Gene expression analysis in rat lungs after intratracheal exposure to nanoparticles doped with cadmium

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Abstract. Silica nanoparticles (NPs) incorporating cadmium (Cd) have been developed for a range of potential application including drug delivery devices. Occupational Cd inhalation has been associated with emphysema, pulmonary fibrosis and lung tumours. Mechanistically, Cd can induce oxidative stress and mediate cell-signalling pathways that are involved in inflammation. This in vivo study aimed at investigating pulmonary molecular effects of NPs doped with Cd (NP-Cd, 1 mg/animal) compared to soluble CdCl₂ (400 µg/animal), in Sprague Dawley rats treated intra-tracheally, 7 and 30 days after administration. NPs of silica containing Cd salt were prepared starting from commercial nano-size silica powder (HiSil™ T700 Degussa) with average pore size of 20 nm and surface area of 240 m²/g. Toxicogenomic analysis was performed by the DNA microarray technology (using Agilent Whole Rat Genome Microarray 4x44K) to evaluate changes in gene expression of the entire genome. These findings indicate that the whole genome analysis may represent a valuable approach to assess the whole spectrum of biological responses to cadmium containing nanomaterials.

1. Introduction
Among nanomaterials, silica nanoparticles (NPs) have found extensive applications in several commercial fields. Recently, their use in biomedical area has been extended to DNA delivery [1-4] and drug delivery [5][5].

Silica exists in several crystalline and amorphous forms all characterised by different physicochemical properties as particle shape and dimension, hydrophilicity, presence of surface radicals. The surface properties also depend on the mechanical, thermal and chemical history of a given dust and on the presence of metallic impurities. The NP fate in body and related adverse effects depend on these properties. Crystalline silica dust inhalation may cause silicosis and lung cancer. Amorphous silicones are not generally associated with progressive lung fibrosis, but animal inhalation studies showed reversible lung inflammation, granuloma formation and emphysema.

Silica NPs may contain metals as contaminants, but still unknown is the effect of metals on NP toxicity [6] and if these NPs act as carriers of toxic metals.

Certain inhaled NPs cause higher lung toxicity (e.g., inflammation, cancer) than larger particles of similar chemical composition at equivalent mass concentration. Besides the NP size, additional factors, such as shape, surface charge, coatings and surface reactivity, and particle aggregation/
disaggregation potential, may considerably contribute to NP toxicity [7-9]. Furthermore, the surface can carry large amounts of adsorbed or condensed toxic air pollutants, including the pneumotoxic metals such as cadmium (Cd).

Silica NPs incorporating cadmium [6] have been developed for potential application in informatics and as drug delivery devices. Fluorescent, radio-opaque, and paramagnetic CdS-containing “quantum dots” have been produced as multifunctional probe for bioimaging.

Cd is toxic to the respiratory tract and other organ systems when inhaled. Inhalation of Cd can result in acute injury such as oedema, or in chronic injuries like emphysema, pulmonary fibrosis or adenocarcinomas. Although, the mechanisms of Cd toxicity are not yet fully understood, several publications reported on interference with essential metals, induction of oxidative stress, disruption of cadherins, inhibition of DNA repair and interference with apoptosis [10]. Also some of the molecular sensors of Cd intoxication are known such as metallothionein (MT), heme oxygenase-1 (HMOX-1), and heat shock protein 70 (HSP70) [11]. Moreover inhaled CdCl₂ resulted in pulmonary inflammatory and cell proliferative responses in vitro and in laboratory animals [12,13].

Nanomaterials fall between molecular species and physical entities and their mode of action may vary greatly based on their physicochemical parameters. The scientific basis for performing a toxicological evaluation of these materials can be different from the corresponding nanoscale chemicals.

Conventional procedures for assessing chemical toxicity are not completely suitable for application to nanomaterials [14].

The present study was focalized on the in vivo effects, in laboratory animals, of the test silica nanoparticles incorporating cadmium compared with the metal cadmium chloride.

Nanomaterial testing was focused on specific endpoints and pathological outcomes of lung tissues. Genomic analyses have been performed to provide information about specific mechanisms at the molecular level.

