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ENVIRONMENT DIRECTORATE
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Working Party on Manufactured Nanomaterials

DRAFT DOSSIER DEVELOPMENT PLANS: REFLECTIONS OF SG7

7th Meeting of the Working Party on Manufactured Nanomaterials taking place at OECD Conference Centre in Paris, France on 7-9 July 2010, starting at 10h00 on the first day

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This text was first developed by SG7 for discussion at the Expert Consultation Meeting on Alternative test Methods in Nanotoxicology [ENV/CHEM/NANO(2010)6]. After consultation with the Bureau, it was agreed to share this document with SG3, SG7 and the WPMN.

The SG7 Expert Meeting highly appreciated this initiative for better information sharing in particular for *in vitro* assays that are being used or are planned to be used by the sponsorship programme.

This document is presented as a discussion paper and it is proposed that it be kept as a living document that is updated when vital information becomes available on *in vitro* and alternative assays, *e.g.* with the additional draft DDPs, SOPs availability etc. The document can also serve as a means for data sharing between SG3, SG4 and SG7.

ACTION REQUIRED: The WPMN is invited to take note of this document and to amend as appropriate.

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INTRODUCTION

1. OECD's Working Party on Manufactured Nanomaterials (WPMN) established a project entitled "Safety Testing of a Representative Set of Manufactured Nanomaterials" (SG3) to identify and test a representative set of manufactured nanomaterials using appropriate test methods that would include OECD Test Guidelines or other internationally agreed methods. The WPMN agreed that the project would move forward in two stages, the first stage being to develop and agree to a priority list of representative Manufactured Nanomaterials (MNs) for inclusion in a set of reference nanomaterials for which development of data would support characterization, measurement, toxicological and ecotoxicological testing, and risk assessment or safety evaluation of MNs. The second stage will develop a programme to create a better understanding of MNs that may be relevant for exposure and effects of nanomaterials by testing representative nanomaterials for human health and environmental effects, as well as physical-chemical properties and environmental fate for a specific set of endpoints. It was agreed that the dataset developed through this programme would be of an exploratory nature. This programme would also be science-based, open to all stakeholders and without pre-defined regulatory consequences for any datasets.

2. At the third meeting of the WPMN (November 2007), the list of representative MNs and list of endpoints were adopted and recommended to be declassified by the Chemicals Committee. At the same time, the WPMN agreed to a recommendation for launching a "sponsorship" testing programme for certain MNs. Such a "Sponsorship Programme" establishes a process to conduct, as appropriate, specific tests for those endpoints on those MNs agreed by the WPMN, which will be described in Dossier Development Plans for each MN. For further information see the "*Guidance manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme*" (SG7 Meeting Document #8).

PURPOSE OF DOCUMENT

3. One important role of the SG7 is to support and advice on further developments of alternative test methods and integrated testing strategies and their validation, for further use in the Sponsorship Programme. This activity requires a feed-back mechanism from SG3 to SG7 with information on what assays are applied, the protocols and the results and data from concluded testing for the individual MNs. Since testing has basically just started and no information have been available prior to the submission of the revised draft DDPs for CeO2, ZnO, SiO_2, Fullerenes, SWCNTs and MWCNTs, respectively, in mid-April 2010, the SG7 have not had a possibility to review any information from SG3 yet. However, the draft DDPs have given the SG7 a possibility for a first dissemination of the testing done and what is proposed to be done in the near future. This document aims at providing extracted information from the DDPs on what tests are being used for the different MNs to SG7, in addition to also providing a tool for communication between SG7 and SG3 on individual assays, available SOPs, etc., in the further development of new alternative tests.

ITEMS FOR DISCUSSION

4. Not to disclose any confidential information from the DDPs prior to their publication, the Secretariat have extracted the most central information relating to human health toxicity testing and this information has been compiled and is available as Annex 1 to this document. Participants will be invited to discuss the approaches and proposed testing schemes for each individual MN and give further recommendations regarding the selected test methods, available alternatives and proposed integrated testing strategies. The issue whether Standard Operating Protocols (SOP) are available for any of the proposed *in vitro* assays should also be discussed by the SG7 meeting.

I. Test Guidelines Used in the Sponsorship Programme

5. A draft compilation of the individual Test Guidelines used for the different MNs is available in Annex 2 and the SG7 is invited to discuss the selected Test Guidelines and especially the fact that from Annex 2 it can be deducted that only three Test Guidelines (TGs 403, 412 and 471) have so far been used/proposed for all six MNs.

II. Available Alternative Test Guidelines

6. It should be noted that a number of new alternative OECD Test Guidelines are available that were not considered in the SG4 Test Guideline Applicability Review, since at that time they were still in the process of development. These comprise the fully adopted TGs:

TG 432 on "*In vitro 3T3 NRU phototoxicity test*"

TG 435 on "*In vitro skin corrosivity*"

TG 436 on "*Acute inhalation: acute toxic class*"

TG 437 on "*Bovine corneal opacity and permeability (BCOP) test method: an in vitro method for identifying ocular corrosives and severe irritants*"

TG 438 on "*Isolated chicken eye (ICE) test method: an in vitro method for identifying ocular corrosives and severe irritants*".

7. There are also five new draft TGs that have been adopted by the WNT and are now submitted to the Joint Meeting (JM) for adoption and there is a very low likelihood that any major changes to these protocols will be made. These TGs are:

Draft **TG 439** on "*In Vitro Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method*"
Draft **TG 487** on "*In Vitro Mammalian Cell Micronucleus Test (MNvit)"*
Draft **TG 442A** on "*Skin Sensitization: Local Lymph Node Assay: DA Version*"
Draft **TG 442B** on "*Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA*"
The updated **TG 429 (**LLNA).

There is also a draft Guidance Document on "*Using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests*" that have been submitted to the JM for declassification that may be applicable for use in the Sponsorship programme.

III. Development of New Alternative Test Methods

8. Due to limited time and information, the Secretariat was only available to extract a few new non-OECD assays out of the DDPs, however, the SOPs and the exact endpoints used are not precisely described for these methods and the SG7 meeting should discuss these methods further. It seems, however,

that there are at least three assays (A549, HaCaT and THP-1) that have been used for all five MNs but their exact endpoints are difficult to conclude and they should be evaluated for the potency of being further developed and standardised.

OUTCOME OF THE SG7 MEETING

9 The draft "*Discussion document for the Bureau, SG7 Expert Consultation Meeting, Sponsorship Programme and WPMN7: The draft Dossier Development Plans for six manufactured nanomaterials in the Sponsorship Programme (SG3)",* was discussed, and the meeting highly appreciated this initiative for better information sharing in particular for *in vitro* assays that are being used or are planned to be used by the sponsorship programme.

10. The meeting expressed a need for setting up a sufficiently easy-to-read excel spreadsheet, clearly describing the work on alternative methods performed in SG3 in a manner to become able to more quickly compare the work highlighted in the DDPs. A small drafting group was formed to work in the margin of the meeting and come up with a suggested template containing all pertinent information needed by the SG7 for review and prioritization of new *in vitro* methods, and this draft template has been attached as annex 3, for further discussions by the SG3 and WPMN.

11. The meeting agreed that annex 2 of the DDP document should be revised to show the recommended/mandatory endpoints as presented in the Guidance Manual for Sponsors.

12. In addition to the above observations, it was pointed out at the meeting that there is no procedure to review DDPs at the moment and the meeting recommended that an appropriate mechanism of review and updating of the DDPs is established by the WPMN, maybe in collaboration with other SGs. Furthermore, the co-chair of SG4, Juan Riego Sintes recognized that the Document on the Review of the Applicability of Test Guidelines for the testing of MNs needs to be updated, in due course, depending on the new information generated by SGs and other sources, regarding the appropriateness of the methods for MN testing.

13. A *DDP Discussion Document (DDPDD) Working Group* co-ordinated by Christoph Klein and with Mario Goetz, Juan Riego Sintes (the two SG3 chairs are also invited to participate!) was established to further work on proposals how to establish information exchange and collaborations between SG7, SG3, SG4 and SG6, especially with regards to the information provided in the DDPs on alternative assays, as agreed upon by the WPMN bureau. The draft Discussion Document will be put forward to the SG3 and WPMN7 meetings in July 2010 for discussion. Actions to be followed-up:

- Annex 1 should be revised and be endpoint-specific (Secretariat)
- Annex 2 has been updated to also include comparison with the testing requirements described in the Guidance Manual for Sponsors
- A template with detailed assay information needed by SG7 for judging on the relevance and reliability, and possible prioritisations of potential alternative test methods should be attached to the Discussion Document and the draft Meeting Report (Annex 3)(Christoph Klein and the DDPDD Work Group).

NEXT STEPS

14. The DDP discussion document were discussed and approved by the Bureau before being put forward to the SG7 meeting as a room document for discussion. The Secretariat revised the document after

the SG7 meeting and will submit it to the SG3 and WPMN7 meetings for further discussions and considerations.

15. The Secretariat proposes that this document is kept as a living document that is updated when vital information becomes available on *in vitro* and alternative assays, *e.g.* with the additional draft DDPs, SOPs availability etc, and the document can serve as a means for data sharing between SG3, SG4 and SG7.

ANNEX 1. Testing schemes for CeO_2, ZnO, SAS, Fullerenes, SWCNTs and MWCNTs [as extracted from the latest draft DDPs in April 2010].

