Assessment of temporal dose-toxicity relationship of fumed silica nanoparticle in human lung A549 cells by conventional cytotoxicity and ^{1}H-NMR-based extracellular metabonomic assays

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Abstract

As nanoparticles could form aggregates in biological systems, the dynamics of their dispersity drives the temporal effect of nanoparticles *in vitro*. To test this hypothesis, the fumed silica NP (SiNP) that have primary sizes of 7-14 nm and form aggregates in culture medium were selected for toxicity study in human lung A549 cells. The dispersity of SiNP was analysed by dynamic light scattering and transmission of electron microscopy. Cytotoxicity assays including mitochondrial activity, intracellular level of reactive oxygen species (ROS), and membrane damage together with the ^{1}H-NMR-based extracellular metabonomic assay were conducted to determine the temporal dose-effect relationship of SiNP. In cell culture medium SiNP dispersed well initially at 25-100 µg/ml, however, they sedimented rapidly in a concentration dependent manner. SiNP caused a dose-dependent increase of intracellular ROS and cell membrane damage at 4 hour and a loss of cell viability after 48 hour. SiNP also induced an elevation of extracellular glucose, lactate, phenylalanine, histidine and tyrosine levels in a time- and concentration-dependent manner. The dose-effect patterns at 4 hour were different from that at 12 and 24 hour as assessed by both cytotoxicity and metabonomic assays. Both fitted better with polynomial regression than linear regression, implying multimode action of SiNP at different concentrations. The early NP-cell interaction and the late sedimentation could be attributable to the temporal effects of SiNP. The extracellular ^{1}H-NMR-based metabonomics demonstrated a potential as a robust non-destructive tool for monitoring temporal effect of NP that tend to aggregate in nature.

Keywords: Fumed silica nanoparticles, A549 cells, cytotoxicity, 1H-NMR metabonomics, dose-effect relationship.

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 (Fruijtier-Pölloth, 2012,Kumar *et al.*, 2010,Selvan *et al.*, 2010). As fumed SiNP are more likely to become airborne, therefore posing a higher risk for environmental exposure, studies are deemed necessary to establish the toxicity potential of the fumed SiNP.

There are previous studies using different *in vivo* and *in vitro* models suggesting that fumed 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, which have a major effect on NP stability in biological media. In vitro nanotoxicity studies have so far been mainly conducted using conventional

cytotoxicity assays which are generally endpoint-based, destructive, and low throughput, providing little insights about the molecular pathways of toxicity, It is imperative to develop testing strategies that are more robust, allowing non-destructive and mechanism-based assay. Metabonomics 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). Metabonomics, utilizing advanced techniques such as nuclear magnetic resonance (NMR) spectroscopy combined with multivariate statistics, is a high-throughput platform requiring only a small amount of sample while providing insight into the effect of toxins on metabolite pathways. Moreover many intracellular metabolites are secreted in significant amounts from cells, allowing metabonomic analysis of cell culture medium or other forms of biofluid in a non-destructive way.

As nanoparticles could form aggregates in biological systems, it is possible to distinguish the dose-effect relationships between NP and their aggregates by analysis of the temporal effect in relation to the dynamics of nanoparticle aggregation. To test this hypothesis, this study utilized both *in vitro* conventional cytotoxicity assays and ^{1}H-NMR-extracellular metabonomic assay to establish early and late dose-effect relationship of fumed SiNP in human lung A549 cells, one of the in vitro models that has been widely used to study the cellular and molecular mechanisms of lung injury (Ahamed 2013, Gonzalez *et al*., 2010, Lanone *et al*., 2009)

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 (caboxy-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 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, UK). The physicochemical properties of the 4 SiNP, as described in supplier's data sheet, 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, UK) unless otherwise stated.

Measurement of size distribution and zeta potential of SiNP in culture medium

The Dynamic light scattering (DLS) technique, which is the most common method to measure hydrodynamic size and distribution pattern of NP in aqueous solutions (Murdock *et al.*, 2008), was employed to determine the size distribution of the SiNP in culture medium.

