An in vitro toxicity study of a colloid silver health product and atomic quantum clusters of silver and gold

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Preface

This study was carried out at The National Research Centre for Working Environment (NFA), in the nano science group. NFA has also financed the experimental work.

I will like to acknowledge my supervisors senior researcher Ulla Vogel, NFA, and Professor Håkon Wallin for their excellent help in designing the experiments. I appreciate the constructive criticism in the writing phase of this thesis by my internal supervisor associate professor Anne-Marie Heegaard MD, PhD, Faculty of Pharmaceutical Sciences at Copenhagen University and senior researcher Keld Jesen, NFA. The technical staff in my laboratory group: Anne-Karin Jensen, Birgitte Korsholm, and Lourdes Pedersen, have been a wonderful help when I needed to familiarize with the equipment and standard operating procedures.

Finally, I will like to thank Ulla og Mogens Folmer Andersens fund - I am very grateful for their financial support.

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Mads Bred Georgsen

Table of content

Abbreviations

A549	Type 2 alveolar lung epithelial cell A549
Ag	Silver
AQC	Atomic Quantum Clusters
Au	Gold
cDNA	Copy deoxyribonucleic acid
Ct_{RF}	Reference gene cycle threshold
Ct_{TG}	Target gene cycle threshold
ΔCt	Normalised cycle threshold
EDTA	Ethylenediamine tetraacetic acid
F-12	Ham's F-12 cell medium
FBS	Foetal Bovine Serum
IL	Interleukin
LDH	Lactate dehydrogenase
mRNA	Messenger ribonucleic acid
NFA	The National Research Centre for Working Environment
NP	Nano particles
NSP	Nano scale particles
ROS	Reactive Oxygen Species
SRM	Standard reference material
Triton X-100	p-(1,1,3,3-tetramethylbutyl)-phenyl ether
QRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction

Abstract

Metallic atomic quantum clusters are defined as monodisperse agglomerates of two to fifty metal atoms. Unlike the bulk material counterparts, the clusters posses several special features including a small size, an extreme surface-to-mass ratio, and the influence of quantum confinement effects. The consequence is a unique set of physical-chemical properties with many possible applications, but also unknown risk factors for the ecosystems and human health.

Ionosil is a colloidal silver health drink sold with only limited toxicological analysis.

The aim of this study is to investigate the toxicity of atomic gold and silver quantum clusters and Ionosil in the human alveolar lung epithelial cell line A549. The cytotoxicity, inflammation and DNA damage were measured after 3, 24, and 48 hours by cytotoxicity detection kit, quantitative real time reverse transcriptase polymerase chain reaction of interleukin 6 mRNA, and Comet assay respectively. Ionosil and silver 2-3 atoms/cluster were tested in concentrations up to 1 mg/L and silver and gold 3-5 atoms/cluster were tested in concentrations up to 10 mg/L.

This study did not detect toxicity in any samples. A minor anti-inflammatory effect of atomic silver quantum clusters was detected after 48 hours of exposure, as measured by a significant reduction in interleukin 6 mRNA expression.

Keywords: *Atomic Quantum Clusters, toxicology, in vitro, A549, LDH Assay, Comet Assay, and Real time PCR.*

Sammenfatning

Metalliske atomare kvante clusters er monodisperse agglomerater af 2 til 50 metalatomer og er et nyere aspekt af nanoteknologien. Clusters adskiller sig fra metallerne som vi ellers kender dem, ved blandt andet deres lille størrelse, ekstreme overflade-masse ratio og den direkte indvirkning af kvante effekter. Dette giver dem unikke fysisk kemiske egenskaber med mange potentielle anvendelsesmuligheder. De nye egenskaber er dog også en potentiel og ukendt risici kilde for miljø og det humane helbred.

Ionosil, kolloide sølvnanopartikler suspenderet i vand, markedsføres som kosttilskud, hvor for eventuel toksicitet kun er undersøgt i begrænset omfang.

Dette studie har undersøgt toksiciteten af atomare guld og sølv kvanteclusters samt Ionosil i en *in vitro* model med lunge epithel celler A549. Parametrene cytotokcisitet, inflammation og DNA skade er målt ved henholdsvis cytotoxicity detection kit (LDH), quantitative real time reverse transcriptase PCR af interleukin 6 mRNA og comet assay. 3, 24 og 48 timers eksponeringer blev udført. Sølv 2-3 atomer/cluster og Ionosil blev testet i koncentrationer op til 1 mg/L. For guld og sølv 3-5 atomer/cluster var højeste testdosis 10 mg/L. Ionosil blev testet i en factor 10 fortynding svarende til 1 mg/L.

Dette studie kunne ikke påvise nogen toksicitet. Der er dog indikationer på en antiinflammatorisk effekt af atomare sølv kvante clusters målt som reduceret interleukin 6 mRNA ekspression efter 48 timer.

Introduction

Contrary to the belief of many, nanotechnology is not a new concept. Nano-materials have always existed: For example, nano-sized particles are formed by gas-phase atmospheric reactions, volcanic eruptions or recombination in sediments. Human generated nano-sized particles have occurred since the fumes from the first man-made fire.

In the recent years, two things have however changed: public awareness for the special properties of nano-materials has arisen and the technology to specifically design materials at the nano-scale level has developed [Scenihr 06].

Compared to larger particles, nano particles (NP) have unique physical-chemical properties. Their surface to mass ratio is extremely high and the nano-scale quantum confinement effects may affect the characteristics of the clusters or particles [Kurath & Maasen 06, Oberdorster *et al.* 05B]. Interactions with cells and sub-cellular structures are therefore likely to be different from those observed for larger particles [Oberdöster *et al*. 05A]. These special and today unpredictable attributes are reasons for the attractiveness of nanotechnology; but also constitute a potential toxicological risk for mankind and the environment. The environmental regulations and occupational health guidelines concerning particulate matter have not yet been fully adapted to the special properties for these materials and it is still debatable whether they need to be [Maynard *et al.* 06].

When entirely new potential risk materials are identified an extensive analysis of their toxicological properties is necessary. Proper population-risk assessment and safety analysis can only be implemented at this basis, so we may enjoy the full benefits of nanotechnology while avoiding the downfalls [Scenihr 06, Shields 06]. The following is an overview of the key areas related to the toxicology of NP and metallic atomic quantum clusters (AQC) in the present study.

Definitions

The precise definitions of the terms "AQC" and "NP" are still being debated [Kurath & Maasen 06]. Thus, in this thesis AQC are defined as monodisperse atom clusters consisting of 2 to 50 atoms, approximately 0.3 nm to 1.4 nm in diameter [Kuo & Clancy 05]. NP are defined as homogeneous mono-disperse particles with at least two dimensions in the range of 50 atoms to 100 nm. Particles of polydisperse and chemically complex nature less than 100 nm in all three dimensions are considered ultra fine particles [Kurath & Maasen 06, Oberdorster *et al*. 05B]. Nano scale particles (NSP) is used as a collective term that includes AQC, NP and ultra fine particles.

Metallic atomic quantum clusters

AQC, by some also called "nano-clusters", are a new promising material type in the field of nanotechnology. AQC are atom aggregations too small to be considered particles. Recently, it has been confirmed that specific types of AQC suspended in water or an organic solvent can be stable at -20 to +20 ºC for several weeks [Kou & Claucy 05, López del Río T (NanoGap), personal correspondence]. Coupled with the unique optical, electrical, and magnetic properties of NP, this stability can be exploited in a diversity of applications, such as catalysts, fuel cells and biosensors [NanoGap 07].

Nanoparticle and nanocluster toxicology

Free NP are the subgroup of nano-materials of which there is most concern with respect to health risks, because they are directly available for exposure [Scenihr 06]. The AQC in this study belong to the free fragment class, but information on their toxicity is very limited. In the theoretical discussion of their possible toxic properties, it is assumed that these properties are comparable to NP toxicity.

Three key factors when considering NP toxicology are dose, dimension and durability. Studies indicate that the factors dose and dimensions combined: the overall surface area, may be the critical parameter in determining toxicity. Particle surface area as a function of particle mass increases exponentially when particle diameter is reduced. This has been found to correlate to the increased toxicity of small particles. [Oberdorster *et al*. 05 A, Oberdorster *et al*. 05 B, Monteiller *et al*. 07].

It has been shown *in vitro* that a range of different low solubility low toxicity nano and fine particles all resulted in interleukin (IL) 8 responses and a depletion of intracellular glutathione at equal surface area concentrations: the particle surface area relative to the free type 2 alveolar lung epithelial cell A549 (A549) surface area was for all tested particles between 1 and 10 (figure 1) [Monteiller *et al*. 07]. Compared to NP, AQC are more extreme regarding the dose and dimension parameters. Due to the small size, almost all atoms in the clusters are potentially reactive surface atoms. Furthermore, the small cluster mass makes the dose expressed as number of clusters per weight unit enormous.

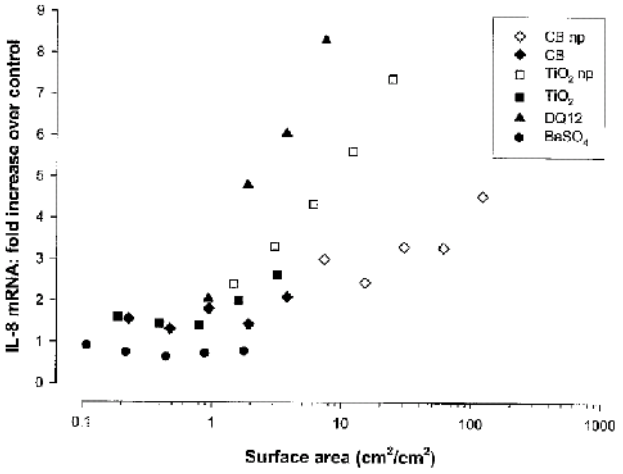

Figure 1. Correlation between particle-to-cell surface area and IL-8 expression. The relative expression of IL-8 as a function of the particle surface to free cell surface ratio in A549 cells after 6 hours of exposure. All the particles elicit an increase in IL-8 expression in the range 1 – 10 cm^2 particle surface / cm^2 cell surface quantified by a semi-quantitative reverse transcriptase polymerase chain method (from Monteiller et al. 07).
Abbreviations: CB-np – carbon black nano particles, CB – carbon black, DQ12 – Quartz, IL-8 – interleukin 8, TiO_2-np – titanium dioxide nano particles, TiO_2 – titanium dioxide.

The durability influences the extent of toxicity: a prolonged exposure may lead to more extensive damage. Opposite, if the AQC degrades before biomolecular interaction with the exposed organism, this is a reason why an inherent toxicity might be insignificant. Information about how the nano-scale in general affects *in vivo* durability has not been available. Slow degradable particles should generate greatest concern and attention should be paid to their long term *in vivo* effects on metabolic and cellular activities [Scenihr 06].

In addition to the three key factors aforementioned, surface chemistry appears to be very important. Surface molecules may influence the biological effects of the NSP in a significant way [Elechiguerra *et al*. 05, Maynard *et al*. 06]. Schins (2001) has shown that coal dust or fibers can act as a carrier and deliver mutagens and carcinogens to the respiratory tract. It is therefore important to analyse the potential presence of impurities or pollutants in the safety studies. This must include impurities from the production and adherence of particles or molecules appearing in the working environment [Scenihr 06].

Nano scale particles toxicity pathways

Cytotoxicity caused by mutagenic NSP carcinogens is described as a unique type of apoptosis mediated by reactive oxygen species (ROS). It has been proposed that ROS are generated by a reaction between the particle and the cell membrane. This produces oxidative stress that may cause a breakdown of membrane lipids, followed by an intracellular calcium homeostasis imbalance, metabolic pathway alterations, and finally apoptosis [Wang *et al*. 06].

For particle-induced genotoxicity, two general mechanisms have been proposed: 1) Particles are *per se* genotoxic; ROS may be generated extra cellularly due to their surface's catalytic properties or intracellular via mitochondrial activation. 2) NSP are indirectly genotoxic by inducing an inflammatory reaction in the tissue [Donaldson *et al.* 05]. Chronic inflammation is also linked to increased production of ROS [Aggerwal *et al.* 06]. ROS are highly reactive and will damage DNA upon contact. DNA damage may lead to mutations either by the inherent error rate of the repair mechanisms or by overloading the repair mechanisms [Dybdahl *et al.* 04, Wang *et al*. 06]. The accumulation of mutations is the usual cause of cancer: 10 to 20 mutations are usually present when a lung carcinoma develops [Croce *et al*. 99]. Short-term *in vivo* and *in vitro* toxicity tests are believed to be sufficient to test the genotoxic potential of carcinogenic compounds [Lima & Van der Laan 00].

Inflammation is the *in vivo* response most frequently observed after NSP exposure and it primarily occur after lung deposition of the NSP. Alveolar epithelial cells and alveolar macrophages are the first line of defence in the lungs [Kurath & Maasen 06]. Foreign

bodies deposited in the alveolar cavity are usually engulfed by the macrophages, which might lead to the production and release of early pro-inflammatory molecules called cytokines, the most prominent being Tumour Necrosis Factor (TNF), IL-1α/ß, IL-6 and IL-8. The cytokines upregulate the excretion of chemokines that lead to the secondary response phase marked by recruitment of other inflammatory cells and the development of inflammation [Heinrich *et al.* 03].

