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Surface area is the biologically most effective dose metric for acute nanoparticle toxicity in the lung

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ABSTRACT

In this study we provide guidance on the biologically most relevant dose metric for pulmonary toxicity of biopersistent, spherical nanoparticles (NPs). A retrospective analysis of nine *in vivo* studies on particle-induced, acute pulmonary toxicity in animal models (mouse, rat) was performed encompassing five different types of nanomaterials (polystyrene, titanium dioxide, carbonaceous materials, transition metal oxides (Co, Ni, Zn) and hydrothermally synthesized α-quartz) with a wide range of primary particle diameters (9–535 nm) and mass-specific BET surface areas (6–800 m^2/g). The acute influx of polymorphonuclear neutrophils (PMNs) into the lungs after intratracheal instillation of NPs was chosen as a toxicological endpoint for acute lung inflammation. The allometrically scaled toxicological data were investigated with respect to various dose metrics, namely (primary) particle number, joint length, BET and geometric surface area, volume and mass.

Surface area is identified as the biologically most relevant dose metric for spherical NPs explaining about 80% of the observed variability in acute pulmonary toxicity ($R^2=0.77$). None of the other dose metrics explains more than 50% of the observed variability in pulmonary inflammation. Moreover, using surface area as the dose metric allows identification of material-based toxicity classes independent of particle size. Typical materials without intrinsic toxicity – here referred to as low-solubility, low-toxicity (LSLT) materials – show low surface-specific toxicity with an EC_{50} dose of 175 m^2/g-lung (geometric mean; $\sigma_g=2.2$), where EC_{50} represents the dose inducing 50% of the maximum effect (here 30% PMN). In contrast, transition metal oxides (here Co, Ni, Zn) – materials known for their intrinsic toxicity – display a 12-fold enhanced surface-specific toxicity compared to LSLT particles ($EC_{50}=15$ m^2/g-lung).

This analysis implies that surface-related modes of action are driving acute pulmonary toxicity for the types of NPs investigated here. The relevance of other dose metrics such as number and volume is acknowledged in the context of different modes of action, namely shape-induced toxicity (fiber paradigm) and extremely high particle lung burden (overload conditions), respectively. So which dose metric should be monitored by aerosol scientists involved in health related aerosol exposure measurements? The short answer is – all of them (except length), but there is a strong preference towards surface area.

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1. Introduction

Epidemiological studies have described correlations between the mass concentration of ambient particulate matter (PM) and increased morbidity and mortality in adults and children (Salvi, 2007; Stone, Johnston, & Clift, 2007). The tremendous economic success of consumer products containing nanomaterials (e.g. sun screen, tooth paste, tires) has raised concerns regarding consumer exposure to inhaled nanostructured materials. However, very little epidemiological data are available for engineered nanomaterials. The few studies performed at industrial sites of nanoparticle (NP) production (mainly carbon black) did not show conclusive evidence of particle-induced health effects (Boffetta et al., 2004). However, most of these studies suffer from a lack of accurate exposure data or even personalized lung-deposited dose information (Peters, Ruckerl, & Cyrys, 2011).

Improved exposure studies are under way, but it is still unclear which of the possible dose metrics is best suited for predicting the adverse health outcome of NP exposure. While most (ambient) epidemiological studies were based on mass as the dose metric, there are also a few short-term epidemiological studies indicating that early, transient exacerbations are associated with the number concentration of ultrafine ambient particles rather than the mass of ambient PM (Peters et al., 2011). In toxicology, mass has been used as the dose metric, since mass is the biologically effective dose metric for soluble toxins. However, for non-soluble (or poorly-soluble) soot or engineered NPs only the molecules detached from the surface or located at the surface of the NPs are interacting with the biological fluids and tissue. Hence, surface area is likely to be a biologically more relevant dose metric for biopersistent nano-/microparticles than mass. This was confirmed by numerous cell-based in vitro and animal-based in vivo toxicity studies (Oberdörster, Oberdörster, & Oberdörster, 2005; Stoeger, Schmid, Takenaka, & Schulz, 2007; Stoeger et al., 2009; Waters et al., 2009). However, some toxicological studies have also suggested particle number or volume as the most relevant dose metric for NPs (Donaldson et al., 2013; Pauluhn, 2011).