Nano-Cerium Oxide (CeO_2) draft DDP 15 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
1	Pharmacokinetics/toxicokinetics (ADME)	Establishment of an expert group needed.	The Test Guideline 417 on '"*Toxicokinetics*" has recently been updated and sent to the Joint Meeting for adoption, it is unlikely that any major changes to the protocol will be introduced. Even though it has not been developed for the direct testing of nanomaterials it may be applicable.
	Acute toxicity		
2	Oral acute toxicity: TG 420, TG 423, TG 425	To be confirmed	
3	Inhalation acute toxicity: - TG 403 - 5-day inhalation study with 28/90 days post experimental monitoring and periodical Broncoalveolar Lavage (BAL and ADME assessment	The studies will be extended studies of diesel exhaust with and without nano CeO_2 fuel additive. No data available.	
4	*In vitro* testing for cytotoxicity, oxidative stress and inflammation - Human lung cells - Mouse macrophages Testing of human skin cells and immune cells (+/-) UV radiation for cytotoxicity, cytotoxicity mechanisms, oxidative stress and cytokine	SOPs available.	Would the SOPs be available for review by SG7?

	production		
	Repeat Dose Toxicity		
5	Inhalation: - TG 412 (28/14) + studies on blood and brain vessels - TG 413 (90)	To be confirmed.	
6	Other relevant information on *in vitro* methods Alternative test methods/approaches are being conducted on various nanoCeO_2 particles using non-cellular assays to examine the surface properties of nanomaterials to assess their reactivity and interactions with proteins and biochemicals. Non-cellular interactions and surface properties may contribute and regulate nanomaterial cellular uptake and toxicity, and the following assays are applied: TBARS, DCFH assays; antioxidant and second messenger depletion assays; DMPO spin trapping; protein modification assays.		Would the SOPs be available for review by SG7?
	Chronic Toxicity		
7	- TGs 451, 452, 453	To be confirmed.	
	Reproductive and developmental toxicity		
8	- TG 414, TG 415, TG 416, TG 421	Considered if available!	
	Genetic toxicity		
9	In vitro: - TG 471, TG 473, TG 486 In vivo: - TG 474, TG 475, TG 486 Other relevant information (if/where available!) Alternative methods using *in vitro* toxicity testing has been initiated to evaluate various nanoCeO_2 particles for pulmonary, vascular, neurological, mutagenic, cardiac, reproductive toxicities. Objectives of these *in vitro* toxicity testing studies are: 1) assist in designing *in*	Considered if available. TG 471 proposed to be used initially together with the comet assay and the draft TG 487 *in vitro* Micronucleus (MNvit) Testing initiated and some testing finished, publication expected in Environ. Toxicol.	Park et al (2008) and Park and Martin (2009) publications comprise an integrated testing strategy (ITS) and hazard/risk assessment schemes for evaluation of CeO_2 when used as a fuel additive, called Envirox™. The use of the product has been approved by UK regulatory authorities based on the ITS and its individual components and maybe this strategy

	vivo toxicity tests; and 2) identify *in vitro* tests that correlate with *in vivo* toxicity responses for future test validation efforts. *In vitro* toxicity test screening include: - pulmonary toxicity (human airway epithelial cells; macrophages; alveolar epithelial cells) - cardiac toxicity (rat cardiomyocytes) - vascular toxicity (human endothelial cells) - neurotoxicity (rodent glial cells; neuronal cells) - reproductive toxicity (whole rodent embryo culture) - occular toxicity (HLE B-3; APRE 19) - genotoxicity (BEAS2B; Caco2; NCM460). *In vitro* endpoints include: cytotoxicity, cell death mechanism, oxidative stress, cytokine production, cellular uptake of CeO_2, molecular profiling (proteomics, genomics) and specific cellular functional alterations. Testing the cell lines A549, HaCaT and THP-1 for cell viability, colony forming ability, oxidative stress and apoptosis has finished and will be published in Environ. Toxicol.		should be further explored by SG3 in their further testing. The use of a "*rat lung slice assay*" was discussed in detail by the SG7 after a presentation by JP Morin and will be further considered by the SG7. Please note that a draft TG 487 on in vitro micronucleus is available for use.

Nano-Zink Oxide (ZnO) draft DDP 15 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
1	Pharmacokinetics/toxicokinetics (ADME) Skin penetration *in vitro*: The dermal penetration *in vitro* of ZnO containing cosmetic formulation (10.3 % Z-Cote) through dermatomed pig-skin (thickness 400 - 700 µm) was assessed by single topical application of a target dose of about 4 mg per cm² of the test emulsion (corresponding to approximately 400 µg/cm² ZnO or 360 µg/cm² Zn) to pig-skin preparations mounted on Franz-type diffusion cells. The methods used are in line with the guidelines for this study type published by Diembeck et al. (1999), the OECD guideline no. 428 (2004) and Guidance document no. 28 (2004). Skin penetration with sunburnt skin: Assessment of the dermal penetration in vitro of ZnO containing cosmetic formulation (5 % Z-Cote HP1) through intact and sunburnt pig-skin by single topical application of the test emulsion to pig-skin preparations. The pharmacokinetics of zinc from uncoated nanoscale ZnO, coated nanoscale ZnO, and zinc carbonate will be determined in female Fischer 344N/Nctr rats following gavage administration. The rats will have been maintained on a low-zinc diet (zinc free AIN-93 supplemented with 0.38 mg/kg zinc carbonate). The gavage dose of zinc carbonate, Z-Cote and Z-Cote HP-1 will be equivalent to the daily consumption of zinc from a diet with standard levels of zinc. The distribution of zinc in blood and tissues will be determined by inductively-coupled plasma mass spectrometry (ICP-MS) and if needed, the presence of zinc oxide nanoparticles will be conducted using transmission electron microscopy. The dermal penetration *in vivo* (OECD 427) will be analysed (and	Status finished Status ongoing Status: in development Status: in development	The Test Guideline 417 on '"*Toxicokinetics*" has recently been updated and sent to the Joint Meeting for adoption, it is unlikely that any major changes to the protocol will be introduced. Even though it has not been developed for the direct testing of nanomaterials it may be applicable. May consider using the TG 432 on photoxtoxicity!

	in vitro (TG 428), if appropriate).		
	Acute toxicity		
2	Acute dermal toxicity *in vivo* in the rat (TG 402): *In vitro*: skin corrosion/irritation test; human skin model test (OECD 431) The programme is focusing on the inhalative and dermal exposure path (oral path generally is considered as not equally relevant as the other two ones). Acute inhalation testing was not included as the more comprehensive study designs of a 14-day and a 90-day inhalation study can deliver more mechanistic aspects of ZnO-induced effects.	Status planned:	Maybe consider using TG 435 for corrosives and/or TG 439 for in vitro skin irritation.
	Repeat Dose Toxicity		
3	5-day inhalation study, up to 28 day post exposure including Lavage To determine the pulmonary toxicity with Z-Cote HP1 and Zinc oxide, powder < 1µ in rats, this study with short term bioassay including bronchoalveolar lavage with clinico-chemical and cytological evaluation of lavage fluid, pathological examination of the lung and pulmonary cell proliferation measurements was performed. Seventeen male Wistar rats per test group and time point were head-nose exposed to respirable dusts on 6 hours per day, on 5 consecutive days. The target concentrations were from Z-Cote HP1: 0.5, 2.5 and 12.5 mg/m3 and from Zinc oxide, powder < 1µ: 12.5 mg/m3. A concurrent control group was exposed to conditioned air. Animals were sacrificed on study day 4 and 25. 14-day inhalation (TG 412): 5 concentrations (Z-COTE HP1, Z-COTE, micronised ZnO). This 14-day test will be used as a DRF study for the subsequent 90-day study., obligatory endpoints according to OECD guideline (adapted from OECD 412), additional endpoint Bronchoalveolar lavage, toxicokinetics, TEM/SEM	Status: finished (SOP available) Status ongoing:	

	analysis. 90-day inhalation (TG 413): 3 concentrations, 1 concentration fine-size reference material obligatory endpoints according to OECD guideline (adapted from OECD 413), Bronchoalveolar lavage, Cell proliferation analysis, (left lung lobe), Toxicokinetics (OECD 417) (CEFIC) A 90-day subchronic toxicity study will be conducted in accordance with U.S. FDA guidelines (GLP) and U.S. National Toxicology Program (NTP) guidelines. The endpoints will be complete histopathological examination in accordance with U.S. NTP guidelines. 5 day dermal absorption, organ distribution and urine excretion study on hairless immune-competent mice using custom formulated sunscreens containing zinc oxide particles enriched with a stable zinc isotope for tracing purposes (three formulations tested: ZnO nanoparticles, ZnO particles >100nm, and formulation only (no ZnO)); blood, urine, skin and internal organs will be assayed for ratios of zinc isotopes using multi-collector inductively coupled plasma mass spectroscopy.	Status planned: Status: in development Status: 5-day study completed; measurements by mc-ICPMS in progress	
	Chronic Toxicity		
4	The DDP should describe any relevant existing chronic study results of nano-zinc oxide (e.g., results from toxicokinetics and repeated-dose toxicity studies) including carcinogenicity studies. The dossier should report the routes of administration, frequency of dose certification and characterization of nanomaterial, any early endpoints to accompany the chronic toxicity test (e.g., micronucleus test at 1 year), and a list of all tissues histopathologically examined at the end of the study. Consistency of the reported results to OECD TGs 451, 452, 422, and 453 should be considered, and the dossier should contain any descriptions of the study that are different than		

	these guidelines. A chronic toxicity/carcinogenicity study is proposed, depending on the outcome of the subchronic oral toxicity study with Z-Cote and Z-Cote HP-1. This study may be withdrawn in the event there is no evidence that nanoscale ZnO is absorbed as a nanomaterial and that zinc pharmacokinetics is not dependent or altered due to nanoscale ZnO when compared to zinc carbonate. A 2-year chronic study would consist of incorporation of zinc carbonate (control), uncoated nanoscale ZnO (Z-Cote), and coated nanoscale ZnO (Z-Cote HP-1) into the diet at dose levels to be established based on the outcome of the subchronic study. It is expected that the dietary levels will be 38, 125, and 250 mg/kg diet. The test animal will be male and female Fischer 344/N/Nctr rats (48/sex/test group). The study will be conducted under the guidelines of the U.S. Food and Drug Administration (GLP) and U.S. National Toxicology Program (NTP).	Status: proposed	
	Reproductive and Developmental toxicity		
5		Considered if available!	
	Genetic toxicity		
6	In vitro: TG 471 In vivo: TG 474, Erythrocyte MN test in the 14-day study hOOG1-modified comet assay in the inhalation 14-day study AMES mutation frequency test to establish the potential mutagenic effect in bacteria (TG 471) *In vitro* Mammalian Cell Gene Mutation Test (TG 476) *In vitro* Mammalian Chromosome Aberration Test (TG 473) hOGG1-modified comet assay with BAL cells from healthy, unexposed animals, with cells pre-cultured for 24 h prior to *in vitro*	Status; Ongoing Status: Planned	Please note that a draft TG 487 on in vitro micronucleus is available for use.

