Fraunhofer
ITEM

**Final Report**

# Measurement of the DNA-damaging and cytotoxic potential of synthetic amorphous silica (NM-200) in cultured primary rat alveolar macrophages

**Fraunhofer ITEM Study No. 17N11540**

**Original 2 of 2**

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Study director (1 copy)

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# List of abbreviations

| | |
|---|---|
| $Al_2O_3$ | Aluminium oxide |
| ALS | Alkali-labile site |
| AM | Alveolar macrophage |
| BAL | Bronchoalveolar lavage |
| DSB | DNA double-strand breaks |
| EMS | Ethyl methanesulfonate |
| Exp. | Experiment |
| hOGG1 | Human 8-oxoguanine-DNA-glycosylase 1 |
| FBS | Foetal bovine serum |
| $KBrO_3$ | Potassium bromate |
| N | Number of independent cultures/slides |
| $Na_2EDTA$ | Ethylenedinitrilotetraacetic acid disodium salt dihydrate |
| NaOH | Sodium hydroxide |
| NM-200 | Synthetic amorphous silica |
| ROS | Reactive oxygen intermediates/species |
| SD | Standard deviation |
| SCG | Single-cell gel |
| SSB | DNA single-strand-breaks |
| U | Unit |

# 1 Preface

## 1.1 General information

| | |
|---|---|
| **Fraunhofer ITEM Study No.:** | **17N11540** |
| **Study Location:** | Fraunhofer ITEM, L.122A, L.123, L.124 |
| **Test Item:** | Synthetic amorphous silica (NM-200) |
| **Study Staff:** | |
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| **Study Dates:** | |
| Experimental starting date: | November 07, 2011 |
| Experimental completion date: | November 24, 2011 |
| Final report: | See page 1 |

## 1.2 Objective

*In vitro* particle studies focus in particular on pulmonary cells like alveolar macrophages (AM) or epithelial cells which are in the first line of defence against inhaled particles. Especially AMs engulf particles and produce biological mediators including reactive oxygen intermediates (ROS = reactive oxygen species) or pro-inflammatory cytokines such as TNFα.

During the process of respiratory burst, molecular oxygen is converted into ROS. ROS include super oxide anion radical ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\cdot}OH$). These extracellular highly reactive oxygen species cause many biological effects such as destruction of bacterial cells, promotion of inflammation and modulation of the immune reaction, but may also damaged cellular structures like membrane, proteins, and also the DNA.

Objective of the present study was to investigate the potential of synthetic amorphous silica (NM-200) to induce DNA-damage and in particular oxidative DNA-damage in cultured primary rat alveolar macrophages, whereby alveolar macrophages represent a first line of defence against inhaled particles. It was of special interest, whether the study results are in line with the respective data obtained *in vivo* and whether this model system exhibits the potential to serve as a meaningful *in vitro* test system to screen new nanomaterials.

### 1.3 Guideline for conduct of study

This study was performed at the Fraunhofer ITEM, Department of Genetic Toxicology. The Comet assay was conducted following the valid standard operating procedures of Fraunhofer ITEM and the principles outlined in: Tice et al. (2000) Single Cell Gel/Comet assay: Guidelines for In Vitro und In Vivo Genetic Toxicology Testing. Environ. Mol. Mutagen. 35(3):206-221. The used hOGG1-modified variant of the Comet assay is based on Smith et al. (2006). The work was not inspected by the Quality Assurance Unit (QAU) of the Fraunhofer ITEM and no report audit was performed. Nevertheless, the study was conducted in accordance with the basic requirements of Good Laboratory Practice GLP (German Chemicals Law § 19a, Appendix 1, July 2, 2008). The study followed the regulations of the German Animal Protection Law (Tierschutzgesetz, May 18, 2006).

## 2 Material

### 2.1 Test item

| | |
|---|---|
| **Name:** | Synthetic Amorphous Silica (NM-200) |
| **CAS No.:** | 112926-00-8 |
| **Molecular weight:** | 60.74 g/mol |
| **Appearance:** | White, fluffy, amorphous powder |
| **Lot No.:** | Masterbatch |
| **Purity:** | ≥ 97.0 % $SiO_2$, 2.8 % $Na_2SO_4$ |
| **Specific surface area according to BET:** | 230 $m^2/g$ |
| **Storage:** | At room temperature in a well closed container, dry and dark place |
| **Expiry Date:** | March 31, 2013 |

NM-200 was supplied by the European Commission Joint Research Center (JRC, Ispra, Italy). The identity and characterization of the test item was documented by the supplier. A sample of the test item was sent back to the supplier for re-analysis. The supplier is responsible for the identity and purity of the test item. Safety precaution, storage and handling of the test item NM-200 was done according to the supplier's information. For certificates of analysis and re-analysis (copies) see Appendix 2.

Before preparation of the stock solutions, NM-200 was sterilized by heating to 180°C for 1 h. Stock solutions (1 mg/ml) were then prepared in the respective incubation medium (see 2.2 Reference item, vehicle control). To minimize agglomeration of the NM-200 particles, the NM-200 stock

solutions were stirred for 24 h at room temperature in the dark prior to cell incubation. This procedure enabled homogeneous dosing suspensions. Nevertheless, high degree of agglomeration of existing test item aggregates was noted with measured particle sizes in the incubation medium containing 10% FCS of $x_{10.3}$ = 3.854 µm, $x_{50.3}$ = 8.130 µm, and $x_{90.3}$ = 16.475 µm, respectively (see Appendix 4, particle characterization).

Fraunhofer IKTS (Dresden, Germany), as a DIN EN ISO/IEC 17025:2005 accredited laboratory performed some physico-chemical characterization of the NM-200 stock solutions. Particle characterization was originally performed for the chromosomal aberration test with NM-200, ITEM study No. 17G11019, which used the same incubation medium and preparation method for the test item stock suspensions (however a fivefold higher stock solutions) comprising particle size distribution after dispersion (Laser light diffraction; Mastersizer 2000 [Malvern Instruments GmbH]) and zeta potential (Microelectrophoresis; Zetasizer Nano ZS [Malvern Instruments GmbH]). Particle dissolution in the two different incubation media (10% and 2% FBS) was analyzed in the respective supernatants by ICP OES (Ultima [HORIBA Jobin Yvon]) after centrifugation of the NM-200 stock solutions directly after the 24 h stirring period and 24 h later (for data see above and Appendix 4).

The test item will be physico-chemically analyzed more extendedly within the OECD Sponsorship Programme for $SiO_2$. The stability of the test item per se and of the test item in the incubation medium was not determined as part of this study. Analysis of the test item concentrations in the incubation media were not performed either.

## 2.2 Reference items

**Reference item # 1:**

Growth medium and vehicle control:

**Name:** Dulbecco`s-minimum essential medium (D-MEM) with high glucose (4.5 g/l), GlutaMaxTM and sodium pyruvate (110 mg/l) (GIBCO/Invitrogen, Karlsruhe, Germany), supplemented with 10 % foetal bovine serum (FBS, BIOCHROM AG, Berlin, Germany) and penicillin (100 units/ml) / streptomycin (100 µg/ml) ((GIBCO/Invitrogen).

