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PRELIMINARY GUIDANCE NOTES ON SAMPLE PREPARATION AND DOSIMETRY FOR THE SAFETY TESTING OF MANUFACTURED NANOMATERIALS

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PRELIMINARY GUIDANCE NOTES ON SAMPLE PREPARATION AND DOSIMETRY FOR THE SAFETY TESTING OF MANUFACTURED NANOMATERIALS

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division

2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. This programme promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

The WPMN recognised that it was essential to develop a guidance document on sample preparation and dosimetry, based on the discussion held in preparing the *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials* [ENV/JM/MONO(2009)21]. It called special attention to this guidance as crucial in using test guidelines when considering the unique chemical and physical characteristics of nanomaterials.

It is important to note that this document, *Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials*, is a "living document" and as such, it will be updated and amended based upon knowledge accumulation and evolving communication and as experience is gained with nanomaterial testing.

The Working Party endorsed this document at its 6th Meeting on October 2009. This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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ABOUT THE WORKING PARTY ON MANUFACTURED NANOMATERIALS (WPMN)

The Working Party on Manufactured Nanomaterials[1] was established in 2006 to help member countries efficiently and effectively address the safety challenges of nanomaterials. OECD has a wealth of experience in developing methods for the safety testing and assessment of chemical products.

The Working Party brings together more than 100 experts from governments and other stakeholders from: a) OECD Countries; b) non-member economies such as China, the Russian Federation, South Africa, Singapore and Thailand; c) observers and invited experts from UNITAR, FAO, UNEP, WHO, ISO, BIAC[2] and TUAC[3]; and d) environmental NGOs.

Although OECD member countries appreciate the many potential benefits from the use of nanomaterials, they wished to engage, at an early stage, in addressing the possible safety implications at the same time as research on new applications is being undertaken.

The Working Party is implementing its work through specific projects to further develop appropriate methods and strategies to help ensure human health and environmental safety:

- OECD Database on Manufactured Nanomaterials to Inform and Analyse EHS Research Activities;
- Safety Testing of a Representative Set of Manufactured Nanomaterials;
- Manufactured Nanomaterials and Test Guidelines;
- Co-operation on Voluntary Schemes and Regulatory Programmes;
- Co-operation on Risk Assessment;
- The role of Alternative Methods in Nanotoxicology;
- Exposure Measurement and Exposure Mitigation; and
- Environmentally Sustainable Use of Nanotechnology.

Each project is being managed by a steering group, which comprises members of the WPMN, with support from the Secretariat. Each steering group implements its respective "operational plans", each with their specific objectives and timelines. The results of each project are then evaluated and endorsed by the entire WPMN.

This document was prepared by the WPMN steering group four leading the work on Manufactured Nanomaterials and Test Guidelines and was endorsed at the 6th meeting of the Working Party.

[1] Updated information on the OECD's Programme on the Safety of Manufactured Nanomaterials is available at: www.oecd.org/env/nanosafety

[2] The Business and Industry Advisory Committee to the OECD

[3] Trade Union Advisory Committee to the OECD.

ABOUT THE PROJECT ON MANUFACTURED NANOMATERIALS AND TEST GUIDELINES

The OECD Guidelines for the Testing of Chemicals (Test Guidelines) are a collection of the most relevant internationally agreed testing methods used by government, industry, and independent laboratories to assess the safety of chemical products. To date, the OECD has published 136 test guidelines which are organised in five sections:

- Section 1 – Physical Chemical Properties
- Section 2 – Effects on Biotic Systems
- Section 3 – Degradation and Accumulation
- Section 4 – Health Effects
- Section 5 – Other Test Guidelines

These Guidelines are an important component of the system of Mutual Acceptance of Data (MAD), which has legally binding implications for OECD member countries (and those non-members who have adhered to MAD). MAD is based on an original OECD Council Decision with subsequent additions.

As part of its Programme of Work, the Working Party on Manufactured Nanomaterials (WPMN) [ENV/MONO(2008)2] established, in 2006, a project entitled "**Manufactured Nanomaterials and Test Guidelines**" to review the published Test Guidelines to assess whether or not they are suitable for manufactured nanomaterials. The project identifies the need for new Test Guidelines or amendments to existing Test Guidelines or develops guidance would describe how existing Test Guidelines could be applied to nanomaterials. This work involves close collaboration with OECD's Working Group of the National Coordinators of the Test Guidelines Programme (WNT). The project was carried out by a steering group 4 (SG4) which comprises members of the WPMN, with support from the Secretariat.

The first task of the project was to identify questions regarding dosimetry, including what new measurement techniques will be needed to understand internal doses, and how to prepare and administer dosing material for *in vivo*/ *in vitro* studies for toxicity as well as for ecotoxicity, and fate and behaviour in the environment. The project also gathered existing information about the unique characteristics of manufactured nanomaterials and how such characteristics could impact on testing approaches. This activity was supported by a review of "white papers" or reports published by 2007.

Following the previous activities, the project undertook the review of OECD Test Guidelines for their applicability to manufactured nanomaterials (MN). The review was conducted by four sub-groups under the steering group corresponding to the four sections of OECD Test Guidelines: Section 1 – Physical Chemical Properties; Section 2 - Effects on Biotic Systems; Section 3 - Degradation and Accumulation; and Section 4 - Health Effects. The *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials* [ENV/MONO(2009)21] combined the results from the review of the four sections of the OECD Test Guidelines.

Based on the discussion held in preparing the *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials*, the WPMN SG4 recommended the WPMN to develop a guidance document on sample preparation and dosimetry. It called special attention to this guidance as crucial in using test guidelines when considering the unique chemical and physical characteristics of nanomaterials.

The first draft of this document was prepared by a drafting group, under the SG4, comprising delegates from the WPMN and WNT, and was presented at the 5th meeting of the WPMN (March 2009). The 5th WPMN provided some inputs and also recommended that the document be forwarded to the WNT for their comments. The second version was prepared for the 21st meeting of the WNT. In addition, the 5th WPMN also acknowledged that it would be valuable to get inputs from the wider research community especially from laboratory researchers; therefore, all delegations to the WPMN and WNT were encouraged to circulate this document as widely as feasible to enhance input to the document. The third version was presented at the 6th meeting of the WPMN (28-30 October 2009) and the fourth version was presented at the 22th meeting of the WNT (March 2010). Since the 6th meeting of the WPMN, delegations to the WPMN as well as the WNT had had a further opportunity to provide inputs to this guidance and have agreed to declassify this document.

The "*Preliminary"* was attached to the title of this guidance notes by the WPMN at its 6th meeting, because the specific methods for the safety testing of nanomaterials has not been fixed and various challenges to developing safety testing, including the OECD Sponsorship Programme (see following the *Executive Summary*), are on their way. Therefore, this guidance notes is susceptible to change until the test methods for nanomaterials are established, so it is important to note that this document, *Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials*, is a "living document" and as such, it will be updated and amended in an iterative manner based upon knowledge accumulation, evolving communication and as experience is gained with the testing of nanomaterials.

EXECUTIVE SUMMARY

The unique properties of manufactured nanomaterials raise the question of whether the current OECD Test Guidelines are adequate to appropriately address their characterisation and the assessment of their toxicological properties. Based on the discussion held in preparing the *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials* [ENV/JM/MONO(2009)21], it was recognised that it is essential to develop a guidance document on sample preparation and dosimetry. It called special attention to this guidance as crucial in using test guidelines when considering the unique chemical and physical characteristics of nanomaterials.

The purpose of this document, *Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials*, is primarily to assist sponsors as they conduct testing in support of the WPMN's exploratory testing programme (OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials[4]) as well as other users involved in the testing of manufactured nanomaterials. This guidance includes general and common issues (Section I to Section IV) as well as specific consideration (Section V) on sample preparation and dosimetry for the safety testing of manufactured nanomaterials.

As a general point, dosimetry should always report mass concentration, but for nanomaterials, the results may be better expressed as a function of surface area or particle number because particle size and specific area may play a major role in determining the toxicity of nanomaterials. So any size distribution measurements and surface area measurements would need to be done for each dose. Also, the soluble nanomaterials are unlikely to need different sample preparation techniques, therefore these guidance notes refer and apply to water insoluble manufactured nanomaterials.

The section on specific considerations is composed of 4 parts: i) physical chemical properties (Section V ; A); ii) ecotoxicity studies (Section V ; B); iii) degradation, transformation and accumulation (Section V ; C); and iv) health effects (Section V ; D). These parts may give researchers specific orientation to those issues that, at present, seem most promising for yielding meaningful and reproducible test results.

[4] The OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials aims to test selected nanomaterials for their physical-chemical properties, environmental degradation and accumulation, environmental toxicology, and mammalian toxicology in the "*List of Manufactured Nanomaterials and List of Endpoints for Phase one of the OECD Testing Programme*" [ENV/JM/MONO(2008)13/REV] available at: www.oecd.org/env/nanosafety.

SECTION I: GENERAL INTRODUCTION

In its review of the OECD harmonised test guidelines, Steering Group 4 of the Working Party on Manufactured Nanomaterials (WPMN) recommended the development of guidance on sample preparation and dosimetry for tests using manufactured nanomaterials. Such guidance would be a separate document from the OECD's existing guidance on difficult substances [*No. 23: Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures,* ENV/JM/MONO(2000)6] and would be written, amongst other things, primarily to inform the WPMN's exploratory testing programme (OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials) as well as other users involved in the testing of manufactured nanomaterials. Drawing from expertise both among the WPMN membership as well as within the member delegations, a drafting group developed the guidance during 2008-2009.

The purpose of this guidance is primarily to assist sponsors as they conduct testing in support of the WPMN exploratory program. Its scope is therefore focused on the kinds of tests that address the endpoints and the types of nanomaterials being tested under the sponsorship programme. Nevertheless, the WPMN hopes that this guidance will also be of use to the scientific community at large, in particular to those investigators conducting tests to advance understanding of the environmental, health, and safety implications of manufactured nanomaterials. It is recognised, however, that due to the wide variety of nanomaterials, it is difficult to develop specific or detailed advice applicable to all nanomaterials; accordingly, the performer of a study will have to exert some judgment on a case-by-case basis on the applicability of the recommendations given in these Guidance Notes to their particular material.

These Guidance Notes refer and apply to the water insoluble manufactured nanomaterials as the WPMN considered that soluble nanomaterials are unlikely to need different sample preparation techniques than other chemicals, other than precautions dictated by the specific reactivity of each material. However their size will still affect where they are being deposited e.g. in the lung.

Because few, if any, standard testing approaches have been developed for nanomaterials, this guidance is not a "cookbook" for preparing samples and administering doses, but rather outlines – often in a general or descriptive manner – considerations based on early results with nanomaterials or other experience with chemicals and particulates. It is a guide in the most basic sense, designed to point researchers in directions that at present seem most promising for yielding meaningful and reproducible test results.

This guidance should be considered a living document, subject to amendment and refinement as researchers gain greater understanding of how to handle nanomaterials in test situations. A significant benefit of the WPMN exploratory program will be the knowledge gained in preparing test samples and administering doses across a wide range of testing scenarios and material types. Accordingly, regular updates of these Guidance Notes can be anticipated. As experience is gained through both the sponsorship programme and other efforts, the WPMN will consider at what point and in what form amending this guidance.

SECTION II: TERMINOLOGY

Dispersion versus solubility

Most dosing techniques require the test material to be in a liquid phase (generally aqueous) for delivery and (eco) toxicologists sometimes use the terms "in solution" or "solubility" to infer this. However, in particle chemistry these terms are often inappropriate. The introduction of an insoluble nanomaterial to a liquid or other aqueous medium with the intention of making a stock "solution" will involve dispersion. A stable dispersion of a nanomaterial in a liquid is referred to as a colloidal dispersion. The term "colloid" applies to particles or other dispersed material in the 1 nm - 1μm size range (IUPAC 1997). Colloids are dispersed rather than *dissolved* in a medium, and the term "dispersion" rather "solubility" or "solution" is used in this document to mean the addition of nanomaterial to a liquid phase, where the solid and liquid phases co-exist. Some metal nanoparticles may release ions from the surface into the surrounding water (corrosion/degradation) and it is therefore possible that these nanomaterials will eventually dissolve or degrade completely. The term "dissolved" is used in other OECD documents and historically in toxicology to mean the component of a liquid sample that has passed through a 0.45 μm (or similar) filter. However, as (colloidal) dispersions of nanoparticles might also pass through such filters, it is recommended that use of the term "dissolved" should be restricted to the formation of true nanomaterial solutions, and where both liquid and particulates are present the term "dispersed" should be used. The terminology used in this document comes directly from colloid science and may need revision for more complex (second generation and beyond) nanomaterials.