2. Materials and Methods

2.1. Preparation of NP of silica containing Cd salt.
Impregnation of SiO₂ (silica nanosize HiSilTM T700 commercial Degussa, average pore size 20 nm, surface area 240 m²/g) with cadmium nitrate dehydrate was obtained from a preparation of aqueous solution of CdNO₃ 3.56 x 10⁻² M, in which the silica was dispersed in concentration ratios which produces a sample containing 40% Cd by weight. Dispersion was brought to dryness and treated at 600 °C for 2 hr in air. This heat treatment leads to the decomposition of nitrate Cd to Cd oxide, dispersed in silica. Powder was later subjected to grinding mills with high energy (200 rpm for 1.5 hr, 400 rpm for 1.5 hr, 600 rpm for 2 hr) to get the most equal distribution of particle size and/or aggregates of particles. The thermal treatment followed by heating the particles at higher temperatures (ca. 500-600 °C) was performed in order to remove the residual organic groups. Attention was devoted to tune the thermal treatments in order to retain the grains dimensions, pores size, and distributions, and particles surface areas.

Cd concentration released from NP of silica in the physiological solution was determined by Flame-atomic absorption analysis for trace metals. Quantitative analysis was also performed on the NPCd sample for the presence of trace metals.

2.2. In vivo study.
Sprague Dawley male adult rats were treated with a single intratracheal instillation (i.t.) as following: (i) Control group (100 µL physiological solution-ps); (ii) Cadmium Chloride (CdCl₂: 400 µg/rat/100 µL ps), (iii) NP doped with Cadmium (NP-Cd: 1 mg/rat/100 µL ps). Animal lung evaluations were performed 7 and 30 days after i.t. administration.

All experimental procedures involving animals were performed in compliance with the European Council Directive 86/609/EEC on the care and use of laboratory animals (European Council directive
of 24 November 1986 on the approximation of low, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and scientific purposes. Off. J. Eur. Communities L358, 1-28)

2.3. RNA Preparation
Rat lung samples were collected and stored at -80 °C in RNA Later (SIGMA Aldrich) according to manufacturer instructions.

Samples of about 30 mg were homogenized by adding a stainless steel bead followed by 600 ml of Buffer RLT (Quiagen, Germantown, MD, USA) using the TissueLyser (Quiagen, Germantown, MD, USA) at 30HZ for 5 min. The lysate was centrifuged at maximum speed for 5 min and the supernatant was transferred to a G-Column (Quiagen, Germantown, MD, USA) to remove DNA and RNA was then purified using the RNeasy Plus kit (Qiagen, Germantown, MD, USA). RNA was quantified using a ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies), and the integrity of the RNA was assessed with the Agilent 2100 Bioanalyzer (Agilent), according to the manufacturer’s instructions. RNA samples used in this study all had a 260/280 ratio > 1.9 and a RNA Integrity Number (RIN) > 8.

2.4. Microarray expression profiling.
The microarray experiment was designed to perform three replicates for each treatment. All the sample-labeling, hybridization, washing, and scanning steps were conducted following the manufacturer’s specifications. In brief, Cy3-labeled cRNA was generated from 500 ng input total RNA using Quick Amp Labeling Kit, One-color (Agilent).

For every sample 1.65 µg cRNA from each labeling reaction (with a specific activity above 9.0) was hybridized using the Gene expression Hybridization Kit (Agilent) to the Agilent Whole Rat Genome Oligo Microarray (Agilent), which is a 4 x 44k 60mer slide format where each of the 4 arrays represents about 44,000 unique rat genes and transcripts. After hybridization, the slides were washed and then scanned with the Agilent G2565BA Microarray Scanner (Agilent). The fluorescence intensities on scanned images were extracted and preprocessed by Agilent Feature Extraction Software (v10.5.1.1).

Quality control and array normalization was performed in the R statistical environment (http://www.r-project.org) using the Agi4x44PreProcess package downloaded from the Bioconductor web site (http://bioconductor.org/). The normalization and filtering steps were based on those described in the Agi4x44PreProcess reference manual.

In order to detect expression differences among different treatments a moderated t test was applied. Moderated t statistics were generated by limma bioconductor package [15]. Modulated genes were chosen as those with a fold of induction greater than 1.5 and a p-value smaller than 0.01 (NP doped with Cadmium versus control and Cadmium versus control). All of the above computations were conducted using the R statistics programming environment available at www.r-project.org.