	exposure.		
7	Other relevant data Testing of human skin cells and immune cells (+/-) UV radiation for cytotoxicity, cytotoxicity mechanisms, oxidative stress and cytokine production *In vitro* testing of ZnO using A549 cell line (carcinomic human alveolar basal epithelial cell line) and multiple cytotoxic endpoints, ROS, GSH/GSSG and interleukin 8 release. These experiments were designed to evaluate the cell type specific sensitivity to ZnO exposure. Up to eleven cell lines representing different routes and different lines of exposure were exposed (A549, CaCo2, HaCaT, MDCK, MDCK II, NIH3T3, NRK52E, RAW267.4, RLE-6TN, CaLu3, T84). Cellular processes such as rapid oxidative stress response, metabolic activity and cell death were determined by standardised LDH release (cell death), DCF (ROS formation) and MTT (metabolic activity) assays. Using a set of different cell lines in order to measure apoptosis Caspase-3 is a key regulator of apoptosis as its activation inevitably leads to programmed cell death. Eight cell lines were tested for the activation of Caspase-3. Furthermore, the integrity of cell-cell contacts in monolayers of MDCK II and NRK52E cells in the presence of ZnO was studied. A certain type of cell-cell contacts (tight junctions) prevent the diffusion of ions across cell layers and thereby establish an electrical resistance across cell monolayers. This electrical resistance is referred to as TransEpithelial Electrical Resistance (TEER). *In vitro* Testing of Pro-inflammatory Effects of ZnO in Coculture Systems In order to study possible pro-inflammatory effects of ZnO *in vitro*, a co-culture model of A549 and the monocyte/macrophage-like cell line Mono-Mac-6 (MM6) was developed. This system provides a relatively simple model of the alveolar response to nanoparticle exposure. An existing inflammatory state can be simulated in the	Status: Planned Status: Finished Status: Finished (SOPs available) Status: Finished Status: Finished	May consider using TG 432 on phototoxicity! Are there any available SOPs that could be shared with SG7?

	coculture model by LPS activation of the monocytes/macrophages. The optimum cell culture conditions, controls, stimulation conditions, cell numbers and ratios as well as the most useful inflammatory parameters to be measured were determined previously in a series of studies or selected according to literature, respectively. In the A549/Mono-Mac-6 coculture model, the proinflammatory potential of ZnO was determined by measuring the release of the inflammatory mediator IL-8 upon 24 hours of exposure. <u>In vitro testing of ZnO</u> Using cell lines of A549, HaCaT, and THP-1, a set of endpoints of cell viability, oxidative stress, DNA injury, colony forming ability, gene expression of cytokine and apoptosis were examined.	Status: Finished	

Synthetic Amorphous Silica (SAS) draft DDP 20 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
1	**Pharmacokinetics/toxicokinetics (ADME)** - 14/90 day Inhalation study (TG not specified; 412/413? - Toxicokinetics (TG not specified; updated 417?)	No test data available yet!	
	Acute toxicity		
2	Acute Inhalation (TG not specified; 403/436?) Acute oral toxicity (TG not specified; 420/423/425?) Acute dermal toxicity (TG not specified; 402?)	No test data available yet! Literature review ongoing for inhalation and oral route.	
	Repeat Dose Toxicity		
3	Sub-acute: Inhalation (TG not specified; 412?) Oral route (TG not specified; 407? Dermal route (TG not specified; 410?) Sub-chronic: Inhalation (TG not specified; 413?) Oral route (TG not specified; 407? Dermal route (TG not specified; 411?)	No test data available yet! Literature review ongoing for the inhalation route.	
	Chronic Toxicity (if available!)		
4	Inhalation, oral route and dermal route	No test data available yet!	
	Reproductive and Developmental toxicity (if available!)		
	Local tolerance- Skin sensitization		
5	Skin irritation (TG not specified; 404/406/439?) Eye irritation (TG not specified (404/437/438?)	No test data available yet! Literature review ongoing.	
	Genetic toxicity		

6	*In vitro:* Reverse mutation (TG not specified; 471?) Mammalian cell micronucleus test (TG not specified; draft 487?) Mammalian cell gene mutation assay (TG not specified; 476?) Mouse lymphoma assay *In vivo:* In vivo germ cell mutagenicity (TG not specified; 485?)	No test data available yet! Literature review ongoing.	

Literature review of available *in vitro* test methods (Table I.3 from draft DDP)

Tested organism	**Reference**	**SAS**	**Size[1] of tested particles**	**Main conclusion in Article**
Mouse monocytes (J774), human bronchoalveolar carcinoma (A549), human endothelial cells (EAHY926)	Lison D, 2008	Ludox HS-40 Stöber silica nanoparticles	29.3+-4. nm (TEM), 35nm (DLS)	Look at the relevance of the dose metric that ought to be used in vitro. They found that the cellular response was determined by the total mass/number/SA of particles as well as their concentration and conclude that the nominal dose remains the most appropriate metric for in vitro toxicity testing of insoluble SNP dispersed in aqueous medium.
Human endothelial cells (EAHY926)	Napierska D, 2009	Ludox L-14/L-15 Stöber silica nanoparticles	14, 15, 16, 19, 60, 104 and 335 nm (checked by TEM and DLS)	The results indicate that exposure to silica nanoparticles cause cytotoxic damage and a decrease in cell survival in the EAHY926 cell line in a dose-related manner.
Rat lung epithelial cells (L2), rat alveolar macrophages, cocultures of the two	Sayes CM, Reed KL, Warheit DB, 2007	Zeofree 80	Aggregates of 1-3 µm (DLS)	The in vitro results do not correlate very well with the in vivo results obtained in the same study. Here SAS was lightly cytotoxic and induced an inflammatory response in macrophages.
Human bronchoalveolar carcinoma (A549)	Lin W, et al., 2006	15 and 46 nm particles from	TEM measurements: 15+-5nm	SAS nanoparticles induce a dose-dependent cytotoxicity that is closely correlated to increased oxidative stress

[1] The size values reported are the measured, mean values of a size distribution.

		Degussa Unspecified	46+-12nm	
Rat (WKY/NHsd) alveolar macrophages	Refsnes M, et al., 2006	Fesil microsilicea (! High contents in Fe)	300nm (no description of the characterisation)	The silica used did not induce proinflammatory markers but was highly cytotoxic
Mouse macrophages (RAW264,7)	Park E-J, 2009	12 nm SAS particles from Degussa Unspecified	12 nm (according to manufacturer 'specifications)	SAS nanoparticles generate ROS and trigger pro-inflammatory responses in vitro
Human fibroblasts (WS1, CCD-966sk, MRC-5, A549, MKN-28, HT-29)	Chang JS, Chang LB, 2007	Precipitated from Na silicate	SEM analysis: 10-15 nm	The cytotoxicity of silica to human cells depends strongly on their metabolic activities but that it could be significantly reduced by synthesizing silica with chitosan.
Human bronchoalveolar carcinoma (A549)	Jin Y, 2007	purposedly made dye doped silica 50 nm nanoparticles (from TEOS)	50+-3nm (no characterization described)	Developing a labelling reagent, the investigators looks for non cytotoxic levels and they show no significant toxic effects due to the luminescent nanoparticles at the molecular and cellular levels below a concentration of 0.1 mg/mL
Human intestinal epithelial cells(CaCo-2)	Wahl, B, 2008	Aerosil 200 and unspecified 15 nm silica nanoparticles from Merck	Aerosil: aggregates of 12 nm primary particles (manufacturer 'specifications) 15nm nanoparticles analysed by	LDH assay showed strong interactions with the tested silica particles. These findings suggest that even well characterized assay systems need a careful evaluation of the particle assay interactions when working with nanoparticles. Furthermore, particles based on the same material exhibit different biological properties depending on whether the material is used in micro- or nanometer range.

			TEM: 29.5+-0.5 nm	
Human hepatic cells (L-02)	Yiyi Ye, 2010	SAS colloids from the Center of Analysis and Test Research (Shangai, China)	21, 48 and 86 nm (no characterisation described)	SAS caused cytotoxicity in size, dose and time dependent manners. Oxidative stress and apoptosis were induced by exposure to 21 nm SiO2.
Human keratinocyte cell line (HaCaT)	Xifei Yang, 2010	SAS from Wang Jung New Material Co.	13+-3.8 nm 20+-3.5 nm 50+-9.2 nm 365+-79 nm (DLS)	Proteomics: SAS has size dependent effects on the expression of proteins involved in oxidative stress, cytoskeleton, chaperones, apoptosis, tumour and metabolism
Human bronchial epithelial cell (Beas-2B)	Hyun-Jeong Eom, 2009	Fumed silica (7 nm) Porous silica (5-15 nm) From Sigma	Aggregates in test medium (DLS): Fumed silica : 400 nm Porous silica : 20 nm	From the overall results, silica nanoparticles exerted toxicity via oxidative stress, cells exposed to porous silica nanoparticles showed a more sensitive response than those exposed to fumed silica
Human lung epithelial cells (A549 and L-132), human epithelial cells (HeLa) and human osteocarcoma cells (MNNG/HOS)	Choi SJ, 2009	Aerosil200 (14 nm)	15+-1 nm (SEM)	Silica nanoparticles had little toxic effects on the proliferation or viability of the cells from the 4 cell lines. But, silica significantly generated ROS, induced release of LDH release and IL-8 production from A549 cells.
Human pleural mesothelial cells (Met5A)	Brown SC, 2007	SAS synthesized for the study	DLS and SEM analysis: 98/113 nm and 190/210	SAS particle exposures in the presence of physiological stretch induce increased LDH release and IL-8 expression regardless of shape. Moreover, it is evident that shape-induced aggregation may play a significant role in mitigating particle

		(100 and 200 nm spheres and rods)	nm	clearance pathways.
Human epithelial cells (HeLa)	Fang Lu, 2009	Purposely prepared mesoporous silica	DLS and TEM charcterization: 30, 50, 110, 170 and 180 nm	Uptake of mesoporous silica by HeLa cells is particle-size-dependent and the maximum uptake by cells occurs at a nanoparticle size of 50 nm. It is expected that the size effect on cell uptake would lead to size-dependent biochemical responses.
Rabbit red blood cells	Slowing II, 2009	Purposely prepared mesoporous silica (MSN) and unspecified SAS from Sigma Aldrich	MSN : 100-300 nm (TEM), ~300nm (DLS) SAS from Sigma: centered at 459 and 1720 nm (DLS)	The authors show that, contrary to the known cytotoxicity of amorphous silica towards RBCs, MSNs exhibit a high biocompatibility at concentrations adequate for potential pharmacological applications. We demonstrated that the hemolytic properties of MSNs are related to the number of silanol groups accessible to the cell membranes of RBCs
Mouse embryonic fibroblast cells	Barnes CA, 2008	Commercial colloidal and laboratory synthesized silica	*currently waiting for a copy of the paper*	No significant genotoxicity was observed for the nanoparticles tested under the conditions described, and results were independently validated in two separate laboratories, showing that in vitro toxicity testing can be quantitatively reproducible.

