ENPRA Newsletter – Issue 5

Newsletter Contents

ENPRA News & Events

- **Fifth EONS meeting**

Fifth EONS meeting *(Paris - October 13, 2011).*

The **fifth expert panel meeting** of the **European Observatory on NanoSafety (EONS)** took place in Paris on October 13th. Gathering ENPRA partners from IOM, UCL, Vrije Univ. Brussels and JRC as well as French experts from the Observatoire des Micro & Nanotechnologies (OMNT), this meeting once again provided the panel of experts with the opportunity **to collectively discuss the latest nanosafety key trends and research progresses**.

A selection of studies and reviews from the recent nanoEHS literature was presented and commented upon. Among the topics of interest, the panel highlighted the latest recommendations from the US National Institute for Occupational Health and Safety (NIOSH) in terms of nanomaterial occupational exposure (*i.e.* the novel Intelligence Bulletin on titanium dioxide and the draft Intelligent Bulletin on carbon nanotubes and nanofibers). The experts also underlined the latest review on nanomaterial occupational exposure from the Institute of Energy and Environmental Technology (IUTA) and the Technische Universität Dresden (TUD) which provide evidence of nanoparticle release into the air for a range of occupational scenarios. The panel also emphasized a selection of experimental studies, including papers on nanotoxicology, nanogenotoxicity and methodological approaches for nanomaterial testing.

As part of the dissemination strategy of the ENPRA project, EONS reports contribute to inform stakeholders of the latest nanoEHS research advances thanks to the expert survey of the domain. A **summary report** of this meeting (5th EONS report) has been published by the OMNT. The PDF version of the full report can be accessed via this link. Summary excerpts of previous EONS reports are available on the ENPRA website.

The **next EONS meeting** is to be held on May 2, 2012 in San Sebastian (Spain), in conjunction with the 3rd ENPRA/JRC Stakeholder workshop.

- **ENPRA workpackage progresses**
  - **WP2: EU-US collaboration**

The contribution for this WP comes from our US partners, EPA and NIOSH. Specifically, EPA is preparing to send their *in vitro* toxicology data to ENPRA to be used in WP6 QSAR (quantitative structure activity relationship) modelling (lead by the JRC). NIOSH will also offer their exposure data for exposure modelling in WP6 to be undertaken by IOM. Collaboration has been ongoing between NIOSH and IOM concerning PBPK (physiologically-based pharmacokinetic) modelling. More recently data that has been generated by US partners using particles provided by ENPRA, as well as others, has begun to be delivered to the ENPRA database. This data, where appropriate, will be used in QSAR modelling.

- **WP3: Hazard Identification - characterization of the physico-chemical properties of ENP**

After completion of the primary characterisation of the panel of engineered nanoparticles (ENPs) selected for the project and establishment of a database compiling the physical and chemical parameters of the ENPs, WP3 partners have initiated biodurability and hydrochemical reactivity studies. These studies aim to assess the potential residence time of the different ENPs in the respiratory tract and their inherent oxidative potential. In parallel, WP3 partners have developed analytical protocols for the determination of ENP i) in solution (dispersion protocol, repeatability and stability of ENP, agglomeration of sedimentation behaviour under various conditions), and ii) in biological tissues (from in vitro and in vivo experiments). As part of the transatlantic collaboration between ENPRA EU and US partners, the US-EPA is now testing the dispersion protocol set up by WP3 EU partners. In the meantime, complementary ICP-MS analyses of the catalyst impurities in CNT are underway at Duke University.

- **WP4: Dose-response assessment I - Development of in vitro models for assessing the potential hazards of ENP**

After establishment of standard operating procedures (also shared with the NanoImpactNet network) for the *in vitro* protocols applied to the multiple target systems, WP4 partners have tested the cytotoxic effect of the 10 ENPs selected for the project in relevant target cell types (i.e. respiratory, cardiovascular, hepatic, renal and embryonic cells). Based on the first results, LC50 values have been determined and assembled to enable strategic decisions for subsequent *in vitro* and *in vivo* experiments. Cytotoxicity, inflammatory and immune response results on the different targets have been submitted to data analysis and database generation. The ENPRA database is being constructed and uses templates for data collection created in FP7 NANOMMUNE.

