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

S²NANO

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FINAL REPORT

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

S²NANO

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SUMMARY

Sustainable development of nanotechnologies requires the simultaneous anticipation of both potential opportunities and concerns, but there is still much that is not known about these. Opportunities are the possibility to solve or reduce significant economic, social and environmental challenges. Concerns include potential environmental and health risks. While some nanomaterials may be used safely, others uses may cause harm to people or the environment. Intelligent and robust ways to anticipate and balance these opportunities and concerns are needed.

The possible adverse health effects of nanomaterials represent a serious cause of concern that may contribute to limit or hamper an adequate economical development of nanotechnologies. The capacity of robustly assessing these health hazards is a serious challenge for regulators and scientists. The production of sound scientific data will, therefore, support the development of sustainable nanoproducts and may contribute to provide industrials and regulators with evidence-based guidelines for the production, assessment and control of safer materials. In line with the concept of sustainable development, the acceptance of a new product, indeed, depends on the capacity of the industrials to integrate (eco)toxicological implications early in the development of nanoproducts. It is, therefore, of great socio-economical value to anticipate (eco)toxicological issues (anticipatory governance). At EU-level, particularly in the frame of REACH, and at USA-EPA level, recommendations are urgently needed to adequately adapt, modify or replace the existing test methodologies or protocols for hazard assessment of nanomaterials.

The objective of the S²NANO program was to understand how nanomaterials exert toxic effects and to identify the physico-chemical determinants of their toxicity.

The S²NANO program was run by a consortium of 4 academic laboratories with strong and complementary expertises to meet these objectives :

- the laboratory of prof. J. Martens (COK, KULeuven) is specialized in the synthesis and characterization of nanomaterials, especially silica-based assemblies
- the research group of prof. P. Hoet (LUNG, KULeuven) is leading research on the mechanisms of the pulmonary toxicity of chemicals, including particles and nanoparticles, using in vitro and in vivo methods
- the team of prof. M. Kirsch-Volders (CEGE, VUB) has developed expertise and validated methods to assess the genotoxic effects of chemicals and particles using cytology and molecular approaches
- the coordination of the consortium was provided by the laboratory of prof. D. Lison (TOXI, UCL) who has extended experience in studying the toxicity of inhaled particles, including metals and carbon nanotubes.

Silica-based nanoparticles (SNPs) were selected as experimental model because they can be produced with an almost infinite variety of properties. An additional advantage of using SNP was that silicon/silica can be measured quite easily and with a sufficient sensitivity in biological tissues, which allowed tracing the biodistribution of SNP in cells and tissues. Finally, a number of SNP are used for industrial applications as fillers or binders to control the viscosity of tooth paste, and certain food products as well as to improve wear resistance of, for example car tyres, which made SNP industrially relevant candidates for toxicological studies.

The S²NANO project has contributed to :

- develop a Belgian's research community through a unique and fruitful inter-disciplinary collaboration,
- advance basic science,
- develop new methods and tools,
- better understand and evaluate the potential health risks of nanoparticles and associated uncertainties.

The environmental impact of nanomaterials was deliberately not covered.
The contributions of the S²NANO consortium were significant both in terms of publications (11 peer-reviewed articles) and invitations to workshops.

The main achievement of the S²NANO project is the building of an excellent and fruitful interdisciplinary network of investigators. A real dialogue has been established between physico-chemists and biologists. This issue is really crucial when conducting nanotoxicological studies for which a detailed physico-chemical characterisation of the material is essential. This unique collaboration has allowed developing an original approach of NP toxicology, which can finely examine the role of a single characteristic of NP separately. This design 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.

The S²NANO project has provided a number of original scientific results that directly contribute to understand how nanomaterials exert toxic effects and to identify the physico-chemical determinants of their toxicity. The main scientific achievements of the S²NANO project include :

1. a unique set of SNP has been specifically designed and prepared for (geno)toxicology studies,
2. adapted methodologies have been developed to assess the hazard of nanoparticles,
3. tools were also developed and applied to investigate the mechanism(s) by which nanoparticles interact with cells and tissues (cell uptake, oxidative stress, cell proteins and genome).

Application of these tools and methodologies by the S²NANO project allowed to draw a number of conclusions :

1. SNP are a useful model to study and decipher the physico-chemical determinants of the toxicity of low solubility nanoparticles.
2. zeolite nanoparticles have a low cytotoxic potential.
3. the nominal concentration in culture medium is a useful descriptor of the dose for SNP in in vitro assays.
4. size and surface area are important determinants of the cytotoxic activity of SNP.
5. microporosity is an additional parameter which appears to lower the cytotoxic activity of SNP in macrophages.
6. the aggregation of SNP does not modify the cytotoxic activity.
7. oxidative stress is not the main mechanism of (geno)toxicity for silica nanoparticles.
8. the cytokinesis block micronucleus assay can be adapted to assess the genotoxic activity of SNP.

These results 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 size.

Thus, BELSPO, through its research programme "Science for a Sustainable Development" (2005-2009) - SSD has provided a strong impulse to build a unique interdisciplinary expertise in nanotoxicology in Belgium. This consortium is now scientifically mature and has produced solid scientific data, contributed to train young scientists in the field of nanotoxicology, and participated in national and international regulatory committees. The expertise of the S²NANO consortium is competitive internationally.

1. INTRODUCTION

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. According to ISO, a nano-object is a material with one, two or three dimensions in the nanoscale (roughly 1 – 100 nm). This general term includes nanoparticles (all three external nanodimensions), nanoplates (only one external nanodimension) and nanofibres (nano-objects with two similar nanodimensions, and a third dimension that is significantly larger) (ISO TS 27 687, 2008). However, the scientific and political communities have increasingly come to understand that a definition in terms of dimensions may not be sufficient and that a number of other factors determine not only the novel properties of a material, but also their potential to cause harm. A recent European report considers that no scientific data are available to indicate that a specific size associated with special properties due to the nanoscale can be identified for nanomaterials in general and that there is no scientific evidence in favor of a single upper size limit SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks, 2011).

Nanomaterials possess outstanding properties, such as unique quantum size effect, large surface area and catalytic properties, which allow the development of a variety of innovating industrial applications. They are already impacting on virtually all types of industrial and domestic products and their production and marketing will increase sharply in the coming years. Nanomaterials are already being used in many objects, including window coatings, sunscreens and other cosmetics, textiles, paint, cutting boards, socks, etc. They may also be found in Organic Light Emitting Diodes (OLEDs) for displays and ear implants, to name a few. The nanomaterials are being incorporated into new generations of drug-delivery vehicles, contrast agents, and diagnostic devices, some of which are currently undergoing clinical investigation or have been approved by the Food and Drug Administration (FDA) for use in humansKim, B. Y. et al. (2010). The most commonly used nanomaterials in consumer products are nanosilver, carbon nanotubes, nanosized metal oxides (titanium dioxide, zinc oxide), silica and gold. Other engineered nanomaterials used in consumer, medical and industrial products include additional forms of nanocarbon, cerium oxide, nickel, aluminium oxide and the nanoclays, copper oxide, iron oxide and quantum dots. A recent survey conducted on the European market identified 475 products as containing nanomaterials in 2010, while only 151 products were recorded in 2009 in a similar survey (BEUC 2010). The need for a regulatory framework for the marketing of nanomaterials and their traceability has been recently addressed at the European level during the Belgian presidency (2010).

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 generally 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 public at large, may be exposed to these materials, and an assessment of their toxic potential (hazard) as well as of their entire life cycle is required.

The technological developments in the field of nanomaterials have, indeed, largely out-paced research efforts to assess their possible health and environmental risks. While efforts have largely been directed at developing applications of nanomaterials, their implications have been ignored to a large extent. This emerging challenge has generally been recognised by governments, regional and international regulatory agencies and scientists, and several research programs are currently funded to examine the (eco)toxicity of nanomaterials.

The existing scientific data raise concerns about the safety of nanomaterials and harmful effects have already been reported. The main potential target organs include the respiratory tract, the brain, the cardio-vascular system, the skin, and the liver. It is generally agreed that the same properties that make nanomaterials so attractive for technological developments and applications, i.e. their small size and their high reactivity, are sources of concern for adverse heath effects. The nanoscale size of these objects indeed implies that they can theoretically distribute to the smallest biological structure, access and interact with fine cellular and molecular targets. The toxic activity of nanomaterials may also significantly differ from that of bulk material with the same chemical composition because of their greater surface reactivity. Typically, the biological activity of particles increases as the particle size decreases (Hoet, P. H. et al. , 2004)

Assessing the toxic potential of low-solubility nanomaterials presents, however, several challenges, including the difficulty of designing proper experimental procedures to reliably test their toxic potential, the necessity to reconsider the definition of the dose (the mass dose does not seem to be the most appropriate for solid toxicants in general and for nanomaterials specifically), the need to develop exposure metrics relevant for nanomaterials (e.g. measuring particle surface area rather than mass concentration) and the importance of monitoring the uptake. An additional challenge is to address the myriad of different nanomaterials that are designed every day (for instance more than 12 different forms of zinc oxide exist in the nanorange). It will therefore be virtually impossible to test each and every nanomaterial for its toxic potential, and a generic assessment scheme is needed. This approach is, however, only possible if common properties that drive toxicity can be identified.

While we already have some hints of the physico-chemical properties of nanomaterials which determine their toxicity, these are mainly based on empirical observations and knowledge remains largely fragmentary. It is generally admitted that the biological reactivity of nanomaterials probably 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 dose in contact with the biological systems. Thus, the physico-chemical properties of the particulate surface are likely to play an important role in the biological effects. We may, however, expect significant differences in the extent and mechanism 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 in these studies were often insufficiently characterized.
The gap between the rate of innovation in nanotechnologies and our capacity to assess the human and environmental implications of these innovations also implies a number of societal challenges and addresses the need for some anticipatory governance (Philbrick, M. , 2010).

