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A novel platform for pulmonary and cardiovascular toxicological characterization of inhaled engineered nanomaterials

GEORGIOS A. SOTIRIOU¹, EDGAR DIAZ², MARK S. LONG², JOHN GODLESKI², JOSEPH BRAIN², SOTIRIS E. PRATSINIS¹, and PHILIP DEMOKRITOU^{2,*}

¹Particle Technology Laboratory, Institute of Process Engineering, Department of Mechanical and Process Engineering, Swiss Federal Institute of Technology Zurich (ETH Zurich), Sonneggstrasse 3, CH-8092, Zurich, Switzerland ²Department of Environmental Health, Harvard School of Public Health, Harvard University, 401 Park Drive, 02215 Boston, MA U.S.A.

Abstract

A novel method is presented which is suitable for assessing in-vivo the link between the physico-chemical properties of engineered nanomaterials (ENMs) and their biological outcomes. The ability of the technique to generate a variety of industry-relevant, property-controlled ENM exposure atmospheres for inhalation studies was systematically investigated. The primary particle size for Fe₂O₃, SiO₂, Ag and Ag/SiO₂ was controlled from 4 to 25 nm, while the corresponding agglomerate mobility diameter of the aerosol was also controlled and varied from 40 to 120 nm. The suitability of the technique to characterize the pulmonary and cardiovascular effects of inhaled ENMs in intact animal models is also demonstrated using in-vivo chemiluminescence (IVCL). The IVCL technique is a highly sensitive method for identifying cardiopulmonary responses to inhaled ENMs under relatively small doses and acute exposures. It is shown that moderate and acute exposures to inhaled nanostructured Fe₂O₃ can cause both pulmonary and cardiovascular effects.

Keywords

Engineered Nanomaterials; in-vivo inhalation; nanotoxicology; cardiovascular and pulmonary effects; nanotoxicology; iron oxide

INTRODUCTION

The use of engineered nanomaterials (ENM) in household products, textiles, industrial processes, medical devices and therapeutics is widespread and increasing exponentially. Environmental and occupational exposures are considered by experts to be “inevitable” (National Research Council 2008; National Science and Technology Council Committee on Technology 2008; Powell et al. 2010). Preliminary evidence demonstrates the potential for ENM to cause adverse biological effects (Colvin 2003; Nel et al. 2009; Service 2000). Indeed, the potential of nanoparticles and nanofibers to translocate through the air-blood barriers, and thus to reach the pulmonary connective tissues, lymphatic system, or even to reach the circulating blood and thus have access to other critical organs, is of concern. Nano-sized particles (NPs) may enter cells, and be more biologically active than their micro-sized counterparts due to their small size and large surface to volume ratio (Hamilton et al. 2009;

*Corresponding author: pdemokri@hsph.harvard.edu.

Larsen et al. 2010; Li et al. 2010; Muller et al. 2005; Poland et al. 2008; Rossi et al. 2010; Service 2004; Witzmann and Monteiro-Riviere 2006) and increased release of ions (Sotiriou and Pratsinis 2010).

Many in-vitro assays and novel aerosol generation methods suitable for inhalation studies have been developed and contributed significantly to improving our understanding of biological mechanisms related to atmospheric particle health effects (Demokritou et al. 2002; Demokritou et al. 2003; Gupta et al. 2004). However, these currently available particle exposure methods can not be applied in the nanotoxicology field, primarily because of the unique properties of ENM. For in-vivo ENM inhalation studies, an important limiting factor is the difficulty in aerosolizing and dispersing commercially available nanopowder ENM down to the nano-size level, as they would exist in many relevant inhalation exposures (Schmoll et al. 2009). Some common aerosol generators used in the past to disperse nanopowders for inhalation studies, including nebulizers, fluidized beds, and other venturi aspirator type systems, have limited applicability for nanotoxicology (Schmoll et al. 2009). For example, such nanopowder aerosol generation systems have difficulty producing consistent nanosized aerosol distributions and more importantly, fail to control physico-chemical properties in order to elucidate the link between properties and toxic biological responses (Fischer and Chan 2007; Schmoll et al. 2009).