During the last few months, special emphasis has been given to the impact of serum or surfactant addition in the experimental medium, when looking at the reactive oxygen generation, the genotoxicity or the inflammatory and immune responses developed by the respiratory cells exposed to the different ENPs. In this respect, the interesting findings on the impact of serum on proliferation and genotoxicity are further developed in the following focus article.

Cardiovascular toxicity induced by sub-toxic concentrations of the different ENPs has been assessed using an optimised tri-culture system (endothelial cells + respiratory epithelial cells + activated monocytes). In addition, first results on reactive oxygen species (ROS) generation induced by the different ENPs in the hepatic system have been accepted for publication[1]. Renal toxicity of the ENPs has also been investigated. Finally, in parallel of the development of a high-throughput method for testing the embryonic toxicity of ENPs, WP4 partners have assessed the sensitivity of embryonic stem cells to short and long term exposure to ENPs.

- **WP5: Dose-response assessment II - Using in vivo models for a kinetics study and verification of in vitro results**

Kinetic studies after inhalation and instillation of different ENPs have been completed and compiled with additional (external) results on injection; these investigations have provided valuable data on the fate of ENPs in the organism that are essential for the implementation of the *in silico* studies developed in WP6 (e.g. PBPK modelling).

In vivo experiments for determination of the acute effects (24hours) after lung exposure to the full panel of ENPs have been performed and data analyses have allowed establishment of dose-response

[1] Kermanizadeh A, *et al.* In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. Nanotoxicology 2012 (*In Press).*

relationships for a range of endpoints. Among the panel of ENPs tested, the two types of ZnO nanoparticles showed the highest level of toxicity; these results are in accordance with the *in vitro* data obtained in WP4. Complementary *in vivo* experiments are currently underway in order to collect further data on early lung tissue changes after lung exposure to both types of ZnO nanoparticles. Since interesting findings have been obtained in the *in vitro* experiments with regard to the composition of the cell culture medium on ENPs-induced toxicity, additional *in vivo* studies are planned in order to test the role of serum in the dispersion medium on ENPs-induced pulmonary toxicity; in particular, this would enable to determine whether the presence of serum can significantly influence ENPs surface-induced toxicity.

WP5 partners have also completed a round of *in vivo* experiments on compromised animal models. To investigate the impact of MWCNT on the development of atherosclerosis, Apo-E mice (animals prone to develop atherosclerotic plaques in the aorta) were exposed by repeated instillation to two different types of MWCNT; data analysis on plaque progression and pulmonary inflammation revealed different impacts of the two types of CNT; additional investigations will determine the potential relationship between the observed effects and the physical-chemical properties of the CNT.

Finally, *in vivo* experiments are currently in progress in rapid aging mice to test the effects of (selected) nanoparticle exposure on aging/elderly subjects. A similar repeated instillation protocol as for the ApoE mice is being applied.

- **WP6: Risk assessment and risk analysis**

Within WP6 work is well underway in analysing the data being provided by WP3, 4 and 5 partners. The database is being populated with this data and preparations for uploading of this data in NanoHub.

The data thas been made available to date has been extracted in order to be able to use it for *the in-vivo in vitro* extrapolation (IVIVE) and this work is also underway, where benchmark dose and concentration (BMD and BMC) values have been determined for all of the end-points for which there are *in vitro* and *in vivo* data. These values will be used for examining whether there are any possible correlations between *in vivo* and *in vitro* end-points.

This data will also be utilised by the QSAR modelling, in order to investigate the effects that physical-chemical characteristics of the particles may have on the biological activity. Further work has been carried out on the PBPK modelling in order to extrapolate the results to the TiO_2 and ZnO particles used within ENPRA in order for it to be sued in the final risk assessment of ENPRA. The development of an exposure model is also ongoing; this involves looking to make it nano-specific, allowing for agglomeration of particles in the air.

