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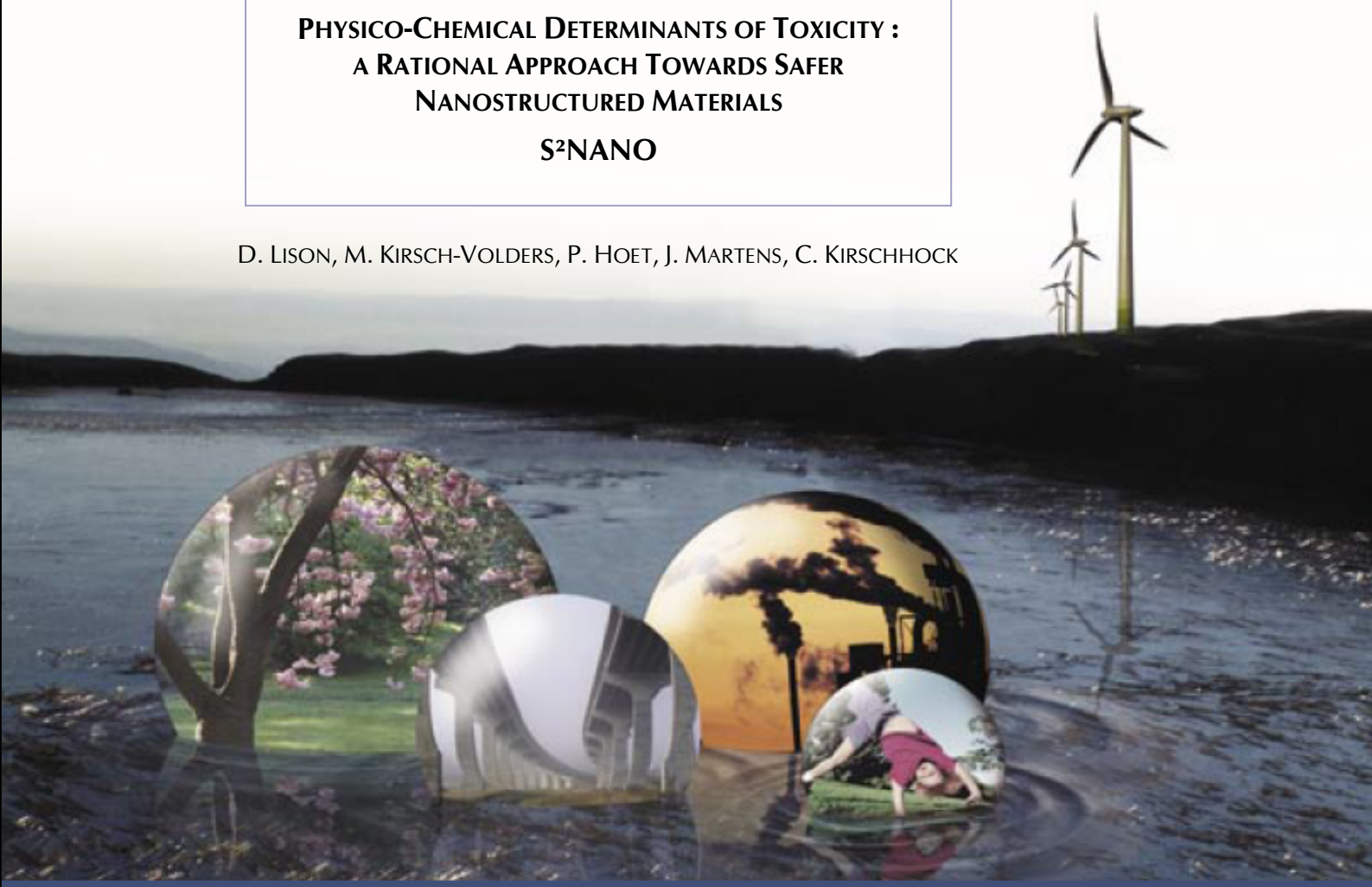
PHYSICO-CHEMICAL DETERMINANTS OF TOXICITY : A RATIONAL APPROACH TOWARDS SAFER NANOSTRUCTURED MATERIALS

S²NANO

D. LISON, M. KIRSCH-VOLDERS, P. HOET, J. MARTENS, C. KIRSCHHOCK



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FINAL REPORT PHASE 1

PHYSICO-CHEMICAL DETERMINANTS OF TOXICITY : A RATIONAL APPROACH TOWARDS SAFER NANOSTRUCTURED MATERIALS

S²NANO

SD/HE/02A

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ACRONYMS and ABBREVIATIONS

A549	human type II lung epithelial cell line
BET	Brunauer, Emmett and Teller surface adsorption
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle medium
EAHY926	human endothelial cell line
ELISA	enzyme-linked immuno-sorbent assay
FTIR	Fourier transformed infra-red spectroscopy
ICP-MS	inductively coupled plasma-mass spectrometry
J774	mouse monocyte-macrophage cell line
LDH	lacticodehydrogenase
MTT	MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
NMR	nuclear magnetic resonance
NP	nanoparticles
SAXS	small angle X-rays scattering
SEM	scanning electron microscopy
SNP	silicon-based nanoparticles
TC50	concentration causing 50% cytotoxicity
TG	thermogravimetry
TNF-a	tumour necrosis factor alpha
U937	human monocyte-macrophage cell line
WST1	a variant MTT methodology relying on soluble formazan products
XRD	X-ray diffraction

1. INTRODUCTION

1.1 Context

Nanomaterials represent a broad class of small-scale (<100 nm) entities formed by molecular level engineering to achieve unique mechanical, optical, electrical or magnetic properties. They are already impacting on virtually all types of industrial and domestic products and their production and commercialisation will increase sharply in the coming years. In many cases, nanostructured materials are components of large-scale products such as nano-composites, surface coatings and electronic circuits, and the likelihood for direct exposure of the consumer is expected to be low. However, during the manufacture, treatment, recycling or degradation of these materials, workers producing or processing nanomaterial-containing devices, and possibly the consumer at large, may be exposed to these materials, and an assessment of their toxic potential (hazard) is required.

