

Assessment of fumed silica nanoparticle toxicity in human lung A549 cells by combining conventional cytotoxicity assays and extracellular metabonomics

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Abstract

The suitability of conventional toxicological assays for manufactured nanoparticles (MNP) is questionable due to MNP tendency to aggregate, resulting in concentration-dependent dispersion patterns in biological matrices as opposed to their bulk counterparts. This study employed both conventional assays and $^1\text{H-NMR}$ -based profiling of extra cellular metabolites to identify the dose-toxicity relationship of silica nanoparticles *in vitro*. A group of fumed amorphous silica nanoparticles (SiNP) with primary size between 7-14 nm were assessed at 10-100 $\mu\text{g/ml}$ in human lung epithelial A549 cells. All SiNP formed polydisperse agglomerates (<100 nm to $\sim 10 \mu\text{m}$) in cell culture medium. The polydispersity was concentration dependent. All SiNP induced an increase of intracellular reactive oxygen species at all the concentrations tested, and cell membrane damage at 25 $\mu\text{g/ml}$ and higher concentrations. A metabonomic assay on one of the SiNP showed that SiNP treatment altered glucose, lactate, ethanol, phenylalanine, histidine and tyrosine levels in the extracellular medium. Simulation of the dose-effect relationship using the multivariate calibration technique of partial least squares resulted in two distinct relationship models: linear and cubic polynomial models with correlation coefficients of 0.9235 and 1.000 respectively, and a root mean square error of prediction 15.3 $\mu\text{g/ml}$, implying that SiNP dose-effect relationship could be better predicted by the cubic polynomial model. Our study suggests that although conventional cytotoxicity studies could provide insight into nanotoxicity mechanisms, metabonomics can serve as a robust and non-destructive tool for recognising *in vitro* dose-effect relationships and toxicity biomarkers of MNP with polydisperse nature.

Introduction

Naturally occurring silicon dioxide or silica is the most abundant earth mineral present predominantly as alpha-crystalline quartz. Man-made silica, however, is predominantly in the form of amorphous particles. Based on the methods of synthesis, man-made silica nanoparticles (SiNP) can be distinguished as different forms including fumed silicas, which are produced as dry aggregates under high-temperature flame (Pratsinis, 1998), and precipitated, colloidal, or mesoporous silicas, which are made via molecular condensation of silanol groups in aqueous solution or under hydrothermal conditions, as reviewed by Fruijtier- Pölloth (Fruijtier-Pölloth, 2012). These silicas have been manufactured at industrial scale to meet an ever-increasing demand for a wide range of applications including construction materials, cosmetics, food, and medicine (Kumar et al., 2010; Selvan et al., 2010; Fruijtier-Pölloth, 2012). As fumed SiNP are more likely to become airborne, therefore posing a higher risk for environment exposure, studies are deemed necessary to establish the toxicity potential of fumed SiNP.

There are previous studies using different *in vivo* and *in vitro* models suggesting that amorphous SiNP are toxic and the toxicity is associated with cytokine production, inflammatory gene expression, and genotoxicity (Zhang et al., 2012; Napierska et al., 2010; McCarthy et al., 2012). Unlike toxicity studies on bulk materials, it is the aggregate state in different testing systems that complicates the interpretation of the dose-effect relationship of nanoparticles (NP). For example, it is easy to define the toxic dose with well dispersed NP, such as colloid NP. However, in environmental and biological media, fumed SiNP are polydispersed due to the formation of different sized agglomerates. It is imperative to develop tools that recognize dose-effect patterns of NP which form aggregates in nature.

The conventional cytotoxicity assays are mechanism based but they are low in robustness, whilst metabolomics is the simultaneous quantification of the response of all metabolites to a stimulus in a biological system (Nicholson and Lindon, 2008; Dekker et al., 2012; MacIntyre et al., 2011). Metabolomics, utilizing advanced techniques such as nuclear magnetic resonance (NMR) spectroscopy combined with multivariate statistics, is a high-throughput platform requiring only small amounts of sample while providing insight into the effect of toxins on cellular functions. Moreover many intracellular metabolites are also present in significant amounts outside of cells, allowing metabolomic analysis of cell culture medium or other forms of biofluid, which has proved robust and non-destructive in recognizing dose-effect relationship of toxicity in biological models. However, the suitability of metabolomics for *in vitro* nanotoxicological studies has only scarcely been investigated. In this study, we examined the toxicity potency of four fumed SiNP at dose range of 10-100 µg/ml using human lung epithelial A459 cells. By using conventional cytotoxicity assays and profiling of extracellular metabolites by ¹H-NMR, we aimed to define a reliable dose-effect relationship of fumed SiNP in the chosen *in vitro* model.

Materials and Methods

Materials

Dulbecco's modified eagle medium F-12 (HAM) (DMEM) was purchased from GIBCO (Paisley, UK). Trypsin (0.25%), sterile phosphate buffer saline, and carboxy dichlorofluorocein (carboxy-DCF), were purchased from Fisher Scientific (Loughborough, UK). Fumed amorphous SiNP of 4 variations were sourced as follows: Aerosil 200 (hydrophilic) and Aerosil 974 (hydrophobic), each with an average size of 12 nm, were sourced from Evonik Degussa Polska (Poland) through Professor Krzysztof Pielichowski (Department of Chemistry and Technology of

Polymers, Cracow University of Technology (Poland); silica nanoparticles 7 nm (SiNP 7) and 14 nm (SiNP 14), both hydrophilic, were from Sigma-Aldrich (Gillingham Dorset, UK). The physiochemical properties of the 4 SiNP are presented in Table 1. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), and all other materials were purchased from Sigma-Aldrich (Gillingham Dorset, UK) unless otherwise stated.