Finally a strategy has been developed which will make use of the results of each of these tasks, as well as other information available, in order to carry out a risk assessment of the particles used within ENPRA.

Third ENPRA/JRC stakeholder workshop

As part of the dissemination strategy of the ENPRA project (WP7), the **3rd ENPRA stakeholder workshop** will be held as a **Joint Dissemination Event** with the FP7 projects NEPHH, HINAMOX, NANOPOLYTOX, and in collaboration with the **Joint Research Centre (JRC) Enlargement and Integration Programme**. This major international event on '**Safety issues and regulatory challenges of nanomaterials**' will take place in **San Sebastian, Spain on May 3-4, 2012**.
Additional information (program, registration, venue…) will be soon available on the JRC website.

Focus article: In vitro hazard assessment of engineered nanoparticles - First findings from the Vrije Universiteit Brussel on genotoxicity and developmental toxicity of nanomaterials (WP4)

Research conducted in the ENPRA WP4 aims at improving the understanding of the potential hazard of engineered nanoparticles (ENPs) through the development of relevant *in vitro* models. Among the 11 European partners involved in this WP, researchers at the Vrije Universiteit Brussel (VUB) are investigating the impact of ENPs on genomic stability and embryonic development. In the following interview, **Dr. Laetitia Gonzalez**, post-doctoral researcher of the Fund for Scientific Research (FWO) Flanders in the laboratory of Cell Genetics at VUB, and **Sara Corradi**, PhD student at VUB are presenting the on-going research and the recent findings obtained by the team.

1- What are your main objectives within WP4?

Laetitia Gonzalez: Our team involved in the ENPRA project at the laboratory of Cell Genetics (VUB) consists of two **Professors Micheline Kirsch-Volders** and **Luc Leyns**, **Sara Corradi** (a PhD student) and myself. Furthermore a number of graduate students have been working on this project. In 2012 our "nano"-group will be reinforced by a post-doctoral researcher and technician in the frame of Brussels Research (Innoviris). Within ENPRA we are involved in the **assessment of developmental toxicity and genotoxicity of engineered nanoparticles** (ENPs), the fields of expertise of Prof. Luc Leyns and Prof. Micheline Kirsch-Volders, respectively.

Within the ENPRA project we have two main objectives:

- To **investigate whether ENPs have the potential to induce developmental toxicity**, and in particular have the ability to **alter differentiation patterns** of mouse embryonic stem cells. As task leader for the assessment of developmental toxicity we are responsible for the testing of ENPs using different in vitro assays, the **embryonic stem cell test** (EST), validated by the European Centre for the Validation of Alternative Methods (ECVAM), and **mouse embryonic differentiation assays**. In both assays mouse embryonic stem (ES) cells are used as cell models. These cells have the unique property to pluripotency, meaning that they can be differentiated towards all cell types. In the former assay the effects of ENPs are measured as inhibition of differentiation of mouse embryonic stem cells towards cardiac muscle cells. The mouse embryonic differentiation assays are based on altered gene expression of some marker genes specific for mesodermal and neural differentiation;

- To **assess the genotoxic potential of the ENPs**, more specifically by considering both **direct** genotoxic effects, e.g. the generation of reactive oxygen species that target directly the DNA as well as the **indirect genotoxic effects** that can be induced by ENPs when non-DNA targets are involved. In particular, as we demonstrated for monodisperse amorphous silica nanoparticles that the experimental conditions of the in vitro micronucleus assay were determinant for the assay outcome[2]; we also addressed these issues for the ENPRA ENPs. Therefore the hypothesis that **serum** had an **influence on the micronucleus formation and cell proliferation** was investigated. Accumulating evidence indicates that ENPs (e.g. monodisperse silica) can bind covalently proteins depending on the charge and size[3]. Our working hypothesis is that binding with extra- (e.g. serum