In addition, nanoparticles may interact with the liquid phase components, partially or totally yielding soluble or dispersed transformation products (as well as some solubilised nanomaterial itself) that may influence the overall toxicity and fate processes. This possibility needs to be taken into account when selecting the media and procedures as well as in the assessment of the results of any experiment.

The formation of other colloidal systems as emulsions (dispersed liquid drops in another immiscible liquid) have not yet been considered in these Guidance Notes, although they will become more relevant as manufactured nanomaterials are further modified and functionalised.

Consideration of stability in sample preparation

Many nanoparticles are presented in the form of aqueous dispersion (some may exist in the form of organic or oil based dispersions). The particles may be stabilised by surfactants (either added or not) or surface charges. Generally, three different forces are encountered in normal dispersions of particles: electrostatic and steric repulsions, and Van der Waals attraction, and, for magnetic particles, an additional magnetic attraction force. The stability of the dispersion depends on the net effect of all these forces, which is determined mainly by the properties of the particle and the dispersing medium and particle surface properties, i.e. surface chemistry. For example, particles sterically stabilised by non-ionic surfactants would be less sensitive to pH and electrolyte conditions than those that are only electrostatically stabilised. For particles stabilised by (extra) surfactants, dilution during sample preparation may lead to desorption of

the surfactant from the particle surface and hence agglomeration of particles may occur. For those particles stabilised by surface charges, pH and ionic strength in the medium used for sample preparation may cause agglomeration of the particles, in which case the test results may differ from what would otherwise be the case. In such cases, suspension chemistry is important to create kinetically stable suspensions. It is also important to consider the fundamental relevance of surface treatment or modifications of nanomaterials. In absence of these, nanomaterials with a primary particle size < 100 nm will tend to form large agglomerates and the primary particles will not disperse in water. Sonication or stirring can break up agglomerates into smaller sizes and can result in their temporary suspension in solution. However, once sonication or stirring is stopped, the smaller agglomerates will tend to re-agglomerate into larger ones and will tend to re-precipitate out of solution.

The zeta potential (at a specified pH and ionic strength) and/or the isoelectric point of the particles (in case the particles are stabilised by surface charges) should be determined and provided so it can be used for the fate assessment. For sterically stabilised particles, the zeta-potential may not be a suitable parameter to estimate the fate of the particles *a priory*.

Agglomerate (Working definition from ISO TS27687 2008)[5]

Agglomerate: collection of weakly bound **particles, aggregates** or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

- NOTE 1. The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.
- NOTE 2. Agglomerates are also termed secondary **particles** and the original source particles are termed primary particles.

Nanoparticles are not necessarily primary particles.

Aggregate (Working definition from ISO TS28687 2008)[6]

Aggregate: particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

- NOTE 1. The forces holding an **aggregate** together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.
- NOTE 2. **Aggregates** are also termed secondary **particles** and the original source **particles** are termed primary particles.

Nanoparticles are not necessarily primary particles.

[5] These definitions differ from the ones described in the British Standards Institution Standard (BS 2955: 1993) and Nichols, Gary, et al. A Review of the Terms Agglomerate and Aggregate with a Recommendation for Nomenclature Used in Powder and Particle Characterization, Journal of Pharmaceutical Science, Vol 91 2103-2109, 2002. The more up-to-date and currently more widely accepted definitions from ISO are preferred in this document.

SECTION III: CONSIDERATIONS ON APPROPRIATE DOSE-METRICS

Dosimetry should always report mass concentration (e.g., mg/l), but for some nanomaterials the results may be better expressed as a function of surface area or particle number because particle size and specific area may play a main role in determining the toxicity of nanomaterials. Indeed this seems to be the case for many nanomaterials and there is a trend in toxicology to relate potential toxic effects of nanomaterials with these properties individually or in combination with each other. Accordingly, measurements that enable expression of data in this way should be made where and whenever possible (e.g., particle number counts in test media and surface area measurements on the dry nanomaterial). Particle size distributions are a function of particle mass concentration, and so any size distribution measurements or surface area measurements would need to be done at each dose. It is also recognised that surface area measurements are made in dry state and assuming that no aggregation but only agglomeration occurs.

Agglomeration in the suspensions may be a slow process especially at low mass concentrations. Hence, it is recommended that the determination of the particle size be repeated at regular intervals to ensure that dynamic changes of the dose are detected and recorded.

The fact that specific surface area of particles in water is not directly accessible for nanoparticles the derivation of surface area from size measurements has to be done with great care. Most sizing techniques will report a fraction of the outer diameter of existing or forming agglomerates (depending on which technique is used). The back-calculation from this diameter to a surface area may be highly erroneous.

SECTION IV: COMMON ISSUES REGARDING SAMPLE PREPARATION AND DOSIMETRY

There are some common features of sample preparation and dosimetry that apply to both toxicology and ecotoxicology, as well as different routes of exposure/delivery methods. These common aspects are outlined below, with deviations from these described in the relevant section of this document. One aspect that always deserves particular attention is the fact that small impurities can have a strong impact on the physico-chemical properties of nanomaterials. Therefore, it is important to pay attention to the presence of impurities. Substances with different impurities may behave very differently, even if the main constituent is the same.

Further, there is considerable interest in producing nanomaterials with specific surface functionalities/modifications. Such modifications can significantly affect the chemical reactivity of a nanomaterial, and thereby its potential effects. In addition, certain modifications (e.g. DNA or protein functionalisation) have been shown to have an effect on the uptake of nanoparticles into cells in vitro. Therefore, the surface functionality of a nanomaterial may have a strong impact on its behaviour. It is important to note that the surface functionality can determine the size of the entity that is dispersed in solution. Surface functionalisation is often employed to minimise nanomaterial agglomeration. Thus the size of the entity dispersed may differ by more than an order of magnitude for a given nanomaterial depending on whether or not it has been surface functionalised.

Other common features include:

1. Storage and stability of test material

Nanomaterials should be stored according to the manufacturer's recommendations, but some general issues are highlighted when manufacturer's information is limited. The usual considerations for storing chemicals will apply, including avoiding extremes of temperature, sunlight, and moisture. Nanomaterials that are supplied as dry powders or dispersions should be stored so that they remain dry or under liquid respectively. Clearly, experimenters will need to make stock dispersions from the original material supplied by the manufacturer. These dispersions should be stored taking into account the usual considerations above for any chemical, but also considering the reactivity of the material. For example, photoreactive materials should be kept in the dark. Once stock dispersions are prepared, and a full characterisation of the freshly prepared stock has been made, additional checks should be done to confirm the shelf life of the material. Two key aspects need to be investigated: (i) whether or not the nanomaterial gradually dissolves or transforms such that the solid material disappears (e.g., for metal particles that form free metal ions in the external medium) (ii) any temporal changes in the particle size distribution and surface charge in the stock dispersion. If changes occur, then protocols should be developed to restore the particle size distribution (e.g. re-sonicating the dispersion just before dosing in the case of aggregation). If the stock dispersion cannot be restored, it should be made fresh from the same batch number of the test material and re-characterised. If a different batch number of test material is used, then additional physico-chemical characterisation will be required.

2. The chemical composition of the test media

The chemical composition of the test media will affect particle aggregation/agglomeration. The following parameters should therefore be measured for nanomaterials ecotoxicology studies, or salines used in mammalian studies (in additions to other routine measurements):

- **Ionic strength**- it is likely that many types of nanomaterials will agglomerate in very dilute brackish water, or any saline conditions, where studies have shown that even 2 % seawater will do this. Thus, for any marine or estuarine studies the salinity and NaCl concentration in the water should be recorded. In natural fresh water or seawater, NaCl is likely to be the bulk electrolyte. Similarly, for salines used in mammalian studies, the composition should be given so that the ionic strength can be calculated. It is also highly likely that the typical salt concentrations in physiological salines (e.g., 0.9 % NaCl) and culture mediums would cause agglomeration of some nanomaterials.
- **Calcium concentration and hardness**- divalent metal ions can also have a big effect on agglomeration, especially at low salinity (freshwater). Therefore, in all freshwater ecotoxicology studies the calcium (Ca) concentration of the water should be measured. In addition, magnesium (Mg) concentration, and total hardness would be useful. In mammalian studies, if drinking water is used to deliver nanomaterials then Ca, Mg and total hardness of the water should similarly be measured. These procedures are well known. In mammalian studies special attention should be given to reporting the Ca and Mg concentrations in salines, including the anion (e.g., whether $MgSO_4$ or $MgCl_2$ was used in their preparation).
- **pH**- This should be routine in any experiment. pH affects agglomeration of *charged nanomaterials. Physiological saline*s usually contain pH buffering, and the buffers should be reported precisely (e.g., the specific type of Tris buffer with the full chemical name, or exact details of phosphate buffers). Where commercially available buffer tablets or ready-made solutions are used, the full composition of the buffer should be reported.
- **Dissolved organic matter**- it is very clear that the precise type of organic matter, and the ligands it presents, will have potentially large effects on agglomeration and dispersion of nanomaterials. It would therefore be prudent to have some general information about the organic matter in any water. This could be something simple like a measurement of total organic matter, or dissolved organic carbon. This would at least give an overview of the general type of water. For mammalian studies, the addition of bovine serum albumin (BSA) and antibiotic preparations to salines represent a source of organic matter. It would be prudent to use high purity reagents in these cases (e.g., fatty acid-free BSA or similar) rather than cheaper reagents. Since any charge-carrying organic substance which may be adsorbed on the surface of the nanoparticles will change the charge properties of the surface, and hence the dispersion behaviour, all the organic substances (proteins, antibiotics) added should be stated.
- **Alkalinity**- this may affect agglomeration and similar arguments to pH apply. This is a routine measurement in ecotoxicology, but not in mammalian toxicology. This will be especially important where bicarbonate buffers are used as the main method to control pH in salines.
- **Dispersing agents**- In case an added surfactant is used to stabilise the dispersion, it would probably normally be of high concentration, considering the high specific surface area presented by many nanomaterials. Distribution of the dispersing agents between the aqueous phase and the particle surfaces would occur. Therefore, information regarding structural formula and concentration of the agent should be provided. The use of strong dispersing or stabilising agents may modify the bioavailability of the nanomaterial and, in addition, if an added agent has been

used to stabilise the stock solution, this may not be appropriate for studies that investigate the fate and behaviour of nanomaterials in natural conditions. Accordingly, much care should be paid in the conduct of tests and the interpretation of the test results when the use of such agents is unavoidable.

3. Characterisation of stock dispersions

In addition to routine water quality measurements in ecotoxicity testing, or reporting saline quality in mammalian studies, there is some essential information required about the nanomaterials (discussed in Handy et al., 2008; Crane and Handy, 2007; Crane et al., 2008). The following would apply to stock dispersions and arguably, this list could be common to human health and ecotoxicology studies:

i) Any manufacturer's information on the test material.

ii) Measured mean primary particle size (for example by electron microscope). The method of particle size determination should be described and the character of the mean (number, volume, z- or intensity) must be given. If a certain given mean/average value is calculated from a primary data (e.g. volume average derived from dynamic light scattering z-average) the calculation procedure should be described.

iii) Particle size distribution and indications of mono or polydispersity (e.g., by dynamic light scattering or similar optical method), or other attempt to describe aggregates, agglomerates or ranges of particle sizes in the stock dispersion, including distribution of primary particles. The methodology to derive this size distribution either must be standardised or must be described together with the applied procedures. If a buffer or saline is used to make the dispersion, then the exact composition of the medium, measured pH, temperature and any aeration or gassing of the dispersion should also be reported as this may affect particle size distributions. The method of dispersion (stirring, sonication) should be fully described (duration, stir speed, sonication power etc.)

iv) Mass concentration (measured) in the stock dispersion (e.g., mg/l).

v) Surface area measurements of the primary particle will allow results to be calculated on a surface area basis, but may have limited validity for the aqueous dispersions.

vi) For some charged particles, surface charge may be critical to the agglomeration process and so the surface charge may be indirectly assessed via measurements of zeta potential. Since the deviation of the zeta-potential is a function of the ionic strength and composition of the dispersing medium, the conditions during determination should be standardised or reported. It would also be important to measure or fix other abiotic factors that might alter this, such as solution pH and ionic strength.

vii) Any other measurement that is particularly relevant for a specific particle type, for example, aspect ratio for fibres, length of nanotubes, surface functionality.