As described above, the first stage of the inflammatory response is the release of cytokines. Cytokines are a group of small molecules regulating haematopoiesis, acute phase reactions and immune responses. Cytokines have autocrine, paracrine, and in some cases endocrine function. IL-6 is a typical endocrine tri-purpose cytokine and IL-6 has been shown to act as a paracrine growth factor for multiple myeloma, different sarcomas and renal cell carcinoma [Aggerwal *et al.* 06, Heinrich *et al. 03*].

Non-genotoxic compounds may however still be cancerous. Carcinogenic non-genotoxic compounds induce tumour development without direct DNA interaction. This group of compounds are involved in the gene regulation and may help mutated cells to proliferate and possibly undergo malignant transformation. Common features of the non-genotoxic action are tissue or species specificity, the existence of a threshold, and the necessity of continual exposure. The mechanisms include, but are not limited to, chronic cell injury, immunosuppression, receptor activation and cytochrome P450 regulation. The diversity of mechanisms and the lack of adequate understanding of the cellular and molecular events involved have however hindered the development of quick and reliable screening tests [Lima & Van der Laan 00]. The adherence of silver (Ag) NP to bio-molecules has been documented and a disturbance of cellular activity has been suggested [Elechiguerra *et al.* 05]. If the bio-molecules are involved in the processes, the AQC may exhibit carcinogenicity through these mechanisms.

Bioavailability

A key element in toxicity is bioavailability, which has not been investigated for AQC. It has been demonstrated that ultra fine particles are able to penetrate pulmonary cells and enter the systemic circulation. The mechanisms facilitating this translocation have not yet been ascertained [Kurath & Maasen 06, Peters *et al*. 06]. It can therefore not be known if these mechanisms also facilitate AQC penetration. The gaps in the tight junctions of epithelial cell layers are less than 2 nm wide effectively blocking the passage of NP, but probably not AQC being less than 2 nm in diameter [Garnett & Kallinteri 06].

Atomic quantum cluster characteristics

All AQC consists of metallic zero-valence atoms. Bulk metals are built up by a uniform metal grid structure with delocalised outer shell electrons. A study of Goldsmith *et al*. (2005) indicates that gold (Au) AQC cores are chiral. A chiral structure cannot be explained by a metal grid structure, leaving the structure of the AQC unknown [Goldsmith *et al*. 05]. This is another indication that AQC cannot be considered simply as smaller versions of the bulk metals; they are a unique group possessing unique properties. It is uncertain what properties the bulk metals and NSP do share. Increasing evidence indicates that transition metals present in particulate matter might induce ROS levels after inhalation [Karlsson *et al*. 06]. AQC of the same transition metals could have the same effect.

AQC have been developed from a variety of transition metals including Ag, Au, platinum and cobber [NanoGap 07]. This thesis focuses on Ag and Au.

Silver toxicology

Ag has a long history as a medical remedy mostly due to its well-documented bacteriostatic properties. Recently, the refinements of Ag NP have given Ag a renaissance as a bacteriostatic component in consumer goods ranging from sports socks to antibacterial cellular phones [Stuer-Lauridsen *et al.* 07]. High exposure to Ag may result in local or generalised argyria, a benign permanent blue or bluish-grey skin discolouration. This results from the extracellular binding of Ag to collagen and reticular fibres in the connective tissue.

Ag toxicity is believed to be a result of the release of Ag ions, which exert their toxic effect by binding to the functional groups in proteins, RNA, and DNA molecules. Metallic Ag is easily oxidised in the organism and colloidal Ag NP is considered a potential danger if it passes the epithelial membranes, because it might end up also binding to functional groups in the bio molecules. The toxicity seems to be present until the Ag ions accumulates as inert Ag-sulphur/Ag-selenium crystals in the lysosomes. These crystals do not seem to redistribute [Dansher & Stoltenberg 06, Rungby 90].

Elechiguerra *et al*. (2005) have documented that Ag NP from 1 to 10 nm with three different surface chemistries: foamy carbon, poly(N-vinyl-2-pyrrolidone) and bovine serum albumin adhere to glycoproteins of the HIV-1 virus (figure 2). The adherence effectively reduces viral infectivity at Ag concentrations of 6 mg/L for carbon-coated NP and 25 mg/L for the latter two. Particles larger than 10 nm do not bind. [Elechiguerra *et al*. 05]. The tested particle range includes large size AQC, which indicates that these are able to inhibit biological activity. Most relevant, it has been shown that acute toxicity of Ag is dependent on the concentration of proteins in the medium [Rungby 90]. It has also been found that colloidal silver in the range 0.1 to 1 mg/L decrease ROS generation and cytotoxicity of the organic compound naphthazarin in astrocytes [Kim *et al.* 07].

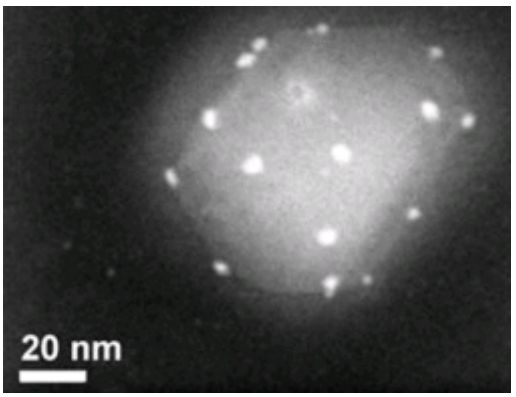

Figure 2. Silver Nano particles bound to the surface of a HIV-1 virus. This is photographed by the use of high angle annular dark field scanning transmission electron microscopy (from Elechiguerra et al. 05).

Elechiguerra *et al*. (2005) found 95% cytotoxicity at 10.5 mg/L for the carbon coated, 11.5 mg/L for poly(N-vinyl-2-pyrrolidone), and 14 mg/L for bovine serum albumin coated NP mentioned above in the MT-2 T-cell line. No significant differences were found between 3 and 24 hours of exposure (figure 3). Their hypothesis was that the greater potency of carbon coated Ag NP was due to the partially uncovered silver surface compared to those effectively encapsulated by poly(N-vinyl-2-pyrrolidone) and bovine serum albumin.

Figure 3. Silver NP induced cytotoxicity in the T-cell line MT-2. Carbon, PVP, and BSA coated silver nano particles induced cytotoxicity after 3 and 24 hours exposure (left and right picture, respectively). The carbon coated silver nano particles are the most potent particles: a reduction in viability is observed at 3 mg/L and reaches 95% at 10.5 mg/L. Analysed by the use of the trypan blue exclusion assay (from Elechiguerra et al. 05).
Abbreviations: BSA – bovine serum albumin, PVP - poly(N-vinyl-2-pyrrolidone).

Gold toxicology

The pharmacological and toxicological properties of Au vary profoundly with its oxidation state. Metallic Au is the least corrosive and the most biologically inert metal available. It has been used as a medical agent and jewellery for millennia. Allergic reactions are very rare and those reactions documented seem to be due to nickel impurities and not the gold component itself [Merchant 98].

A review of Au toxicology by Merchant (1998) suggests that any direct toxicity of Au is unlikely. For example has human promyelocytic leukemic HL-60 cells been exposed to Au metals plates for 10 minutes without inducing any toxic damages [Yamazaki *et al.* 06]. It has however been documented that minute amounts of metallic Au in contact with saliva amino acids are converted to absorbable Au(I)-ions. These ions can then interact with phagocytic and/or antigen presenting cells and be converted to Au(II)-ions, thereby generating immunosuppressive effects [Merchant 98]. If the conversion is linked to particle surface area, the minute amounts in the reaction may be significantly increased in Au AQC due to the high surface-mass ratio. In addition, nano-structured Au may act as catalysts for biological reactions. Experiments have shown high heterogeneous and homogeneous catalytic properties including CO oxidation, NO reduction and oxidation of hydrocarbons at temperature as low as -30 ºC [Xu *et al*. 06].

Cationic and anionic functionalised Au AQC and Au NP have previously been shown to be cytotoxic at concentrations around the range of 220 nM [Goodman *et al.* 04]. The functionalisation makes it impossible to directly compare these results to the present study, but it indicates that Au NP might have cytotoxic properties when it interacts with organic molecules.

Thesis

AQC are available for sale but have not reached extended use. This leaves time for a proactive approach to health safety analysis of these new inventions. Colloidal Ag solutions are already on the market as health drinks without any proper safety studies. Based on the previous mentioned studies by Monteiller *et al*. (2007) and Elechiguerra *et al.* (2005) a toxic effect might be expected at around 10 mg/L for Ag AQC and Au AQC. The objective of this thesis is to study the *in vitro* cytotoxicity, inflammatory and DNA damaging effects of the Au AQC, Ag AQC and colloidal Ag at varying doses. The influence of variable exposure times from 3 to 48 hours will also be evaluated.

Methods and materials

This section consists of two parts: An introduction to the methods followed by a description of the standard procedures. The cytotoxicity was investigated by studying LDH release, inflammation by measuring the *IL-6* messenger ribonucleic acid (mRNA) expression, and DNA damage by the Comet Assay. Three experiments have been designed and their set-up and endpoints are outlined in figure 4.

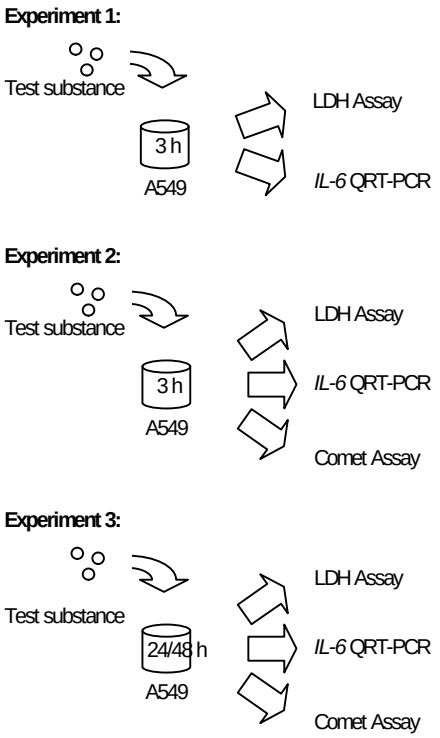

Figure 4. Overview of experimental designs. Experiment 1 and 2 were replicated three times and experiment 3 once. All exposures were run in triple in each replicate. Experiment 1 included Ionosil and Ag AQC 2-3 atoms/cluster. Experiment 2 included Au AQC and Ag AQC 3-5 atoms/cluster. Experiment 3 included all test substances in highest doses.
Abbreviations: LDH – lactate dehydrogenase, IL-6 QRT-PCR – Interleukin 6 quantitative real-time reverse transcriptase polymerase chain reaction, h – hours, A549 – type 2 alveolar lung epithelial cells A549.

Introduction to methods

Validated screening methods for AQC or NP safety analysis have not been developed at this point [Maynard *et al*. 06]. The assays used in this study have been selected because they have been extensively used in ultra-fine particle research at NFA [Allermann & Poulsen 01, Dybdahl *et al.* 04, Saber *et al.* 05, Saber *et al.* 06].

The cell line model

This *in vitro* model is based on A549 (LGC Promochem). The cell line was established post-mortem from a lung carcinoma in a 58-year-old Caucasian man in 1972. A549 is an adherent large cell lung cancer with similarities to type II alveolar epithelial cells [Croce *et al.* 99, Giard *et al*. 73]. The cell line has lost the tight junctions of the alveolar cells. However this function is not considered important for toxicological purposes [Forbes & Ehrhardt 05]. Type II alveolar epithelial cells have been estimated to constitute 60% of all alveolar epithelial cells and cover 5% of the alveolar surface in adult persons. But there are concerns about the reliability of the method used to obtain these results [Fehrenbach 01].

A steady expression of “reference genes” (discussed in a later section) in A549 is found when the cells are in a logarithmic growth phase. This phase is characterized by a well defined uniform cell growth and sufficient cell density to enable adequate intracellular chemical communication. These two requirements are fulfilled when confluence lies between 2.5% and 90%. This span allows 5 cell generations of around 22 hours between each reseeding [Giard *et al*. 73, LGC Promochem].

Studies have documented provoked release of cytokine IL-6 and IL-8 in A549 after exposure to toxic compounds. However, TNF-α and IL-1 are not secreted from A549 [Allermann & Poulsen 01, Dybdahl *et al.* 04, Monteiller *et al.* 07].

Controls

For validation purposes, three types of controls were included in this experiment: a positive particle control, cell medium, and vehicle controls. Furthermore, assay-specific controls have been included in the three assays, as described in the section concerning the individual assays.