[2] Gonzalez L, *et al.* Adaptations of the in vitro MN assay for the genotoxicity assessment of nanomaterials. Mutagenesis (2011)26:185-191.
[3] Wang J, *et al.* Soft interactions at nanoparticles alter protein function and conformation in a size dependent manner. Nano Lett. (2011)11:4985-4991.

proteins, membrane receptors) and intra-cellular (DNA repair enzymes, cytoskeleton) proteins can indirectly modulate genotoxic effects, both chromosome breakage and/or malsegregation[4].

Therefore the vitro micronucleus assay (**Figure 1**), a methodology that we have developed, validated and harmonised at international level (OECD guideline 487)[5], was chosen as it can detect both direct (chromosome breakage) and indirect (chromosome loss) genotoxic effects.

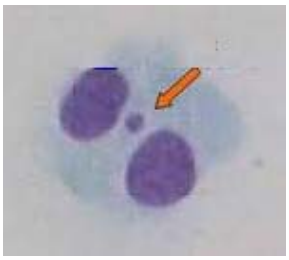

Figure 1: **The in vitro micronucleus assay**
Micronuclei are formed when, during mitosis, chromosome fragments or entire chromosome are lagging and not incorporated into the daughter nuclei. At the end of mitosis these chromosome fragments and/or chromosomes are surrounded by a nuclear membrane leading to the formation of a small nucleus or a micronucleus. The *in vitro* cytochalasin-B micronucleus assay is based on the addition of cytochalasin-B, an actin inhibitor that allows nuclear division but inhibits cytokinesis. This methodology allows the discrimination between cells that did not divide during culture (mononucleated cells), cells that divided once (binucleated cells) and cells that divided more than once (multi-nucleated cells).

2- What are your progresses so far in the evaluation of the developmental toxicity of ENPs?

Sara Corradi: Up to now, we are aiming to develop a **high-throughput method based on the embryonic stem test** (EST). In fact the EST, validated by ECVAM is a time consuming procedure, and not handy especially to test ENPs, as it requires a lot of manipulation and refreshing of medium. Moreover the **high-throughput method** is implemented by using an ES cell line fluorescent for mesoderm and cardiomyocyte markers to make **easy and quick the screening of beating structures**.

In parallel, we assessed the **sensitivity of ES cells** (in comparison to somatic cells) **on short** (24h) and **long term** (10 days) exposure to ENPs. 10 days toxicity gives us the range of concentration that needs to be used for differentiation assay. Preliminary results showed high toxicity with ZnO and Ag ENPs both at 24 h and 10 days, as we already observed in A549 lung epithelial cells. No toxicity is observed at 24 hours for TiO_2 at the highest concentration tested, while at 10 days decrease in percentage of viable cells is detected. Therefore long exposure to ENPs seems to have an effect on cell viability and more results on TiO_2 as well as MWVNT, ZnO and Ag will be available soon.

3- As mentioned by Laetitia, experimental conditions may directly affect the outcomes of in vitro toxicological assays and careful setting of the methodological approach is required in order to ensure proper investigations. Regarding the assessment of the genotoxic potential of ENPs, what did you learn from the first ENPRA studies?

SC: We wanted to assess **whether serum can influence the proliferation and genotoxicity of cells exposed to ENPs**. To address this we used:

- An epithelial lung carcinoma cells line, A459 chosen as the lung represent one of the **target organ for inhalation** and because we previously showed that A549 are capable to grow in the absence of serum[6];
- An **in situ method** without trypsinization step, that allow **direct morphological and cytotoxicological analyses** of the samples[7];

[4] Gonzalez L, *et al.* Induction of chromosome malsegregation by nanomaterials. Biochemical Society Transactions (2010) 38:1691-7.
[5] Kirsch-Volders M, *et al.* The in vitro MN assay in 2010: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance. Archives of Toxicology (2011) 85:873-99.
[6] Gonzalez L, *et al.* Methodological approaches influencing cellular uptake and cyto-(geno) toxic effects of nanoparticles. J Biomed Nanotechnol. (2011)7:3-5.

