical properties important for toxicity are size and shape, purity, agglomeration state and surface charge (Murdock et al., 2008).

Another important property is tendency to agglomerate where some studies have reported increased toxicity with agglomerated fibers (Murray et al., 2012). However, the thin single fibers that are associated with asbestos-like pathogenicity are believed to have greater effects due to their capability of reaching deep within the lungs (Murphy et al., 2011). Regarding fibers used in our study, CNT-1 is more often found in bundles and has a significantly less proportion of single fibers/µg than CNT-2. With the physico-chemical properties in mind the appropriate dose metric to assess toxicity must be considered. In conventional toxicological studies the mass of the sample is the dose metric most often used, but that may not be appropriate for CNTs and other NPs. Other studies have tried factoring in other parameters than mass by measuring toxicity related to surface area (Oberdorster et al., 2005), size and fiber concentration (Jiang et al., 2008). We therefore recalculated the cytotoxicity data from mass concentration to fiber concentration to take into account the effect of number of single
fibers. Here CNT-2 which contained the highest number of single fibers showed less toxicity than CNT-1 and asbestos. However, CNT-2 fibers are shorter in length and thinner in diameter. Additionally, they are less likely to form agglomerates and were more often found as straight and needle-like structures.

Some studies have found that the longest CNTs tested are the most toxic (Poland et al., 2008). CNT-1 is also considerably longer than CNT-2, so length may be an important parameter for the toxic affects as well as other molecular effects.

The toxic effects observed by the cytotoxicity assays can have serious consequences. Apart from the direct toxicity on epithelial cells, this injury may also induce inflammation and affect other cells in the lung. However, one must take in mind that doses used in this assay are much higher than one would expect during occupational exposure. Results from cytotoxicity assays alone are also inadequate for any conclusions to be made, and must be considered in conjunction with other experiments.

4.3 Pro-inflammatory potential of CNTs

Epithelial cells are capable of producing several pro-inflammatory mediators and are together with macrophages regarded as regulators of airway inflammation (Takizawa., 1998). The lung epithelium plays a key role in inflammatory processes by releasing inflammatory cytokines such as IL-1 and TNF-α (Herzog et al., 2009). We therefore investigated gene expression of IL-1α, IL-1β and TNF-α using qPCR to see if CNTs could induce these pro-inflammatory mediators in vitro. We also investigated the tumor suppressor gene TP53 which is a stress-responsive gene and is important for the induction of programmed cell death under stressful situations. Furthermore, TP53 may play a role in inflammatory responses induced by NPs through the oxidative stress.

IL-1α was in our study found to be overall unregulated by CNT-1 and asbestos, but was at two time points downregulated by CNT-2. The fact that IL-1α is also unregulated by the crocidolite asbestos that has been shown to be upregulated in some studies (Shukla et al., 2009; Tsuda et al., 1997) may indicate methodological differences such as primer design or differences in cell lines used. An in vitro study that exposed human bronchial epithelial cell line BEAS-2B to SWCNTs found significantly elevated levels of IL-1α (Park et al., 2014).
However, unlike our CNTs, they used SWCNTs containing a large amount of iron (36%), which could be the inducer of pro-inflammatory cytokines through oxidative stress and ROS production. In addition, SWCNTs are thinner than MWCNTs which could affect toxicity. However, in studies comparing SWCNTs to MWCNTs using the same methods and route of administration they were found to have corresponding toxicity (Porter et al., 2010; Mercer et al., 2011).

*IL-1β* was downregulated by the CNTs and by asbestos. Another study using BEAS-2B cells also found *IL-1β* to be significantly downregulated after MWCNT exposure (Tsukahara and Haniu, 2011). Similar to our study they also used purified MWCNTs without metal contaminants. This may explain why there is no upregulation of this gene as *IL-1β* in other studies has been seen activated by ROS associated with metal contaminants (Zhou et al., 2003). Some studies have also found downregulation or suppression of other inflammatory mediators. One study reported suppression of *IL-6*, *IL-8* and *TNF-α* after exposure of normal human bronchial epithelial cells (NHBE) as well as to type II alveolar epithelial cells to SWCNTs (Herzog et al., 2009). Even though upregulation of pro-inflammatory mediators is linked to fiber pathogenicity, downregulation may also result in adverse effects. Suppression of immune responses makes the immune system less reactive towards possible ROS created by the same particles or infections created by other agents. In an *in vivo* study with mice exposed to SWCNTs and subsequently infected with *Listeria monocytogenes* bacteria, there was increased bacterial activity and decreased bacterial killing (Shvedova et al., 2008a).

There was a strong upregulation of *TNF-α* by both CNTs, with the strongest induction after CNT-1 exposure. Other studies have also seen upregulation of *TNF-α* in other MWCNT-exposed cells like monocytes (Brown et al., 2007) and macrophages (Kagan et al., 2006). In addition, several *in vivo* studies in mice have reported significant upregulation of *TNF-α* after MWCNT exposure (Poland et al., 2008; Shvedova et al., 2008b). Murphy *et al* reported in an *in vitro* study that *TNF-α*, as well as IL1-β secreted by both macrophages and endothelial cells are the most likely candidates to drive the pro-inflammatory effects seen in mesothelial cells (Murphy et al., 2012). *TNF-α* can also stimulate lung epithelial cells to produce IL-8, which is a chemotactic cytokine that attracts neutrophils to the target site. The strong upregulation of *TNF-α* seen in our results may indicate induction of an inflammatory response leading to pathogenicity associated with fiber induced chronic inflammation.
A study using microarray analysis on SWCNT exposed human Hek293 kidney cells reported upregulation of \textit{TP53} followed by apoptosis (Cui et al., 2005), providing the link between CNT exposure, \textit{TP53} induction and apoptosis. In our study there was a significant upregulation of \textit{TP53} by CNT-1 after 3 hours, but little or no change at other concentrations and time points.

