Cranfield UNIVERSITY

Advances and Controversies in Fibre Toxicology

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ABSTRACT BOOKLET

Oral Papers

The production and applications of High Temperature Insulating Wool

Ron Wainwright, Morgan Thermal Ceramics, UK

The paper discusses the evolution of High Temperature Insulating Wool from RCF, developed in the 1950's, to later forms including Alkaline Earth Silicate Wools and Polycrystalline Wools. This process of development has been driven on the one hand by the desire to reduce the risk associated with inhaled fibrous dust in the workplace and on the other hand by the desire to have products with higher levels of performance.

The application performance of HTIW products in high temperature furnaces is determined by the chemistry of the fibre and by the fibre manufacturing process. Chemistry is important as it determines the response of the fibres to high temperature. The manufacturing process is important as it determines the fibre diameter distribution and also the shot content. These are fundamental properties in determining insulation performance. However, the chemistry of the fibre also determines bio-persistence and the fibre diameter determines the tendency to produce respirable dust during handling. The paper therefore draws out the degree to which high performance and reduced carcinogen hazard are conflicting requirements.

Vitreous HTIW products (RCF and AES types) are subject to crystallisation at high temperatures. This phenomenon is discussed in terms of the possible health concern related to the formation of crystalline silica and also to the way in which crystallisation may affect the lifetime of a furnace installation.

Manufacture of high aspect ratio nanoscale fibres and structures

Rob Dorey, University of Surrey, UK

The majority of nanomaterials that are produced are equiaxed yet there exist many examples of high aspect ratio nanoscale structures that have been manufactured and which are finding applications in a variety of systems. This talk will review some examples of these materials highlighting their morphological characteristics and potential applications.

CARE: European monitoring program for RCF/ASW

L. Daniel Maxim, Everest Consulting Associates, USA

Klaus Kamps, Unifrax GmbH, Teichwolframsdorf, Germany; Dean Venturin, Unifrax 1 LLC, Tonawanda, New York, USA

The companies that manufacture refractory ceramic fiber (RCF)/alumino silicate wool (ASW) have operated an integrated product stewardship program since 1990. A key component of this program entails monitoring respirable fiber concentrations at both firms operated by RCF/ASW producers and their customers. The monitoring program now covers operations in Asia, Europe, and North and South America. The European program is known as CARE an acronym for Controlled and Reduced Exposure. A stratified random sampling plan (SRSP) is used to collect data; strata are defined as functional job categories (FJCs). The exposure

data are analyzed in various ways including; (i) longitudinal analysis to detect time trends and measure progress in reducing exposure, (ii) cross sectional analyses to identify FJCs with higher exposures, (iii) job- and plant-specific analyses to identify jobs where respirators are required, (iv) special emphasis sampling to evaluate the effectiveness of controls, and (v) benchmarking analyses to identify plants and FJCs with relatively low exposures to highlight best practices. Examples of each type of analysis are included in the presentation. Overall, the data show that exposures have been reduced substantially since 1990 at both producers and customers. In recent years, progress has slowed because the most attractive control options have already been implemented. Present operations show high rates of compliance with applicable OELs and also the industry's recommended exposure guideline (REG).

Perspectives on RCF carcinogenicity

Helmut Greim, Technical University, Munich, Germany

Mark J. Utell, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA; L. Daniel Maxim and Ron Niebo, Everest Consulting Associates, Cranbury, New Jersey, USA

In 2011 the Scientific Committee on Occupational Health (SCOEL) classified refractory ceramic fibres (RCF) as a carcinogen and supported a practical threshold. Inflammation was considered the predominant manifestation of RCF carcinogenicity. Intrapleural and intraperitoneal implantation induced mesotheliomas and sarcomas in rats and hamsters. Chronic nose-only Inhalation bioassays indicated that RCF exposure in rats increased the incidence of lung cancer and mesothelioma in hamsters. Although the epidemiological studies in the US (University of Cincinnati) and in Europe (IOM) showed an increased prevalence of respiratory symptoms and pleural plaques, no interstitial fibrosis, mesotheliomas, or increased numbers of lung tumors were seen during 31 years of follow-up. Since the latency of asbestos induced mesotheliomas is up to 50 years according to some reports, the relationship between RCF exposure and respiratory malignances has not yet been fully determined. RCF and rock/slag wool have similar airborne fibre dimensions and biopersistence. Therefore, it is likely that these fibres have similar toxicology. Rock/slag wool has been the subject of numerous cohort and case control studies. For rock/slag wool, IARC (2002) concluded: "The results from these studies provide no evidence of an increased risk for pleural mesotheliomas or any other tumours." Almost 10 years later Marsh et al (2011) confirmed this conclusion in a (US) study of over 32,000 workers followed as long as 47 years. RCF producers have developed a wide ranging product stewardship program to measure and control fibre concentrations and to maintain an ongoing mortality study.