Fullerenes (C60) draft DDP 15 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
	Pharmacokinetics/toxicokinetics (ADME)		
1	There is no existing data of ADME using C60 with no surface modification radio active label, therefore ADME of the principal fullerenes needs to be examined. Test Design (1): In the sponsorship program, the half life of the principal fullerenes in lung, accumulation in other organs and discharge will be examined after single intratracheal instillation to rats. Test Design (2): The study of intratracheal administration with C60 was conducted, in order to analyze the absorption and distribution in rats. Test Design (3): An appropriate method to address via an oral route and an *in vitro* method for the dermal route will be also developed.	Status: test 1 and 2 finished	The Test Guideline 417 on '"*Toxicokinetics*" has recently been updated and sent to the Joint Meeting for adoption, it is unlikely that any major changes to the protocol will be introduced. Even though it has not been developed for the direct testing of nanomaterials it may be applicable.
	Acute toxicity		
2	State of Understanding Nelson M. A. et al. studied the acute toxic effect of fullerenes applied in benzene by epidermal DNA synthesis and the induction of ornithine decarboxylase activity in the epidermis. No effect on either DNA synthesis or ornithine decarboxylase activity over a 72 hour time course after treatment. The subchronic effects of the fullerenes as a mouse skin tumor promoter was assessed by repeatedly applying the chemical to the skin after initiation with the polycydic aromatic hydrocarbon, 7,12-dimethlybenz-anthracene (DMBA). Repeated administration of the fullerenes for up to 24 weeks post-initiation did not result in either benign or malignant skin tumor formation, whereas promotion with the phorbol ester,		

	12-0-tetradecanoyl-phorbol-13-acetate (TPA) resulted in the formation of benign skin tumors. This study indicates that fullerenes applied in benzene at a likely industrial exposure level do not cause acute toxic effects on the mouse skin epidermis. Huczko A. et al.(2) assessed whether fullerene matter can induce any significant skin hazards by two methods. a) Thirty volunteers reporting various irritation and allergy susceptibilities were subjected to a patch test during 96 hours. b) The modified Draize rabbit eye test was carried out. One eye of each rabbit was instilled with 0.2 ml of water suspension of fullerene soot while the other eye was a reference. The rabbits were controlled after 24, 48 and 72 hours. From these two studies, it is unlikely that working with fullerene soot is associated with any risk of skin irritation and allergy. Thus, no special precautions have to be taken while working with fullerene nanostructures. Since the past studies above were conducted using different materials, the acute test with the principal fullerenes needs to be executed. A test for skin irritation will be conducted based on OECD TG404. <u>Test Design of skin irritation TG 404 (1)</u> The test is conducted using white rabbits. The pelage of the back is shortly cut at 24 hours before the examination. The pulverized test material is contacted well to the skin. The test material is applied at a small area of the skin and covered with gauze fixed with non-pungency tape. The test material sticking to the skin is removed after 4 hours' exposure. The applied part of the skin is compared with non-applied area. The irritating reaction of the skin is observed at 1, 24, 48 and 72 hours after the removal of the material. In particular, the erythema and the edema in all tested animals are observed macroscopically and scored following the guideline of Ministry of Agriculture, Forestry and Fisheries of Japan. When the skin irritation is found, the observation of lesion (reversibility) is	Status: Planned/ongoing	May consider using draft TG 439 on in vitro skin irritation! There is an OECD Guidance Document on histopathological lesions that is available upon request to the Secretariat.

	extended to 14 days after removing test material at most. Test Design of skin irritation TG 404 (2) Another skin irritation study is planed according to the OECD TG404. Pulverized powder and/or oil based suspension of C60 are applied to skin in the back of rabbit. Test Design of skin sensitization TG 406 (1) The test consist of four groups: a) test material administered group (20 guinea pigs are administered in both sensitization and elicitation test), b) negative control group (10 guinea pigs are administered only in elicitation test), c) positive control group (10 guinea pigs are administered with the positive material in both sensitization and elicitation test), d) negative control group to the positive material (5 guinea pigs are administered with the positive material only in elicitation test) Test Design of skin sensitization TG 406 (2) Another skin sensitization study is planned according to the OECD TG406 (Buehler method). Test Design of eye irritation TG 405 The test is conducted using white rabbits. The both eyes of the testing animals are confirmed as normal within 24 hours prior to the test. The test material is crushed and applied 0.1g at most. A conjunctiva sac of a eye is applied with the test material and compared with a non-treated eye as a control. At 1 hour, 24 hours, 48 hours and 72 hours after dosage, the irritating reactions of the eyes are observed. In particular, the irritation change of the cornea, the iris and the conjunctiva are observed with a slit lamp and scored following the guideline of Ministry of Agriculture, Forestry and Fisheries of Japan. When the persistent cornea disorder or other eye irritations are found, the observation of lesion (reversibility) is extended to 21 days at most after dosing. Test Design of acute oral toxicity Acute oral toxicity has been completed under the repeated dose toxicity test. Six-week old male mouse in 35.7 g weight was used as	Status: Planned Status: Planned/ongoing Status: Planned Status: Planned/ongoing Status: Finished	May consider using the updated LLNA draft TG 429, or the new non-radioactivelly labelled LLNA draft TGs 442A&B. May consider using the TG 437 and TG 438 on in vitro eye irritation/corrosion!

	a test subject. After five mice were exposed in each dose concentration level (22, 45, and 88 mg/kg by two times), 24 hours was taken for the post dose observation period. Death or abnormal behavior was not observed. Test Design of acute inhalation Acute inhalation toxicity will be addressed under the test for repeated dose toxicity.	Status: Planned	
	Repeat Dose Toxicity		
3	State of Understanding Baker, G. L. et al. reported the first inhalation toxicity study of fullerenes comparing nanoparticle (55nm) generated by vaporization and condensation process and micro particle (0.93 μm) exposing for 3h/days for consecutive 10 days at an aerosol concentration of approximately 2 mg/m3. The assessment of toxicity found minimal change in the toxicological endpoints examined. Additional toxicological assessments involving longer duration are needed. Since the above study was conducted by using a different material and relatively short period, an inhalation test with the principal fullerenes for longer duration needs to be examined. In the sponsorship program, an inhalation test will be conducted using the principal fullerenes based on OECD TG 412. Test Design TG 412 (1) 15 Wistar male rats (9 weeks old at the beginning of the test) for each observation period are exposed to aerosol fullerenes for the duration of 6h/d*5d/w*4w and the observation periods are 3 days, 1 month and 3 months after the exposure. The rats are divided into three groups as exposure to fullerenes, non-exposure (negative control) and exposure to nickel oxide (positive control). Lung weights, biomarkers in blood and BAL representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen) are		

	examined. Test Results (1) Three days after the exposure to fullerenes, the lungs tissue showed transient inflammation degree increase by pathological examination, then returned to the same level with a negative control. Other than this observation, no change or abnormality was observed in lung wet weight, HO-1 gene expression (a gene about inflammation fiber) and other organs (the cerebrum, the cerebellum, the nasal cavity, the spermary, the liver, the kidney and the spleen). In conclusion, inhalation exposure of 0.12 mg/m3 of C60 at 6h/d*5d/w*4w resulted in no death or abnormal behavior of Wister rats. Test Design TG 410(2) A test for dermal toxicity has already started, based on TG410. Test design oral toxicity(3) A test for oral toxicity will also be planned based on the findings of the test for ADME.	Status: Finished Status: Ongoing Status: Planned	
	Chronic Toxicity, Reproductive and Developmental toxicity		
4		Considered if available!	
	Genetic toxicity		
5	State of Understanding Dhawan A. et al. evaluated genotoxicity of stable aqueous suspensions of colloidal C60 fullerenes with respect to human lymphocytes using Comet assay. The assay demonstrated genotoxicity with a strong correlation between the genotoxic response and nC60 concentration, and with genotoxicity observed at concentrations as low as 2.2μg/L for aqu/nC60 and 4.2 μg/L for EtOH/nC60. Since the past study exemplified above used a different material from the principal fullerenes, further tests for *in vitro* genotoxicity needs to be conducted.		May consider using the new draft TG 487 on in vitro micronucleus.

	Test Design Ames test has been completed based on OECD TG 471. Test Results (a): In conclusion, the mutagenicity for bacteria of the fullerene without the light irradiation was negative. Mammalian chromosomal aberration test has been completed based on OECD TG 473. Test Results (b): In conclusion, the fullerenes without light irradiation did not cause the chromosome aberration regardless of the pro-metabolism activation in this experiment using the CHL/IU cell strain of the Chinese hamster lungs origin. In vivo Somatic Cell Genotoxicity: State of Understanding Since there is no existing data, micronucleus assay of marrow cell from orally administrated ICR mice have been completed based on OECD TG 474 in the sponsorship program. Test Design TG 474: The fullerenes dispersion is administered to ICR SPF mice by oral route using a stomach sonde at the start of experiment and at 24 hours later. Also, bone marrow stained samples are made and observed as a micronucleus assay. Test Results: In a toxicity test, neither abnormality nor decease was observed in both male mice group and female mice group within 48 hours after a single dose. In conclusion, the nucleus induction characteristics (chromosome aberration induction characteristics) of the fullerene were negative in this experiment condition using ICR mice.	Status: Finished Status: Finished Status: Finished	
	Other relevant test data:		
6	Intratracheal instillation: State of Understanding Sayes C. M. et al. (5) compared pulmonary toxicity of derivatized (water-soluble) and underivatized (nano-C60) fullerenes by		

	intratracheal instillation test. Both derivatized and underivatized fullerenes showed little or no difference of toxicity compared with control. Exposure of both type of fullerenes produced transient inflammatory and cell injury effect only at one day postexposure. Since the past study exemplified above used different material from the principal fullerenes, a single intratracheal instillation test using the principal fullerenes at various level dosages and an observation for long period (from 3 days to 24 months) has been completed. Test Design The dispersions of fullerenes are intratracheally instilled in a single dose to Wistar male rats. On 3 days, 1 week, 1, 3, 6, 12 and 24 months after instillation, lung weights, biomarkers in blood, BAL, and lung representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen) are examined. Test Results Lung wet weight increase was observed only at 1 week after instillation. As finding of BAL examination, total cell number has increased until 1 week; and the number of neutrophil cell has increased until 3 months after instillation. For 3.3 mg/kg dose group, the increase was extremely small compared with negative control group, which could be considered toxicologically negligible small. Stehenia is observed until 1 week as HO-1 gene expression in lung tissue. Inflammation degree has increased until 3 days for 0.33 and 0.66 mg/kg dose group and until 1 week for 3.3 mg/kg dose group then returned to the same level with negative control group. As inflammation in blood, no increase is observed for number of eukocyte and neutrophil. No abnormal finding was observed in other organs for the cerebrum, the cerebellum, the nasal cavity, the spermary, the liver, the kidney and the spleen.	Status: Finished	
7	*In vitro* tests: *In vitro* tests using three kinds of cell lines, (i) *human lung*		Are there available SOPs that could be forwarded to the SG7?

	adenocarcinoma cell line (A549); (ii) *human keratinocyte cell line (HaCaT)*; and (iii) *human acute monocytic leukemia cell line (THP-1)*, have been executed to examine cell viability, oxidative stress and apoptosis as endpoints. Along with the endpoints, absorption of protein and stability of dispersion have been measured as a material characterization. HaCaT and A549 cells were exposed for 6 and 24 hrs and viabilities of cells were measured by MTT assay. LDH release, level of oxidative stress (ROS level in cells, lipid oxidation level) and various apoptosis-associated markers (Caspase-3) were also measured. In addition, analysis of DNA damage and TEM observation were conducted. Test Results In conclusion, C60 did not influence viabilities and LDH activities of HaCaT and A549 cells. Apoptosis was not increased but cell proliferation rate slightly decreased. The oxidative stress marker and DNA damage were observed. There was no evidence that C60 will cause adverse effect, but possibility of the long-term effect due to the DNA damage can not be denied.		