A detailed analysis of the composition of the stock dispersion should be undertaken with special attention to the possible impurities in it. Contaminants can be incorporated into the nanomaterial at any point, during production, handling and dispersing. Examples include iron contamination of carbon nanotubes during fabrication (Jurkschat et al., 2006), THF contamination of fullerene during solvent-exchange dispersion preparation (Markovic et al., 2007), and endotoxin contamination during manufacturing and handling (Vallhov et al., 2006). In some cases these “contaminants” are intrinsic components of the nanomaterial likely to be encountered during real world exposure, such as polyaromatic hydrocarbons on diesel exhaust particles (Xia et al., 2004), in which case their quantities should be measured to compare their impact

across studies. In other cases, such contaminants may not be intrinsic to the original nanomaterial (e.g., contaminants in dispersing agents) and be accounted for in controls. Ultrasonication processes sometimes produce contaminating particles by ablating probe tip and vessel. Alternatively, a purification step may need to be added to stock dispersion preparation.

While a test of the material for metal impurities is relatively easy to perform, a test for unknown organic impurities is often not feasible. Here the information from the manufacturers about additives and possible by-products is indispensable but encompasses both technological and policy implications.

4. Characterisation of samples (prepared from stock dispersions) prior to administration/testing

The general recommendations about the characterisation of stock dispersions (above) should be followed.

The key point is to know the exact composition of the prepared sample, and to report how it was made. As particle size and concentration may vary with depth after stock dispersions are free-settled for a certain time, a consistent sampling point for very heterogeneous samples over time could provide better precision (Ma and Bouchard 2009). The following information is required:

i) Volumes prepared, type of water or solvents used.

ii) pH and use of any buffers.

iii) Exact details of any sonication times (or preferably energy input in J/L) given in terms of durations/intensity/instrument used/frequency of ultrasound.

iv) Exact details of how long after sonication or mixing/stirring before test dispersion was added to test vessels. Any extra (precautionary) period of mixing or sonication (e.g., 30 min or preferably energy input) immediately prior to dosing to the experimental model may be helpful and should be recorded. Re-characterisation of subsamples from stock suspensions after pH modification, sonication or other treatments should be considered.

v) Exact details of volumes added to tanks or test vessels, and how they were mixed in the tanks/test vessels. For example, passively by diffusion, stirred in, allowed to mix with air bubbling around the system. Details about the depth of the liquid under treatment in tanks or vessels or details on the depth of the probe that is inserted under the liquid surface should be recorded and kept constant in all related tests.

vi) pH, ionic strength, dissolved organic matter

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SECTION V: SPECIFIC CONSIDERATIONS

A. PHYSICAL CHEMICAL PROPERTIES [6]

In recognition of the unique properties of manufactured nanomaterials, several regional, national and supranational organisations formed Physical Chemical expert panels for the purpose of issuing recommendations concerning the applicability of existing standardised test procedures (e.g., U.S. EPA Series 830 [U.S. EPA, 2007b] and the OECD Series 100 [OECD, 2007] test guidelines) to these materials. These workgroups have identified a large number of standardised test guidelines that are unlikely to be directly applicable to insoluble manufactured nanomaterials (for example, test guidelines for aqueous solubility and octanol/water partition coefficients); these same issues have ramifications for standardised test procedures in the areas of ecotoxicology, human health effects and in assessing the environmental fate (transport, degradation and accumulation) of these materials. In addition, due to the unique properties of manufactured nanomaterials, some new physical/chemical characterisation procedures may require development in order to allow them to be adequately characterised and to assist in assessing the risks associated with intentional or unintentional exposure of humans or the environment to these materials. Characteristics requiring determination might include (but are not limited to): particle size, size distribution, aggregation, agglomeration state, shape, chemical composition, surface area, surface chemistry, dissociation constant, crystal structure, surface charge, zeta potential, Hamaker constant, interfacial tension, and porosity (Oberdorster *et al.*, 1994; Hunt *et al.*, 1996; Oberdorster *et al.*, 2002; Kreyling *et al.*, 2004; Oberdorster *et al.*, 2005; Nel *et al.*, 2006; Champion and Mitragotri, 2006; Elder and Oberdorster, 2006; Zhu *et al.,* 2006; Warheit *et al.*, 2007; Limbach *et al.*, 2007; Ji *et al.*, 2007; Teeguarden *et al.,* 2007; Murdock *et al.*, 2008; ISO, 2008; Utterback *et al*., 2008; OECD, 2008; Loux and Savage, 2008). A recent review highlights the difficulties associated with the characterisation of more complex nanomaterials that cannot be considered as simple colloids. (Richman, E. K.; Hutchison, J. E., The Nanomaterial Characterisation Bottleneck. ACS Nano 2009, 3 (9), 2441-2446.) The relevance of these characteristics will depend on the specific nanomaterial(s) considered.

Observations from the several groups that have addressed the problem of sample preparation for physical-chemical characterisation often do not distinguish between characterisation appropriate in order to assess human health effects and ecotoxicology. Therefore, the term (eco)toxicology in this section is generally used unless a clear distinction is necessary. Relevant findings are in three main areas:

1. Sample Preparation: When a procedure for generating nanomaterial preparations intended for (eco)toxicological studies is employed, a great attention should be paid to minimise any alteration of the physical, chemical or (eco)toxicological properties of the substrate (Crane et al, 2008). For example, grinding agglomerates may lead to the fracturing of individual particles which in turn

[6] Note: It is recommended to characterising both stock dispersion and the diluted/prepared dispersions for dosing, as appropriate.

can expose new sites of enhanced reactivity. Aqueous nanomaterial dispersions may require the use of surfactants, solvents or sonication, which in turn can alter the degree of agglomeration, fracture individual particles, or alter the bioavailability and toxicity of the parent compound. Interaction with organic material or other constituents contained within the supporting medium also should be taken into account. In order to practically and meaningfully extrapolate laboratory findings to environmental and physiological systems, the difference between sample preparation techniques compatible with the test protocol and the anticipated environmental/physiological processes should be considered. It has to be recognised that the interaction of a nanomaterial with testing media will always influence, if not alter, its properties, as is the case for any other chemical. Another important point for the preparation of aqueous dispersions of nanoparticles is the disequilibrium after mixing and slow reaction (mainly surface chemistry) towards equilibrium. It could be helpful to allow some time for dispersions to equilibrate before they are dosed in an experiment. For example, distilled and/or deionised water is in disequilibrium with atmospheric CO_2, but CO_2 dissolution into the dispersion and adsorption to the surface of freshly dispersed NPs is an important process altering the surface charge of many NPs and the pH of the dispersion. The same holds true for all surface reactions of the NPs with any substance in the used medium (e.g. BSA).

2. Dosing: (Eco)toxicological studies typically employ dosage procedures intended to be both reproducible and quantitative. However, aqueous nanomaterial dispersions may be very sensitive to the techniques employed in their preparation and they may not necessarily follow the principles of equilibrium partitioning. In particular, significantly more empirical data may be required in order to develop methods designed to ensure reproducible and quantitative dosimetry (especially with aqueous dispersions).

3. (Eco)toxicological characterisations: Human and environmental toxicologists seek to develop rigorous mechanistic understandings of their findings for the purposes of elucidating: a) the toxicological response, b) possible antagonisms and synergisms with other toxicants, and c) predictive methodologies useful for assessing the risks posed by new products with limited characterisations. This last aspect is perhaps the least understood of these three main areas.

A.1 Tentative Guidance Relevant to Sample Preparation and Dosimetry for Physical Chemical Characterisation

A.1.1 Particle size, shape, size distribution, and degree of agglomeration

There exists a suite of standardised procedures (e.g. by EPA, OECD and other organisations) for physically characterising particles, however, many of them have minimum size cut off thresholds that exceed the 1 nm to 100 nm size range, although, agglomerates/aggregates may form from the primary particles to make larger secondary particles. Of all of the standardised characterisation procedures, these are perhaps the most easily modifiable through incorporation of more recent technological advances in areas such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), confocal microscopy, light scattering, atomic force microscopy, etc. Researchers also should recognise that dimensional values obtained from physical inspection might differ from hydrodynamic estimates. Thus, values for particle size, shape, size distribution and degree of agglomeration will depend both on the employed methodology as well as on the properties of the medium supporting the sample under consideration. In particular, care should be exercised in extrapolating properties observed under high vacuum conditions (as with SEM and TEM measurements) to aqueous and physiological dispersions. Lastly, nanomaterial physical properties that may influence any of these properties (e.g., magnetisation) also should be characterised.

A.1.2 Chemical description (composition and identification)

A thorough chemical description of the nanomaterials comprising both purity and coating or surface modification(s) is essential. This issue encompasses both technological and policy implications. It is likely that nanomaterial preparations will contain impurities and might receive surface treatments or coatings designed to generate desirable interfacial properties (Alexandre and Dubois, 2000). In addition, the nanomaterials may contain residues of catalysts or other materials used in its production. Although there are a number of standardised test guidelines addressing the purity issue, it may be necessary to adjust them to focus more on the issue of surface coatings. Additional guidance also may be needed to specifically address surface coated nanomaterials.

A.1.3 Specific surface area

Standardised procedures are available for measuring colloidal particle specific surface areas that are likely applicable to manufactured nanomaterials (e.g., BET procedures, dye adsorption, negative ion adsorption, particle morphology etc.). However, in many cases specific surface area measurements are derived quantities that depend on the nature of the probe molecule (Klobes *et al.,* 2006). Nevertheless, in comparison with some of the other characterisation procedures, measurement of the specific surface area of a given sample is relatively straightforward. In addition, investigators may wish to evaluate whether the particle size distributions (and surface areas) of sparingly soluble manufactured nanomaterials are altered through ripening and/or phase alteration phenomena (Ohman *et al*., 2006; Lefevre *et al.,* 2006). Lastly, according to Klobes *et al.* (2006), the measurement of the specific surface area might most efficiently be conducted concurrently with measurements of pore size, pore size distribution, porosity and perhaps even particle density as these properties will most probably have an important influence on the (eco)toxicological properties of the material.

A.1.4 Surface chemistry

The surface properties of nanoparticles are critically important with respect to the agglomeration, aggregation, and toxicity behaviour of these particles. In application, the expression *surface chemistry* may need to be considered in more detail or perhaps in a hierarchical manner. For example, this issue has been examined by experts in the fields of spectroscopy, interfacial analysis (ISO, 2008), toxicology (reactive oxygen species generation; Oberdorster *et al.,* 2005; Nel *et al.,*2006; Balbus *et al.,* 2007), surface complexation modelling (Loux and Savage, 2008), and colloid chemistry (colloidal particle stability; Shaw, 1992).

One aspect of surface chemistry that may be particularly relevant is the surface acidity related to dissociation constants of surface ionisable sites. Ionisable sites may influence the surface charge which has been considered significant in toxicological studies (Oberdorster *et al.,* 2005; Nel *et al.,* 2006). Surface ionisation also may play a major role in colloidal particle stability (Shaw, 1992) and may even inhibit migration into hydrophobic phases (e.g., octanol/water partition coefficients). Because of its significance, surface ionisation will be discussed in more detail in this section.

Many metal oxide nanomaterials possess surface ionisable sites (e.g., >SOH groups) that exhibit surface complexation reactions with the hydronium ion (and other soluble ions) of the following forms (where >SOH designates a reactive bound site):

$$>SOH <==> >SO^- + H^+$$

$$>SOH_2^+ <==> >SOH + H^+$$

Historical mass action expressions that are sometimes used to describe these reactions are:

$$K_{a1} = \frac{[>SOH][H^+]EXP(-e\Psi/kT)}{[>SOH_2^+]}$$

and

$$K_{a2} = \frac{[>SO^-][H^+]EXP(-e\Psi/kT)}{[>SOH]}$$

Where the subscripted K's represent intrinsic acidity constants, the species in brackets represent the concentrations of reacting species, e is the charge of the proton, Ψ is the surface potential, k is the Boltzmann constant and T is the absolute temperature.