We also analyzed the protein expression and phosphorylation of the MAPK protein ERK. MAPKs induce phosphorylation of proteins such as transcription factors NF-\kappa B and AP-1 that regulate expression of many pro-inflammatory genes including \textit{IL-1} and \textit{TNF-\alpha}. The activation of MAPKs is regulated by phosphorylation which activates the target proteins by phosphorylation of the respective proteins (Johnson and Lapadat., 2002). Different stimuli such as growth factors, cytokines, virus infection and carcinogens are known to activate the ERK pathway (Johnsen and Lapadat, 2002). In our study phosphorylation of ERK decreased after 24 hours followed by an increase after 48 hours in cells exposed to both CNTs. This could just be a delayed expression because proteins take longer time to be synthesized and expressed than mRNA. An increase in ERK phosphorylation can lead to an increase in pro-inflammatory transcription factors and an onset of inflammation. One study reported significant phosphorylation of ERK in human mesothelial cells after exposure to SWCNTs (Pacurani et al., 2008). The same study also found activation of NF-\kappa B and AP-1 using ELISA assay in the same cell line. An increase in ERK in our study may indicate a similar induction of pro-inflammatory transcription factors in the lung cells.

4.4 Effects of CNTs on intercellular communication

Several carcinogens are known to interfere with gap junctions and disrupt intercellular communication through downregulation of \textit{Cx43} (Yamasaki et al., 1999). One study compared the GJIC in primary human mesothelial cells and asbestos-associated malignant mesothelial cells by the scrape loading method (Linnainmaa et al., 1993). The GJIC capability in tumor cell lines were significantly decreased compared to normal mesothelial cells. These findings suggest that asbestos may interfere with GJIC. To our knowledge the effects of CNTs on GJJC has not been investigated before.

We used three approaches to study the effects of CNTs on intercellular communication. Firstly, we studied gene expression of \textit{Cx43} and \textit{Cx26} by qPCR after exposure to both CNTs
and asbestos. We found a decrease of \( Cx43 \) expression for both CNTs after 3 hours and no changes in expression of \( Cx26 \). Secondly, we also studied the protein expression of \( Cx43 \) by western blotting. A small decrease (20%) in protein expression after 24 hours, and a large decrease (90%) after 48 hours were found for both CNTs.

Finally, using the scrape loading assay we attempted to directly visualize the activity and status of GJIC in the exposed cells. In the scrape loading assay Hela cells which overexpress \( Cx43 \) were used as a model system and as a reference for the results obtained with the HBEC-3KT cell line. The Hela cells showed a significant downregulation of cell-cell communication. These findings indicate that CNTs may be capable of interfering with gap junctions and thus decrease cell-cell communication. We also investigated the effects of CNTs on the HBEC-3KT cells. In this cell line cell-cell communication was unregulated after 6 hours, but downregulated after 24 hours. This indicates that CNTs may also have an effect on intercellular signaling in lung epithelial cells. An important issue in this method is high background signals. Necrotic and apoptotic cells will take up the dye and be counted as communicating cells. Based on the cytotoxicity results one would assume more cell death in the CNT-exposed cells and thereby more background signal that skew the results toward more communication. However, the MWCNT concentrations were very low in the scrape loading assay (1 \( \mu g/ml \) and 2 \( \mu g/ml \)), which was done to minimize confounding effects associated with cell death.
5 Conclusion and future perspectives

The cytotoxicity assays related to mass of the fibers showed a clear dose dependent cytotoxicity for both MWCNTs. Both were also more than 3-fold more toxic than asbestos. Even though the two MWCNTs possess differences in toxicological important physical features they showed similar toxic effects by this dose metric. With respect to number of fibers the CNT-1 was considerably more toxic than CNT-2. Cytotoxicity results obtained in this study raises concerns about pathological affects of CNTs on human lung cells.

TNF-α was the only pro-inflammatory mediator where CNT exposure resulted in a raised expression and may suggest a role for TNF-α in CNT induced inflammation. Small up-regulation was found in protein expression of the MAPK ERK. Together the results in this study are inconclusive concerning pro inflammatory effects of CNTs on human lung cells.

The gap junctional gene Cx43 was investigated by three methods. Concerning gene and protein expression, both gave reduction in Cx43 expression. Intercellular communication was also inhibited by CNTs, visualized by the scrape loading assay. Combined, these three methods point towards possible down regulation of gap junctions via Cx43 after CNT exposure.

Even though this study could not confirm effects of CNTs on IL1 expression, inflammation is still the area where focus should be held in the future. One should clarify the lack of IL-1α, and IL-1β induction, either by the use of different time points or by the use of metal contaminated MWCNTs. CNT induced ROS formation is another area of great interest and its effects on pro-inflammatory genes should be investigated. This can be investigated directly, by measuring ROS levels, comparing gene expression after exposure to pure and metal contaminated CNTs. Other cell lines important in inflammation like macrophages could also be incorporated in the in vitro model.

Another area of interest is chronic exposure to CNTs. Experiments with small doses over a long period could elucidate both pathological outcomes and degree of bioaccumulation of CNTs after long term exposure. It would also be interesting to investigate the ecotoxicological impact of CNTs on the ecosystem concerning waste disposal.
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