Single-walled Carbon Nanotubes (SWCNTs) draft DDP 15 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
	Pharmacokinetics/toxicokinetics (ADME)		
1	Status of Understanding Wang, Haifang et al. investigated the distribution of hydroxylated carbon single-wall nanotubes with radioactive 125I atoms in mice. After administrating into the abdominal cavity of mice, hydroxylated SWCNTs moved easily among the compartments and tissues of the body except in brain. 80% of SWCNTs are excreted within 11 days. Cherukuri, Paul et al. (2) monitored single-walled carbon nanotubes intravenously administrated to rabbits. The characteristic near-infrared fluorescence spectra indicated that blood proteins displaced the nanotube coating of synthetic surfactant molecules within seconds. The nanotube concentration in the blood serum decreased exponentially with a half-life of 1.0 +/- 0.1 h. No adverse effects from low-level nanotube exposure could be detected from behavior or pathological examination. At 24 hours after intravenous administration, significant concentrations of nanotubes were found only in the liver. These results demonstrate the absence of acute toxicity and promising circulation persistence suggests the potential of carbon nanotubes in future pharmaceutical applications. Studies exemplified above used different material from the principal SWCNTs, therefore the ADME test on the principal SWCNTs will be conducted. Test Design: In the sponsorship program, test method for a quantitative analysis of CNTs in the biological tissues is developed. Using such a method, intratracheally instilled SWCNTs will be examined on its half life in lungs and transferring to the other organs such as brain and liver.	Status: Planned	The Test Guideline 417 on '"*Toxicokinetics*" has recently been updated and sent to the Joint Meeting for adoption, it is unlikely that any major changes to the protocol will be introduced. Even though it has not been developed for the direct testing of nanomaterials it may be applicable.

	Acute toxicity		
2	Acute oral toxicity will be conducted based on OECD TG 420 (Fixed Dose Procedure). However, the maximum dose of 2000 mg/kg required by the guideline may be impracticable, because of very high specific volume of SWCNT.	Status: Planned	
	Two tests for skin irritation will be conducted based on OECD TG404.	Status: Planned	May consider using draft TG 439 on in vitro skin irritation, the updated LLNA draft TG 429, or the new non-radioactivelly labelled LLNA draft TGs 442A&B or the TG 437 and TG 438 on in vitro eye irritation/corrosion!
	Two tests for skin sensitization will be conducted based on OECD TG406 using guinea pigs and the Bueler test, respectively.	Status: Planned	
	A test for eye irritation will be conducted based on OECD TG405. In particular, the irritation change of the cornea, the iris and the conjunctiva are observed with a slit lamp and scored following the guideline of Ministry of Agriculture, Forestry and Fisheries of Japan. When the persistent cornea disorder or other eye irritations are found, the observation of lesion (reversibility) is extended to 21 days at most after dosing.	Status: Planned	
	Acute inhalation toxicity will be addressed utilizing the repeated inhalation test data (TG 412) and also an instillation method to address inhalation toxicity will be conducted.	Status: Planned	
	Testing of Alternative SWCNTs: Alternative SWCNTs will be examined for acute pulmonary toxicity and immunotoxicity by using OECD TG 403 and by intratracheal instillation (rats) or pharyngeal aspiration (mice). Pulmonary toxicity will be evaluated by biochemical and cellular analysis of bronchoalveolar lavage samples.	Status: Planned	
	Cardiovascular toxicity as well as alterations in cardiac and vascular physiology and toxicity following acute (TG403) inhalation exposure and/or intratracheal instillation will be studied. These studies are critical since currently no OECD or US EPA OPPTS Harmonized Test Methods to assess cardiovascular toxicity to nanomaterials exist and inhalation exposure could produce	Status: Pla Status: Planned/ongoing	

	cardiovascular toxicity. It is anticipate this testing will ultimately provide guidance to establish nanomaterials cardiovascular toxicity testing methods.		
	Repeat Dose Toxicity		
3	State of Understanding Z. Li et al. demonstrated the ability of multi-doses of SWCNT given by pharygeneal aspiration induced cardiovascular disease in mice. Since no study is reported on the inhalation test of SWCNTs, the inhalation test of the principal SWCNTs needs to be examined. Test Design The repeat dose inhalation test will be conducted from the middle of 2009 to the middle of 2010 based on OECD TG 412. A new technique for supplying SWCNTs aerosol with a stable concentration including sample preparation is being developed. This technique is applied for realizing the stable exposure for four weeks, which enables sufficient observation period. The inhalation test will be executed under the following conditions: (a) exposure period is 6h/d*5d/w*4w; (b) observation periods are 3 days, 1 week, 1 month and 3 months after the exposure; (c) 15 male Wistar rats (9 weeks old at the start of the test) are used for each observation periods; (d) aerosol concentration is according to the results of sample preparation method; (e) non-exposure group, solvent exposure group and nickel oxide exposure group are also tested as controls; and (f) lung weights, biomarkers in blood and BAL representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen) are examined at each observation period. Test for repeat oral toxicity study will be conducted based on TG 407. However, the maximum dose of 1000 mg/kg required by the guideline may be impracticable, because of very high specific volume of SWCNT.	Status: Ongoing Status: Planned	

	A test for dermal toxicity will be conducted based on OECD TG412. Methods to address sub-chronic inhalation toxicity using repeated intra-tracheal instillation are ongoing. Methods to address inhalation toxicity by intra-tracheal instillation are on going. Testing of alternative SWCNTs SWCNTs will be examined for Pulmonary Toxicity and Immunotoxicity following repeated pulmonary deposition (TG 412) of various doses of each SWCNT by intratracheal instillation (rats) or pharyngeal aspiration (mice). Pulmonary toxicity will be evaluated by biochemical and cellular analysis of bronchoalveolar lavage samples. Information from these studies will be use to evaluate the repeated dose toxicity of a specific SWCNT by OECD TG412. Cardiovascular Toxicity as well as alterations in cardiac and vascular physiology and toxicity following repeated (TG 412) inhalation exposure and/or intratracheal instillation of SWCNTs will be evaluated. These studies are critical since currently no OECD or US EPA OPPTS Harmonized Test Methods to assess cardiovascular toxicity to nanomaterials exist and inhalation exposure could produce cardiovascular toxicity. It is anticipate this testing will ultimately provide guidance to establish nanomaterials cardiovascular toxicity testing methods.	Status: Planned Status: Planned Status: Planned Status: Planned Status: Planned	
	Chronic Toxicity, Reproductive and Developmental toxicity		
4	---	Considered if available!	
	Genetic toxicity		
5	*In vitro genotoxicity*: Since there is no existing data, Ames test will be conducted based on TG 471 and a mammalian chromosomal aberration test will be conducted based on TG 473.	Status: Planned	May consider using the new draft TG 487 on in vitro micronucleus test.

	In vivo Somatic Cell Genotoxicity: Since there is no existing data, micronucleus assay of marrow cell from orally administrated ICR mice will be conducted based on OECD TG 474.	Status: Planned	
	Other relevant test data:		
6	Intratracheal instillation test- State of Understanding Warheit, D. B. et al. investigated the acute lung toxicity of intratracheally instilled SWCNTs in rats. Exposures to SWCNTs produced transient inflammatory and cell injury effects. The observation of SWCNT-induced multifocal granulomas is inconsistent with (1) lack of lung toxicity by assessing lavage parameters; (2) lack of lung toxicity by measuring cell proliferation parameters; (3) an apparent lack of a dose response relationship; (4) nonuniform distribution of lesions; (5) the paradigm of dust-related lung toxicity effects; and (6) possible regression of effects over time. Considering very low aerosol SWCNTs exposures at the workplace, the physiological relevance of these findings should ultimately be determined by conducting an inhalation toxicity study. Lam, Chiu-Wing et al. investigated the acute lung toxicity through intratracheal instillation test to mice using three different SWCNTs made by different processes and containing different types and amount of residual catalytic metal. All nanotube products induced dose-dependent epithelioid granulomas. In some cases, interstitial inflammation, peribronchial inflammation and necrosis that had extended into the alveolar septa were observed. The results show that, if SWCNTs reach the lungs on an equal-weight basis of the test, they are much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures. Shvedova, Anna A. et al. demonstrated that pharyngeal aspiration of SWCNTs elicited unusual pulmonary effects in mice that combined an acute inflammation with progressive fibrosis and granulomas. An early neutrophils accumulation followed by lymphocyte and macrophage influx, was accompanied by early	Status: Finished	

	elevation of proinflammatory cytokines followed by fibrogenic transforming growth factor. A rapid progressive fibrosis found in mice exhibited two distinct morphologies: 1) SWCNT-induced granulomas mainly associated with hypertrophied epithelial cells surrounding SWCNTs aggregates; and 2) diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNTs. The intratracheal instillation test is useful to examine the pulmonary toxicity. Since past studies exemplified above used different material from the principal SWCNTs and the observation period is relatively short, the instillation test using the principal SWCNTs will be conducted from middle of 2009. This test consists of single instillation and long period (from 3 days to 24 months) observation. Test Design The sample preparation procedure is now being developed. The inhalation test will be performed under the conditions of: (a) 9 weeks old of 15 male Wistar rats are used foe each observation period; (b) the dose amount is 0 (control), 0.2 and 1mg/rat. After a single intratracheal instillation, the rats are anatomized in 3 days, 1 week, 1 month, 3 months, 6 months, 12 months and 24months to examine lung weights, biomarkers in blood and BAL representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen).		
7	In vitro tests *Test Design (1):* three kinds of cell lines, *human lung adenocarcinoma cell line (A549), human keratinocyte cell line (HaCaT) and human acute monocytic leukemia cell line (THP-1)* will be used to examine cell viability, oxidative stress and apoptosis as endpoints. Along with the endpoints, absorption of protein and stability of dispersion have been measured as a characterization of the material. *Test Design (2):* the neurotoxicity and gliotoxicity of SWCNTs		Are there any available SOPs that could be forwarded to the SG7?