31b. These reactions are analogous to the acidity behaviour of a diprotic acid in aqueous solution with two exceptions: 1) the use of concentrations instead of chemical activities, and 2) the exponential Boltzmann term which converts bulk solution hydronium ion concentrations into interfacial values. The interfacial potential (Ψ) can be related to the zeta potential obtained from electrokinetic studies which in turn can in part be used to develop predictions of colloidal particle stability. In addition, geochemical speciation model predictions of surfacial ionised site concentrations can be used to interpret (eco)toxicological findings when the surface charge is considered a significant variable.

Unfortunately, although they display a vast potential for interpreting (eco)toxicological findings, there are a multitude of incompatible surface complexation models available for simulating these reactions (e.g., diffuse layer models; constant capacitance models; Gouy-Chapman-Stern-Graham models; triple layer models etc.). In addition, there is no general consensus within the environmental research community as to which model is demonstrably superior. The minimum dataset needed to utilise models of this nature then includes intrinsic acidity constants, site densities (requiring values for total concentrations of reactive sites and specific surface areas), solution composition and a multitude of binding constants useful for describing reactions with those dissolved ions commonly found in aqueous media. Some of these models (e.g., constant capacitance and triple layer models) may also require one or more capacitance terms. In summary, the area of surface complexation modelling is a decades-long research area and in the short term, it may be preferable to develop empirical datasets of the driving variables obtained from inquiries in this area: solution-composition-dependent surface charge densities and zeta potentials.

A.1.5 Surface charge, zeta potential and Hamaker constant

The toxicological role of surface charge is discussed in Oberdorster *et al.* (2005) and Nel *et al.* (2006). The surface charge of manufactured nanomaterials in aqueous suspension will likely result from two phenomena: 1) isomorphic substitution of an ion with one valence by an ion of a different valence in the crystalline structure and 2) surface site reactions with the proton and other ions in aqueous solution (Loux

and Savage, 2008; Hemraj-Benny *et al.*, 2008). In particular, the surface charge of a given particle may be dependant both on pH and solution composition. Clearly, those (eco)toxicologists conducting inquiries in this area will need to insure that it is measured within the bounds of the fluid properties likely to occur in the medium of interest.

Associated with a surface charge is a surface potential (in volts). The surface potential plays a major role in such phenomena as: 1) surface complexation with other ions in solution (Loux and Savage, 2008), 2) interfacial pH and oxidation/reduction potentials (Loux and Anderson, 2001), and 3) the stability of colloidal particle dispersions in water (Shaw, 1992; Loux and Savage, 2008). Although it is difficult to measure the surface potential on nonconductive surfaces, it can be related to a zeta potential obtained from widely applicable electrokinetic procedures (Hunter, 1981; Delgado *et al.*, 2007). If one can obtain dispersion-composition-dependent zeta potentials for particles in aqueous dispersions, one can them employ Poisson-Boltzmann charge/potential relationships to obtain estimates of the charge density at the beginning of the diffuse layer. In conjunction with a specific surface area measurement, one can then estimate a total charge on the surface.

Along with the zeta potential, the Hamaker constant (which may be obtained from a variety of procedures; Visser, 1972; Bergstrom, 1997; Ackler *et al.,* 1996) can be used to predict whether manufactured nanomaterials are likely to agglomerate in natural waters (Nowack and Bucheli, 2007; Loux and Savage, 2008). Predictions of this type of agglomeration will be limited to homo-aggregation of the particles since the data needed to predict the deposition with a heterogeneous set of natural surfaces is often not available (e.g. Hamaker constants and zeta potentials). Agglomeration is considered to play a role in (eco)toxicological phenomena; this property also may be useful for toxicological interpretations.

A.1.6 Influence of water chemistry on nanomaterial properties and dispersion behaviour

Although not rigorously tested yet, Derjaguin-Landau-Verwey-Overbeek (DLVO) based theories exist for predicting the agglomeration behaviour of nanomaterial dispersions in water. For example, Shaw (1992), Ross and Morrison (1988) and Overbeek (1952) derived expressions for predicting the minimum ionic strength in water (or the Critical Coagulation Concentration [CCC]) needed to lead to the onset of room temperature colloidal particle agglomeration (Loux and Savage, 2008):

Shaw (1992):

$$CCC = \frac{3.84E\text{-}39\ \gamma^4}{A^2 z^6} \quad (\text{mol dm}^{-3})$$

Ross and Morrison (1988)

$$CCC = \frac{8.74E\text{-}39\ \gamma^4}{A^2 z^6} \quad (\text{mol dm}^{-3})$$

Overbeek (1952):

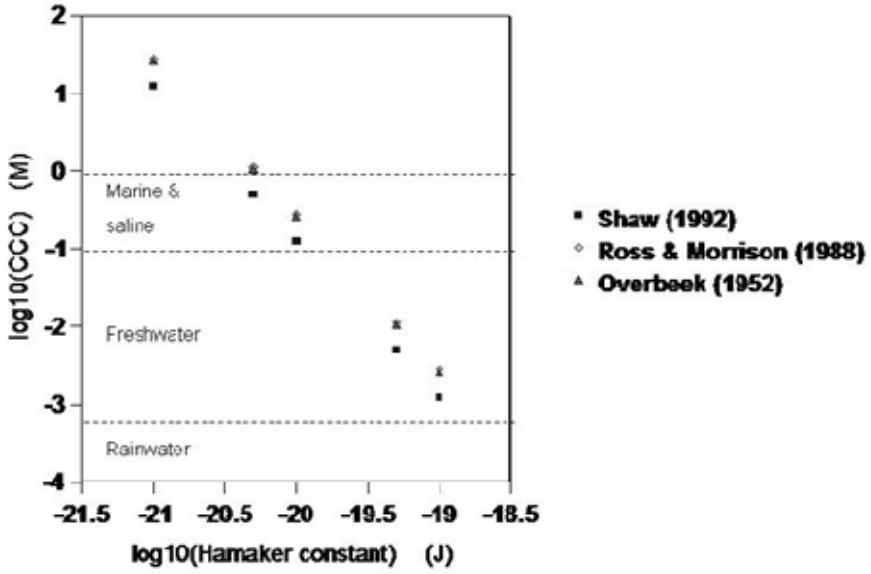
$$CCC = \frac{8.1E\text{-}39\ \gamma^4}{A^2 z^6} \quad (\text{mol dm}^{-3})$$

CCC – Critical coagulation conc.	A – Hamaker constant (Joules)
k – Boltzmann constant	z – counter ion valence
Ψ – zeta potential	T – absolute temperature
e – proton charge	γ – (EXP(zeΨ/2kT)-1)/(EXP(zeΨ/2kT)+1)

In application, given values for both the zeta potential and Hamaker constant of a colloidal (or nano-) particle in aqueous dispersion, these calculated CCC values can be compared with the solution ionic strength (I; where $I = 1/2\Sigma[c_i z_i^2]$; c_i is the concentration and z_i is the valence of dissolved ion i) and predictions can be made as to whether these colloidal particles are likely to form agglomerates. By definition, this approach requires knowledge of the concentration of the major ions in the aqueous medium (i.e., the *Water chemistry*). This is usually defined in most test guidelines.

Figure 1 compares estimated critical coagulation concentration (CCC) values obtained using the above three equations for particles with an absolute zeta potential of 0.025 V in a room temperature aquatic medium. Based on these simulations, particles with a Hamaker constant of 1E-19 J (e.g., iron and aluminium oxides; Loux and Savage, 2008) are predicted to remain in a stable dispersion only in low ionic strength freshwaters and rainwater. In contrast, particles with a Hamaker constant of 1E-21 J (e.g., some natural organic matter or organic matter coated particles; Loux and Savage, 2008) are predicted to remain in a stable dispersion even in hyper saline waters. Other possible influences on colloidal particle stability such as pH and organic matter are further discussed in parts C and D as well as in the introduction of this guidance document.

Figure 1. Estimated critical coagulation concentration (CCC) values for a room temperature system with particles possessing zeta potential of +/- 0.025 V



A.1.7 Preparation of liquid dispersions; octanol/water partition coefficients (K_{ow}s)

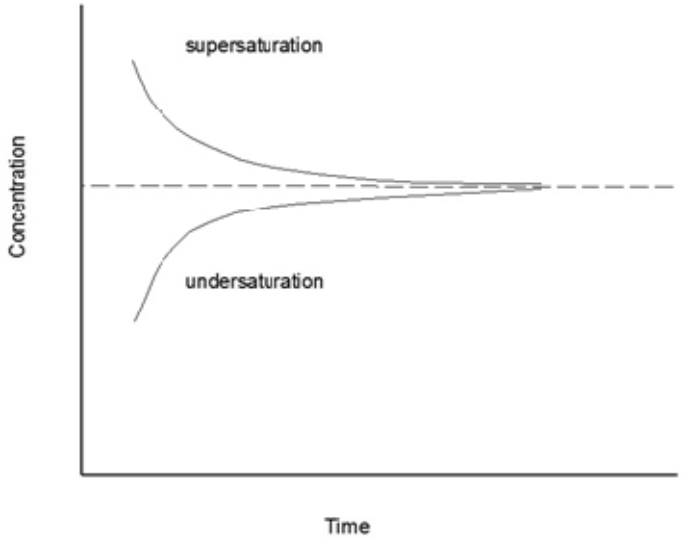
Both the EPA (Utterback et al., 2008) and OECD (OECD, 2008) Physical Chemical workgroups identified difficulties in the application of standardised procedures designed to measure solubility for estimating the likelihood that insoluble manufactured nanomaterials will form stable dispersions in either water or octanol. An octanol/water partition coefficient (K_{ow}) has an equilibrium thermodynamic basis and is predicated on the assumption that the solute has equal chemical potentials in both liquid phases under equilibrium conditions. As will be discussed later, particles suspended in fluids may well be governed by kinetic limitations rather than thermodynamic considerations and therefore, under these circumstances, the application of a standardised concept such as an octanol/water partition coefficient may be inappropriate in this type of system. Nevertheless, K_{ow}s play a key role in assessing the hydrophobicity of truly dissolved chemicals and it may be necessary to develop surrogate procedures to acquire information of this nature with liquid dispersions.

A point raised within workgroup deliberations concerned the need to definitively characterize solutions/dispersions used in (eco) toxicological studies. Specifically, there is no evidence to support (or refute) a contention that true solutions (derived from parent nanomaterials), insoluble nanomaterial dispersions or dispersions of insoluble nanomaterial agglomerates and/or aggregates are likely to engender the same toxicological response. Hence, the lack of information of this nature may compromise the interpretation of any subsequently obtained (eco) toxicological results.

A recent publication (Jafvert and Kulkarni, 2008) demonstrated great promise for extending the traditional concepts of equilibrium solubility/partitioning theory to buckminsterfullerene colloidal dispersions. It is not at all clear that similar successes will be achieved with larger, higher molecular weight manufactured nanomaterials. For example, Figure 2 illustrates the type of expected solubility behaviour for a compound immersed in a liquid as a function of time. Generally speaking, an undersaturated solution will dissolve more of the solute with time until it reaches a maximum value. In contrast, an oversaturated system (e.g., possibly obtained through the evaporation of solvent) will precipitate the solute onto particles

with time until it reaches a minimum value. Therefore, with a compound exhibiting true thermodynamic solubility, one anticipates that one will observe a common equilibrium value in both under- and oversaturated systems given sufficient time. However, the required time to reach equilibrium may have implications for the environmental fate of these products because the kinetics of solubility phenomena is sensitive to the diffusive properties of the solute. In addition, it is known that very high molecular weight products can take extended time periods to reach equilibrium. Hence, even if a given liquid dispersion is being governed by thermodynamic equilibrium processes, the length of the equilibration period may be such that the local equilibrium assumption may be inappropriate in these systems.

Figure 2. Expected concentrations as a function of time for a system exhibiting true thermodynamic solubility behaviour.



There are standardised methods for estimating particle size distributions via the preparation of time-dependent aquatic suspensions in the field of soil science (e.g. Amezketa, 1999; Fristensky and Grismer, 2008) and the adaptation of these existing methodologies may prove to be an efficient means of achieving this goal.