Positive control

The Standard Reference Material (SRM) 2975 (National Institute of Standards and Technology) is included as a positive control. This standard consists of diesel particulate matter from a diesel-powered forklift [May & Trahey 00]. The inflammatory and DNA damaging effects of diesel particulate matter in A549 have previously been described [Dybdahl *et al.* 04, Saber *et al. 05,* Saber *et al. 06,* Don Porto Carero *et al.* 01]. Diesel particulate matter is poly-disperse with particle diameters from 1 nm to several micrometers [Ono *et al*. 07]. The National Institute of Standards & Technology (USA) list some characteristics as well as references and certified values for several organic constituents in the sample [May & Trahey 00]. The main composite is carbon particles with a variety of substances adsorbed including heavy metals and organic compounds.

Negative control

The pure test culture medium (test medium control reference) reflects the basal response of A549 and provides information about the day-to-day variations.

Vehicle control

The vehicle control enables the effects of the vehicle to be quantified separately from the AQC and increases sensitivity.

Cytotoxicity detection by LDH Assay

Membrane integrity was quantified by an enzymatic method. The release of the intracellular lactate dehydrogenase (LDH) to the medium was used as a measure for cytotoxicity and quantified by the cytotoxicity detection kit (Roche A/S). The assay measures the absorbance of a coloured formazan product formed in a reaction cycle with LDH activity as the rate-limiting step. Cytotoxicity is expressed as the viable cell fraction in the sample. This is found by subtracting the spontaneous LDH release from the sample value and divide by the difference between the spontaneous and maximal LDH release. The formula is:

$$\text{Viability (\%)} = 1 - \text{Cytotoxicity (\%)} = 1 - \frac{\text{sample} - \text{spontanous}}{\text{maximal} - \text{spontanous}}$$

Full cell lysis and hereby maximal LDH release may be induced in eukaryotic cells by the use 1 % of the non-ionic surfactant polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100) with an average of 9.5 ethylene oxide units per molecule. Triton X-100 lyses the cells by increasing the permeability of the eukaryotic cell membrane [Sigma-Aldrich 99].

LDH is a cytoplasmic enzyme released in high quantities at cell lysis. Cytotoxicity usually results in cell lysis. Figure 5 illustrates the assay reaction cycle. LDH converts lactate to pyruvate, which generates NADH (nicotinamide adenine dinucleotide). The NADH is used as the energy source for the diaphorase catalysed break of a nitrogen-nitrogen binding in the pale yellow tetrazolium molecule. The result is the formation of the water-soluble red formazan molecules, which may be photometric quantified at 492 nm. The delta optical density is calculated by normalising to the absorbance at a wavelength above 600 nm to correct for inter-sample variations. The absorbance curve approximates linearity when the optical density delta values are in the range 0.3 to 2.75 [Roche Applied Sciences 05].

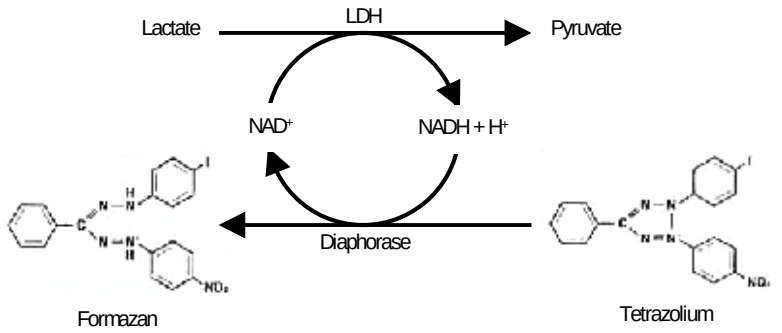

Figure 5. The cytotoxicity detection kit reaction cycle. The kit is designed with the conversion of lactate to pyruvate by LDH as the rate-limiting step. Formazan is dark red and tetrazolium is pale yellow and the increase in absorbance at 492 nm is finally photometric quantified (Adapted from Roche Applied Sciences (2005)).
Abbreviations: LDH – Lactate dehydrogenase, NAD - Nicotinamide adenine dinucleotide.

The cytotoxicity detection kit measures LDH in the medium, which also contains the test substance. It is therefore important to investigate interactions between the assay and the test substances.

Quantitative real-time reverse transcriptase PCR of mRNA

The amount of IL-6 was determined as mRNA expression using quantitative real-time reverse transcriptase polymerase chain reaction (QRT-PCR). QRT-PCR is a powerful method to quantify gene expression [Livak & Schmittgen 01]. The process consists of four steps: RNA isolation, DNase treatment, creating a deoxynucleic acid copy (cDNA) of the mRNA and synthesis of PCR products (figure 6).

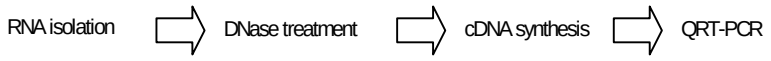

Figure 6. Flow diagram for preparation of lysed cell samples for QRT-PCR.
Abbreviations: cDNA – copy deoxynucleic acid, QRT-PCR – real-time reverse transcriptase polymerase chain reaction.

The RNA isolation method is based on selective binding of RNA to a silica membrane [QIAamp RNA Blood Mini Kit].

In principle QRT-PCR products double each cycle, and it is this increase which is quantified. All quantifications of target genes must be normalised to a reference gene with a stable continuous expression in the cell to account for variable amounts of cDNA. This study uses *ß-actin* as reference. The *ß-actin* encodes a cytoskeleton protein and is expressed at moderately abundant levels in most cells [Bustin 00].

The normalised cycle threshold (ΔCt) is found by subtracting the reference gene cycle threshold (Ct_{RF}) from the target gene cycle threshold (Ct_{TG}): $\Delta Ct = Ct_{TG} - Ct_{RF}$. The efficiency of a properly optimized setup will approach 100 percent, and the number of DNA copies double in each cycle. This being the case, the relative expression of the target gene may be calculated by the formula: $2^{-\Delta Ct}$ [Livak & Schmittgen 01].

The TaqMan hydrolysis probes applied in the QRT-PCR setup consist of an oligonucleotide with a 5'-reporter fluorescence dye and a 3'-quencher dye. As long as the probe is intact, the quencher reduces the fluorescence by half. During the annealing phase, the probe attaches to the DNA downstream of the primer. The DNA polymerase elongates the primer in the extension phase. When it reaches the probe, the probe will be cleaved by the 5'-3' exonuclease activity of the DNA polymerase complex. When the probe is cleaved the quenching effect disappears, and an increase in fluorescence is observed [Bustin 00, Giulietti *et al.* 01]. The thresholds are defined where the intensity of the fluorescence is considered significant compared to the baseline level (figure 7).

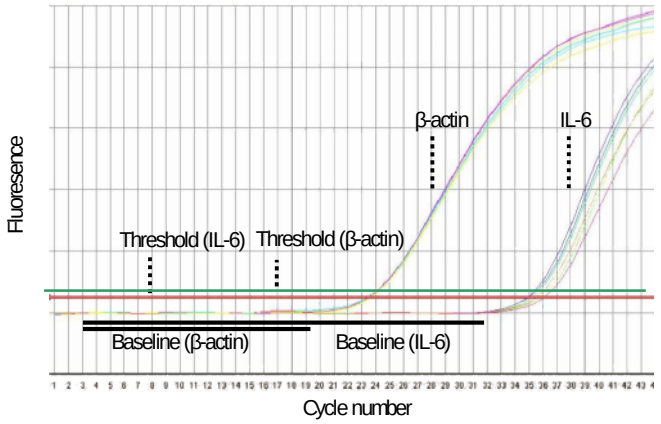

Figure 7. An example of the QRT-PCR output with the critical parameters: baseline and threshold marked. The fluorescence exceeds the baseline after about 24 doublings for the reference β-actin and 35 for the target IL-6 (logarithmic y-axis). This illustration is the 4000 mg/L positive diesel control sample run in sextet.

The results are expressed as absolute numbers. The relative expression, to the medium control, is calculated for any sample with a statistically significant increase in *IL-6* expression.

DNA Damage by comet assay

The single layer comet assay enables the detection of DNA damage which is visualised as the migration of individual cell DNA forming a "comet tail" (figure 8) [Tice *et al*. 00].

Figure 8. The Comet Assay output as seen in the fluorescence microscope. To the left is undamaged DNA visible as a nucleoid and to the right damaged DNA visible as a “comet”. The tail length for the damaged DNA is marked at the drawing.

The principle is to embed the cell DNA in a gel film and apply a voltage difference to attract the negatively charged DNA towards the anode. The migration speed depends on DNA fragment size, with the shortest strands moving the fastest. The DNA is visible by the use of fluorescence dyes and the length of the tail may be studied to quantify the relative amount of short DNA segments. The type of damages observed can be regulated by the pH of the electrophoresis. Neutral pH will only reveal double strand breaks, while single strand breaks, DNA-DNA cross linking, and alkali-labile sites may also be detected at pHs above 13 [Tice *et al*. 00].

Test materials

Following this paragraph is a list of the tested substances. In the first period of the experimental phase was only Ionosil and Ag AQC 2-3 atoms/cluster with silver ions available. At a later point were the Ag and Au AQC 3-5 atoms/clusters delivered in concentrations 10 times higher than the Ag AQC 2-3 atoms/cluster and without the silver ions. The tested solutions were the only ones Ion Silver and NanoGap could deliver at the time of the experimental work.

List of test substances

10 mg/L Ag AQC 2-3 atoms/cluster + 40 mg/L silver ions (Ag^+, NO_3^-), NanoGap.
5 mg/L Ag AQC 2-3 atoms/cluster + 20 mg/L Ag^+ (NO_3^-), NanoGap.
40 mg/L Ag^+ (NO_3^-), NanoGap.
20 mg/L silver ion solution (Ag^+,NO_3^-), NanoGap.
100 mg/L Ag AQC 3-5 atoms/cluster, NanoGap.
100 mg/L Au AQC 3-5 atoms/cluster, NanoGap.
Milli-Q water, NanoGap.
Ionosil Kollodialt Silver 10 mg/L, Ion Silver (99.99% pure particulate (colloidal) silver in distilled water. Colloids: 1 - 6 nm; colloid agglomerates 2 - 24 nm).

Standard procedures

A549

A549 was cultivated in a culture medium consisting of 89% Ham's F-12 cell medium (F-12) (Invitrogen Life technologies), 1% penicillin-streptomycin and 10% heat-inactivated Foetal Bovine Serum (FBS) (Invitrogen Life technologies) in an incubator at 37 °C, 5% CO_2/95% atmospheric air, and 80-90% relative humidity. Stock cells were grown in 25 cm^2 T25 Nunc Nunclon surface, 75 cm^2 T75 Corning, and 150 cm^2 T150 Nunc Nunclon surface flasks. Culture medium was changed every two or three days.

The cells were reseeded every 5 days or when 80% confluence was reached [LGC Promochem]. Confluence was determined as percentage of the container surface covered by cells by visual examination. The cells were detached from the container surface before every reseeding: 5 minutes of trypsin exposure (trypsin 500 mg/L + 200 mg/L ethylenediamine tetraacetic acid (EDTA), Cambrex Bio Science Vierviers) followed by 10 hard slaps on the side of the flask. The cells were centrifuged for 10 minutes at 300 G and

4 °C. The cells were then re-suspended in new culture medium and the concentration adjusted to 10^6 cells/mL. A volume containing the desired cell number was seeded in the new container.

Positive control

The diesel particles were stored at -20 ºC. Four or forty milligram of the SRM 2975 was used for the positive reference. Two millilitre of test medium was added to the reference material followed by 4 minutes of sonification on ice bath (10 seconds pulses, 10 seconds intermissions). Additionally, 2 mL medium was added and the mix was sonifed for 4 minutes. The diesel concentration was adjusted to 400 or 4000 mg/L. The 200 or 2000 mg/L diesel control was prepared by mixing 5 mL 400 or 4000 mg/L with 5 mL medium.

Exposure settings

$8 \cdot 10^4$ A549 cells were seeded in each well (Multidish 24 wells, Nuncleon Surface, Nunc A/S) and incubated for 48 hours until approximately 70-80% confluence was obtained. The cells were exposed in grouped triplets. Approximately 10 minutes of the exposure time was in the laminar airflow bench and the rest in the incubator (37 °C, 5% CO_2/95% atmospheric air, and 80-90% relative humidity). At each well plate, the lower left triplet was reserved for the test medium control reference. In the other wells, the test solution was never allowed to exceed 10% of the total test medium. The test culture medium consisted of F-12 in the 3-hour study and of 95% F-12 and 5% FBS in the 24- and 48- hour studies.

The medium was removed for cytotoxicity studies immediately after end of exposure. In experiment one (number according to figure 4), the cells was washed twice with phosphate buffer (pH 7.4) and lysed with 400 µL Buffer RTL™ (Qiagen) incl. 1% ß-Mecaptoethanol (Merck). The triplets were pooled and immediately frozen at -80 ºC. In experiment two and three the cells were detached from the well surface by the addition of 100 µL trypsin (twofold diluted with phosphate buffer) and 4 minutes incubation at 37 °C, 5% CO_2/95% atmospheric air, and 80-90% relative humidity. The enzyme was then neutralized with 500 µL culture medium. 100 µL suspension from each well was reserved for the comet assay. This volume was centrifuged at 300 G (10 minutes, 4 °C) and re-suspended in freezing medium and cooled to -80 °C at a rate of 1 degree Celsius pr. minute in isopropanol boxes.