Assessing the impacts of implementing changes to REACH regulations for nanomaterials: learnings from a recent EU activity

Camilla Pease, ENVIRON UK Ltd, UK.

It has been reported that 500-2000 different nanomaterials are on the marketplace in Europe and that number is destined to grow rapidly. A handful of materials have been formally registered as nanomaterials in currently submitted REACH dossiers. Controversially, REACH regulation is considered by many stakeholders interviewed as sub-obtimal, in terms

of the way nanomaterials and nanofibres are handled. Also different EU regulations include different definitions. The problem is considered by a broad range of stakeholders to be a current perceived lack of clarity regarding technical requirements for the toxicology testing and characterisation of nanomaterials within REACH. Results for the Commission's Formal Public Consultation Exercise found that 68% of stakeholders considered guidance to be "unclear" and a further 18% "very unclear". The consequence is that nanomaterials dossiers are unlikely to provide sufficient evidence using current testing methods to ensure protection of human health and the environment. In this presentation, learnings and perspectives will be shared from a recent EU exercise, that built on the Joint Research Centre Nanosupport project, in which a range of technical and policy options were evaluated regarding potential changes to REACH requirements. We have recently surveyed the ability of GLP laboratories across the EU to offer characterisation and toxicology testing for nanomaterials. Our findings will also be put into the context of developments that have recently been published e.g. from EU collaborative research activities and bodies such as SCENIHR, SCCS (covering nanomaterials in cosmetics), EFSA (including nanomaterials in novel foods) and the EMA.

Determinants of Fibre Pathogenicity to the Lung and Pleura

Tom Hesterberg, Consultant, USA

A series of chronic fibre inhalation studies in rats and hamsters examined the potential pathogenicity of 3 different types of asbestos and 9 types of Synthetic Vitreous Fibres (SVFs). These inhalation studies revealed a broad range of fibre pathogenicities to the lung and pleura. Exposure to some fibre types produced lung fibrosis, lung cancer and mesothelioma, while other fibre types produced no lung disease. In addition to lung pathogenicity, the biopersistence of fibres was examined by determining how rapidly they cleared from the lung. Some fibre types dissolved, leached, and broke into shorter segments, resulting in rapid clearance from the lung over a period of days or weeks. Other fibre types were biopersistent in the lung and remained for long periods of time, some indefinitely. These studies showed that there was a good correlation between the biopersistence of fibres and their pathogenicity to the lung and pleura.

From these and other studies it was determined that the characteristics of fibres that are important to their pathogenicity are; 1) dose to the deep lung, 2) fibre dimensions (length and diameter), and 3) durability or biopersistence of the fibres in the lung and pleura. During long-term exposure to fibres, a steady-state dose of fibres in the lung is reached, which is determined by the rate of deposition minus the rate of dissolution, breakage and macrophage-mediated clearance. Fibre diameter is important, because thin fibres, which line up with the flow of air, can more readily deposit in the deep lung. Long fibres are more pathogenic, because they are not completely phagocytized by macrophages and other cells in the lung, resulting in "frustrated" phagocytosis and leakage of oxidants, chemokines, cytokines, etc. This results in toxicity, inflammation and cell proliferation in the lung and pleura, which can eventually result in fibrosis and tumour formation. Fibres that are biopersistent result in a higher steady-state dose and resultant pathogenicity to the lung and pleura. However, fibres that are biosoluble can also be pathogenic to the lung and pleura if exposures are very high, resulting in a high steady-state dose (deposition-minus-clearance) in these tissues.

Nano-fibers and asbestos – lessons to be applied

David Bernstein, Consultant, Switzerland

The evolution of our understanding of fiber toxicology has evolved significantly. The importance of dose, dimensions and durability which originally which were developed through studies on synthetic vitreous fibers have more recently been seen to apply in differentiating chrysotile from amphibole asbestos. Studies nano-fibers suggest that similar principles apply in understanding the toxicological potential of these fibers. The importance of dose, fiber length and durability in the design and interpretation of fiber inhalation toxicology studies are discussed.

High aspect ratio nanoparticles, the lungs and the pleura: What do we know and what don't we know?

Craig Poland, Institute of Occupational Medicine, UK

The state of understanding surrounding carbon nanotube pathogenicity has advanced significantly in the last decade since the initial rodent lung instillation studies which focused attention on the potential respiratory hazard. During the intervening time, in addition to numerous in vitro as well as in vivo instillation studies; inhalation studies showing dose related respiratory effects of carbon nanotubes have provided high quality data of the type required for derivation of exposure limits. This has led to the proposition of several exposure limits for carbon nanotubes to ensure their responsible handling, safe use and protection of workers. Such exposure limits understandably focus on the observed response to exposure which, in the case of carbon nanotubes as well as other high aspect ratio nanomaterials, has been predominantly inflammation, granuloma formation and fibrosis. However such responses are heavily influenced by the experimental design (e.g. duration and dose) and may not show all eventualities associated with exposure. Such problems are inherent in any experimental design and endpoints such as the induction of mesothelioma are particularly challenging to address. Nonetheless, there is mounting evidence suggesting that carbon nanotubes could cause effects other than localized lung toxicity characterized by granulomatous inflammation which could have implications on proposed exposure limits.