SiNP were dispersed in cell culture medium with 10 % foetal bovine serum (FBS) at 25 µg/ml and100 µg/ml and the DLS measurements were taken immediately. The samples were then left in cell culture incubator at 37°C. After 2 and 24 hour (h), the top part of the suspension was taken for the assay. Two measurements were made for each sample under wavelength of 633 nm and power of 4 mW by the Malvern Zetasizer (Worcestershire, UK). Cell culture medium without SiNP was also analysed by the DLS assay for the presence of nanosized aggregates. For the zeta potential

assay, the NP solutions were injected into the Malvern Zetasizer. An average zeta potential was taken from 30 cycles of measurements.

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 on 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 culture medium (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/cm^2 in flasks and incubated at 37°C under a humidified atmosphere with 5% CO_2 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 $\times 10^3$ cells/well in 96-well plates and left to attach overnight.

The cells were treated with 200 µl of culture medium containing NP at the desired concentrations. Cells without treatment were used as negative control, whilst cells treated with hydrogen peroxide (H_2O_2, 200 µM) were used as positive controls. The cells were then assessed for toxicity at different time intervals.

Cell viability assay (MTT test)

Cell viability was assessed at 24 and 48 hours of SiNP treatment by the MTT assay as described previously (Irfan *et al.*, 2013). Briefly, after treatment cells were washed three times with PBS. The plate was incubated with 50 µl of MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution (1 mg/ml) for 2 h at room temperature. The formazan salt crystals were then solubilised with 100 µl of DMSO. After 20 minutes further incubation at room temperature, the absorption was recorded using a Thermo Scientific Varioskan plate reader 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 was 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.

For assessing the dose-effect relationship of SiNP induced membrane damage, the data from the LDH assay was normalized as (Liu *et al.*, 2013):

$$LDH\ (toxicity\ index) = \frac{(NP - NC)}{(PC - NC)} \qquad (2)$$

where NP refers to NP treatment, NC to negative control (cells without treatment), and PC to positive control in which cells were treated with H_2O_2. The dose-effect

relationship was displayed as the smooth scatter graph with trend line using the Microsoft Excel programme.

Intracellular reactive oxygen species (ROS) assay

The assay of intracellular ROS was described previously (Irfan *et al.*, 2013,Fede *et al.*, 2012) using Carboxy-H2DFFDA, 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate, which when in contact with ROS is converted to fluorescent dicholorofluorocein by oxidation. The supernatant in the 96-well plate was removed and the wells were washed three times with PBS. A fresh 50 µl of culture medium and a 50 µl of Carboxy-H2DFFDA, 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate solution at 5 µM were added to each well. The plate was incubated for 40 minutes before reading with a Thermo Scientific Varioskan plate reader for fluorescence intensity (excitation at 490 nm and emission at 530 nm).

^{1}H-NMR spectroscopy

The preparation of samples and ^{1}H-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 µl of supernatant was mixed with 200 µl D_2O phosphate buffer containing 1 mM of trimethylsilyl propanoic acid (TSP) as internal standard and sodium azide (an NMR silent antimicrobial). ^{1}H-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 ^{1}H-NMR spectra were acquired under automation using a 1-dimension nuclear overhauser effect spectroscopy (1D-NOESY) water/solvent pre-saturation assay. Due to poor water suppression, sample 26 (treated with 10 µg/ml of SiNP at 4 h) and sample 73 (treated with 100µg/ml of SiNP at 12 h) were removed together with the residual water peak (δ4.67-4.86) from further analyses. A constant receiver gain (rg: 128) was maintained throughout automation.