Composition of the resulting growth and incubation medium:

500 ml D-MEM
50 ml FBS (synonym FCS, Biochrom AG, Berlin, Germany)
50.000 µg streptomycin sulfate*
50.000 U penicillin G, sodium salt*

*Added to the growth medium as 5 ml of a ready to use solution of 10 mg/ml streptomycin sulfate and 10.000 U penicillin G, sodium salt (Biochrom AG)

**Reference item # 2:**

Particulate negative control:

| | |
|---|---|
| **Name:** | Aluminium oxide |
| **Sum formula:** | $Al_2O_3$ |
| **Supplier:** | Sigma-Aldrich (Taufkirchen, Germany) |
| **CAS No.:** | 1344-28-1 |
| **Molecular weight:** | 101.96 g/mol |
| **Particle size:** | Geometrical mean, weighing by mass: 4.09 ± 1.77 µm |
| **Purity:** | p.a. |
| **Appearance:** | Solid, powder |
| **Colour:** | White |
| **Storage conditions:** | It was stored dry and sealed at room temperature. |

**Reference item # 3:**

Particulate positive control:

| | |
|---|---|
| **Name:** | Quartz DQ12, middle size Dörentrup (87% α-quartz, 13% amorphous silica) |
| **Sum formula:** | $SiO_2$ |
| **Supplier:** | Bergbauforschung Essen, Germany |
| **CAS No.:** | 99439-28-8 |
| **Molecular weight:** | 60.08 g/mol |
| **Particle size:** | Geometrical mean, weighing by mass: 2.99 ± 1.53 µm |
| **Appearance:** | Solid, crystals |
| **Colour:** | Colourless |
| **Storage conditions:** | It was stored dry and sealed at room temperature. |

**Reference item # 4:**

Positive control for oxidative DNA-damage:

| | |
|---|---|
| **Name:** | Potassium bromate ($KBrO_3$) |
| **Sum formula:** | $KBrO_3$ |
| **Supplier:** | Merck, Darmstadt, Germany |
| **CAS No.:** | 7758-01-2 |
| **Molecular weight:** | 167.01 g/mol |
| **Appearance:** | Solid, crystals |
| **Colour:** | White |
| **Storage conditions:** | It was stored at 4 – 6°C, with stock solutions being prepared directly before use. |

# 3 Test system

In this study cultured primary rat alveolar macrophages from bronchoalveolar lavage (BAL) were used as a model system to investigate in parallel DNA-damage (hOGG1-modified Comet assay) and membrane integrity/cytotoxicity (LDH-release) of NM-200 *in vitro*. Due to their high sensitivity towards particle and fibre treatment, primary rat alveolar macrophages are a useful cell type for Comet assay studies with particulate and fibrous test items.

# 4 Study performance

## 4.1 Bronchoalveolar lavage for isolation of primary rat alveolar macrophages

About 12 weeks old healthy untreated Wistar rats (male, strain Crl:WU) were killed painlessly. Lungs were lavaged (bronchoalveolar lavage, BAL) five times, each time with 5 mL of 0.9 % NaCl (room temperature). Cells were recovered by centrifugation (256 x g for 10 min at 4 °C), re-suspended in growth medium (see 2.2) and counted. The resulting BAL cells were then plated at a density of 1.5 x $10^5$ cells in 0.5 ml of growth medium per well on 24-well plates with hydrophobic culture surface (NUNC, Langenselbold, Germany).

## 4.2 Culture and exposure of alveolar macrophages to test and reference items

Cells were pre-cultured for 24 h in 500 µl growth medium consisting of D-MEM with high glucose (4.5 g/l), GlutaMax™, and sodium pyruvate (110 mg/l), supplied by GIBCO/Invitrogen (Karlsruhe,

Germany), supplemented with 5 ml of a ready to use solution of 10.000 µg/ml streptomycin sulphate and 10.000 U penicillin G, sodium salt per 500 ml growth medium and 10 % FBS (both Biochrom AG) at 37°C, 5 % $CO_2$ and 90 % humidity using an incubator.

For cell exposure, twofold concentrated particle suspensions (for preparation of NM-200 stock solutions see 2.1) containing the desired particle mass per well were prepared by diluting the stock solutions (1 mg/ml) with growth medium. Five hundred µl of the final particle suspensions were added to the cultures without medium exchange, thus resulting in a total incubation volume of 1 ml per well. The particulate negative ($Al_2O_3$) and positive (quartz DQ12) controls were directly suspended in growth medium by using a short sonication procedure in a sonication water bath. Two cultures/wells per treatment condition were incubated with the different test item concentrations and the reference items for 4 or 24 h under cell type specific condition at 37°C, 5 % $CO_2$ and 90 % humidity using an incubator.

Because of their cytotoxicity, $KBrO_3$ and quartz DQ12 were always added to the respective cultures for only 4 h. In addition, there was a need for a medium control and a high control for the LDH-assay. As medium control (background control), wells without cells, but containing growth medium were used. As the high control, BAL cells were treated for about 10 – 15 min with 1 % Triton X-100 to determine the maximum amount of releasable LDH-enzyme activity and to calculate % LDH-release/cytotoxicity.

After the end of exposure, aliquots of 600 µl of the incubation media were taken for the LDH-assay and the macrophages were then placed on ice for 10 min to enable cell detachment without trypsination.

## 4.3 Concentrations of the test item NM-200 and the reference items

To better enable correlation of *in vivo* and *in vitro* data and to obtain meaningful results it is desirable to approximate the *in vitro* mass concentrations to the deposition rates *in vivo*.

For calculation of meaningful *in vitro* mass concentrations the following parameters and numerical values, including parameters taken from the Fraunhofer ITEM study No. 02G11001, were used:

- Minute volume for the rat: 0.2 L/min
- Duration of inhalation in study No. 02G11001: 3.600 min [360 min (6 hours per day) x 10 days (five days per week for two weeks)]
- *In vivo* aerosol concentrations in study No. 02G11001: 1, 5, and 25 mg/m$^3$ NM-200
- Supposed deposition rate: 5 %
- A lung surface of the rat of 4.000 cm$^2$

By using these parameters approximated mass per $cm^2$ deposition values in the lung were calculated, amounting to 9 ng/$cm^2$ (1 mg/$m^3$), 45 ng/$cm^2$ (5 mg/$m^3$), and 225 ng/$cm^2$ (25 mg/$m^3$), respectively. To subsequently define the mass concentrations of NM-200 for *in vitro* use, mass per $cm^2$ culture surface, based on the deposition *in vivo*, was used. For *in vitro* use, it was decided to round the *in vivo* values to 10, 50, and 250 ng/$cm^2$ NM-200 and to use them as main concentrations. However, based on historical data and a current pre-test, the technical positive control DQ12 was used for 4 h at a particle mass concentration of 25 µg/$cm^2$ for both the 4 and 24 h exposure period. For the 24 h exposure period quartz DQ12 was added for 4 h after 20 h of pre-incubation to get an appropriate DNA-damage answer. Based on a pre-test on cytotoxicity, 10 µg/$cm^2$ (4 h of incubation) and 2.5 µg/$cm^2$ (24 h of incubation) were used as cytotoxic maximum concentration (< 50% cytotoxicity) for the test item NM-200, as otherwise, without at least one cytotoxic concentration, the study might be evaluated as genotoxicity study with limited significance due to lack of a sufficiently high, cytotoxic concentration. It seemed reasonable to use these maximum concentrations also for the negative reference item $Al_2O_3$ to try to differentiate between potential particle- and material-specific genotoxic effects.

Based on historical data, the positive control for oxidative DNA-damage, $KBrO_3$, was used in growth medium at a final concentration of 0.5 mM in all experiments. For the long term treatment (24 h), $KBrO_3$ was added 4 h before the end of exposure (20 h of pre-exposure of the cells).