Here we discuss the known and hypothesized effects of carbon nanotubes towards the lungs and mesothelium, its relevance to other high aspect ratio nanoparticles and ask the question – are we ready for an exposure limit for carbon nanotubes?

Inhalation toxicity studies in rats with respirable-sized p-aramid, organic fibers: Correlation of findings with results of *in vitro* biodegradability studies

David Warheit, DuPont Haskell Laboratory, USA

para-Aramid is a commercial organic fiber whose applications include advanced composites as well as fabrics, body armor, and friction materials. Respirable-sized fibrils of p-aramid (RFP) can be released from the fibers in workplaces and become airborne. An extensive body of research has been conducted into the hazards posed by inhaled p-aramid fibrils and its potential mechanism of action in the respiratory tract – the major port of entry. High-dose, chronic inhalation studies in rats have shown that inhaled p-aramid RFP produced

fibrosis and proliferative keratin cysts following 2-year inhalation exposures. However, a variety of peer-reviewed mechanistic toxicology studies have demonstrated that p-aramid fibrils can be long and thin but that the inhaled RFP are not biopersistent in the lung- an essential component of the fiber pathogenicity paradigm. Accordingly, following deposition in alveolar regions, increased postexposure residence time in the lung milieu correlates with fiber shortening, concomitant with complete phagocytosis and effective clearance. This finding of long p-aramid RFPs cleaved into shorter fibrous fragments has been reported by a number of independent investigators. Thus, the p-aramid hazard is low, and this is confirmed in animal studies.

In subsequent studies, we have undertaken efforts to elucidate the mechanism(s) through which inhaled p-aramid respirable-sized fiber-shaped particulates (RFP) are biodegraded in the lungs of exposed rats and hamsters. We have postulated that lung fluids coat/activate p-aramid RFP following deposition in the alveolar regions of the lung, thus predisposing the RFP to enzymatic attack and consequent shortening. This process enhances the rate of RFP clearance. To test this hypothesis, we have conducted both *in vivo* and *in vitro* cellular and acellular investigations. As one defining example, cultures of rat lung epithelial cells, alveolar macrophages, or co-cultures of epithelial cells and macrophages were treated with p-aramid RFP for 1 h, 1 day, or 1 week to determine whether RFP shortening occurs directly in the phagocytic cells. The lengths of fibrils were measured using scanning electron microscopy (SEM) techniques. The results demonstrated that (1) no shortening occurred in the epithelial cell cultures at any time point; however, (2) in the macrophage and co-cultures, cleavage of p-aramid RFP was observed at 1 day and 1 week postexposure. Our data suggest that components of lung fluids coat and catalyze the p-aramid RFP as a prerequisite for enzymatic cleavage. This process could play a significant role in facilitating the transverse cleavage or shortening of inhaled p-aramid RFP in the lungs of exposed rats and hamsters. Finally, a set of *in vitro* studies with similar experimental designs was undertaken to determine whether shortening mechanisms of p-aramid RFP biodegradability are also operative in human lung cells. Cultures of human A549 lung epithelial cells (A549), primary alveolar macrophages (HBAL) (collected via bronchoalveolar lavage [BAL]) from volunteers), and co-cultures (Co) of the A549 and HBAL were incubated with p-aramid RFP for either 1 h, 1 day, or 1 week to assess RFP shortening. Lengths of RFP were measured using SEM. Similar to findings with rat lung cells, only slight RFP shortening was measured in A549 cultures at 1-day and 1-week post-incubation. More importantly, in HBAL and Co groups, greater transverse cleavage of p-aramid RFP was measured at 1-day and 1-week postexposure compared to 1-h HBAL or Co groups, or in any A549 groups. In contrast, cellulose RFP, a biopersistent reference control fiber, were not measurably shortened under similar circumstances. Similar to our findings with rat lung cells, components of human lung fluids coat the p-aramid RFP as a prerequisite for subsequent enzymatic cleavage by human phagocytic lung cells and this finding reinforces the concept that inhaled p-aramid RFP are likely to be biodegradable in the lungs of humans.

Biodistribution of ^{60}Co-labelled Carbon Nanotubes (CNT) Following an Acute Inhalation in Rats

Otto Creutzenberg, Germany

Anja Hackbarth, Dirk Schaudien, Bernd Bellmann(deceased), Albrecht Leonhardt, Fraunhofer Institute for Toxicology & Experimental Medicine, Hannover, Germany, and Leibniz Institute for Solid State and Materials Research, Dresden, Germany.