The freezing medium consisted of culture medium with 10% sterile filtered dimethyl sulfoxide (Merck). The remaining suspension was prepared for *IL-6* measurements: in experiment 2, the triplets were pooled and in experiment 3 kept separate. The suspensions were centrifuged for 1 minute at 5 kG, the medium removed, and respectively 400 µL and 200 µL Buffer RTL™ buffer incl. 1% ß-Mecaptoethanol was added to the pooled and unpooled samples. Both were immediately frozen at -80 °C.

LDH Assay

The LDH release was measured in the medium either immediately after the end of exposure or the medium was stored at -80 °C until analysis. Carried out in doublet, 2.17 µL kit solution 1 (Diaphorase & NAD^+) and 97.83 µL kit solution 2 (sodium lactate & iodotetrazolium chloride) was added to 100 µL medium in a 96 well plate. The positive diesel control was first centrifuged at 5 kG. The mixture was incubated for 30 minutes under shaking rapped in aluminium foil. Absorbance was read at 492 nm with 630 nm as the reference wavelength using an ELISA-reader.

Maximal release controls were produced simultaneous with each study. A549 were incubated with 1 mL 1% Triton X-100 (Merck) in test culture medium (v/v) for 30 minutes on a 24-well plate. In experiment three, the maximal release controls were incubated with 900 µL test culture medium. 100 µL 10% Triton X-100 was added 30 minutes before end of exposure.

In the LDH kit-AQC interactions test, the cells were incubated 30 minutes with culture medium containing 10% test solution (v/v) and 1% triton X-100 (v/v). The medium was prepared for optical density measurements as described above. The result is expressed as the mean delta optical density.

IL-6 QRT-PCR

As previously mentioned, the *IL-6* QRT-PCR analysis consists of four steps: RNA isolation, DNase treatment, cDNA synthesis, and QRT-PCR. Care was shown to mix and spin all samples in all operation steps except in the RNA isolation. To reduce costs, the relative *IL-6* expression was only measured for the highest exposure concentration. If these

results gave a statistically significant increased response (relative to the medium control), then the other concentrations were analysed.

RNA isolation

RNA was isolated with the QIAamp RNA Blood Mini Kit (Qiagen). All of the samples were added to the QIAshredder® and centrifuged for 2 minutes. The filtrate was mixed 1:1 with ethanol and added to the QIAamp® tube. The tube was washed once with 700 µL Buffer RW1™ and twice with 500 µL Buffer RPE™ followed by 15, 15, and 180 seconds of centrifugation, respectively. The RNA was centrifuged of the membrane with 2 x 30-50 µL RNase free water, the first time with 15 seconds, and the second time with 60 seconds of centrifugation. The force in all centrifugations was in the range 16 – 20 kG.

The purity of the mRNA was determined by measuring the 260 nm to 280 nm absorbance in buffer (10 mM (hydroxymethyl)$_3$-aminomethan, Sigma-Aldrich, 1 mM EDTA, Sigma-Aldrich, pH 7.6). The Absorbance$_{260nm}$ to Absorbance$_{280nm}$ ratios between 2.0 to 2.1 was accepted. The mRNA quantity was determined from the absorbance at 260 nm (1 optical density unit = 44 µg/mL).

DNase treatment

21.6 µL mRNA was incubated 30 minutes at 37 ºC together with 3.38 µL mix (table 1), and the reaction was stopped by incubating the sample at 65 ºC with 2.50 µL mM sterile EDTA.

Table 1. The components in the DNase treatment mix.

50 mM (hydroxymethyl)$_3$-aminomethan, Sigma-Aldrich
5 mM $MgCl_2$, unknown origin
1 mM dithiothreitol, unknown origin
3.7 U/µL RNAse inhibitor, Applied Biosystems
0.25 µL DNAse I, RNAse free, Qiagen

cDNA synthesis

Approximately 400 ng mRNA was adjusted to a volume of 7.70 µL with RNase free water. TaqMan Gold RT-PCR™ mix (table 2) was added to a total volume of 20 µL. In each batch a control sample was included without the addition of the MultiScripe Reverse Transcriptase Enzyme™ as a process control of the cDNA synthesis and QRT-PCR steps.

The reverse transcription cycle included 10 minutes incubation at 25 ºC, 30 minutes transcription at 48 ºC, and inactivation of the enzymes with 5 minutes at 95 ºC.

Table 2. TaqMan Gold RT-PCR mix used in cDNA synthesis. All reagents are provided by Applied Biosystems.

1x TaqMan RT buffer™
5.5 mM $MgCl_2$
500µM each Deoxynucleotide triphosphate
2.5 µM Random Hexamers
4 U/µL RNAse inhibitor
1.25 U/µL MultiScripe Reverse Transcriptase™

QRT-PCR amplification

5.00 µL cDNA was added to a mix of 53.0 µL TaqMan universal master mix™ and 47.0 µL milli-Q water. 47.5 µL was added to two tubes containing 2.50 µL Human *IL-6* FAM (6-carboxyfluorescein) and human *ß-actin* VIC® primer/probe mixes, respectively. Both probes used TAMRA (6-carboxytetramethylrhodamine) as quencher. 15.0 µL of each sample (for composition see table 3) was run on the ABI-PRISM 7500 sequence detector (Applied Biosystems) with standard thermal cycle conditions in triplet. The variance between different PCR setups in the same experiment was not allowed to vary more than 30% and is checked by comparing a repeated positive control run at every plate. If the standard deviation exceeded 15% an attempt to locate and eliminate the potential outlier was performed or the run was discarded. The C_t threshold was set at 0.1 for *IL-6* and 0.15 for *β-actin*. Amplification efficiency and optimal thresholds for the primer & probes mixtures had been determined by the NFA staff and found acceptable.

Table 3. PCR amplification mixture. Each sample run in the ABI-PRISM 7500 sequence detector contained 15 µL.

47.9 % TaqMan Universal Master Mix, Applied Biosystems
42.5 % RNase free water, NFA
4.5 % cDNA
5.0 % Primer and probe mixture: human IL-6 FAM or ß-actin VIC, Applied Biosystems

Comet Assay

All comet procedures were conducted under reduced light. 0.75% (w/v) low melting agarose (GibcoBRL, life technologies Inc.) was melted in a microwave oven (600 W) and stabilised at 37 ºC in a water bath. The cell suspension was thawed quickly in a 37 ºC

water bath. 25.0 µL of cell suspension was mixed with 225 µL agarose. An eight-holed polyethylene mould was set up at the hydrophilic side of a levelled GelBond film (Agarose gel support medium, Cambrex Bio Science Rockland Inc.) and 130 µL of mixture was applied to each hole (19.5 mm in diameter). The gels rested 10 minutes at 4 ºC before the form was removed.

The gel bond films were placed in 4 ºC lysis buffer (table 4) for one hour, followed by three washes with milli-Q water. Four films at a time were alkalised for 40 minutes in a levelled electrophoresis tank containing fresh prepared electrophoresis buffer (table 4) at 3-5 ºC. The electrophoresis was run for 20 minutes (25 V, 292-296 mA, 3-5 ºC). A final pH 7.5 was obtained by treating the slides with neutralization buffer (table 4) twice for 5 minutes each. The gel bond films were rinsed in water, dehydrated in 96% ethanol (De Danske Spritfabrikker) for 1.5 hour, and air-dried overnight. Two GelBond films at a time were stained with 100 mL staining solution for 10 minutes (table 4), each cut in four, and two samples were placed on each object glass. All samples were blinded and randomised by an external person.

Fluorescence was measured in the microscope at 400x magnification. Tail length for 50 randomly selected cells was scored using the software Komet 3.0 (Kinetics imaging Ltd.). DNA damage was expressed as tail length. To ensure conformity, the first batch including 20 samples and 12 controls was scored by separately by both the author and a trained technician.

The assay specific positive controls and negative control were made for the assay by incubating A549 cells with 30 µM and 0 µM H_2O_2, respectively, in an isotonic phosphate buffer system for 30 minutes at 4 ºC. The cells were detached with trypsin and centrifuged (4 ºC, 300 G, 10 minutes). Cell density was adjusted to $2 \cdot 10^5$ cells/mL with freezing medium and frozen at -80 ºC, the first night in isopropanol boxes. The controls were analysed in parallel with the samples. 50.0 µL of cell suspension was mixed with 450 µL agarose and 130 µL and analysed in duplicate as previously described for the samples.

Table 4. Comet assay solutions. Four solutions were used in the comet assay: lysis, electrophoresis, neutralisation buffer and a staining solution. All solutions are on basis of Milli-Q water.

Lysis buffer (pH 10)
2.5 M NaCl, Merck
0.1 M Na_2EDTA, Sigma-Aldrich
10 mM Tris, Sigma-Aldrich
1% Na-sarcocinat (w/v), Sigma-Aldrich
10% Dimethyl sulfoxide (v/v), Merck
1% Triton X-100 (v/v), Merck
Electrophoresis buffer (pH 13.2)
0.3 M NaOH, Merck
1 mM Na_2EDTA, Merck
Neutralization buffer (pH 7.5)
0.4 M Tris-base, Sigma-Aldrich
Staining solution (pH 8)
10 mM Tris-HCl, Merck
1 mM EDTA, Merck
0.005% SYBR Gold (v/v), Molecular Probes, Invitrogen

Statistics

All results are presented by mean and standard deviation. The statistical analysis was performed in SAS 9.1 for Windows, SAS Enterprise.

Normality of data was verified using Shapiro-Wilk statistics (SAS command: “Proc univariate normal”, $\alpha = 0.05$) and equality of variances controlled using F-statistics (SAS command: “proc ttest”, $\alpha = 0.05$). Results that do not hold the assumptions for parametric testing were logarithmic transformed and this will be mentioned in the results section. The samples were compared by the use of the Student t-test (SAS command: “proc ttest”, $\alpha = 0.05$).

Instruments & assay Kits

The following is a list of the applied instruments and assay kits.

Cell culturing & exposure preparations

Centrifuge: Minifuge T, Hereaus.
CO_2 incubator: ASSAB, Kebo Biomed Sweden.
LAF-bench: VFB 1206 BS, Dan Lap.
Microscope: Leica DMIL.
Pump: Heto, High Technology of Scandinavia.
Sonifier: Branson Digital Sonifier.
Whirl mixer: Vidrofix NF1 electronic, Janke & Kunkel Ika-labortechnik.

LDH Assay

Assay kit: Cytotoxicity Detection kit (LDH), Roche Applied Science.
ELISA-reader: ELX 808, Bio-Tek Instruments Inc.
Shaking table: HS 250 Bacis, Ika labortechnic.

IL-6 mRNA QRT-PCR

cDNA synthesis kit: TaqMan Gold RT-PCR mix.
Centrifuges: Centrifuge 5417, Microcentrifuge 157MP, Ole Dich Instrument Makers APS, Twister www.gtf.se; Costar mini centrifuge; Varifuge 3.0, Heraeus, Eppendorf.
LAF-benches: VFB 1206 BS, Dan Lap; HLBC 2448 BS.
Milli-Q water: Milli-Q water purification system, Millipore
QRT-PCR: ABI-PRISM 7500 sequence detector, Applied Biosystem, w/ Dell PC.
Reverse transcriptase PCR: PTH-100 Programmable Thermal controller, MJ research Inc.
RNA purification kit: QIAamp RNA Blood Mini kit, Qiagen.
Spectrophotometer: HP UV/VIS spectrophotometer 8453 with HP Vectra XA PC.
Waterbath: No-name with Heto, Intermed thermostat.

Comet Assay

Centrifuges: Microcentrifuge 157MP, Ole Dich Instrument makers APS.
Electrophoresis tank: Maxicell EC360M electrophorec gel system, E-C Apparatus Cooperation.
Microscope: Leica DM BL with a 450-490 nm excitation filter and a LP520 suppression filter.
Milli-Q water: Milli-Q water purification system, Millipore
Stirring: Ikamag Ret., Janke & Kunkel Ika-labortechnik.
Shaker: Cello Shaker variospeed, Electrophoresis and membrane technologies, Chemtron Products.
Voltage supply: EC250-90, E-C Apparatus Cooperation.
Waterbath: No-name with Heto, Intermed thermostat.

Results

The result section is subdivided into three parts. First, the results from the cytotoxicity analyses, then the results from the inflammation analyses and finally the results from the DNA damage analyses.

All the results from the analysis of the different compounds are when possible summarized in a single graph to provide the best possible overview.

Cytotoxicity

A549 were exposed to Ag 2-3 atoms/cluster, its vehicle, and Ionosil in 4 concentrations in the interval from 0.016 to 1 mg/L for 3 hours and no reductions in viable cell counts were observed (figure 9). The experiment was repeated with slightly larger AQC of 3-5 atoms/cluster consisting of Ag and Au. These were tested in 4 concentrations ranging from 0.3 to 10 mg/L (figure 10). No significant cytotoxicity was found in any of these samples.