## 4.4 hOGG1-modified Comet assay

DNA-strand breaks and oxidative DNA damage (8-OH-dG) were analysed in primary rat alveolar macrophages after 24 h of pre-culture and 4 or 24 h of incubation with the test and reference items, using the human 8-hydroxyguanine DNA-glycosylase 1 (hOGG1)-modified alkaline Comet assay, based on the alkaline version of the Comet assay.

The single-cell gel (SCG)/Comet assay represents a test principle for identifying agents with genotoxic activity in mammalian cells and for further characterizing types of DNA damage. The assay is based on electrophoretic mobility of DNA fragments in agarose gels on slides. Evaluation unit is the single cell. The alkaline version (pH>13) of the Comet assay is able to detect DNA single- (SSB) and double-strand breaks (DSB), DNA-DNA and DNA-protein cross-links, alkali-labile sites (ALS) and SSBs associated with incomplete DNA excision repair. DNA damage is detected as DNA migrating out of the cell nucleus during single-cell electrophoresis, resembling a comet tail. Tail length and tail intensity are proportional to the number of DNA strand breaks. The Comet assay may employ cultures of established cell lines, cell strains or primary cell cultures, and also primary cells *ex vivo*; in this study cultured primary rat alveolar macrophages (BAL cells) were used as a model system.

There exist various modifications of the methodology in order to specify positive data of the alkaline version of the Comet assay, including the hOGG1-modified Comet assay to detect oxidative DNA-damage in terms of 8-hydroxy-2-deoxy-guanosine (8-OHdG), an important marker of DNA base oxidation. In brief, in the present study, after incubation with the test and reference items and detachment from the culture surface by cold-treatment, two cell aliquots per treatment condition (150.000 cells in a volume of 1 ml) were centrifuged, re-suspended in pre-heated 0.75 % low melting agarose, applied to agarose pre-coated slides, and lysed overnight at 4°C to liberate the DNA. One of the two slides per treatment condition was subsequently be incubated for 15 min with 0.16 U/ml of hOGG1 (incubation volume 100 µl) to detect oxidative DNA-damage. The other slides only received enzyme buffer. In both cases, DNA-unwinding (to obtain single stranded DNA and to express ALS as SSB, 20 min) and electrophoresis (20 min, 24 V, 290 - 300 mA) were done on ice in 4°C cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). After electrophoresis, DNA was stained with ethidium bromide (20 µg/ml) and analyzed using the Comet assay III Software (Perceptive Instruments, UK). As the main endpoint the tail intensity of 100 nuclei per slide and treatment (with or without hOGG1 incubation) was determined per experiment with 3 independent experiments in total. The tail intensity is a direct measure for the number of broken pieces that can be standardized among various studies. Nevertheless, tail length and tail moment were also determined and documented in the raw data. A significant increase in tail intensity on the hOGG1-treated slides, as compared to the slides treated with enzyme buffer only, is indicative for the occurrence of the oxidative base lesion 8-OHdG. All methodological steps, following cell detachment, were performed under red light to avoid unspecific DNA-damage due to UV-irradiation.

**Table 1**: Study design for the Comet assay experiments with NM-200

| Treatment | Concentrations | Incubation times |
|---|---|---|
| Growth medium | - | 4 and 24 h |
| $Al_2O_3$ | 10 µg/cm$^2$ | 4 h |
| $Al_2O_3$ | 2.5 µg/cm$^2$ | 24 h |
| $KBrO_3$ | 500 µM | 4 h* |
| DQ12 | 25 µg/cm$^2$ | 4 h* |
| NM-200 | 10, 50, 250 ng/cm$^2$ and 10 µg/cm$^2$ | 4 h |
| NM-200 | 10, 50, 250 ng/cm$^2$ and 2.5 µg/cm$^2$ | 24 h |

* See 4.3 for further information concerning the particulate positive control DQ12 and $KBrO_3$

## 4.5 Determination of lactate dehydrogenase (LDH) activity

Release of LDH is widely used to assess changes in cell viability due to test item exposure. LDH is an intracellular protein that is released into the cell culture supernatant after disintegration of the cell membrane. Its activity is measured by conversion of a tetrazolium salt to formazan which is formed proportionally to the amount of necrotic cells.

Changes of LDH activity were determined in the culture supernatants of the Comet assay (see 4.4) to be able to differentiate between unspecific DNA-damaging effects due to cytotoxic activity and particle specific DNA-damage. Two cultures per treatment were analyzed and each sample was measured in triplicate. For determination of LDH-activity a commercially available enzymatic assay, obtained from Roche (Mannheim, Germany) was used. One hundred µl of each cell supernatant were transferred to a well of a 96-well plate and mixed with 100 µl of freshly prepared LDH-reagent. The mixture was then incubated in the dark for 25-35 min at room temperature. Absorbance was finally measured at 490 nm using an ELISA reader. Absorbance at 650 nm served as the background reference wavelength.

Real absorbance and thus LDH-enzyme activity in the samples was calculated by subtracting the 650 nm background values from the measured 490 nm data. The average absorbance values of the two samples per treatment each measured in triplicate were subsequently calculated. All absorbance values were further corrected by an average medium control value without cells. Percent LDH-release/cytotoxicity was finally calculated over the corrected high control value (1% Triton X-100, complete LDH-release), multiplied by 100 % using the corrected sample values.

## 4.6 Disposal

Biological waste materials was collected in special containers and disposed of in compliance with local, state and federal regulations. Any other used materials was also collected and disposed according to law regulations.

## 4.7 Calculation of the results and statistical analyses

Data in the figures and tables are given as means ± standard deviation (SD). Statistical analysis was performed by using the SigmaStat 3.1.1 software in combination with the SigmaPlot 9.0 software (Systat Software GmbH, Erkrath, Germany). Differences between NM-200- and particulate reference item-exposed samples and the vehicle control (unpaired testing) or the respective slides without hOGG1-incubation (paired testing) were considered as statistically significant at the level of $*p \leq 0.05$. Data were judged as highly significant if $**p \leq 0.01$ or $***p \leq 0.001$.

# 5 Storage and retention of records and materials

Irrespective of the fact, that the present study was performed as non-GLP study, the overall project consisted also of several GLP-studies. For this reason archiving of the present study will be handled like for the related GLP-studies. After completion of the study and issuance of the final report, one original of the study plan, the raw data, one original of the final report, a sample of the test item (already archived in the frame of Fraunhofer ITEM study No. 02G10031), and all other relevant materials will be transferred to the archives of Fraunhofer ITEM. All records and materials will be properly indexed, catalogued and stored for a minimum period of time in compliance with the Principles of Good Laboratory Practice (15 years for records). The sponsor has to inform Fraunhofer ITEM if further archiving is required.

# 6 Results

## 6.1 hOGG1-modified Comet assay

There are 3 different relevant endpoints to evaluate the hOGG1-modified Comet assay. All three endpoints tail intensity (TI), tail length (TL), and tail moment (TM) were measured and documented in the raw data of the study. However, in the results section and in Appendix 1 only the tail intensity (TI, %DNA in tail/proportion of damaged DNA) is presented and served as a basis for evaluation of the DNA-damaging potential of NM-200 (see Figures 1 and 3 and Appendix 1, Tables 2 and 4). TI is currently the most accepted endpoint for the Comet assay in Genetic Toxicology. In contrast to TM, which is calculated from TL and TI, TI is a direct measure and is linearly related to DNA break frequency up to about 80 % in tail, which defines the useful range of the assay. In addition, as TI is a clearly defined and direct measure, TI data can be better compared between different Comet assay analysis systems.