The objective of the S²NANO program was to understand how nanomaterials exert toxic effects and to identify the physico-chemical determinants of their toxicity.

This approach contributes
- to better understand the mechanisms influencing the interactions of nanomaterials with the cell and tissues,
- 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 was expected, through this research effort, to improve the scientific bases for the development of sustainable nanoproducts and to provide industrials and regulators with some 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 indeed depends 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 focused on human health effects and did not address environmental impacts of nanomaterials.

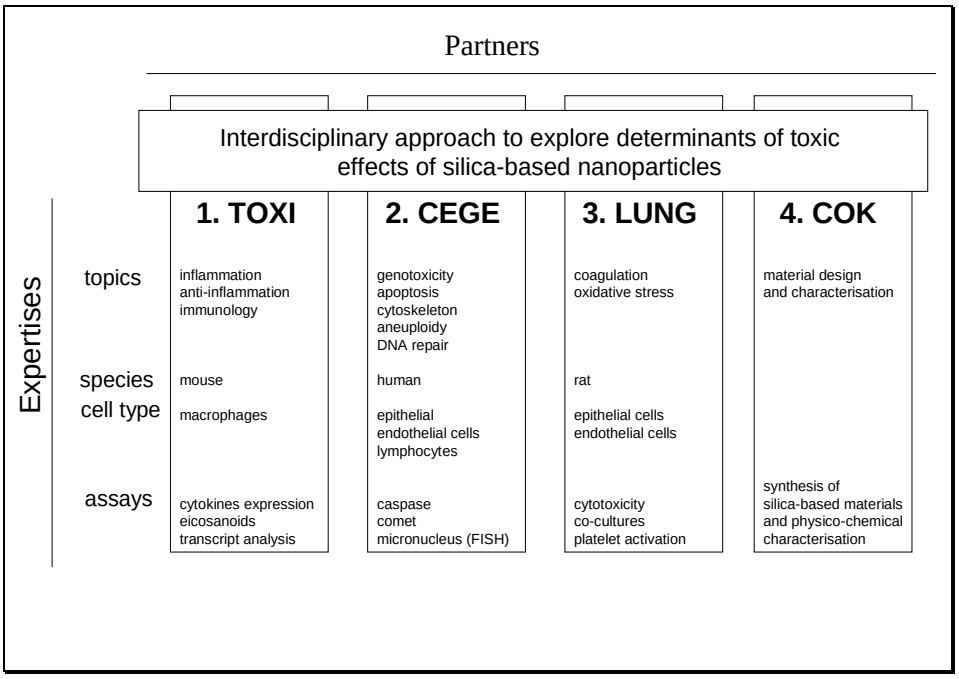
The S²NANO program was run by a consortium of 4 academic laboratories with strong and complementary expertises to meet these objectives. The laboratory of prof. J. Martens (COK, KULeuven) is specialized in the synthesis and characterization of nanomaterials, especially silica-based assemblies. The research group of prof. P. Hoet (LUNG, KULeuven) is leading research on the mechanisms of the pulmonary toxicity of chemicals, including particles and nanoparticles, using in vitro and in vivo methods. The team of prof. M. Kirsch-Volders (CEGE, VUB) has developed expertise and validated methods to assess the genotoxic effects of chemicals and particles using cytology and molecular approaches. The coordination of the consortium was provided by the laboratory of prof. D. Lison (TOXI, UCL) who has more than 15-year experience in studying the toxicity of inhaled particles, including metals and carbon nanotubes.

2. METHODOLOGY AND RESULTS

2.1. Experimental strategy

Given the wide range of disciplines and analytical techniques associated with nanotechnology, a multidisciplinary collaboration between synthesis, analysis and toxicology, offering state-of-the-art capabilities, was needed to achieve the goals of S²NANO. The program was run by 4 leading research groups in Belgium, combining strong physico-chemical and toxicological expertises (Figure 1). The network has worked in a truly collaborative manner to develop a common understanding and to tune the synthesis and production of NP according to the needs and contraintts of the investigators performing biological tests (amounts needed, purity, sterility, fluorescent labelling, …).

FIGURE 1



2.2. Nanoparticle preparation and characterization.

Silica-based nanoparticles are a useful model to explore physico-chemical determinants of toxicity

To identify physico-chemical determinants of toxicity in the nanosize we decided to work with a model nanomaterial that can be easily engineered and tailored to selectively vary one single physico-chemical property at the time. We choose silica-based nanoparticles (SNPs) that can be produced with an almost infinite variety of properties as experimental model. An additional advantage of using SNP was that silicon/silica can be measured quite easily and with a sufficient sensitivity in biological tissues, which allowed tracing the biodistribution of SNP in cells and tissues.

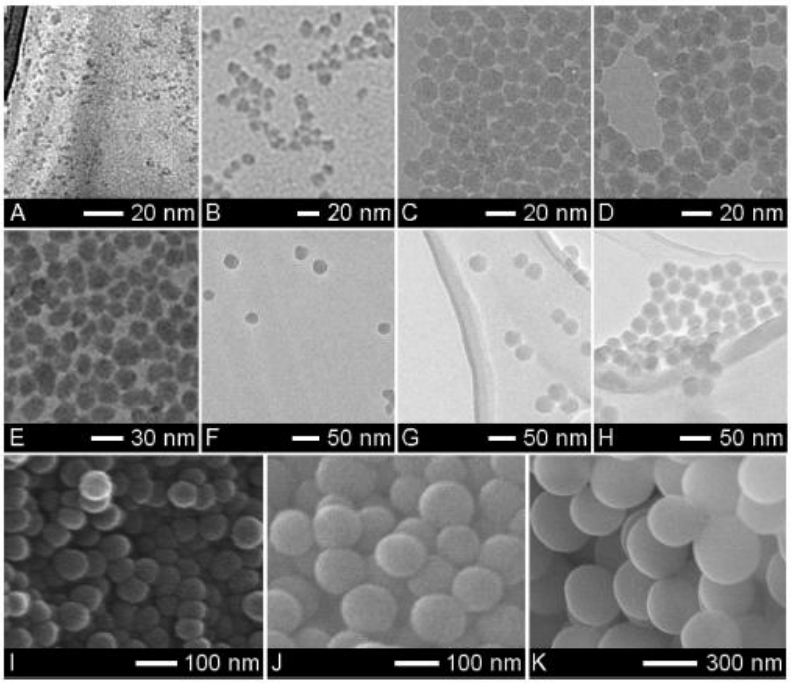
Finally, a number of SNP are used for industrial applications as fillers or binders to control the viscosity of tooth paste, and certain food products as well as to improve wear resistance of, for example car tyres, which made SNP industrially relevant candidates for toxicological studies.

The idea was to design model nanoparticles based on silica nanostructures to explore systematically and rationally the determinants of nanoscale particle toxicity. 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, often commercially available, with uncertain characteristics and with a combination of several physico-chemical variations from which it is often difficult to decipher the critical determinants of toxicity.

Different silica nanoparticles have been prepared and the nanoparticle properties tuned to address experimentally several research questions.

A very significant asset of the S²NANO project has been the fruitful dialogue between physico-chemists and toxicologists. The COK partner successfully prepared stable monodisperse suspensions (sols) of SNP in the nanosize range. These sols could be tuned with a variety of properties depending on the research questions to be tested. This yielded an array of NP with varying dimensions (Figure 2) that were then prepared and stabilised specifically for use in cell culture tests.

FIGURE 2 : Some examples of SNP prepared by S²NANO



This preparation was achieved through extensive dialysis under sterile conditions against purified endotoxin-free water. The monodisperse preparations were shown to be stable, in the absence of any dispersant, for several months in water, and at least for several days when diluted in DMEM culture medium.

This unique collection of tailored made SNP is original and innovative in the field of nanotoxicology and constitutes a significant achievement of the program. With these tools, the S²NANO consortium was, therefore, able to completely eliminate or control the issues of endotoxin contamination and agglomeration/aggregation of NP which represent significant challenges for most investigators who generally use dry preparations that need to be dispersed in cell culture medium with some varying degree of success.

TABLE I : Variety of silica nanoparticles prepared by the S²NANO consortium

Amorphous silica nanoparticles

- Variation in size : 2-335 nm
- Variation in synthesis procedure :
  - Applied catalyst: Stöber versus Lysine SNP
  - Applied silica source: Ludox (sodium silicate) versus Stöber and Lysine (Tetraethylorthosilicate)
- Variation in porosity : Stöber particles (0-100 µl/g)
- Role of oxidative stress in cytotoxicity : Stöber particles doped with iron
- Tracing cellular fate of SNP : Stöber particles labeled with fluorescein isothiocyanate
- Influence of aggregation on cytotoxicity : controlled aggregates of Ludox SNP with increasing aggregate size and/or decreasing amount of primary particles and small aggregates

Crystalline zeolite nanoparticles

- Variation in crystal structure: nanozeolite Y and A with FAU and LTA topology, respectively.

The S²NANO SNP were fully characterized physico-chemically.

A careful physico-chemical characterization of the material used is essential in every toxicological studies and even more in nanotoxicology Powers, K. W. et al. (2006). This need was especially crucial for the S²NANO program since its objective was to identify the physico-chemical parameters that determine the toxicity of a nanomaterial.

The physico-chemical characteristics of the S²NANO SNP were verified and documented by electron microscopy, nitrogen sorption, X ray diffraction and dynamic light scattering (DLS). The purity of these preparations was assessed by ICP-AES.

All these preparations were distributed to the partners and their physico-chemical characteristics were thoroughly discussed and commented. This constant dialogue between physico-chemists, biologists and toxicologists was a very significant asset of the S²NANO program.