A.1.8 Crystal structure

Standardised powder X-ray diffraction procedures exist for determining the crystal structures of colloidal particles (at least larger ones). Physical inspection also may provide valuable information in this area. Crystal structure determination is useful for distinguishing among different crystal phases of materials of the same chemical composition. In turn, this can lend insight into whether historical data can be used to further characterize a given product. A major concern however is whether a given manufactured nanomaterial has been derivatised; in particular, an amorphous surface coating will not be revealed by the use of x-ray diffraction.

A.1.9 Interfacial tension

Thus far, the discussion has focused largely on water insoluble nanomaterials. Some manufactured nanomaterials will likely exhibit sparingly soluble behaviour. If the solubility or the transformation in the aqueous media of a sparingly soluble nanomaterial leads to aqueous toxicant concentrations in excess of (eco)toxic levels, then these products may also be of concern.

Due to their extremely small size, manufactured nanomaterials possess an extremely high specific surface area and, relatively speaking, also possess an extremely high fraction of atomic/molecular constituents on the surface (compared to the number of constituents contained internally). As these surface species have fewer bonds with adjacent species than do internal constituents, it takes less energy to remove surface species from the particle. A consequence of this phenomenon is that in comparison with bulk material of the same composition, many of these nanoparticulate species will have lower melting points and enhanced solubility or degradation in solvents. For nanomaterials that can degrade in solution, e.g. metal nanoparticles, a quantitative expression relating aqueous "solubility" to the particle specific surface area (SSA) of the solid phase and the solid/water interfacial tension (γ) is given below (Stumm and Morgan, 1981):

$$\log(K_{sp,\,SSA}) = \log(K_{sp,\,SSA=0}) + (2/3)\gamma(SSA)/2.303RT$$

where $K_{sp,SSA}$ is the solubility product of a material with a specific surface area SSA, $K_{sp,SSA=0}$ is the solubility product of the bulk material, γ is the solid/water interfacial tension, R is the ideal gas constant and T is the absolute temperature. In most cases, finely divided materials are significantly more soluble than large, bulk products of the same composition. Similarly, with some materials, larger particles will grow larger at the expense of smaller particles in a given dispersion; the net result is that the aqueous solubility of a material also will decrease with time due to this "ripening" phenomenon. Within the context of the present discussion, many nanomaterials are likely to display enhanced aqueous solubilities when compared to the bulk material. Alternatively, given a value for the interfacial tension, one also can calculate the solubility of a nanomaterial provided that the temperature and solubility of the larger bulk material also is known.

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B. GUIDANCE ON PREPARING SAMPLES OF NANOMATERIAL IN EXPOSURE MEDIA FOR ECOTOXICITY STUDIES

B.1 Introduction

There is currently no broad consensus on the best approaches for preparing nanomaterial samples in media for ecotoxicity studies. Review of the current literature reveals that suspension methods have involved use of strong solvents (e.g. tetrahyrofuran, THF), dispersion or stabilising agents (e.g. TWEEN™, citrate, etc.), bath or probe ultrasonication, stirring for a broad range of time periods, bead milling, etc (Klaine et al. 2008, Handy et al 2008). Adding to the difficulty in evaluating these various techniques is evidence that some solvents may interact with, and alter nanomaterial properties and toxicity, and the fact that in most cases characterisation of tested nanomaterials has been limited to working or stock dispersions rather than in post-dilution exposure media (e.g. sediment, exposure media). There is also some probability that the methods reported might not produce similar results for all nanomaterials, or forms of a specific nanomaterial, for example anatase dominated or rutile dominated titania, or surface and non-surface treated titania.

Variability in nanomaterial properties, most notably agglomerate or aggregate size, has been shown to depend consistently and significantly on media pH, ionic strength, and concentration of dissolved organic matter (DOM; Domingos et al. 2009, French et al. 2009)). Even within a narrow range of 3.4 to 13.3 mol/L, well within the range typical of freshwaters, agglomerate or aggregate size can vary two to three fold (French et al. 2009, Stolpe and Hassellöv, 2007). Because total surface area for a given volume of material increases as a square function of decreasing particle size, and the interaction of nanomaterials and biotic systems occur at the particle surface, these ionic strength effects have clear implications for exposure in ecotoxicity testing of nanomaterials. For DOM, the extensive literature describing its interactions with chemical contaminants (e.g. metals) suggests that nanomaterial properties, fate, and toxicity might be strongly affected by these substances. For example, DOM has been shown in several studies to stabilise particles in suspension, and to maintain them at smaller sizes (Hyung et al. 2007, Loux and Savage 2008). The interaction of nanomaterials and DOM might also alter bioavailability and rates of uptake by test organisms. It is also important to recognise that DOM itself is highly variable among water sources, is difficult to characterize, and can contain a broad range of aromatic, lipid, protein, and other constituents; all factors that are likely to produce variation in their effects on nanomaterials.

Control and measurement of these factors is highly desirable to assure that sponsorship programme data are as consistent and comparable as possible. Every effort should be made to quantify these factors at time intervals sufficient to fully describe exposure conditions, most importantly their effect on bulk concentration and particle size over the duration of testing. Where possible, it is also suggested that additional efforts be made to determine how these factors affect particle behaviour and properties. For example, simple beaker tests might be done over a range of pH values typical for a test system to quantify the effects on particle size (*See also the Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme ENV/JM/MONO(2009)20*]*.]*. In the absence of broadly applicable methods for producing exposure media for biotic effects assessment, the following advice is generally intended to minimise intra- and inter-laboratory variability within a sponsorship group.

B.2 Aquatic Media Preparation

B.2.1 Methods of suspension

Dispersion of nanomaterial might include stirring, sonication, grinding, use of solvents, and stabilising agents. The advantages and disadvantages of these methods are outlined in Crane et al. (2008). This suggested that some nanomaterials are significantly altered by sonication and grinding (e.g. carbon nanotubes can be shortened) and that the interaction of solvents with some nanomaterials might result in toxic by-products. The method of dispersion will also depend on the specific material to be tested and whether or not it has been surface treated. Best scientific judgment should be used in selecting the methods, and where there is evidence or an indication that a dispersion method might significantly alter toxicity, those effects should be controlled or quantified. The general goal of dispersion efforts is to produce consistent particle sizes with reasonable polydispersity and to maintain those sizes over the exposure period (assuming renewal schedules currently described in test guidelines). In some cases, in order to remove the larger particles, filtration with 0.45 um or 0.22 um filters is employed (e.g. Ma and Bouchard, 2009). This approach should be altered where information is available concerning environmentally relevant particle sizes. This might include manufacturer information on specific sizes produced and incorporated into products, and unlikely to undergo post-production processes that might alter particle size or distribution. It is assumed that tests will be conducted using periodic renewal approaches to avoid expense and waste production. Whatever is the way of nanomaterial dispersion and dosage, the test media quality (pH, ionic strength, DOM concentration) should be as harmonised as far as possible between comparative studies. It is especially important that the conditions and the quality of the media be recorded throughout the study in order to enable possible retrospective analysis of the results.

B.2.2 Media quality

It is essential that pH, ionic strength, and DOM concentrations be quantified and made as uniform as possible among tests (and replicates), endpoint measurements, and sponsor laboratories. Careful consideration should be given to assuring that physical-chemical properties determinations are representative of all other test conditions , e.g. a full suite of physico-chemical determination of a material in de-ionised water are very unlikely to accurately predict many of those values in high ionic strength media. [Accordingly, it is recommended to perform the physical-chemical characterisation in the actual test media, whenever possible]. Media quality determinations should be made at intervals sufficient to determine their variability, both in stock solutions and in test media. Typically, intervals described (and the methods used) in current guidelines should be sufficient, however, more determinations should be made where there is evidence or indication of increased variability, perhaps due to tested nanomaterials, e.g. interaction of DOM with fullerenes or carbon nanotubes.

B.2.3 Physical/Chemical Characterisations

Particle size distribution and bulk concentration should be assessed at intervals sufficient to demonstrate consistent exposures. Because few nanomaterial studies have measured these parameters across dilution series or at intervals during exposure, it is difficult to prescribe a specific approach. However, at a minimum and where possible, these determinations should be made immediately prior to, and after, media renewal. It is also desirable to have particle size determined using two or more methods, e.g. dynamic light scattering and SEM or TEM, possibly cryo-TEM [see section A.1.1 regarding comparability of results using different methods and annex III *of the Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme ENV/JM/MONO(2009)20*]. These

confirmatory measurements might be made in preliminary studies, or in a subset of tests or treatment levels to establish the comparability of measurement techniques. It is recognised that many physico-chemical properties cannot be determined in wet media, most notably surface area, which relies upon dry samples.

B.3 Non-Aqueous Media Preparation

B.3.1 Method of nanomaterial introduction

This section covers media preparation for all non-aqueous tests, including sediment (e.g. OECD TG 218), soil (e.g. OECD TG 222), and direct application (e.g. OECD TG 214) testing. Given the lack of methods for detecting or quantifying many nanomaterials in these more complex media, these tests will necessarily be relatively more exploratory compared with aquatic testing. In general, however, it is recommended that where possible, materials be delivered to test media in the form of water-based dispersions (as opposed to mixing dry materials with media) produced in a manner identical as those for aquatic tests. It is hoped that this approach will provide for comparability between aquatic and terrestrial or sediment tests, and is an approach described in many test guidelines. If the nanomaterial is introduced and homogenised directly in solid form to the media, care should be taken in homogenisation so that the test material is not unintentionally damaged, and details of homogenisers/speeds should be reported

B.3.2 Media quality

All of the media quality issues discussed for aquatic tests apply as well to preparation of dispersions for delivery to other media. In addition, all guidance on characterising these media described in appropriate OECD test guidelines should be followed. Best scientific judgment should be used in determining whether nanomaterial testing might require additional, or more frequent measurement. All efforts should be made to minimise variation in these media variables between tests and sponsor laboratories. One approach to address this issue is to homogenise and distribute natural media among all researchers, or to use a single batch of laboratory-constructed media (e.g. constructed sediments or soils; as in Tier-1 testing), following test guideline recipes and procedures.

B.3.3 Physical/Chemical Characterisations

It is recognised that methods for many physico-chemical properties, most importantly, particle size, have yet to be developed for complex media. However, where possible, and using best scientific judgment, methods for doing so should be investigated, e.g. identifying and perhaps measuring carbon nanotubes using microscopy techniques. Where methods exist for digesting or extracting materials for determination of bulk concentration, these measurements should be made. The intervals for such measurement should be sufficient to document accurate and consistent delivery of materials to test media.

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C. GUIDANCE ON PREPARING NANOMATERIAL SAMPLES FOR DEGRADATION, TRANSFORMATION AND ACCUMULATION STUDIES

C. 1 Introduction to Existing Knowledge

This part presents a recent scanning of the existing published literature on environmental behaviour and fate studies.

C. 1.1 Environmental behaviour

In general, it is accepted that, in the natural environment, MNs are unlikely to stay dispersed in water, except perhaps in some very soft freshwaters that are high in certain types of organic matter. Depending on their chemistry and the receiving environment, interaction between the nanomaterials and the natural organic matter (NOM, which may enhance agglomeration, and thus sedimentation, or lead to dispersion) and sediment is likely. Depending on the nanomaterial type and receiving environment, the interaction with NOM may lead to enhanced (e.g. Hyung et al. 2007) or reduced (Baalousha et al. 2008) bioavailability for pelagic organisms. As discussed, fate of nanomaterials in the marine environment is likely to be characterised by enhanced agglomeration and thus sedimentation (Klaine et al, 2008, Stolpe and Hassellöv 2007). The increased salt content and ionic strength tend to lead to agglomeration and thus sedimentation. Although bioavailability could be diminished, it is possible that biological systems may become clogged and thus their activity impaired (e.g. Nielsen et al. 2008). This considers only nanoparticles of the first generation, without specific surface functionalisation and only possible electrostatic interaction based on surface charge and the variation of the surface charge by interaction with e.g. NOM. The ability of NOM to stabilise or flocculate unfunctionalised particles depends on many factors, still to be quantitatively assessed. Non-charge effects as steric/entropic stabilisation through e.g. polymers attached to the surface, will make the dispersion stability more independent from simple electrostatic effects, hence more independent from ionic strength or the presence of NOM. It is not yet clear if those NPs considered as "second generation" particle types will be prone to quick aggregation in e.g. salt water / marine conditions. Reactions like bridging flocculation caused by e.g. natural polysaccharides, seem to be very effective (more effective than simple increase in ionic strength) and still not quantitatively understood for complex systems such as surface waters.