Full resolution (32k data points) ^{1}H-NMR spectra were automatically referenced to TSP. The raw NMR spectra were subjected to baseline correction via a polynomial curve fitting algorithm developed in-house. In order to reduce the variability in peak positions and thus improve local alignment, recursive segment-wise peak alignment (RSPA) was applied to the NMR spectra (Veselkov et al., 2009), followed by uniform binning. The bin width was 3.42×10^{-4} which is calculated from the ppm range (-1 to 10) divided by the number of variables in the data matrix (32126), i.e. 11/32126. The NMR spectra were exported into an Excel (.xlsx) file, which was subsequently imported to MATLAB (2011a, MathWorks Inc., USA) for data analysis.

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 was repeated three times. For each run, the independent (NMR profiles) and dependent toxicity index (from Equation 2) at the respective SiNP concentrations were 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). Scaling of the NMR dataset included: 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 metabonomics); 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 unit variance scaling 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 ± 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

Dispersity of SiNP in culture medium

The size, surface area and the surface property of the SiNP used in this study were provided by the suppliers and presented in Table 1. Further characterization of the SiNP in the in vitro testing system was carried out by the DLS and TEM assays.

DLS profiling of complete cell culture medium revealed two overlapping peaks with average size 6.5 nm and 24 nm respectively (Figure 1a, P1 and P2), and a third peak with size exceeding nanoscale, suggesting that some components in the culture medium formed aggregates. The polydispersity index (PDI) was measured as 0.44.

DLS profiles of SiNP at different concentrations were acquired at three time points after suspension in culture medium. As measured immediately after suspension, SiNP at 25 µg/ml showed similar DLS profiles as culture medium with a slight increase of intensity in P2 (Figure 1b, d, f and h). For Aerosil 200 an increase of intensity in the region > 100 nm was also detected (Figure 1 f) at all timepoints, suggesting that this sample formed more aggregates in suspension than the other two hydrophilic SiNP. For Aerosil 974, a peak with size exceeding nanoscale was detected (Figure 1 h), which was consistent with the hydrophobic nature of the sample. The low intensity in the DLS profiles of SiNP at 25 µg/ml suggests that this concentration is too low to be detectable by the instrument. SiNP at 100 µg/ml exhibited profiles with separate P1 and P2 (Figure 1 c,e,g and i) as measured

immediately after suspension (0 h). While P1 intensity remained relatively consistent among the hydrophilic SiNP, P2 increased to different extent, suggesting that P1 was mainly formed by the components of culture medium, whilst P2 dictated the presence of SiNP (~ 60 nm). At the high concentration, a third peak with size distribution of 105-255 nm was also detected in Aerosil 200 sample (Figure 1g, P3). The high intensity of P2 in all SiNP samples as detected immediately after suspension suggested that nanosized particles/agglomerates were present at all concentrations used in this study at the start point of the experiments. The polydispersity index was 0.33, 0.36, 1.86 and 0.57, for SiNP 7, SiNP 14, Aerosil 200 and Aerosil 974 respectively, suggesting that larger aggregates were present in the latter two samples.

After incubation for 2 h and 24 h at 37^{o}C, the particle intensity in the SiNP suspensions of 25 µg/ml decreased to the level slightly higher than that in culture medium , and slightly further decreased at 100 µg/ml for all the NP samples, apart from SiNP 7, in which peaks with relatively high intensity were detected at 2 h. Although Aerosil 974 exhibited 2 peaks (P1 and P2) with intensity comparable to that in the hydrophilic Aerosil 200 sample, to which they share the same primary particle size of 12 nm (Figure 1, compare h with f and i with g), this sample lacked P3 as detected in Aerosil 200 suspension. The overall low particle intensity of Aerosil 974 in suspension was consistent with its hydrophobic surface property.

Using the TEM technique it was also confirmed that at100 µg/ml, all 3 hydrophilic SiNP dispersed well in culture medium with some in clusters (Figure 2 a-c). For the hydrophobic Aerosil 974 sample (Figure 2 d), larger NP were observed, which was in agreement with the poor water solubility of the sample. The TEM results were

consistent with the DLS assay, showing lower intensity and larger particle aggregates for Aerosil 974 as compared with other SiNP.