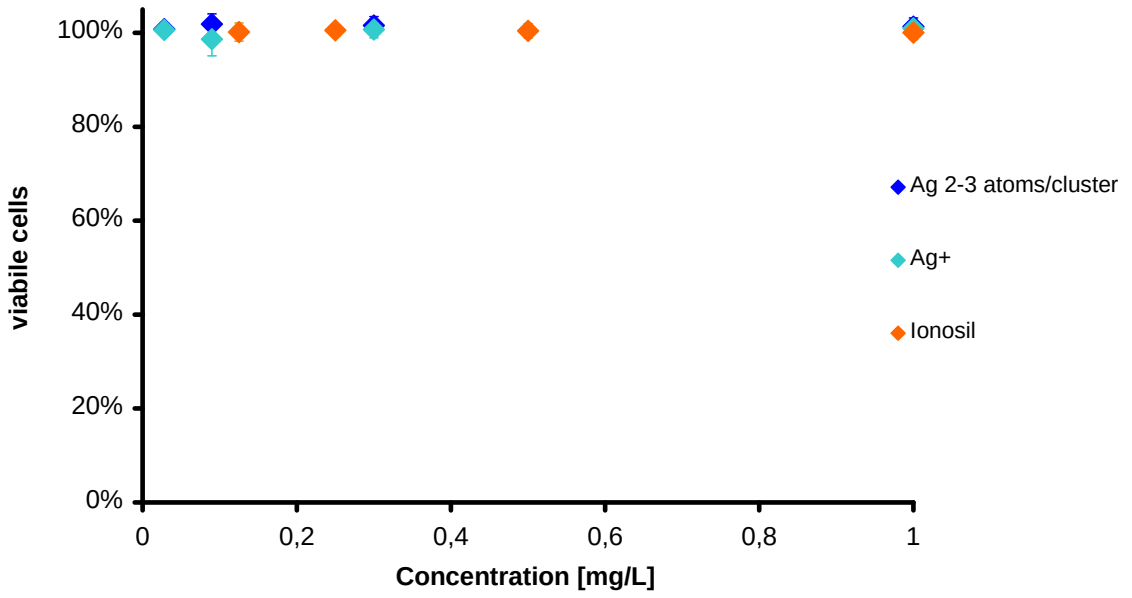

Figure 9. Cell viability after 3 hours exposure to Ionosil and Ag atomic quantum clusters 2-3 atoms/clusters. No group have viability counts different from 100%. Ag^+ is the vehicle for Ag 2-3 atoms/cluster, data points are set at the concentrations corresponding to the cluster for whom they represent the vehicle. (mean ± standard deviation, n=9 (3 replicates)).

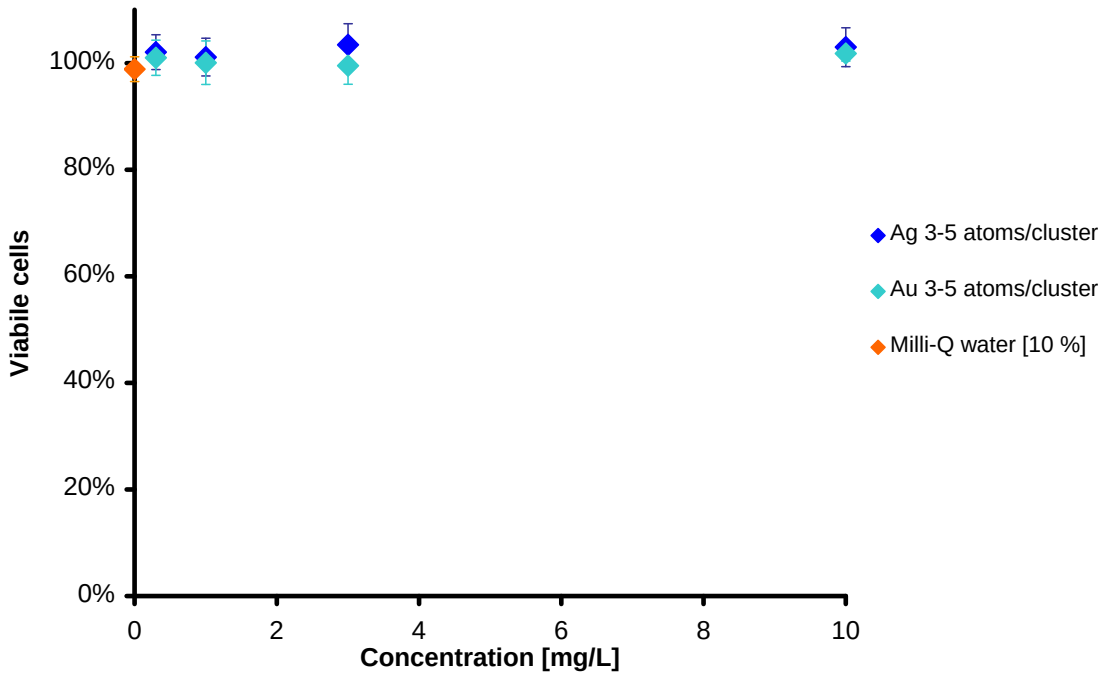

Figure 10. Cell viability after 3 hours of exposure to Au and Ag atomic quantum clusters 3-5 atoms/cluster. No group have viability counts different from 100%. The cytotoxicity for the milli-Q water vehicle was measured at 10% (v/v) as present in the 10 mg/L QAC (mean ± standard deviation, n=9 (3 replicates)).

To observe the effect of exposure time on the test substances cytotoxicity additionally exposures of 3, 24, and 48 hours were performed for test substances at the highest available concentrations: Ag 2-3 atoms/cluster 1 mg/L, Ag 3-5 atoms/cluster 10 mg/L, Au 3-5 atoms/cluster 10 mg/L, and Ionosil 1 mg/L. The milli-Q water vehicle and the Ag^+ vehicle were also tested. The positive diesel control was tested at 200, 400, 2000 and 4000 mg/L. The results are presented in figure 11 and as it can be observed, no significant cytotoxicity was found for any of the groups.

The Milli-Q water vehicle for Ag and Au AQC 3-5 atoms/cluster from NanoGap was visible microbiological contaminated after 48 hours of exposure and this group was discarded.

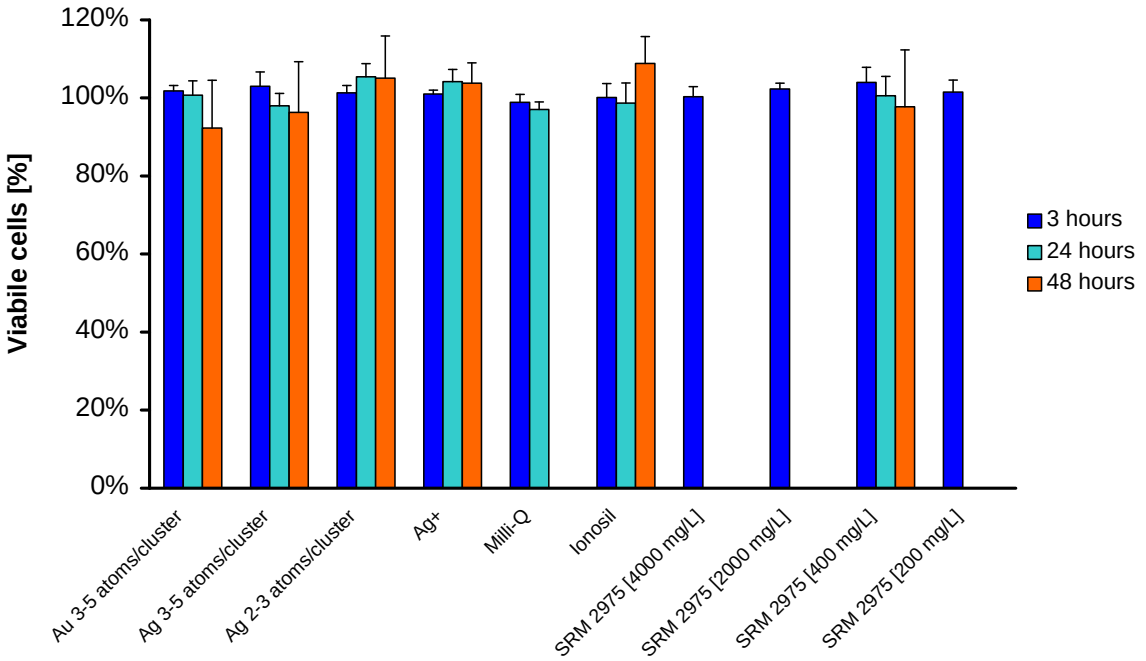

Figure 11. Cell Viability as a function of exposure time for all test substances. No group displayed statistical significant cytotoxicity. The test substances were analysed in the following concentrations: Au 3-5 atoms/cluster 10 mg/L, Ag 3-5 atoms/cluster 10 mg/L, Ag 2-3 atoms/cluster 1 mg/L, Ag^+ 4 mg/L (corresponding to Ag 2-3 atoms/cluster 1 mg/L), Milli-Q water 10 %, and Ionosil 1 mg/L (mean + standard deviation, n= 9 for 3 hours (3 replicates) and n=3 for 24 and 48 hours (1 replicate)).

The LDH assay measures the release of LDH to the medium, which also contains the AQC. The AQC has not been tested with the LDH assay before and a test for interaction must therefore be employed. No significant difference in absorbance was observed in the interaction test between LDH measurements for the cells lysed with 1% triton X-100 when Au or Ag AQC 10 mg/L were present (Figure 12).

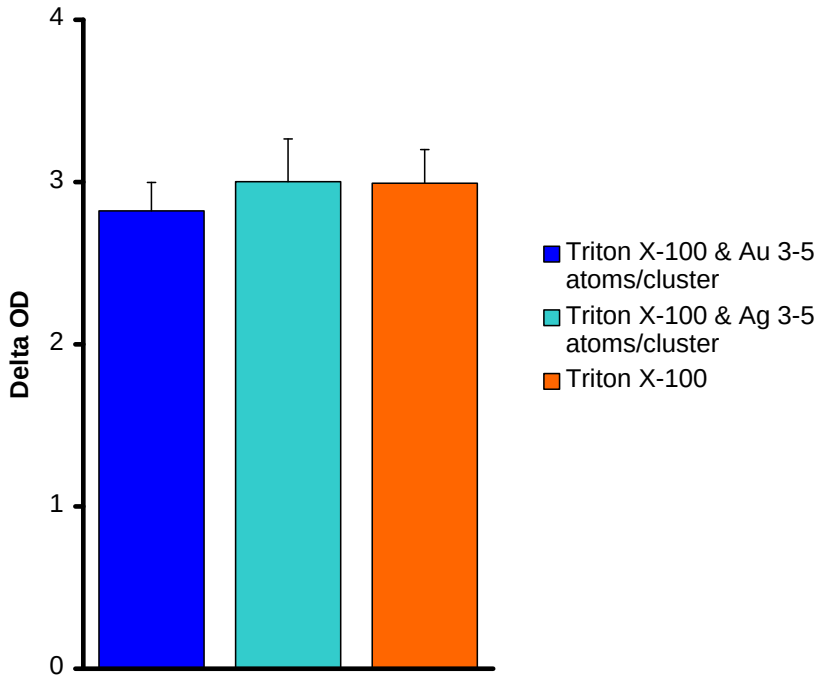

Figure 12. LDH assay – AQC Interaction test. No significant differences were observed for the three groups. An assay – test substance interaction is therefore unlikely. Triton X-100 and the atomic quantum clusters were at concentrations of 1% (v/v) and 10 mg/L respectively and exposure time was 3 hours. The y-axis is the delta optical density found by reading the absorbance at 492 nm and normalising to the absorbance at 630 nm (mean + standard deviation, n = 6 (2 replicates)).

Inflammation

To test whether AQC have any inflammatory effects the *IL-6* gene expression was analysed for all substances at the highest exposure concentration. It was decided not to test any of the lower concentrations to keep costs low. For the same reason, only one sample from each replicate was analysed when 3 were available.

The results are summarized in figure 13. None of the test substances induced statistically significant *IL-6* expression compared to the medium control. There is a significant decrease in mRNA expression after 48 hours for Ag 2-3 atoms/cluster 1 mg/L ($p = 0.006$) and Ag 3-5 atoms/cluster 10 mg/L ($p = 0.042$) compared to the medium control. The positive diesel control at 400 mg/L results in a significant 4-fold ($p = 0.049$, logarithmic transformed) and 7-fold ($p = 0.002$, logarithmic transformed) increase in mRNA expression after 3- and 24-hours respectively. At the concentration of 4000 mg/L there was a significant 36-fold increase ($p < 0.001$, logarithmic transformed) and at 2000 mg/L an 11.5 fold increase ($p < 0.001$, logarithmic transformed).