Rats were exposed to carbon nanotubes (MWCNT) in a single 4-hr inhalation study. The MWCNT used showed a mean diameter of approx. 40 nm and a mean length of 5-10 µm (WHO fibres) and was aerosolized by nebulization of an aqueous suspension (actual concentration: approx. 4 mg/m3). Purification assured that the solubility of the catalyst Co was <5%. The objective of the study was to investigate the deposition and clearance of MWCNT in lungs and the distribution and excretion following translocation from the respiratory tract. In the first part of the project the exposure was performed using unlabelled MWCNT. In lung lavage fluid inflammatory reactions were observed 1 day after exposure but not 28 days after exposure. Individual MWCNTs were detected in liver, kidneys and in the agarose cast of the pleural cavity using a high resolution light microscope. - In the second part Co-60 labelled MWCNT were used for single exposure (gamma tracer). The deposition in lungs and in the trachea-bronchial tract was approx. 10% and approx. 6%, respectively, after end of exposure (day 0). The activity analysed in several organs showed the highest amounts in liver (approx. 2%) and kidneys (approx. 1%).

The analysis of the lung lavage fluid has shown, that already 1 day after exposure most MWCNTs were phagocytized by macrophages. By analysis of the agarose cast a fast translocation to the pleural cavity was detected on the day of exposure and 1 day after exposure (approx. 0.3%). Thereafter a fast decrease of MWCNT concentration in the pleural cavity was measured in the post-inhalation period (day 14 and day 28).

Induction of malignant mesotheliomas by intraperitoneal injection of carbon nanotubes in rats

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Biological effects of tailor-made multi-walled carbon nanotubes (MWCNTs) were investigated *in vivo* in a 2-year carcinogenicity study in a project funded by the German Federal Ministry of Education and Research (contract no. 03X0109A).

Fifty Wistar rats per group (total of 500 rats) were treated once by intraperitoneal (i.p.) injection of a low (1x10e9 WHO fibers) or high (5x10e9 WHO fibers) dose of different MWCNTs (MWCNT1, 2, 3, and 3a) suspended in artificial lung medium that was also used as negative control. Amosite asbestos (0.1x10e9 WHO fibers) served as positive control. Moribund rats were sacrificed and necropsy comprising all organs was performed. Histopathological classification of tumors and, in addition, immunohistochemistry were

conducted for a variety of tumor markers to compare the induced tumors with mesotheliomas occurring in humans.

The treatments induced mesotheliomas in all dose groups, whereas incidence and time to tumor were different between the groups. Rats treated with MWCNT3 (L=8.57 µm; D=0.085 µm) and MWCNT3a (L=9.3 µm; D=0.062 µm) were killed moribund between 8 and 12 months after treatment and exhibited high tumor incidences in the low- and high-dose groups. The survival time of rats treated with MWCNT2 (L=10.24 µm; D=0.04 µm) was even longer, but resulted also in high tumor incidences in both dose groups in relation to the positive control. For MWCNT1 (L=7.9 µm; D=0.037 µm) the survival time of the low-dose group was similar to the amosite asbestos (L=13.95 µm; D=0.39 µm) positive control. Overall, tumor incidences for MWCNT1 were lower than for MWCNT2, 3, and 3a. Most tumors were histologically and immunohistochemically classified as malignant mesotheliomas comparable to those in humans.

Long-fibre Carbon Nanotube- and Asbestos-induced Pleural Lesions exhibit a Common Molecular Signature

Fiona Murphy, MRC Toxicology Unit

Exposure to asbestos fibres causes pathological changes in the pleural cavity including malignant mesothelioma. The structural similarity between some carbon nanotubes (CNT) and asbestos has raised concerns that CNT may pose a similar hazard. Studies have shown CNT display asbestos-like pathogenicity after direct injection into the pleural and peritoneal cavities however the molecular changes driving the pathogenicity are not understood. Using a model of direct injection into the pleural cavity we compared the molecular changes which occur at the mesothelium after exposure to short and long asbestos fibres (SFA, LFA) and short and long CNT (SNT, LNT) at 1 and 12 weeks post-injection. Exposure to LFA and LNT induced an inflammatory response and fibrosis on the parietal pleura; no inflammatory changes were detected after exposure to SFA and SNT. Furthermore, whole genome array analysis showed a common pattern of gene expression changes in both LFA- and LNT-induced lesions. Kinome profiling and immunostaining showed activation of pro-oncogenic signalling pathways that was sustained in the pleurae of animals exposed to long but not short fibres. The acute and sustained kinase activation in both LFA- and LNT-exposed pleurae was strikingly similar in terms of both specific pathways activated and degree of activation. The lesions induced by LFA and LNT were further examined at 6 months post-injection where key pro-oncogenic molecular events (oxidative DNA damage and increased proliferation) were identified. This study highlights a commonality in the hazard mechanism of pathogenic fibres at the molecular level. Since information on the molecular changes that occur during the long latency period from asbestos exposure to mesothelioma development in humans is lacking, these findings provide important new insights into the early molecular events in fibre-induced pleural pathogenesis.