SiNP induced cytotoxicity as determined by conventional cytotoxicity assays

In order to determine the toxicity potential of the fumed SiNP, cell viability, intracellular ROS and membrane damage were assessed after NP treatment. In cell viability assay, the absorption value of negative control cells (without NP treatment), increased in a time dependent manner, indicating normal cell growth during the 48 h of the MTT study. The effect of NP on cell viability was not detected at 24 h but became significant after 48 h for all the SiNP at all the concentrations tested (Figure 3A). For the positive control H_2O_2 (200 µM), a time-dependent reduction of cell viability was detected at 24 h and 48 h.

An elevation of ROS was detected in cells treated with hydrophilic SiNP of the highest concentration at 4 h, and lower concentrations at 12 h (Figure 3B). The ROS increase was also induced in cells treated with the hydrophobic SiNP Aerosil 974 at all the concentrations at 12 h. The positive control H_2O_2 induced a significant elevation of intracellular ROS at both 4 and 12 h. The ROS level in SiNP treated cells returned to the level as negative control at 24 h.

The LDH leakage was detected at 4 h of SiNP treatment (Figure 3C). The effect was significant in comparison with control (cells without treatment) for all the SiNP at concentration of 25 µg/ml and above as measured at 12 h, and also at 24 h apart from Aerosil 974. The positive control H_2O_2 induced LDH leakage at all the time points tested.

Although the changes in both intracellular ROS and membrane integrity were detected as early as 4 h after cellular exposure to SiNP (Figure 3 B and C), their dose-effect patterns appeared different, suggesting that these two cellular events may not be interrelated or only partially related. Moreover, the differences of the time course and dose-response patterns as detected by the LDH and MTT assays support the hypothesis that mass concentration of SiNP was not the driving force for these toxicity outcomes.

The toxicity of SiNP as detected by the LDH assay was further analysed for dose-effect relationship with SiNP 7 as a model NP. It can be seen in Figure 4 that the coefficient of determinations (R^2) was higher for polynomial models than linear models (Figure 4 compare the R^2 value in e and f with that in b and c), indicating that the dose-effect of SiNP 7 could be better predicted by polynomial regression than linear regression.

SiNP7 induced alterations of extracellular metabolites

To assess whether the extracellular metabonomic changes can predict the cytotoxicity of SiNP, the ^{1}H-NMR-based metabonomic profiling technique was utilized and SiNP7 was selected as a model SiNP. To examine the temporal dose-effects, the extracellular metabonomics was performed at the same time points as for the cytotoxicity study. As shown in Figure 5, SiNP caused an early onset of elevation in some metabolites in culture medium in a concentration-dependent manner. The most affected metabolites include glucose and lactate 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 μg/ml at 4 h and both 50 and

100 µg/ml at 12 and 24 h. Some other metabolites also responded to the treatment but their identities have yet to be determined (Figure 5).

Principal components analysis (PCA) revealed a clear distinction between the samples acquired at different time points after treatment (Figure 6A). 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 6B).

Dose-effect relationship of metabonomic response and toxicity initiated by SiNP

The multivariate calibration technique of SIMPLS was used to establish mathematical models for predicting dose-effect relationship of SiNP on extracellular metabonome. It was shown that the cubic polynomial models were better than linear models for predicting metabonomic changes in relation to SiNP dose, consisting with the dose-effect patterns as derived from the LDH assay at the same timepoints. As seen in Figure 4, SiNP induced a sharp rise in LDH release at 10 µg/ml, followed bya decrease to zero at 25 µg/ml and then a steady linear rise at 50 and 100 µg/ml; yet for 12 h and 24 h, the toxicity index was zero at 10 µg/ml with a sharp rise at 25 µg/ml, followed by a shallow rise at 50 and 100 µg/ml. The decrease in toxicity at higher concentrations (25-50 µg/ml) as compared to the lower concentration could be due to the formation of nanoparticle aggregates. These patterns of dose-toxicity initiated by SiNP at all the time points also fitted the polynomial models better than their respective linear models derived from the metabonomic modelling (Figure 7), suggesting that alterations in the extracellular metabonome could be an indication of cytotoxicity.

