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**COMMITTEE ON, CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT. (COT/COC/COM)**

**OVERVIEW OF NANOMATERIAL TOXICOLOGY (March 2005)**

**Introduction.**

1. The risk assessment of nanomaterials has been identified by COT/COC/COM as an area of interest during horizon scanning discussions for 2004. The Committee's interest in this area was prompted by the publication of the royal society review of nanotechnology. (<http://www.nanotec.org.uk/>) The appended documents have been drafted to provide baseline toxicology information for all three committees. These comprise; The objective is to collect initial views from the COC (21 April), COM (26 May ) and COT (12 July) and draft a short statement.

(Annex 1) The HSE review: health effects of particles produced for nanotechnologies

(Annex 2) An update review prepared by the secretariat which is based on the structure of the HSE review but also provides some further information, for example on structure activity of photooxidative effects of fullerenes and the oral administration of nanomaterials.

(Annex 3) Appended selected references. These provide some more information on potential novel structures of nanomaterials.

*For members of COC, an overview of carcinogenic effects of particles by Dr U Henrich (Fraunhofer Insititute, Hannover, Germany) is appended as background information. Members may recall reviewing the animal carcinogenicity data on diesel exhaust and carbon black in 1996. An extract from the COC annual report is appended. One further additional specific reference is appended concerning a skin tumour promotion study undertaken with a fullerene extract (C60:C70 6:1 Nelson et al Tox indust health, vol 9, no 4, 623-630). Additional specific references will be appended for COM and COC.*

**Scope of nanomaterials considered in COT/COC/COM review.**

2. The review is limited to nanomaterials produced by nanotechnologies as considered in the HSE document. Nanomaterials designed for use in human medicines or for medical devises have been excluded. Information from recent studies of chemically defined nanoparticles which have used in studies to simulate ultrafine particles found in air pollution have been included. The current review was not intended to look at studies of ultrafine particle in air pollution which are the responsibility of COMEAP. It is notable that the terminology used by research groups to describe particulates of <100nm does vary with the terms nanoparticle or ultrafine particle used to describe the

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same or very similar materials. There were no studies of carcinogenicity with nanomaterials retrieved. Members may wish to just consider the summary of the HSE review the introduction section of Annex 2 (para 1-6), the summary sections in Annex 2 (paras 12 (ADME), 25 (effects of nanoparticles), 34 (nanotubes), 38 fullerenes)) and the discussion section of Annex 2 (paras 39-48). The generic questions posed below have been drafted for all three committees. A specific question for COC is given in para 5.

3. The Government response to the Royal Society and Royal Academy of Engineering report “Nanoscience and nanotechnologies opportunities and uncertainties..” which was published in February 2005 asks relevant advisory committees (COT/COC/COM are listed in an Annex to this report) asks relevant advisory committees to consider “..issues as they arise and seek to ensure that nanotechnologies will be explicitly mentioned in their terms of reference.”

### Generic questions arising from the review

4. These are outlined in the update review Annex 2, (para 48, page 17) and are reproduced below for ease of reference.

“The Committees are asked to consider the questions in para 48 (as appropriate to each terms of reference) using the subdivision of nanomaterials suggested in the HSE hazard assessment document (i.e. nanoparticles, carbon nanotubes, fullerenes, nanodots, carbon nanofoam) but also including the need to consider the potential for adverse effects following oral exposure.

- (i) What are members views on the approaches used for hazard identification? Are there any adaptations to standard toxicological test methods or novel approaches that members would suggest should be considered?
- ii) From the available information, are there any comments/conclusions that could be reached regarding potential hazards and priorities for future evaluation?
- iii) Given the potential for development of novel structures with potential applications that cannot be currently identified, what approach to monitoring the literature would the committees suggest? Is it possible to make any comments on the potential hazards of such materials.”

### Additional question for COC

5. There were no studies of carcinogenicity with nanomaterials retrieved. Given the absence of relevant data, are members able to make any comments on likely carcinogenic potential from the available information ( e.g the limited studies of effects of SWCNTs on the lung in rodents or the evidence for oxidative/free radical induction with fullerenes).

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**Secretariat March 2005.**

**COMMITTEES ON TOXICITY, CARCINOGENICITY AND MUTAGENICITY  
IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT.  
(COT/COC/COM)**

**OVERVIEW OF NANOMATERIAL TOXICOLOGY (March 2005)**

**Introduction**

2. The risk assessment of nanomaterials has been identified by COT/COC/COM as an area of interest during horizon scanning discussions for 2004. The Committee's interest in this area was prompted by the publication of the royal society review of nanotechnology (<http://www.nanotec.org.uk/>). This overview is based on the recently published review from the Health and Safety Executive (HSE. Hazard assessment document EH75/6, December 2004 entitled 'Health effects of particles produced for nanotechnologies'. (Appended Annex 1 prefaced with a 5 page summary for members convenience) The HSE Hazard assessment document focuses on the occupational health perspective. A literature search was undertaken to identify any further studies published during 2003/4 and those post dating the HSE hazard assessment document which might be of relevance to public health (which could potentially include inhalation, dermal and/or oral exposure to nanomaterials). A number of topics have been identified in this review for consideration. These include the relative importance of particle size, surface chemistry and adsorbed chemicals in determining the potential toxicological effects and whether the studies using defined ultrafine particulates (UFPs) which have been undertaken to investigate the potential effects of ultrafine particles present in air pollution provide information of relevance to the evaluation of nanomaterials produced intentionally from nanotechnologies. A further aspect relates to the observation of the rapid development of new and novel nanomaterials with differing physical and chemical properties and whether any information or generic advice on hazard identification can be derived from the available information on structure and *in-vivo* and *in-vitro* studies.
3. The format of this review paper uses the HSE document as a template to help in the presentation of data. A brief overview of the HSE hazard assessment document is given followed by an overview of additional information and finally a section which identifies possible questions for discussion. A short section is given below to define which nanomaterials are included in this review. The literature review was conducted up to February 2005. The Government response to the Royal Society and Royal Academy of Engineering report "Nanoscience and nanotechnologies

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opportunities and uncertainties..” which was published in February 2005 asks relevant advisory committees (COT/COC/COM are listed in an Annex to this report) asks relevant advisory committees to consider “..issues as they arise and seek to ensure that nanotechnologies will be explicitly mentioned in their terms of reference.” The current review establishes a baseline of information for the COT/COC/COM but as noted in the discussion section there are elements of expertise relating particularly to nanoparticles which are likely to be represented in other advisory committees, in particular COMEAP (Committee on Medical Effects of Air Pollution) .