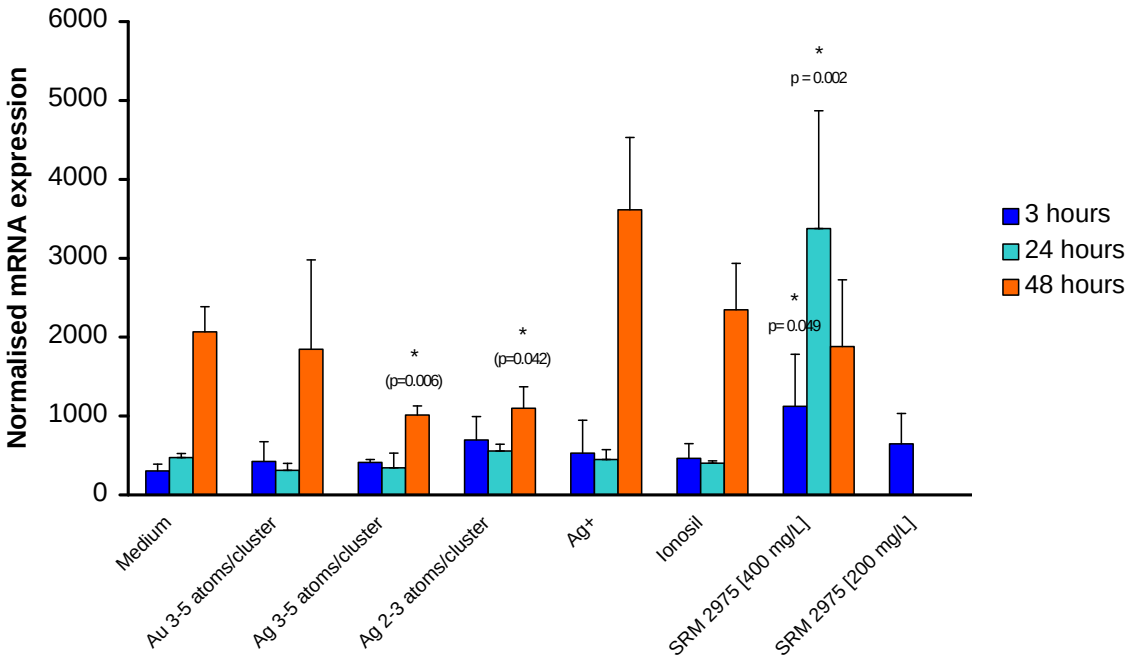

Figure 13. IL-6 expression normalised to β-actin as function of exposure time. Asterisks mark statistical significant results. Test concentrations were: Ag 2-3 atoms/cluster 1 mg/L, Ag^+ 4 mg/L (corresponding to Ag 2-3 atoms/cluster 1 mg/L), Au 3-5 atoms/cluster 10 mg/L, and Ag 3-5 atoms/cluster 10 mg/L (mean + standard deviation, n=3 (3 replicates at 3 hours with 1 from sample each, 1 replicate at 24 and 48 hours)).

The microbiologically contaminated milli-Q water from NanoGap had an *IL-6* expression at level with the medium after 3 hours exposure. A significant 473-fold increase relative to the medium control was however observed after 24 hours of exposure ($p < 0.001$, logarithmic transformed).

When considering all measurements at 24 hours as one group and all measurements at 48 hours as a second group (excluding all results significantly different from the medium control), the 48 hour group has statistically significant increased *IL-6* expression compared to 24 hours ($p < 0.001$, logarithmic transformed).

DNA Damage

The results for DNA damage assay are presented in table 5. The tail lengths for Au AQC 3-5 atoms/clusters 10 mg/L, Ag AQC 2-3 atoms/cluster 1 mg/L, Ag AQC 3-5 atoms/cluster 10 mg/L and Ionosil 1 mg/L did not vary significantly from the medium control. Neither did the tail lengths for the two vehicles: Milli-Q water (vehicle for Au AQC 3-5 atoms/clusters 10 mg/L and Ag AQC 3-5 atoms/cluster 10 mg/L) and 4 mg/L silver ions (vehicle for Ag AQC 2-3 atoms/cluster) differ significantly from the medium control. No DNA damage could therefore be observed for any of these samples.

The positive diesel control at 400 mg/L resulted in statistically significant increased tail length after 3 hours of exposure ($p = 0.043$), but not after 24 and 48 hours of exposure. The comet assay specific high controls were statistically significant ($p < 0.001$). The values for both the high and low assay specific controls were at level with NFA logbook values.

Table 5. Average Tail lengths in Comet Assay. SRM2975 400 mg/L after 3 hours of exposure and the 30 µM assay control had significant increased tail lengths as marked by asterisks. None of the other samples were significant (mean ± standard deviation, n = 2-6 (2-3 replicates with 1-2 samples from each, sample sizes in parenthesis).

Treatment	3 hours	24 hours	48 hours
Medium control	30.3 ± 3.5 (5)	29.4 ± 3.7 (3)	25.1 ± 9.4 (3)
Au AQC			
3-5 atoms/cluster [10 mg/L]	31.7 ± 6.4 (6)	23.9 ± 4.8 (3)	25.5 ± 6.8 (3)
Ag AQC			
2-3 atoms/cluster [1 mg/L]	36.1 ± 10.2 (6)	32.5 ± 5.3 (2)	
3-5 atoms/cluster [10 mg/L]	33.6 ± 8.8 (6)		21.2 ± 10.2 (2)
Ionosil			
- 1 mg/L	29.3 ± 7.7 (3)	28.4 ± 3.9 (3)	23.4 ± 3.6 (3)
SRM 2975			
- 400 mg/L	41.4 ± 8.6 (6)*	27.2 ± 8.9 (3)	25.8 ± 3.5 (3)
- 200 mg/L	38.4 ± 7.7 (6)		
Milli-Q Water			
- 10%	28.6 ± 1.9 (3)		
Ag^+			
- 4 mg/L	27.1 ± 4.9 (3)		
Assay controls			
- 30 µM H_2O_2	57.3 ± 10.9(5)*		52.6 ± 0.5 (1)*
- 0 µM H_2O_2	27.6 ± 4.7 (5)		29.5 ± 4.8 (1)

Discussion

Ag 2-3 atoms/cluster and Ionosil were analysed in concentrations up to 1 mg/L. Ag 3-5 atoms/cluster and Au 3-5 atoms/cluster were analysed in concentrations up to 10 mg/L for 3, 24 and 48 hours. It was not possible to document any cytotoxicity, inflammatory, or DNA damaging effects of the test suspensions at these concentrations.

Negative results in toxicological studies should always be interpreted with caution. The essence of the problem is the inherent difficulty in ensuring that the negative result is due to non-toxic concentrations of the compound and not due to an inappropriate choice of model or detection methods.

A statistical significant reduction in IL-6 mRNA expression was observed for the two Ag AQC suspensions, but only after 48 hours of exposure. This suggests that Ag AQC might have anti-inflammatory effects and corresponds well with the findings by Kim *et al.* (2007) that colloidal silver 0.1 mg/L and 1 mg/L reduced ROS generation and cytotoxicity after naphthazarin exposure, perhaps by reducing the oxidative stress level in the cells. None of the samples in the present study displayed cytotoxicity properties. The LDH assay would thus not be able to detect any viability-preserving possible associated with the potential anti-inflammatory effect of Ag AQC.

There was a statistical significant increase in *IL-6* expression after 48-hours of exposure compared to 24-hours indicating that 48-hours of exposure *per se* is a stress inducer. The observed anti-inflammatory effect might be that Ag AQC counteracts this experiment-induced stress or that the Ag AQC affects cell growth. The effect is not observed after 3 and 24 hours because the cells stress level is already at base level or that cell growth has not progressed sufficiently, respectively. Ionosil did not show any anti-inflammatory effects, the reason could be that the highest tested concentrations expressed as surface area is very low compared to the AQC. Au is well known for its anti-inflammatory effects [Merchant 98]. However, this effect was not shown by this study, where no anti-inflammatory effects were detected for Au AQC in the 48-hours exposure. The reason may be that the Au effect is mediated by pathways different from those observed for Ag and not

detectable by the present assay. The data material is very limited and a very conservative approach to the stated hypotheses is recommended.

The vehicle for the Ag and Au AQC 3-5 atoms/cluster: The milli-Q water from NanoGap was contaminated with micro organisms and can therefore not be considered a valid vehicle control. This is not a serious problem, because none of the AQC samples had toxic effects and a vehicle effect is therefore very unlikely.

Study Design

In the assessment of the study design validity four key areas are identified: the dosage, model, exposure settings, and assays. The following pages are devoted to evaluating these key areas.

Dose

"Poison is in everything, and no thing is without poison.
The dosage makes it either a poison or a remedy."

This quote by Paracelsus PA (1493-1541) should be kept in mind when deciding dosages scheme for toxicological experiments. An experiment could be designed with one of two purposes in mind: to test dosages in concentrations comparable to the expected dosage in an occupational health scenario or to find the toxicity threshold. The present study focused on the occupational health risks. When *in vitro* studies are conducted at extreme concentrations, there is a risk that toxic responses are due to a cell-overload scenario not applicable to environmental *in vivo* exposure settings [Oberdorster *et al.* 05 B]. Environmental regulations usually require carcinogen exposure levels not to exceed levels causing cancer in one out of 10^5-10^6 individuals [Shields 06]. The Danish threshold limiting values for non-carcinogenic dust in the working environment is in the 2-10 mg/m^3 range [Jensen 02]. The commercial applications of AQC have not been disclosed. Therefore, it is not possible to discuss the appropriateness of the doses used in the present study in an occupational health risk context.

Elechiguerra *et al.* (2005) found 95% percent cytotoxicity at 10.5 mg/L for carbon coated Ag NP in the MT-2 T-cell line and their hypothesis were that the potency correlated to

percentage of free Ag metallic surface. The AQC in this study is produced with a completely free metal surface. If the Ag AQC toxicity is comparable to Ag NP, a cytotoxic response should be expected at the highest tested concentration. A reason for the lack of correlation might be because MT-2 cells are more sensitive than A549.

Monteiller *et al.* (2007) have hypothesised that poorly soluble, low toxicity nano particles should elicit inflammatory responses at concentrations of 1 – 10 cm^2 particle surface/cm^2 A549 surface. The cluster surface to A549 surface has been conservatively estimated to 40 cm^2/cm^2 (calculations in Appendix A) at 10 mg/L: a dosage that should have induced an inflammatory response. This calculation does however not take into consideration that probably only a fraction of the AQC is in direct contact with the A549 cells.

It is a weakness of this study that an independent verification of the declared concentrations of the NSP test samples was not available. The author of the present study has had access to their characterisation reports for both the Ion Silver and NanoGap products, documenting the stated content [Lopez del Rio T (NanoGap) and Stålbrand L (Ion Silver), personal communication]. It is problematic that the fates of the colloidal silver and the AQC after administration have not been investigated. Ideally, the materials should be characterized after administration to check what dosages that were actually tested [Oberdorster *et al.* 05 A]. Commercially available instruments reach a lower detection limit at 3 nm, this does not allow AQC detection [Scenihr 06].

In summary, the missing cytotoxicity might be due to differences in the toxic properties of AQC compared to NP or an unidentified loss of the AQC in the model.

The A549 model

In vitro cell line models are simplifications of the *in vivo* systems. The advantages in toxicity testing include low costs, high speed, and avoidance of the ethical considerations linked to *in vivo* investigations. Disadvantages include a risk for over-simplicity and non-involvement of the complete inflammatory responses due to lack of the important *in vivo* intracellular interactions in mono cell type models [Oberdorster *et al.* 05 A].

Cell lines might have an altered phenotype compared to *in vivo* human cells [Forbes & Ehrhardt 05]. In this study, it has been assumed that the ability to induce toxicological effects in A549 correlates to the effects observable in humans if they were exposed to the same materials. Good *in vitro-in vivo* correlation for the A549 model has been reported when assessing toxicity of fire combustion chemicals [Lestari *et al.* 05]. Only after epidemiology studies may this assumption be verified in the relation to AQC. The mutation rate in immortalised cell lines is also generally high. All exposures with the same chemical were performed within 3 passages to minimize the risk of phenotypic cell line changes.

The epithelium is the first barrier that confronts fine particles and NSP after inhalation. Oberdorster *et al.* (2005 A) recommend that both bronchial and alveolar epithelial cell lines are considered when designing *in vitro* experiments of inhalation toxicology. Deposition of inhaled NSP is due to random collisions with the air molecules and following displacement. At the nano-scale, the inertia of NSP decrease with size and density. Of 1 nm particles 90% are deposited in the nasopharyngeal compartment, 10% in the tracheo-bronchial region, and essential none in the alveolar region [Oberdorster *et al.* 05 B]. AQC are around 1 nm in diameter and the use of the alveolar A549 cell line must therefore be considered an inaccurate choice for the study of AQC inhalation toxicology if the AQC are inhaled as individual units. According to the expected deposition and assuming exposure to individual AQC units, the bronchial cell line would simulate the *in vivo* situation more accurately.

The media might oxidise the clusters. Reduced oxidation of ascorbic acid has been associated with F-12 compared to range of media including Dulbecco's Modified Eagle's, McCoy’s, and Williams medium [Kirkland *et al.* 07]. A549 cells may be grown in different media, but F-12 must be considered the safest, because oxidation is a potential risk for metals of zero valences. F-12 is also the medium recommended by the cell line distributor [LGC Promochem].