Dynamic light scatter (DLS) assay of NP in culture medium

The Dynamic light scattering (DLS) technique, which is the most common method to measure hydrodynamic size distribution pattern of NP in aqueous solutions, was employed to determine the size distribution of the SiNP in culture medium. SiNP were analysed at 4 concentrations: 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. Two measurements were taken for each sample under wavelength of 633 nm and power of 4 mW by the Malvern Zetasizer (Worcestershire, UK).

Transmission electron microscopy (TEM)

Particles of silica 7, silica 14, Aerosil 200 and Aerosil 974 were dispersed in culture medium at 100 µg/ml. A droplet of each solution was allowed to dry on a TEM grid overnight. The samples were examined the following day with TEM (CM20, Philips) at an operating voltage of 200kV.

Cell culture and treatment

Human lung epithelial A549 cell line was purchased from ECACC (European collection of cell culture) via Sigma-Aldrich, and suspended in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). The cells were seeded at a density of 1×10^4 cells/cm² in flasks and incubated at 37 °C

under a humidified atmosphere with 5% CO₂ and 95% air. The cells were sub-cultured every 3 days by trypsinisation with trypsin coupled with ethylene diamine tetraacetic acid (trypsin-EDTA) at 0.25%. For NP treatment, cells were seeded at a density of 5-10 x 10³ cells/well in 96-well plates and left to attach overnight.

The cells were treated with 200 µl of DMEM containing NP at the desired concentrations. Cells without treatment were used as negative control, whilst cells treated with hydrogen peroxide (H₂O₂, 200 µM) were used as positive controls. The cells were then assessed for toxicity at different time intervals.

Cell viability assay (MTT test)

MTT was performed at 24 and 48 hours after SiNP treatment. The supernatant of 150 µl was removed from each well and 50 µl of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (1 mg/ml) was added. After incubation for 2 hours at room temperature, 100 µl of DMSO was added to solubilise the formazan salt crystals. After 20 minutes further incubation at room temperature, the plate was placed into a Thermo Scientific Varioskan plate reader and absorption was recorded at a wavelength of 570 nm.

Lactate dehydrogenase (LDH) assay

LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity is determined by a coupled enzymatic reaction. LDH oxidizes lactate to pyruvate which then reacts with a tetrazolium dye to form a coloured soluble formazan derivative.

The formazan solution was detected by a Thermo Scientific Varioskan plate reader at 490 nm. A background reading was taken at 690 nm as instructed by the

manufacturer; the final value was obtained by subtracting the reading at 690 nm from the reading at 490 nm.

Intracellular reactive oxygen species (ROS) assay

The increase of intracellular ROS, an indication of oxidative stress, was measured using carboxy dichlorofluorocein (caboxy-DCF), which when in contact with ROS is converted to fluorescent dicholorofluorocein by oxidation. After NP treatment, the supernatant in the 96-well plate was removed and a fresh 50 µl of medium was added to each well. Carboxy-DCF solution (50 µl at 5 µM) was also added to each well. The plate was left in an incubator for 40 minutes before reading with a Thermo Scientific Varioskan plate reader for fluorecence intensity (excitation at 490 nm and emission at 530 nm).

¹H-NMR spectroscopy

The preparation of samples and ¹H-NMR spectroscopy were performed according to the protocols described previously (Ellis et al., 2011). Briefly, cells were cultured in 6-well plates to reach 80% confluence and then treated with SiNP 7 at 10, 25, 50 and 100 µg/ml. At different intervals, the culture medium was transferred into a sterile eppendorf and the supernatant was collected by centrifugation at 4 °C, 1500 rpm for 5 minutes, and stored at -40 °C until further use.

For NMR analysis, 400 μ l of supernatant was mixed with 200 μ l D₂O phosphate buffer containing 1 mM of trimethylsilyl propanoic acid (TSP) as internal standard and sodium azide (an NMR silent antimicrobial). ¹H-NMR spectra were acquired using a Bruker DRX600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at a frequency of 600.13 MHz and a temperature of 300 K. A total of 75 ¹H-NMR spectra were acquired under automation using a nuclear overhauser effect spectroscopy (NOESY) water pre-saturation assay. Sample 26 (4hrs, 10 μ g/ml SiNP) and sample 73 (12hrs, 100 μ g/ml SiNP) were found to have poor water suppression and removed prior to further analyses. The residual water peak (δ 4.67-4.86) was also removed. A constant receiver gain (rg: 128) was maintained throughout the automation. Full resolution (32k data points) ¹H-NMR spectra were automatically referenced to TSP, phase and baseline corrected and imported into a Matlab file.

Multivariate data analysis and dose-effect modelling

The exploratory data analysis technique of principal components analysis (PCA), which is both an unsupervised data reduction technique, and a multidimensional data reduction projection method (Wold et al., 1987), was initially employed to detect major sources of variation and biological or analytical clustering patterns within a given data set.

For dose-response relationship modelling, the multivariate calibration technique of SIMPLS (statistically inspired modification of partial least squares) was employed (De Jong, 1993). The data was randomly split into training and testing sets. The mathematical models were created using the training set and then validated using the test set via the leave-one-out cross-validation (LOO-CV) approach (Brereton R

G., 2003), which were repeated three times. For each run, the independent (NMR profiles) and dependent (doses) variables were also individually scaled, in conjunction with increasing the number of latent variables (LVs) from 1 to 20. The optimum conditions were deduced by the highest correlation coefficient and the lowest root mean square error of prediction (RMSEP).