### **Nanomaterials included in the review**

4. This review covers nanomaterials as identified in the HSE review (i.e. insoluble or low water soluble particles with  $\mu$ metre counterparts, nanoparticles of existing materials and novel nanoparticles (nanotubes, fullerenes, nanodots, and carbon nanofoam). Additional references retrieved provided some information on the physico-chemical characteristics of carbon nanotubes where fluorine or nitrogen are included in the carbon structure of the nanomaterials. (Bettinger 2003, Yadov 2004). A recent review on the synthesis of rare-earth nanowires, nanotubes and fullerene-like nanoparticles reported that it was possible to produce an enormous range of structures and to modify these by inclusion of other rare earth ions or coating with metal nanoparticles with consequent opportunities for use in biology, as catalysts and in optoelectronics. (Wang 2003) Thus the nanomaterials included in any current review can only consider a small minority of possible structures. The information retrieved includes additional information on kinetics following inhalation exposure and in-vitro and in-vivo toxicity data which might potentially have value in hazard identification. Of particular interest is whether the toxicological properties of nanoparticles are a function of size or surface chemistry or of both properties and what, if any conclusions can be reached on the influence of particle size and chemistry on hazard assessment. The HSE hazard assessment document provides evidence to suggest that surface chemistry and particle size both affect the toxicological properties of nanomaterials. The HSE hazard assessment document reviews these properties with respect to the potential effects of nanoparticles on the lung following inhalation exposure. Colvin (2003) argued that the available toxicological data on ultrafine particles (UFPs) derived from occupational exposure situations and air pollution represented chemically heterogeneous materials and would be unlikely to be of relevance with respect to engineered nanoparticles. An overview of some studies where chemically defined poorly or insoluble UFPs have been investigated has been included in this review. Studies which have been based on concentrated air pollution particles have not been reviewed since the exposure material can not be fully defined.

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[The size definition for UFP and engineered nanoparticles (NPs) used in this review is taken from the Royal society report, i.e. one dimension less than 100 nM. As far as is possible the terminology used by study investigators has been used in this review.]

### **Nanomaterials not included in the review.**

5. There is a considerable published literature on the use of nanoparticles to aid or enhance pharmaceutical delivery. Some of these studies have investigated potential toxicity to cell cultures and presented data on kinetics following oral dermal or parenteral routes of administration. These materials include polyalkylcyanoacrylate nanoparticles, (Meisha MS 2005, Gibaud 1994, Maul 1995), chitosan nanoparticles (deCampos 2004), and lipid nanoparticles (Cons 2003, Olbrich 2004). These materials have not been reviewed and neither have some recently proposed specialised medical uses of nanomaterials (e.g microfabricated silicon needles (Chabri 2004). Most authors report altered kinetics for pharmaceuticals adsorbed to nanoparticles compared to standard or microemulsion formulations giving improved bioavailability and in some cases altered toxicity. Most authors attribute altered toxicity to changes in kinetics of the pharmaceutical induced by adsorption to the nanoparticle. However in one instance improved analgesia in mice associated with the oral administration of Dalargin adsorbed to polybutylcyanoacrylate nanoparticles was associated with a toxicological effect of the nanoparticle altering the Blood Brain Barrier rather than enhanced gastric absorption of Dalargin (Olivier JC et al 1999). A number of papers which investigated potential toxicological effects of novel nanomaterials where there is potential for biomedical applications have been included where the data provide some insight into structure activity relationships.
6. There is also a considerable literature on the use of nanomaterials in medical devices such as use of nanoparticulate hydroxyapatite (Huang 2004), titanium/cobalt alloys (Webster 2004) and nanosilical fused whiskers (Xu 2004) to enhance adhesion to bone and stimulation of osteoblast activity. The published data on toxicological investigations of these materials has not been reviewed.
7. Studies on the potential toxicological effects of lithographic produced nanoscale topographies have not been reviewed (examples of *in-vitro* studies, Rice 2003, Anderson 2003, Cousins BG 2004).

### **Additional information on ADME of nanoparticles**

8. The HSE hazard assessment document presents an overview of the available published information on ADME undertaken with

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nanoparticles. A model of deposition in the lung is presented predicting greater alveolar deposition of nanoparticles compared with particles of >100 nm. Two published studies from the Departments of Medicine and Environmental Medicine, University of Rochester, Rochester, U.S.A. report results for respiratory deposition of UFP carbon particles in humans during rest and exercise and in subjects with asthma. (Daigle et al 2003, Chalupa DC et al 2004). Exposures were to carbon particles generated from graphite electrodes (Count Median Diameter in the study for health subjects was 26 nm (SD 1.6 nm) and in the study in asthmatics was 23 nm (SD 1.6nm)) using a mouthpiece inhalation system. Study parameters and results are briefly tabulated below. The authors showed high fractional deposition of UFP carbon particles which increased as the size of particle decreased. They reported that exercise increased deposition by an amount greater than predicted by model calculations. They also reported increased deposition in asthmatics (with clinically diagnosed mild to moderate asthma which included response to albuterol or hyperresponsiveness to methacholine challenge) compared to healthy subjects. In the discussion, the authors noted that enhanced fine particle deposition (i.e. >100nm <1µM) had been previously reported to be increased in patients with chronic obstructive pulmonary disease compared to healthy individuals, but no investigations of COPD using UFPs were retrieved for this review.