TABLE II : Methods used to characterize the S²NANO SNP

Standard techniques applied on all SNP :

- Quantification: inductively coupled plasma- atomic emission spectrometry (ICP-AES)
- Sizing, morphology : Transmission/Scanning Electron Microscopy (TEM/SEM)
- Sizing in suspension and stability : Dynamic Light Scattering (DLS)
- Surface charge : Zeta potential analysis
- Surface area and micropore analysis : Nitrogen Sorption analysis
- Crystallinity : X-Ray Diffraction (XRD)
-

Techniques applied to answer specific research questions

- Elemental analysis to detect the presence of trace elements and contaminants: inductively coupled plasma-mass spectrometry (ICP-MS), X-Ray Fluorescence (XRF)
- (Surface) Silanol characterization : Infrared spectroscopy
- Surface reactivity : production of hydroxyl radicals from hydrogen peroxide: Spin trapping with DMPO and Electron Spin Resonance spectroscopy
- Sizing and intensity measurement of fluorescent SNP : Fluorescence correlation spectroscopy (FCS)
- Fluorescent intensity and fluorescent decay of fluorescent SNP: Confocal fluorescence microscopy

The list of all SNP prepared during the S²NANO program and their main physico-chemical characteristics are summarized in Table III.

The overall strategy and methods for the design, purification, preparation and characterisation of these SNP is described in details in a publication in Langmuir (IF 2009 : 3.898) (Thomassen, L. C. et al., 2010). This paper has already been cited 6 times (December 2010).

Table III : S²NANO silica nanoparticles

Sample	Timing	Type	Concentration (mg/ml)	Hydrodynamic diameter in DMEM, DLS (nm)	Particle diameter, EM (nm)	BET surface area (m^2/g)	External surface area alphas (m^2/g)	Micropore volume alphas (µl/g)	Zeta potentials in KCl (1 mM)	Zeta potential in DMEM
S1	2007	Stober silica	3.69	38.09	29.3	NA	NA	NA	NA	NA
S2	2007	Stober silica	3.24	25	16.4	220	183	22	-67	-13
S3	2007	Stober silica	2.54	26	19.4	139	145	34	-8	-7
S4	2007	Stober silica	4.63	75	60.4	42.1	33	0	-24	-13
S5	2007	Stober silica	44.66	104	74.5	167	57	71	-34	1
S6	2007	Stober silica	5.13	139	104	41.2	28	2	-23	4
S7	2007	Stober silica	3.96	566	335	16.4	8	3	-40	-15
HS	2007	Ludox	124.36	22	14.7	255	179	0	-7	-10
LS	2007	Ludox	134.39	22	13.8	275	196	0	-31	-6
SM*	2007	Ludox	167.82	63	10.3	343	250	0	-30	-2
SM2	2007	Ludox	44.48	21	11.0	241	179	0	-23	-3
F1	2007	Lysine silica	11.12	12	2.1	325	232	2	-8	-3
F2	2007	Lysine silica	2.09	28	25.7	141	121	0	-2	-8
F3	2007	Lysine silica	5.79	38	33.6	80	71	0	-27	-4
F4	2007	Lysine silica	10.59	40	35.7	89.4	73	0	-19	-9
S10	2008	Stober silica	1.09	30	18.1	279	206	3	-7	-5
S11	2008	Stober silica	0.84	33	15.1	385	244	33	-4	-3
S12	2008	Stober silica	3.32	37	16.7	422	261	40	-13	2
C3L1	2008	Fluorescent stober silica	2.74	33	NA	233	167	7	-2	2
C3L2	2008	Fluorescent stober silica	1.12	24	NA	258	199	0	-2	-8
C3L3	2008	Fluorescent stober silica	1.02	33	NA	136	112	0	-12	-6
C3L4	2008	Fluorescent stober silica	4.38	42	NA	156	96	15	-31	-10
B10d	2009	Silicalite-1	68.14	85	60.3	112	80	3	NA	NA
B10c	2009	Silicalite-1	-	1550	56.9	417	113	136	NA	NA
S13	2009	Stober silica	2.87	28	NA	259	185	8	NA	NA
S14	2009	Stober silica	3.70	59	NA	85	59	3	NA	NA
S15	2009	Stober silica	4.51	174	NA	12	9	0	NA	NA
SMagg0	2009	Ludox	90.92	22	NA	301	206	0	NA	NA
SMagg1	2009	Ludox	9.38	37	NA	298	220	1	NA	NA
SMagg2	2009	Ludox	7.59	56	NA	285	230	2	NA	NA
SMagg3	2009	Ludox	6.87	134	NA	289	227	3	NA	NA
SFe1	2010	Iron doped Stober silica	2.2	24.6	NA	392	254	28	NA	NA
SFe2	2010	Iron doped Stober silica	2.8	41	NA	125	80	11	NA	NA
SFe3	2010	Iron doped Stober silica	2.5	76.4	NA	112	76	6	NA	NA
SFe4	2010	Iron doped Stober silica	1.2	126	NA	71	48	4	NA	NA
SMagg8	2010	Ludox	10.17	183	NA	331	249	0	NA	NA
SMagg10	2010	Ludox	4.67	182	NA	314	257	1	NA	NA
SMagg11	2010	Ludox	2.33	188	NA	283	233	0	NA	NA
SMagg10sup	2010	Ludox	2.61	46	NA	294	230	0	NA	NA
C3S110	2010	Fluorescent stober silica	10.8	78	NA	NA	NA	NA	NA	NA
C3S210	2010	Fluorescent stober silica	12.7	168	NA	NA	NA	NA	NA	NA
C3S310	2010	Fluorescent stober silica	10.1	60	NA	NA	NA	NA	NA	NA
Zeolite Y	2010	Zeolite Y	9.8	140	25-50	375	NA	NA	-40	-16
Zeolite A	2010	Zeolite A	7.3	159	45-100	241	NA	NA	-45	-23

* primary particles and ~90 nm aggregates, NA: not available

2.3. Toxicology studies

The toxicological studies were first conducted *in vitro* with cellular models relevant for the health effects of concern upon inhalation exposure, namely macrophages, pulmonary epithelial and endothelial cell as well as platelets. The macrophage is a key player in orchestrating particle clearance, inflammation, fibrosis and indirect genotoxicity Geiser, M. (2010). Epithelial and mesothelial cells are important targets for genotoxicity and carcinogenesis Knaapen, A. M. et al. (2004). Endothelial cell and platelet activation, aggregation and coagulation are central to the cardio-vascular effects associated with inhalation exposure to particles (Nemmar, A. et al., 2002). These cellular models were developed to examine the physico-chemical parameters that may be critical for one, some or all these toxicity endpoints.

In a second stage, specific issues were addressed *in vivo* and *in vitro* to explore the cellular and biochemical mediators involved in the organ responses.

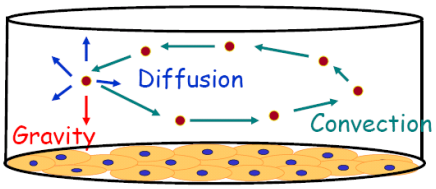
This approach is consistent with the recommendations of an international panel of experts convened by the ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group(Oberdorster, G. et al. , 2005) .

The in vitro cytotoxic activity of SNP is related to their nominal dose in the culture medium.

The attention of the S²NANO investigators was attracted by a publication that appeared in Toxicological Sciences (Teeguarden, J. G. et al., 2007) while the COK laboratory was preparing the first SNP. These authors suggested that, because of the virtual absence of impact of the gravitation force on SNP 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 an *in vitro* experiments, i.e. the dose introduced in the assay (expressed in µg/ml of culture medium or µg/cm² of culture well), may not reflect the biologically effective dose, and additional efforts to better deliver SNP to the cells would have been needed. The SNP developed by the COK laboratory offered an ideal model to test this hypothesis by measuring the amount of silica/silicon that reached the cells in parallel with a measurement of the cytotoxic response.

The 4 laboratories involved in the S²NANO project decided, therefore, to investigate this issue, in view of its potentially critical impact on the design and interpretation of the assays that were planned in the research program. With a Stöber SNP prepared and characterized by COK (29 nm), a series of experiments were 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 SNP in suspension in the culture medium contributed to the cytotoxic response, even if they did not sediment. This might be explained by convection forces that are almost always present in suspensions, allowing the particles to collide with target cells (Figure 2). Based on these results, it was concluded that there was no need to modify the *in vitro* experimental protocols that were originally proposed.

FIGURE 2 : Different forces responsible for nanoparticle movements in cell culture

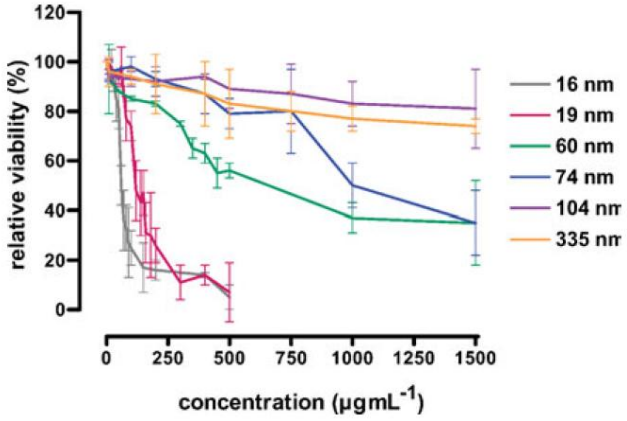


A manuscript summarising these observations has been published in Toxicological Sciences (IF 2009 : 4.814) (Lison, D. et al., 2008); and this paper was already cited 22 times in December 2010.

SNP size and surface area are important determinants of the cytotoxic activity.