As a result of the challenges and limitations, the majority of nanotoxicity research has focused on in-vitro cellular or in-vivo instillation studies using commercially available ENM nanopowders and nanopowder liquid suspensions; however, there is a consensus among scientists that data will require verification from animal inhalation experiments (National Science and Technology Council Committee on Technology 2008; Schmoll et al. 2009). There are also serious shortcomings related to the physiological relevance of this particle to cell delivery approach using liquid ENM suspensions. The shortcomings of these in-vitro and instillation approaches are twofold: First, commercial ENM are limited in diversity of physicochemical and morphological properties – usually to a few sizes for a given composition – making it impossible to perform comparative parametric toxicological studies of ENM properties (size, surface, composition, shape, charge, etc.). Second, nanoparticles suspended in culture media flocculate or dissolve, and interact with serum components (Fadeel and Garcia-Bennett 2010; Jones and Grainger 2009; Verma and Stellacci 2010), which can alter their biological properties and effects. Furthermore, comparison of particle doses delivered in suspension to those administered by inhalation is difficult, which can result in large differences in effective dosage between in-vivo and in-vitro studies. These limitations may explain some of the disparities reported in the literature between in-vivo and in-vitro ENM studies (Fadeel and Garcia-Bennett 2010; Fischer and Chan 2007; Sayes et al. 2007). It is apparent that new methods and systems suitable for both in-vitro and in-vivo inhalation toxicological characterization need to be developed.

Here, a novel technique is presented which is suitable for both ENM in-vivo inhalation and in-vitro toxicological characterization studies. The ability of this technique to generate a variety of industry-relevant, property-controlled exposure atmospheres for inhalation studies was systematically investigated. The suitability of the technique to characterize the pulmonary and cardiovascular effects of inhaled ENM in intact animal models was also demonstrated in an in-vivo study involving Sprague-Dawley rats, using freshly generated nano-iron oxide (Fe_2O_3) as a test aerosol. We demonstrated both pulmonary and systemic toxicity using in-vivo chemiluminescence of heart and lung (Bartoli et al. 2009; Clarke et al. 1999; Clarke et al. 2000; Godleski et al. 2002; Gurgueira et al. 2002; Rhoden et al. 2004; Saldiva et al. 2002). This novel platform will make it possible for toxicologists to link physico-chemical properties of inhaled ENMs to biological outcomes and help the industry to develop safer ENM.

METHODS

Versatile engineered nanomaterials generation system (VENGES)

Figure 1 illustrates the Versatile Engineered Nanomaterial Generation System (VENGES) (Demokritou et al. 2010). The system consists of four main components: ENM synthesis; particle sampling and collection system; animal exposure system; and exposure monitoring system.

ENM synthesis—The synthesis of ENMs is based on the industry-relevant flame spray pyrolysis (FSP) method, as most of today's ENMs are made by gas-phase techniques (Pratsinis 2010). An inflammable liquid precursor, containing dissolved organometallic compounds, is pumped through a stainless-steel capillary tube (ID 0.41 mm; OD 0.71 mm). Immediately surrounding the capillary tube is a narrow annular gap of adjustable cross-sectional area that supplies oxygen, which is used to disperse the liquid precursor into fine droplets. A small pilot flamelet (premixed CH₄ and O₂ at 1 and 2 L/min, respectively) ignites the droplets so that the organic parts of the precursor combust, while the metals are oxidized, and form particles that grow by coagulation and sintering in the flame. The precursor liquid feed is metered by a mass rate-controlled syringe pump, and all gas flows are controlled by mass flow controllers (Madler et al. 2002; Teleki et al. 2008).

Sampling and off line characterization of generated ENM—The flame-generated particles are collected using a water-cooled, stainless-steel filter housing supporting a glass fiber filter (Whatman GF6, 25.7 cm in diameter, USA), with exhaust gases drawn by a vacuum pump (Busch, Seco SV 1040C, Netherlands). Collected particles can be extracted from the filter as nanopowder, and used for off-line physico-chemical and morphological characterization, as well as for in-vitro or in-vivo instillation studies. Off-line ENM characterization included N₂ adsorption (5-point isotherm, Micromeritic Tristar, Switzerland). ENM Samples were degassed under N₂ at 150 °C for 1.5 hours. The average primary particle diameter d_{BET} was calculated from their specific surface area SSA : $d_{BET} = 6000/(\rho \cdot SSA)$, where ρ is the materials density. X-ray diffraction (XRD, Bruker D8 Advance, Cu K_α, Switzerland) was also performed over scan range $2\theta = 20 - 70^\circ$. Morphology of the generated ENM was also obtained using transmission electron microscopy and scanning transmission electron microscopy (STEM, instrument for both: Tecnai F30, Switzerland). The samples were dispersed in ethanol and then placed on a carbon coated copper grid.