Contribution of pro-inflammatory cells in pro-oncogenic alteration of normal mesothelium

Tanya Chernova, MRC Toxicology Unit

Malignant mesothelioma (MM) is an aggressive, fatal tumour of the pleura or peritoneum and strongly related to asbestos exposure. Malignant pleural mesothelioma (MPM) is the most common and occurs with a latency of up to 40 years. The mechanism of MM carcinogenesis is not well understood and the heterogeneity of the tumour is considered to be a major barrier to successful therapy. Several studies have identified changes in the expression and activity of defined cell signalling pathways in mesothelial and stromal cells, but the relationship between different cell types in the process of tumorigenesis has not been studied. To examine the pro-oncogenic role(s) of different cell populations, the effect of primary fibroblasts from human mesotheliomas and fibre-activated macrophages on cellular signalling in normal untransformed mesothelial cells was monitored using imaging and immunoblotting techniques. Paracrine signalling from activated fibroblasts or macrophages increased the levels of proliferation and motility in normal mesothelial cell cultures. Activation of pro-oncogenic signalling was demonstrated in the cultures subjected to a cross-talk with pro-inflammatory cells. Normal mesothelial cells co-cultured or treated with conditioned media from fibre-activated macrophages also displayed signs of epithelial-to-mesenchymal transition. The levels of growth modulators and the survival rates were altered in these cells. Thus, non-mesothelial cells instigate alterations in cellular signalling in mesothelial cells. Further, integral examination of the aberrant signalling pathways, especially at early stages of neoplasia, will provide new insights into the mechanisms underlying malignant mesothelioma oncogenesis.

Interaction and bioreactivity of carbon nanotubes with primary human lung parenchymal cells in vitro.

Terry Tetley, National Heart and Lung Institute, Imperial College London

The synthesis and application of carbon-based nanomaterials for use in numerous aspects of everyday life is increasing rapidly. One concern is that structurally they resemble asbestos, and inhalation might induce similar pathology, such as mesothelioma and fibrosis. Carbon nanomaterials are being produced in many different shapes and sizes and may be functionalised to exhibit a range of surface properties, depending on the intended use. A high proportion of inhaled nanomaterials will reach the respiratory alveolar region and parenchymal lung tissue.

We have established a number of models of human parenchymal cells for in vitro studies of nanomaterials, to determine overt toxicity, and to establish mechanisms of toxicity. These include primary human lung alveolar epithelial cells, macrophages fibroblasts and microvascular endothelial cells. We are studying commercial carbon nanotubes (CNTs) and CNTs synthesised in-house to better understand their potential bioreactivity.

These studies show that the effect of CNTs on parenchymal lung cells critically depends on the function of the target cell. For example, relatively short (<2 microns long) CNTs are avidly internalised by, and ~3% translocate to the basolateral surface of, alveolar type 1 epithelial cells, major target cells that covers 95% of the respiratory epithelium. However,

alveolar epithelial type 2 cells rarely internalise nanoparticles, but CNTs cause mediator release and affect surfactant metabolism. The shorter CNTs are also particularly bioreactive with pulmonary microvascular endothelial cells, and acid oxidation, commonly used to solubilise carbon nanomaterials, enhances their reactivity. In contrast, alveolar macrophages are more susceptible to long CNTs (>15 microns), which cause cytotoxicity and are pro-inflammatory. All types of CNTs stimulate fibroblast growth and collagen production. These studies show that inhalation of CNTs could potentially affect parenchymal homeostasis and may be pathogenic.

Genotoxic and immunoregulatory effects of carbon nanotubes in vitro

Martin Clift, Adolphe Merkle Institute, Switzerland.

Due to their electrical, thermal and spectroscopic properties, in addition to increased strength, stiffness and durability, carbon nanotubes (CNTs) pose as an advantageous component for numerous applications. The same characteristics however, have raised heightened concerns regarding their potential risk towards human health, due to their inevitable human exposure (i.e. via inhalation). Despite this, a mechanistic understanding of how different CNTs may cause adverse health effects, including their potential carcinogenicity, is limited. The aim of this study therefore, was to systematically evaluate a panel of CNTs that exhibit fundamentally different physical properties (i.e. aspect ratio, stiffness, morphology) as to their potential to cause genotoxicity in a multicellular in vitro model the human epithelial airway-barrier. At non-cytotoxic, yet (pro)-inflammogenic (TNFalpha/IL-8) concentrations (0.005-0.02mg/mL) mediated by oxidative stress (decrease in GSH/increase in ROS) within the co-culture model, all CNTs were observed to be inside the macrophage cache only (none seen in the epithelial or dendritic cells), yet not within the cell nucleus of any cell after 24hrs. The onset of any potential CNT-associated genotoxicity was therefore assumed to be via secondary means. Subsequent investigation at 4 and 24hrs showed short nanotubes to cause a significant decrease (p