The same unique physico-chemical properties that make nanomaterials so technologically attractive may, however, also present potential challenges to human health. Unfortunately, technological developments have out-paced research efforts to assess these health and environmental risks. This emerging challenge has, to some extent, been recognised by governments, regional and international regulatory agencies and scientists, and several research programmes are funded to examine the (eco)toxicity of nanomaterials.

The available data raise concerns about the safety of nanomaterials. Data have already been reported indicating that nanomaterials can exert harmful effects. The main potential target organs include the respiratory tract, the brain, the cardio-vascular system, the skin, and the liver. The nanoscale size of new materials implies that they can theoretically access and interact with cellular and molecular targets and structures to a larger extent than their bulk analogues. The toxic activity of nanomaterials may significantly differ from that of bulk material with the same chemical composition. Typically, the biological activity of particles increases as the particle size decreases and there is a need to find exposure metrics that would be relevant for nanomaterials.

While there already exists some hints of the physico-chemical determinants of toxicity for nanomaterials, these are mainly based on empirical observations and knowledge remains, therefore, largely fragmentary because it is not possible to test each and every nanomaterial. The biological reactivity of nanomaterials likely involves surface chemistry reactions taking place at the interface between the particle and the biological environment. The extent of these reactions will theoretically depend on the surface area in contact with the biological systems. Thus, the physico-chemical properties of the particulate surface have been shown to play an important role in the biological effects. This, however, implies that we might expect significant differences in the extent and mechanisms of toxicity of nanomaterials that differ in solubility in the biological and environmental conditions. Moreover, research efforts to explore the toxic potential of nanomaterials have been mainly limited to descriptive studies, often leading to contradictory observations. This reflects the fact that nanotoxicology is still in its infancy, but is also attributable to a lack of co-ordination of the research efforts and standardisation of the materials tested. It is indeed highly probable that apparent discrepancies reported among published studies result, in part, from subtle or major differences in the physico-chemical characteristics of the material tested. It is, however, often impossible to identify these differences because the materials used were poorly characterised.

These specificities have critical implications for the characterisation of the hazard of nanomaterials: (1) qualitatively, the toxic properties of nanomaterials cannot easily be deduced from existing knowledge for similar bulk or soluble materials, and (2) quantitatively, because we do not know exactly how to express the dose of nanomaterials. It is also difficult to assess exposure, and hence to conduct risk assessment and control measures. There is, therefore, a need to understand which characteristics of nanomaterials are critical for toxicity.

1.2 Objectives and expected outcomes

The objective of this programme is to understand how nanomaterials exert toxic effects and to identify the **physico-chemical determinants of their toxicity**.

This approach will contribute
(1) to better understand the **mechanisms** influencing the interactions of nanomaterials with the cell and tissues,
(2) to improve the **metrological** approach of nanoparticles based on parameters other than mass, e.g. surface area or number of particles, particle size distribution and morphology.

It is expected to produce sound scientific bases to support the development of sustainable nanoproducts and to provide industrials and regulators with evidence-based guidelines for the production and control of safer materials. In line with the concept of **sustainable development**, the acceptance of a new material will, indeed, depend on the capacity of the industrials to integrate (eco)toxicological concerns early in the development of nanoproducts. It is, therefore, of great socio-economical value to anticipate (eco)toxicological issues.

This programme focuses on human health effects and does not address environmental impacts of nanomaterials.

1.3 Workplan

Several health effects are likely to occur upon inhalation exposure to nanoparticles, including pulmonary inflammation, pulmonary fibrosis, chronic obstructive pulmonary diseases (emphysema, chronic bronchitis), lung and mesothelial cancers and possibly systemic effects (mainly cardio-vascular and coagulation diseases).

The toxicological studies were conducted with in vitro models (screening tests) relevant for the health effects mentioned above, namely (1) production of cytokines in macrophage cultures, (2) genotoxicity in pulmonary epithelial cells, and (3) platelet aggregation and coagulation. The macrophage is a key player in orchestrating particle clearance, inflammation, fibrosis and indirect genotoxicity. Epithelial are important targets for genotoxicity and carcinogenesis. Platelet activation, aggregation and coagulation are central to the cardio-vascular effects associated with inhalation exposure to particles.These models have been used to screen the physico-chemical parameters that may be critical for toxicity. Integration of the results obtained in vitro is relevant for most health effects considered. For instance, the potential to induce cardio-vascular diseases upon inhalation of nanoparticles will depend on their capacity to elicit an inflammatory response in the lung, to damage epithelial and endothelial DNA and to affect platelet and coagulation homeostasis.