State-of-the art aerosol technologies for real-time measurement of all moments of the size distribution (number, length, surface area, volume/mass) of compact aerosols are available (Kulkarni, Baron, & Willeke, 2011). Moreover, the number, joint length (Wittmaack, 2002), surface area and volume/mass of loosely agglomerated NPs ("soot-like" agglomerates/aggregates) can be determined in real-time by multi-instrument approaches (Wang et al., 2010). For non-hygroscopic, compact NPs (<300 nm) the lung-deposited surface area concentration can be monitored directly in real-time by electrometer based measurement techniques (Fissan, Neumann, Trampe, Pui, & Shin, 2007). However, for optimized design of future aerosol exposure measurements related to workplace safety or other health-related issues, it is important to identify the biologically most effective dose metric.

In this retrospective analysis of selected animal studies on acute pulmonary toxicity of intratracheally instilled NPs we provide guidance on the biologically most relevant dose metric for acute lung inflammation. We investigated the correlation between acute pulmonary inflammation as evidenced by the influx of inflammatory cells into the lung and pulmonary (primary particle) number, joint length, (BET and geometric) surface area, volume and mass as dose metrics. Published data were compiled from nine different studies conducted by various laboratories using different animal models (mouse, rat). The data cover some of the most commonly used types of NPs (polystyrene, titanium dioxide, various carbonaceous materials including aged diesel soot, quartz, and transition metal oxides) covering a wide range of primary particle diameters (9–535 nm) and mass-specific BET surface areas (6–800 m^2/g). Retrospective analysis of this unified data set provides insight into the relevance of the various dose metrics for predicting NP-induced pulmonary toxicity.

2. Materials and methods

The study presented here is based on animal studies on acute pulmonary toxicity due to intratracheal instillation of NPs into the lung. More than 60 animal studies on acute pulmonary toxicity published between 2001 and 2009 were screened and nine of them were selected for matching all of the following criteria:

(1) Use of mouse or rat as animal models
(2) Particles were applied to the lungs via intratracheal instillation
(3) Data on the influx of polymorphonuclear cells (PMNs) into the lung as hallmark of inflammation are available for at least one (acute) time point (between 16 h and 24 h after particle application). The number of PMNs and the total number of cells on the lung epithelium was determined via differential cell counting of the bronchoalveolar lavage (BAL) fluid.
(4) Non- or poorly-soluble, smooth, nano-sized (<535 nm) primary particles of spherical shape were used. The state of agglomeration in the applied NP suspension is typically not reported and therefore not considered as criterion here.
(5) Lung-deposited (instilled) mass dose, mass-specific BET surface area and material density are reported (or available).
(6) Accurate information on the primary particle diameter is provided for conversion of the pulmonary mass dose into other dose metrics, namely number, (joint) length, geometric surface area.

As mouse and rat are the most frequently used animal models for particle toxicity studies, we included both rat and mouse data here. NPs were delivered to the lungs via intratracheal instillation, i.e. the anesthetized animals were intratracheally intubated and a defined volume of a NP suspension was squirted directly into the lungs with a syringe via the trachea. While this route of application is physiologically not realistic (not an aerosol, but a liquid bolus of a NP suspension is

applied), the method is technologically simple, widely used and delivers an accurate and reproducible dose of NPs to the lungs of animals. To account for the large difference in body weight of mice and rats the applied NP doses were allometrically scaled. As we are focusing on pulmonary effects we chose the lung weight as allometric scaling factor. For 7–12 weeks old adult mice lung weights between 0.14 and 0.25 g with an average of about 0.18 g were observed for various strains (Kida, Fujino, & Thurlbeck, 1989). For rats an average value of 1.3 g was reported for the frequently used Fischer F344 rats (1.1–1.5 g; male, 7–20 weeks old; (Tillery & Lehnert, 1986)). The choice of 1.3 g and 0.18 g of lung weight for rats and mice, respectively, is also consistent with the widely used allometric dose scaling based on body surface area (Reagan-Shaw, Nihal, & Ahmad, 2008).