Discussion

Silica NP are representatives of nanoparticles that are manufactured in a large quantity for different applications, therefore likely to interact with humans in many ways. Although it has been well documented that some nanoparticles 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 NP 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 of NP toxicity may not be suitable for prediction of nanoparticle toxicity. This study attempted to define the temporal dose-toxicity relationship of fumed SiNP by combining conventional cytotoxicity assays and metabonomics *in vitro* with characterization of NP dispersion property in culture medium.

As shown by DLS and TEM assays (Figure 1 and 2) , SiNP at concentrations used for toxicity study dispersed well in culture medium but form aggregates very quickly, which could largely be due to their low zeta potential as shown in Table 1. The differences in disperse patterns and intensities of different SiNP at the same mass concentration suggested that the intensity of SiNP in suspension was not merely determined by the mass concentration. The size, zeta potential and water solubility should all be considered in interpreting their behaviour in biological systems. Although the fumed SiNP used in this study share very similar primary sizes (7-14 nm), they exhibited distinctive time- and concentration-dependent dispersion patterns, suggesting that the source of SiNP suppliers should be evaluated for

variations in relation to physiochemical characteristics due to the difference in SiNP synthesis.

SiNP 7, SiNP 14 and Aerosil 200 all induced a significant increase in ROS generation at the highest concentration at 4 hours, however, the corresponding toxicity was not so significant as compared with the low concentrations as assessed by the LDH and MTT assays, suggesting that ROS increase was not the sole cause of cytotoxicity in A549 cells. The effects of SiNP seemed not to be concentration dependent, particularly as assessed by LDH at 4 h and MTT at 48 h. All the hydrophilic SiNP showed a consistent level of toxicity whilst the hydrophobic Aerosil 974 exhibited a relatively low level of effect as assessed by MTT, ROS and LDH assays. These results could be explained as that the poor solubility in culture medium resulted in less interactivity of Aerosil 974 with cells as compared with hydrophilic SiNP. Alternatively, the interaction between Aerosil 974 and proteins in the culture medium reduced Aerosil 974 toxicity potential.

This study was to some extent in agreement with some published work using the same *in vitro* model. It was suggested by Ahamed (2013) that SiNP induced cytotoxicity in both A549 and A431 (human skin epithelial cell) cells through ROS generation and oxidative stress. Panas *et al*. (2013) reported a selective toxicity of SiNP in A549 cells rather than in RAW264.7 macrophages, and that the toxicity was most pronounced in the absence of foetal calf serum as compared with some other metal oxide nanoparticles. Their study suggested that the protein corona formed on the surface of SiNP suppresses their toxicity, which needs careful consideration while extrapolating *in vitro-in vivo* studies.

Further analysis of the acute cytotoxic data from the LDH assay resulted in polynomial models that fitted with the actual data pattern much better than linear models, although the trend was more predictable after 12h (Figure 4). Interestingly, a recent study conducted by Berg *et al.* (2013) showed a nonlinear pattern of cellular response to SiNP at a range of concentrations using a number of assays in A549 and MeT-5A pleural mesothelial cell lines, although the implication of this pattern of dose-effect relationship was not further discussed by the authors (Berg *et al.*, 2013). Based on the results from different groups, the non-linear dose-effect pattern could be due to the multimode action of nanoparticles at different concentrations. At relatively low concentrations, the reactive NP surface could be covered by protein corona. When NP concentration reached the level beyond the corona formation capacity, the reactive NP surface becomes accessible, resulting in either NP-cell or NP-NP interactions. The former could lead to direct cytotoxicity, whilst the latter could lead to NP sedimentation and subsequent cellular stress and toxicity. Therefore the relationship of NP dose-effect could not be simply linear. As suggested by the DLS assay in this study, the NP were detectable in suspension at an early time point at all concentrations tested, however the intensity decreased with time in a concentration dependent manner, suggesting the size of the aggregates and sedimentation speed of SiNP at different concentration could all be attributable to the temporal effect of SiNP at different concentrations.