Recently, Jafvert and Kulkarni (2008) have studied the octanol-water partition coefficient (log Kow) of fullerene (C60) and its aqueous dispersability. They obtained a value for log Kow of 6.7, and a value for the solubility of C60 in water-saturated octanol of 8 ng/L. Hence based on this high Kow- value, it is expected that C60 has high affinity for lipids and organic matter. This indicates that in the natural environments, C60 will tend to adsorb to solid phases.

Some modelling work has been published for TiO_2 and silver nanoparticles and carbon nanotubes (Mueller and Nowack, 2008; Boxhall et al., 2007). However, current knowledge of the behaviour of nanoparticles in natural waters does not provide sufficient basis for the full assessment of environmental exposure concentrations or amounts of dispersed nanomaterials.

In terrestrial systems some nanomaterials may preferentially bind to NOM and thus become less bioavailable (e.g. Li *et al.* 2008), although sediment and soil ingesters (e.g. earthworm) may be able to take up these nanomaterials (in fact they may preferentially ingest them if they are associated with NOM (e.g. see Roberts *et al.* 2007) and strip de-associate them within the gastrointestinal tract (GIT)). The organisms

selected for toxicity tests should address both type of exposure, i.e. in liquid matrix (in dispersion/solution) or associated to solid matrix (particle bound).

Determining the agglomeration/aggregation and sorption characteristics of the nanomaterials can provide valuable information when developing new testing guidelines, using existing test guidelines with modifications or interpreting the results from existing test guidelines. The capacity of nanomaterials to adsorb chemicals and work as toxicant carriers should be verified in this category of environmental behaviour.

Methods for environmental analyses are now in development for various materials and environmental matrices (Hassellöv *et al*. 2008) and these methods could provide the basis for environmental fate testing.

C. 1.2 Degradation and transformation

Degradation, transformation and persistence of nanomaterials in the environment depend on their chemical composition, of both core and surface material. It is likely that most nanomaterials which are currently available will be persistent in their original particulate form, though levels of agglomeration/aggregation can be expected to be different. Some nanomaterials might have biocidal effects on microorganisms and hence affect the biodegradation. There is lack of data in this area, although, the organic coatings could be degraded or transformed by environmental factors.

C. 1.3 Bioaccumulation

Current work assessing uptake has focussed on exposures in media with different nanomaterial loads over a specific time interval, followed by total body burden assessment, especially if individuals are small, such as *Daphnia* species, copepods, *Lumbriculus* or *Eisenia* species (Roberts et al, 2007, Fernandes et al 2007, Petersen et al, 2008). For larger organisms, specific studies have focussed on detection, following exposures, of loads within specific organs, such as liver, kidney, muscle, gills (e.g., Ti in trout, Federici et al., 2007). In terms of detection, it may not always be possible to identify the form of such material. This may be particularly important for materials that may tend to transform and get into solution promptly such as silver.

The first step in the uptake and possible accumulation on substance at least in aquatic environment is often the adsorption and agglomeration of the material onto the surface of the organism (Handy and Eddy 2004; Fernandes *et al.* 2007, Nielsen *et al.* 2008). This has also been shown by the agglomeration of single wall carbon nanotubes on the gill mucus of rainbow trout (Smith *et al*. 2007).

As first generation nanomaterials tend to follow colloidal chemistry and colloids can eventually agglomerate, these nanomaterial agglomerates will end up in the sediments (Klaine et al. 2008). Thus, bioaccumulation studies on sediment organisms would be especially important.

Petersen *et al.* (2008) have indicated that CNTs were not readily bioaccumulated by the earthworm *Eisenia foetida* with results indicating bioaccumulation factors 2 orders of magnitude smaller than those measured for pyrene. Lee *et al.* (2008) have detected bioaccumulation of insoluble copper nanoparticles in cells of emerging and growing plants when tested on agar plates.

Not much work has been published on potential food chain effects of nanomaterials, although fish that drink water containing nanomaterials show gut pathology (Federici et al., 2007; Smith et al., 2007). A

recent study (Holbrook *et al*., 2008) on the possible transfer of quantum dots in a simplified aquatic food chain has found that these materials can be transferred to rotifers through dietary uptake of ciliated protozoans. Although there was transfer across these levels, bioconcentration (accumulation from surrounding environment) in the ciliates was limited and no biomagnification (enrichment across levels) in the rotifers detected. This study indicates potential for transfer across food chain levels but this would depend on material type and food chain, as is mostly the case for other studies of chemicals. Also Fortner *et al.* 2005 have observed that fullerene nanoparticles accumulate in microbial cells, in worms eating those microbes and possibly in animals higher up the food chain.

C. 2 Test Method Applicability and Dosimetry

C. 2.1. Environmental distribution

C. 2.1.1 Methods

It is likely that the OECD test methods for a number of physico-chemical properties for environmental distribution are applicable, and their applicability has been assessed by SG4-1. Furthermore, as with other test methods, dosage for the testing and the detection, analysis and quantification of the nanomaterials are the most challenging issues. Methods for the characterisation of key properties of nanomaterials have been identified in several publications (e.g. Klaine *et al*., 2008, Hasselov *et al*,. 2008, Tiede *et al*. 2008).

Dispersion and solubility

Dispersion and possible solubility/transformation of nanomaterials are important properties that have been already addressed in the general part of this document (section 3). It is unclear to date to what extent can the effects observed be attributed mostly to the soluble form or to a combination of soluble and particle form, and to the size of the particulate form or to degradation products such as metal ions from metal based nanomaterials (Franklin *et a*l., 2007, Navarro *et al*., 2008). Although work in this area is increasing now, the results will depend on the material under consideration. The OECD assay on water solubility (OECD TG 105) may be useful in this context, but many of the organic based materials (such as fullerenes) are so water insoluble that specialised methods are likely to be needed in order to measure or estimate solubility. For example, fullerene solubility is usually estimated by measuring solubility in alcohols and extrapolating to a zero carbon alcohol, i.e. water (Jafvert and Kulkarni, 2008).

Water/octanol partitioning

The measurement of the K_{OW} (OECD 107, 117, 123) is problematic given that many organic nanomaterials have such low water solubility that measuring their concentration in the aqueous phase is not a straightforward procedure. However, Jafvert and Kulkarni (2008) have studied the octanol-water partition coefficient (log K_{ow}) of Buckminster fullerene with method modifications.

C. 2.1.2 Dosage and sample preparation for physical-chemical fate studies

The methods presented in section A on assessing the physico-chemical properties of nanomaterials could be generally followed for a dispersion.

C. 2.2 Degradation and transformation

C. 2.2.1 Methods for degradation

Biodegradation

Again, the main technical challenges in degradation and transformation studies are the detection and characterisation of nanomaterials in the various environmental media. The existing test protocols (e.g. OECD test guidelines) seem to be as appropriate for nanomaterials as for the comparable bulk material.

Purely inorganic nanomaterials will not require testing in any of the biotic degradation tests. Therefore, it is necessary to examine first whether the nanomaterial contains carbon that can be used as an energy and nutrient source for microorganisms. Secondly, the physical-chemical and compartmentalisation properties of the material can provide insight into whether some of the simulation tests are necessary. For example, if the material is unlikely to reside in the water column or if it is not soluble in water (e.g. fullerenes and carbon nanotubes), any testing in surface water may be unnecessary.

OECD methods have been developed and validated principally for assessment of organic compounds. The nanomaterials addressed now under this test programme are principally inorganic; indeed even carbon-based nanomaterials tend to be of an inorganic nature. Hence, they will be most probably considered persistent against biodegradation. In principle, the methods measuring carbon dioxide production or oxygen uptake are applicable, but they require large amounts of test material. It is also important to consider whether carbon based nanomaterials such as fullerenes and nanotubes can be degraded at all under any conditions. However, limited data have indicated that fullerenes could be taken up by wood decay fungi, suggesting that the carbon from fullerenes could be metabolised (Filley *et al*., 2005).

If several conclusive aerobic degradation tests indicate very low or negligible degradation, then other aerobic degradation tests will most likely also be negative and it may be useless to proceed with additional tests. For example, if the result of a ready biodegradation test is below 10%, then the chances are that the simulation test in surface water will also be very low and it may be better to decide to skip the more elaborate test, and conclude that the substance is not biodegradable.

Simulation tests for biological degradation in various environmental compartments are also in principle applicable, but again the detection of the nanomaterials is the challenge. The possible degradation to carbon dioxide (mineralisation) integration into biomass or other partition could be followed by labelled test material. The advantage of using labelled substances would be to allow the testing of low concentrations and to provide degradation kinetics and mass balance on the fate of the carbon from the tested material. However, radio-labelled nanomaterials can only be used with great caution: the label must be distributed uniformly on the nanomaterial. This very complicated issue requires further input from radiochemistry experts.

Abiotic degradation

Likewise, for hydrolysis testing, the chemical structure of the material and whether it contains groups which could be subject to hydrolysis dictate whether this test is necessary or appropriate.

In view of the sometimes very long lifetime of nanomaterials in the environment, the photodegradation studies might be considered relevant. The OECD proposal for photodegradation and transformation in water could be an applicable method for this purpose.

C. 2.2.2 Dosage and sample preparation for degradation studies

Similarly to the testing of physico-chemical properties or biotic effects, the dispersion methods for degradation studies could include ultra sonication and/or stirring for longer periods. Especially in biodegradation tests measuring carbon dioxide production or oxygen consumption, the use of organic solvents is not possible, as remnants of the solvent will interfere with the nanomaterial degradation. In the simulation tests using radiolabelled materials, the use of solvent carrier or detergent could be possible.

The detection of biodegradation in standard screening tests is usually followed by measuring the carbon dioxide produced or oxygen consumed by the degraders. As nanomaterials are normally not soluble, the measurement of dissolved organic carbon might not be relevant. Of course, in certain test systems the decrease of total amount of carbon could be assessed. In simulation testing ^{14}C labelling and chemical analysis and characterisation would be the means of detecting the degradation.

C. 2.3 Bioaccumulation

C. 2.3.1 Methods for bioconcentration and bioaccumulation

Aquatic studies

Many of the possible ideas for exposure for bioaccumulation studies originate from, and are informed by effect studies.

For simple organic chemicals, there is an established relationship between octanol water partition coefficient (K_{ow}) and bioaccumulation or bioconcentration factor (BCF). However, this relationship may not hold true for many nanomaterials. The studies of Jafvert and Kulkarni (2008) have shown log K_{ow} of 6.7 for fullerene, and it is hence expected that C_{60} has high affinity for lipids and organic matter. However, more data are needed to judge this.

The main challenge once again in testing the bioaccumulation of nanoparticles is their detection and characterisation in tissues and body fluids. Radiolabelling could make detection and quantification easy but it has also limitations; for example, the labelled material depending on the labelling method, e.g. if a tethered label is used, can behave differently from the non-labelled particles. One novel possibility could be neutron activation of metal and metal oxide nanoparticles (Oughton *et al.* 2008). This enables both localisation and quantification within tissues or organisms. Also more traditional chemistry e.g. ICP-MS analysis for metals could provide valuable information on the total amounts of material accumulated by the organism.

Standard BCF testing protocols such as OECD 305 (OECD 1996) may have limitations for determining bioaccumulation of nanoparticles. It has been observed for substances dissolved in water that a large molecular size (MW > 600, or effectively a diameter size > 0.5 nm) effectively limits direct uptake. It is likely that in most cases the large size (1-100 nm) of nanoparticles compared to dissolved molecules limits their direct uptake by carrier-mediated transport in fish gills, but uptake by endocytosis cannot be excluded (Handy et al., 2008b). Fish dietary BAF testing (Fisk *et al.* 1998; Stapleton *et al.* 2004) is not yet a standard OECD testing protocol. This spiked food method is suitable for testing of poorly soluble large molecules and might be suitable for testing several classes of nanoparticles, either as the only test or in combination with the OECD 305. Fish do eat diets contaminated with nanomaterials by this method, and toxic effects have been observed (Ramsden et al., 2008). However, more data using a harmonised OECD dietary protocol, especially for testing nanomaterials, are needed. The testing results of human health endpoints, if available, should also be taken into consideration when generating environmental testing plans for specific nanomaterials. Uptake studies from mammalian studies may give valuable basic information of uptake characteristics, rates and mechanisms of nanoparticles also for non-mammalian species.