After removal of redundant genes, figures-of-merit analysis was applied to define the optimal number of clusters [16]. Gene trends were organized by K-means clustering using squared Pearson correlation by mean of TIGR MultiExperiment Viewer (Mev) [17][17]. Each clusters generated by K-mean analysis was analyzed in the Metcore package to identify genes with similar functions. The MetaCore software (GeneGo, St. Joseph, MI) is a computational resource that uses logic operations for identifying biological processes that are altered because of changes in gene expression [18].

3. Results and Discussion

3.1. Evaluation of the release time of Cd from silica nanoparticles (NP-Cd).
Cd concentration released from NP of silica in the physiological solution was linear during a period of 16-232 hr (evaluated by linear of regression; y= 473.24+0.7235x, R2=0.9975). The maximum release (about 15%) was observed after 16 hr in physiological solution.
3.2. Purity of NP doped with cadmium.
Quantitative analysis was performed on the NPCd, to verify the amount of doping effectively dispersed in the silica and the presence of any metal pollutants from possible impurities in the reagents or the grinding process in jars of tungsten carbide alloy with cobalt. The presence of Cd and Si has been highlighted (32.5% and 24.1% respectively), the main pollutants were Ca (0.3%), Na (0.2%), K (0.2%), Fe (0.04%) and Mn (0.001%). Other metals were present in quantities less than 1 ‰.

3.3. Preliminary characterization of NPCd.
The crystalline structure of the product was investigated by X-ray powder diffraction; an amorphous compound was obtained. The diffractogram shows only a broad band in the range of 10-35° angle/2θ, typical of components of low crystallinity and low particle size (Fig. 1). NPCd showed stability in suspension > 30 days; amorphous structure; spherical form; average size of 20 nm; size distribution mono-modal, asymmetric, with modal value of 10 nm; 200 m²/g surface area; tendency to form aggregates and agglomerates of about 150 nm; and zeta potential of -30 mV (in dehyonized water).

Figure 1. X-ray diffractogram of a NPCd sample.

3.4. Pulmonary Toxicogenomic data.
The study approach was not to investigated the well know effects of Cd toxicity for which the bulk of experimental and epidemiological data have abundantly addressed them till to define the limit value in the human exposure scenario[19], but that to evaluate the effects of engineered NPs (ENPs) namely cadmium-doped NPs using a new model of screening toxicity study, such as whole genome analysis, by means of the Cd as a tool of lung toxicity model. Specifically, the aim of the present work was to use Cd, a well know pneumotoxicant, as a tool to investigate its counterpart-doped nanoparticles. Consequently, we employed a Cd dosage that is known to induce lung toxicity in rodents. The dose selected was based on previous experiments showing lung dysfunction, cytotoxicity, edema and fibrosis after application of cadmium chloride [20-22]. In particular, lung injury induced by CdCl₂ given i.t. at the dose of 400 µg/rat (≡ 245 µg Cd) was shown to represent a good model of human interstitial lung disease [22]. In other studies, the dose of 0.3 mg Cd corresponding to 490 µg CdCl₂,
caused leukocytic infiltration and edema in lung 24 hr after i.t. dosing in guinea pig [21], and 400 µg of i.t. administered CdCl₂ caused various forms of intraluminal fibrosis 4 and 7 days after treatment in rat [22]. Persistent lung damage has been reported in an animal model following a single i.t. exposure to 25, 100, or 400 µg CdCl₂/kg bw [23] with histopathological alterations including inflammation and fibrosis still present 90 days post-exposure and the incidence and severity of the lesions greater at 90 days compared to 28 days.

Differences exist in the dose rate and distribution of material delivered by instillation versus inhalation, however, the database on pulmonary toxicity of various particulate materials indicates that the two modes of exposure yield qualitatively similar results for a variety of biological endpoints, including pulmonary inflammation, fibrosis, susceptibility to infection, allergic sensitization and lung cancer in rats after exposure to poorly soluble particle [24]. In particular for Cd, as discussed in Oberdörster [25-27], the pulmonary retention half-time of both inhaled and intratracheal instilled CdCl₂ resulted in similar Cd retention and similar clearance kinetics (i.e. 60-85 days) in rats.