Table 1:

Study group	Exposure parameters	Exposure concentrations	Fractional Deposition (Number)	Fractional Deposition (Mass)
Healthy (Rest N=12 (6m/6f))	2 h (10 min rest at end of first hour) minute ventilation 9.0±1.3 L/min	10 µg/m <sup>3</sup>	0.66±0.11  0.80±0.09 (for CMD 8.7 nm)	0.58±0.13
Healthy (Exercise) (n = 7 (2f/5m))	4x 15 min cycles exercise/15 min rest. (Target ventilation 25 L/min/m <sup>2</sup> body surface area) Achieved mean 38.1±9.5 L/min)	10 or 25 µg/m <sup>3</sup>	0.83±0.04  0.94±0.02 (for CMD 8.7 nm)	0.76±0.06
Asthmatics (Rest n= 16 (number of male/females not reported))	(As in the study in health subjects) minute ventilation 13.3 ±2.0 L/min	10 µg/m <sup>3</sup>	0.76±0.05  0.84±0.03 (for CMD 8.7 nm)	0.69±0.07
Asthmatics (Exercise n=16 (number of males/females not reported))	(As in healthy subjects)  41.9 ±9.0 L/min	10 µg/m <sup>3</sup>	0.86±0.04  0.93±0.02	0.79±0.05

- One important area of research described in the HSE hazard assessment document is the potential for systemic translocation of inhaled particles deposited in the lung. There was evidence from the reviewed studies for both rapid translocation and for negligible translocation. No overall conclusion could be reached and a need

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for further studies was identified. The long term clearance kinetics of inhaled UFP iridium (15-20 nm) in rats has been investigated in a recently published study. (Semmler M et al 2004) Clearance was studied at 3 weeks, 2 months and 6 months post exposure of rats (via a cannula for 1-1.5h). The most prominent fraction was retained in the lungs at each time point (26%, 15%, 6% respectively). Clearance occurred via the urine and faeces. Extrapulmonary particle uptake did not continue to increase but decreased with time in the liver, spleen, heart and brain. The authors reported that long term lung retention was similar to previous studies undertaken with insoluble µmetre sized particles. Overall it was concluded that the pattern of fractional clearance of UFP iridium particles was similar to that of µmetre sized particles. (Semmler M et al 2004)

10. No additional information on dermal uptake was identified for this review.
11. A number of studies have investigated oral uptake of nanoparticles. These investigations have been performed as part of studies designed to investigate the uptake of pharmaceuticals. Florence reviewed the literature on oral particle uptake. (Florence 2004) It is likely that much of the data considered by Florence concerns particles developed for pharmaceutical uses which are not the subject of this review. Oral uptake of NPs will depend on the physicochemical properties of the particle, and the processes of aggregation of particles, and adsorption and adhesion to the gastrointestinal tract. Processes of absorption can include uptake by M cells or through enterocytes and may involve diffusion through gap junctions. Translocation to systemic tissues will occur via the lymph or blood. The author notes experiments undertaken with 500nm sized polystyrene particles (i.e particles which are above the definition for NP material) which showed translocation as single particles and agglomerates. The author also states that uptake of nanoparticles may be rapid occurring in as little as 1 hour, although no data were presented to substantiate this view and this conclusion may well refer to materials not included in this review lipid NPs used for pharmaceutical delivery. It is possible that absorbed NPs will be rapidly taken up by elements of the RES system and in particular Kupffer cells, although no data relating to particles considered in this review are available. The oral uptake of µmetre sized particles has been demonstrated in a large number of species of mammals (Thanos et al 1999).

[A recently identified study on oral absorption of a single walled carbon nanotube is summarised in para 27 of this overview.]

12. Some evidence for translocation of nanoparticles to the brain via the olfactory nerve have been published. No additional information was retrieved for this review other than one study which used reported retrograde transport of fluorescent latex nanospheres in the cerebral cortex of monkey following intracranial injection. Sato Y

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et al 2004). This study is not considered any further in this discussion paper.

### Summary additional data on ADME

13. The available published studies of inhalation exposure report information for uptake consistent with the conclusions reached in the HSE hazard assessment document. One recent study in rats suggests that uptake and extrapulmonary distribution and clearance of inhaled nanoparticulate iridium is similar to  $\mu$ metre sized iridium. This information adds to the data summarised in the HSE hazard assessment document. There is evidence from studies in volunteers that disease status may affect uptake of ultrafine particles via the respiratory tract. There are very little relevant data on oral and dermal kinetics relating to nanoparticles considered in this review. [Some preliminary *in-vivo* data on a SWCNT is summarised below in para 27]

### **Review of toxicology of nanomaterials**

#### *Overview of HSE hazard assessment document: Comparison of NPs with $\mu$ metre counterparts*

14. Inhalation exposure to nanoparticulate  $\text{TiO}_2$  produced comparatively more respiratory tract toxicity than the equivalent mass of  $\mu$ metre-sized material. When expressed on a surface area basis the comparative respiratory tract toxicity was similar. There was no useful information to compare the systemic toxicity of NP material following inhalation exposure with  $\mu$ metre sized counterparts. The available evidence suggests that NP material is not absorbed dermally.