Pulmonary inflammation was chosen as the toxicological endpoint, since this represents one of the most sensitive cellular responses and is also considered one of the key adverse effects induced by particulate air pollution (Donaldson, Mills, Macnee, Robinson, & Newby, 2005). The most relevant hallmark of acute pulmonary inflammation is the influx of inflammatory leukocytes, especially polymorphonuclear neutrophils (PMNs) (Henderson, 2005), into the airspace of the lungs which can be determined by bronchoalveolar lavage (BAL) of the lung (rinsing the epithelial surface of the lung with saline solution) and subsequent counting of the number of PMNs in the recovered BAL fluid. A healthy, noninflamed lung contains virtually no PMNs, so that the resident alveolar macrophages will be the only leukocyte population recovered by BAL. Species-, strain- and handling-specific differences in the total number of PMNs retrieved from the lungs are accounted for by normalizing the number of PMNs to the total number of cells in the BAL fluid. As PMN influx into the lung is a time dependent process with a maximum response typically around 24 h only data obtained between 16 and 24 h after NP application were included in this study.

The pulmonary particle dose was assumed to be the nominal dose, i.e. it was derived from the concentration and the nominally applied volume of the NP suspension. This is an approximation, since a certain fraction of the nominal dose will remain in the instillation tools. In our laboratory ca. 85 $\pm$ 10% of nominal dose is delivered to the lung (Barapatre et al., 2015). This systematic (negative) dose bias of about 15% with an uncertainty of about $\pm$10% was neglected, since it is expected to be similar for instillation-studies and it is much smaller than the about $\pm$2.2-fold dose variability within a given toxicity class of particles (see below).

Only materials with low or poor solubility in aqueous media were included, since biopersistent particles were the focus of the present study. Moreover, for calculation of the various dose metrics, namely number (of primary NPs), joint length, geometric and BET surface area as well as volume and mass of the applied NPs, only studies providing the following information were included: diameter of the (spherical) primary NPs (d_p), material density, applied total NP mass (M) and mass-specific BET surface area (sa_{BET}), i.e. the surface area per mass according to the gas adsorption method described by Brunauer, Emmet, and Teller (1938).

From this information the primary particle number (N_p), joint length (L_p) and geometric surface area (SA_{geom}) of the applied NPs was calculated according to

$$N_p = \frac{M}{\rho_p\left(\pi d_p^3/6\right)}, L_p = N_p d_p \text{ and } SA_{geom} = N_p \pi d_p^2, \tag{1}$$

respectively, where M and ρ_p represent the applied NP mass and the material density, respectively. Moreover, the BET surface area dose SA_{BET} was calculated from

$$SA_{BET} = sa_{BET} M, \tag{2}$$

where sa_{BET} is the mass-specific BET surface area (m^2/g). The volume dose V is given by

$$V = \frac{M}{\rho_p f} \tag{3a}$$

where f=0.74 is the volume filling fraction of closely packed spheres, which accounts for the void spaces between the primary particles. The assumption of a close packed structure is justified by the toxicological mode of action related to volume as dose metric. As discussed in more detail below for chronic exposures, if the volume of NPs, which is taken up (phagocytized) by alveolar macrophages, exceeds a limiting (overload) dose, macrophage-mediated clearance of the nanoparticles from the alveolar region is inhibited and PMN release into the lung is observed (Pauluhn, 2011). It has been shown that even loosely packed NP agglomerates are strongly compacted inside macrophages justifying the assumption of a close packed NP structure for estimating the effective volume dose (Takenaka et al., 2012). For interpretation of the volume data with respect to the so-called overload conditions, it is important to normalize the phagocytized NP volume to the total volume of the alveolar macrophages in the lung, which is given by Eq. (3b) for rats (Pauluhn, 2011)

$$V_{alv,mac} = 7 \times 10^{10} \mu m^3/kg \times 0.3\ kg = 2.1 \times 10^{10} \mu m^3. \quad \text{(for rats)} \tag{3b}$$

This equation can be allometrically scaled for mice

$$V_{alv,mac} = 7 \times 10^{10} \mu m^3/kg \times 0.3\ kg \frac{0.18\ g}{1.3\ g} = 2.9 \times 10^{9} \mu m^3, \quad \text{for mice} \tag{3c}$$

based on lung and body weight as justified above (assumed body weight for rat/mouse: 300 g/20 g; lung weight: 1.3 g/0.18 g).

Table 1
Physico-chemical characteristics of the different types of (agglomerated) nanoparticles (NPs) included in the retrospective study presented here. Particles were applied directly into the lungs of mice and rats via intratracheal instillation (i.e. as liquid bolus not as aerosol).