Another pattern recognition technique, partial least squares discriminant analysis (PLS-DA) which is a supervised technique (Barker and Rayens, 2003), was used for classification of samples after the construction of mathematical models. The samples were classified for percentage of specificity and sensitivity.

Prior to modelling and classification, the data were subject to scaling treatments. These include 1) mean centring (MC): subtracting the average of every column in a given dataset from each value in the respective column; 2) auto scaling (AS): dividing MC by the standard deviation of each column in the dataset (normally referred to as unit variance scaling in metabolomics); 3) pareto scaling (PS): dividing MC by the square root of the standard deviation of each column in the dataset; 4) range scaling (RS): setting the minimum to be either 0 or -1 and the maximum to be 1 and forcing every element to be scaled between the two values; and 5) normalisation: dividing each value in a respective column by the square root of the sum of the squared values in each column (van den Berg et al., 2006). In practice, pareto scaling can be a good compromise with AS as it enhances medium intensity observations and limits incorporation of noise into the dataset.

Statistics

For MTT, LDH and ROS assays, the experiments were performed at least three times. The data were expressed as mean values of three replicates with standard

deviation (mean $\pm$ SD). The results were represented as a percentage of the negative control (cells without treatment). One-tailed unpaired student's t-test was used for comparison of differences between samples and control. The differences were considered significant when $p \leq 0.05$.

Results

SiNP form polydispersed agglomerates in culture medium

The DLS profiling of complete cell culture medium revealed that 2 peaks with size < 100 nm exists, indicating the presence of nanosized molecules and their aggregates (Figure 1A a). All the SiNP exhibit a polydispersion pattern in the culture medium. The polydispersity and size of SiNP increases with increased concentration (Figure 1A b-e), suggesting the formation of agglomerates. For Aerosil 947, the intensity of the peaks is relatively low, which is due to the poor water solubility of the particles. Using the TEM technique it was also shown that all SiNP exhibit heterogynous size distribution at the highest concentration, suggesting the co-presence of primary SiNP and their different sized aggregates (Figure 1B a-d). The size and shape of the aggregates in SiNP 7 samples are relatively more regular and smaller than that in the rest of the samples, which is consistent with the results from the DLS assay. Different crystalline structures were formed during sample preparation (Figure 1B a).

SiNP induced cytotoxicity as determined by conventional cytotoxicity assays

In order to determine the toxicity potential and associated mechanisms of the fumed SiNP, the cell viability and membrane damage were assessed in A549 cells after NP treatment. The effect of SiNP on cell viability was not detected at 24 hours (h) and

became detectable only after 48 h as assessed by MTT assay (Figure 2A). The LDH leakage was detectable at 4 h of SiNP treatment (Figure 2B). The effect was significant in comparison with control for all the SiNP at concentration of 25 µg/ml and above as measured at 12 h, and also 24 h apart from Aerosil 974. The differences were significant when compared with control but not significant between the effective doses for both LDH and MTT assays. H₂O₂ induced both LDH leakage and loss of cell viability at the time points tested.

SiNP also induced an elevation of intracellular ROS (Figure 2C), with the effect detected as early as 4 h for the highest concentration of all 3 hydrophilic SiNP. The effect reached maximum for the rest concentrations at 12 h and became undetectable at 24 h, apart from SiNP 7 at 25 µg/ml. The positive control H₂O₂ also caused a significant elevation of intracellular ROS at 4 and 12 h.

SiNP7 induced alterations of extracellular metabolites

In order to assess the effect of SiNP on cellular metabolism, ¹H-NMR metabonomic profiling of culture medium from cells with and without SiNP treatment was performed. SiNP 7 was used as a model fumed SiNP. Principal components analysis (PCA) revealed a clear distinction between the samples acquired at different time points after treatment (Figure 3A). Further PCA at each time point revealed a clear separation across PC2 of the higher concentrations 50 and 100 µg/ml from controls at 24 h of treatment (Figure 3B).

PCA is an unsupervised technique, meaning that it may not be sufficient to recognize all distinctions between samples. Therefore the more robust, supervised technique of partial least squares discriminant analysis (PLS-DA) was employed. In this instance, PLS-DA learns the relationship between the NMR profiles of different treatments.

With this knowledge it can distinguish the difference between a control and a given dose of treatment.

A 2-way classification using PLS-DA resulted in a dose and time related scores for sensitivity, specificity and overall effect of SiNP 7 treatment. As shown in Table 2, SiNP 7 of 50 $\mu\text{g/ml}$ retained the highest overall score at all the time points assessed. SiNP 7 of 100 $\mu\text{g/ml}$ scored the same as 50 $\mu\text{g/ml}$ at 12 and 24 h, whilst 10 and 25 $\mu\text{g/ml}$ scored relatively low at all the time points tested, suggesting that the effects of SiNP treatment is concentration dependent at all the time points tested.

The latent variable (LV) numbers in Table 2 give indications of the dimensions of dose and effect relationship; the higher the number, the more complex the dose-effect relationship becomes. At 4 h, the LVs ranged from 2 to 6, suggesting the possibility that NP of different doses elicited distinctive cellular responses at early time point, which may reflect the initial polydispersion pattern-associated effect. At 12 and 24 h, however, the LVs ranged from 2 to 4 across all doses, indicating that similar cellular response patterns could be shared among NP of different doses.