#### *Additional data retrieved*

##### *In-vivo study*

15. A number of intratracheal studies have been undertaken using polystyrene UFPs (60nm) which are either uncharged, (negatively charged (carboxylated or positively charged (amine modified) compared to  $\mu$ metre sized equivalent particle polystyrene particles (400 nm, uncharged or amine modified). Doses of 5  $\mu\text{g}$ , 50  $\mu\text{g}$  or 500  $\mu\text{g}$  were administered intratracheally to groups of male and female hamsters. In separate experiments lung tissue was taken 1 h post dose for histology or Bronchoalveolar lavage (BAL) was undertaken. A separate group of animals was anaesthetised, the femoral vein exposed. Thrombi induced by Rose Bengal administration were monitored by microscopic examination of the femoral vein (under trans illumination) 1 hour after dosing with NP or 400 nm polystyrene. The authors reported that unmodified and negative nanoparticles did not modify thrombosis or BAL indices. Positive nanoparticles increased thrombosis at 500  $\mu\text{g}/\text{animal}$

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(+341± 96%) and at 50 µg/animal (+533 ± 122%) but not at 5 µg/animal. Neutrophils, LDH (at all dose levels), and histamine were increased in BAL at 50 and 500 µg /animal but protein concentrations were only increased at 500 µg/animal. Positive 400 nm particles (500 µg /animal) did not affect thrombosis, although they led to neutrophil influx and an increase in BAL proteins and histamine. . Additional *in-vitro* studies reported that positive nanoparticles and 400 nanometre particles led to the activation of hamster platelets. The authors concluded that positive nanoparticles but not 400 nm polystyrene particles enhanced thrombosis in this animal model. (Nemmar et al 20030

### Summary: Comparison of NPs with umetre sized particulates

16. Thus the additional retrieved information provides evidence for systemic effects (potential thrombogenesis) following intratracheal administration on a positively charged nanoparticle but not following dosing with the equivalent µmetred sized particle.

### *Overview of HSE hazard assessment document: Differences/similarities between NPs of existing materials*

17. Differences in pulmonary toxicity were reported following intratracheal administration of NP Co, Ni or TiO<sub>2</sub>. This may be due to differences in oxidative damage induced by these materials or disagglomeration following instillation. There were no studies which compared different nanoparticle material when administered dermally.

### *Additional data retrieved.*

### Study in human volunteers.

18. The research group from the Department of Medicine and Department of Environmental Medicine, University of Rochester, Rochester, U.S.A. have published the first investigation of exposure to UFPs in human volunteers. The study involved an investigation of the effects of UFP carbon (CMD 25 nM, MMAD 35 nM) on pulmonary function, diffusing capacity, and inflammation in healthy and asthmatic subjects exposed using similar protocols to those reported in table 1 above. Healthy subjects were exposed to 10 µg/m<sup>3</sup>, 25 µg/m<sup>3</sup> or 50 µg/m<sup>3</sup> whilst asthmatics were exposed to 10 µg/m<sup>3</sup>. lung function and airways inflammation were unaffected in healthy and asthmatic subjects except for some findings in healthy subjects. A reduction in midexpiratory flow rate (-4.34± 1.78% (UFPs) vs +1.08 ± 1.86% (air) p= 0.042) and carbon dioxide diffusing capacity (-1.76 ± 0.66 ml/min/mmHg (UFPs) vs -0.18 ± 0.41 ml/min/mmHg (air) p=0.40) at 21 h after exposure (but not at 45 h after exposure) were recorded. The authors noted there were no effects on FEV<sub>1</sub> and considered the data were not clinically

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significant. There were no consistent differences in symptoms, induced sputum, exhaled nitric oxide parameters. The results suggested mild transient small airways dysfunction and impaired alveolar gas exchange in normal subjects. The effects did not appear to be related to airways inflammation. The authors considered that additional studies were required to confirm these findings in normal subjects and to compare with additional susceptible patient populations. This study did not provide any comparative investigations with other UFP particles and there are no human volunteer studies in the HSE hazard assessment document to compare these data with. (Pietrapoli et al 2004)

### Studies in experimental animals

#### *Respiratory tract effects*

19. Halothane anaesthetised Female Wistar rats (250-300 g) were given an intratracheal dose of either 62.5 µg or 125 µg UFP carbon black suspended in sterile saline with or without 100 µM or 500 µM ferric chloride. The lungs were excised 18 h post dosing and a saline lavage undertaken. A dose of 125 µg UFP carbon black induced a significant inflammation as indicated by increase in the number of neutrophils in the lavage fluid. The 100 µM ferric chloride did not induce neutrophil influx compared to controls (saline). The authors reported that instillation of both UFP carbon black and ferric chloride (100 µM) resulted in a significantly increased neutrophil numbers in lavage fluid compared to UFP carbon black alone. (P,0.05, data for 62.5 µg UFP carbon black or 500 µM ferric chloride not reported). This study did not provide information on different nanoparticle materials. (Wilson et al 2002)

#### *Systemic effects*

20. The effects of inhaled UFP carbon particles (CMD 36 nM, 150 µg/m<sup>3</sup> for 6h) on systemic inflammatory cell oxidant stress, the acute phase response, lung inflammation and coagulability were measured in aged (23 months) F344 rats or hypertensive (SH, 11-14 months of age) rats treated with lipopolysaccharide (LPS, 2mg/kg, i.p.), immediately prior to inhalation exposure to UFP which was intended to simulate a compromised cardiopulmonary system. Twenty-four hours after treatment with LPS did not result in an influx of neutrophils (PMNs) into the alveolar space but did increase the number of circulating PMNs and the concentration of plasma fibrinogen in both strains of rats. Inhaled UFP (in rats not treated with LPS) did not induce lung inflammation in either strain of rat but was reported to decrease the number of blood PMNs, increase intracellular oxidation of 2,7 dichlorodihydrofluorescein, (i.e formation of reactive oxygen species), increased the formation of thrombin-anti-thrombin complexes (in SH rats but not aged F344 rats), increased fibrinogen concentration in F344 rats (decreased in SH rats). The lack of an inflammatory response in the lung is in

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contrast to studies of nanoparticles reviewed in the HSE Hazard Assessment document and also previous studies in both young and old rats undertaken using UFP carbon particles by the same research group. Limited weight in terms of helping to identify potential hazards for NP assessment has been placed on these data given the lack of consistency between studies undertaken by these authors and with the data in the HSE review. (Edler 2004)