For the present study the i.t. was applied as a useful method to compare the lung effects in rats of a new material (i.e., counterpart-doped nanoparticles) against similar compounds (i.e. CdCl₂) for which an extensive inhalation database is available [19]. Moreover, i.t. model may serve to a screening tool to determine the approximate dose range that may be appropriate for later inhalation studies as well as to address specific endpoints regarding the respiratory toxicity of nanomaterials [24].

Based on the physiology of the human respiratory tree, a comprehensive model to predict the kinetics of inhaled cadmium in humans [28] has suggested that only about 5% of particles >10 µm in diameter will be deposited, up to 50% of particles <0.1 µm will be deposited, and between 50 and 100% of cadmium deposited in the alveoli will ultimately be absorbed.

Since, nanoparticles have unique physicochemical properties and functionalities that are different from their bulk counterparts, their mode of action may vary greatly based on their physicochemical parameters. This is therefore crucial to determine the appropriate way to compare physicochemical properties with fate and toxicity. Moreover, once they have entered the body (e.g. through inhalation), engineered NPs have the ability to translocate and to be distributed to distant target organs. The harmful effects associated with this process have not yet sufficiently investigated. Translocation is also an important component in the toxicity of Cd which has been shown to accumulate in kidney and affect renal function after chronic inhalation exposure other than produce toxic effects on the primary target organ, i.e. lung, being the site of entry.

The detrimental effects of silica NPs containing metals as contaminants are still unknown [29] and still largely undefined is whether these NPs act as carriers of toxic metals. However, engineered cadmium-containing silica NPs show great promise for targeted drug delivery [30].

Transcriptomics was applied to study whole genome expression in the lung tissues collected from particle-exposed animals in order to identify early adverse events at transcriptional level. Such early changes may be predictive of delayed irreversible responses and may be useful as early biomarkers of organ toxicity.

After 7 days we identified 22 probes regulated by cadmium and 71 by NP doped with cadmium, whereas 138 probes are regulated by cadmium and 156 by NP doped with cadmium after 30 days. The few genes regulated at 7 days were still modulated at 30 days, therefore, we decided to further analyse only the set of genes regulated at 30 days.

After removal of duplicated genes and probes without Entrez ID, 129 genes were identified as the union set of genes regulated. This set was subjected to figures-of-merit analysis, where K-means clustering performed optimally for 4 clusters, with no increase in the predictive value of the algorithm for additional behavioral categories (data not shown). Pearson correlation was used as similarity measurement, and generating a total of 4 gene clusters (Figure 2). To gain insight into the biological processes involved, each cluster was then subjected to GO (gene ontology) analysis as included in the Metacore package (www.genego.com).

Cluster I is identified by genes that are up-regulated by both stimuli, and as a result of meta-analysis the most overrepresented category was circadian rhythm, that is represented by Per1, Per2,
Per3 and Nr1d1. Circadian variation has been associated with inflammatory response as it has been described in patients affected by asthma [31][15], therefore such an unexpected result could actually fit with the induction of inflammation in our model. The second category represented in terms of relevance is unfolding protein response, confirming previous published data on cadmium as an inducer of endoplasmic stress and of unfolding protein response [32].

Cluster II contains genes that are upregulated only by cadmium, the main categories enriched in this cluster are inflammation, blood coagulation and phagocytosis. This is probably due to the cellular damage induced by Cadmium, that ultimately causes inflammation and macrophage recruitments.

Cluster III represents genes that are upregulated only by NP doped with cadmium. This group is enriched in genes involved in the inflammatory process. Neutrophils and macrophages are probably activated by damaged cells through the expression of chemokines: interestingly in this cluster CXCL9 and CXC3 are represented. It has been described that CXCL9 interaction with cells expressing CXCR3 has an important role in the recruitment of mononuclear cells [33].

Cluster IV is populated by genes that are downregulated by both stimuli. Again, we can observe here an enrichment of genes involved in circadian rhythm Arnt1 and Npas2. This category is also enriched in cluster I where Per1, Per2, Per3 and Nr1d1 are upregulated. This pathway will be further investigated since the data suggest that cadmium and NP doped with Cd have an assorted effect on this pathway. Moreover, genes involved in cell cycle regulation are downregulated and this can probably induce inhibition in the cell cycle processes.