As shown in Figure 4, a number of metabolites were affected by SiNP 7 treatment. The most affected metabolites include glucose, lactate and ethanol in the aliphatic region (A), and histidine, phenylalanine, and tyrosine in the aromatic region (B). The effects were most pronounced for the treatment of 50 $\mu\text{g/ml}$ at 4 h and both 50 and 100 $\mu\text{g/ml}$ at 12 and 24 h. Some other metabolites also responded to the treatment but their identities have yet to be determined. For example, the peaks at 3 ppm could be acetate-methylene protons of EDTA (Han et al., 2007). The singlet peak at around 3.4ppm could well be oxidised nicotinamide, derived from cellular excretion of nicotinamide N-oxide (Atac et al., 2011). The peaks just below 4ppm are likely to

be amino acid or potentially sugar protons. According to the experimental metabonomic spectra data in the human metabolome database, the possibility of these peaks being folic acid, riboflavin, N-methylnicotinamide (Human Metabolome Database Version 3.0 a-c) or niacin could can be excluded (Atac et al., 2011).

The modelling of dose-effect relationship, derived from the SIMPLS model attained at LV7, thus resulted in two best prediction models. Figure 5A illustrates the linear relationship model attaining a coefficient of determination (R^2) of 0.8528 (correlation coefficient of 0.9235). Figure 5B presents the cubic polynomial relationship model with the coefficient of determination (R^2) of 1.000 (correlation coefficient of 1.000). The root mean square error of prediction (RMSEP) was 15.3 $\mu\text{g/ml}$. It can therefore be deduced that a linear relationship exists up to the dose of 50 $\mu\text{g/ml}$.

Discussion

Silica NP are representatives of nanoparticles that are manufactured in large quantity for different applications, therefore likely to interact with humans in many ways. Although it has been well documented that some smaller particles are more toxic than their larger counterparts (Zhang et al., 2012; McCarthy et al., 2012), it is extremely challenging to study the toxicity potential of small MNP, such as SiNP < 20 nm that have a very strong tendency to aggregate or agglomerate in the air, in biological media, and inside cells. Therefore, the conventional approach for the study of dose-effect relationship may not be suitable for prediction of nanoparticle toxicity. This study attempted to define the dose-toxicity relationship of a group of fumed SiNP by combining conventional cytotoxicity assays and metabonomics *in vitro*.

As shown by DLS and TEM assays, the SiNP exhibit a concentration-dependent size distribution and polydispersion pattern in culture medium. The polydispersity increases with increased concentration.

Among the four SiNPs, SiNP 7 and SiNP 14 induced a significant increase in ROS generation at the highest dose at 4 hours, which was not correlated with a significant high level of toxicity as assessed by LDH and MTT. In addition, 10 µg/ml SiNP induced a similar level of ROS generation as higher concentrations, but failed to induce cellular membrane damage, suggesting that ROS increase was not sufficient to cause acute cytotoxicity in A549 cells. In most cases, the effects appeared not significantly different between effective doses as assessed by ROS and LDH at later time points. This pattern of toxicity effect could not be interpreted as concentration dependent.

Metabonomics, combined with multivariate statistics, helps define complex dose-effect relationships and biomarkers of toxic agents, and can also generate models for toxicity prediction. ¹H-NMR -based assay on extracellular metabolites requires minimal sample preparation, thus minimizing artefacts and allowing better correlation with *in vivo* studies (Dekker et al., 2012; MacIntyre et al., 2011). The ¹H-NMR metabonomic assay revealed that SiNP 7 induced pronounced effects on glucose, lactate, histidine, phenylalanine, and tyrosine at early time points when cell viability was not impaired. The effects were detected at 4 hours of SiNP 7 treatment at 50 µg/ml and 12 hours treatment at 50 and 100 µg/ml. Importantly, some effects were also detected at later time points in cells treated with the two lower concentrations.

Although the mechanisms for SiNP induced metabolite changes were not further investigated, the increase in some metabolites could in turn cause cytotoxicity or

could serve as indications of cell damage. For instance, high concentration of glucose could result in oxidative stress and cell damage (Li et al., 2011). As lactate is produced from glucose through glycolysis in the cytosol and then enters mitochondria to be oxidized via mLDH to pyruvate (Luo et al., 2012; Passarella et al., 2008), the increase of lactate could indicate the damage of mitochondria. It has also been reported that lactate can activate macrophages (Samuvel et al., 2009; Nareika et al., 2005). A recent study showed that in rat, zinc oxide NP induced an increase in lactate and glucose in urine, which is associated with kidney toxicity (Yan et al., 2012). Our results showed that SiNP at doses 25 µg/ml and above induced both lactate increase and membrane damage in the A549 cells. Taking all these together, lactate production could be a sensitive and global marker for cell damage and toxicity. In addition, SiNP 7 could also induce an increase of nicotinamide N-oxide, a potential biomarker of CYP2E1 activity that can be induced by ethanol and play a critical role in ethanol induced oxidative stress and toxicity (Cederbaum, 2009). The increase of nicotinamide-N-oxide coincided with the increase of ethanol suggests that the CYP2E1 pathway could be involved in SiNP 7 induced toxicity. The increase in extracellular amino acids also suggests that SiNP may impair the mechanism of amino acids catabolism, preventing cells from utilizing phenylalanine, tyrosine and histidine.