Material (nanoparticles)					Dose	Animal	Reference
Type	Primary diameter (nm)	Density (g/cm^3)	Mass-specific BET[a] surface area (m^2/g)	Ratio geometric/BET surface area	Mass dose per animal (mg)	Species	
Polystyrene	65	1.05	89.3[b]	1[b]	0.125, 1	Rat	Brown, Wilson, MacNee, Stone, and Donaldson (2001)
	202		28.3[b]	1[b]	0.125, 1		
	535		10.7[b]	1[b]	0.125, 1		
Titanium dioxide	20	3.9	50	1.5	0.032, 0.125, 0.5	Rat	Oberdörster et al. (2005)
	250		6.5	0.95	0.125, 0.5, 2		
	20	3.9	50	1.5	0.005, 0.025, 0.1	Mouse	Oberdörster et al. (2005)
	250		6.5	0.95	0.025, 0.1, 0.4		
	300	3.9	6	0.85	0.275, 1.375	Rat	Warheit, Webb, Sayes, Colvin, and Reed (2006)
	20	3.9	50	1.5	0.125	Rat	Dick, Brown, Donaldson, and Stone (2003)
	25		50	1.2	1, 6	Rat	Hohr et al. (2002)
	180		6.5	0.85	1, 6		
Hydrophobic (methylation)	25		50	1.2	1, 6		
	180		6.5	0.85	1, 6		
Carbonaceous materials							
Printex 90	14	2	272	0.79	0.184	Rat	Lu et al. (2009)
				0.79	0.125	Rat	Dick et al. (2003)
				0.79	0.005, 0.02, 0.05	Mouse	Stoeger et al. (2006)
5 Other types of carbonaceous materials[c]	9, 11, 12, 25, 51	2	800, 441, 268, 108, 43	0.42, 0.62, 0.93, 1.1, 1.4	0.005, 0.02, 0.05	Mouse	Stoeger et al. (2006)
Transition metal oxides							
Co_3O_4-nano	20	–	37	–	0.125	Rat	Dick et al. (2003)
Ni-nano	20	–	36	–	0.125		
ZnO-nano	60	5.6	12.1	1.5	0.25, 1.25	Rat	Sayes, Reed, and Warheit (2007)
ZnO-fine	–[d]	5.6	9.6	–	0.25, 1.25		
Silica							
Nanoquartz[e]	12, 50	2.65	91, 31	2.1, 1.4	0.275, 1.375	Rat	Warheit et al. (2007)

[a] Surface area characterized by gas adsorption according to the method described by Brunauer et al. (1938).
[b] The BET surface area was calculated from the geometric diameter.
[c] In addition to Printex90 (carbon black), spark-discharge generated elemental carbon as well as four other types of combustion-derived soot types were investigated, namely PrintexG (carbon black), diesel exhaust particles, gas flame soot with low and high organic carbon content.
[d] Diameter of ca. 1000 nm according to manufacturer's information; however, since the accuracy is uncertain, it was not included in analysis of dose metrics relying on primary particle diameter for calculation of number, joint length, geometric surface area.
[e] α-Crystalline silica (SiO_2); was synthesized hydrothermally in NaOH, but not mined and milled as most commercially available quartz types (e.g. Min-U-Sil).

3. Results

In this comparative study in vivo animal data from our laboratory (Stoeger et al., 2006) and from eight other studies were combined to investigate acute pulmonary inflammation for five different material types, a wide range of primary particles in the nano-size range (diameter: 9–535 nm), mass-specific BET surface areas (6–800 m^2/g) and dose range (several orders of magnitude) (see Table 1). The five different types of biopersistent materials comprise polystyrene, two types of titanium dioxide (TiO_2), six types of carbonaceous materials (including flame soot, diesel soot and carbon black (Printex90)) as well as transition metal oxides (Co-, Ni-, and Zn-oxides) and hydrothermally synthesized α-quartz (α-crystalline silica, SiO_2). A total number of 53 data points were included in this study.