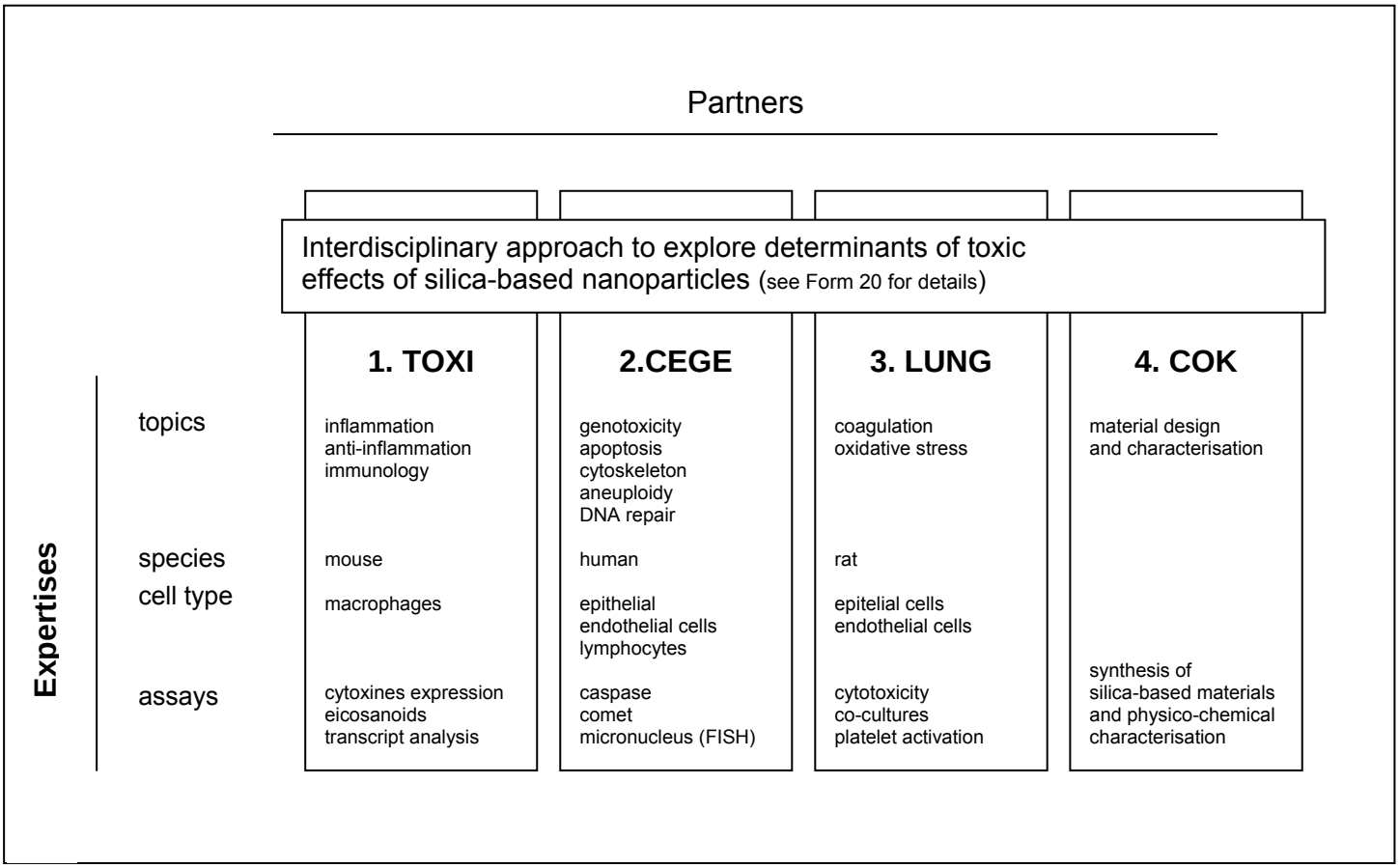
The first phase of the initial work programme (years 1 and 2) comprised several tasks :

1. Synthesis, optimisation, characterisation of silica-based nanoparticles
2. Development and application of *in vitro* screening assays :
   - genotoxicity in epithelial cells
   - platelet function and endothelial modulation of coagulation
   - inflammatory mediator production in macrophages

This work programme has been executed according to the initial schedule, except *in vivo* studies that are planned to start in the first half of 2009 because of the need to prepare new batches of endotoxin-free nanoparticles (see below).
An additional task has been included to address a dosimetry issue that was raised soon after the initiation of the programme.

2. COLLABORATION AND SYNERGY WITHIN THE NETWORK

The programme relied on expertise and techniques existing in the 4 collaborating laboratories (Figure 1).



Their competencies are fully complementary, and they have, in the past, already conducted several collaborative and interdisciplinary projects, including on inhaled particulates. During this first phase, the network has been working in a truly collaborative manner since the early beginning of the programme. A kick-off meeting was organised to secure a common understanding among the teams and especially to tune the synthesis and production of NP according to the needs and constraints of the investigators performing biological tests (amounts needed, purity, sterility, fluorescent labelling, …). Frequent and informal contacts have been quickly set up among young investigators involved in the project and exchange of information, protocols and data occurred on an almost daily basis. More formal meetings involving all the partners (junior + senior investigators) were organized on a regular basis to examine the progress of the work, to raise and examine how to test hypotheses, to set priorities, shape, articulate and organise the collaborative work in the laboratories. When a specific issue needed to be addressed by several teams in parallel, a lead scientist was appointed to manage the coherence and progress of the work.