The effective dose range of SiNP on metabonomics is consistent with that causing cytotoxicity as assessed by multiparametric toxicity assessment, suggesting that metabonomic modelling could be a useful tool for prediction of toxicity. Metabonomic modelling of the dose-effect using the technique of multivariate calibration suggests that the cubic polynomial model is better than a linear model for prediction of the dose-effect of SiNP 7 toxicity. In this model, the linear relationship was attained up to 50

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3 µg/ml. However when the concentration reaches 100 µg/ml, the linear relationship is
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5 no longer retained, which coincides with an apparent increase of aggregates larger
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7 than 1µm, suggesting that larger particles/nanoparticle aggregates exhibit different
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9 toxicity potential. The cubic polynomial model of dose-toxicity prediction could be
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11 applicable for the rest of the fumed SiNP or any other NP which tend to aggregate
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13 and exhibit different dispersion patterns in a concentration-dependent manner.
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20 **Conclusions**

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23 Our study demonstrated that fumed SiNP exert their cytotoxicity via causing
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25 metabolism disturbance, oxidative stress, damage to cell membrane, and possibly
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27 also to mitochondria. Although conventional cytotoxicity studies could provide insight
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29 into nanotoxicity mechanisms, they are not sufficient to recognize dose-effect
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31 patterns. In this regard, metabonomics could prove to be a robust and non-
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33 destructive tool for recognising toxicity markers and dose-effect relationship of NP
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35 with polydispersity nature *in vitro*. To our best knowledge, this is the first report on
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37 ¹H-NMR- metabonomics application for modelling of nanotoxicity *in vitro*.
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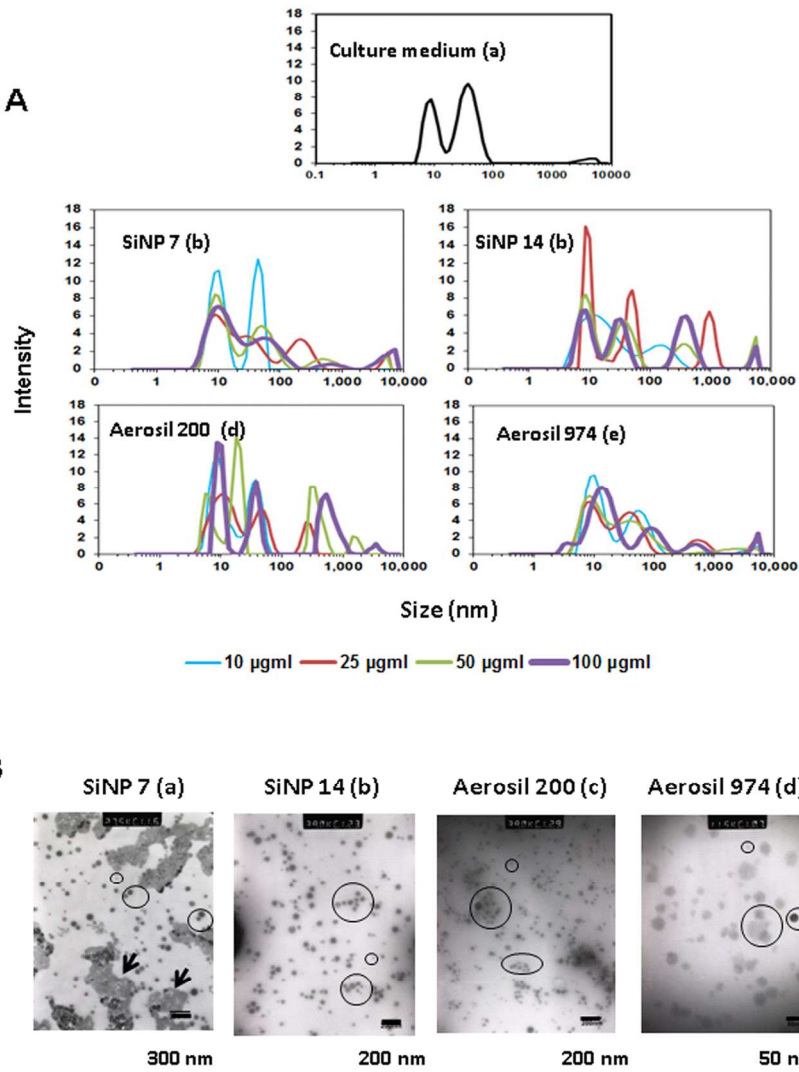


Figure 1. SiNP form agglomerates in culture medium in a concentration dependent manner. A: DLS profiles of cell culture medium and SiNP at different concentrations. The complete cell culture medium (a) exhibits 2 peaks, indicating the presence of nanosized molecules and their aggregates. All the hydrophilic SiNP (b-d) formed agglomerates in a concentration dependent manner. For Aerosil 947 (e), the intensity of the peaks is relatively low due to the poor water solubility of the particles. B: TEM confirmation of the formation of aggregates of Aerosil 200 and Aerosil 974 at 100 µg/ml. The bars represent the length as indicated. 151x163mm (300 x 300 DPI)