Multiwalled carbon nanotubes induce DNA damage and cellular senescence in human peritoneal mesothelial LP9 cells

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Multiwalled carbon nanotubes (MWCNTs) are nanomaterials with immense potential in many technological applications, but their toxic and genotoxic effects are still unclear. Notably, there are indications that certain MWCNTs cause malignant mesothelioma, a cancer derived from mesothelial cells and particularly linked to asbestos exposure. We thus investigated the *in vitro* toxic and genotoxic activity of various tailor-made MWCNTs in primary human peritoneal mesothelial LP9 cells, in the BMBF-funded project CarboTox (contract no. 03X0109A). LP9 cells were exposed for 24 h to 0.3-5.0 µg/cm^2 MWCNTs of different length, diameter, and morphology. Long amosite asbestos served as positive and milled MWCNT3 as material control. We found that certain more straight MWCNTs (i.e.

MWCNT3: L=8.57µm, D=0.085µm; MWCNT3a: L=9.30µm, D=0.061µm; MWCNT2: L=10.24µm, D=0.04µm) exhibited marked anti-proliferative, membrane-damaging and genotoxic potential, and impaired tubulin integrity, compared to milled MWCNT. DAPI-staining of respective cell nuclei frequently showed nuclear fragmentation, condensed chromatin, and senescence-associated heterochromatin foci, reminiscent of apoptotic or senescent cells. Such abnormal nuclei displayed pan- or bright-staining with gamma-H2AX, also indicative for cellular senescence. Furthermore, we observed senescence-associated, increased *CDKN2A* and repressed *LMNB1* gene expression in MWCNT-treated cells. Interestingly, the *in vitro* most active MWCNTs also induced, with similar order of potency, mesothelioma in a parallel two-year carcinogenicity study with rats. Altogether, our findings suggest marked genotoxic potential of straight MWCNTs with wider diameters, leading to cellular senescence as a possible molecular mechanism in mesothelioma development after MWCNT exposure.

Epidemiology of aluminosilicate wools (refectory ceramic fibers, RCF): Results to date and plans for continued study

Mark Utell, University of Rochester, USA

Animal studies on the toxicity of RCF have raised a number of questions about possible adverse respiratory effects of these fibers in humans. To answer these questions, epidemiologic studies were initiated at the University of Cincinnati in the United States in the mid-1980s and the Institute of Occupational Medicine in Europe. In brief, ongoing epidemiological studies demonstrate that exposure to RCF causes selected respiratory symptoms and pleural plaques. Specifically, pleural changes were seen in 2.7% of non-asbestos exposed workers and related to latency and cumulative exposure. However, these studies have failed to demonstrate progressive decrements in lung function, interstitial fibrosis, excess lung cancer, or mesothelioma. Potential confounding by cigarette smoking, age, and asbestos exposure have been included in the models, but it should be recognized that they remain major risk factors for serious pleural and parenchymal lung disease. The mortality study has not shown any increase in death rate (all deaths), cancer deaths, or respiratory deaths. A statistically significant increase in cancers of the urinary organs was found, but the biological significance of this finding is uncertain. Although mortality studies have not revealed significant increases in respiratory related mortality, there are plans for continued follow-up as the overall mortality rate increases because of aging of the cohort. As the RCF industry continues to focus on lowering workplace exposure and the production of lower biopersistent fibers, potential lung impact related to RCF exposures should further diminish in the future.

The risk of mesothelioma and lung cancer due to asbestos exposure - and update including recent epidemiology.

Andrew Darnton, HSE, UK

Britain has one of the highest mesothelioma rates worldwide with about 2,000 deaths per year in men and 400 in women currently. Men born between 1935 and 1955 have the highest rates, a consequence of past occupational asbestos exposures, which peaked between 1950 and 1975 then fell sharply. By 1980, crocidolite and amosite use had ceased

in the UK, and chrysotile use had fallen by 90%, but a large amount of asbestos remains in many older buildings, and there is ongoing concern about the potential for environmental exposure to building occupants and occupational exposure during maintenance, renovation and demolition. In the light of this, it would be useful to be able to predict mesothelioma and asbestos-related lung cancer risks for current-day asbestos exposure scenarios. To what extent are risk models derived from epidemiological studies of asbestos exposed cohorts useful in doing this? What are the uncertainties and unresolved issues in the epidemiology that affect the application of these models, particularly when extrapolating to lower exposures that are typically of interest today? Are there other approaches that may be useful in assessing the extent to which asbestos remaining in many buildings continues to pose a risk? This presentation will explore these questions, including the perspective of HSE's WATCH committee which recently considered these issues.