High quality of the allometric dose scaling approach used here is a prerequisite for combining rat and mouse data in this retrospective-analysis. This issue was examined using the five TiO_2 data sets for mice (1) and rats (4) obtained in different laboratories (see Table 1). As seen from Fig. 1A there is excellent agreement between the dose–response curves from all five data sets. The logarithmic fit of PMN number versus BET surface area dose explains 91% of the variability of the toxicological

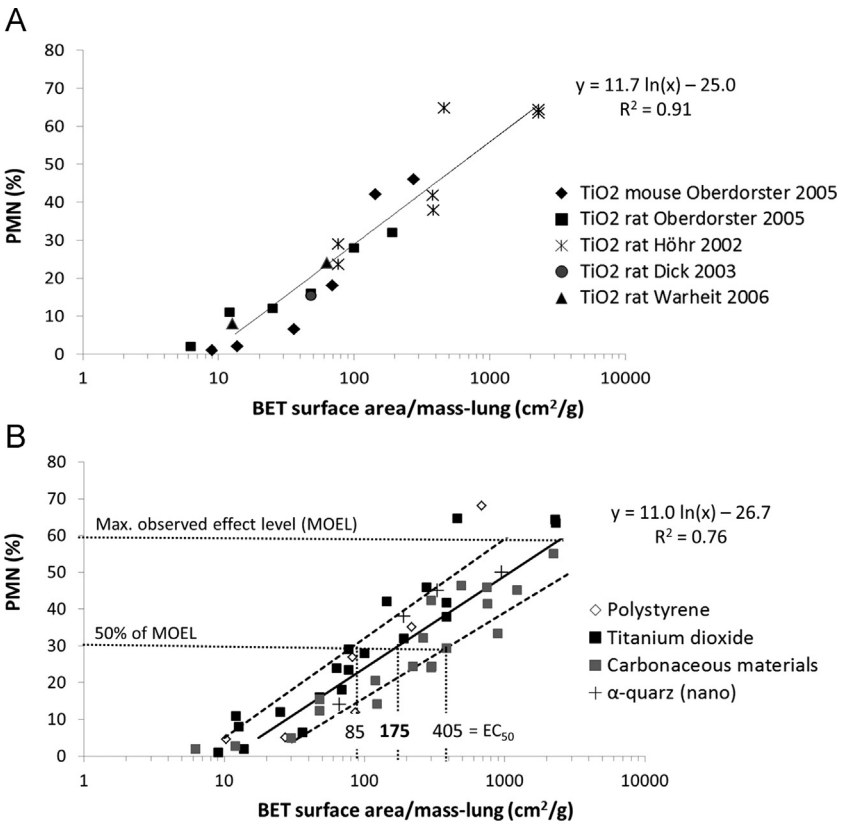

Fig. 1. Dependence of acute lung inflammation on BET surface area dose for various types of nanoparticles (NPs). Lung inflammation is expressed in terms of the number of inflammatory cells (PMNs, normalized to total number of cells) recovered from the lung 16–24 h after intratracheal instillation of NPs into the lung. (A) Allometric scaling of mouse and rat data provides consistent results unifying five different TiO_2 data sets ($R^2=0.91$). Inter-species and inter-laboratory differences in the dose–response curves are reasonably small as indicated by the tight clustering of all data about the fit line. (B) Four different types of low-solubility, low-toxicity (LSLT) materials show relatively tight clustering of the dose–response curves (dashed lines indicate the $\pm 1\sigma$ levels of (solid) best fit line). In spite of significant differences in primary particle diameter and mass-specific BET surface area (see Table 1) there is good correlation between inflammation and logarithmic BET surface area dose ($R^2=0.76$). The EC_{50} dose (dose inducing 50% of the maximum observed effect level (MOEL); here: 30% PMN) is 175 cm^2/g-lung (geometric mean; 85 and 405 cm^2/g-lung corresponds to $\pm 1\sigma$ dose levels).

response ($R^2=0.91$). Hence, normalizing the pulmonary particle dose to the lung weight allows comparison of (normalized) PMN data from rats and mice on the same dose-scale. Moreover, Fig. 1A also shows great consistence of data from different laboratories which is another prerequisite for reliable retrospective-analysis of data from different laboratories.

The dose–response data presented in Figs. 1 and 2 show a sigmoidal shape with a no observed effect level (NOEL) between 0% and 5% (no inflammation) for low doses and an asymptotic maximum response level between 50% and 70% (maximum (100%) observed effect level (MOEL)) for high doses. As both the asymptotic NOEL and MOEL are not far enough explored for sigmoidal curve fitting, we performed logarithmic dose–response fits over the entire data set and compared particle-specific toxicities based on EC_{50} values, i.e. effect concentration (or dose) at 50% of MOEL (=30% PMN) as seen in Fig. 1B. As the logarithmic fit is not valid for PMN values below 5% and above 60%, the fit curves are truncated at these PMN values.