21. The potential for systemic UFPs (carbon black) to induce thrombogenic and/or inflammatory changes in the liver were studied in mice 2h after intra-arterial administration ( $1 \times 10^7$  and  $5 \times 10^7$  NPs, 60% <100nm) to anaesthetised mice. Platelet and leukocyte endothelial interactions were assessed by intravital microscopy of the excised left liver lobe. Cells were labelled chromogenically (e.g. platelets with rhodamine G) and infused. Sinusoidal permeability and fibrin deposition were assessed by FITC microscopy. The phagocytic activity of Kupffer cells were assessed using FITC labelled latex particles. Liver tissue was subsequently taken and immunohistology for fibrinogen, P-selectin and von Willerbrand factor undertaken. Apoptosis was assessed using TUNEL. The authors reported significantly enhance platelet accumulation on the endothelium of post sinusoidal venules and sinusoids. Platelet adhesion was associated with fibrin deposition and increased vWF expression on the endothelial surface. In contrast inflammatory factors and the number of apoptotic cells were unaffected by treatment, Kupffer cell function was not altered by NP treatment. The authors concluded that this study had demonstrated for the first time that carbon black nanoparticles could accumulate in the liver, induce a procoagulatory effect in the absence of an inflammatory reaction. (Khandoga et al 2004)

### *In-vitro studies*

22. Exposure of Mono Mac 6 human monocytes overnight to UFP carbon black (14 nM, 15 µg/ml) resulted in significant induction of intracellular reactive oxygen species (ROS, cf approximately 30 fold compared to control) formation as measured by 2,7 dichlorofluorescein intensity, whereas incubation with fine carbon black particles (260 nM, 15 µg/ml) had no effect. (Wilson 2002)
23. The effect of a number of nanoparticulate materials on microvascular isolated juvenile foreskin endothelial cell viability, proliferation (Ki67 immunohistochemistry), the release of the proinflammatory marker IL-8 and cell structure (intracellular F-actin organisation) was measured following a 72 hour incubation (37°C using passage 4 cells). Nanoparticles tested were;

SiO<sub>2</sub> 14 nM (4-40nm)  
TiO<sub>2</sub> 70 nM (20-160 nm)  
Co 120 nM (50-200 nm)  
Ni 50 nM (agglomerated to 40-420 µm)

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PVC 130 nM (60-170 nm)  
(at 0.5, 5, 50 µg/ml)

24. The authors noted that all nanoparticles tested were internalised by endothelial cells with the exception of Ni. Most internalised particles were found in autophagic vacuoles. No other morphological changes were noted with the exception that for CO a number of vacuoles appeared enlarged containing whorl shaped membrane complexes with vacuoles (probably as a result of vacuole fusion). A number of annular shaped electron dense material was noted in Co containing vacuoles which probably represented agglomerations. There was evidence for a dose related decrease in cell viability in cells treated with Co. Evidence for decreased cell proliferations was reported for Co (13% at 5 µg/ml and 50% at 50 µg/ml ) and with SiO<sub>2</sub> (10% at 50 µg/ml). Exposure to 50 µg/ml Co or SiO<sub>2</sub> resulted in significantly increased release of IL-8. A small statistically significant and but reproducible effect was documented for 50 µg/ml TiO<sub>2</sub>. Evidence for F-actin reorganisation was seen with 50 µg/ml Co (an effect which was similar in a number of respects to that induced by TNFα). Overall the authors considered that the effects seen in cells treated with NP Co were similar to those which could be attained using a solution of Co ions. Proinflammatory effects were seen with Co, SiO<sub>2</sub> and minor effects were seen with TiO<sub>2</sub>. The effects seen with Co occurred in the presence of reduced cell viability. The authors remarked on the small increase in release of IL-8 seen with NP TiO<sub>2</sub> in the context of the known biocompatibility of this large particle sized TiO<sub>2</sub> (Peters K et al 2004).
25. UFP carbon black (14nM) was incubated with a confluent layer of murine macrophage cell line J774 (at 0.39 µg/mm<sup>2</sup>) for 4h at 37<sup>0</sup>C. In a number of studies the cells were also exposed to ferrous sulphate or ferric chloride (from 0-1000 µM). A UFP concentration related increase in tumour necrosis factor-α production was observed which was not affected by the presence of iron. (These cells sequestered or chelated iron without any toxicity.) (Wilson MR et al 2002).

### Summary: Comparisons between different nanoparticles

26. Thus overall these studies provide evidence that intratracheal administration of nanoparticulate carbon black and ferric oxide together resulted in a greater pulmonary toxicity than either of these two material separately. However the relevance of these data to potential human exposure are unclear. Intra-arterial administration of UFP CB to rats demonstrated that accumulation in the liver with subsequent effects may occur. The relevance of this route of administration to routes of potential human exposure limits the value of these findings. An *in-vitro* comparative study of different nanoparticles cultured with microvascular isolated juvenile foreskin showed that Co, SiO<sub>2</sub> and to a lesser extent TiO<sub>2</sub> could induce a

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proinflammatory response which was not seen with PVC nanoparticles suggesting that the specific properties of different nanoparticles may be important regarding potential local effects. These latter data support the findings reported in the HSE hazard assessment document regarding effects on the respiratory tract. No conclusions could be reached regarding NP Ni from the in-vitro studies since the agglomerated test material was not internalised by cells. [One additional study not retrieved in the HSE review in human subjects exposed to fine/ultrafine magnesium oxide fume is reported below in para . There was no evidence in these individuals for pulmonary inflammation.]

### *Overview of HSE hazard assessment document: Novel Nanomaterials.Nanotubes.*

27. Intratracheal administration of a SWCNT to rats resulted in pulmonary inflammation and granuloma formation similar to SiO<sub>2</sub> which was not reported in equivalent studies using CB. *In-vitro* studies suggest that SWCNT have the potential to induce oxidative damage and to cross cell membranes.