Given the tendency of nanomaterials to agglomerate, and thus their likelihood to end up associated with sediments (Klaine et al. 2008), bioaccumulation studies on sediment organisms would be especially important. OECD adopted in 2008 a new method TG 315 (OECD 2008a) for the bioaccumulation in sediment worms e.g. using *Lumbriculus variegatus*. This method could be relevant to be used in a test battery for risk assessment as OECD has also published recently a toxicity test OECD TG 225 (OECD 2008b), based on the same species, which could provide effects data.

Soil and terrestrial studies

Petersen *et al* (2008) have indicated that CNTs were not readily bioaccumulated by the earthworm *Eisenia foetida* with results indicating bioaccumulation factors 2 orders of magnitude smaller than those measured for pyrene. Scott-Fordsmand *et al.* (2008) have detected effects on the reproduction of earthworms (*Eisenia veneta*) when the worms were exposed to double-walled carbon nanotubes in food. A validated OECD method is currently under preparation to assess bioaccumulation of chemicals in earthworms.

Effects of ingested nano-sized titanium dioxide on enzymatic activity of terrestrial isopods (*Porcellio scaber*) have been detected by Jemec *et al.* 2008. The TiO_2 nanoparticles were dispersed in distilled water with and without sonication and pipetted on to homogenised hazelnut tree leaves. The isopods were then fed with the leaves. The particle location and its composition were analysed by transmission-electron diffraction pattern.

Lee *et al.* (2008) detected bioaccumulation of insoluble copper nanoparticles in cells of emerging and growing plants when tested on agar plates. The particles were well characterised and the homogenous distribution of Cu particles in the agar media was evaluated by SEM. The distribution and accumulation of Cu particles in the plant cells was characterised by TEM and energy-dispersive spectroscopy.

C. 2.3.2 Dosage, exposure and sample preparation for bioaccumulation studies

The methods of sample preparation and dosage for bioconcentration and bioaccumulation studies could be similar to those for assessing biotic effects. These might include ultra sonication, stirring for various periods, use of solvents and introducing stabilising agents. There is still limited information to

prioritise the dispersion methods for bioaccumulation studies, but probably the same preference could be valid as for testing biotic effects. The aim would be to achieve a stable and homogenous dispersion over the exposure period. Often the smallest particle size would be expected to provide high accumulation but the exposure conditions should be always decided depending on the nanomaterial tested and the aim of the study. Whatever the method of test material dispersion and dosage, the test media quality (pH, ionic strength, DOM concentration) should be as harmonised as possible between comparative studies. It is especially important that the conditions and the quality of the media be recorded throughout the study in order to enable possible retrospective analysis of the results.

Depending on the test, the exposure to the test nanomaterial could be via water, sediment and pore water, soil and pore water or ingestion and food. For aquatic studies, the methods for dosage mentioned above are relevant but e.g. for soil studies the nanomaterial can be introduced directly to the media in solid form and homogenised. Care should be taken in homogenisation so that the test material is not damaged, and details of homogenisers/speeds should be reported.

In bioconcentration and accumulation studies, both the accumulation and depuration phases are important. It must be noted that the nanomaterial could be excreted in a different form from which it was taken into the organism. Hence the characterisation methods for the test material are important, not just the total amount measured e.g. by total metal content of metallic nanomaterial.

Detection of the overall amount of, e.g., ^{14}C labelled material is rather easy in the tissues and in the whole organism. In addition, neutron activation of metal and metal oxide nanoparticles (Ag, Co or Co_3O_4 and CeO_2) can be an option. These could enable both localisation and quantification within tissues or organisms. Of course, traditional chemical analysis ICP-MS for metals and various HPLC methods can be useful for the measurement of the total amount of the nanomaterial accumulated. Electron microscopy provides means both for the detection and analysis of the materials in the exposure media and inside the organism.

C. 3 References

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D. HEALTH EFFECTS AND DOSIMETRY

D.1 Knowledge transfer from environmental chemistry and stock dispersion preparation.

The environmental chemistry community has been working on colloids and particle chemistry for many years, and this knowledge has recently been placed in context for nanomaterials ecotoxicology (Handy et al., 2008 and references therein). There is a clear list of abiotic factors that can have substantial effects on particle agglomeration (and therefore bioavailability), as outlined in section 5 on dosimetry. Since these are fundamental properties, they should also be considered for the preparation of salines and other media for mammalian studies. The characterisation suggested (section 5) and information (volumes, sonication times, etc) reporting precisely on how a stock dispersion was made should be provided.

D.2 Measurements on test dispersions or salines during experiments

This is in addition to routine checks on pH, temperature etc:-

i) **Some attempts at confirming the exposure concentration** where techniques are currently available. For example, measured metal concentrations in water or salines containing metal nanomaterials. This is problematic for carbon-based materials as the methodologies are not necessarily sensitive enough to measure environmentally relevant low microgram concentrations in water and the background levels of DOC may overwhelm the actual concentration of carbon based NMs. In salines containing protein (e.g., BSA) any carbon measurement is likely to be overwhelmed.

ii) **Change the water/test media** to maintain exposure concentration if necessary (semi-static test method). The frequency of media changes may need to be derived empirically, but target doses should be maintained (e.g., within 80 % of the target concentration). Flow through methods can create a waste disposal problem, semi-static methods reduce this and the occupational exposure risk. Nevertheless, in particular for in vitro methods, attention needs to be paid to possible detachment of cells and to possible removal of cells grown in suspension.

iii) **Particle size distribution and agglomeration rate will change with dose.** This is a fundamental problem (see collision theory in Handy et al., 2008). We must accept that agglomeration and dispersion will vary with each concentration in the test system. The total surface area available to the organisms will therefore not be the same at each dose in the test design. Similarly, the total surface area available to the organisms might not follow the predicted, mathematical dependency on the doses in the test design, but will be also influenced by the agglomeration and dispersion taking place at each dose level. It is a huge amount of work to measure these processes in every

test vessel or saline, and one may not have control of this anyway because of different rates of ligand secretion by the organisms at each dose (e.g. mucus production). However, some simple optical methods to indicate general dispersion (e.g. turbidity, colour etc) might at least give indications for the dispersions used. This might be especially important in mammalian studies in vitro where saline media are re-circulated or re-used, and cellular secretions inevitably build up in the media. This is also very important for inhalation studies both when using gas phase exposure or direct delivery techniques.

iv) **Dispersants and vehicle controls**-this would be the same as any other experiment. These controls must be included. However, the dogmatic approach of standardising dispersant levels in all treatments should be avoided. Excess dispersant can change particle shape (see discussion in Smith et al., 2007), and so some thought needs to be given as to whether such an excess is appropriate or not, or if individual dispersant controls are needed for each dose (as this poses an ethical dilemma regarding reduction of animal usage, wherever possible, existing data should be used). If dispersion cannot be reasonably achieved, then some standard protocol of sonication or mixing, "immediately" before dosing may be required to at least give a known amount of nanomaterial /unit volume of saline. A certain degree of uncertainty would anyway be unavoidable in this case.

D.3 Special considerations for physiological salines used in mammalian studies

In addition to the details on stock dispersions above, the high ionic strength of physiological salines may present specific problems for dosimetry, including instantaneous agglomeration of the test material. The reason for using these salt solutions in the first place is to match the ionic environment in the airway or gut (for example). The use of saline therefore cannot be avoided. It may be better to make stock dispersions in ultrapure water, and then disperse smaller volumes in the saline to be dosed into the animal. If this second step is taken, then all the characterisation may need to be done again for the saline. It may also be helpful to add dispersion agents (such as Tween or DMSO) to the saline to improve dispensability/dispersion handling of the test material. However, this must be fully justified for practical reasons (i.e., impossible to handle the nanomaterial in saline phase without it), reflected in the dispersant controls, and with some appreciation of how the dispersing agent is working (e.g., coating the surface of the nanomaterial). Tween, triton, and other similar products should be obtained at the best available analytical grade to minimise spurious effects of contaminants in these products on the nanomaterial chemistry. In addition the inherent toxicity of the dispersion agent has to be kept in mind, in order to avoid high toxicity (Zhu, S. et al. 2006, Monteiro-Riviere, N. and Tran, L. 2007), which might prevent the interpretation of studies even if appropriate vehicle controls are used.

Salines for use in *in vitro* studies with mammalian cells or tissues are often gassed with high concentrations of oxygen and carbon dioxide (e.g., 95 % O_2: 5 % CO_2), and of course may be used at body temperature (37 ^{o}C). It is important to note that these conditions have not been employed in agglomeration chemistry studies/environmental colloid chemistry. Therefore, currently it is assumed that these conditions used in mammalian studies in the above-mentioned systems will not alter the chemistry. This assumption may need to be revised when results of research on the nanomaterial chemistry in 5 % CO_2 at body temperature are available. However, in the meanwhile, it should be reported whether the particle characterisation was done in gassed or air-equilibrated saline, and at which temperature. One should also be mindful of keeping salines in tempered water baths at higher temperatures immediately prior to dosing.

Physiological salines and culture media also contain additional substances that are specific to different types of test. For example, the use of lipopolysaccharide (LPS) as an immune activator, or the addition of metabolic inhibitors in ADME studies. It must be stated whether characterisation was done before or after

adding these extra substances, and preferably with some checks to show this does not have a big effect on particle dispersion. It may also be possible to adjust the experimental protocol to avoid such effects, for example, by pre-infusion of the test animal with LPS-saline, prior to the NP saline.

D.4 Routes of delivery and the behaviour of nanomaterials dispersions in physiological salines in mammalian studies

Mammalian tests can involve inhalatory, oral or dermal exposure, and some consideration of the physical behaviour of the test material is needed for each route. The following delivery methods are routinely employed:

i) Aspiration or instillation of salines, or exposure to nanomaterials in air or gas phase (inhalation studies for which there is no need to produce nanoparticles dispersions in physiological salines but in suitable low toxicity vehicles, if any, which prevent agglomeration as much as possible).

ii. Oral dosing of saline via gavage (acute or repeated oral toxicity testing).

iii. External application of salines, emulsions, or creams (dermal studies)

iv. Use of salines for injection routes (ADME studies for example).

D.4.1 Respiratory tract exposures

Much of the traditional research on particle toxicity have used salines to deliver test material to the lung via intra-tracheal instillation/aspiration. Fewer studies have used inhalation exposure (i.e., breathing of particles dispersed in the air of inhalation atmospheres). Nevertheless, inhalation is the normal route of exposure prescribed in standard OECD Test Guidelines because inhalation is the physiological process during which nanoparticles are deposited in the respiratory tract, allowing for a slow build up of the dose and for normal clearance processes to occur. This is the only way to determine the NOEL for the airborne concentration of suspended dust. However, determination of the administered nanoparticle dose is difficult and its estimation requires careful monitoring of breathing, of the aerosol parameters and of tissue analysis (SCENIHR, 2007).The test atmosphere with e.g. nanoparticle dust should be characterised and reported (primary particle size, particle size distribution, mass concentration and number concentration) carefully so that the results can be useful for hazard and risk assessment and characterisation.

All technical aspects of inhalation toxicology studies including the use of dynamic nose-only inhalation systems are addressed in GD 39 [OECD (2008) Draft Guidance Document on Acute Inhalation Toxicity Testing. Environmental Health and Safety Monograph Series on Testing and Assessment No. 39].

In most cases, a test atmosphere generated from a powder consisting of nano-sized particles will contain aggregates of nano-sized particles. Therefore, in general, the aerosol can be characterised with the usual instruments, i.e. cascade impactor or other instruments based on inertial forces. In the case that single nano-sized particles are expected to be present in an aerosol, these can be characterised with e.g. a SMPS (scanning mobility particle sizer) or an ELPI (electrical low-pressure impactor)..

Prior to the study, the test item should be characterised comprehensively as recommended in previous chapters.

If it is necessary to use a vehicle to generate an appropriate concentration and particle size, water should be given preference. Constancy and homogeneity of atmospheric concentrations of the tested particles should be ensured.

The flow of air through the exposure chamber/system should be carefully controlled, continuously monitored, and recorded at least hourly during each exposure. Details on this and on the exposure chamber conditions can be found in the inhalation toxicity test guidelines.