Before investigating the different toxicity endpoints (inflammation, genotoxicity), cytotoxicity experiments were first designed in absence of serum to address the appropriate expression of the SNP dose (particle number, mass or surface area) and to calibrate the dose range for further experiments. Using the first SNP prepared by the COK laboratory, a detailed study of the dose-effect relationships was first conducted on endothelial cells with spherical SNP of different sizes (Stöber SNP 16, 19, 60, 74, 104, 335 nm, commercial Ludox SNP 14 and 15 nm). It was demonstrated that the cytotoxicity of monodisperse amorphous SNP with the same morphology was strongly related to particle size (Figure 3). Smaller particles showed significantly higher toxicity than the bigger ones when dose was expressed in mass concentration (µg/ml of culture medium). The surface area of tested SNP was an important parameter determining their toxicity. Experiments performed by the LUNG group also indicated that, in endothelial cells, the cytotoxicity of silica NP appeared rapidly, especially with the smallest SNP (after 30 min with 16 nm Stöber SNP). The mechanism of cell death induced by SNP seemed to involve necrosis more than apoptosis in this cell line. The results of this study have been published in Small (IF 2009 : 6.171) (Napierska, D. et al., 2009) and this paper has already been cited 31 times (December 2010).

FIGURE 3 : Cytotoxic response of endothelial cells to SNP (Napierska et al., 2009)



The determinants of SNP cytotoxicity are multiple and vary with cell type

Further studies were then programmed with a larger set of SNP (17 SNP with diameters ranging from 2 to 335 nm) on different cell types. Each laboratory used a different human cell line (CEGE, EAHY926 endothelial cells for LUNG, J774 macrophages and 3T3 fibroblasts for TOXI) and different cytotoxicity endpoints were applied (MTT, LDH release and MTT, and WST1, respectively). The reliability of these cytotoxicity assays in the presence of SNP 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 SNP was strongly related to particle size with TC_{50} values 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 associated 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 a new set of SNP specifically prepared by COK to investigate this issue. The results indicated a protective effect of microporosity in J774 macrophages.

Using multiple regression analysis, we found that the response to these SNP is governed by different physico-chemical parameters which vary with cell type : in J774 macrophages, the cytotoxic activity (WST1 assay) increased with external surface area (αs method) and decreased with micropore volume (r^2 of the model, 0.797); in EAhy926 endothelial and 3T3 fibroblast cells, the cytotoxic activity of the SNP (MTT and WST1 assay, respectively) increased with surface roughness and small diameter (r^2, 0.740 and 0.872, respectively); in erythrocytes, the hemolytic activity increased with the diameter of the SNP (r^2, 0.860). This study showed that it is possible to model with good accuracy the in vitro cytotoxic potential of SNP on the basis of their physicochemical characteristics. It also contributed to enlarge the view of the toxicologists on SNP surface area, indicating that behind the usual measurement of surface area (general assessed by N_2 adsorption with the BET method) other more subtle parameters, including micropores (Figure 4), are involved and deserve further investigations. The results of this study have been published in Nanotoxicology (IF 2009 : 5.744) (Rabolli, V. et al. , 2010). This publication has already been cited 22 times (December 2010).

FIGURE 4 : SNP surface area is more than just surface

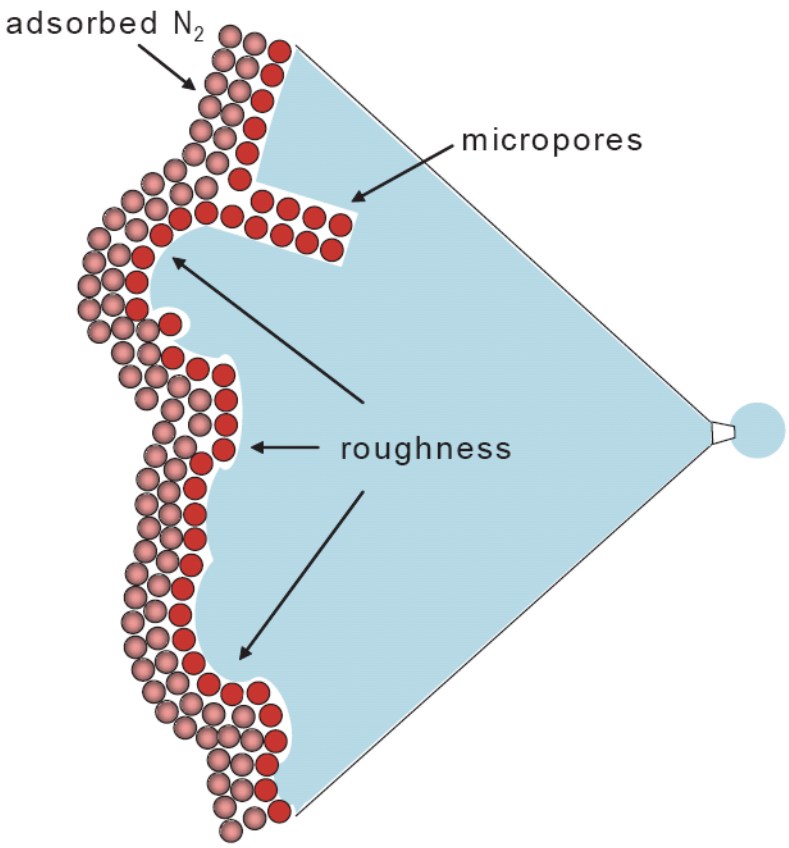


TABLE IV : Multiple regression analyses of SNP cytotoxicity (Rabolli et al., 2010).

J774 macrophages (WST1)	**Independent variables**	**Regression coefficient (SE)**	**P-value**	**Partial r²**
	External surface area (m^2/g)	-0.180 (0.0437)	0.0002	0.439
	Micropore volume (µl/g)	0.668 (0.1381)	0.0001	0.358

r^2 of the model : 0.797
ED50 (µg/ml) = 40.930 - 0,180 * external surface area + 0,668 * micropore volume

EAHY926 endothelial cells (MTT)	**Independent variables**	**Regression coefficient (SE)**	**P-value**	**Partial r²**
	Diameter (nm)	4.260 (1.295)	0.0064	0.656
	External surface area (m^2/g)	-3.072 (1.263)	0.0316	0.084

r^2 of the model : 0.740
ED50 (µg/ml) = 557,550 + 4.260 * diameter - 3.072 * external surface area

3T3 fibroblasts (MTT)	**Independent variables**	**Regression coefficient (SE)**	**P-value**	**Partial r²**
	External surface area (m^2/g)	-0.191 (0.0515)	0.010	0.805
	Diameter (nm)	0.149 (0.0372)	0.007	0.067

r^2 of the model : 0.872
ED50 (µg/ml) = 46.390 - 0.191 * external surface area + 0.149 * diameter

A special attention was paid to the hemolytic assay which has been reported as reflecting the in vivo lung responses to inhaled particles Lu, S. et al. (2009). This assay is quite simple to perform and measures the capacity of the tested material to damage cell membranes of human red blood cells as targets. In this assay, we found, opposite to other endpoints, that smaller SNP were relatively ineffective, only larger particles (diameter >100 nm) being able to damage red blood cells. We found that the hemolytic activity of silica particles (>100 nm) is mediated by the density of surface hydroxyl radicals (Rabolli V. *et al., 2010).*

The cytotoxic activity of SNP is not modified upon aggregation.

The S²NANO consortium had originally decided to work with stable monodisperse SNP to specifically address the effect of single nanoparticles, and not aggregates or agglomerates. However, in real life, humans are rarely exposed to single monodisperse nanoparticles, as aggregates appear rapidly when nanoparticles are dispersed in air or in water (Tsuji, J. S. et al., 2006).We, therefore, designed additional experiments to address the influence of SNP aggregation on the toxic response. To this end, stabilized non-porous SNP aggregates (with increasing size but similar surface area) were prepared and characterized by the COK laboratory and tested in the J774 macrophage and red blood cell models. It was found that the cytotoxic response of macrophages did not vary with the degree of aggregation. This finding was consistent with our previous results, namely that in macrophages the cytotoxic response to non-porous SNP is mainly driven by surface area, which did not vary here. The results are summarized in a manuscript submitted for publication (Rabolli et al., 2011).

In red blood cells, the response was more difficult to interpret because we found that the membranolytic activity was reduced upon aggregation, a finding which appeared in contrast with our previous conclusion that larger particles are more hemolytic than smaller ones. Further studies conducted in collaboration with the group of prof. B. Fubini (University of Torino) have shown that the reduction of the hemolytic activity was related to the consumption of free surface silanol functions upon aggregation of the SNP. These results are summarized in a manuscript which is currently submitted for publication (Thomassen et al., 2011a).

The cytotoxic activity of SNP is not mediated by oxidative stress.

Oxidative stress appears as the quasi universal mechanism proposed to account for the toxicity of nanoparticles (Xia, T. et al., 2006) although this opinion has been recently questioned (Donaldson, K. et al., 2009). A great deal of effort was spent during the S²NANO program to examine the possible role of oxidative stress in the cytotoxic activity of the SNP investigated. Using a battery of acellular assays, we were not able to demonstrate any production of free radicals by the SNP, except for Fe-doped SNP. In cellular systems (macrophages, endothelial and epithelial cells), using an array of biomarkers reflecting the occurrence of oxidative stress (malonedialdehyde, glutathione, heme oxygenase 1 induction) we concluded that, in the different cell types used, oxidative stress is not the main mechanism of toxicity. However, exposure of the cells to SNP containing iron resulted in a significant increase in oxidative stress markers.These results are summarized in a manuscript in preparation (Napierska et al., 2011).

SNP alter endothelial cell adhesion properties.

An additional study was undertaken to examine the effect of SNP of different sizes on endothelial cell function, in the absence or presence of a previously established *in vitro* human airway model consisting of triple cell co-cultures.