Different reference items were included in the hOGG1-modified Comet assay:

- **Vehicle control**: As vehicle/negative control, cells were included, which only received growth medium. In the present study, the vehicle controls demonstrated an acceptably low mean TI (without hOGG1 treatment) of 0.35 ± 0.095 % (4 h of incubation) and 0.70 ± 0.258 % (24 h of incubation), thus documenting accurate performance of the test and adequate viability of the test system. Vehicle control slides incubated with hOGG1 demonstrated slightly higher mean TI than without hOGG1 treatment amounting to 0.57 ± 0.287 % (4 h of incubation) and 1.30 ± 1.256 % (24 h of incubation), indicating some endogenous oxidative stress, in particular obvious in 1 experiment after 24 h of incubation (see Tables 2 and 4).

- **Potassium bromate ($KBrO_3$)**: $KBrO_3$ (0.5 mM for 4 h) served as a positive control for induction of DNA-base oxidation. In the presence of reduced glutathione in cells, $KBrO_3$ induces in particular the oxidative DNA-base lesion 8-hydroxy-2-deoxy-guanosine (8-oxo-G). However, oxidative DNA-base modifications can only be detected in the Comet assay, if the lesion-specific repair enzymes like hOGG1 are used, which, by trying to repair the lesions, induce DNA-strand breaks, which are then detectable. For this reason $KBrO_3$-induced oxidative DNA-base modifications are only seen after incubation of the slides with hOGG1. In the present study, there was no significant difference between the $KBrO_3$-treated and control cells, with mean TI of 0.37 ± 0.090 and 1.13 ± 0.516 % for the 4 and 24 h experiments, as compared to 0.35 ± 0.095 % and 0.70 ± 0.258 % for the vehicle controls, respectively. Thus, no marked direct DNA-strand break induction was observed. In contrast, after incubation with hOGG1, highly significant or marked increase in TI after $KBrO_3$ exposure (34.95 ± 5.113 and 26.44 ± 13.588 %) was noted, if compared to the respective slides without enzyme treatment and the vehicle control cultures with hOGG1 incubation. Human OGG1-induced increase in mean TI indicated sufficient activity of the positive control $KBrO_3$ and the enzyme batch used and pointed to adequate sensitivity of the cell system used towards oxidative DNA-base lesions. The incubation time with $KBrO_3$ was for both incubation times (4 and 24 h) 4 h to assure an appropriate effect of the this positive control by limiting cytotoxicity.

- **Quartz DQ12**: The biologically active quartz DQ12 was used as particulate positive control. Quartz DQ12 represents a crystalline $SiO_2$ species. Like for $KBrO_3$, cells were treated with DQ12 for 4 h for both incubation times, using a concentration of 25 µg/cm$^2$, which was shown in pre-experiments to significantly induce DNA- and oxidative DNA-damage. In the present study, DQ12 induced significant increases in DNA-strand breaks (mean TI 4 h experiments: 10.44 ± 3.848 %; mean TI 24 h experiments: 8.99 ± 2.411 %) and in oxidative DNA-damage (mean TI 4 h experiments: 26.34 ± 6.430 %; mean TI 24 h experiments: 20.77 ± 3.381 %), as compared to both the respective vehicle controls and $Al_2O_3$-treated cultures (particulate negative control) (see Figures 1 and 3 and Tables 2 and 4 in Appendix 1). The DQ12-induced increase in DNA- and oxidative DNA-damage indicated sufficient sensitivity of the cell system towards particle-induced oxidative stress and adequate performance of the test.

- **Aluminium oxide ($Al_2O_3$)**: $Al_2O_3$ was included in the assay as particulate negative control, as it was shown in previous cell culture experiments to be relatively inert, and to exhibit no obvious substance-specific reactivity in the oxidative Comet assay,. In the present study, BAL cells incubated with $Al_2O_3$ demonstrated TI in the range of the vehicle control cells (0.66 ± 0.291 and 0.89 ± 0.613 %), and thus exhibited no genotoxic potential and also no significant induction of oxidative DNA-base lesion (see Figures 1 and 3 and Tables 2 and 4 in Appendix 1).

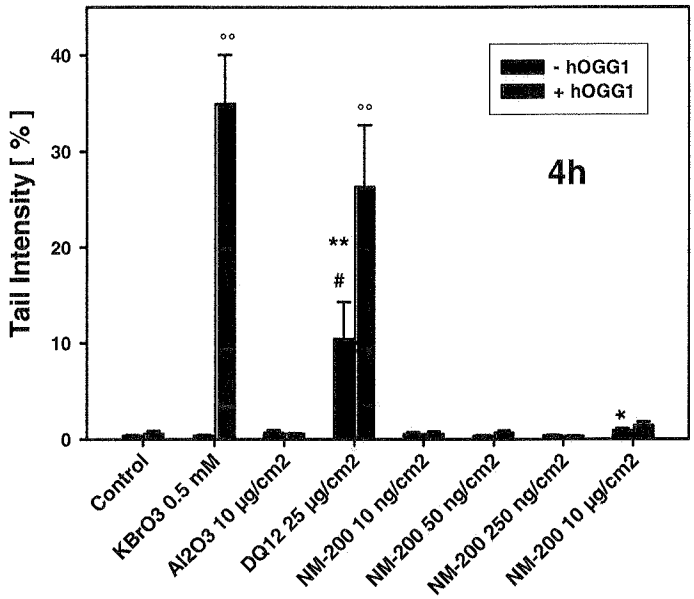


**Figure 1:** Human OGG1-modified Comet assay with NM-200 (4 h of incubation). Induction of DNA-strand breaks and oxidative DNA-damage in BAL cells. Increase in "Tail Intensity" without hOGG1-incubation indicates occurrence of DNA single- and double-strand breaks and alkali labile sites. Increase in "Tail Intensity" with hOGG1-incubation, as compared to the respective hOGG1-untreated slide, indicates oxidative DNA damage. Data represent mean values ± SD of 3 independent experiments. Significantly different from the respective control cells (*), from the respective hOGG1-untreated sample (°), or from the particulate negative control ($^{\#}$): $^{\#}$ $p \leq 0.05$, **/°° $p \leq 0.01$, Student's *t*-test for unpaired (*/$^{\#}$) or paired (°) values.

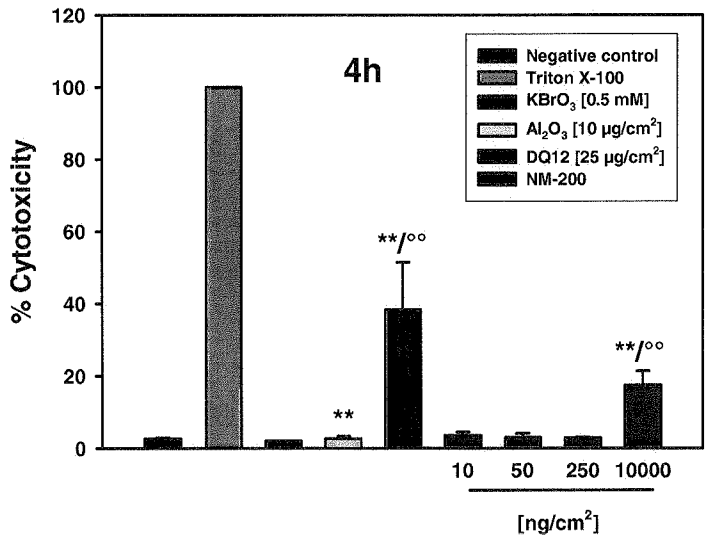


**Figure 2:** Lactate dehydrogenase (LDH)-release assay with BAL cells exposed for 4 h to the test and reference items. Increase in LDH-release indicates disturbed membrane integrity and thus cytotoxicity. Data represent means ± SD of 3 independent experiments, each performed with two cultures, which were measured in triplicate. Significantly different from control (*) or $Al_2O_3$-treated cells (°): **/°° $p \leq 0.01$, Student's *t*-test for unpaired values.