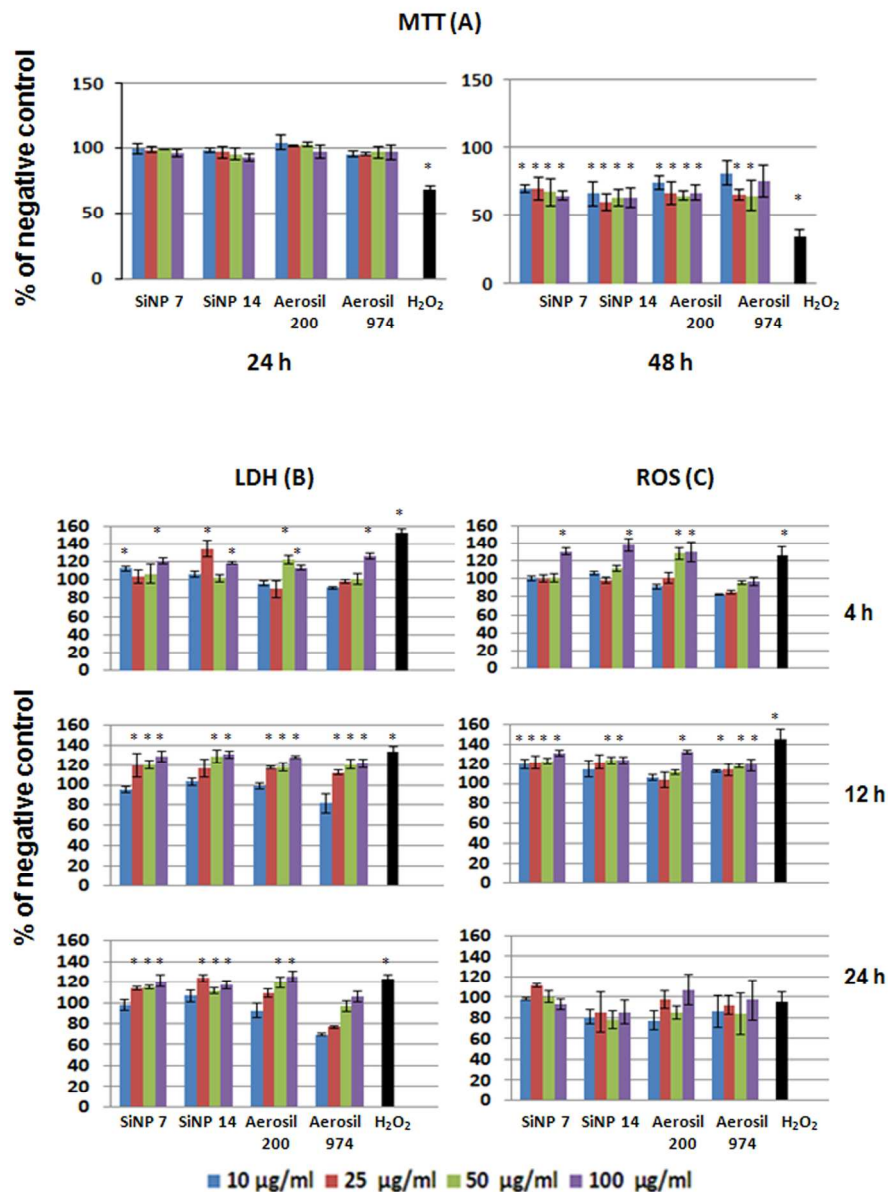


Figure 2. Cytotoxicity assessment of SiNP. Loss of cell viability was detected in cells treated with SiNP at 48 h but not 24 h at all doses (A); LDH leakage detected at 25-100 µg/ml of all time points (B); intracellular ROS increased at 10-100 µg/ml (C) at early time points. The asterisks indicate significant difference ($P < 0.05$) as compared with negative control (without SiNP treatment).

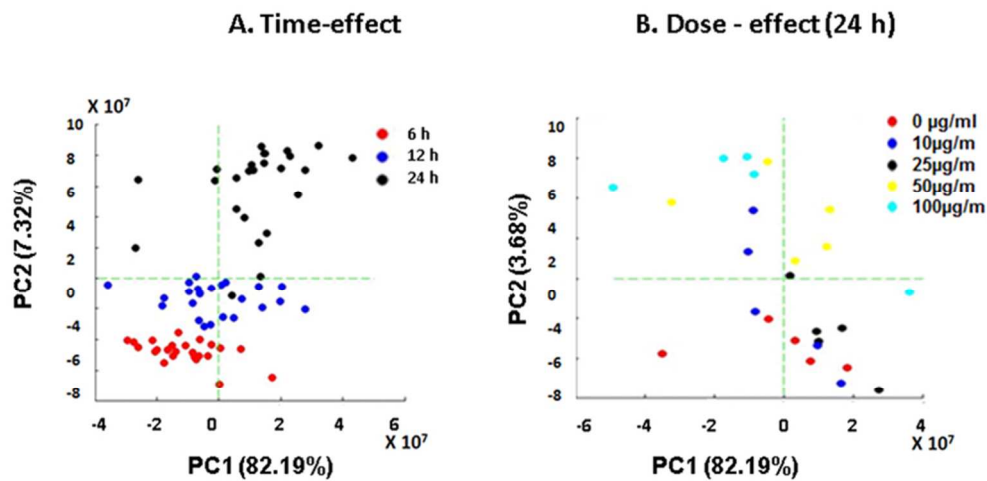
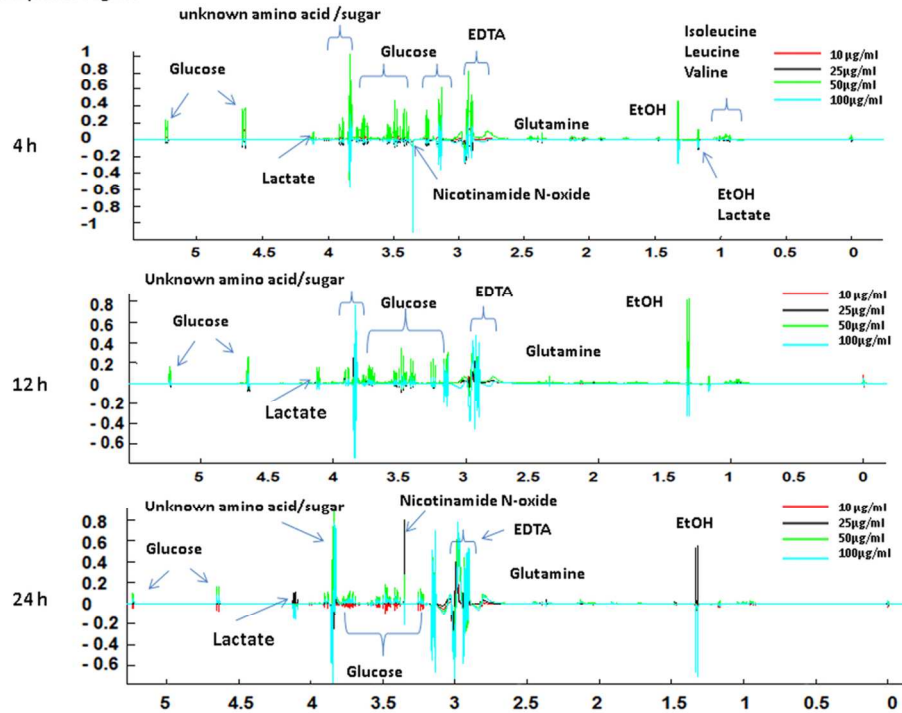