### *Additional retrieved information: Carbon nanotubes*

28. Studies on the biodistribution of a SWCNT (radiolabelled with I<sup>125</sup>) were undertaken using different routes of administration to male KM mice. This potentially important study is appended for COT members. A raw soot of a SWCNT (ca 1.4 nM diameter) was purified to 90%. Groups of five mice were given an i.p. dose of 100µl (15µg/ml, 3.52 x 10<sup>6</sup> cpm/ml) and animals sacrificed at various intervals and levels in tissues, blood determined. In a separate set of experiments groups of animals were given the same dose by i.p, i.v, oral (gavage) or s.c administration. Animals were sacrificed after 3 hours and levels of radiolabel determined in various tissues. Control studies were undertaken using inorganic NaI<sup>125</sup>.
29. The authors reported measurable levels of radioactivity in all tissues studied by all routes of administration with the exception of the brain. The authors remarked on the evidence for retention of material in bone tissue. The authors measured elimination over a period of 11 days following i.p administration and reported that 80% of administered material was eliminated (with 94% in the urine and 6% in the faeces).. These data suggest that SWCNTs can be readily absorbed and distributed. However the purity of the test material was somewhat low in comparison to conventional ADME studies. In addition the authors did not report on the percent oral absorption which limit the usefulness of these data. (Wang H et al 2004)..

### *In-vitro studies SWCNTs*

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30. Samples from commercially produced SWCNT (Carbon nanotechnologies Inc; 0.78 µg/ml up to 200 µg/ml ) were incubated with human embryo kidney HEK 293 cells for periods of 1-5 days. Cell morphology, viability, proliferation, adhesion, flow cytometry, DNA fragmentation, western blot analysis (both in presence and absence of incubation using fetal calf serum), and transcriptomics were undertaken. cRNA transcriptomics was undertaken using a custom made array (100 probes focusing on cell cycle, apoptosis and signal transduction). Treated cultures (25 µg/ml for 2 days) were visualised using Cy-5 whereas control cultures were treated with Cy-3. Detection used Affymetrix 428™ array scanner using appropriate software for background detection and normalisation. Three independent experiments were undertaken.
31. SWCNTs inhibited cell proliferation, decreased cell adhesive ability in a dose-and time-dependent manner. HEK 293 cells secreted 20-30Kd proteins which were wrapped around SWCNTs and produced aggregation of cells attached by SWCNTs and formation of nodular structures. Cell cycle analysis showed that 25 µg/ml SWCNTs induced G<sub>1</sub> arrest and cell apoptosis. Using a cut off value of differential expression of >2.5 compared to controls, the authors reported up-regulation of cell cycle genes such as *p16*, *bax*, *p57*, *hrk*, *cdc42* and *cdc37* and down regulation of cell cycle genes such as *cdk2*, *cdk4*, *cdk6*, and *cyclin D3* and down regulation of signal transduction genes such as *mad2*, *jak1*, *ttk*, *pcdha9* and *erk*. Western blot analysis showed SWCNTs can reduce the expression of adhesion-associated proteins such as laminin, fibronectin, cadherin, FAK and collagen. Overall the authors concluded that SWCNTs can inhibit HEK 293 cell growth by inducing apoptosis and decreasing cell adhesion ability. They reported that no effects were seen in any of the end points measured at a concentration of 1 µg/ml . There are no appropriate studies to confirm these effects *in-vivo* and no information was provided on the structure and/or chemical specification of the SWCNT used. (Cui et al 2005)

*In vitro studies: MWCNTs*

32. Samples of laboratory prepared MWCNTs (produced by plasma enhanced chemical vapour deposition using iron catalysts, approximately 100 nm in diameter, and up to 50 µm in length) were incubated with confluent cultures of pooled neonatal human epidermal keratinocytes for 1,4,8,12,24, or 48 hours. Concentrations used were 0.1,0.2,0.4 mg/ml. Cell morphology was examined and cell viability (neutral red) and release of IL-8 measured. In a number of studies the test material was filtered (0.2µm) to remove aggregates. The morphology of MWCNTs was described as bamboo shoots. Elemental analysis of the test material and cells treated with MWCNTs indicated that all iron had been removed.

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33. Chemically unmodified MWCNTs were reported to be internalised in HEKs showing a dose and time dependent increase. Most were associated with vacuoles, although some were present in the free cytoplasm. The authors reported that these findings could be confirmed in monolayer cultures. MWCNTs in cells were below 3.6  $\mu\text{m}$  in length (although the authors consider that the microscopic approaches used might have missed longer MWCNTs). The authors reported a significant dose-related release of IL-8 at 24 hours treatment from HEKs cells and a slight but dose-related decrease in cell viability at 24h and 48h. Studies which used filtered MWCNTs reported the presence of MWCNTs in only a few isolated cells and no effect on IL-8 release. The authors conclude that MWCNTs showed the potential for localising within and initiating an irritation response in dermal epithelial cells. They acknowledged that HEK cell system used lacked a stratum corneum protective layer. (Monteiro-Riviere NA et al 2005).