Animal exposure system—The continuously produced (in the gas phase) nanoparticles are sampled in-situ over the flame (Q_S, Figure 1), and transferred to inhalation chambers for animal studies through a 1 L aerosol container to stabilize the flow rate. The sampled aerosol can be further diluted with high-efficiency-particulate (HEPA) -filtered dry air (Q_D). The ENM remain airborne, with a very low level of agglomeration, and are drawn directly through the animal exposure chambers (Q_R, Figure 1). For this study, each animal was housed in its own whole body exposure chamber (10 cm in diameter, 18 cm in length). Chambers were made of clear polycarbonate to allow visualization of the animals during the exposure, and used a modified rubber stopper as a cap. Flow through each chamber was maintained constant at 1.5 L/min.

Exposure monitoring system—The animal exposure atmosphere was monitored continuously, using a number of real time instruments. Exposure parameters monitored included total particle counts (P-Track, TSI, USA), aerosol size distribution (FMPS, TSI, USA), NO₂, CO, CO₂, temperature, and relative humidity (Figure 1).

Performance characterization experiments

The versatility and ability of VENGES to generate an array of industrially relevant ENM while controlling important nanoparticle properties (i.e. primary particle size, agglomeration state, surface properties, shape) as well as its suitability for in-vivo inhalation studies was demonstrated in a number of performance characterization experiments, as follows:

ENM generated nanopanel—Four different ENM, silicon dioxide (silica, SiO₂), iron oxide (Fe₂O₃), silver (Ag, nanosilver), and composite nanosilver supported on silica particles (Ag/SiO₂) were generated here. Table 1 summarizes the ENM used in these experiments and their respective liquid precursor characteristics. It is worth pointing out that the FSP synthesis method used in VENGES allows for the generation of a large universe of industrially relevant ENM (Strobel and Pratsinis 2007).

Controlling both the aerosol size (airborne phase) and primary particle size—

In order to investigate whether VENGES can successfully vary both the size of the generated aerosol as well as the primary particle size, a study was performed by varying two important VENGES process parameters: i) liquid precursor feed rate (from now on: x) over the O₂ dispersion flow rate (from now on: y) ratio (x/y); ii) liquid precursor molarity, M (mol/L). Table 1 summarizes for each ENM the x/y ratios and the variable molarity experiments, used in the experiments. For all experiments, the properties of the ENM VENGES generated aerosol were characterized in-situ in real-time. In addition, the physico-chemical and morphological characterization of the ex-situ collected nanoparticles was also performed off line as previously described.

Reproducibility of the generated test aerosols over time—For inhalation toxicological studies, it is important to: a) generate in a reproducible manner test aerosols with constant concentration and size distribution for the duration of the exposure (hours); and b) for dose/response toxicological studies, the number concentration should be able to be controlled. The ability of VENGES to fulfill both requirements was explored by varying the ratio of the sampling to the dilution flow rate (Q_S/Q_D). ENM were synthesized at constant precursor molarity and x/y ratio was kept constant. The Q_S/Q_D ratio varied in a step-wise manner over a period of more than 5 hours of continuous ENM aerosol generation. The mass concentrations were calculated from the FMPS data assuming Fe₂O₃ density of 5.24 g/cm³ for the test aerosol and 1 g/cm³ for indoor particles.

ENM surface modification experiment—The VENGES system also allows for generation of surface modified test aerosols in the gas phase (Madler et al. 2003). At the nanoscale level, surface properties and chemistry may affect the biological properties of ENM and are among the important parameters for toxicological investigation. Surface modification is also one of the emerging methods for safer nanomaterial formulation. Surface modification may result in a less toxic ENM while its important electrical or structural properties of interest remain unchanged. Here, VENGES was used to generate SiO₂ nanoparticles and their surface was modified in-situ by adding nanosilver particles on their surface (Demokritou et al. 2010). The total particle number concentration and size distributions of both the basic SiO₂ and the surface modified Ag/SiO₂ aerosol properties were measured in-situ. Furthermore, ex-situ characterization of the collected ENM was also performed as described above.