Figure 3. PCA plots of 1H-NMR metabolites with respect to time and dosage of SiNP 7 treatment. A: a clear distinction can be seen between samples acquired at different time point of treatment. B: a clear discrimination was achieved between doses 50 and 100 $\mu\text{g/ml}$ with untreated samples and samples treated with lower concentrations of SiNP 7 at 24 h.
68x33mm (300 x 300 DPI)

A. Aliphatic region



104x78mm (300 x 300 DPI)

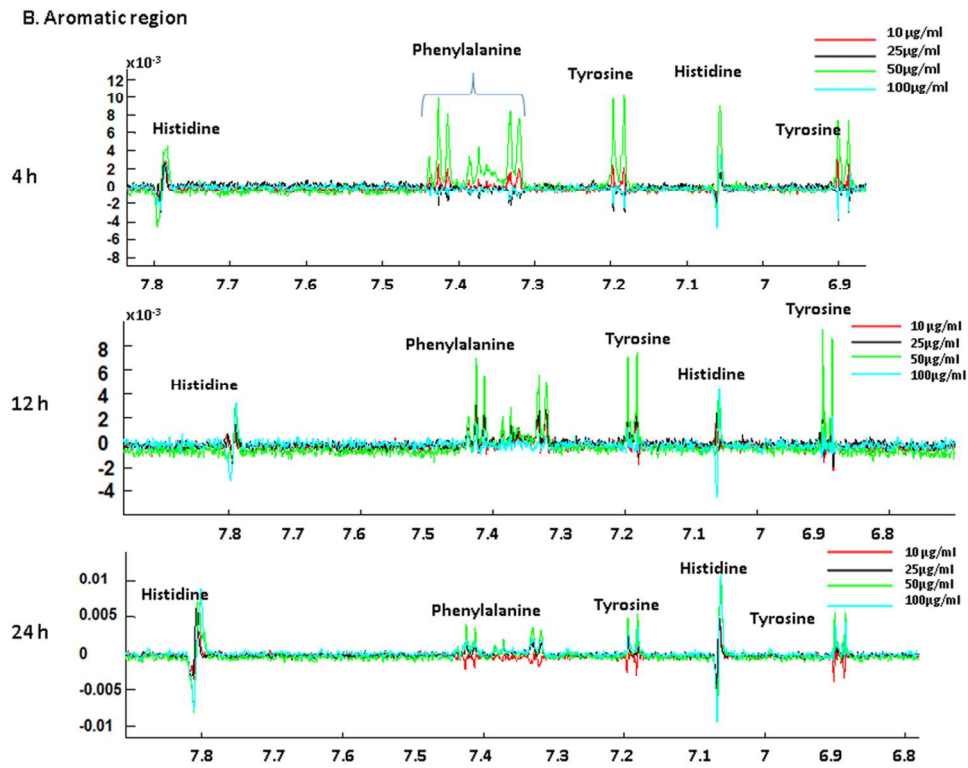
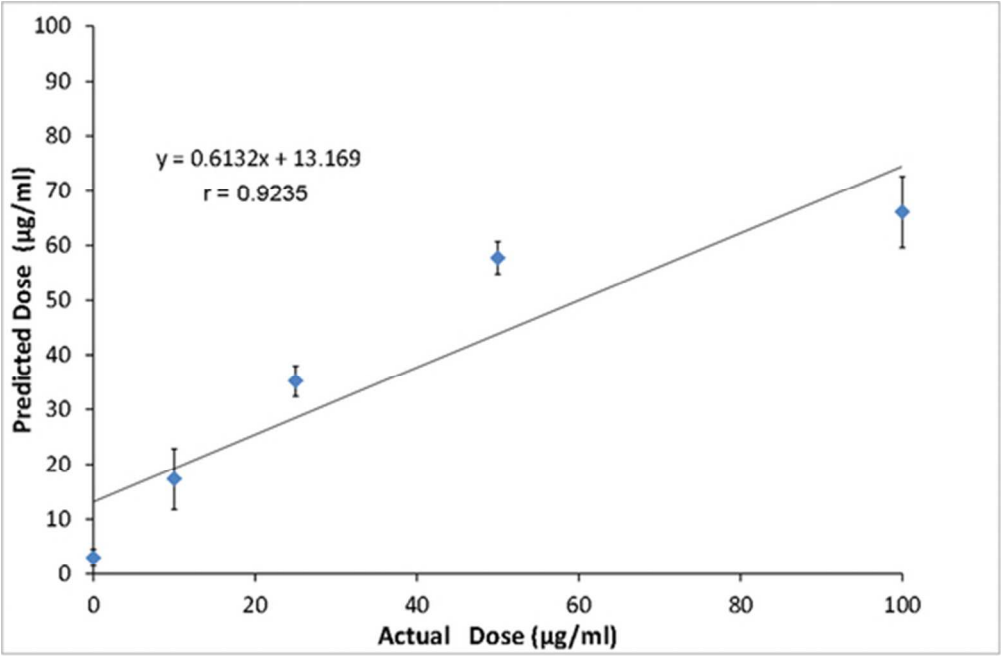