Fig. 1B depicts acute pulmonary inflammation induced by four different types of materials with similar toxicological classification, namely polystyrene, TiO_2, (six types of) carbonaceous materials and hydrothermally synthesized nano-quartz (Table 1). Again there is good linear correlation between (logarithmic) dose and toxicological response ($R^2=0.76$) for BET surface area as the dose metric. The derived EC_{50} dose corresponds to a (geometric) mean surface dose of 175 cm^2/g-lung. Moreover, all data are clustering relatively tightly about the dose–response fit curve resulting in EC_{50} dose variability from 85 to 405 cm^2/g-lung ($\pm 1\sigma$ dose limits). This dose range corresponds to a moderate geometric standard deviation of 2.2 $(=(405/85)^{0.5})$ about the geometric mean, which is similar to the width of typically observed aerosol size distributions such as produced by standard aerosol generators. Consequently, the four types of materials presented in Fig. 1B can be categorized as a specific toxicity class sometimes referred to as low-solubility, low-toxicity (LSLT) materials without intrinsic toxicity.

Fig. 2 compares the toxicity of transition metal oxides (cobalt (Co), nickel (Ni), zinc (Zn)) to that of LSLT materials. As seen in Fig. 2A the EC_{50} dose for the investigated transition metal oxides is 15 cm^2/g-lung indicating that cobalt, nickel and zinc oxide show 12-fold higher BET surface-specific toxicity than LSLT particles ($EC_{50}=175$ cm^2/g-lung). Virtually the same result

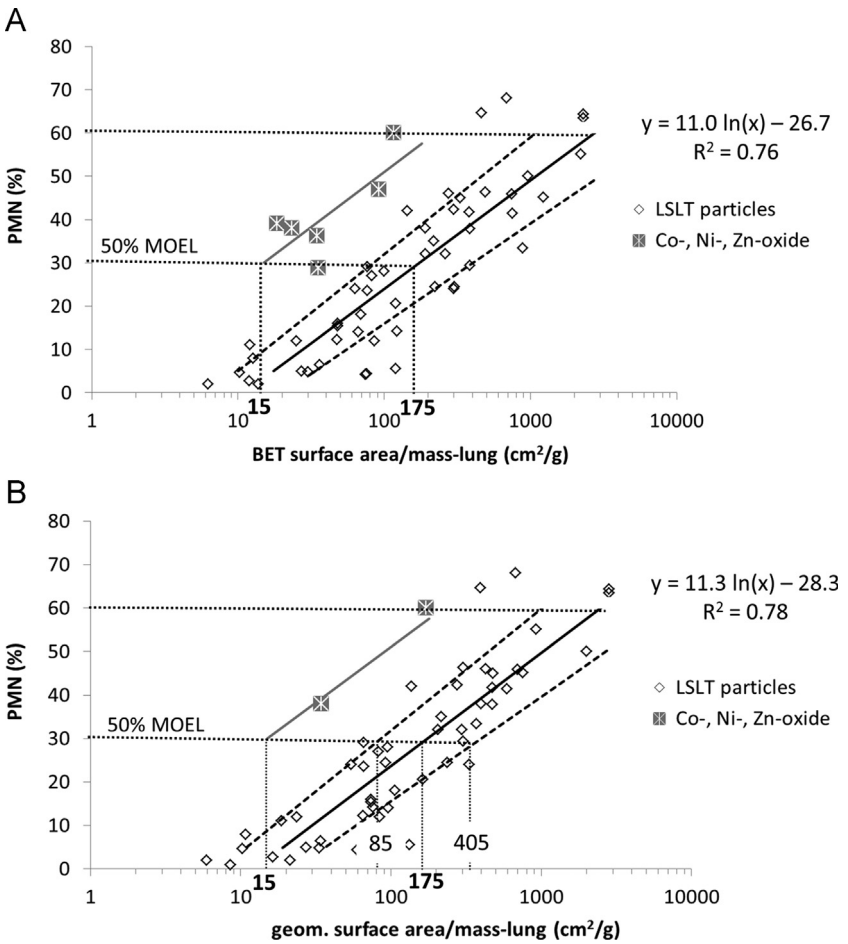

Fig. 2. Comparison of acute lung inflammation induced by low-solubility, low-toxicity (LSLT, see Fig. 1B) and transition metal oxide NPs (here cobalt, nickel and zinc) with BET or geometric surface area as dose metric. For both BET surface area (A) and geometric surface area (B) as the dose metric, transition metal oxides show a 12-fold elevated toxicity compared to the LSLT particles as evidenced by corresponding EC_{50} values of 175 cm^2/g-lung (with 85 and 405 cm^2/g-lung as $\pm 1\sigma$ limits) and 15 cm^2/g-lung, respectively.

is found for geometric (instead of BET) surface area as dose metric with the limitation that only two of the six BET-based transition metal data points could be converted into geometric volume due to a lack of particle-specific information (see Table 1). In light of the wide range of primary particle diameters (9–535 nm), the absence of any significant size-dependent particle toxicity is remarkable and documents the biological relevance of surface area as dose metric.