- Cells exposed to different type of nanomaterials – Lys-SiO_2, TiO_2, ZnO and MWCNT;
- Cell proliferation assessed by **cytokinesis blocked proliferation index** (CBPI), based on cell nuclearity, which is **an indication of cell cycle delay and cell toxicity**.

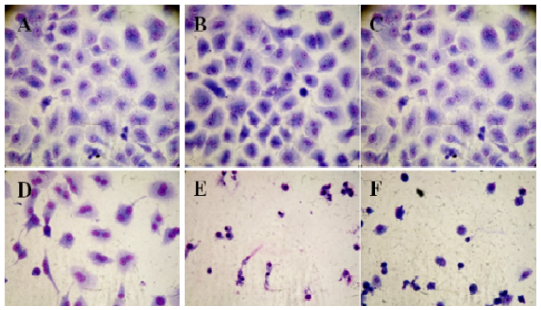

Figure 2: Cell morphology after 40 hours of treatment in presence (A,B and C) and absence (D,E,F) of 10% FBS. A) and D) untreated control. B) and E) 5µg/ml Lys-SiO_2 ENPs. C) and F) 25µg/ml ZnO ENPs.

We observed a statistically significant decrease in CBPI in cells treated with Lys-SiO_2 in the absence of serum and with ZnO both in presence and absence of serum as also reflected also by cell morphology analysis (**Figure 2**). No toxicity was detected for MWCNT and TiO_2. No difference was observed in the frequency of micronuclei (MN), for all the ENPs, except for ZnO, but at high toxic dose.

One of the most interesting results is the evidence of **increased cell toxicity using Lys-SiO_2 suspended in serum-free medium compared to 10% serum**. In fact, after observing that in absence of serum, Lys-SiO_2 induced high toxicity, we tried to further analyse the effect of serum. We **pre-incubated Lys-SiO_2 with 100% serum** to allowed interaction between ENPs and protein serum and we used these serum pre - incubated Lys-SiO_2 to **treat the cells in the absence of serum**. We observed that CBPI induced by serum pre-incubated Lys-SiO_2 was comparable to CBPI of untreated cells (**Figure 3**).

The serum effect could not be observed for TiO_2, ZnO and MWCNT. We observed formation of huge **agglomerations of MWCNT and TiO_2** increasing in size and density **as ENPs dose increased**. And as consequence the microscopical analyses was unreliable. Moreover as these NPs required the presence of 2% serum to be dispersed, following the ENPRA protocol, we tried to disperse the ENPs only **in pure water**, but **MWCNT failed to disperse** and **TiO_2** formed denser **agglomerates**.