Collaboration in the network has been really excellent. This is illustrated by the successful experiments dealing with dosimetry which were triggered by a discussion between biologists and chemists about the behaviour of NP in suspension. The conceptions of toxicologists about the behaviour of NP in suspension were indeed completely erroneous and this was solved by discussions and dedicated experiments to verify hypotheses (see below Dosimetry). Another illustration of this interaction is the work carried out on the role of the roughness and porosity of NP surface triggered by an observation with a single particle (S5) of unexpectedly low

cytotoxicity. The COK partner has synthesized different NP with almost the same diameter but with varying porosity in order to test the hypothesis that high porosity would reduce the toxic potential of NP. There was, therefore, a real feed-back of biological results on the synthesis and preparation of NP. For the COK partner, the project was very challenging because it could apply and develop tools for targeted synthesis of materials other than adsorbents and catalysts that represent the core of its usual scientific work.

Biologists were also greatly benefiting from the discussions with chemists and now understand better how particle characteristics are assessed and what kind of information can be expected from the different physico-chemical techniques available. This issue is really crucial when conducting nanotoxicological studies for which a detailed physico-chemical characterisation of the material is essential, certainly when the main objective of the programme is dedicated to physico-chemical determinants of toxicity.

This unique collaboration in the network has allowed the groups to develop a really original approach of NP toxicology, which can finely examine the role of almost every characteristic of NP separately. This approach is scientifically more powerful than what is done by most other investigators who usually test an array of different materials obtained from different sources but with a combination of several physico-chemical variations from which it is often difficult to decipher the critical determinants.

When submitting their research proposal, the promoters were already convinced that a dialog between physico-chemists and toxicologists was a key to understand how NP cause toxicity. They have now experienced the benefits of this fruitful interaction; the quantity of data already obtained during these 24 months, together with the number of publications, manuscripts and communications are probably the best evidence of the success of this collaboration.

3. RESULTS AND ACHIEVEMENTS

3.1 Synthesis and physico-chemical characterisation of SNPs

As already mentioned, a very significant asset of the S²NANO project is the fruitful dialogue between physico-chemists and toxicologists. During this first phase, the COK partner was able to prepare stable monodisperse suspensions (sols) of amorphous silica particles in the nanosize range. These sols were prepared specifically for being used in toxicological tests by performing all synthesis steps under sterile conditions and eliminating all cytotoxic reagents by dialysis. Efforts were done to avoid the presence of pyrogens, which can cause inflammatory responses. Dry powders were characterised by electron microscopy revealing the shape and size distribution; nitrogen adsorption was giving information about the surface area and microporosity and X ray diffraction confirmed the amorphous or crystalline nature of the particles. The stock suspensions were characterized by dynamic light scattering (DLS) showing the stability of the particles in different media (water, phosphate buffered saline, cell culture media). The concentration of silica in the samples was determined by ICP-AES. Attenuated total reflectance infrared spectroscopy and ICP-MS (TOXI) showed the absence of any other molecules (or toxic agents) in the stock suspension, except water and SiO_2.

The surface reactivity (ability to form radicals from H_2O_2) was investigated by electron spin resonance. Since the OH° radical is very short-live d, a spin trap (DMPO) was added. The magnitude of the DMPO-OH° signal is a quantitative measure for the amount of radicals generated by the particles.

By changing synthesis parameters (concentrations of the reagents, silica source, catalyst, synthesis time and temperature), the production of amorphous silica can be tuned with an almost infinite variety of properties depending on the hypothesis to be tested.

During the first part of the programme several batches of amorphous silica were synthesized and/or prepared in liquid phase:

- 6 batches of Stöber silica NP with a varying diameter and to a lesser extent porosity. Particles with diameters (based on electron microscopy images) of 16, 19, 60, 74, 104 and 335 nm were synthesized.
- 3 batches of Stöber silica NP with a varying porosity (BET surface area of 279, 385 and 422 m²/g) and very small variation in diameters (based on TEM images) from 15 to 18 nm.
- 4 batches of Stöber silica NP with fluorescent labels (diameters from 20 – 40 nm)
- 4 Ludox® NP (diameter from 10 to 15 nm) were obtained from commercial source,
- 4 batches NP were synthesised through the hydrolysis of organic silicon in the presence of lysine (lysine silica NP) and very small particles with diameters down to 2 nm were obtained.

The Stöber procedure is carried out at room temperature and consists of an hydrolysis and a condensation of tetraethylorthosilicate (silicium source) in ethanol in the presence of water and ammonia to catalyze the reaction. This method yielded an array of NP with varying dimensions that were then prepared and stabilised specifically for use in cell culture tests. These monodisperse preparations were shown to be stable for several months. The stability of these stock suspensions was checked at weekly intervals by DLS measurements. Dilutions of these suspensions were obtained in cell culture medium (DMEM). Also in this medium the particles were found stable (no aggregation, no solubilisation) for at least 4 days, which is sufficient to carry out a cell culture experiment. The S²NANO consortium was therefore able to completely eliminate the issue of agglomeration/aggregation of NP which represents a significant difficulty for most investigators who generally use dry preparations that need to be dispersed in cell culture medium with some varying degree of success.

A manuscript summarising the synthesis and purification procedures as well as the full physico-chemical characterisation of these samples, suitable for toxicological studies, is submitted for publication in an international journal.

Additional Stöber NP with varying porosity (micropore volume of 4 to 43 µl/g) were also synthesised and formulated in appropriate conditions for *in vitro* testing of the influence of porosity on cytotoxicity.