The nominal concentration is the mass of nanomaterial consumed during test atmosphere generation divided by the total volume of air passed through the exposure system. In most cases, the amount used is too small to be measured accurately. Nevertheless, as for nano-particle testing, the nominal concentration is not used to characterise the animals' exposure., The nominal concentrations need not to be calculated, especially if particle separation or air dilution systems are used.

The actual concentration, which is the nanomaterial concentration as sampled from the animals´ breathing zones in an inhalation system should be measured and reported. For non-volatile single-component nano-particles, the actual concentrations can, in some cases, be obtained by non-specific gravimetric filter analysis, however in many other cases the mass concentration will be very low and determination by weight not possible. For multi-component aerosols, concentration may also, in some cases, be determined by gravimetric analysis. However, this requires analytical data which demonstrate that the composition of airborne material is similar to that of the starting material. The range of exposure concentrations should be relevant for any anticipated exposure of humans.

The exposure atmosphere should be held as constant as possible. The methods for monitoring this and the allowed deviation ranges are described in the inhalation toxicity test guidelines..

If a vehicle other than water is used, the concentration of the vehicle in the atmosphere should be determined by an appropriate method (e.g. gas chromatography).

The particle size distribution of aerosols should be determined at least once during the study for each concentration level by using an appropriate measurement method. The total mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained during concentration control analysis. To enhance the resolution of measurements in the range of visible particle sizes, an optical particle sizer (OPC) may be used (this would require very high levels of agglomeration, particularly for small nanoparticles).

To further characterise the presence of free nano-particles in the inhalation atmospheres a differential mobility analysing system (DMAS) should be used.

Other exposure techniques involve direct delivery to the respiratory tract in a liquid, so there is a need to disperse the nanoparticles for intratracheal instillation or laryngeal aspiration or for delivery to other sites, such as peritoneal cavity, skin or gut. A number of approaches have been published for preparing salines, including those used for cells/tissues in culture. Some studies, including influential studies from NIOSH have not used any dispersant other than Ca^{2+} + Mg^{2+}-free phosphate-buffered saline (PBS) with sonication (Shvedova et al., 2005; 2008; Warheit et al., 2007). All mammals have albumin as a ubiquitous protein and it is well conserved in evolutionary terms. Dispersal in bovine serum albumin (BSA) has therefore been used for intraperitoneal injection of nanotubes (Poland et al., 2008). One study has used the actual bronchoalveolar fluid (BALF) obtained from normal rats to suspend the nanoparticles in before injecting back into rats (Sager et al., 2008). BALF contains phospholipids (e.g. DPPC), albumin and other proteins and this is an interesting approach. A number of chemical surfactants have also been used to disperse nanoparticles prior to instilling into rat lungs, including Pluronic-F68 (Mangum et al., 2006) and

Tween (Warheit et al., 2004). Aspiration of fine sprays may present some practical problems in terms of blocking spray equipment and achieving a precise quality of spray (and dose). The alternative is to use an instillation (essentially a gavage-like "injection") of saline that gently delivers the dose to the bronchi and lung. There are disputes about the level of penetration of each direct delivery method into the alveolar region of the lung, but the latter direct delivery method has less practical problems, and may be a more pragmatic direct delivery method for comparative hazard assessment.

Nevertheless, this mode of exposure is not physiological in the sense that there is usually a very high dose and application rate and, since the particles are suspended in saline, the lung surface receives particles contained in a liquid, which is likely to affect the defence systems of the lung. The advantage of instillation is that it involves the administration of a more precise nanoparticles dose. Pharyngeal aspiration is a variant of instillation, which still involves a high dose, a high dose rate and the fact that the particles are in suspension, but in this case, the exposure is to suspension droplets that disperse in the lung more readily than with simple instillation. However, two side effects may detract from pharyngeal aspiration, involving unusually high doses to bronchioles and the bacterial rinsing induced alveolar inflammation. Results with instillation and pharyngeal aspiration are rather similar in terms of allowing comparison in toxic potency between particle types and can be used for the oropharyngeal region down to the sterile alveolar region in the context of screening purposes and for mechanistic studies. However, neither method can be used to determine NOEL (SCENIHR, 2007).

D.4.2 Oral Exposure

The acute regulatory tests use a gut gavage dosing (instillation into the stomach) of typically 10 or 20 ml of saline/kg body weight of laboratory animal. This volume is designed to deliver a dose comfortably to the stomach of the animal without dilatation of the stomach (or e.g. initiating the vomiting reflex). There is no reason why these volumes and approaches cannot be used for nanoparticles, and all the issues outlined above for salines or other vehicles would apply to this technique. Chronic studies of dietary exposure are best performed by feeding the nanoparticles in a diet to the animal. Few published studies describe exposure via the digestive tract by dietary intake of NP contaminated food, but the necessity to incorporate the nanoparticles into diet means that considerations of aggregation/agglomeration may be secondary. However, the techniques for manufacturing food often include a step where the test ingredient is sprayed into the feed mixture as it is blended, or used as a top coat on the feed. In either case, a stock dispersion would be required (as above) and the aim would be to ensure a uniform spread of the dose in the resulting food pellets. Where possible, the dose should be measured in the food produced, along with the usual nutritional analysis of the feed. Storage and degradation of the feed may be an issue, especially with oxidising NPs (rancid food). It may also be possible that nanoparticles cause secondary toxic effects by reducing the bioavailability or digestibility of the feed ingredients by adsorption processes. Additionally, the chemistry within the digestive tract needs to be considered. The low pH of the stomach is likely to have effects on any protein pre-coating and the general effects of the stomach milieu on nanoparticles could be to disperse them or agglomerate them, regardless of pre-treatments. The digestive tract is also a high ionic strength environment, containing mucus and other soluble proteins and special microbial environment. The chemistry is likely to be very complex, and not likely to be easy to predict from theory alone, and observational experiments are needed on nanoparticle bioavailability from different food matrices.

D.4.3 Dermal Exposures

Dermal exposure to nanoparticles may occur in the workplace environment or via consumer products (e.g. cleaning agents, cosmetics). In consumer products, the nanoparticles are usually dispersed in some

excipient, such as glycerol, which allows the particles to be applied to the skin (Mortensen et al., 2008). It has been argued that, if nanoparticles are considered to resemble macromolecules of high molecular weight, skin absorption is considered unlikely. This expectation was verified in project Nanoderm (http://www.uni-leipzig.de/~nanoderm/) mostly for TiO2 or studies with nanoparticles in cosmetic formulations (Gamer et al. 2006). Nevertheless, it cannot be excluded that other nanomaterials might penetrate the skin. Indeed the opinion of the Scientific Committee on Cosmetic Products (SCCP, 2007) states for instance that nanomaterial constituents may act as penetration enhancers by penetrating individually into the stratum corneum and subsequently altering the intercellular lipid lamellae within this skin layer. In addition, nanomaterials may serve as a depot for sustained release of dermally active compounds. In addition, follicular openings are compatible with particulate dimensions. Therefore, it is not unreasonable to anticipate a size dependent phenomenon, whereby particles lodged within the appendageal openings may allow increased diffusion for ingredients. Additionally, nanoparticles may have increased substantivity in skin "furrows" and may not be efficiently removed by standard cleaning procedures. It has been demonstrated that spherical and elliptical quantum dots penetrate the stratum corneum and localize within the epidermal and dermal layers. If the skin is exposed to large nanoparticle doses, even small fractions may become important to accumulating secondary target organs.

Methods for performing skin absorption studies are given in OECD Guidelines 427 and 428 and Guidance document 28. . The state of agglomeration is not easily studied in such a situation. However, there are some assumptions worth revisiting. One assumption in the dermal test is that the test material has reasonably good access to the skin under the fur or hair of the animal. It would be up to the experimenter to ensure this is the case. For example, does the test material agglomerate on fur, and not reach the skin in appreciable quantities? Does shaving a small area of skin eliminate this problem, or would this add unnecessary skin sensitisation. This issue of sensitisation may be important given the known inflammatory effects of some particles in epithelial tissues. On the other side, when hairy skin is shaved or depilated before treatment there is an additional risk of damage to barrier function exacerbating further the problem of reliably assessing nanoparticle absorption (SCCP, 2007). The use of hairless animal models (genetically modified) might overcome these practical issues, but would also create a significant ethical issue. If a dispersing agent or solvent has been used to make the test solution or cream (emulsion), then a solvent control should be included to account for any irritating effects of these reagents, or their ability to alter the intrinsic permeability of the skin. The precise location on the skin should be stipulated (e.g., ear, precise region on the abdomen or thorax) because the thickness and sensitivity of the skin will change at different locations. The same location must be used on all the test animals.

D.4.4 Injection routes

Injections into the blood supply, tissues, or body cavities are used as tools generally in ADME studies. This is usually done in the form of dispersion in saline, or in the case of a very hydrophobic material, in lipophilic vehicles like corn oil. In addition to the considerations on salines above, one concern is the behaviour of the nanoparticles in the syringe. Micro bubbles in the syringe can act as precipitation surfaces, and so it would be important to avoid creating bubbles by good dispensing skills, and also not allow the syringes to sit for too long where micro bubbles may form on the inner surface of the syringe. The gauge of needle should be sufficient to enable a smooth injection without blockage of the syringe. It may be that larger needle sizes are needed for some nanomaterials preparations, depending on concentration/viscosity and in this case animal welfare should be paramount, with a prior injection of local anaesthetic to be considered. We must not exclude the possibility that nanoparticle injections may be very painful (e.g., like a heparin injection) because the materials are reactive. A precautionary anaesthesia may be advised regardless of needle size.

D.4.5 Cell cultures and dispersion of NPs in culture media

In vitro techniques are discussed in more detail in documents prepared for SG7 for the OECD working party. However, there are a number of test systems that use in vitro tools (mutagenesis tests, cell/tissue culture screening assays, immunotoxicity assays etc). Most, if not all of these test systems, rely on using a saline solution or a much more complex culture medium. It is inevitable that test materials will agglomerate in some normal culture mediums (e.g., Vevers and Jha, 2008) and increase the direct contact of the cells with the test material. It might be possible to use soluble peptides or other organic ligands to act as dispersing agents in cell culture medium. While this may be a good idea from the perspective of nanomaterial dispersion, it may be very problematic from a biological perspective. Biological systems may see these added materials as "antigens", or the material may change the unstirred layer chemistry on the cell membrane that defines how the material interacts with the cell surface, or even adhesion of the cells to the culture plate (also used as an end point in some tests). Culture media often contain proteins, and it may not be necessary to add a "special" dispersing agent. For example foetal calf serum (FCS) is commonly added to cell culture systems, and contains a ubiquitous mammalian protein called albumin (bovine serum albumin; BSA) and so it is already present at high concentration in cell cultures that contain serum. BSA has been used as a fairly bland protein which, because of its zwitterionic nature (contains both positive and negative charges) is a useful dispersant that changes the balance of protein in a cell culture very little (Bihari et al, 2008; Poland et al., 2008). It is likely that dispersing in a protein like BSA will aid in prevention of false positive toxicity engendered by the adsorption of nutrients from the culture medium onto nanoparticle surfaces and which may cause toxicity by nutrient depletion (Casey et al., 2008). Once dispersed in BSA, the nanoparticle surface should be 'blocked' and much less able to absorb nutrient proteins from solution.

One issue with using serum or BSA is that these materials inevitably contain a number of unknown ingredients (peptides, fatty acids, sugars etc) which vary with the batch of BSA used. It is possible to buy high purity BSA, or chemically defined media that have been manufactured from non-animal sources where all the components of the media are known. Another approach to dispersion in studies with lung epithelial cells is to use a surfactant lipid found in the lung lining fluid, called dipalmitoylphosphatidylcholine (DPPC, Herzog et al., 2008; Wallace et al., 2007) usually as an addition to BSA or serum. This is obviously favoured for studies modelling the lung. Whichever dispersing agent or method is used, a balance must be achieved between the need for dispersion and how this will affect viability measurements in the *in vitro* system, and the quality of the system. There is also an argument that the effects of serum or tissue-specific natural surfactants like DPPC should be accepted as part of the particle behaviour, especially since real body fluids have a myriad of proteins, peptides etc, and additions of serum to culture medium would merely give a better reflection of what would happen *in vivo*.

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