Metabonomics combined with multivariate statistics provides a tool for exploring the metabolism basis of complex dose-effect relationships and biomarkers of toxic agents, and can also generate models for toxicity prediction. ^{1}H-NMR -based assay on extracellular metabolites requires minimal sample preparation, thus minimizing artefacts and allowing better correlation of *in vitro* with *in vivo* studies (Dekker *et al.*,

2012,MacIntyre *et al.*, 2011). The ^{1}H-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 more pronounced for 50 µg/ml at 4 hours and for both 50 µg/ml and 100 µg/ml after 12 hours of treatment, suggesting that SiNP induced different dose-dependent effects with different time course.

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, a high concentration of glucose could result in oxidative stress and cell damage (Li *et al.*, 2011). A recent study in rat showed that the increase in some metabolites including lactate in serum was associated with SiNP-induced liver damage (Parveen *et al.*, 2012). Another study also showed in rat that zinc oxide NP induced an increase in lactate and glucose in urine, which is associated with kidney toxicity (Yan *et al.*, 2012). Feng et al. (2013) reported an early increase of lactate/alanine ratio among other intracellular metabolite changes in human cervical adenocarcinoma HeLa cell line. In the current study SiNP at concentrations 25 µg/ml and above induced both lactate increase and membrane damage in the A549 cells. Taken together, these studies strongly suggest that lactate increase could be a sensitive marker for cell damage and toxicity. Moreover, it has also been reported that lactate can activate macrophages (Samuvel *et al.*, 2009,Nareika *et al.*, 2005), suggesting that lactate could also play a role in inflammation. On the other hand, Tang et al. (2013) reported that TiO_2 nanoparticles-induced liver and kidney damage were associated with a decrease in lactate and an increase in LDH, implying that the alteration of lactate may be nanoparticle-type dependent.

As lactate is produced from glucose through glycoslysis in the cytosol and then enters mitochondria to be oxidized via mL-LDH to pyruvate (Luo *et al.*, 2012,Passarella *et al.*, 2008,Passarella *et al.*, 2008), the increase of lactate could indicate the damage of mitochondrial, which was confirmed in our study by the loss of cell viability as assessed by the MTT assay after 48 h of SiNP treatments. The increase in extracellular amino acids also suggested that SiNP may impair the mechanism of amino acids catabolism, preventing cells from utilizing phenylalanine, tyrosine and histidine.

The concentration range of SiNP effect on extracellular metabonome was consistent with that causing cytotoxicity as assessed by LDH assay. The polynominal models derived from the toxicity and metabonomics assays of SiNP7 effect could also be applicable for the rest of the fumed SiNP or any other NP which tend to form aggregates. A polynomial pattern of NP dose-effect relationship was also reported by Safari and co-workers (Safari *et al.*, 2011), who investigated the pattern of cellular response to NP with varying surface charge.

It is well known that, in the field of nanotoxicology and nanomedicine modelling, the relation between accuracy and biological insight of cellular effect modelling is very complex and difficult to maintain. This is because many alterations in physical and chemical properties and biological processes could occur on the nano/micron scale (Freitas Jr., 2005). In the case of fumed SiNP, the low zeta potential in culture medium determines their low stability in suspension. The distinctive dose-effect patterns of the temporal effects induced by SiNP implicated concentration and time dependent mode of actions. The early effects of NP on cells as assessed by the LDH and ROS assays at 24 h and earlier, could be the outcome of NP interaction with cells . The significant loss of cell viability as assessed by the MTT assay at 48 h, however,

could be mainly due to the sedimentation of nanoparticles that impairs the cellular transport system, leading to the inhibition of nutrient transportation and therefore cell growth.