The Comet assay results for the reference items demonstrated validity of the hOGG1-modified Comet assay experiments with accurate performance of the method, sufficient activity of the hOGG1 enzyme and good viability and sensitivity of the test system.

Test item:

- **NM-200**: The test item NM-200 demonstrated no significant induction of direct DNA-damage in the *in vivo* relevant concentrations of 10, 50, and 250 ng/cm$^2$. Both after 4 and 24 h of incubation TI was not significantly enhanced by NM-200 treatment, as compared to the vehicle and particulate negative controls. There was no significant increase in TI on hOGG1-treated slides, as compared to the slides without hOGG1-treatment, indicating no significant induction of the oxidative base lesion 8-OH-dG. Only in the already cytotoxic concentration of 10 µg/cm$^2$ there was a very slight increase in mean TI after 4 h of incubation (0.90 ± 0.242 %) which reached statistical significance if compared to the vehicle control (0.35 ± 0.095 %), but not to the particulate negative control $Al_2O_3$. NM-200 thus did not show a significant genotoxic potential in BAL cells under the test conditions and the concentrations used (see Figures 1 and 3 and Tables 2 and 4 in Appendix 1).

## 6.2 Determination of LDH-activity

Viability of the test- and reference item-treated cells was determined in parallel to the hOGG1-modified Comet assay to exclude unspecific effects due to high cytotoxicity. For this purpose LDH-activity was measured in the incubation supernatants of the Comet assay. An increase in LDH-activity is indicative for membrane damage and thus cytotoxicity of the test item. In the present study, cytotoxicity was absent for all *in vivo* relevant NM-200 concentrations (10, 50, and 250 ng/cm$^2$), as compared to the vehicle and particulate negative controls. However, highly significant cytotoxicity was noted for the highest NM-200 concentrations with 17.4 ± 3.90 % after 4 h of incubation with 10 µg/cm$^2$ NM-200 and 24.7 ± 5.24 % after 24 h of incubation with 2.5 µg/cm$^2$, as compared to 2.7 ± 0.65 and 4.1 ± 1.91 % for $Al_2O_3$ in the same mass concentrations, respectively. The particulate positive control DQ12 (25 µg/cm$^2$) demonstrated even higher and significant cytotoxicity with 38.3 ± 13.14 % and 35.7 ± 13.02 % in the two sets of experiments, respectively, as compared to the vehicle and particulate negative controls (see Figures 2 and 4 and Tables 3 and 5 in Appendix 1). As cytotoxicity did not amounted to more than 50 %, unspecific DNA-damaging effects due to excessive cytotoxicity can be excluded.

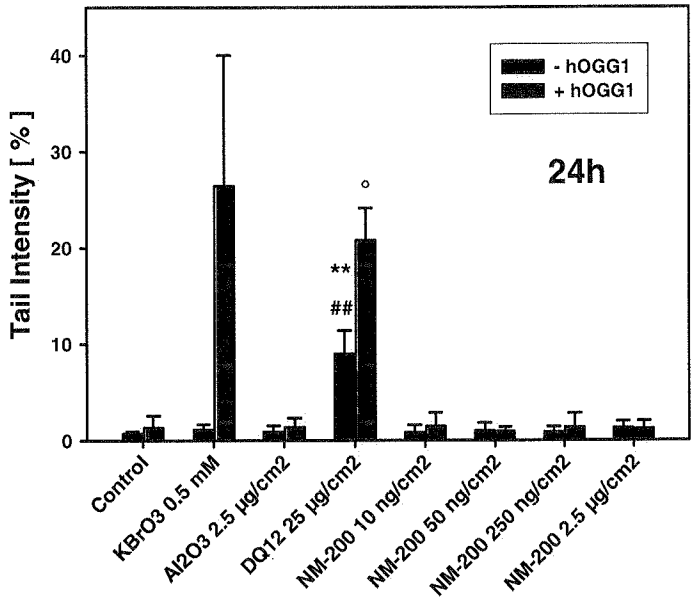


**Figure 3:** Human OGG1-modified Comet assay with NM-200 (24 h of incubation). Induction of DNA-strand breaks and oxidative DNA-damage in BAL cells. Increase in "Tail Intensity" without hOGG1-incubation indicates occurrence of DNA single- and double-strand breaks and alkali labile sites. Increase in "Tail Intensity" with hOGG1-incubation, as compared to the respective hOGG1-untreated slide, indicates oxidative DNA damage. Data represent mean values ± SD of 3 independent experiments. Significantly different from the respective control cells (*), from the respective hOGG1-untreated sample (°), or from the particulate negative control ($^{\#}$): $^{\circ}$ $p \leq 0.05$, $^{**/\#\#}$ $p \leq 0.01$, Student's *t*-test for unpaired ($^{*/\#}$) or paired (°) values.

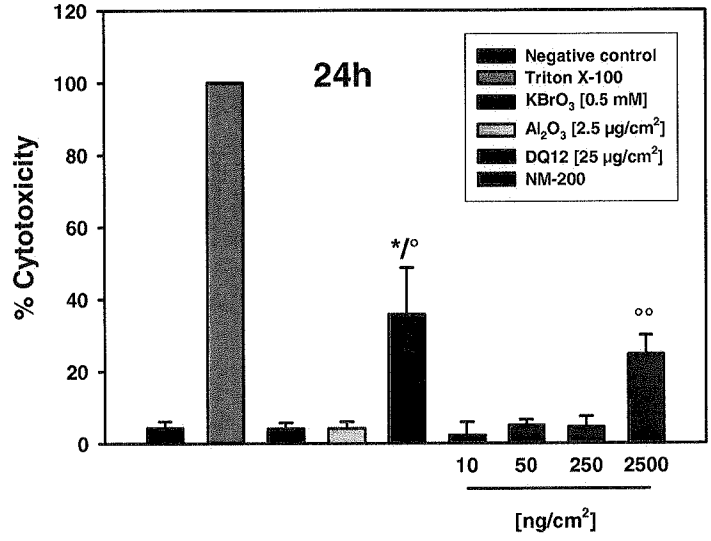


**Figure 4:** Lactate dehydrogenase (LDH)-release in BAL cells exposed for 24 h to the test and reference items. Increase in LDH-release indicates disturbed membrane integrity and thus cytotoxicity. Data represent means ± SD of 3 independent experiments, each performed with two cultures, which were measured in triplicate. Significantly different from control (*) or $Al_2O_3$-treated cells (°):$^{*/\circ}$ $p \leq 0.05$, $^{\circ\circ}$ $p \leq 0.01$, Student's *t*-test for unpaired values.