Stöber NP were also labelled with a fluorescent probe and embedded in a silica shell in order to provide toxicologists with a tool to finely track the fate of NP in the cells. Commercial Ludox® particles with sodium counter ions were purified and characterized before distribution to the partners. Because of their small size (10 to 15 nm) and different synthesis method they were interesting test materials in the S²Nano project. Another synthesis procedure, where tetraethylorthosilicate reacted in an aqueous lysine solution, leaded to extremely small particles down to 2 nanometer. Bigger particles of 26, 34 and 36 nm were obtained by changing the reaction temperature and the concentrations of the reagents.

All these amorphous silica particles were fully characterized physico-chemically in order to explore the most relevant physico-chemical properties leading to toxic effects.

Finally, crystalline silica NP were synthesised in the nanometer dimensions. Synthesis conditions had to be modified to produce stable sols in the DMEM cell culture medium. Normal synthesis procedures yielded crystalline materials which immediately flocculated upon dilution in cell culture medium. Since the project goal is to test fully characterized and monodisperse particles, this aggregation did not fit with our primary aim. All these preparations were distributed to the partners and their physico-chemical characteristics were thoroughly discussed and commented (Table 1 in annex).

3.2 In vitro dosimetry issue

While COK was preparing the first NP, the attention of the network was attracted by a publication that appeared early in 2007 in Toxicological Sciences (Teeguarden *et al*. 95:300- 312). These authors suggested that, because of the virtual absence of impact of the gravitation force on NP in suspension, which is reflected by their ability to form stable suspensions in aqueous media, the major part of nanoparticles introduced in cell culture may never reach the target cells at the bottom of the culture well. If this was true, it would have implied that the nominal dose used in *in vitro* experiments may not reflect the biologically effective dose, and additional efforts to better deliver NP to the cells or to define the effective dose would have been needed.

The 4 laboratories involved in the S²NANO project decided to investigate this issue, in view of its potentially critical impact on the design and interpretation of the screening assays that were planned in the research programme. With a Stöber SNP prepared and characterised by COK (29 nm), a series of experiments was designed in the 3 toxicology laboratories (CEGE, LUNG and TOXI) each using a different cell line and at least one different cytotoxicity endpoint. The 3 teams came to remarkably concordant conclusions, i.e. that, contrary to the concern expressed by Teeguarden *et al*., the vast majority of NP in suspension in the culture medium contribute to the cytotoxic response, even if they do not sediment. This might be explained by convection forces that are almost always present in suspensions, allowing the particles to collide with target cells. A manuscript summarising these observations has been published in Toxicological Sciences (Lison *et al*. 2008).

Based on these results, it was concluded that there was no need to modify the *in vitro* experimental protocols that were originally proposed.

3.3 Cytotoxicty experiments

Before testing the NP prepared by COK for the different toxicity endpoints (genotoxicity, platelet and vascular functions, macrophage inflammatory mediators), cytotoxicity experiments were first designed to calibrate the dose range for the screening experiments and to address the appropriate expression of the NP dose (particle number, mass or surface area). Each laboratory used a different human cell line (A549 type II pneumocytes for CEGE, EAHY926 endothelial cells for LUNG and J774 macrophages for TOXI) and different cytotoxicity endpoints were applied (MTT, LDH release and MTT, and WST1, respectively). The reliability of the used cytotoxicity tests in the presence of silica NP was also carefully addressed; no major interference with the assay systems was found in the range of doses tested. The results of these cell viability assays agreed remarkably among the 3 laboratories, indicating that the cytotoxicity of monodisperse amorphous silica NP was strongly related to particle size, with the dose causing 50% cytotoxicity (TD_{50}) increasing with particle size. No clear difference was found between Stöber, Ludox or lysine NP. One Stöber NP appeared, however, as an outlier because it did only induce limited cytotoxicity in spite of its relatively small size. This was connected with the fact that this sample had a microporous surface, a characteristic that could be associated with low cytotoxicity. This observation was potentially very interesting and has been further investigated in J774 cells with the new ad hoc set of NP prepared to investigate this issue. The hypothesis of an influence of NP porosity on cytotoxicity could, however, not be confirmed when examining the bivariate relation between porosity and TD_{50}.

We have then examined in the different cell lines whether cytotoxicity is more influenced by particle mass, number or surface area. Again, we remarkably came to similar conclusions in the 3 laboratories: surface area appeared, beside mass, as a main determinant of the cytotoxicity of amorphous silica NP. In addition, macrophages (J774 cells) appeared more sensitive to SNP than epithelial and endothelial cell lines, suggesting that phagocytosis might be involved in the cytotoxic response. This hypothesis will be tested during the second phase of the programme.

Experiments performed by the LUNG group also indicated that, in EAHY926 endothelial cells, the cytotoxicity of silica NP appeared rapidly, especially with the smallest NP (after 30 min with 16 nm Stöber NP). The mechanism of cell death induced by silica NP seems to involve necrosis more than apoptosis in this cell line. A first manuscript summarising these observations is in press.