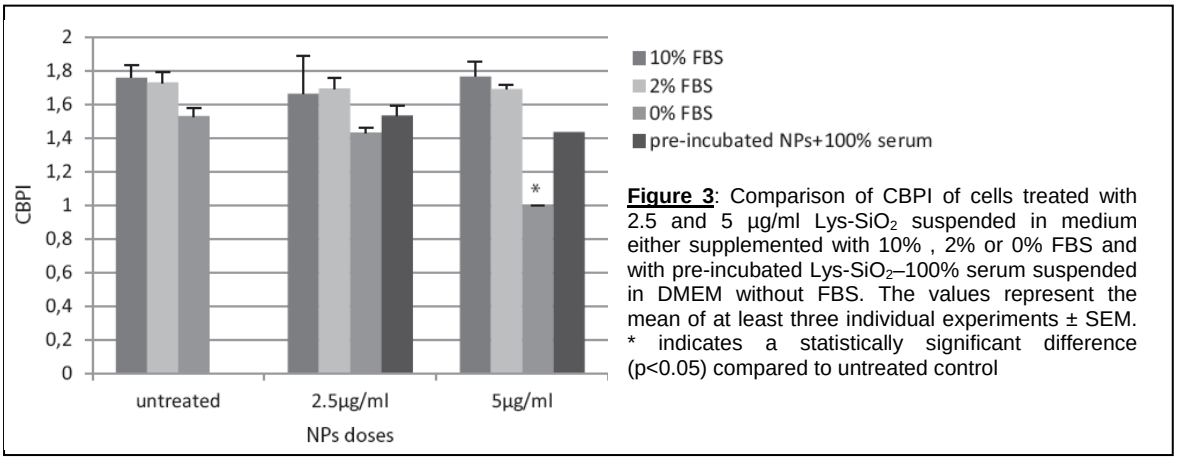

Figure 3: Comparison of CBPI of cells treated with 2.5 and 5 µg/ml Lys-SiO_2 suspended in medium either supplemented with 10% , 2% or 0% FBS and with pre-incubated Lys-SiO_2–100% serum suspended in DMEM without FBS. The values represent the mean of at least three individual experiments ± SEM. * indicates a statistically significant difference ($p<0.05$) compared to untreated control

To conclude, the in situ assay that we proposed is an interesting method to evaluate cell morphology and toxicity. We learned that **applicability of assay need to be adapted to test ENPs**. The microscopical analysis of MN and nuclei is not adequate due to overload of ENPs that mask the MN.

[7] Corradi S, et al. Influence of serum on in situ proliferation and genotoxicity in A549 human lung cells exposed to nanomaterials. Mut Res (2011) *In Press*

Therefore alternative techniques have to be used, such as flow cytometry[8,9]. Moreover we showed that when monodispersed Lys-SiO_2 interact with serum proteins, the toxic effect of silica is minimized possibly because of the corona that is formed around the ENPs.

4- Since appropriate battery of *in vitro* tests are required to minimize *in vivo* testing, to what extent do your findings hold impacts regarding current methodological approaches for safety assessment of nanomaterials?

LG: Our findings, previous and current, clearly demonstrate that the choice of assay and execution of the assay (experimental conditions) are of major importance and that, therefore, the **straightforward application of standardised tests (OECD) might not always be the best option/choice for ENP testing**.

In previous papers from us and others, different experimental conditions, for the *in vitro* micronucleus assay, were analysed and it was shown that the experimental outcome could markedly differ according to the experimental conditions[2,6,10]. From these findings we proposed some **adaptations of the *in vitro* micronucleus assay** that are crucial for its adequacy for the testing of nanomaterials. These adaptations include (1) not to add cytochalasin-B at the same time as the nanomaterial treatment as cytochalasin-B can reduce nanomaterial uptake and (2) the necessity for a treatment during mitosis in particular when dealing with nanomaterials that are not able to cross the nuclear membrane. Furthermore we recommended to test in presence of different serum concentrations[2]. These adaptations or conditions still fall within the OECD guideline for this assay.

In a broader context, for the development of an adequate testing battery for the risk assessment of nanomaterials different general issues should be addressed:

- Interference of ENP with assay components. This has been extensively shown for cytotoxicity assays, but can occur in any type of assay and assay components should be tested for this phenomenon before applying any assay;

- Interference of assay components with the uptake or action of ENP;

- Treatment schedule. Not only acute effects but also chronic exposure should be considered. This is also evidenced by our above-mentioned findings on the cytotoxicity of TiO_2 in mouse ES cells;

Head researchers at the laboratory of Cell Genetics (VUB), involved in ENPRA: **Pr. Luc Leyns** *(left)* and **Pr. Micheline Kirsch-Volders** *(right)*.

- Adequate choice of top dose. This aspect is of major importance for all types of toxicity assays;

- The choice of the assay. As nanotoxicology is a relatively new field, specifically some of its sub-fields (developmental toxicity, ecotoxicology) and the effects are not fully understood it is important to keep an open mind for new cellular targets and endpoints that could be more suitable for ENP testing.