The overall data show a complex regulation induced by nanoparticles doped with cadmium, only partially overlapping with cadmium treated, but modulating some relevant specific pathways.

In summary, the present preliminary results indicate that whole genome analysis of pulmonary tissue may represent a valuable approach to assess early molecular events in response to model NPs in a biological system.

In this context, one strategy in the application of microarray technology is the correlation to toxic end results. Specifically, combining the present gene expression results with biochemical and/or histopathological evaluation at the target tissues could be profitable in providing more direct linkages between a change in gene expression and the type and outcome of the toxic response. In this manner, patterns of gene expression can be used as biomarkers of exposure and as a method of identifying molecular mechanisms of toxicity to predict detrimental effects.

This technology should be further applied to verify its suitability to detect subtle effects for example in low exposure situation mimicking the real world scenario in a context of environmental persistent nanomaterials.
Figure 2. K-mean clustering of genes differentially expressed. Modulated genes were organized in 4 clusters by K-means clustering. Each column corresponds to a single biological replicate. Each row represents a gene, with red and green for high and low expression levels, respectively.
Table 1. Top ranked pathways from GeneGo MetaCore pathway analysis.

| Cluster I                  | pValue            |
|----------------------------|-------------------|
| Neurophysiological process_Circadian rhythm | 1.88324E-05       |
| Protein folding_Response to unfolded proteins | 5.36253E-05       |
| Reproduction_Male sex differentiation | 5.80785E-05       |
| Development_Ossification and bone remodeling | 8.89511E-05       |
| Protein folding_Folding in normal condition | 0.005492635       |
| DNA damage_Checkpoint | 0.00652288        |
| Protein folding_Protein folding nucleus | 0.013729699       |
| Cell adhesion_Amyloid proteins | 0.01859377        |
| Cell cycle_G2-M | 0.02518908        |
| Cell cycle_Meiosis | 0.038738286       |

| Cluster II                  | pValue            |
|----------------------------|-------------------|
| Inflammation_IL-6 signaling | 0.000712405       |
| Transport_Bile acids transport and its regulation | 0.001804934       |
| Blood coagulation | 0.009247253       |
| Inflammation_Kallikrein-kinin system | 0.023610214      |
| Immune response_Phagocytosis | 0.038935501       |
| Regulation of metabolism_Bile acid regulation of lipid metabolism and negative FXR-dependent regulation of bile acids concentration | 0.091342713      |
| Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT | 0.227018485      |
| Development_Ossification and bone remodeling | 0.228417535       |
| Cell adhesion_Platelet-endothelium-leucocyte interactions | 0.250494565      |
| Apoptosis_Anti-Apoptosis mediated by external signals via PI3K/AKT | 0.27200139        |

| Cluster III                  | pValue            |
|----------------------------|-------------------|
| Cell adhesion_Leucocyte chemotaxis | 3.78124E-05       |
| Chemotaxis | 0.000147037       |
| Proliferation_Negative regulation of cell proliferation | 0.007075679       |
| Inflammation_NK cell cytotoxicity | 0.03637402       |
| Reproduction_FSH-beta signaling pathway | 0.046560893       |
| Immune response_TCR signaling | 0.057686767       |
| Reproduction_Feeding and Neurohormone signaling | 0.08168305        |
| Inflammation_IL-13 signaling pathway | 0.144983618       |
| Immune response_Th17-derived cytokines | 0.17433034       |
| Proteolysis_ECM remodeling | 0.186759724       |

| Cluster IV                  | pValue            |
|----------------------------|-------------------|
| Neurophysiological process_Circadian rhythm | 0.013089908       |
| Cell cycle_G0-G1 | 0.017940113       |
| Immune response_Th17-derived cytokines | 0.029541451       |
| Cell cycle_Core | 0.051104212       |
| Chemotaxis | 0.063016076       |
| Cell adhesion_Glycoconjugates | 0.06979147        |
| Cell cycle_G1-S | 0.092543148       |
| Immune response_Antigen presentation | 0.106901061       |
| Proliferation_Negative regulation of cell proliferation | 0.114677047       |
| Cytoskeleton_Actin filaments | 0.11602948        |
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