It was then realised that cytotoxicity is unlikely to be determined by a single physico-chemical parameter and multiparametric correlations studies were performed. It has been shown that the TD_{50} of the particles could be reasonably predicted ($r>0.60$) by an association of physicochemical parameters. For instance, the TD_{50} in J774 cells was influenced positively (i.e. more cytotoxic) by the surface area and negatively by the pore volume (i.e. pore volume protects against cytotoxicity). Quite remarkably, the association of parameters that predicted TD_{50} was not identical across all cell lines and cytotoxicity endpoints (in EAHY926 cells, TD_{50} was predicted by particle diameter, i.e. smaller diameter was associated with lower TD_{50}). This finding is not completely unexpected since it would be naïve to consider that a complex phenomenon such as cytotoxicity would be governed by a single parameter that would be identical for all cells. This may, however, have important implications for the toxicological testing of nanoparticles for which a “one size fits all” approach may not be appropriate. Before coming to this conclusion, it will be necessary to examine other cellular endpoints, including genotoxicity, inflammatory mediator release or platelet activation. This work is actively carried on during the second phase of the programme.

3.4 Genotoxicity

In order to explore the physico-chemical characteristics of SNP that would determine a possible genotoxic activity, an in vitro system for testing SNP was first developed in a relevant cell line (A549 epithelial cell line) in the CEGE laboratory. The genotoxic potential of SNP has been investigated with the micronucleus assay. The *in vitro* cytochalasin-B micronucleus (CBMN) test was selected because it allows the detection of two types of chromosomal damage, i.e.

clastogenic events or chromosome breaks and aneugenic events or chromosome loss. This assay is based on the addition of cytochalasin-B, an actin inhibitor, which blocks cytokinesis and allows discrimination between cells that have divided once or more (bi-or multinucleated cells) or did not divide (mononucleated cells) during the *in vitro* culture. The CBMN assay was applied in A549 cells treated with different concentrations of 3 types of Stöber silica nanoparticles (16, 60 and 101 nm SNPs). A statistically significant increase of micronuclei in binucleated cells was detected after treatment but only with the smallest SNPs, indicative of a size dependent genotoxic effect. However, results showed no dose-dependent effect.

When Fluorescence In Situ Hybridization (FISH) with pancentromeric probes is combined with the CBMN assay, the technology allows discrimination between chromosome breakage and chromosome loss. Results demonstrated that 16 nm SNPs predominantly induced chromosome loss. This evidence together with previous results that indicated the induction of mitotic slippage and mitotic block, suggested that interferences with the microtubules could be the mode of action through which this SNP exerts genotoxic effects.

To understand why no clear dose-effect relationship could be identified in these experiments, Si measurements were performed by ICP-MS (inductively coupled plasma mass spectrometry) to determine the cell-associated dose for the 3 types of SNPs (16, 60 and 101 nm), and this at 4 time points. In one kinetic experiment, ICP-MS measurements were performed after different treatment periods (15 min, 2h, 6h and 24h) with the same SNPs. Results indicated a rapid and dose-dependent increase in cell-associated Si (except for the 101 nm SNP). Furthermore results showed that at the 4 time points tested more 16 nm SNPs were taken up, suggesting that particle number is a major determinant for genotoxic effects induced by SNPs.

In a second experiment Si was measured synchronously with the fixation of the CBMN (40h). These data revealed that after 40 hours of treatment the Si content was similar or lower than at 24 hours, suggesting a role of exocytosis. Moreover the A549 cells were associated with more Si after treatment with 60 nm SNPs than after treatment with 16 nm SNPs. The least Si was found after treatment with 101 nm SNPs. When the 40 hours results were converted in terms of number of cell-associated particles or cell-associated surface area, an almost linear relationship between the fold induction of micronuclei and the cell-associated number of particles or cell-associated surface area was observed. This indicates that these parameters are determinants for genotoxic effects induced by SNPs.

As a conclusion it is suggested that the smallest SNPs have a higher genotoxic potential which results from interference with microtubule dynamics.

3.5 Coagulation and thrombosis (LUNG)

Platelet function was assessed by measuring closure times in the Platelet Function Analyser PFA-100, using cartridges coated with collagen/epinephrine. DMEM medium or DMEM medium containing NP was added to blood for 5 min. In this method, the closure time reflects platelet aggregate formation in a shear stress-dependent manner. These analyses did not reveal a significant shortening of closure times in blood supplemented with negatively charged monodisperse amorphous silica nanoparticles at a concentration up to 25 µg/ml. These results are not unexpected since it is already known that mainly positively (but not negatively) charged particles stimulate platelet function. Silica NP used so far were all negatively charged in the used medium.

The cellular models to assess translocation *in vitro* (primary type II pneumocytes and the human Calu-3 cell line grown on permeable membranes with 0.4-3 µm pores) and endothelial dysfunction (mixed endothelial, epithelial cells and macrophages co-culture) were developed and validated to be used in the second phase of the programme with the silica NP prepared by COK.

3.6 Macrophages and inflammation (TOXI)

The human macrophage model has been developed with U-937 cells differentiated with PMA. The dose of PMA, duration of differentiation, time points for the assessment of inflammatory markers (mainly TNF-a production) both at mRNA and protein levels have been optimised. The model will be applied during the second phase of the programme with NP particles prepared under very strict conditions to avoid endotoxin contamination. This assay is indeed very sensitive to endotoxin that may contaminate the samples, potentially leading to erroneous conclusions.

Considering the complex composition of airways and alveoli, examining the inflammatory response of one single cell type is most probably far from reality. The interactions of epithelial cells and macrophages, i.e. the cells with direct contact with deposited particles, with other important cell types such as endothelial cells, must be important but remain largely unknown. Models have therefore been developed to evaluation the effects of particles on the cytokines expression in single cultures of pneumocytes and macrophages; and then in co-cultures of pneumocytes and macrophages (bicultures); and finally the interaction of these bicultures with endothelial cells.