Acute Pulmonary and cardiovascular effects of inhaled nanostructured Fe₂O₃ using the VENGES platform and IVCL assay

The suitability of VENGES platform for in-vivo animal inhalation studies was demonstrated here. The pulmonary and cardiovascular effects of inhaled Fe₂O₃ nanoparticles were

assessed. Fe_2O_3 nanoparticles have been used widely in a number of applications including drug delivery, bio-imaging and nutrition (Hilty et al. 2010; Lu et al. 2007; Teleki et al. 2009). The toxicological assay used to assess health outcomes in this study was in-vivo chemiluminescence (IVCL) of the lung and heart surface immediately after exposure; this technique has been widely used in our laboratory for ambient particle toxicity studies and is a highly sensitive method for identifying pulmonary and cardiovascular responses to inhaled particles under relatively small doses and acute exposures (Godleski 2006; Gurgueira et al. 2002).

Protocol—Male Sprague-Dawley rats (200–250 g) were obtained from Taconic Laboratories (Rensselaer, NY), housed, and managed according to the NIH guidelines for the care and use of laboratory animals. Upon arrival, animals were assigned a unique identification number, which determined the exposure date and exposure group (Aerosol or Filtered Air) for the animal. Rats were allowed to acclimate to the animal facility for 4–5 days prior to start of experiments. The Harvard Medical Area's Animal Use Committee approved the animal protocols used in this study. Each day two animals were exposed to Fe_2O_3 test aerosol and two animals to filtered air (sham) in individual exposure chambers for 5 hours. Four repetitions (days) with a new set of animals each day were carried out. At the end of each five-hour exposure, two animals from each group had IVCL (Boveris et al. 1980).

RESULTS

Performance characterization experiments

Effect of x/y ratio on both the aerosol size (airborne phase) and primary particle size

Figure 2a illustrates the particle number concentration as a function of mobility diameter for pure SiO_2 . It is worth mentioning that the mobility diameter is correlated to the aerodynamic diameter and volume equivalent (thermodynamic) diameter (Hofmann et al. 2009; Kasper 1982; Peters et al. 1993). Particles that are sized based on their mobility in an electric field are considered for practical purposes classified by their thermodynamic diameter (McCawley et al. 2001; Schiller et al. 1988). Thermodynamic diameter is an important determinant of nanoparticle lung deposition (ICRP 1994). As shown in the Figure, the VENGES generated aerosol has a unimodal and narrow size distribution. As inset in Figure 2a, the mode, mean, median diameters and the geometric standard deviations are presented showing the statistical analysis of the obtained particle size distributions (Hinds 1999). Additionally, the modal diameter increases with increasing x/y ratios, as the SiO_2 aerosol concentration increases. It is clear that VENGES not only has the ability to generate aerosols with unimodal size distributions but also has the ability to adjust the aerosol size. This can be seen more clearly in Figure 2b, where greater modal diameters (circles, left axis) were indicated for larger x/y ratios. This is attributed to the higher particle concentration with higher x/y ratio during their flame formation that leads to larger aggregates/agglomerates. It is noteworthy that all particle size distributions are stable over time, as it will be discussed later on (see Figures 5a and 6b).

Figure 2b compares the average primary particle diameter of the collected ENM as obtained by ex-situ N_2 adsorption with their airborne phase modal diameter. The average SiO_2 primary particle size of the collected ENM decreases with increasing x/y ratios (Demokritou et al. 2010; Madler et al. 2002). However, there is a difference between the primary particle size of the collected ENM determined by N_2 adsorption and the modal diameter of the same particles in their airborne phase. This discordance between the mobility (airborne phase) and the primary particle diameter (nanopowder form) indicates, that the latter is not necessarily a good predictor of the ENM behavior when it becomes airborne. In other words, the

nanopowder-derived primary particle diameter is not a good predictor of its fate, transport and deposition in the environment and in human or animal lungs. This finding may explain some of the discrepancies of the toxicological results reported in the literature between in-vivo and in-vitro ENM studies (Fadeel and Garcia-Bennett 2010; Fischer and Chan 2007; Sayes et al. 2007). The in-vivo validation of in-vitro (cellular) studies is considered an important element of the proposed Environmental Health and Safety strategy recently released by the National Nanotechnology Initiative (NNI).

