REQUEST FOR PROPOSAL

Design of an *in vitro* system to assess the inhalation toxicity of nanomaterials

Submission deadline: 29 May, 2015

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I. GOAL

Development and preliminary assessment of the relevance and reliability of an *in vitro* test to predict the development of pulmonary fibrosis in cells co-cultured at the air-liquid interface (ALI) following exposure to aerosolized multi-walled carbon nanotubes (MWCNTs). In an integrated approach, this test will help address the regulatory safety testing requirements for inhaled nanomaterials (NMs) while maintaining human health protection and reducing the use of animals for this purpose.

II. PROPOSAL SUBMISSION

Please send proposals, or direct any questions, to:
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Applications must be submitted via email by 5:00 p.m. Eastern Daylight Time (EDT) on May 29, 2015.

III. AWARD DATE

A decision on the award will be made by July 10, 2015.

IV. SCOPE OF WORK

To establish and verify the relevance of an *in vitro* cell culture system that can be used to predict fibrogenesis in human lung. This system should allow exposure to aerosols of MWCNTs or positive and negative control atmospheres at the ALI. Cells should be exposed for durations that are sufficient to reflect *in vivo* lung burdens associated with fibrosis. Exposures may, but are not required to, extend over several days, but should not compromise the integrity of the cultures. Test atmospheres should be characterized before exposure for mass, number, and particle size at a minimum, and dosimetry to the cells should be determined by appropriate methods. Endpoints for cytotoxicity (e.g., lactate dehydrogenase [LDH]), inflammation (e.g., tumor necrosis factor alpha [TNF-α], transforming growth factor beta [TGF-β], serum amyloid A3 [SAA3], etc), collagen production (e.g., Sircol assay), fibroblast proliferation, epithelial cell apoptosis, and cytokine expression (platelet-derived growth factor [PDGF], osteopontin [OPN], Tenascin-C and chemokine (C-C motif) ligand 2 [CCL-2]) should be assessed at appropriate timepoints as determined by optimization of the assays and responses. The endpoints should refer to effects observed *in vivo*. Overall, the studies should be aligned with Good Laboratory Practices (GLP) outlined by the Organization for Economic Cooperation and Development (OECD) (The Application of the Principles of GLP to *in vitro* Studies, 2004).

V. BACKGROUND

Engineered NMs, and carbon nanotubes in particular, are being increasingly used in consumer products, necessitating the development of cost- and time-effective methods to assess their toxicity.

With this in mind, the PETA International Science Consortium Ltd. (PISC; PISCLtd.org.uk), recently organized an international workshop (held at the William Jefferson Clinton Building, EPA headquarters, Washington, DC on February 24-25, 2015), which focused on the technical

details to develop an *in vitro* method to assess the mechanism leading to the development of pulmonary fibrosis following exposure to aerosolized MWCNTs. During the workshop, experts from multiple sectors (government, industry, academia and NGO) and disciplines (*in vitro* and *in vivo* inhalation studies of NMs, fibrosis, dosimetry, fluidic models, aerosol engineering, and regulatory assessment) made recommendations on cell types, endpoints, exposure systems, and dosimetry considerations required to develop the *in vitro* model for hazard identification of inhaled MWCNTs.

The method is intended to be included in a non-animal test battery to reduce and eventually replace the use of animals in studies to assess the toxicity of inhaled engineered NMs. The long-term vision is to develop a battery of *in silico* and *in vitro* assays that can be used in an integrated testing strategy, providing comprehensive information on biological endpoints relevant to inhalation exposure to NMs which could be used in the hazard ranking of substances for use in the risk assessment process.

The intent of this RFP is to have interested laboratories provide information regarding their capabilities, expertise, and qualifications for designing an *in vitro* test system that addresses the recommendations set forth by the experts at the workshop. Following are the features that the proposed method should include.

1. **Test Material**: The proposed method development will test two types of MWCNTs, one is the Nanocyl 7000s (JRC NM 400) and the other is Mitsui-7s because of the availability of *in vivo* and *in vitro* studies for both indicating their potential to cause fibrosis. The test material for the study will be supplied to the laboratories selected to develop the system. Positive controls include aerosolization of either long fiber amosite asbestos or crystalline silica and the negative controls either highly soluble refractory ceramic fiber (RCF) or carbon black.

2. **Cell Types**: The project requires developing a co-culture model comprised of primary alveolar epithelial cells, macrophages and fibroblasts cultured at the air-liquid interface. Adequate characterization (morphological and molecular) should be conducted at different time intervals to indicate the stability of the cell system. These parameters include (but are not limited to) transepithelial electrical resistance (TEER) and evaluation of tight junction proteins (immunofluorescence). Comparisons with studies conducted using submerged cell culture should also be performed either within the selected laboratory or using data from other laboratories.

3. **Aerosol generator and exposure module**: The method will involve aerosol exposure of the co-culture model at the ALI. Laboratories with aerosol generators and ALI modules that are transferrable to other laboratories are preferred.