### *In-vitro study of SWCNTs, MWCNTs and fullerenes on potassium ion channels*

34. Laboratory derived SWCNTs (mean diameters reported to be 0.9 nm and 1.3 nm) and MWCNTs (mean diameters (10-15 nm) and fullerene onions (3-5 nm) were incubated with CHO cells carrying transfected cDNA for potassium channels from a variety of sources (Caenorhabditi elegans, EXP-2, KVS-1, human KCNQ1 and Kv4.2 and HERG (human ether a go-go related gene). Electrophysiology was undertaken to investigate the potential of novel nanomaterials to inhibit potassium channels. The authors reported that SWCNT reversibly inhibited all types of potassium channel studied (with the exception of the endogenous channel present in CHO cells) but that effects seen with SWCNTs of diameter 0.9 nm were significantly greater than 1.3 nm material. The evidence suggested that SWCNTs increased the number of inactivated potassium channels (possibly through stabilising the inactive site or accelerating the transition from open to inactive. No effects were seen with MWCNTs of fullerenes. The authors proposed that SWCNTs were a universal class of potassium blocking agents probably working by fitting into the pore. These studies support the view that the nanomolecular structure of nanomaterials influences interaction with potential target molecules in or on cells. (Park K et al 2003)

### Summary Nanotubes

35. Some preliminary evidence in mice has been published since the HSE hazard assessment document to show that SWCNTs are absorbed and distributed. There is evidence of wide distribution to tissues with the exception of the brain and some evidence for

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specific distribution to the bone. Additional *in-vitro* studies using SWCNTs provided evidence for uptake into human embryo kidney cells with result apoptosis and decreased cell viability. Additional studies with MWCNTs using human epidermal keratinocytes showed that internalisation was significantly reduced when the test material was filtered to remove agglomerates. A comparative study of the potential for SWCNTs, MWCNTs and fullerenes to inhibit transfected potassium channels in CHO cells demonstrated that SWCNTs with a diameter of 0.9nm could reversibly inhibit potassium channel function.

### *Overview of HSE hazard assessment document: Novel Nanomaterials.Fullerenes*

36. There are comparatively little data on fullerenes. The evidence suggest these compounds do not induce skin irritation. One study suggested that fullerene (C<sub>60.70</sub>) was not a tumour promoter. There is some evidence that C<sub>60</sub> fullerene may induce oxidative damage and is a bacterial mutagen following irradiation with visible light. "

### *Additional retrieved information on in-vitro studies of fullerenes*

37. C<sub>60</sub> fullerenes are considered as having potentially useful application in biomedical applications (e.g. inhibition of HIV protease, neuroprotection of CNS damage (induced by free radicals), and photodynamic therapy of certain tumours. The C<sub>60</sub> fullerene requires modification to enhance water solubility but this results in enhance toxicity in in-vitro test systems.
38. Some additional retrieved studies help to provide insight into the the structure-activity for photoinduced cytotoxicity. Attachment of various sugar molecules ( a range of mono and bisugar derivatives) to C<sub>60</sub> fullerene resulted in formation of singlet oxidation under laser irradiation (355nm). Monosugar derivatives exhibited photocytotoxicity in HeLa in a study where cells were incubated with the test material (25 µm for 6h), washed and then subject to irradiation. The lack of activity of bisugar derivative was considered to be due to either low potency for singlet oxygen formation or poor uptake into cells. (Mikata Y et al 2003). In separate studies the photocytotoxicity of malonic acid derivatives of C<sub>60</sub> fullerene in HeLa cells was dependent on the number of malonic acid substituents with Dimethyl>Trimethyl>quaternarymethyl malonic acid. Part of the enhanced activity of DMA C<sub>60</sub> was found to be due to inhibition of the cell cycle by this compound (Yang XL et al 2002). In a separate series of *in-vitro* tests the haemolytic properties (in human red blood cells) of a series of bialkyl substituted C<sub>60</sub> fullerenes was demonstrated to be dependent on the number of cations present in the alkyl groups. This correlated well with the predicated surfactant and haemolytic activity of a reference alkyl surfactant and calculated total hydrophobic and hydrophilic surface area. All of the substituted C<sub>60</sub> fullerenes that were haemolytic were also

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cytotoxic (in a range of cells including MCF-7, Hep2G and LLC-PK<sub>1</sub>). However the reference alkyl surfactant was not cytotoxic. The cytotoxicity of the substituted fullerenes occurred at lower concentrations than haemolysis. (Bosi et al 2004).

### Summary Fullerenes

39. These studies provide further evidence to demonstrate phototoxicity of fullerenes in-vitro and some preliminary evidence regarding the structural influence of fullerene side chains in this effect. No appropriate in-vivo studies are available to demonstrate phototoxicity. The potential structure activity for photo- induced mutagenicity has not been studied.

### **Discussion/Questions for consideration**

*Literature reviewed: Additional data from air pollution research groups.*

40. The Objective of this overview has been to provide baseline information for the Committees to reach an initial position on the nanomaterials included in the review particularly with respect to the generic questions outlined in para 48 below. The review hasn't included nanomaterials specifically used in medicines or medical devices as these are the responsibility of the MHRA. Evidence from studies which used chemically defined ultra fine particles where the key objective of the study was to provide information relevant for air pollution have been included in order to assist in the evaluating whether exposure to nanoparticles can result in adverse effects at distant sites from the route of exposure. In this respect evidence from studies using concentrated air pollution (e.g Cambell (Neurotoxicology, 26, 133-140, 2005) and colleagues have recently published a study showing proinflammatory responses in the mouse brain following exposure to concentrated air pollution particles) or diesel particles haven't been summarised since the test materials haven't been defined and the information is less useful regarding the generic questions posed for COT/COC/COM. In addition the evaluation of air pollution is the responsibility of the Committee on Medical Effects of Air Pollutants (COMEAP) who have also been requested to provide advice on nanomaterials.
41. The literature search for this review concentrated on 2003/4 in order to supplement the HSE hazard assessment document. However there are a number of older published papers not cited in the HSE review from research groups (such as Oberdorster and colleagues from the Department of Environmental Medicine University of Rochester, New York, U.S.A. and from Donaldson and colleagues from the Department of Environmental and occupational Medicine, University of Aberdeen) which used defined UFPs predominantly to model the effects of particles in air pollution. These studies provide

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additional information on deposition and uptake following inhalation in humans including individuals with chronic obstructive pulmonary disease. The conclusions reached in the HSE hazard assessment document are consistent with these studies.