Estimating children's vulnerability to asbestos

David H. Phillips, King's College London, UK

It is estimated that 75% of schools in England have buildings that contain asbestos. There is potential for school occupants, including children, to be exposed to asbestos, especially when the material is disturbed or damaged. The Department for Education (DfE) has asked the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) for advice on the relative vulnerability of children to asbestos. The COC considered relevant epidemiological studies, animal studies, levels of exposure which children may experience, and anatomical and physiological differences between children and adults. All forms of asbestos are carcinogenic to humans, causing mesothelioma and cancer of the lung, larynx and ovary. There is good epidemiological evidence that childhood exposure to asbestos can cause mesothelioma in later life. There are respiratory and immunological difference between adults and children, but it is unclear how these might affect inhalation and retention of fibres, or host reaction to them, and thus the impact of such differences on susceptibility of children to asbestos-induced cancer is unclear. Thus the COC advice to DfE [1] is that from the available, albeit limited data it is not possible to say whether children are intrinsically more susceptible to asbestos-related injury than adults. Nevertheless the long latency of mesothelioma and other cancers means that children will have an increased lifetime risk of mesothelioma due to their increased life expectancy. A pragmatic estimate is that a child first exposed at age 5 is at about 3.5 times and 5 times greater risk than adults aged 25 and 30, respectively, at first exposure.

Reference

1. Statement on the relative vulnerability of children to asbestos compared to adults (2013) http://www.iacoc.org.uk/statements/documents/Asbestosinschoolsstatement_000.pdf

Poster Presentations

Prototype risk assessment for bio-persistent fibers on the nano scale using adverse outcome pathway (AOP) approaches.

Paul Fowler, Unilever

In order to evaluate the potential toxicity to the lung of fibrous ingredients in cosmetic aerosol and spray products, many of which have at least one dimension on the nano scale, we present a prototype risk evaluation process based around adverse outcome pathways (AOPs) for key events involved in lung toxicity. A combination of detailed exposure assessment, assessment of bio persistence, altered gene expression/function and cellular effects including apoptosis, inflammation and tissue remodelling have been used to predict downstream lung pathology and eventually lung disease. Several well studied human lung disorders particularly asbestos related mesothelioma have complex and well documented pathologies, data from such studies have been used to ground the in vitro data in biologically relevant outcomes and enabled the link between early biomarkers of lung toxicity and disease state to be made without the use of in vivo models.

In vitro evaluation of the potential genotoxicity and carcinogenicity of fibrous nano materials.

Paul Fowler, Unilever

As novel uses for nanotechnologies develop, there is potential in the future for consumers to be exposed to a range of nanomaterials, including fibrous ingredients with one or more dimension in the nanoscale, the importance of assessing the toxicological profile of these materials is paramount. Traditional in vitro testing strategies for assessing genotoxic hazard of these materials is often inappropriate due to physical factors such as poor solubility, difficulty in dispersion in culture media and lack of intracellular exposure. Human lung disease resulting from exposures to asbestos fibres is well documented and characterised; many authors report standard genotoxicity tests are capable of detecting asbestos and other carcinogenic fibres. We present data from the in vitro micronucleus test (IVM) after exposure to a number of asbestos and organic nano fibres as well as the Bhas42 cell transformation assay. Our data highlight the inability of the IVM to respond to known carcinogenic fibres and whilst the Bhas42 assay did highlight differences between the test fibres it was not as expected. Therefore when attempting to predict likely genotoxicity/carcinogenicity from exposure to fibrous materials the choice of test requires careful consideration.

Development of in vitro methods for determining the potential of biopersistent materials to cause lung fibrosis in humans

Bobbie Bradford, Philip Carthew, **Annette Furniss,** Richard Stark, and Andrew White, **Unilever**

In order to develop an alternative approach to inhalation animal testing for risk assessment of local lung effects from materials such as biopersistent fibres we have undertaken a programme of work to identify the critical responses in lung fibrosis. The selection of relevant in vitro cell systems and gene expression analysis has enabled us to outline a lung fibrosis Adverse Outcome Pathway (AOP), describing the mechanism of response to biopersistent ingredients (including nanomaterials) contained within consumer products which could be inhaled. Cell systems were initially selected based on their observed responses in levels of known biomarkers of pro-fibrotic effect (i.e. collagen, osteopontin, Î±SMA, and specific genes). Transcriptomic studies of in vitro cell lines and of relevant human disease states in vivo has furthered our understanding of the pathways involved. Sensitivity and specificity were tested using previously characterised substances, including multiwalled carbon nanotubes (MWCNTs). Following on from this initial work, we used relevant fibrosis BioMap systems (at BioSeek, DiscoveRX) to identify the key events which are consistently activated in response to known fibrosis-inducing materials. Here we present our findings, with a view to using a fully characterised lung fibrosis AOP for future inhalation toxicity risk assessment.