4. **Endpoints**: The cells will be monitored for markers including those for cytotoxicity (e.g., LDH), inflammation (e.g., TNF-α, TGF-β, SAA3 etc) and collagen production (e.g., Sircol assay), fibroblast proliferation, epithelial cell apoptosis, PDGF, OPN, Tenascin-C and CCL-2).

5. **Material Characterization**: Characterization of MWCNTs will be conducted at the following stages of the method development and testing: (a) prior to NM aerosolization (pristine form); (b) during the generation or delivery of the NMs (administered dose); (c) following NM deposition in the exposure chamber (deposited dose); and (d) within the cells following NM uptake (cellular dose). The NM characteristics that are relevant to some of the aforementioned stages include, but are not limited to, the following:

- Agglomerate structure
- (De)agglomeration potential
- Impurity profile / content
- Effective density
- Bivariate length and diameter distribution (BVD)
- Surface charge
- Surface area
- Rigidity
- Dustiness
- Cellular uptake

6. **Dose and dose metrics**:
The project will involve extrapolation of the dose range previously used in *in vivo* studies to an *in vitro* dose. Surface area, mass and structure (aggregate or fiber) number will be used as dose metrics.

7. ***In silico*** **modeling**: *In silico* modeling using models such as Multiple-Path Particle Dosimetry (MPPD) and *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) models will be conducted to predict the probable biological outcome and to understand dosimetry profile.

VI. REVIEW PROCESS AND APPLICATION SUBMISSION

All submissions will be reviewed by a selected panel of international experts. The panel comprises experts from multiple disciplines who will evaluate the submissions based on the information regarding the technical capabilities and scientific expertise provided in the proposal by the laboratories.

Laboratories with expertise in multiple areas including NM characterization (of aerosols and as associated with cells), aerosol generation and exposure, co-culture development and *in silico* modeling are preferred; however, it is recognized that one laboratory may not have expertise in all of these areas. Because of the multi-disciplinary nature of the project, laboratories can submit proposals according to the following options:

Option 1: a single laboratory able to fulfill all the project requirements can submit an independent application.

Option 2: multiple laboratories with different specialties can form a consortium of laboratories that, together, fulfill all of the project requirements. In this case, a joint application may be submitted with a point of contact (POC) identified for each laboratory.

The applications will be reviewed based on the information provided in the following sections.

VII. QUALIFICATIONS

Please answer the questions providing information that can specifically be used for the review process. If submitting as a consortium, each of the questions below will need to be completed by each laboratory.

1. Please provide the following information:

1.1 Contact person

1.2 Name of organization

1.3 Address

1.4 Telephone number

1.5 E-mail address

2. Provide a general statement of qualifications that responds to the project recommendations given above.

3. Scientific expertise

Please write 'NA' for each point that is not applicable to your laboratory's expertise.

3.1 Do you have expertise to develop co-culture systems? If yes, have you worked with cell types relevant to respiratory system? Please describe.

3.2 Have you cultured cells on membranes (such as Transwells®) or extracellular matrix?

3.3 Have you worked with primary cell types previously? If yes, please list the cell types and their source.

3.4 Do you have a means to obtain human tissues for isolating primary human cells? Do you have experience in extracting primary cells from tissue biopsies? If yes, please describe.

3.5 Have you characterized cells cultured on membranes or extracellular matrix? If yes, what methods have you used (e.g., tight junction staining, transepithelial electrical resistance, other)?

3.6 Have you conducted aerosol generation and exposure experiments? If yes, did you test NMs or other substances? Please list the substances tested.

3.7 Do you have expertise to conduct *in silico* modeling (MPPD, ISDD, other)? If yes, please specify what model(s) have you worked with and whether you have used the model(s) for NMs.

3.8 List publications (if any) that you have relevant to the topic under consideration.

3.9 List the scientists who would be committed to this project. Provide specific information as to their experience on projects similar to this one. Please attach curriculum vitae for the identified scientists.

3.10 Will the project require hiring of additional experts? If yes, what expertise will be required?

3.11 Other expertise relevant to the project.

3.12 What is the biosafety level of your lab?

4. Technical capabilities

4.1 Nanomaterial characterization

4.1.1 Please list the NMs characterization equipment that you have access to that can be used for this project (e.g. BET, SEM or field emission-SEM, TEM [HRTEM], RAMAN, ICP-MS, other).

4.1.2 Have you used the equipment to characterize carbon-based NMs? If yes, please specify what materials you have characterized (MWCNTs, SWCNTs, other)?

4.1.3 Have you characterized carbon-based NMs in their pristine form or in a biological system (e.g., to assess cellular localization or to quantify cellular uptake)?