Historically, different *in vitro* genotoxicity studies of the same compounds have reported variances and even contradictory results [Schins 02, Kirkland *et al.* 07]. Results of non-toxicity should therefore be validated by testing in multiple cell lines and with a diverse

range of assays. Peripheral blood lymphocytes are the most commonly used cell line in genotoxicity testing [Schins 02]. However, it is not suitable for ultra fine particles because these particles do not penetrate the epithelial membranes: AQC free penetration of the epithelium could be expected and other cell lines might therefore be relevant models in further studies. The choice of model should reflect the expected site for bioaccumulations of Ag AQC or target sites for possible toxic effects.

Exposure settings

All experiments were conducted in 24 well plates. Compared to 96 and 6 well plates, each well contains sufficient medium and cells to sample for three assays utilized.

3-, 24-, and 48-hours exposures were chosen to cover both fast and slow induced toxic responses. The *IL-6* expression is significantly raised after 48 hours compared to 24 hours and the standard deviations are also notably increased. In the present study, it was decided to actuate all exposures after 48 hours of acclimatization of the cells and at 80% confluence. The 48-hours exposure was terminated two cell generations after 80% confluence was reached, when free logarithmic growth no longer can be expected. If the free growth assumption are invalidated uniform levels of the internal reference gene, *β-actin*, may not be expected. The results from the 48-hours study must be interpreted with caution. In future studies it should be considered to modify the design in order to account for this. If all exposures were stopped when 80% confluence is reached, the free growth assumption would be valid.

It has been documented that various types of NSP including silver adsorb to proteins [Rungby 90, Elechiguerra *et al.* 05, Oberdorster *et al*. 05 A]. FBS has caused concern when designing the present study. It was estimated that the cells did not need the mix for the 3-hours study, but it was necessary for the 24- and 48-hours studies to obtain reliable results. However, Monteiller *et al.* (2007) conducted their experiments on A549 deprived from FBS for up to 48-hours. It has not been possible to find literature validating this action anyway FBS might be an avoidable confounding factor. Again, if the dosage could be characterized after administration, the role of FBS may be disclosed.

Assays

Cytotoxicity by LDH release, IL-6 mRNA expression by QRT-PCR, and DNA damage by Comet assay are all methods recommended by Oberdorster *et al.* (2005 A) when assessing toxicity in epithelial cell lines.

Lactate dehydrogenase assay

No significant difference in the absorbance was found in the interaction control. This indicates that no interaction between AQC and LDH or the LDH kit is present. The measured absorbance is slightly outside the recommended interval in the kit, but since this study focused on the difference between the groups as opposed to the viability; this was not considered a problem.

Cytotoxicity can be detected with a range of assays including: LDH, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Trypan blue exclusion, and protein. When comparing these assays, the LDH assay has been recognized to have the lowest sensitivity [Arechabala *et al. 99,* Fotakis & Timbrell 06]. When the cytotoxicity assay is only employed as a screening test to ensure no significant cell death before running other analyses, high sensitivity is not an issue. However, in the present study cytotoxicity is an endpoint and in retrospect, a high sensitivity assay should be a preferred choice. Running multiple types of assays will increase reliability of the obtained results [Fotakis & Timbrell 06].

IL-6 mRNA QRT-PCR

The availability of cytokines is primarily regulated at the transcriptional level [Heinrich *et al.* 03]. Veranth *et al.* (2007) have shown that nano-sized particles may adsorb IL-6 proteins and produce artefacts when inflammation is determined at the protein level by the ELISA method. It was therefore decided to quantify IL-6 mRNA (pre translation) in preference to IL-6 proteins (post translation).

DNase treatment is in general not required because the RNA isolation effectively removes most of the DNA [QIAamp RNA Blood Mini Kit]. The gene sequence between the primer and probe sites covers an intron sequence. Thus, only replication of the cDNA results in a

response because polymerases replicating any remaining original DNA will not reach the site of the probe before the end of the cycle. DNase treatment reduces the complexity of the complex DNA mix and thereby improves amplification efficiency [Bustin *et al.* 05]. *IL-6* is a low-abundance target requiring high sensitivity and DNase treatment is therefore a standard routine at NFA for *IL-6* quantifications.

The results would be difficult to interpret if *IL-6* expression is quantified by QRT-PCR in samples with considerable cell death. Cell death was not detected in any of the experiments and was not a problem in this study.

QRT-PCR has become the method of choice for quantifying mRNA. A major source of variation in QRT-PCR tests is fluctuating quality of the rather unstable purified RNA. In this study, mRNA was converted into cDNA using random hexamer priming. According to Bustin *et al.* (2005), this is not a recommended method, since amplification may not be quantitative for mRNA expressed at low levels. A more precise cDNA copy could have been obtained with Oligo-dT priming. Furthermore, QRT-PCR amplification is problematic with low abundance targets (<1000 copies) like *IL-6*. The method has an inherent limitation in high efficiency amplification of low abundance targets in the complex cDNA mixture. To eliminate this problem mRNA specific priming can be used [Bustin 00, Bustin *et al. 05*].

When choosing a reference gene, it should be expressed at about the same level as target gene. The most commonly used references are ribosomal RNA, *glyceraldehyde 3-phosphate dehydrogenase*, and *ß-actin* [Bustin 00, Livak & Schmittgen 01]. Ribosomal RNA is expressed at much higher levels than *IL-6* and studies indicate that *glyceraldehyde 3-phosphate dehydrogenase* is not always expressed at a stable continuous level. *ß-actin* is the best choice for this study, although it was expressed at markedly higher levels than *IL-6* [Bustin 00].

AQC is a new class of components. If AQC illicit an inflammatory response it cannot be known if will be mediated by IL-6. A micro-array analysis would allow a quick screening of gene expression of thousands of genes. This analysis could have identified the most

appropriate marker genes. A micro-array analysis was not conducted in the present study due to its high cost. It is the opinion of the author that future inflammation studies should include this analysis to allow marker genes to be selected at a sound basis and enable a quick screening of a range of different toxicity pathways interfering with gene expression.

Comet assay

The Comet Assay is extensively used as a genotoxicity screening, and have advantages compared to other DNA damage assays because of its low costs, high sensitivity, low cell requirements, and high effectiveness. In the present study, the assay was carried out in accordance with published guidelines [Tice *et al.* 00].

The scoring was carried out in a semi-automatic system acquired in 1993. A large proportion of the Komet 3.0 software suggestions for tail lengths were found to be incorrect, and a manual, possible biased, rectification was necessary. More accurate, fully automated systems have been developed subsequently. These would remove the subjective correction in the scoring phase and be quicker in use [Frieauff *et al.* 01].

Control Particles

It is recommended to include both negative and positive control particles in all experiments. The positive control should be well defined and have well documented significant effects in the assays included in the experiment. If these conditions are met it may serve two purposes: 1) to investigate the sensitivity of the applied assays and validates the capability of the experimental design to detect a positive response. 2) It eases the comparability of the results with other studies. The highest tested dose should give toxic response in all assays [Oberdorster *et al.* 05 A].

It has not been possible to find studies using SRM 2975 in the A549 model. The consequence is that 2975 can not be considered a positive control in this study, because a positive response has never been confirmed. However, studies have been made using SRM 1650, diesel particulate matter from a heavy-duty engine with similarities to SRM 2975 [Don Porto Carero *et al.* 01, Dybdahl *et al.* 04]. It is assumed in the following that SRM 2975 and 1650 have similar toxic properties.

Cytotoxicity

No significant cytotoxicity was observed after 3 hours exposure to 4000 mg/L, 2000 mg/L, 400 mg/L, and 200 mg/L. For 24 and 48 hours of exposure were cytotoxicity analysed for 400 mg/L with no significant results.

No significant cytotoxicity has been found after exposures to SRM 1650 in the concentration interval 10 mg/L to 16000 mg/L. The exposures of up to 500 mg/L have been conducted in 2, 5 and 24 hours and were evaluated by haemocytometer counting and the trypan blue dye exclusion assay. The 48 hours exposures for concentrations up to 16000 mg/L were evaluated by AlamarBlue Assay [Dybdahl *et al.* 04, Don Porto Carero *et al.* 01]. The results of the present study are in agreement with their results.

IL-6 mRNA QRT-PCR

The diesel reference at 400 mg/L resulted in a statistically significantly increased *IL-6* expression after 3 and 24 hours, but was not significant after 48 hours.

A statistically significant induction of *IL-6* expression has previously been observed for 2, 5, and 24 hours at 500 mg/L but not at 100 mg/L [Dybdahl *et al.* 04]. This is in concordance with the results from the present study. It has not been possible to find information about *IL-6* expression in A549 after 48 hours of exposure.

DNA damage

The increase in the mean tail length after SRM 2975 exposure was in the present only significant for the diesel reference at 400 mg/L after 3 hours, the values after 24 and 48 hours of exposure were at level with the medium controls. The tail length for 200 mg/L after 3 hours of exposure was elevated but not statistically significant.

It has been documented that SRM 1650 induce significant DNA damage after 2, 5 and 24 hours of exposure at 100 mg/L and 500 mg/L measured in the comet assay [Dybdahl *et al.* 04]. 48 hours of exposure to 160 mg/L and 1600 mg/L resulted in significant increased mean tail lengths after 48 hours of exposure [Don Porto Carero *et al.* 01]. The result for the 3 hour exposure at 400 mg/L is in concordance with these findings. The elevated, but not significant tail length after 200 mg/L indicates that the sensitivity of the assay in the

present study may be reduced compared to the references or that SRM 2975 is less potent DNA damaging properties compared to SRM 1650.

In the present study, the 400 mg/L diesel exhaust particles after 24 and 48 hours exposures did not induce DNA damage. This indicates a flaw in the experiment execution or design because of the lack of concordance with previously published results for SRM 1650 [Don Porto Carero *et al.* 01, Dybdahl *et al.* 04]. However, this is in contrast to the *IL-6* expression results for the exact same samples after 24 hours of exposure which were statistically significant increased and therefore indicates a valid exposure execution and setup. The assay specific controls, when evaluating the DNA damage after the 24 and 48 hours, were at expected levels indicating that also Comet Assay was performed correctly. Furthermore, the diesel exhaust particles were visible in the fluorescence microscope, annihilating the possibility that the samples have been switched. It is the opinion of the author that the negative results must be due to the special characteristics of SRM 2975, but a repeat of the experiment is necessary to prove this hypothesis.

Recommendations for choice of positive control

Studies in A459 have been made previously with the SRM 1650 and this reference should have been purchased for this experiment to replace SRM 2975. Diesel exhaust particle utilized as positive particle controls have the general weaknesses that they are highly complex, have time-dependant decomposition and are not able to induce a positive response in the cytotoxicity assay. A standard should preferably be of a simple, highly stable composition and give positive responses in all assays. For example, crystalline alpha-silica would be a more appropriate choice for a positive particle control [Fanizza 06 *et al.* 06, Monteiller *et al.* 07, Oberdorster *et al.* 05A].

Negative control

The negative control particle was omitted from the experiment. Medium and vehicle controls were included, and a negative control particle was therefore expected to increase the experiment complexity without providing any important additional knowledge.

Statistics

The 3-hour exposure experiments were replicated three times with all samples in triplets. Cytotoxicity was measured for all 9 replicates of each concentration. Inflammation was determined for the pooled triplet and DNA damage for one sample from each triplet. The 24- and 48-hours experiments were run in singlet. Most of the group consisted of 3 measurements and these small samples result statistical analysis of low power and should raise concern about the sensitivity of the experiments.

This study is a screening for toxicity, and it is the opinion of the author that no-toxicity results only need to be verified with three independent experiments at the highest tested negative dose. The detected potential anti-inflammatory effect after 48 hours of exposure should be confirmed by two independent replicates of the experiment.

The samples in the experiments were not randomised, as is tradition at NFA, this is however not in compliance with standard assumptions of statistical analysis.

Conclusion

Ionosil, Ag AQC, and Au AQC were tested at the highest concentrations up to 1, 10, and 10 mg/L, respectively. They did not induce a cytotoxic response after 3, 24 and 48 hours of exposure and the substances are probably non-toxic in the tested dosages. The results for Ag AQC and Au AQC do not correspond with research in low solubility and low toxicity NP and Ag NP. This indicates that AQC might have different characteristics from both bulk materials and NP.

Recommendations for future work

Replication of all experiments by use of a bronchial cell line would simulate the *in vivo* situation more accurately than A549. More than one cell line should also be used to secure that the non-toxic findings are not a result of special phenotypic characteristics of the A549. It will also be interesting to test the materials in higher doses. Animal testing should be considered to assess AQC and Ionosil interactions with the complex multi-cellular *in vivo* systems.

The observed anti-inflammatory effect should be confirmed by repeating the experiment. It would be interesting to design an experiment with induced *IL-6* levels to investigate the potential and pathways of the observed anti-inflammatory effect. Other inflammation marker genes should also be analysed, preferably by a micro array analysis. *IL-6* could with advantage also be quantified at protein level. It will also be very interesting to investigate the effect at higher dosages.