	(SWCNTs cleaned with acid, SWCNTs cleaned with acid and funcionalised with DNA, SWCNTs functionilised with DNA) using several culture systems (oligodendrocyte cell line OLN 93, primary neuronal cultures, astrocytes and microglial cells obtained from fetal or newborn rat brain) will be used. Endpoints: *in vitro* viability and cytotoxicity (using the following assays CCK 8, MTT, LDH), cell growth, cell proliferation, oxidative stress, measurement of mitochondrial membrane potential, adhesion of cells, gene expression of adhesion molecules, apoptosis, calcium signals.		
8	*Other relevant information: State of Understanding* Radomski et al. demonstrated the ability of SWCNT to induced in vivo vascular thrombosis. PM Raja et al demonstrated the ability of SWCNT to alter aortic smooth muscle growth. Gold labelling of SWCNT documents low uptake by alveolar macrophages and track rapid movement into the alveolar interstitial space. An inhalation exposure of mice to SWCNT resulted in qualitatively similar pulmonary reaction as pharyngeal aspiration; but it was 4 times more potent in causing interstitial fibrosis due to the generation of smaller structures that in the aspiration study. *In vitro* toxicity testing will be performed on Alternative SWCNTs using human airway epithelial BEAS2B cell line (pulmonary toxicity surrogate), human endothelial cells (vascular toxicity surrogate), and macrophage cells (immunotoxicity surrogate). *In vitro* toxicity testing endpoints will include cell growth, cell toxicity, cytokine production, oxidative stress, regulation of nitric oxide expression and signaling, toxicogenomics, and cellular uptake.		

Multi-walled Carbon Nanotubes (MWCNTs) draft DDP 20 April 2010			
	ENDPOINT	**Status of testing**	**Recommendations or questions for specific clarifications by SG7**
	Pharmacokinetics/toxicokinetics (ADME)		
1	State of Understanding Ding et al. investigated biodistribution and translocation pathways of MWCNTs in mice by three different routes, using water soluble 14C-taurine-multi-walled CNTs. After mice were exposed by intravenous injection MWCNTs predominantly accumulated in liver and retained for long time. In the intratracheal exposure, the tracer accumulates largely in the lung and is gradually eliminated. In the stomach, intubation experiment the radioactivity is detectable only in the stomach, small intestine, large intestine and faeces. The study above is the first trial of ADME study of MWCNTs, which used 14C-taurine-MWCNTs. It is unsure that results of the study using 14C-taurine-MWCNTs could also describe the behaviour of pure MWCNTs. Therefore, another method for testing a pure CNT needs to be developed. Test Design (1) A test method for a quantitative analysis of CNTs in the biological tissues is being developed. Using such a method, intratracheally instilled MWCNTs will be examined on its half-life in lungs and transferring to the other organs such as brain and liver. Other tests via the oral or dermal route will be also planned. Test Design (2) Intratracheal administration of MWCNTs is being conducted in order to analyze the absorption and distribution in rats. The injected MWCNTs are suspended with phospholipids and Tween20. The quantitative analysis method is still under development.	Status: Planned Status: Planned	The Test Guideline 417 on '"*Toxicokinetics*" has recently been updated and sent to the Joint Meeting for adoption, it is unlikely that any major changes to the protocol will be introduced. Even though it has not been developed for the direct testing of nanomaterials it may be applicable.
	Acute toxicity		

2	- Acute oral toxicity will be conducted based on TG 420 (Fixed Dose Procedure). However, the maximum dose of 2000 mg/kg required by the guideline may be impracticable, because of very high specific volume of MWCNT. - Two tests for skin irritation will be conducted based on TG 404. - A test for skin sensitization will be conducted based on TG406 using guinea pigs will be performed. - Another skin sensitization study will be conducted according to the TG406 (Buehler method). - A test for eye irritation will be conducted based on TG405 - Acute inhalation toxicity will be addressed utilizing the repeated inhalation test data (TG 413) and also a test using the instillation method to address inhalation toxicity will be conducted. Testing of alternate MWCNTs by several sponsors: Sponsor 1-The following tests have been performed: - Acute toxicity by oral route (TG423) : LD0 > 2000 mg/kg - Acute toxicity by dermal route (TG402) : LD0 > 2000 mg/kg - Eye irritation (TG405) : irritant - Skin irritation (TG404) : slightly irritant - Skin sensitization (LLNA, TG429) : not sensitizer Sponsor 2-The following tests will be conducted: - Acute toxicity by dermal route (not OECD TG) - Skin irritation (TG404) - Skin sensitization (LLNA, TG406) - Eye irritation (TG405) Acute inhalation toxicity test by applying the single intra-tracheal instillation followed by 90-day observation. Sponsor 3 will examine the pulmonary, cardiovascular and	Status: Planned Status: Planned and finished	May consider using draft TG 439 on in vitro skin irritation, the updated LLNA draft TG 429, or the new non-radioactivelly labelled LLNA draft TGs 442A&B or the TG 437 and TG 438 on in vitro eye irritation/corrosion!

	immunotoxicity associated with acute inhalation exposure (403) and intratracheal instillation. The following tests have been addressed: - Acute toxicity, oral (TG 423) - Acute toxicity, dermal (TG 402) - Primary skin irritation (TG 404) - Primary eye irritation (TG 405) - Skin sensitization (TG 406) Sponsor 4 has performed an acute inhalation toxicity study: Inflammatory endpoints in broncho-alveolar lavage (BAL) were determined on post-exposure days 7, 28, and 90. The deposition of cobalt (Co) was determined in lungs, lung-associated lymph nodes (LALN), brain, kidneys, testes, and liver. Histopathology revealed an increased cellularity in the bronchiolo-alveolar region with focal septal thickening and focal septal collagen depositions at 241 mg/m3. Despite a concentration-dependent increase of Co in lung tissue, determinations in the remaining tissues were unobtrusive. The following tests have been performed: - Acute toxicity by oral route (Modified TG 420): No toxicity - Acute toxicity by dermal route (Modified TG 431) : No toxicity - Ingestion (Modified TG 420) : No effect - Skin irritation (Modified TG 431) : No irritation and no corrosion		
	Repeat Dose Toxicity		
3	State of Understanding Li et al. compared inhalation with intratracheal instillation using female Kunming mice. CVD pristine MWCNTs (Shenzhen Nanotech Port, O.D.50nm, length 10μm, specific surface area 280m2/g, C>95%, amorphous carbon <3%, 0.2%>La, Ni,) was aerosolized to mean concentration of 32.61 mg/m3 for 90 min at 4 times/day and 5, 10 and 15 days exposure. The aggregations of MWCNTs induced proliferation and thickening of alveolar walls. The preliminary study showed a difference in lung pathological		

	lesions induced by instilled MWCNTs and inhaled ones which may be due to the different size and distribution of MWCNTs in lung. NIOSH has conducted pharyngeal aspiration of Mitsui MWNT-7 in mice. Results indicate transient inflammation and damage peaking 7 days post exposure. One day post exposure, NIOSH found induction of inflammatory markers which decreased to control thereafter. A manuscript is in preparation. Mitchell L.A. et al. studied the systemic immune suppression through inhalation test of MWCNTs. Male C57Bl/6 mice were exposed to 0, 0.3 or 1mg/m3 dispersible MWCNTs (Shenzhen Nanotech Port Co.) for 6 hours per day for 14 consecutive days in whole-body inhalation chambers. Only those exposed to a dose of 1mg/m3 presented suppressed immune function; this involved activation of cyclooxygenase enzymes in the spleen in response to a signal from the lungs. Spleen cells from exposed animals partially recovered their immune function when treated with ibuprofen, a drug that blocks the formation of cyclooxygenase enzymes. Knockout mice without cyclooxygenase enzymes were not affected when exposed to MWCNTs. Those findings suggest that signals from the lung can activate signals in the spleen to suppress the immune function of exposed mice. Since the past studies above were conducted using different materials without sufficient characterization, an inhalation test with the principal MWCNTs needs to be executed. <u>Test Design</u> An inhalation test is scheduled from the end of 2008 to the middle of 2010. A new technique for supplying MWCNTs aerosol with a stable concentration has been developed. This technique is applied for realizing the stable exposure for four weeks, and then sufficient observation period will be ensured. The inhalation test will be carried out under the following condition: (a) exposure period is 6h/d*5d/w*4w; (b) observation periods are 3 days, 1 week, 1 month and 3 months after the exposure; (c) 15 male Wistar rats (9 weeks old at the start of the test) are used for each observation periods; (d)		

	aerosol concentration is 0.47+0.18mg/m3 (mass based concentration) or 0.37+0.18mg/m3 (elemental carbon concentration); (e) non-exposure group, solvent exposure group and nickel oxide exposure group are also tested as controls; and (f) lung weights, biomarkers in blood, BAL, and lung representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen) are examined at each observation period. Test Results The lung wet weight increased significantly only at third day after the exposure, but it was a transient change. The significant increase of the total number of cells and neutrophils in BALF was not observed. The heme oxygenase -1 (HO-1) genes in BALF and in lung tissue, which is related to the inflammatory fibrosis reaction, increased significantly only at third day after the exposure, but it was a transient change. In addition, no significant increase of inflammation area is observed by the histopathological evaluation of lungs at all the exposure periods. For the MWCNTS exposure group (0.37mg/m3), the lungs weight and HO-1 gene in BALF and lung tissue showed a transient change only at the third day after the exposure, but no significant change was observed about other observation items. On the other hand, the nickel oxide exposure group (0.2mg/m3) in reference test showed a sustained change in the lung weights, the inflammation cell counts in BALF, HO-1 genes in BALF and lung tissue and the inflammation of the lung tissue and it is quite different from the reaction by the exposure of MWCNTs. - Test for oral toxicity will be conducted based on TG 407. However, the maximum dose of 1000 mg/kg required by the guideline may be impracticable, because of very high specific volume of MWCNT. - Another inhalation test will be done based on TG 412.		