Similarly, Figure 2d show the effect of x/y ratio on both the primary particle and the mobility diameter of Fe_2O_3 . Figure 2c also shows the XRD patterns of the ex-situ collected and characterized nanoparticles as a function of the x/y ratio. This indicates that larger Fe_2O_3 particles are formed with higher x/y ratio, in agreement with literature (Demokritou et al. 2010; Teleki et al. 2009). This is due to their larger residence time in higher temperature zones that result in larger crystals. It is worth pointing out that the XRD-estimated average crystal particle sizes (filled triangles, Figure 2d) are consistent with their average primary particle sizes (open triangles, Figure 2d), with the latter having slightly larger values indicating polycrystalline or aggregated nanoparticles. The modal mobility diameter for the Fe_2O_3 aerosol (Figure 2d, circles, left axis) is not significantly affected by the x/y ratio, as for most ratios it is fairly constant. Furthermore, as it is shown above in the case of SiO_2 , there is a difference between mobility diameter (airborne phase) and the primary particle diameter (nanopowder form).

Effect of precursor molarity on both the aerosol size and primary nanopowder particle size

Figure 3 indicates the effect of another important process parameter of VENGES, the metal precursor molarity, on both the aerosol size (mobility diameter) and primary nanopowder particle size for SiO_2 , Fe_2O_3 and Ag ENM. Figure 3a shows for the SiO_2 aerosol, the particle number concentration as a function of mobility diameter (for a constant 2/7 ratio) for different metal precursor molarities with their corresponding diameters and geometric standard deviation as inset. It can be seen that the mobility size distribution shifts to larger sizes with an increased precursor molarity. It is worth mentioning that there is an overlapping among the different particle size distributions presented in the figure. However, the aerosol size distributions were randomly selected to prove the point that aerosol properties can be adjusted. Whether this size distribution differences are clinically relevant, it remains to be seen in future toxicological studies.

Similarly, this pattern can be seen also in Figure 3b, where the mode mobility diameter (circles, left axis) is also increasing for higher precursor molarity. This could be attributed to the higher concentration of the particles at an early stage during their synthesis that would lead to formation of larger agglomerates. In contrast to the behavior of the SiO_2 particles in airborne phase, the average primary particle diameter (d_{BET} , triangles, right axis) does not significantly vary with precursor molarity (Jossen et al. 2005) as sintering rather than coagulation determines the primary particle size in contrast to mobility diameter that is determined by coagulation and particle concentration levels.

Similarly for Fe_2O_3 nanoparticles, the mobility size distributions shift to larger sizes for higher precursor molarities (Figures 3c and 3d). The average primary particle (Figure 3d, d_{BET} , open triangles, right axis) and the crystal (Figure 3d, d_{XRD} , filled triangles, right axis) diameter seem to slightly increase for higher precursor molarity, forming polycrystalline nanoparticles, as with SiO_2 . The TEM image of Fe_2O_3 nanoparticles shown in Figure 3e shows that there are many agglomerates/aggregates composed of smaller primary particles, in agreement with the results obtained by XRD, N_2 adsorption and mobility size distribution analysis. Finally, Figure 3f indicates that the mobility size distribution of nanosilver (Ag) can be also shifted to larger values for higher precursor molarity.

Control of concentration/Reproducibility of the generated test aerosols over time—

We have demonstrated above that VENGENS has the ability to control both the aerosol or agglomerate size (airborne phase) and the primary particle or crystal size (nanopowder form), in a highly reproducible way. However, for inhalation toxicological studies, aerosol generation methods need to be able to i) adjust the concentration level (dose/response studies); and ii) deliver test aerosols with constant properties for hours. Figure 4a shows the total particle number concentration C_T (solid line) and the modal (broken line) and median (dotted line) mobility diameters of the nanosilver aerosol (2/7, 0.02 M precursor concentration) as a function of exposure time. The dilution of the sampled aerosol (Q_S/Q_D , Figure 1) was periodically adjusted in a stepwise manner during the experiment to control the concentration level. As expected, for higher Q_S/Q_D ratio, higher C_T is reached. Most importantly, when a constant Q_S/Q_D ratio is used, both the aerosol total particle concentration (C_T) and the size distribution indicated by the modal and median (dotted line) mobility diameters remain constant. This is clearly an indication that VENGENS can control the aerosol concentration levels and deliver test aerosols with constant size distributions over time.