[8] Lukamowicz M, *et al.* In vitro primary human lymphocyte flow cytometry based micronucleus assay: simultaneous assessment of cell proliferation, apoptosis and MN frequency. Mutagenesis. (2011)26:763-70.
[9] Lukamowicz M, *et al.* A flow cytometry based in vitro micronucleus assay in TK6 cells--validation using early stage pharmaceutical development compounds. Environ Mol Mutagen (2010)52:363-72.
[10] Doak SH, *et al.* Confounding experimental considerations in nanogenotoxicology. Mutagenesis. (2009)24:285-93.

Upcoming events

You will find below announcements of a selection of future nano EHS events.

- **NanoImpactNet – Q Nano Conference**

The multidisciplinary European network on the health and environmental impact of nanomaterials **NanoImpactNet** and the European Union-funded infrastructure for nanomaterial safety testing **Q-Nano** will hold a joint conference entitled "**From theory to practice - development, training and enabling nanosafety and health research**" from **27 February to 2 March 2012**, in **Dublin**, **Ireland**. The event will consist of a three-day integrating conference (including a special stakeholder session) and two one-day training schools. Topics include:

- Materials for the future
- Eco-Hazard Assessment
- From Production to Exposure
- Beyond non-specific hazards
- Characterisation in situ following exposure
- Stakeholder needs and Risk Assessment

For more information click here.

- **Nanotoxicology 2012**

The **6th International Conference on Nanotoxicology** (Nanotoxicology 2012) will be held on **September 4th** (Tuesday) **- 7th** (Friday), **2012** in **Beijing, China**.

With the rapid development of nanotechnology applications, the safety assessment of nano-products has become important than ever before. The conference will hence provide a timely international forum for presentation and discussion of current and emerging sciences of all-round. Conference themes are:

- Nanotoxicology and human toxicology (NanoTOX)
- Nano Environmental Health and Safety (Nano EHS)
- Nanomedicine, Pharmacokinetics and Particokinetics (Nano PK)
- Nanobiotechnology, Nano-bio interface &Nanobiomaterials (NanoBio)
- Nano-bioinorganic Chemistry (NanoBioChem)
- Exposure scenarios and risk assessment of nanomaterials (ESRA)
- Nano-bioanalytical sciences and nanostandardization (NanoAnalysis)

Deadline for abstract submission is **April 30, 2012**. For more information, please click here.

- **SENN 2012**

The NANODEVICE project partners and the Finnish Institute of Occupational Health organize the "**International Congress on Safety of Engineered Nanoparticles and Nanotechnologies**" to be held on **28–31 October 2012** in **Helsinki**, **Finland**.

The goal of the SENN2012 Congress is to summarize and share the latest knowledge on the safety of engineered nanomaterials and nano-related technologies. The emphasis is on producing solutions to the safety challenges related to engineered nanomaterials and nanotechnologies. Another aim is to enable commercial opportunities for the safe use of these materials and technologies.

The Congress will provide a forum for reporting and demonstrating findings, methods, tools, and approaches to safety and health at workplaces using nanoparticles and nanotechnologies. The plenary and free communication sessions will be designed to facilitate interaction between participants and presenters.

Deadline for abstract submission is **April 1st 2012**. For more information, please click here.

- **Nanosafe 2012**

After the success of Nanosafe 2008 and Nanosafe 2010, the next edition **Nanosafe 2012** will be held from **13th** to **15th November 2012** in Minatec, **Grenoble, France**.

The objectives of the conference will be to make available the major progresses and future trends in the domain of the safe production and use of nanomaterials. Topics of the conference are:

- Exposure assessment
- Characterization, Detection and Monitoring
- Nanomaterials life cycle
- Toxicology
- Environmental impact
- Nanoparticle release from consumer products
- Personal protection equipment
- Secure industrial production
- Safety parameters evaluation
- Standardization, Regulations

Deadline for abstract submission is **July 30, 2012**. For additional information, please click here.