Cell proliferation measurement as early detection method of carcinogenic potential of carbon nanotubes

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Cell proliferation measurement as early detection method of carcinogenic potential of carbon nanotubes Multiwall carbon nanotubes (MWCNT) are discussed to have a toxic potency depending on their length and fiber-like shape. To investigate potential early recognition of carcinogenic behavior of MWCNT, they were injected intraperitoneally in rats and the cell proliferation rate was measured at the diaphragmatic peritoneum in a project funded by the German BMBF. Tailor-made MWCNT (1, 2, 3) with different lengths and diameters were produced, suspended in artificial lung medium and injected intraperitoneally in rats in two dose groups (low: $1x10^9$ WHO-fibers; high: $5x10^9$ WHO-fibers). Long amosite asbestos ($0.1x10^9$ WHO-fibers) served as positive, ground MWCNT and Printex 90 (5 mg/rat) as particle negative controls. Three, 6 and 12 months after injection of fibers, the animals were necropsied, the thickness of the peritoneum was measured, and the cell proliferation rate was determined using the BrdU method. The acquired data were correlated with a parallel carcinogenicity study. There was a time-independent significant increase in the cell proliferation rate of MWCNT 1(high) (length=7.9µm; diameter=0.037µm), MWCNT 2(low/high) (length=10.24µm; diameter=0.04µm) and MWCNT 3(low/high) (length=8.57µm; diameter=0.085µm) comparable to amosite asbestos (length=13.95µm; diameter=0.39µm). In the parallel carcinogenicity study, MWCNT 2 and 3 showed a higher mesothelioma incidence than MWCNT 1. Some MWCNT mediate enhanced proliferation of peritoneal cells of the diaphragm in rats which correlated well with results of the parallel carcinogenicity study. Early detection of carcinogenic potential of CNT via cell proliferation measurement of the diaphragmatic peritoneum after intraperitoneal injection in rats is possible.

Nanoregulation needs: Integrating science to social demands.

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An engineered nanomaterial may present different hazards due to small changes in structure such as the presence of traces of contaminants and functional groups attached to its surface. Some nanomaterials, such as carbon derivatives are difficult to obtain in pure form, without the presence of such changes, which may present different toxicological profile. If this is the rule rather than the exception, such materials should impose changes on the basis of the regulatory logic in at least four requirements : a) the hazard assessment and its regulatory consequences are likely to be made in terms of classes /general categories of materials, assuming uncertainties higher than for non nanoscale material, b) the difficulty of monitoring the rate of production of new nanomaterials and the differences already mentioned should increasingly and faster include the incorporation of alternative methods with no animals for assessment, which in silico modeling become not an option but an imposition to the existence of sufficient data to hazards evaluation; c) the classification of products as composed of nanomaterials should not be a result of their percentage in the product under review, but the theoretical potential hazard they present, including analysis in silico of the life cycle; d) the evaluation priorities, in terms of what materials must first be assessed, should be a function of four initial parameters: prediction of the current volume of products, the potential dangers posed to workers who produce them, analyzes of the dangers associated with disuse products, implying consume studies and integrate analyzes of the life cycle, incorporating data from the social sciences to in silico prediction.

Cristobalite in heated alkaline earth silicate wools does not cause increased cyto- and genotoxicity in short-term *in vitro* assays

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Alkaline earth silicate (AES) wools are low-biopersistence high-temperature insulation wools which may produce cristobalite and other crystalline silica (CS) polymorphs (classified as carcinogenic to humans) after prolonged heating at high temperatures.

This study investigated the cytotoxic and genotoxic significance of cristobalite in heated AES wools. Primary rat alveolar macrophages were incubated *in vitro* for 2h with 200µg/cm^2 unheated/heated calcium magnesium silicate wools (heat-treated for 1week at, or 4weeks 150°C below, their classification temperatures) or magnesium silicate wool (heated for 24h at 1260°C). Chemical and morphological characterization was done by x-ray diffraction and electron microscopy. For toxicity screening, lactate dehydrogenase release and comet assays ± aluminum lactate (quencher of CS effects) were used.

Cristobalite content of the wools increased with heating temperature and duration, paralleled by decreases in fibre length and changes in fibre shape. No marked cytotoxicity

and only slight DNA-strand break induction was noted compared to the CS-negative control Al_2O_3, whereas the CS-positive control DQ12 was highly active. Some samples induced minor oxidative DNA-damage, but no biological endpoint significantly correlated with free CS, quartz, or cristobalite content.

In conclusion, heating of AES wools mediates changes in CS content and fibre length/shape. While fiber morphology can impact biological activity, cristobalite content appears to be of minor or no relevance to the intrinsic toxicity of heated AES wools in short-term assays with rat alveolar macrophages.