It is noteworthy that when the Q_S/Q_D ratio is adjusted back to its initial value, the aerosol size distribution returns to its initial one, a clear indication of the reproducibility of the method. Similarly, for Fe_2O_3 , Figure 4b shows this constant aerosol particle concentration and size distribution over time for different Q_S/Q_D ratios.

ENM surface modification—The surface of ENMs can be modified in order to add desired attributes such as dispersibility (Teleki et al. 2008) (e.g. SiO_2 -coated Ag) (Sotiriou et al. 2010) or antibacterial activity (Sotiriou and Pratsinis 2010) (e.g. SiO_2 supported nanosilver). It may also be a useful concept for the formulation of safer ENM (Demokritou et al. 2010; Sotiriou et al. 2010). Recently, it was shown that doping ZnO nanoparticles with Fe can reduce the release of Zn ions (George et al. 2010). Figure 5a illustrates the particle number concentration as a function of mobility diameter for both pure SiO_2 and for the surface modified nanocomposite Ag/ SiO_2 (Ag-content 10 wt%). Both size distributions are almost identical (Figure 5a) as verified also by the mode, median, mean diameters and the geometric standard deviations (inset). This indicates that VENGENS has the ability to generate surface modified nanoparticles while maintaining the intended size distribution. This will be useful when studying the comparative toxicity of surface modified nanoparticles in-vivo. By controlling the size, we can also influence control the particle deposition in the lungs. In addition, ENMs can also be used as a “vehicle” for delivery of drugs in-vivo. Figure 5b shows a STEM image of the composite Ag/ SiO_2 nanoparticles. The nanosilver particles (bright spots) are homogeneously dispersed on the amorphous SiO_2 (diffuse gray) surface (Demokritou et al. 2010; Sotiriou and Pratsinis 2010).

Pulmonary and cardiovascular effects of inhaled nanostructured Fe_2O_3 using the VENGENS platform

Figure 6a indicates the total particle number concentration as a function of time for the 5 hour exposure period for the Fe_2O_3 aerosol (2/7 ratio, 0.02 M precursor molarity). As shown in this figure, the concentration levels remained fairly constant for the whole animal exposure (the 5 minute stops indicated in the Figure were necessary in order to replace the syringe with liquid precursor). For this exposure scenario, VENGENS was tuned to generate a test aerosol of a total number concentration of $2-3 \cdot 10^5$ particles/cm³ (this would correspond to approximately to 100-200 $\mu\text{g}/\text{m}^3$), approximately 10-20 times higher concentration of indoor conditions (10 $\mu\text{g}/\text{m}^3$). The temperature, relative humidity, CO_2 , CO and NO_2 concentrations of the test aerosol were identical to the room conditions, ensuring no interference with the in-vivo toxicological results. Additionally, Figure 6b shows the

averaged mobility size distribution of the test aerosol. The error bars correspond to the standard deviation of the particle concentration for each particle size, over the exposure period. This clearly shows that VENGES can generate a highly reproducible exposure aerosol with constant properties (size, size distribution and concentration) for toxicological inhalation studies.

Figure 7 shows the in-vivo chemiluminescence (IVCL) difference (counts per second-cps/cm²) of lungs and hearts, which corresponds to the relative reactive oxygen species (ROS) concentration in those organs of both exposed (red) and unexposed (blue) groups of animals. The error bars correspond to the standard deviation of the data points and the significance level of the measured data is $P < 0.001$. The IVCL measurements in the lungs of the exposed animals were about 60 times higher than for the unexposed animals, indicating that the Fe₂O₃ test aerosol increased ROS in the lungs. In addition, oxidative stress was present in the heart of the animals (11-fold increase in the chemiluminescence of the heart).