Study of the cellular and molecular mechanisms of toxicity in vitro is an integral part of mechanism-based risk assessment. While in vivo NP interaction with cells may occur, NP sedimentation is unlikely to occur. Further study of the mechanisms of NP-induced acute toxicity is required for development of reliable dose-effect modelling tools for NP toxicity prediction.

Conclusions

This study demonstrated that fumed SiNP caused both acute and delayed cytotoxicity in A549 cells. The early cytotoxicity could be due to the interaction of NP with cells, leading to oxidative stress, damage to cell membrane, and mitochondria dysfunction; the later cytotoxicity could be mainly due to sedimentation of NP aggregates, leading to further mitochondrial dysfunction and loss of cell viability. It also demonstrated that SiNP-induced extracellular metabonomic alteration and toxicity both followed cubic polynomial dose-response trend better than linear trend. Although conventional cytotoxicity studies could determine the toxicity potency of a given substance, they are mainly endpoint-based and provide little insight about the mechanisms of toxicity. In this regard, ^{1}H-NMR provides a robust and non-destructive tool for identification of toxicity metabolism pathways and predicting toxicity *in vitro*. Due to the ever-increasing application of nanomaterials in many areas, the scale of nanotoxicology tests is expected to expand. The *in vitro* toxicity tests in combination with better characterization of NP physiochemical property in

biological systems will play a pivotal role in our understanding of cellular and molecular mechanisms of nanotoxicity and the development of high-throughput and hypothesis-driven toxicology assays.

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Figure legends

Figure 1. DLS profiles of culture medium and SiNP. Complete cell culture medium exhibited 2 peaks, indicating the presence of nanosized molecules and their aggregates (a); SiNP at 25 µg/ ml exhibited similar profiles as culture medium with a slight increase of peak intensity (b, d, f and h); SiNP at 100 µg/ml exhibited 2-3 separate peaks (c,e, g and i). The samples were analysed at 0 h (thick line), 2 h (thin line), and 24 h (dotted line) after sample suspension in culture medium. The labels P1, P2 and P3 denote major peaks as detected at 0 h.

Figure 2: TEM confirmation of SiNP dispersion in culture medium at100 µg/ml. All the hydrophilic SiNP samples (a-c) dispersed well in culture medium, although some loose clusters (highlighted) in circles formed. Larger particles were observed in the hydrophobic Aerosil 974 (d) samples. The bars represent the length as indicated. Figure 3. Cytotoxicity assessment of SiNP. Loss of cell viability was detected in cells treated with SiNP at 48 h at all doses but not 24 h (A); intracellular ROS increased at 10-100 µg/ml at 4 h and 12 h (B); LDH leakage detected at 25-100 µg/ml at 4 h, 12, and 24 h for all hydrophilic SiNP(C). The asterisks indicate significant difference ($P<0.05$) as compared with negative control (without SiNP treatment).The results were representatives of 3 independent experiments for each assay.

Figure 4. Dose-effect relationship of SiNP cytotoxicity as detected by LDH assay. The dose-effect relationship of SiNP induced membrane damage was analysed using the smooth scatter graph with trendline method. The plot (grey) and the linear and polynomial trendlines (black) with the highest coefficient of determination (R^2) were derived for each time point. The R^2 for linear trendline at each time point (a-c) was less than its respective polynomial trendline (d-f).

Figure 5. 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. The asterisks denote unidentified metabolites.

Figure 6. PCA plots of ^{1}H-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 µg/ml with untreated samples and samples treated with lower concentrations of SiNP 7 at 24 h.

Figure 7. Correlation of metabonomic dose-effect models (black lines) and dose-toxicity (grey lines) as determined by LDH assay. The polynomial metabonomic dose-effect models correlated better than the linear metabonomic models with cytotoxicity at all the 3 time points, as indicated by the higher coefficient of determination (R^2) value in d-f as compared with that in a-c.

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