Figure 4. NMR profiles normalised against the TSP internal standard and subtracted from control. A: SiNP 7 induced changes of metabolites at the aliphatic region; B: SiNP 7 induced changes of metabolites at the aromatic region.
108x83mm (300 x 300 DPI)



49x32mm (300 x 300 DPI)

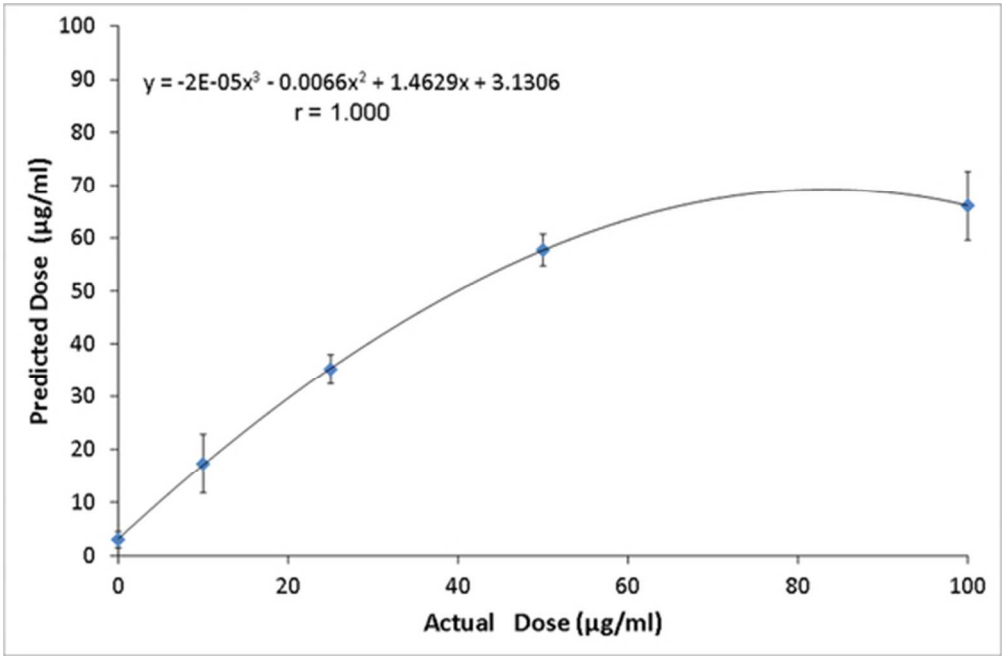


Figure 5. Prediction of SiNP 7dose-effect relationship at 4, 12 and 24h. A: linear response with coefficient of determination R2=08528 (correlation coefficient = 0.9235); B: cubic polynomial response with a coefficient of determination R2=1.000 (correlation coefficient = 1.000). Data pre-treatment was via mean-centring for dependent and range-scaling (0 to 1) for independent variables, and 7 LVs were optimal for the model. 49x32mm (300 x 300 DPI)

Table 1. Physiochemical properties of fumed silica NP used in the study

Silica NP	Supplier	Primary size (nm)	Surface area (m ² /g)	Behaviour in water	Zeta potential (mV)	
					in water	in culture medium
SiNP 7	Sigma Aldrich	7	395±25	Hydrophilic	-29.9	-9.8
SiNP 14	Sigma Aldrich	14	200±25	Hydrophilic	-30.1	-8.6
Aerosil 200®	Evonik	12	200±25	Hydrophilic	-27.2	-12
Aerosil 974®	Evonik	12	200±25	Hydrophobic	-31	-17

Table 2. Summary of the 2-way classification using PLS-DA of the NMR profiles of all doses at different time points.

Time (h)	Dose (µg/ml)	Scale	LV	Overall %	Specificity %	Sensitivity %
4	10	RS1	2	66.7	80.0	50.0
4	25	RS1	6	80.0	80.0	80.0
4	50	Norm	3	100.0	100.0	100.0
4	100	PS	3	90.0	100.0	80.0
12	10	PS	2	80.0	80.0	80.0
12	25	AS	4	80.0	100.0	60.0
12	50	RS1	3	100.0	100.0	100.0
12	100	NS	2	100.0	100.0	100.0
24	10	NS	2	70.0	80.0	60.0
24	25	MC	4	80.0	80.0	80.0
24	50	PS	2	100.0	100.0	100.0
24	100	PS	2	100.0	100.0	100.0
ALL	10	PS	3	79.3	86.7	71.4
ALL	25	RS1	5	93.3	93.3	93.3
ALL	50	Norm	6	100.0	100.0	100.0
ALL	100	RS1	9	100.0	100.0	100.0

Scaling Key: NS = no scaling; MC = mean-centred; AS = Auto-scaled; RS1 = Range-scaled (0 to 1); Norm = Normalisation; PS = Pareto scaling