An immortalized human endothelial cell line (EA.hy926) and primary human pulmonary artery endothelial cells (hPAEC) were seeded in inserts (apical compartment) which were introduced or not above triple cell co-cultures in the baso-lateral compartment (pneumocytes (A549), macrophages (THP-1), and mast cells (HMC-1)). Endothelial cells (EC) were incubated with SNP (28, 59 and 174 nm). Exposure of EA.hy926 cells to 28 and 59 nm particles at non-toxic concentrations induced a significant increase of U937 monocyte adhesion and decrease in endothelin-1 secretion (up to 2-fold). Both adhesion and ET-secretion were increased when EC exposure to SNP was performed in the presence of triple cell co-cultures. Exposure to all three SNP induced expression of intercellular adhesion molecule-1 (ICAM-1) but no vascular cell adhesion molecule-1 (VCAM-1) in the EA.hy926 cells, and both ICAM-1 and VCAM-1 in hPAEC cultures.

These results suggest that exposure of human endothelial cells to amorphous SNP influences adhesive properties of the studied cells, which may have an impact in terms of cardio-vascular events and/or diseases. . These results are summarized in a manuscript in preparation (Napierska et al., 2011).

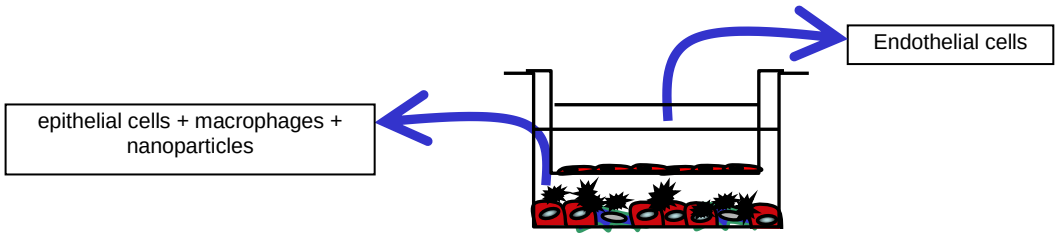
Zeolite nanoparticles have a low cytotoxic activity.

Beside amorphous SNP, efforts were also done to produce crystalline nanoparticles of silica and compare their response to that of the amorphous SNP investigated.

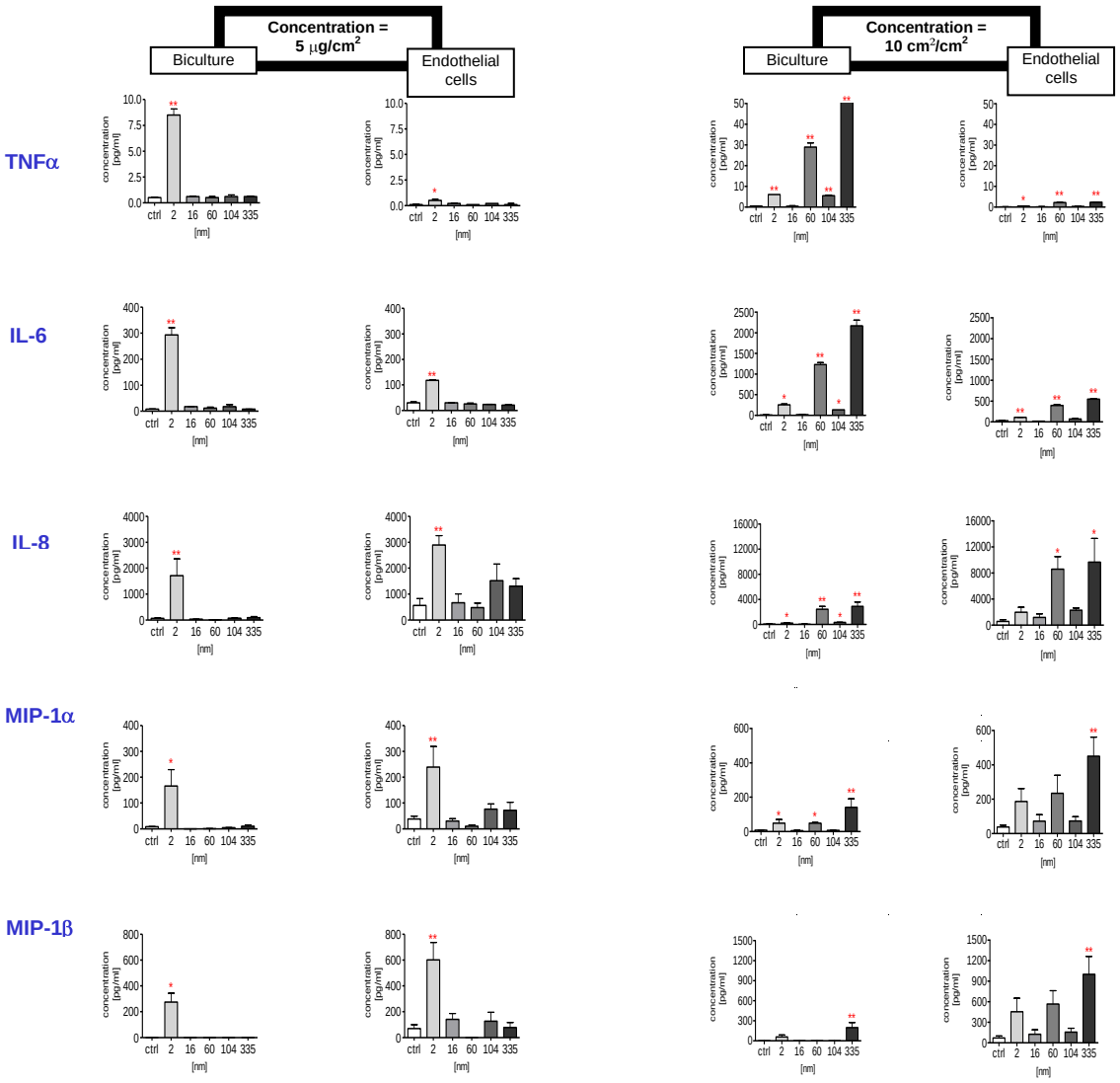
Nanozeolites Y and A with particle sizes of 25-100 nm and an adequate colloidal stability for in vitro cytotoxicity experiments were synthesized and characterized. The cytotoxic response of macrophages, epithelial and endothelial cells to these nanoparticles was assessed by determining mitochondrial activity (MTT assay) and cell membrane integrity (LDH leakage assay) and compared to the response of an amorphous SNP of similar size. After 24 h of exposure, no significant cytotoxic activity was detected up to 500 µg zeolite/ml, indicating that, despite their large specific surface area, these materials are of low toxicity compared to other nanosilicas of similar size. The results of these experiments have been summarized in a manuscript which is currently under revision (Thomassen et al., 2011b).

Alternative co-culture models.

We also tested the influence of SNP size on the cytokines expression *in vitro* using co-cultures of pulmonary epithelial cells with macrophages and endothelial cells in a two compartment system. Particles were incubated with biculture (epithelial cells and macrophages) and supernatants were recovered from both compartments. Quantification using the FACS array system showed significant increase for TNFα, IL-6, IL-8, MIP-1α for the 2 nm SNP in all conditions tested. The larger particles did not induce cytokine expression at 5 µg/cm^2, but at dosing 10 cm^2 particle surface area/cm^2 (and thus a large mass) also the larger particles induced a response. This work showed again the role of size and surface area in response to SNP.



	Concentration (mass) 5 µg/cm2	**Concentration (surface area, SA) 10 cm2/cm2**
diameter [nm]	SA [cm2/cm2]	mass [µg/cm2]
2	15.5	3.3
16	8.5	5.8
60	2	24.8
104	1.5	33.4
335	0.5	136.6



2.4. Genotoxicology studies.

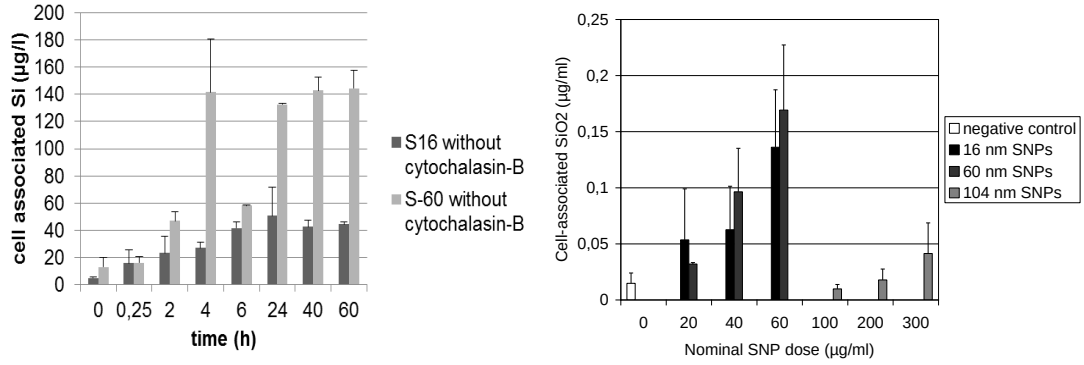
In view of the capacity of nanomaterials to access almost every cellular structure including the cytoskeleton, the nucleus and the genome, associated with their assumed great reactivity, the potential mutagenic/carcinogenic activity of nanomaterials is a source of great concern. Thus assessing the genotoxic activity of nanomaterials is an important step in the characterization of their carcinogenic potential. The project focused on A549 human lung epithelial cells, as an in vitro model for lung genotoxicity

Uptake of SNPs by lung epithelial cells

Since the targets of mutagenic events are essentially intracellular, the potential genotoxic activity of insoluble nanparticles is likely related to their uptake in the chosen cell line. The intracellular fate of SNP has been investigated, in collaboration with the laboratory of prof E. Cundari (CNR Rome), in A549 cells with fluorescent SNP prepared by the COK laboratory.