4. PRELIMINARY CONCLUSIONS

The scientific community, and toxicologists in particular, are currently facing the challenge of characterising the health risks associated with the production, uses and potential release of nanomaterials. Obtaining sound scientific bases to support the sustainable development and public acceptance of nanomaterials is indeed critical. The issue is, however, extremely complex and there is a need to develop and validate new methodologies. Efforts are made worldwide to address this issue, but it would be somewhat naïve to expect a simple and rapid answer. Even for data-rich nanomaterials such as TiO_2, a clear picture of the health risks is not yet available.

After 24 months of research, the S²NANO group has produced :

- stable monodisperse and sterile suspensions of SNP with selected physico-chemical characteristics, in a form suitable for toxicological investigations,
- *in vitro* toxicity tools and models to assess the impact of SNP on relevant health endpoints (genotoxicity, coagulation, vascular dysfunction and inflammation),
- the demonstration that the nominal dose introduced in an *in vitro* system remains an appropriate expression of the dose for toxicological studies with SNP.

These tools and data will allow to explore in the coming months the determinants of SNP toxicity. We have already found that particle surface area (not number) is determining the cytotoxic response. We also realised that cytotoxicity is determined by a multidimensional vector of physico-chemical parameters. It remains to examine whether metric applies to other more subtle cellular read-outs of genotoxicity, vascular dysfunction and inflammation.

Thus, we confirmed so far that, in the nanometer range, the smallest particles are the most toxic and that, for insoluble silica nanoparticles, a combination of physico-chemical parameters determine their cytotoxic activity.

While it is, at this stage, not yet possible to formulate recommendations that would be directly useful for industrials, bridging and translating our research data into information and/or guidelines remains the final objective. The expertise build in the frame of the first phase of the S²NANO programme will also be useful to provide guidelines or protocols to test new nanomaterials. The review of genotoxicity studies conducted by L. Gonzalez *et al.* (2008) included recommendations for a minimal set of quality criteria for (geno)toxicology studies and is a first step in this direction.

5. FOLLOW-UP COMMITTEE

The follow-up committee comprises members from academia, industry, regulatory bodies and administrations. Most members of the follow-up committee attended the 3 meetings and expressed their interest for the programme. The partners to the S²NANO project want to thank the members of the follow-up committee for their help, support, interest and valuable suggestions during this first phase.

During the first meeting (July 2007) the aims and objective of the programme together with the respective competencies and responsibilities of the partners were mainly presented with relatively limited exchanges with the follow-up committee.

Very fruitful exchanges took then place during the second (December 2007), third (June 2008) and fourth (October 2008) meetings, several members provided useful comments and recommendations for the conduct of the research.

This final report was submitted to the Committee for advice, and the following comment were received :

"the report …. shows that all the participants have worked hard and that contribution from all groups were vital to achieve good results. The preparation of the particles tested clearly required cute approach, an extremely long and difficult laboratory work which in the present case appears quite successful. The Thomassen et al. paper describes well this important part of the study.. The results obtained in different laboratories with different approaches and cell lines confirm the requirement of collective effort in such a complex system."

"The systematic, multidisciplinary approach will definitely contribute to a better understanding of the determinants of toxicity of nanoparticles. The success of the project is reflected by the number of publications and presentations."

Yseult Navez	Ministère de la Communauté française
Linda Wouters	FOD Werkgelegenheid, Arbeid en Sociaal Overleg
Bice Fubini	University of Torino - Italy
Bert Swennen	Umicore sa
Peter Blomme	Bekaert n.v. – Bekaert Technology Center
Daniel Marzin	Université Pasteur Lille - France
Janos B. Nagy	Facultés universitaires N-D de la Paix
Philippe Martin	CEC
Bruno Schmit	Solvay sa
Peter Priem	Solvay sa

6. PUBLICATIONS / VALORISATION

6.1 Peer reviewed publications (attached in annex page 18)

All publications are co-publications of the whole S²NANO consortium (COK, CEGE, LUNG and TOXI)

D. LISON, L. C. J. THOMASSEN, V. RABOLLI, L. GONZALEZ, D. NAPIERSKA, J. W. SEO, M. KIRSCH-VOLDERS, P. HOET, C.E. A. KIRSCHHOCK, J. A. MARTENS. Nominal and effective dosimetry of silica nanoparticles in cytotoxicity assays. Toxicol Sci (2008)104:155-62.

D. NAPIERSKA, L. THOMASSEN, V. RABOLLI, D. LISON, L. GONZALEZ, M. KIRSCH-VOLDERS, J. MARTENS, and P. HOET. Size-dependent cytotoxicity of monodisperse silica nanoparticles in human endothelial cells.
Small (in press)

L. GONZALEZ, D. LISON and M. KIRSCH-VOLDERS1 Genotoxicity of engineered nanomaterials: a critical review.
Nanotoxicology 2:252-273

L.THOMASSEN, V. RABOLLI, D. LISON, L. GONZALEZ, M. KIRSCH-VOLDERS, D. NAPIERSKA, P.H. HOET, C.E.A. KIRSCHHOCK, AND J.A. MARTENS. Stable Silica Nanoparticle Dispersions in Cell Culture Media for Toxicity Testing.
Submitted

6.2 Other activities

Presentations at scientific meetings :