	- A test for dermal toxicity will be conducted based on TG 412. Testing Alternative MWCNTs by several sponsors Sponsor 1 will address repeated dose toxicity by 5-, 28- and/or 90-day inhalation exposure studies. Sponsor 2 will examine the pulmonary, cardiovascular and immunotoxicity associated with repeated inhalation exposure (TG 412, and possibly TG 413). Sponsor 3 will address repeated inhalation and intratracheal instillation toxicities, in addition to examining repeated dose dermal toxicity. Sponsor 4 has performed a repeated dose inhalation toxicity by a 90 days exposure study (TG 413). The focus of study was on respiratory tract and systemic toxicity, including analysis of MWCNT biokinetics in the lungs and lung-associated lymph nodes (LALNs). The time-course and concentration-dependence of pulmonary effects were examined by bronchoalveolar lavage (BAL), and histopathology up to 6 months postexposure. Particular emphasis was directed to the comparative characterization of MWCNT structures prior to and after micronization and dry powder dispersion into inhalation chambers. These determinations were complemented by additional analyses in digested BAL-cells. Animals were exposed on 6 hours/day, five days per week for 13 consecutive weeks to 0, 0.1, 0.4, 1.5, and 6 mg/m3. The subchronic exposure to respirable solid aerosols of MWCNT was tolerated without effects suggestive of systemic toxicity. Kinetic analyses demonstrated a markedly delayed clearance of MWCNT from lungs at overload conditions. Translocation into LALNs occurred at 1.5 and 6 mg/m3 and required at least 13 weeks of study to become detectable. At these exposure levels the lung and LALN weights were significantly increased. Sustained elevations in BAL PMNs and soluble collagen occurred at these concentrations with borderline effects at 0.4 mg/m3. Histopathology revealed principal exposure-related lesions at 0.4 mg/m3 and above in the upper		

	respiratory tract (goblet cell hyper- and/or metaplasia, eosinophilic globules, focal turbinate remodeling) and the lower respiratory tract (inflammatory changes in the bronchiolo-alveolar region, increased interstitial collagen staining). Granulomatous changes and a time-dependent increase of a bronchiolio-alveolar hyperplasia occurred at 6 mg/m3. All endpoints examined were unremarkable at 0.1 mg/m3 (NOAEL). In summary, this study demonstrates that the induced pathological changes are consistent with overload-related phenomena. Hence, the etiopathological sequence of inflammatory events caused by this type of MWCNT appears to be related to the high displacement volume of the low-density MWCNT-assemblage structure rather than to any yet ill-defined intrinsic toxic property. Thus, the hypothesis of study is verified, namely common denominators between carbon black and MWCNT do exist. Sponsor 5 performed the inhalation toxicity of MWCNTs. A 90-day inhalation toxicity study with Nanocyl NC 7000 according to TG 413. Wistar rats were head-nose exposed for 6 hours/day, 5 days/week, 13 weeks, total 65 exposures, to MWCNT concentrations of 0 (control), 0.1, 0.5 or 2.5 mg/m3. Inhalation exposure to MWCNT produced no systemic toxicity. However, increased lung weights, pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis were observed in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m3. These effects were accompanied by slight blood neutrophilia at 2.5 mg/m3. Those effects were concentration-related. At 0.1 mg/m3, there was still minimal granulomatous inflammation in the lung and in lung-associated lymph nodes; a no observed effect concentration was therefore not established in this study.		
	Chronic Toxicity		
4	One sponsor will address carcinogenicity by long-term inhalation exposure study with an alternative MWCNT.	Considered if available!	
	Reproductive and Developmental toxicity		

4	----	Considered if available!	
	Genetic toxicity		
5	**<u>In vitro Genotoxicity: State of Understanding</u>** Zhu et al. assessed the DNA damage response to MWCNTs in mouse embryonic stem cells. They found that MWCNTs can accumulate and induce apoptosis in mouse ES cells and activate the tumor suppressor protein p53 within 2 hours of exposure. A mutagenesis study using an endogenous molecular marker showed that MWCNTs increased the mutation frequency by 2-fold compared with the spontaneous mutation frequency in mice ES cells. Wirnitzer et al. performed a chromosome aberration test using V79 cells (TG 473) and a Salmonella microsome test (TG 471) using different concentrations of "baytubes" (Bayer Material Science, macro-agglomerates of engineered MWCNTs). Particle size distribution was determined under the incubation conditions of the in vitro studies. The tested MWCNTs did not induce bacteriotoxicity or mutations in a set of five tested Salmonella strains in concentrations up to 5000µg/plate, both with and without metabolic activation using S9 mix. Furthermore the MWCNTs were not cytotoxic and not clastogenic in concentrations up to 10 µg/ml in the absence or presence of S9 mix in the chromosome aberration test. Since the past study exemplified above used a different material from the principal MWCNTs, further tests for *in vitro* genotoxicity needs to be conducted: <u>Ames test and the chromosomal aberration test.</u> <u>Testing of Alternate MWCNTs by several sponsors</u> The following tests have been performed by <u>sponsor 1</u>: - Gene mutation in bacteria test (Ames test) : negative - Gene mutation in mammalian cells: negative - Chromosome aberration test: negative		May consider using the new draft TG 487 on in vitro micronucleus test.

	Sponsor 2 has completed cytotoxicity and chromosomal aberration test in mammalian cells, and will conduct cell transformation test. Sponsor 3 performed a chromosome aberration test using V79 cells (TG 473) and a Salmonella microsome test (TG 471). Under the conditions used and in the concentration range tested there were no bacteriotoxic and no mutagenic effects. ***In vivo* Somatic Cell Genotoxicity:** Since there is no existing data, micronucleus assay of marrow cell from orally administrated ICR mice will be conducted based on TG 474 and an *in vivo* somatic cell genotoxicity test will be performed.		
	Other relevant test data:		
6	Instillation test: State of Understanding Haczko et al. studied the pulmonary toxicity by intratracheal instillation method. Guinea pigs were intratracheally instilled with 15 mg of various MWCNTs. Histopathological evaluation after 90 days revealed perivascular, peribronchial, and interstitial infiltration of inflammatory cells, central and peripheral atelectasis and emphysema and alveolar exudation regardless of instilled materials. Muller et al. also studied by intratracheal instillation method. NaOH purified CVD MWCNTs (5.9μm long) and ground ones (0.7μm long) were administered intratracheally (0.5, 2 or 5mg) to SD rats and estimated lung persistence, inflammation and fibrosis. Both CNTs stimulated the production of TNF-α in the lung and were still present in the lung after 60 days and induced inflammatory and fibrotic reactions. Ground CNTs were better dispersed in the lung parenchyma and induced granuloma in the interstitial tissue. The intratracheal instillation test is useful to study pulmonary effects but limitations of this approach need to be recognized (ENV/Chem/Nano(2009)8)(16). Since past studies exemplified above used different materials as the principal MWCNTs, the instillation test using the principal MWCNTs has been conducted.	Status: Finished	

	Test Design Such instillation test into the trachea has been executed since the middle of 2008. The test consists of single instillation and long period (from 3 days to 24 months) observation. The raw MWCNTs are solidified with fructose and pulverized by vibration ball mill and/or a planetary ball mill. The pulverized MWCNTs are dispersed by sonication in Triton solution and centrifuged. The supernatant is used for the test. The dispersion state is checked by TEM and SEM. The test is done under the following conditions: (a) 9 weeks old of 15 male Wistar rats are used foe each observation period; and (b) the dose amount is 0 (control), 0.2 and 1mg/rat. After a single intratracheal instillation, the rats are anatomized in 3 days, 1 week, 1 month, 3 months, 6 months, 12 months and 24 months to examine lung weights, biomarkers in blood and BAL representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen). Test Results The significant increase of the lung wet weight was observed only at third day after the MWCNT administration in the 0.2 mg/rat dosing group. As for the 1mg/rat dosing group, the significant increase of the lung wet weight was observed until six months after the dosage. In both 0.2 and 1 mg/rat dosing groups, the total number of cells and neutrophils in BALF showed significant increase only at the third day after the dosage. Hem oxygenase -1 (HO-1) in BALF, which relates to inflammatory fibrosis, showed no significant increase in in both 0.2 and 1 mg- MWCNT /rat dosing groups, but that in lungs organization showed significant increase 1 mg/rat dosing group until one month after the dosage. The 0.2 mg/rat dosing group showed the transient inflammation until 1 week after the dosage, and the persistent inflammation was observed in 1 mg/rat dosing group until 3 months by the histopathological evaluation of lungs. In neither groups, the fibrosis		

	of the lung tissue was observed. In 0.2 mg of MWCNT/rat dosing group, a transient change in the lung wet weight, the inflammation cell count in BALF and the inflammation of the lung tissue was observed at third day or in a week after the dosage. With 1 mg/rat dosing group, the transient increase of HO-1 genes in lung tissue was observed, and the inflammation cell count in BALF and the inflammation of the lung tissue increased until three months after the dosage. On the other hand, the nickel oxide dosing group (0.2 mg/rat) of the past experiment, lung wet weight, inflammation cell count in BALF and inflammatory fibrosis-related gene showed sustained increase. In addition, the sustained inflammation of the lung tissue was observed. The inflammation by MWCNT is minimal and transient comparing with that by nickel oxide. The hazardous by the MWCNT exposure will be totally assessed after a long term observation of two years.		
7	In vitro tests In vitro test using three kinds of cell lines, *human lung adenocarcinoma cell line (A549), human keratinocyte cell line (HaCaT) and human acute monocytic leukemia cell line (THP-1)* will be done. The test examines cell viability, oxidative stress and apoptosis as endpoints. Along with the endpoints, absorption of protein and stability of dispersion will be measured as a characterization of material.		Are there available SOPs that could be forwarded to SG7?
8	State of Understanding Radomski et al. (2005) demonstrated the ability of MWCNT to induced in vivo vascular thrombosis. The study showed the need to evaluate the cardiovascular toxicity of pulmonary deposited MWCNTs. Poland et al.(2008) injected long or tangled MWCNT into the peritoneal cavity of mice (50µg/animal). After 24 hours and 7 days mice were killed and the peritoneal cavity was lavaged. The authors found asbestos-like, length-dependent, pathogenic behavior of MWCNTs: At 24 hours, an inflammatory response to the long		

	MWCNTs was detected comparable to that found for long fiber amosite (an amphibole form of asbestos used as positive control), but no significant inflammation was found for the tangled MWCNTs. At 7 days granulomas formation of the mesothelial surface as a sign of a pathogenic response was detected upon exposure to long fibers of MWCNTs and long fiber amosite, but not upon exposure to tangled MWCNTs. Takagi et al. (2008) demonstrated the mesotheliomagenesis potency of Mutsui MWNT-7 (Alternate MWCNT, fiber length 1 - 20μm, ratio of fiber > 5μm is c.a. 25%) by intraperitoneal administration (3mg/animal) to p53+/- mouse, which have been shown to be sensitive to oxidative stress-mediated carcinogenesis. The overall incidence of mesothelioma after first incidental case found at day 84 were about 90% in MWCNT group. The dose of 3 mg/mouse corresponds to about 100 mg per kg of animal, and also corresponds to about 1,000,000,000 fibers longer than 5 micrometer per animal. This is a high level, and near the maximum amount among the study reports for man-made mineral fibers. So another intraperitoneal injection study at dosages that are lower than this study by 10 times (high-dose group: 300 μg/animal), 100 times (medium-dose group: 30 μg/animal), and 1000 times (low-dose group: 3 μg/animal) is just finished. As preliminary results, peritoneal tumors were induced in nearly 100% of the high-dose group, about 60% in medium-dose group and about 20% in low-dose group. The observation was terminated at one year, and now analyzing the results. The potency of mesotheliomagenesis by intrascrotal (nearly equal to "intraperitoneal") administration with MWNT-7 was confirmed by using genetically intact animal (F344 rat) (Sakamoto et al., (19)). Meanwhile Muller et al. (13) indicated that a single intraperitoneal administration of the shorter MWCNT (length: < 1 μm on average) at dose of 20 mg/animal could not induce mesothelioma in rat 2-year bioassay. These results suggested that fiber length was important factor for the mesotheliomagenesis.		