A qualitative analysis, using fluorescent SNPs of 60 nm and 168 nm SNPs, showed cellular uptake of both SNPs by A549 cells in presence and absence of serum. A decreased or slower SNP uptake was observed in presence of serum. Preliminary results indicate that the route of SNP uptake is through clathrin-dependent endocytosis. Quantitative analysis by ICP-MS using 16, 60 and 104 nm SNPs showed a dose-dependent increase of cell-associated SNPs as well as a time-dependent increase reaching a plateau after 6-24 hours.

FIGURE 5: Time and dose dependency of Si uptake without cytochalasin-B



Assessing the genotoxic potential of nanomaterials

When the S²NANO program was launched, several investigators had already attempted to assess the genotoxic potential of nanomaterials, but these early studies were generally incomplete and their results appeared inconsistent. The group led by prof. M. Kirsch-Volders decided, therefore, to reconsider the potential cellular targets of nanomaterials and to conduct a review of the existing literature to systematically and critically assess these studies against a framework of quality criteria.

No general conclusion could be drawn concerning the genotoxic activity of nanomaterials, essentially because of the limited number of data, incomplete physico-chemical characterization of materials examined and shortcomings in experimental approaches. This evaluation revealed gaps that should be considered in future studies (e.g., one-sided approach focusing mainly on ROS as mode of action and neglecting their aneugenic potential) and the need to develop adequate positive controls for genotoxicity assays when conducted with nanomaterials. This study was published in Nanotoxicology (IF 2009 5.744) (Gonzalez, L. et al., 2008) and has already been cited 14 times.

The cytokinesis-block micronucleus assay can be adapted to assess the genotoxic potential of nanomaterials.

There exist several tests to assess the genotoxic activity of a chemical and each of them has its own merits and drawbacks. In the S²NANO program, the option was taken to mainly investigate the usefulness of the cytokinesis-block micronucleus (MN) test to assess the genotoxic potential of nanomaterials using SNP as a model. There were several good reasons for focusing on the MN test, including (1) the fact that this test assesses stable mutations that are predictive of an increased cancer risk as demonstrated in human biomonitoring studies, (2) the capacity of this assay to capture several modes of genotoxicity (clastogenic and aneugenic), (3) the recent validation of the assay at OECD level, (4) the possibility to automate the reading of this assay, and (5) the great expertise of the laboratory of prof. M. Kirsch-Volders in the development and validation of this assay Decordier, I. et al. (2006).

While standardized protocols are available for the execution of the MN test with soluble chemicals, these needed to be assessed and validated for solid materials, especially in the nanosize range. The first efforts of the S²NANO program were, therefore, devoted to investigate the possible applicability of the MN assay to SNP, trying to examine the methodological adaptations needed to adapt the test successfully. The main findings were that :

1. treatment with SNP in the absence of serum leads to higher levels of cytotoxicity and cellular uptake,
2. the use of cytochalasin-B generally lowers the uptake of SNPs in A549 cells,

These results led to the publication of recommendations for the in vitro testing of nanomaterials with the MN asssay (Gonzalez, L. et al., 2011) in Mutagenesis (IF 2009 3.541) and Gonzalez et al. in Journal of Biomedical Nanotoxicolgy (2011a; submitted)

SNP display a weak genotoxic response in the in vitro MN assay.

In a second step, the MN assay (manual reading) in presence of serum (as recommended by OECD) and including our modifications was applied to assess the genotoxic response of lung epithelial cells to a set of 3 SNP with increasing size (16 , 60 and 104 nm). The results indicated that SNP induced a weak non-statistically significant genotoxic response with the smallest SNP being apparently more active.

When considering MN data of the three SNPs together a statistically significant positive correlation between the fold MN frequency and particle number or total surface area, but not mass dose. The best descriptors of this genotoxic response were the particle number or the surface area, and using either the nominal or the cell-associated dose of SNP did not modify these relationships. These results have been published by Gonzalez, L. et al. (2010b) in Nanotoxicology (IF 2009 : 5.744).

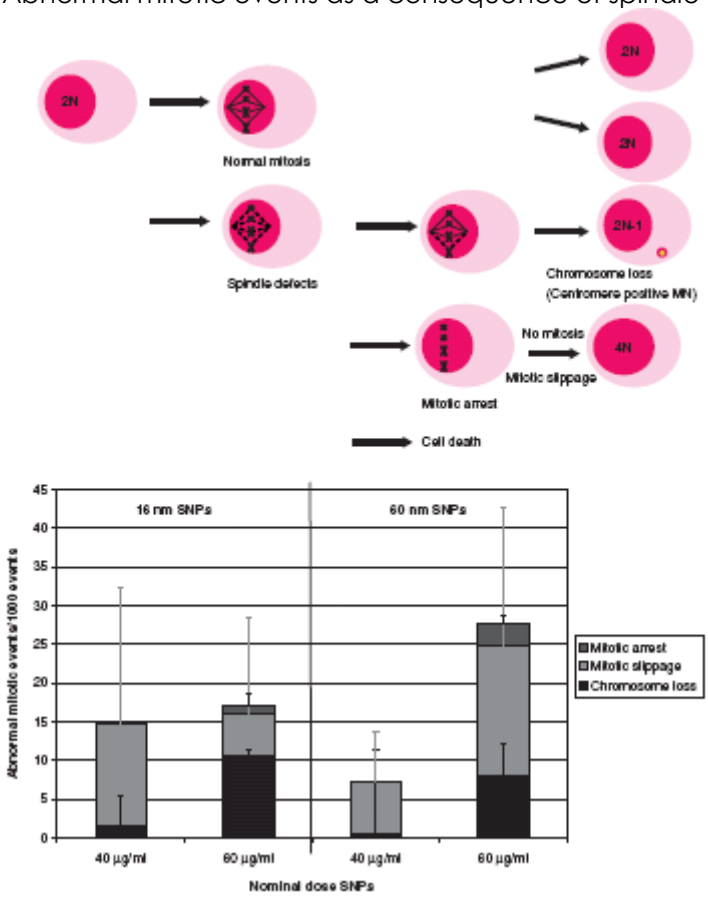
Then, the laboratory decided to address the influence of serum proteins on the in in vitro genotoxicity of SNPs. In collaboration with investigators at Novartis (Dr Elhajouji), the response of a large range of SNPs (12-174 nm) in the MN test was also investigated with a FACS analysis method on lung epithelial cells in presence and absence of serum. These investigations confirmed a higher toxicity in absence of serum and a weak but in some cases statistically significant induction of MN by the SNPs. The results of these experiments have been summarized in a manuscript which is currently in preparation (Gonzalez et al., 2011d).

Induction of apoptosis by SNPs

In collaboration with investigators at Novartis (Dr Elhajouji), the induction of apoptosis in lung epithelial cells by the same range of SNPs (12-174 nm) as studied in the flow MN assay was investigated with a FACS analysis method; these investigations confirmed a higher induction of apoptosis in absence of serum and found that smaller SNPs size show a higher induction of apoptosis at the same mass dose. The results of these experiments have been summarized in a manuscript which is currently in preparation (Gonzalez et al., 2011d).

Exploring the mechanism of the possible genotoxic activity of SNPs

FIGURE 6: Abnormal mitotic events as a consequence of spindle defects



In a first review paper (Gonzalez, L., Lison, D., and Kirsch-Volders, M. 2008), it was suggested that besides ROS production aneugenic events might play a major in the induction of genotoxicity by nanomaterials. Therefore both modes of action were studied. Induction of oxidative stress was assessed with the alkaline Comet assay with and without fpg and FISH probing with pancentromeric probes. Little evidence for the participation of oxidative stress was found. The aneugenic potential of SNPs was investigated with 2 approaches:

1) FISH probing with pancentromeric probes, mitotic slippage and mitotic arrest. The results iindicate a weak prevalence of effects such as chromosome malsegregation and modulation of progression through mitosis was also observed, but without reaching statistical significance (Figure 5).

The results of these experiments have been published by Gonzalez, L., Lison, D., and Kirsch-Volders, M. (2008) in Nanotoxicology (IF 2009 5.744) and compared with similar studies by Gonzalez, L. et al. (2010a) in Biochemical Society Transactions (IF 2009 3.378)

(2) effects on microtubule depolymerisation and repolymerisation of a range of SNP. A stronger repolymerisation (after cold treatment) of tubulin was recorded after treatment with 28, 59 and 174 nm SNPs compared to untreated controls. This is still further investigated in collaboration with the laboratory of prof. E. Cundari (CNR, Rome). The results of these experiments have been summarized in a manuscript which is currently in preparation (Gonzalez et al., 2011c).

Assessing the in vivo genotoxicity of SNP.

Inhaled particles, and probably also nanoparticles, may induce a genotoxic response in respiratory epithelial cells by a primary genotoxic activity (the particles are also genotoxic *in vitro*) and/or a secondary mechanism resulting from the production of reactive radicals by recruited leucocytes when these particles are inflammogenic (Schins, R. P. F. et al., 2007).

Preliminary experiments have been conducted to assess the capacity of 2 sizes of SNP (28 and 59 nm) to induce a genotoxic response in rat epithelial cells, using an *in vivo* protocol applied successfully by us in the past with hard metal particles De Boeck, M. et al. (2003) and carbon nanotubes (Muller, J. et al., 2008). This issue requires further investigations to secure a proper positive control material and to elicit a robust inflammatory response to SNP in the rat lung.

2.5. Platelet activation.

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 SNP 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. Most SNP used in the S²NANO program are negatively charged in the used medium.

2.6. Macrophages and inflammation

The capacity of SNP to induce the production of pro-inflammatory cytokines by leucocytes was investigated with PMA-differentiated U937 cells and mouse peritoneal macrophages. A particular attention was paid to the capacity of SNP to activate the NALP-3 inflammasome, in view of recent publications indicating that micrometric crystalline silica particles were strong activators of this pathway. The first results indicated that nanometric silica nanoparticles did not activate the NALP-3 inflammasome as virtually no IL-1β was detected upon incubation with the SNP. These studies require, however, further investigations because it was realized that SNP strong bind IL-1β and might obscure a real activation of the inflammasome pathway.