# 7 Discussion

In the present *in vitro* Comet assay experiments with BAL cells, NM-200 mediated no cytotoxicity in the *in vivo* relevant concentrations of 10, 50, and 250 ng/cm$^2$ or 19, 95, and 475 ng/ml, respectively. This was in line with a study of Uboldi et al. (2011) who didn't observe any cytotoxic effect of NM-200 (1, 10, and 100 µg/ml) after 72 h of incubation in complete growth medium with 10 % FBS by using a colony formation assay with Balb/3T3 cells. However, at the maximal concentrations used in the present experiments (10 and 2.5 µg/cm$^2$ for 4 h and 24 h, respectively, or 19 and 4.75 µg/ml) some increase in LDH-activity and thus disturbance of membrane integrity was noted. Observed cytotoxicity may be based on substance-specific effects, due to the soluble fraction of the particles, or also on mechanical effects. But more likely, cytotoxic activity in higher concentrations might represent cellular stress due to absorption of nutrients on the particle surface. In line with this hypothesis, significant cytotoxicity of different amorphous silica species *in vitro* was only observed in serum-reduced and thus nutrient deprived conditions. Gehrke et al. (2012) demonstrated a cytotoxic potential of pyrogenic amorphous silica in human HT29 colon carcinoma cells in serum reduced (1 % FBS), but not in complete growth medium (10 % FBS).

In general, cytotoxic activity of amorphous silica particle species *in vitro* seems to be cell type-specific and to depend on physico-chemical parameters like particle type, particle diameter, external surface, micropore volume, and surface roughness as described for a series of monodisperse amorphous silica nanoparticles (Rabolli et al., 2010). In addition, particle uptake, ROS-generation, and also FBS content of the incubation media were discussed as critical determinants. Because of these complex set of parameters, different studies in the field are very difficult to compare, because of the different cell types and particles used, various incubation media, concentrations, and treatment times, divergent suspension generation, and in particular differences in the aggregation and agglomeration state of the particles.

In the present study, NM-200 under the test conditions, and in the cell type used (BAL cells, pre-cultured for 24 h) did not induce neither DNA-strand breaks nor oxidative DNA-base lesions in the chosen concentrations, relevant for the rat lung *in vivo*, both after 4 and 24 h of incubation. Only at the highest concentration used (10 µg/cm$^2$, 4 h of incubation) a very slight, but statistically significant increase in TI was noted, when compared to the negative control. However, if compared to the particulate negative control (same mass concentration of 10 µg/cm$^2$), no significant increase in TI was obvious. Absence of significance concerning the particulate negative control indicates that the biological relevance of the observed small effect is questionable and represents more likely a particle and not a material-specific effect or is of incidental nature. This is supported by the facts that the negative control in the present study exhibited a very low mean TI with a very small SD value and that the mean TI of 0.9 % at 10 µg/cm$^2$ lay within the range of historical negative controls.

Interestingly, up to now, uptake of amorphous silica nanoparticles into the nucleus, as a pre-requisite for direct DNA-damage, was not demonstrated, even for well dispersed colloidal synthetic amorphous silica (Uboldi et al., 2011, Gonzalez et al., 2011, and Gehrke et al., 2012). Also for NM-200 particles it is very unlikely that theparticles reach the cell nucleus, as NM-200 exhibits a high agglomeration state of the existing aggregates with mean measured particle sizes clearly in the µm range (see Appendix 4). For this reason, it is not very probable that direct clastogenicity/ interaction of NM-200 particles with the DNA may have accounted for the slight induction of DNA-strand breaks at 10 µg/cm$^2$ (4 h of incubation) and that this small effect is more likely of unspecific or incidential nature. This is supported by the fact that a pyrogenic amorphous silica species, also with a high tendency towards agglomeration, did not mediate DNA strand break induction in the Comet assay (Gehrke et al., 2012).

Irrespective of the fact that Nabeshi et al. (2011), who observed ROS generation and induction of 8-OH-dG by colloidal amorphous silica nanoparticles in HaCat cells (keratinocytes), postulated that particle-induced ROS generation may take part in DNA-damage by amorphous silica particles, we observed no DNA damage and no induction of 8-OH-dG in pre-cultured BAL cells due to treatment with *in vivo* relevant NM-200 concentrations, indicating cell and especially particle type specificity. Independent of the calculated primary particle size of NM-200 in the nm range and a long stirring period to minimize agglomeration, NM-200 suspensions consisted predominantly of agglomerated aggregates with particles sizes in the µm range (see Appendix 4), thus making it difficult to draw conclusions from other studies with in particular stabilized colloidal amorphous silica nanoparticle suspensions.

In summary, irrespective of a cytotoxic potential of the highest NM-200 concentration used, there was no significant clastogenic potential or potential to induced oxidative DNA base lesions (8-OH-dG) in primary rat alveolar macrophages, both after 4 and 24 h of incubation.

# 8 Conclusion

Due to the absence of a significant increase in TI in NM-200 treated BAL cells, as compared to the particulate negative control, NM-200 is judged, under the test conditions used, to not exhibit a clastogenic and 8-OH-dG-inducing potential in concentrations, which are relevant for the rat lung.

# 9 References

**Principles of Good Laboratory Practice**
German Chemicals Law, §19a, Appendix 1, July 02, **2008**

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# Appendix 1: Tables 2 – 5

**Table 2:** hOGG1-modified Comet assay, 4 h of incubation, tail intensities of 3 experiments.

**Table 3:** LDH assay, 4 h of incubation, 3 experiments.

**Table 4:** hOGG1-modified Comet assay, 24 h of incubation, tail intensities of 3 experiments.

**Table 5:** LDH assay, 24 h of incubation, 3 experiments.

**Table 2:** hOGG1-modified Comet assay, 4 h of incubation, tail intensities of 3 experiments.

| Treatment | Experiment 1 | | Experiment 2 | | Experiment 3 | | -OGG1 | | +OGG1 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | -OGG1 | +OGG1 | -OGG1 | +OGG1 | -OGG1 | +OGG1 | Mean | SD | Mean | SD |
| **Control:** Medium | 0.25 | 0.64 | 0.44 | 0.25 | 0.36 | 0.81 | **0.35** | 0.095 | **0.57** | 0.287 |
| **$KBrO_3$:** 0.5 mM | 0.28 | 36.21 | 0.37 | 39.31 | 0.46 | 29.32 | **0.37** | 0.090 | **34.95°°** | 5.113 |
| **$Al_2O_3$:** 10 µg/cm² | 0.63 | 0.54 | 0.38 | 0.55 | 0.96 | 0.61 | **0.66** | 0.291 | **0.57** | 0.038 |
| **DQ12:** 25 µg/cm² | 14.86 | 33.70 | 8.62 | 23.48 | 7.84 | 21.83 | **10.44**/Δ** | 3.848 | **26.34°°** | 6.430 |
| **NM-200:** 10 ng/cm² | 0.69 | 0.40 | 0.27 | 0.34 | 0.58 | 0.83 | **0.51** | 0.218 | **0.52** | 0.267 |
| **NM-200:** 50 ng/cm² | 0.42 | 0.57 | 0.24 | 0.48 | 0.36 | 0.94 | **0.34** | 0.092 | **0.66** | 0.244 |
| **NM-200:** 250 ng/cm² | 0.42 | 0.30 | 0.32 | 0.37 | 0.31 | 0.27 | **0.35** | 0.061 | **0.31** | 0.051 |
| **NM-200:** 10 µg/cm² | 0.87 | 1.40 | 0.68 | 1.80 | 1.16 | 1.06 | **0.90*** | 0.242 | **1.42** | 0.370 |