4.1.4 Please list the equipment that you have used specifically to characterize each of the following NM properties:

- Agglomerate structure
- (De) agglomeration potential
- Impurity profile / content
- Effective density
- Bivariate length and diameter distribution (BVD)
- Surface charge

- Surface area
- Rigidity
- Dustiness
- Cellular uptake

4.1.5 Please list any other NM properties, not mentioned in 4.1.4, and the equipment used to characterize it that will be appropriate to add to the proposed study.

4.1.6 Other comments.

4.2 Aerosol generation equipment

4.2.1 Do you have an aerosol generator? If so, please indicate type of generator (e.g., nebulizer, fluidized bed, acoustic, other).

4.2.2 Have you used the aerosol generator for carbon-based NMs? If yes, please specify which type of materials (MWCNTs, SWCNTs, other?).

4.2.3 Have you used the aerosol generator for *in vitro* or *in vivo* studies?

4.2.4 Is your laboratory set up for exposing *in vitro* systems to aerosols?

4.2.5 Other comments.

4.3 Aerosol characterization equipment

4.3.1 Please list the aerosol characterization equipment that you have (APS, SMPS, Nano-MOUDI, other).

4.3.2 Have you characterized aerosolized NMs before? If yes, please list the types of NMs previously characterized?

4.3.3 Have you characterized aerosolized carbon-based NMs before? If yes, please specify which ones (MWCNTs, SWCNTs, other).

4.4 Exposure modules

4.4.1. What type of exposure module do you own? If you have a commercially available exposure module, please indicate the specific manufacturer, model and date of purchase (e.g. VitroCell [Trumpet or CLOUD], Cultex [RFS or RFS compact], other).

4.4.2 Have you used the exposure model to expose cells at air-liquid interface? If yes, please indicate what type of cells?

4.4.3 Have you used the exposure modules to expose cells to NMs? Is yes, what kind of NMs (carbon-based, metallic or metal oxides)?

4.4.4 Have you quantified the nanomaterial deposition? If yes, what method have you used (e.g., quartz microbalance, TEM grid, other)?

4.4.5 Equipment for characterizing the co-culture model (e.g., TEM sectioning, confocal microscopes, other).

4.4.6 Other capabilities.

4.5 What additional equipment, not currently owned, will be required to support the project?

5. Please list any features that you think could help make the project design better. Explain your suggestions with scientific evidence (e.g., relevant publications, past experience).

VIII. PROPOSED BUDGET AND MILESTONES

The following milestones are to be reviewed by the review committee as they are achieved. PISC will provide funding for the method development with amounts to be specified at the time of award.

a. Proposed budget

A proposed budget breakdown for each milestone listed below should be included as an attachment in submissions.

Phase I
Milestone: culturing and characterization of cellular model (primary alveolar epithelial cells, macrophages and fibroblasts at the air-liquid interface)
Milestone: aerosolization and characterization of Nanocyl 7000s, Mitsui-7s, positive and negative controls
Milestone: dosimetry and *in silico* modeling (e.g., MPPD and ISDD)

Phase II: Progression to phase II is contingent upon successful completion of phase I
Milestone: detection of pro-fibrotic markers following exposure to aerosolized positive control, no increase in pro-fibrotic markers following exposure to the negative control, and results with MWCNTs (includes lifecycle characterization)

b. Other

Would your participation in the project require special legal formalities through your organization?

IX. PROPOSAL ACCEPTANCE

Upon acceptance and before work begins, a legally-binding contract will be signed by the involved parties. Below are the basic terms that will be in the contract.

a. Data Ownership

All results of the study and other data and information developed by the laboratory related to the method development studies (collectively, the "Results") shall be the joint property of the laboratory and PISC. Each party shall not publish or otherwise disclose such Results, nor use the same for its own benefit or the benefit of any third party, without the prior written consent of the other party, such consent not to be unreasonably withheld.

b. Deliverables

Timeline for method development: The method development is intended to be completed within a 12 month period from the designated start date of the project.
Quarterly plans and reports: The selected laboratory is required to submit a project plan and a quarterly report in the beginning and end of each quarter, respectively. Quarterly reports must describe the progress in method development towards achievement of milestones under Phase I.
Final report: A written report describing the method development, protocols used and raw and analyzed data is required at the end of the 12 month period.
Teleconferences: A representative from the selected laboratory will be required to participate on quarterly conference calls with the review committee to update on progress.
Presentations/ publications: The results are to be presented at a workshop(s) and subsequently published in a scientific journal. Investigators will participate in national and international meetings to discuss the results and participate in the drafting of the publication. Reasonable travels costs will be covered.

c. Budget and Payment Schedule

PISC will provide funding for the method development which will be strictly contingent upon the timely submission of quarterly project plans, reports, teleconference calls and progress made towards the method development. Amounts will be specified at the time of award. Failure to comply with the project goals and time frame will lead to withdrawal of the funding.

Payment schedule (subject to modification)

30% at signing of contract
15% when system capability is demonstrated (aerosol generated, cells exposed, aerosol measured, dose quantified)
15% at the end of experimental phase
30% upon receipt of draft final report
10% on finalization of report