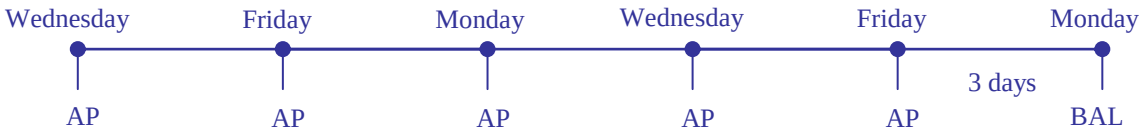
2.7. In vivo experiments.

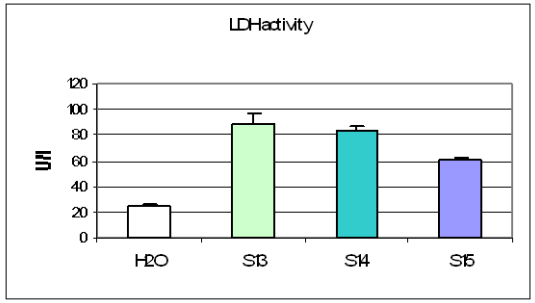
The initial program of the S²NANO project included an *in vivo* toxicological assessment of the SNP lung toxicity.

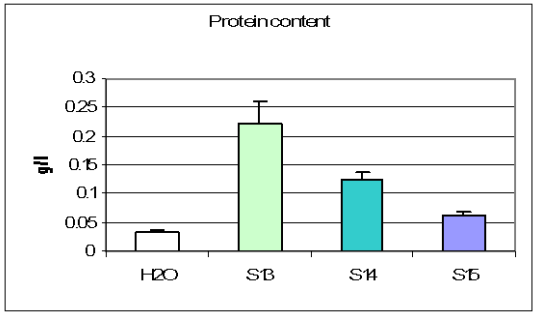
Extensive efforts have been made to obtain endotoxin-free preparations of SNP (dialysis under endotoxin-free conditions). A limited series of experiments have been conducted both in rats (see genotoxicity) and in mice using intratracheal instillation or pharyngeal aspiration to deliver these SNP to the lungs. In mice, the SNP (S13-S15; 16 ,60 and 180 nm, respectively) instilled in aqueous suspension at the maximal concentration available) did not induce much inflammation as assessed by broncho-alveolar lavage 3 days after a single administration. In another series of experiments using repeated administrations (5 aspirations over a week), some limited signs of toxicity (elevation of LDH activity, protein content and inflammatory cells) were recorded with the smallest SNP (Figure 7).

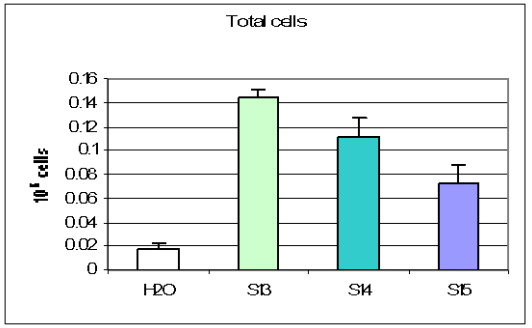
Similar results were obtained in the peritoneal cavity of the mouse (not shown).
These investigations need to be completed by assessing the elimination kinetics of SNP in the lungs.

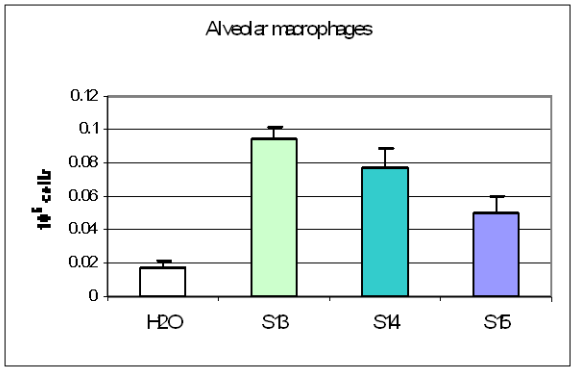
FIGURE 7 : IN VIVO LUNG RESPONSE TO SNP IN C57B6/BL MICE AFTER 5 INSTILATIONS

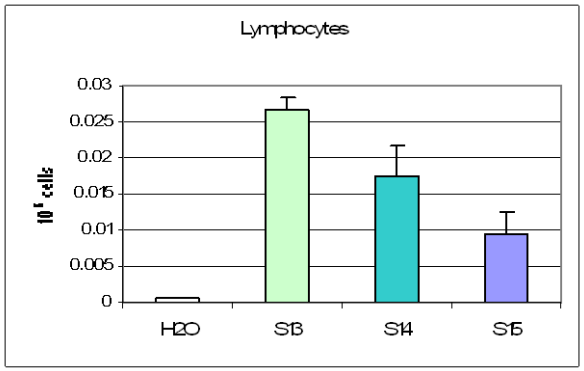


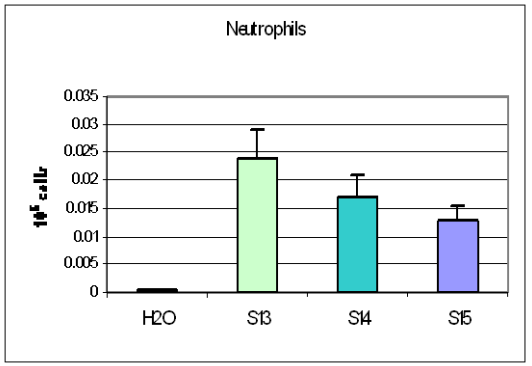












2.8. Applications to industrial nanoparticles.

The findings of the S²NANO program are valid for monodisperse silica nanoparticles and should be verified with other nanomaterials. An industrial member of the follow-up committee provided the consortium with 2 samples of nanomaterials to be tested for their genotoxic activity with the tools developed by the laboratory of prof. M Kirsch-Volders during this programme.

2.9 Conclusions and perspectives

The main achievement of the S²NANO project is the building of an excellent and fruitful interdisciplinary network of investigators. A real dialogue has been established between physico-chemists and biologists. For the former, this project was a challenge to target synthesis of materials according to the constraints of (geno)toxicology assays (monodisoperse suspension, sterility, endotoxin-free, fluorescent labeling). A constant dialogue was established among the investigators and the experimental tools were adapted from this feed-back. Biologists greatly benefited from the discussions with chemists and understand now 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.

This unique collaboration in the network has allowed to develop a really original approach of NP toxicology, which can finely examine the role of a single 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.

The S²NANO project has produced a number of original scientific results that directly contribute to "understand how nanomaterials exert toxic effects and to identify the physico-chemical determinants of their toxicity". The main scientific achievements of the S²NANO project include :

1. a unique set of silica-based nanoparticles has been specifically designed and prepared for (geno)toxicology studies,
2. adapted methodologies have been developed to assess the hazard of nanoparticles,
3. tools were also developed and applied to investigate the mechanism(s) by which nanoparticles interact with cells and tissues (cell uptake, oxidative stress, cell proteins and genome).

The application of these tools and methodologies by the S²NANO project allowed to draw a number of conclusions :

1. silica-based nanoparticles are a useful model to study and decipher the physico-chemical determinants of the toxicity of low solubility nanoparticles.
2. zeolite nanoparticles have a low cytotoxic potential.
3. the nominal concentration in culture medium is a useful descriptor of the dose for SNP in in vitro assays.
4. size and surface area are important determinants of the cytotoxic activity of SNP.
5. microporosity is an additional parameter which appears to lower the cytotoxic activity SNP in macrophages.
6. the aggregation of SNP does not modify the cytotoxic activity.

7. oxidative stress is not the main mechanism of (geno)toxicity for SNP.
8. the cytokinesis block micronucleus assay can be adapted to assess the genotoxic activity of SNP.

These results 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 size.

As for any research project, however, important issues are still open and further work is needed to better understand the mechanisms of nanoparticle toxicity which were already addressed, to some extent, by the S²NANO project :

1. can the results obtained with SNP be extrapolated to other low-solubility nanoparticles ?
2. how are nanoparticles taken up by/in cells ?
3. how do nanoparticles alter cell cycle control ?
4. how do (extracellular) proteins affect the toxicity of nanoparticles ?

An important effort is also needed to address the in vivo (geno)toxicity of nanoparticles and to examine how it relates to in vitro toxicity.

Thus, BELSPO, through its research programme "Science for a Sustainable Development" (2005-2009) - SSD has provided a strong impulse to build a unique interdisciplinary expertise in nanotoxicology in Belgium. This consortium is now scientifically mature and has produced solid scientific data, contributed to train young scientists in the field of nanotoxicology, and participated in national and international regulatory committees. The expertise of the S²NANO consortium is competitive internationally.

3. POLICY SUPPORT

Responsible development of nanotechnologies requires the anticipation of both potential opportunities and concerns. There is still much that is not known about the potential benefits (applications) and risks (implications) of nanotechnologies and sustainable development requires an early understanding of both. Opportunities are the possibility to solve or reduce significant economic, social and environmental challenges. Concerns include potential environmental and health risks. While some nanomaterials may be used safely, others uses may cause harm to people or the environment. Intelligent and robust ways to anticipate and balance these opportunities and concerns are needed.
The possible adverse health effects of nanomaterials represent a serious cause of concern that may contribute to limit or hamper an adequate economical development of nanotechnologies. The capacity of robustly assessing these health hazards is a serious challenge for regulators and scientists.
The production of sound scientific data supports the development of sustainable nanoproducts and may contribute to provide industrials and regulators with evidence-based guidelines for the production, assessment and control of safer materials. The acceptance of a new product, indeed, depends on the capacity of the industrials to integrate (eco)toxicological implications early in the development of nanoproducts. It is, therefore, of great socio-economical value to anticipate (eco)toxicological issues (anticipatory governance) as discussed by Philbrick, M. (2010).
The S²NANO project has contributed to this aim; it has contributed to :

- develop the Belgian's research community through a unique and fruitful inter-disciplinary collaboration,
- advance basic science,
- develop new methods and tools,
- better understand and evaluate the potential health risks of nanoparticles and associated uncertainties.