	As for the possible risk of mesotheliomagenesis after pulmonary exposure, two studies were reported. Poter et al. (2010) reported an aspiration exposure study of male C57BL/6J mice by MWNT-7 dispersed in dispersion medium at dose of 10, 20, 40 or 80 μg/animal. Histopathological studies determined that MWCNT exposure caused rapid development of pulmonary fibrosis by 7 days post-exposure, that granulomatous inflammation persisted throughout the 56-day post-exposure period, and also demonstrated that MWCNT can reach the pleura after pulmonary exposure. However, the authors indicated more extensive investigations are needed to fully assess if pleural penetration results in any adverse health outcomes. Ryman-Rasmussen et al (2009). showed that multiwalled carbon nanotubes reach the subpleura in mice after a single inhalation exposure of 30 mg/m3 for 6 h. Nanotubes were embedded in the subpleural wall and within subpleural macrophages. Subpleural fibrosis unique to this form of nanotubes increased after 2 and 6 weeks following inhalation. MWCNTs of 'standard' length (0.5–50 μm) synthesized by carbon vapour deposition with nickel and lanthanum catalysts were purchased from Helix Material Solutions. Testing of Alternate MWCNTs by sponsors The following tests will be executed by sponsor 1: - Cardiovascular Toxicity Following Pulmonary Deposition Sponsor 1 will examine the cardiovascular toxicity as well as alterations in cardiac and vascular physiology following acute (TG 403) and repeated (TG 412) inhalation exposure and/or intratracheal instillation. - Immunotoxicity Following Pulmonary Deposition Sponsor 1 will examine immunotoxicity following acute (TG 403) and repeated dose TG 412 inhalation exposure and/or intratracheal instillations. - Acute and Repeated Dose Toxicity Pulmonary TGs 403 and 412, including bronchoalveolar lavage		

	with clinical and cellular assays to assess edema and inflammation will be performed. - Alternative Test Methods *In vitro* toxicity testing will be conducted using human airway epithelial BEAS2B cell line and human endothelial cells. In vitro toxicity testing endpoints will include cell growth, cell toxicity, cytokine production, oxidative stress, regulation of nitric oxide expression and signaling, toxicogenomics, and cellular uptake. The following tests will be executed sponsor 2: Alternate MWCNT was administered at a dose of 5 mg/animal (a dosage volume of 0.5 mL/animal, 12 rats/group). The test consists of single instillation and 3 months observation (Wako et al. (2010)). After a single instillation, the total cell count, neutrophil ratio, LDH activities and Protein concentration of the BALF are analysed in days 2, 8, 29, 92. and lungs tissues were examined histopathologically under a light microscope Sponsor 3 will perform tests with MWCNTs using human lung cells (A549) to determine endpoints like proliferation and vitality of cells, inflammation factors and immune response. So far, no acute toxicity was detected. A 3D skin model was developed to investigate a potential penetration of MWCNTs through the skin and to monitor an irritative effect upon chronic exposure. So far, no penetration of MWCNTs was found. A 3D trachea model is under development. Sponsor 4 explored the carcinogenic potential of alternative MWCNTs in the peritoneal cavity of rats. The incidence of mesothelioma and other tumors was recorded in three groups of 50 male Wistar rats injected intraperitoneally with a single dose of MWCNT with defects (2 or 20 mg/animal) and MWCNT without defects (20 mg/animal). Two additional groups of 26 rats were used as positive (2 mg UICC crocidolite/animal) and vehicle controls. After 24 months, although crocidolite induced a clear carcinogenic		

	response (34.6% animals with mesothelioma vs. 3.8% in vehicle controls), MWCNT with or without structural defects did not induce mesothelioma in this bioassay (4, 0, or 6%, respectively). The incidence of tumors other than mesothelioma was not significantly increased across the groups. The initial hypothesis of a contrasting carcinogenic activity between MWCNT with and without defects could not be verified in this bioassay. We discuss the possible reasons for this absence of carcinogenic response, including the length of the MWCNT tested (< 1 mm on average), the absence of a sustained inflammatory reaction to MWCNT, and the capacity of these MWCNT to quench free radicals.		

ANNEX II.Draft Compilation of Test Guidelines used, or proposed to be used, for five Manufactured Nanomaterials.

A "+" indicate that the test is employed for any of the six defined and characterized MNs, an "A" means it is tested in an alternative NM. The "?" in the SAS column indicate that the endpoints are selected but no specific Test Guidelines were listed. (*) indicate mandatory tests as described in the Guidance manual, and (¤) indicate tests where data have to be presented, if existing data is available.

Assay	CeO2	ZnO	SAS (SiO2)	Fullerens	SWCNT	MWCNT
Intratracheal instillation				+	+, A	+, A
TG 402*-Acute dermal		+	?			+, A
TG 403*-Acute inhalation	+	+	?	+	+, A	+, A
TG 404-In vivo skin irritation/corrosion	+		?	+	+	+, A
TG 405-In vivo eye irritation/corrosion			?	+	+	+, A
TG 406-Skin sensitization			?	+	+	+, A
TG 407*- Repeated Dose 28-day Oral Toxicity Study in Rodents			?		+	+
TG 409*- Repeated Dose 90-Day Oral Toxicity in Non-Rodents						
TG 410*- Repeated Dose Dermal Toxicity: 21/28-day Study		+	?	+		
TG 411*- Subchronic Dermal Toxicity: 90-day Study			?		+	+, A
TG 412*- Subacute Inhalation Toxicity: 28-Day Study	+	+	?	+	+	+, A
TG 413*- Subchronic Inhalation Toxicity: 90-day Study	+	+	?		+	+, A
TG 414¤- Prenatal Development Toxicity Study	+					
TG 415¤- One-Generation Reproduction Toxicity Study	+					
TG 416¤- Two-Generation Reproduction Toxicity	+					
TG 417*- Updated Toxicokinetics (ADME* and pharmacokinetics*)			?			
TG 420*- Acute Oral Toxicity - Fixed Dose Procedure	+		?		+	+, A
TG 421- Reproduction/Developmental Toxicity Screening Test	+					
TG 422- Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test		+				
TG 423*- Acute Oral toxicity - Acute Toxic Class Method	+		?			+
TG 425*- Acute Oral Toxicity: Up-and-Down Procedure	+		?			
TG 427- Acute Oral Toxicity: Up-and-Down Procedure		+				
TG 428- Skin Absorption: In Vitro Method		+				
TG 429- Skin Sensitisation: Local Lymph Node Assay						A

TG 431- In Vitro Skin Corrosion: Human Skin Model Test		+				**+, A**
TG 432- In vitro phototoxicity						
TG 436- Acute inhalation Acute Toxic Class						
TG 435- In vitro skin corrosion						
TG 437- In vitro eye irritation (BCOP)						
TG 438- In vitro eye irritation (ICE)						
TG 439- In vitro skin irritation (approved by WNT, submitted to JM)						
TG 442A- Non-radioactive LLNA: DA version						
TG 442A- Non-radioactive LLNA: BrdU version						
TG 451¤-Carcinogenicity study	+	+				
TG 452¤- Chronic Toxicity Studies	+	+				
TG 453¤- Combined Chronic Toxicity/Carcinogenicity Studies	+	+				
TG 471¤- Bacterial Reverse Mutation Test	+	+	**?**	+	+	+
TG 473¤- In vitro Mammalian Chromosome Aberration Test	+	+		+	+	+
TG 474¤- Mammalian Erythrocyte Micronucleus Test	+	+		+	+	+
TG 475¤- Mammalian Bone Marrow Chromosome Aberration Test	+					
TG 476¤- In vitro Mammalian Cell Gene Mutation Test		+	**?**			
TG 485- Genetic toxicology, Mouse Heritable Translocation Assay						
TG 486¤- Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells in vivo	+					
TG 487- In vitro mammalian Micronucleus test (MNvit)						
Non-OECD In vitro assays						
BEAS2B	+				+	+
Caco2	+	+				
A549	+	+		+	+	+
HaCaT	+	+		+	+	+
THP-1	+	+		+	+	+

ANNEX III. TEMPLATE FOR INFORMATION ON ALTERNATIVE TEST METHODS FOR PURPOSES OF REVIEW AND PRIORITIZATION BY SG7

Laboratory name, Funding project	US EPA, ToxCast / ENPRA / JRC cooperation		
Methodology used:			
Assay Name	HCS		
Technology	Fluorescent cellular imaging		
Contractor/Project	US TOXCAST-Cellumen		
Cell/Tissue Type	HepG2		
Additional Assay Detail			
Exposure Times	1,24,72 hr		
Determined parameters	Cell loss, cell cycle arrest, nuclear size, oxidative stress, stress kinase ,DNA damage, mitochondria membrane potential, mitochondria mass, mitotic arrest, microtubule stability		
SOP available?	Yes, attached		
Validation status?	pending		
IPR/patent?	NO		
	NM type, sample IDs	NM type, sample IDs	
TiO2	NM-101, NM-103, NM-104, NM-105		
SiO2	NM-200, NM-201, NM-202, NM-03, NM-204		
CNT	NM-400		
ZnO	NM-110, NM-111		
nanoAg Ceria	NM-300, NM-300DIS		
iron			
carbon black			
fullerenes			