DISCUSSION

A promising technological platform suitable for both in-vitro and in-vivo toxicological characterization of engineered nanomaterials, with emphasis on the cardiovascular and pulmonary effects of inhaled ENM, is presented in this study. ENM are produced continuously in the gas phase allowing their continuous transfer to inhalation chambers, with minimal alterations in their state of agglomeration. Defining properties of the generated aerosols (i.e. primary and aerosol particle size, concentration, shape, state of agglomeration, surface chemistry) can be easily modified by adjusting simple process parameters allowing for both in-vitro and in-vivo investigations of toxicity. The ability of the developed technique to generate a variety of industry relevant, property controlled exposure atmospheres for inhalation studies was systematically investigated and documented in the previous section.

The suitability of the technique to characterize the pulmonary and cardiovascular effects of inhaled ENM in intact animal models was also demonstrated here using the highly sensitive IVCL assay. IVCL measures the reactive oxygen species generation (ROS). ROS and free radical generation is considered one of the primary mechanisms of nanoparticle toxicity; it was shown in many ambient particle health effect studies that ROS generation may result in oxidative stress, inflammation, and damage to proteins, membranes and DNA (Bartoli et al. 2009; Clarke et al. 1999; Clarke et al. 2000; Godleski et al. 2002; Gurgueira et al. 2002; Rhoden et al. 2004; Saldiva et al. 2002). ROS generation has been also found in many ENMs including carbon based ENMs (fullerenes, carbon nanotubes) and metal oxides (Nel et al. 2006). Our results indicate that moderate acute exposures to inhaled nanostructured Fe₂O₃ can generate ROS and oxidative stress and cause both pulmonary and cardiovascular effects. The IVCL measurements in the lungs of the exposed animals were about 60 times higher than for the unexposed animals, indicating that the Fe₂O₃ test aerosol increased ROS in the lungs (Figure 7). This oxidative stress was also present in the heart of the animals showing that the inhalation of ENM influences not only the respiratory but also the cardiovascular system with an 11-fold increase in the chemiluminescence of the heart. This substantial effect was found with a moderate mass exposure concentration of 200 µg/m³, a concentration approximately 20 times the fine particle concentration of room air. The substantial IVCL response observed in our study, indicates that these particles reached deep in the lung and evoked a toxicological effect. The increased IVCL response of the heart may indicate a direct effect on the heart (Godleski 2006) but also may be a manifestation of indirect effects via the autonomic nervous system (Ghelfi et al. 2008; Rhoden et al. 2005). It should be noted, however, that the proposed platform is not only limited to the evaluation of pulmonary and cardiovascular effects. It can also be used to assess other biological

outcomes related to inhaled ENM and it can be a powerful tool to understand the link between certain ENM properties and their bioavailability and toxicity.

CONCLUSIONS

In conclusion, this novel approach enables us to generate industry-relevant, property controlled ENM exposure atmospheres suitable for inhalation toxicological studies and assess the link between ENM physico-chemical properties and specific biological outcomes. In addition, the documented in the study ability of the technique to alter in-situ ENM surface properties can be one of the ways to further explore the formulation of safer ENM. Furthermore, this technological platform can be a powerful tool for validation of in-vitro screening assays with adverse biological effects in intact animals, an important element of the strategy recently proposed by the National Nanotechnology Initiative (NNI) on Environmental Health and Safety of Engineered nanomaterials. Its future use will help to assess the cardiovascular, pulmonary and other toxicological effects of inhaled ENM and improve our understanding on our central hypothesis that physical and chemical characteristics of ENM determine their bioavailability, redistribution, and toxicity in the lungs and elsewhere.

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Figure 1.

Overview of VENGES. All different parts of VENGES are presented. Engineered nanomaterials (ENM) are generated by flame synthesis. The nanoparticles are collected on a filter for further ex-situ characterization. Directly above the flame, in-situ sampling of the test aerosol is performed (Q_s). Immediately after, the ENM aerosol stream can be further diluted with HEPA filtered room air (Q_D) and directed into the exposure animal chambers (Q_A) for in-vivo toxicological testing. Simultaneously, the test ENM aerosol is in-situ real-time characterized (Q_P). The total particle number concentration is measured, as well as the mobility particle size distribution. Finally, the CO_2 , CO , NO_2 , relative humidity (RH) and temperature (T_2) are continuously monitored.