42. There are also a number of older published papers not cited in the HSE review which investigated inhalation/intratracheal administration of defined UFPs to experimental animals and *in-vitro* experiments using mammalian or human cells which were not cited in the HSE review. These studies provide additional information on the inflammation, particle uptake, oxidative stress and conformational changes in proteins. One paper worthy of specific mention is the investigation by Baggs RB et al (from Oberdorsters' group) who investigated the regression of pulmonary lesions produced by inhaled titanium dioxide ( 20 nm or 250 nm at approximately 23 mg/m<sup>3</sup> for 6h/day, 5 days/week for 3 months followed by sacrifice at 6 months or 12 months after completion of exposure). (Silicon dioxide 800 nm (1.3 mg/m<sup>3</sup> was used as a positive control). The degree of pulmonary fibrosis was SiO<sub>2</sub>>TiO<sub>2</sub> (20 nm)>TiO<sub>2</sub> (250 nm). After one year fibrosis had decreased in the SiO<sub>2</sub> group but was still present, whereas fibrosis had largely returned to control levels in the TiO<sub>2</sub> groups (i.e at both 20 nm and 250 nm). (Baggs RB et al Vet Pathol, 34, 592-7, 1997.)
43. A further study from Kuschner WG et al (Environmental Health Perspectives, 105, 1234-7, 1997) reported a lack of evidence for pulmonary inflammation in a small number of subjects following exposure to magnesium oxide fumes (containing ultrafine particles). There would be appropriate expertise regarding these studies on COMEAP.

### *Limited data for oral exposure*

44. The lack of appropriate toxicological studies for nanomaterials was cited in the HSE hazard assessment document and this is also evident from the available information presented in this review. The lack of data appears to most evident for oral exposure possibly because the most evident areas of exposure to nanoparticles are occupational or via air pollution. Thus there is evidence to suggest that airborne agglomerates of nanomaterials fragment into many different nonparticulate forms in the human respiratory tract (Murr LE and colleagues have recently published information for a range of manufactured nanoparticles and air pollution (J of materials Science: Materials in Medicine, 15, 237-247, 2004)). However there is no reliable information regarding disagglomeration for nanomaterials considered in this review following oral exposure. Florence suggests in her review of nanomaterials predominantly used for medicinal applications that agglomeration in the gastrointestinal tract is likely to occur. One preliminary report using a SWCNT provides evidence for widespread distribution in mice

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following administration via a number of routes including oral and s.c administration.

45. However the potential that ultrafine particles might be involved in adverse effects following oral exposure can't be discounted. One published paper was retrieved which reported on measurements of immune potentiation (release of interleukin-1) from peripheral blood mononuclear cells and colonic biopsy specimens from patients with ulcerative colitis, Crohn's disease and healthy controls. Samples (*ex-vivo*) were treated with lipopolysaccharide (LPS from *E.coli*), or TiO<sub>2</sub> (200 nm, i.e just outside the upper limit used for defining nanoparticle) or TiO<sub>2</sub> adsorbed onto LPS. The authors recorded a 30-60 fold stimulation of peripheral blood mononuclear cells using TiO<sub>2</sub> adsorbed onto LPS compared to either material alone. In intestinal cultures there was no increase in IL-1 secretion when using TiO<sub>2</sub> or LPS alone and a 2-3 fold increase in healthy subjects and patients with ulcerative colitis using TiO<sub>2</sub> adsorbed onto LPS. The authors suggests that ultrafine TiO<sub>a</sub> may be an important adjuvant in the gastrointestinal tract. (Powel JJ et al, J of Autoimmunity, 14, 99-105, 2000, appended for COT members)

### *Novel structures.*

46. The information summarised provided does aid in the evaluation of relative contribution of particle size/structure and chemistry regarding toxicological effects of the materials studied showing that both can be important. However the range of new materials likely to be produced onto the market could potentially include some quite novel structures. Some examples are given in the appended papers (Annex 4) from Cohen and Mahadevan (PNAS, 100, 12141-12146, 2003) and Needleman DJ et al (PNAS, 101, 16099-1613, 2004). It is also possible that nanomaterials may induce interactions with mammalian biological systems which may not be predicted from currently available toxicological data. Thus Sommer (J Proteome Research, 3, 667-669, 2004) undertook some *in-vitro* studies with 60 or 200 nM polystyrene spheres may self assemble on suitable surfaces in the body. Although there are no appropriate *in-vivo* studies to evaluate the possibility of such a response, the information is provided to indicate that there are potential uncertainties in the hazard evaluation of novel nanomaterials.

### *Limited information on mutagenicity testing.*

47. The COM considered this aspect during its horizon scanning exercise. Little additional information has been retrieved (some data for fullerenes are identified in the HSE hazard assessment document). Currently , there is little data on the genetic toxicity of nanoparticles. Micronized zinc oxide (0.2µm) has been shown to be more clastogenic than unm micronized chemical (SCCNFP/0649/03). Similarly, Rahman et al (2002) provides

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evidence that ultrafine titanium dioxide, a normally inert compound, induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. It is postulated that nanoparticulates stimulate phagocytosis, thereby generate the production of reactive oxygen species

### *Questions for committees*

48. The Committees are asked to consider the questions in para 48 (as appropriate to each terms of reference) using the subdivision of nanomaterials suggested by the HSE hazard assessment document (i.e. nanoparticles, carbon nanotubes, fullerenes, nanodots, carbon nanofoam) but also including the need to consider the potential for adverse effects following oral exposure.
49. (i) What are members views on the approaches used for hazard identification? Are there any adaptations to standard toxicological test methods or novel approaches that members would suggest should be considered?
- ii) From the available information, are there any comments/conclusions that could be reached regarding potential hazards and priorities for future evaluation?
- iii) Given the potential for development of novel structures with potential applications that cannot be currently identified, what approach to monitoring the literature would the committees suggest? Is it possible to make any comments on the potential hazards of such materials.

**Secretariat March 2004**

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