Finally, it should be kept in mind that human toxicological studies are only half of the required safety analysis for new substances. Eco-toxicological analyses are equally important.

Reference list

Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: How hot is the link? Biochem Pharmacol. 2006 Nov 30;72(11):1605-21. Epub 2006 Aug 4.

Allermann L, Poulsen OM. Inflammatory potential of dust from waste handling facilites measured as IL-8 secretion from lung epithelial cells *in vitro*. Ann Occup Hyg. 2000 Jun 44(4):259-69.

Arechabala B, Coiffard C, Rivalland P, Coiffard LJ, de Roeck-Holtzhauer Y. Comparison of cytotoxicity of various surfactants tested on normal human fibroblast cultures using the neutral red test, MTT assay and LDH release. J Appl Toxicol. 1999 May-Jun 19(3):163-5.

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2000 Oct 25(2):169-93.

Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR - a perspective. J Mol Endocrinol. 2005 Jun 34(3):597-601.

Cakmak GD, Schins RP, Shi T, Fenoglio I, Fubini B, Borm PJ. In vitro genotoxicity assessment of commercial quartz flours in comparison to standard DQ12 quartz. Int J Hyg Environ Health. 2004 Feb;207(2):105-13. [abstract only]

Croce MV, Colussi AG, Price MR, Segal-Eiras A. Identification and characterization of different subpopulations in a human lung adenocarcinoma cell line (A549). Pathol Oncol Res. 1999;5(3):197-204.

Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ. Nanotoxicology.Occup Environ Med. 2004 Sep;61(9):727-8.

Danscher G, Stoltenberg M. Silver enhancement of quantum dots resulting from (1) metabolism of toxic metals in animals and humans, (2) *in vivo*, *in vitro* and immersion created zinc-sulphur/zinc-selenium nanocrystals, (3) metal ions liberated from metal implants and particles. Prog Histochem Cytochem. 2006;41(2):57-139. Epub 2006 Aug 7.

Don Porto Carero A, Hoet PH, Verschaeve L, Schoeters G, Nemery B. Genotoxic effects of carbon black particles, diesel exhaust particles, and urban air particulates and their extracts on a human alveolar epithelial cell line (A549) and a human monocytic cell line (THP-1). Environ Mol Mutagen. 2001;37(2):155-63.

Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, MacNee W, Stone V. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol. 2005 Oct 21;2:10.

Dybdahl M, Risom L, Bornholdt J, Autrup H, Loft S, Wallin H. Inflammatory and genotoxic effects of diesel particles *in vitro* and *in vivo*. Mutat Res. 2004 Aug 8;562(1-2):119-31.

Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, Lara HH, Yacaman MJ. Interaction of silver nanoparticles with HIV-1. J Nanobiotechnology. 2005 Jun 29;3:6.

Fanizza C, Ursini CL, Paba E, Ciervo A, Di Francesco A, Maiello R, De Simone P, Cavallo D. Cytotoxicity and DNA-damage in human lung epithelial cells exposed to respirable alpha-quartz.

Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res. 2000;2(1):33-46. Epub 2001 Jan 15.

Forbes B, Ehrhardt C. Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm. 2005 Jul 60(2):193-205. Epub 2005 Apr 21.

Fotakis G, Timbrell JA. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride.Toxicol Lett. 2006 Jan 5;160(2):171-7. Epub 2005 Aug 18.

Frieauff W, Hartmann A, Suter W. Automatic analysis of slides processed in the Comet assay. Mutagenesis. 2001 Mar 16(2):133-7.

Goodman CM, McCusker CD, Yilmaz T, Rotello VM.Goodman CM, McCusker CD, Yilmaz T, Rotello VM.Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjug Chem. 2004 Jul-Aug 15(4):897-900.

Garnett MC, Kallinteri P. Nanomedicines and nanotoxicology: Some physiological principles. Occup Med (Lond). 2006 Aug 56(5):307-11.

Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst. 1973 Nov 51(5):1417-23.

Goldsmith MR, George CB, Zuber G, Naaman , Waldeck DH, Wipf P, Berantan DN. The chiroptical signature of achiral metal clusters induced by dissymmetric adsorbates. Phys Chem Chem Phys. 2006 Jan 7;8(1):63-7.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods. 2001 Dec;25(4):386-401.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J. 2003 Aug 15;374(Pt 1):1-20.

Jensen Jens. At-vejledning – grænseværdier for stoffer og materialer. København: Arbejdstilsynet. 2002 Oct.

Kagan VE, Bayir H, Shvedova AA. Nanomedicine and nanotoxicology: two sides of the same coin.Nanomedicine. 2005 Dec 1(4):313-6.

Karlsson HL, Ljungman AG, Lindbom J, Moller L.Comparison of genotoxic and inflammatory effects of particles generated by wood combustion, a road simulator and collected from street and subway. Toxicol Lett. 2006 Sep 10;165(3):203-11.

Kim DW, Hong GH, Lee HH, Choi SH, Chun BG, Won CK, Hwang IK, Won MH. Effect of colloidal silver against the cytotoxicity of hydrogen peroxide and naphthazarin on primary cultured cortical astrocytes. Int J Neurosci. 2007 Mar 117(3):387-400.

Kirkland D, Pfuhler S, Tweats D, Aardema M, Corvi R, Darroudi F, Elhajouji A, Glatt H et al. How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. Mutat Res. 2007 Mar 30;628(1):31-55. Epub 2007 Jan 13.

Kuo CL, Clancy P. Melting and freezing characteristics and structural properties of supported and unsupported gold nanoclusters. J Phys Chem B. 2005 Jul 109(28):13743-54.

Kurath M, Maasen S. Toxicology as a nanoscience? - disciplinary identities reconsidered. Part Fibre Toxicol. 2006 Apr 28;3:6.

Lima BS, Van der Laan JW. Mechanisms of nongenotoxic carcinogenesis and assessment of the human hazard. Regul Toxicol Pharmacol. 2000 Oct 32(2):135-43.

Livak JK, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec 25(4):402-8.

Lestari F, Hayes AJ, Green AR, Markovic B. *In vitro* cytotoxicity of selected chemicals commonly produced during fire combustion using human cell lines. Toxicol *In vitro*. 2005 Aug 19(5):653-63.

LGC Promochem [homepage on the internet]. A549 product description. Teddington, UK: The company; c2006 [cited 2007 May 25]. LGC Promochem: cell biology collection [About two screens]. Available from: http://www.lgcpromochem-atcc.com/common/catalog/numSearch/numResults.cfm?atccNum=CCL-185#Propagation.

May WE, Trahey NM [editors]. Certificate of analysis - standard reference material 2975 diesel particulate matter (industrial forklift). The National Institute of standards and technology. 07 Nov 2000. Available at: https://srmors.nist.gov/certificates/2975.pdf?CFID=9417083&CFTOKEN=826effc7022e9cc5-3E211486-BCBB-0BF7-9349A478A366C0AD&jsessionid=b43019a67a4d1359e6c4

Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, Philbert MA, Ryan J *et al*. Safe handling of nanotechnology. Nature. 2006 Nov 16;444(7117):267-9.

Merchant B. Gold, the noble metal and the paradoxes of its toxicology. Biologicals. 1998 Mar;26(1):49-59.

Monteiller C, Tran L, Macnee W, Faux SP, Jones AD, Miller BG, Donaldson K. The Pro-Inflammatory Effects Of Low Solubility Low Toxicity Particles, Nanoparticles and Fine Particles, on Epithelial Cells *In vitro*: the Role of Surface Area. Occup Environ Med. 2007 Apr 18; [Epub ahead of print].

NanoGap [Home page on the internet]. Spain: The company; [Cited 2007 May 29] Edificio FEUGA C/ Lope Gómez de Marzoa s/n. Available from: http://www.nanogap.es.

Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B *et al*. Principles for characterizing the potential human health effects from exposure to nanomaterials: Elements of a screening strategy. Part Fibre Toxicol. 2005 A Oct 6;2:8.

Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles.Environ Health Perspect. 2005 B Jul ;113(7):823-39.

Ono N, Oshio S, Niwata Y, Yoshida S, Tsukue N, Sugawara I, Takano H, Takeda K. Prenantal exposure to diesel exhaust impairs mouse spermatogenesis. Prenatal exposure to diesel exhaust impairs mouse spermatogenesis.

Peters A, Veronesi B, Calderon-Garciduenas L, Gehr P, Chen LC, Geiser M, Reed W, Rothen-Rutishauser B *et al*. Translocation and potential neurological effects of fine and ultrafine particles a critical update. Part Fibre Toxicol. 2006 Sep 8;3:13.

Qiagen. QIAamp RNA blood Mini Handbook. Jan 1999. Cat. No. 52304. [Manufactures instructions]

Roche Applied Sciences. Cytotoxicity Detection Kit (LDH). Mannheim, Germany: Roche Diagnostics Gmbh. July 2005. Cat. No. 11644793001. [Manufacturers instructions]

Rungby J. An experimental study on silver in the nervous system and on aspects of its general cellular toxicity. Dan Med Bull. 1990 Oct 37(5):442-9.

Saber AT, Bornholdt J, Dybdahl M, Sharma AK, Loft S, Vogel U, Wallin H. Tumor necrosis factor is not required for particle-induced genotoxicity and pulmonary inflammation. Arch Toxicol. 2005 Mar;79(3):177-82. Epub 2004 Nov 11.

Saber AT, Jacobsen NR, Bornholdt J, Kjaer SL, Dybdahl M, Risom L, Loft S, Vogel U, Wallin H. Cytokine expression in mice exposed to diesel exhaust particles by inhalation. The role of tumor necrosis factor. Part Fibre Toxicol. 2006 Feb 20;3(1):4.

Scenihr: Scientific Committee on Emerging and Newly Identified Health Risks. The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies. Health & consumer protection directorate-general, European Commission: Directorate C – Public Health and Risk Assessment. 10th plenary meeting: 2006 Mar 10.

Schins RPF. Mechanisms of genotoxicity of particles and fibers. Inhal Toxicol. 2002 Jan;14(1):57-78.

Shields PG. Understanding population and individual risk assessment: the case of polychlorinated biphenyls. Cancer Epidemiol Biomarkers Prev. 2006 May;15(5):830-9.

Sigma-Aldrich. Triton X-100. Apr 1999. CAS #: 9002-93-1. [Product information]. Available at: http://www.sigmaaldrich.com/sigma/product%20information%20sheet/t8532pis.pdf

Stuer-Lauridsen F, Kamper A, Borling P, Petersen GI, Hansen SF, Baun A. Kortlægning af produkter der indeholder nanopartikler eller er baseret på nanoteknologi. Miljøministeriet, Miljøstyrelsen. Kortlægning af kemiske stoffer i forbrugerprodukter. 2007 Jan 81:1-90.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen. 2000 35(3):206-21.

Yamazaki T, Yamazaki A, Hibino Y, Chowdhury SA, Yokote Y, Kanda Y, Kunii S, Sakagami H, et al. Biological impact of contact with metals on cells. In Vivo. 2006 Sep-Oct;20(5):605-11.

Wang JJ, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO(2) particles in cultured human lymphoblastoid cells.Mutat Res. 2007 Apr 628(2):99-106.

Xu C, Su J, Xu X, Liu P, Zhao H, Tian F, Ding Y. Low temperatures CO Oxidation over unsupported nanoporous gold. J Am Chem Soc. 2007 Jan 129(1):42-3.

Appendix A

If it is estimated that a cluster is spherical and has the diameter of 2 atoms, this equals 0.6 nm. A cluster consists that of 4 Au atoms weigh 787.87 atomic mass units, equal to $7.64 \cdot 10^{17}$ atoms/mg (1998 weight definitions). Au clusters are the densest atoms of the tested materials and gives the most conservative estimate. The surface area per mass can now be calculated:

Diameter = 0.6 nm

Surface area $4 (D/2)^2 \cdot \pi = 4 \cdot (0.6 \text{ nm} / 2)^2 \cdot \pi$ = 1.131 nm^2/cluster

Au cluster weight = n · atom weight

= 4 atoms/cluster · 196.967 U/atom = 787.868 U/cluster

Cluster density = 1.66053873E-21 mg/U · 787.868 U/cluster

= $1.308 \cdot 10^{-18}$ mg/cluster

Clusters pr mass = 1 mg/mg / $1.308 \cdot 10^{-18}$ mg/cluster

= $7.6435 \cdot 10^{17}$ clusters/mg

Surface area pr mass = $7.6435 \cdot 10^{17}$ clusters/mg * 1.131 nm^2/cluster

= $8.645 \cdot 10^{17}$ nm^2 / mg / 10^{14} nm^2/cm^2

= 8644.70 cm^2/mg

Surface area pr volume 10 mg/L= 8644.70 cm^2/mg 10 mg/L

= $9 \cdot 10^1$ cm^2 /mL

Surface area A549 in well = $(1.6/2)^2 \cdot \pi$ = <u>2.011 cm^2/well</u>

Surface area pr cm^2 A549 = $9 \cdot 10^1$ cm^2 /mL · 1 mL / 2.011 cm^2/well

= <u>43 cm^2/cm^2</u>