- L. Thomassen. Poster presentation : “Tailor-made nanosilica for toxicity testing” at the international conference ‘Nanoparticles for European Industries II’, London, 24 and 25 October 2007

- L. Gonzalez. Poster presentation at the annual meeting of Beltox (Belgian Society for Toxicology and Ecotoxicology) on 28 November 2007, Namur

- D. Lison. Oral presentation "Nanoparticles Dosimetry for *in vitro* toxicity assays" at the annual meeting of Beltox (Belgian Society for Toxicology and Ecotoxicology) on 28 November 2007, Namur

- L. Gonzalez. Invited oral presentation : Dosimetry, toxicity and genotoxic effects of Stöber silica nanoparticles in A549 human lung carcinoma cells. International Conference on Nanomaterial Toxicology (Icontox 2008) 5-7 February 2008, Lucknow, India

- L. Gonzalez. Poster presentation “Evaluation of dosimetry, toxicity and genotoxic effects of Stöber silica nanoparticles in A549 human lung carcinoma cells” at the SETAC Europe 18th Annual Meeting. 25-29 May 2008, Warsaw, Poland.

- D. Napierska, L. Thomassen, L. Gonzalez, Virginie Rabolli, D. Lison, B. Nemery, P.H.M. Hoet. Poster presentation: " Size dependent cytotoxicity of nanosized monodisperse Stöber silica particles in human endothelium cells". International Conference of American Thoracic Society, Toronto, 16-21 May 2008

- D. Napierska. Poster presentation : Concentration- and size-dependent cytotoxicity of nanosized monodisperse silica particles. 11th International Inhalation Symposium "Benefits and Risks of Inhaled Engineered Nanoparticles", Hannover, 11-14 June 2008

- M. Kirsch-Volders. Invited oral presentation : Effets mutagènes des nanoparticules. Congrès de la Société française de pharmaco-toxicologie cellulaire. Paris 29-30 mai 2008.

- D. Lison. Invited oral presentation : Dosimétrie des nanoparticules dans les tests *in vitro* : dose nominale ou dose effective ? Congrès de la Société française de pharmacotoxicologie cellulaire. Paris 29-30 mai 2008.

- L. Gonzalez. Poster presentation : Genotoxicity of engineered nanomaterials: a critical review. 38th Annual Meeting of the European Environmental Mutagen Society. Cavtat, Croatia 21-25 september 2008

6.3 Support to the decision

Partners of the S²NANO programme contributed to several national and international taskforces involved in nanotoxicology :

ECETOC Task force on nano(geno)toxicology Symposium at the European Environmental Mutagen Society (EEMS) meeting in Basel, August 2007 (M. Kirsch-Volders)

ECETOC Task force for the coming nano(geno)toxicology Symposium at the 1Oth International Conference on Environmental Mutagens (ICEM) meeting in Firenze, August-September 2009, (M. Kirsch-Volders)

nanoBE, the Federal Belgian platform about nanomaterials (P. Hoet, M. Kirsch-Volders, L. Gonzalez)

saisine AFSSET « Evaluation des risques des Nanotubes de Carbone » (D. Lison)

ANNEX 1

Table 1 : Handover S²Nano samples, names and characteristics

date	sample	sample name	type	structure	concentration (mg/ml)	DLS hydrodynamic diameter in DMEM (nm)**	EM diameter (nm)	EM calculated external SA*** (m²/g)	BET surface area (m²/g)	external surface area alphas (m²/g)	micropore volume alphas (µl/g)
20070425	S1	*	Stober silica	amorphous	3,69	38,09	29,3	102			
20070904	S2	S-16	Stober silica	amorphous	3,24	25	16,4	183	220	183	22
20070904	S3	S-19	Stober silica	amorphous	2,54	26	19,4	155	139	145	34
20070904	S4	S-60	Stober silica	amorphous	4,63	75	60,4	50	42,1	33	0
20070904	S5	S-74	Stober silica	amorphous	44,66	104	74,5	40	167	57	71
20070904	S6	S-104	Stober silica	amorphous	5,13	139	104	29	41,2	28	2
20070904	S7	S-335	Stober silica	amorphous	3,96	566	335	9	16,4	8	3
20070904	HS	L-15	Ludox	amorphous	124,36	22	14,7	204	255	179	0
20070904	LS	L-14	Ludox	amorphous	134,39	22	13,8	217	275	196	0
20070904	SM	L-10*	Ludox	amorphous	167,82	63	10,3	291	343	250	0
20070213	SM2	L-11	Ludox	amorphous	44,48	21	11,0	273	241	179	0
20070213	F1	Lys-2	Lysine silica	amorphous	11,12	12	2,1	1429	325	232	2
20070213	F2	Lys-26	Lysine silica	amorphous	2,09	28	25,7	117	141	121	0
20070213	F3	Lys-34	Lysine silica	amorphous	5,79	38	33,6	89	80	71	0
20070213	F4	Lys-36	Lysine silica	amorphous	10,59	40	35,7	84	89,4	73	0
20080526	S10	S-25	Stober silica	amorphous	1,09	30,39	18,1	166	279	206	3
20080526	S11	S-22	Stober silica	amorphous	0,84	33,28	15,1	199	385	244	33
20080526	S12	S-28	Stober silica	amorphous	3,32	36,61	16,7	179	422	261	40

* ~90 nm aggregates

** : @ day 1

*** : perfectly round sphere