**$KBrO_3$ = Potassium bromate (positive control for oxidative DNA-damage); $Al_2O_3$ = Aluminium oxide (particulate negative control); DQ12 = Quartz DQ12 (positive control); NM-200 = Synthetic amorphous silica (test item); OGG1 = Human 8-oxo-guanine DNA glycosylase. Significantly different from control/$Al_2O_3$ treated cells (*/Δ) or from the respective hOGG1 untreated sample (°): */Δ $p \leq 0.05$, **/°° $p \leq 0.01$, Student's *t*-test for unpaired (*/Δ) or paired values (°).**

**Table 3:** LDH assay, 4 h of incubation, 3 experiments.

| Treatment | Cytotoxicity [% of positive control Triton X-100] | | | | |
|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 | Mean | SD |
| **Control:** Medium | 2.5 | 2.9 | 2.9 | **2.8** | 0.23 |
| **Triton X-100:** 1 % | 100.0 | 100.0 | 100.0 | **100.0** | 0.00 |
| **$KBrO_3$:** 0.5 mM | 1.9 | 2.0 | 2.0 | **2.0**** | 0.06 |
| **$Al_2O_3$:** 10 µg/cm² | 3.4 | 2.7 | 2.1 | **2.7** | 0.65 |
| **DQ12:** 25 µg/cm² | 23.1 | 46.0 | 45.7 | **38.3**/°°** | 13.14 |
| **NM-200:** 10 ng/cm² | 4.3 | 3.9 | 2.4 | **3.5** | 1.00 |
| **NM-200:** 50 ng/cm² | 4.3 | 2.6 | 2.1 | **3.0** | 1.15 |
| **NM-200:** 250 ng/cm² | 3.1 | 2.7 | 2.8 | **2.9** | 0.21 |
| **NM-200:** 10 µg/cm² | 21.3 | 13.5 | 17.4 | **17.4**/°°** | 3.90 |

**$KBrO_3$ = Potassium bromate (positive control for oxidative DNA-damage); $Al_2O_3$ = Aluminium oxide (particulate negative control); DQ12 = Quartz DQ12 (positive control); NM-200 = Synthetic amorphous silica (test item). Significantly different from control cells (*) or from $Al_2O_3$ treated cells (°): **/°° $p \leq 0.01$, Student's *t*-test for unpaired values.**

**Table 2:** hOGG1-modified Comet assay, 24 h of incubation, tail intensities of 3 experiments.

| Treatment | Experiment 1 | | Experiment 2 | | Experiment 3 | | -OGG1 | | +OGG1 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | -OGG1 | +OGG1 | -OGG1 | +OGG1 | -OGG1 | +OGG1 | Mean | SD | Mean | SD |
| **Control:** Medium | 0.66 | 0.70 | 0.47 | 0.45 | 0.98 | 2.74 | **0.70** | 0.258 | **1.30** | 1.256 |
| **$KBrO_3$:** 0.5 mM | 1.17 | 14.16 | 0.59 | 24.13 | 1.62 | 41.04 | **1.13** | 0.516 | **26.44** | 13.588 |
| **$Al_2O_3$:** 2.5 µg/cm² | 0.60 | 1.45 | 0.47 | 0.37 | 1.59 | 2.27 | **0.89** | 0.613 | **1.36** | 0.953 |
| **DQ12:** 25 µg/cm² | 10.59 | 19.28 | 10.17 | 24.64 | 6.22 | 18.39 | **8.99**$^{**/\Delta\Delta}$ | 2.411 | **20.77**° | 3.381 |
| **NM-200:** 10 ng/cm² | 0.52 | 0.96 | 0.28 | 0.32 | 1.69 | 3.06 | **0.83** | 0.754 | **1.45** | 1.433 |
| **NM-200:** 50 ng/cm² | 0.70 | 0.97 | 0.31 | 0.40 | 1.92 | 1.35 | **0.98** | 0.840 | **0.91** | 0.478 |
| **NM-200:** 250 ng/cm² | 0.57 | 0.73 | 0.50 | 0.33 | 1.51 | 3.04 | **0.86** | 0.564 | **1.37** | 1.463 |
| **NM-200:** 2.5 µg/cm² | 1.17 | 0.89 | 0.65 | 0.55 | 2.05 | 2.13 | **1.29** | 0.708 | **1.19** | 0.832 |

**$KBrO_3$ = Potassium bromate (positive control, oxidative DNA-damage); $Al_2O_3$ = Aluminium oxide (particulate negative control); DQ12 = Quartz DQ12 (particulate positive control); NM-200 = Synthetic amorphous silica (test item); OGG1 = Human 8-oxo-guanine DNA glycosylase. Significantly different from control/$Al_2O_3$ treated cells ($^{*/\Delta}$) or from the respective hOGG1 untreated sample (°): ° $p \leq 0.05$, $^{**/\Delta\Delta}$ $p \leq 0.01$, Student's *t*-test for unpaired ($^{*/\Delta}$) or paired values (°).**

**Table 3:** LDH assay, 24 h of incubation, 3 experiments.

| Treatment | Cytotoxicity [% of positive control Triton X-100] | | | | |
|---|---|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 | Mean | SD |
| **Control:** Medium | 6.2 | 2.6 | 4.1 | **4.3** | 1.81 |
| **Triton X-100:** 1 % | 100.0 | 100.0 | 100.0 | **100.0** | 0.00 |
| **$KBrO_3$:** 0.5 mM | 5.7 | 2.2 | 4.2 | **4.0** | 1.76 |
| **$Al_2O_3$:** 2.5 µg/cm² | 5.9 | 2.1 | 4.4 | **4.1** | 1.91 |
| **DQ12:** 25 µg/cm² | 21.2 | 39.5 | 46.4 | **35.7**$^{*/°}$ | 13.02 |
| **NM-200:** 10 ng/cm² | 0.0 | 3.7 | 4.9 | **2.9** | 2.55 |
| **NM-200:** 50 ng/cm² | 6.5 | 3.2 | 4.9 | **4.9** | 1.65 |
| **NM-200:** 250 ng/cm² | 6.9 | 1.2 | 5.4 | **4.5** | 2.95 |
| **NM-200:** 2.5 µg/cm² | 28.5 | 18.7 | 26.8 | **24.7**$^{°°}$ | 5.24 |

**$KBrO_3$ = Potassium bromate (positive control for oxidative DNA-damage); $Al_2O_3$ = Aluminium oxide (particulate negative control); DQ12 = Quartz DQ12 (positive control); NM-200 = Synthetic amorphous silica (test item). Significantly different from control cells (*) or from $Al_2O_3$ treated cells (°): $^{*/°}$ $p \leq 0.05$, $^{°°}$ $p \leq 0.01$, Student's *t*-test for unpaired values.**

# Appendix 2: Certificates of Analysis and Re-Analysis

Certficate of Analysis:

## Certificate of analysis

Print date:
13.01.2011
Purchase Order/Date

Delivery item/Date

Order item//Date

Customer number

Truck number/Seal number
/

Batch NM-200 Expiry date 11.05.2011 / Quantity

| Characteristic | Unit | Value | Lower Limit | Upper Limit |
|---|---|---|---|---|
| Country of origin of material | | | | |
| Sample reference | | | | |
| pH (5g/ 100 mL H2O) | UpH | 6,9 | 6,5 | 7,5 |
| Moisture loss at 105°C(TGA, 2hrs) | % | 5,6 | 0,0 | 8,0 |
| Ignition loss at 1000°C (TGA) | % | 9,7 | 0,0 | 12,5 |
| Silica/ anhydrous product | % | 97,0 | 95,0 | 100,0 |
| Laser particul size>=51um | % | 2,1 | 0,0 | 4,0 |