Figure 2.

Controlling particle size distribution by precursor liquid feed rate (x) over O₂ dispersion flow rate (y) ratio (x/y). (a) Mobility size distributions of SiO₂ for a varying x/y ratio and the mode, median, mean diameters and the geometric standard deviation are shown. (b) The mode mobility (circles, left axis) and primary particle diameter (triangles, right axis) as a function of the x/y ratio. (c) XRD patterns of Fe₂O₃ nanoparticles for different x/y ratios. (d) Mode mobility (circles, left axis), primary particle (open triangles, right axis) and crystal (filled triangles, right axis) diameters as a function of the x/y ratio. The precursor molarity was 0.5 M and the total particle number concentration (C_T) $2 \cdot 10^5$ #/cm³ for both SiO₂ and Fe₂O₃ samples.

Figure 3.

Controlling particle size by precursor molarity. Mobility size distributions (a,c,f) of SiO₂ (a), Fe₂O₃ (b) and Ag (nanosilver, c), for different precursor molarities. The mode, median, mean diameters and the geometric standard deviation are also shown. (b,d) Mode mobility size distributions (circles, left axis) primary particle (open triangles, right axis) and crystal (filled triangles, right axis) diameters for SiO₂ (b) and Fe₂O₃ (d). (e) TEM image of Fe₂O₃ nanoparticles for precursor molarity 0.5 M and 2/7 x/y ratio.

Figure 4.

Controlling total particle number concentration and mobility size distribution by dilution. Total particle number concentration (C_T , solid lines, left axis), mode (broken lines, right axis) and median (dotted lines, right axis) mobility diameters of nanosilver (a) and Fe_2O_3 (b) nanoparticles for different Q_s/Q_D ratios.

Figure 5.

Surface modification of SiO₂ with nanosilver. (a) Mobility size distributions of SiO₂ (triangles) and composite Ag/SiO₂ nanoparticles (circles, Ag-content 10 wt%) for 0.10 M precursor molarity and 2/7 x/y ratio for a total particle number concentration $C_T = 2 \cdot 10^5 \text{ \#/cm}^3$. The size distribution remains the same, as seen also in the inset, where the mode, median, mean diameters and the geometric standard deviation are also shown. The error bars correspond to the standard deviation of the particle concentration corresponding to each particle size over time. (b) STEM image of the Ag/SiO₂ nanoparticles. The nanosilver particles (bright spots) are homogeneously dispersed on the amorphous SiO₂ matrix (diffuse gray).

Figure 6.

(a) Total particle number concentration (C_T) of the test ENM aerosol containing Fe_2O_3 nanoparticles monitored continuously over the whole period of exposure for in-vivo toxicological characterization. The C_T remains constant at approximately $2\text{--}3 \cdot 10^5$ #/cm³. Every 25 minutes there was a 5 minutes break during the system was filled up (gray area). (b) Mobility size distribution averaged over the periods that the C_T was constant. The mode, mean diameters and the geometric standard deviation are also shown. The error bars correspond to the standard deviation of the particle concentration corresponding to each particle size over time.

Figure 7.

In-vivo chemiluminescence of the lungs and hearts of the exposed to the test aerosol for 5 hours rats (red) and of the ones to filtered room air (blue). The error bars correspond to the standard deviation of the data and the significance level P is also shown.

Table 1

Synthesized ENM, their corresponding precursors and solvent, as well as their process parameters (precursor molarity and x/y ratio).

ENM	Metal precursor	Solvent	Precursor molarity (M)	Liquid feed rate (x)/ O ₂ dispersion flow rate (y)
SiO ₂	Hexamethyl disiloxane	Ethanol	0.1-1	2/7 - 6/4
Fe ₂ O ₃	Iron acetylacetonate	Acetonitrile/2-ethylhexanoic acid (1:1)	0.01-0.5	2/7 - 6/4
Ag	Silver nitrate	Ethanol/diethylene glycol monobutyl ether (1:1)	0.06-0.31	2/7
Ag/SiO ₂	Silver nitrate/Hexamethyl disiloxane	Ethanol/diethylene glycol monobutyl ether (1:1)	0.1	2/7