The environmental impact of nanomaterials was deliberately not covered.
At EU-level, in particular in the frame of REACH, and at USA-EPA level, recommendations are urgently needed to adequately adapt, modify or replace the existing test methodologies or protocols for hazard assessment of nanomaterials. The S²NANO contributions were significant both in terms of publication and invitations to workshops.

A recent high level event "Towards a regulatory framework for nanomaterials' traceability", was held in Brussels (14 September 2010) during the Belgian presidency of the Council of the European Union. This conference brought together representatives of various associations (consumers, environmental protection, workers, industrial federations), scientists, regulatory experts as well as national and European regulatory bodies, in order to address this emerging issue. The objectives and results of the S²NANO program are completely in line with the recommendations put forward after this conference (2010b), and more specifically with the recommendation "to take up responsibilities at the Member States level" and "to consider research in toxicology and ecotoxicology of nanomaterials, as well as their fate along the whole lifecycle as a high priority".

4. DISSEMINATION AND VALORISATION

The outputs and outcomes of the S²NANO program have been widely disseminated in the scientific and regulatory communities through conferences, communications, reviews and publications.

The S²NANO program was also a unique opportunity for a multidisiciplinary training of young researchers in the field of nanotoxicology, which leads to the presentation of

- 3 PhD in 2011 : Leen Thomassen and Dorota Napierska at the KULeuven and Laetitia Gonzalez at the VUB.
- 5 master thesis : Catherine Princen (UCL),), Nele Lievens (KULeuven) , Tombeur (KULeuven), Despina Papoutsi (VUB) and David Triest (VUB).

The expertise developed during the S²NANO program allowed 3 laboratories of the consortium join a FP7 funded research project dealing with the assessment of the health effects of engineered nanomaterials (ENPRA) which assembles 23 leading European research groups.
Several research groups have shown their interest in the S²NANO projects and its unique set of silica-based nanoparticles after reading publications or attending presentations at international conferences. Contacts were established in particular with the laboratory of spectrochemistry and catalysis (ENSICAEN, France), BioMedimplat (Hannover, Germany), Institute of Physics (Muenster, Germany), Chemistry and Biochemistry department of the University of California Santa Barbara (USA).

Presentations at scientific meetings :

Oral presentations

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

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

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

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

D. Lison. Nanotechnologies : How worried should the occupational physician be ? Journées nationales de medicine du travail, Charleroi, October 2008.

D. Lison. Toxicology of Nanomaterials : Silica nanoparticles and Carbon nanotubes. 11th SAC Seminar Moscow, 24 September 2008

M. Kirsch-Volders. Overview on the genotoxicity of nanoparticles. 40th Annual Meeting of the Environmental Mutagen Society, St. Louis, Missouri, October 2009.

L. Gonzalez, Thomassen L, Plas G, Rabolli V, Napierska D, Decordier I, Hoet P, Kirschhock C, Martens J, Lison D and Kirsch-Volders M. Aneugenic and clastogenic effects of amorphous silica nanoparticles in A549 lung carcinoma cells: Size matters?. EMS meeting in St. Louis, Missouri, USA, 24-28 October 2009

D. Lison. Toxicology of Nanomaterials : Silica nanoparticles and Carbon nanotubes. Gaiker COST conference on the Toxicology of Nanomaterials, Bilbao, September 2009.

D. Lison. Should we worry about the health risks of nanos ? IMEC Leuven, December 2009

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Thomassen LCJ, Napierska D, Rabolli V, Gonzales L, Kirch-Volders M, Hoet PH, Lison D, Kirschhock CEA, Martens JA. Nanotoxicology: the marriage of chemistry and biology. Systematic investigation of silica nanoparticle toxicity. At Dipartimento di chimica I.F.M., Turin, Italy,19 April 2010.

Kirsch-Volders M Nanoparticles can interfere with the mitotic machinery and induce chromosome malsegregation. EMBO Workshop Chromosome segregation and aneuploidy, Edinburgh, June 19-23, 2010.

L. Gonzalez, Thomassen L, Plas G, Rabolli V, Napierska D, Decordier I, Hoet P, Kirschhock C, Martens J, Lison D and Kirsch-Volders M Uptake, Cellular trafficking and mitotic disturbances by nanoparticles in A549 human lung carcinoma cells". EMBO Workshop on Chromosome segregation and aneuploidy in Edinburgh, Scotland, 19 – 23 June 2010

Kirsch-Volders M Exploring the genotoxic potential in the nanosize: theoretical approach. 3rd International Symposium on Genotoxicity in Aquatic Systems: Causes, effects and future needs, Freiburg, Germany, 22-24 September 2010

Gonzalez L. Exploring the genotoxic potential in the nanosize: experimental approach. 3rd International Symposium on Genotoxicity in Aquatic Systems: Causes, effects and future needs, Freiburg, Germany, 22-24 September 2010.

D. Lison. Respiratory toxicity of inhaled nanoparticles. NanoBioMed conference, Créteil, (France) June 2010.

D. Lison. Toxicology of Nanomaterials : Silica nanoparticles and Carbon nanotubes. "First Cross Border Conference on NanoSciences and Materials for Health" Pont-à-Mousson (France) June 2010.

M. Kirsch-Volders. Exploring the genotoxic potential in the nanorange. Inaugural conference, XIX Congreso de la sociedad Española de Mutagénesis, A Coruña, October 20-22, 2010.

L. Gonzalez and Kirsch-Volders M. Adaptations of the in vitro micronucleus test for nanoparticle testing: amorphous silica nanoparticles as an example. XIX Congreso de la SEMA in A Coruña, Spain, 20-22 October 2010

M. Kirsch-Volders. Integration of new technologies : which ones and when are they needed ? Genotoxicity of nanomaterials : refining strategies and tests for hazard identification workshop. ILSI/HESI Nanogenetox Working Group and the EMS Regulatory Task Force, Texas, October 23-27, 2010.

D. Lison. Toxicology of Nanomaterials : Silica nanoparticles and Carbon nanotubes. At Dipartimento di chimica I.F.M., Turin (Italy) July 2010

D. Lison. Nanotechnologies, Nanomaterials, Nanoparticles, Health risks ? Journée nationale de médecine du travail. Luxembourg 19 November 2010.

M. Kirsch-Volders. Exploring the genotoxicity of nanomaterials. International Symposium on the safe use of nanomaterials Nanomaterials. Lucknow, India 1-3 February 2011

D. Lison. Dosimetric considerations for exploring the cytotoxicity of silica nanoparticles in vitro. SOT annual conference, Washington, March 2011.

Poster presentations

2007

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) Namur, 28 November 2007

2008

L. Gonzalez, Lison D and Kirsch-Volders M Genotoxicity of engineered nanomaterials: a critical review". 38th Annual Meeting of the European Environmental Mutagen Society in Cavtat, Croatia, 21-25 September 2008

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. Warsaw, Poland, 25-29 May 2008,

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

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

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L. Gonzalez, Thomassen LCJ, Plas G, Rabolli V, Napierska D, Decordier I, Hoet PH, Kirschhock CEA, Martens JA, Lison D and Kirsch-Volders M.Aneugenic and clastogenic effects of amorphous silica nanoparticles in A549 lung carcinoma cells: Does size matter?. 10th ICEM Conference, Firenze, Italy, 20-25 August 2009

L. Gonzalez, Thomassen LCJ, Rabolli V, Napierska D, Hoet PH, Kirschhock CEA, Martens JA, Lison D and Kirsch-Volders M. Induction of aneugenic events by Stöber silica nanoparticles in A549 human lung carcinoma cells. Mitosis and Cancer Symposium in Amsterdam, Nederland, 26-27 February 2009

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L. Gonzalez, Thomassen L, Plas G, Rabolli V, Napierska D, Decordier I, Hoet P, Kirschhock C, Martens J, Lison D and Kirsch-Volders M. Aneugenic and clastogenic effects of amorphous silica nanoparticles in A549 lung carcinoma cells: Size matters?. EMS meeting in St. Louis, Missouri, USA, 24-28 October 2009

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L. Gonzalez, Thomassen LCJ, Plas G, Rabolli V, Napierska D, Decordier I, Roelants M, Hoet PH, Kirschhock CEA, Martens JA, Lison D and Kirsch-Volders M. Exploring the aneugenic and clastogenic potential in the nanosize range : A549 human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models. Nanotoxicology, Edinburgh, Scotland, 2-4 June 2010

L. Gonzalez, Thomassen LCJ, Plas G, Rabolli V, Napierska D, Decordier I, Hoet PH, Kirschhock CEA, Martens JA, Lison D and Kirsch-Volders M. Uptake and mitotic disturbances by nanoparticles in A549 human lung carcinoma cells. EEMS meeting, Oslo, Norway, 15-18 September 2010

L. Gonzalez, Sanderson BSJ and Kirsch-Volders M.. Adaptations to the in vitro Cytochalasin-B micronucleus assay for nanoparticle testing" 41st Annual EMS meeting, Fort Worth, Texas, 23-27 October 2010

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Members of the S²NANO consortium were involved in several advisory committees in relation with the (geno)toxicology of nanoparticles and nanomaterials.

ECETOC Task force on nano(geno)toxicology Symposium at the European Environmenatl 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)

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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.

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6. ACKNOWLEDGMENTS

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Dr A. Elhajouji, Novartis Institutes for Biomedical Research, Basel, Switzerland
Prof. J. Hofkens, Molecular and nanomaterials, KULeuven, Belgium

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