Certificate of Test Item Identity (Re-Analysis):

EUROPEAN COMMISSION - Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
TP 202
Attn.: Mr. Christoph KLEIN, Ph.D./Mrs. Kirsten Rasmussen

Via E. Fermi 2749
I-21027 Ispra (VA), Italy

Tel. +39 0332 78 6101
Fax +39 0332 78 5388
e-mail: christoph.klein@ec.europa.eu

**Re-Analysis of the Test Item *NM-200***

Batch no.: Masterbatch (JRC, Ispra)

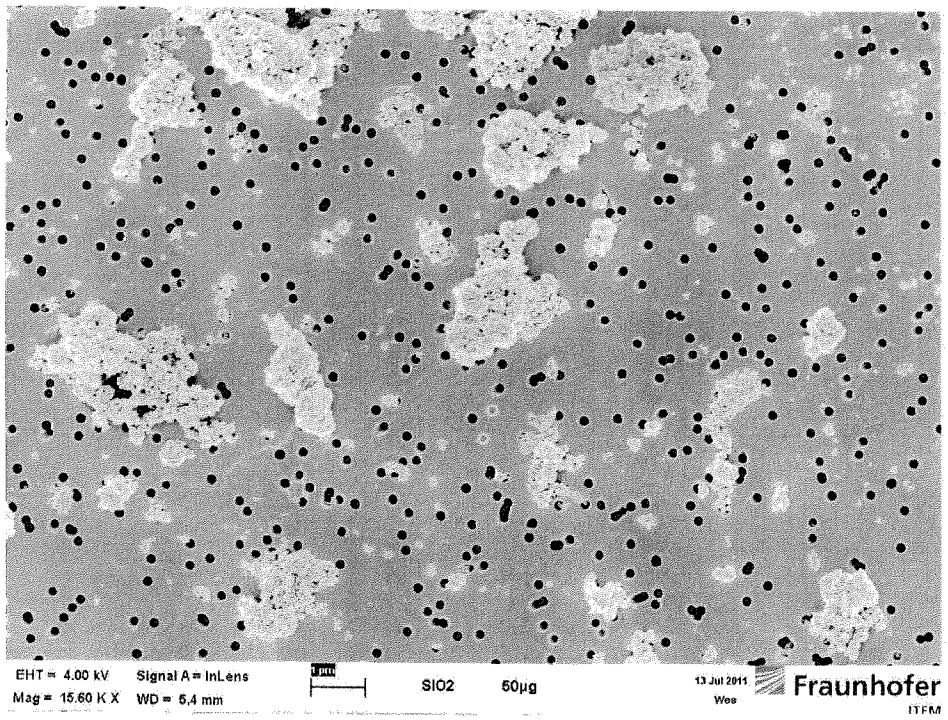
Herewith I confirm that we have checked the identity of the sample sent back to us by the study director (date of receipt: 29/3/11 1.36 p.m.) and that this sample is identical with the test item ***NM-200***

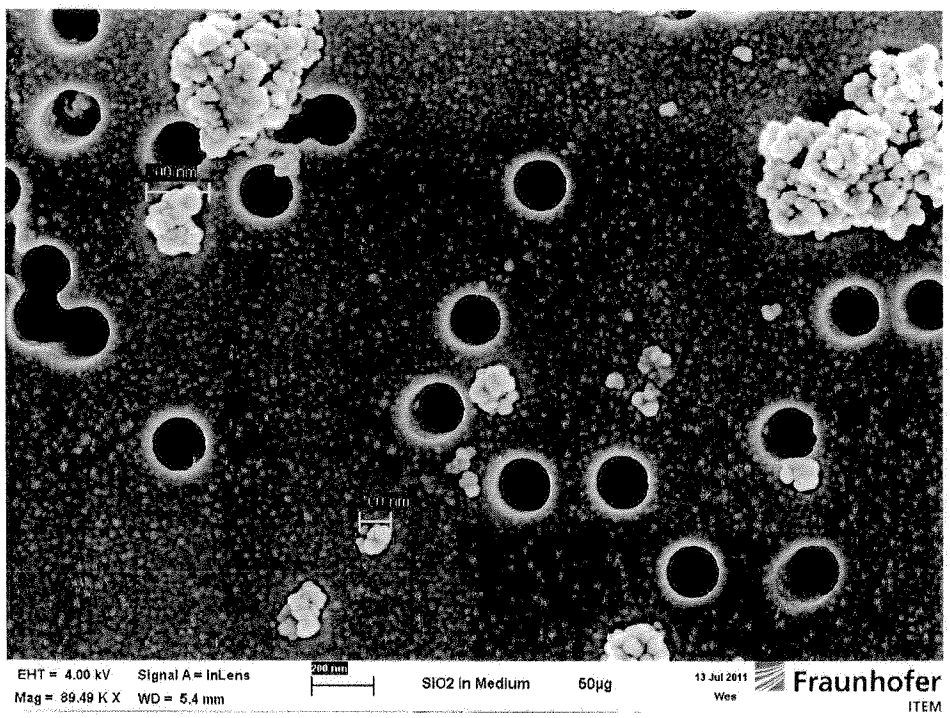
p.o.

Christoph Klein, Ph.D.

Date

# Appendix 3: Electron Microscopic Pictures of NM-200



# Appendix 4: Particle Characterisation

## Suspensions with 10% FBS:

### Datenblatt für NM-200 (Synthetisches amorphes $SiO_2$)-Suspension

**Auftragnehmer**

Fraunhofer IKTS
AG Pulver- und Suspensionscharakterisierung
Winterbergstraße 28
01277 Dresden
Deutschland

**Suspensionskennzeichnung**

| | |
|---|---|
| Auftrag: | Chromosomenaberrationstest |
| Projektnummer: | 17G11019 |
| Inkubationsmedium: | DMEM + GlutaMAX™-I mit 4,5 g/l D-Glucose und Pyruvat; Antibiotika und 10% fötalem Kälberserum |
| Dispergierung: | 5 g/l NM-200 in Inkubationsmedium, 24h Rühren |
| Dispergierdatum: | 23.09.2011 |

**Suspensionsparameter nach Dispergierung**

| | | | |
|---|---|---|---|
| Partikelgröße: | $x_{10,3}$ = 3,854 µm | $x_{50,3}$ = 8,13 µm | $x_{90,3}$ = 16,475 µm |
| Zetapotenzial: | -11 mV | | |
| Löslichkeit $SiO_2$ 24 h: | 83,2 mg/l | | |
| Löslichkeit $SiO_2$ 48 h: | 90,1 mg/l | | |

**Verwendete Chemikalien**

**NM-200** (Synthetisches amorphes $SiO_2$): Masterbatch (JRC, Ispra), Verfallsdatum 31.03.13

**Kulturmedium**: DMEM + GlutaMAX™-I mit 4,5 g/l D-Glucose und Pyruvat, Invitrogen Gibco®, Life Technologies GmbH (Darmstadt, Deutschland)

**Fötales Kälberserum**: Charge 0310 L, Verfallsdatum 3/12, Anbruch 4/11, Biochrom AG (Berlin, Deutschland)

**Antibiotika**: Penicillin G, Natriumsalz (Endkonzentration: 100.000 U/l) / Streptomycinsulfat (Endkonzentration: 100.000 µg/l), Biochrom AG (Berlin, Deutschland)