In addition to surface area other dose metrics were investigated. Fig. 3 shows the same data as depicted in Fig. 2, but presented in terms of mass, volume, number, and joint length. Using mass and volume as the dose metric shows a correlation coefficients of $R^2 = 0.49$ and 0.52, respectively (Fig. 3A and B), which is significantly lower than the 0.76 (or 0.78) for BET and geometric surface area, respectively (Fig. 2). Moreover, the higher toxicity of transition metals compared to LSLT materials is not evident for mass and volume as the dose metric. Expressing the dose–response curves in terms of number and joint length of the primary NPs (only LSLT particles were considered), yields even lower dose–response correlation coefficients of 0.19 and 0.41, respectively (see Fig. 3C and D). The relatively high correlation coefficient of joint length compared to number concentration in spite of very similar scatter plots is a result of the fact that the relative *x*-axis range is much narrower for length than for number (ca. 3 and 5 orders of magnitude, respectively). However, both metrics display a size-dependent stratification of the data, with larger primary particle diameters inducing larger PMN influx. However, this is an artefact due to the larger surface area per particle for larger sizes. Hence neither number nor joint length is a suitable dose metric for LSLT particles.

4. Discussion

The retrospective-analysis presented here identifies surface area as the biologically most relevant dose metric for non- or low-solubility NPs of spherical shape. However, this analysis is based on non-physiologically applied NPs using intratracheal

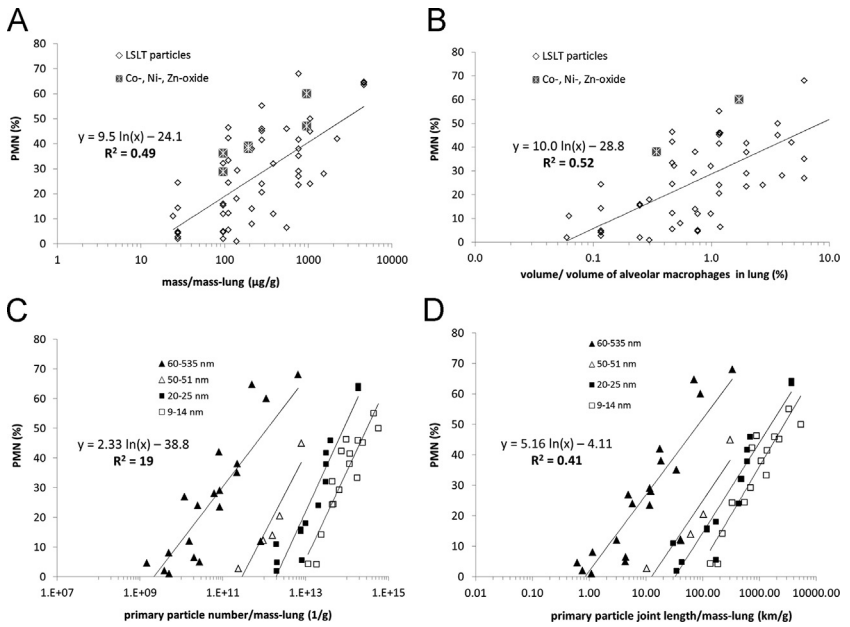

Fig. 3. Acute lung inflammation data from Fig. 2 presented in terms of four different dose metrics. (A) and (B) Mass and volume (normalized to macrophage volume) show a moderately good correlation with inflammation (R^2=0.49 and R^2=0.52, respectively), but – in contrast to surface area – there is no clear distinction between the toxicity of LSLT and transition metal oxide NPs possible. (C) and (D) For number and joint length of the primary particles as the dose metric, the correlation with inflammation reduces to R^2=0.19 and 0.41, respectively, (only LSLT data are considered). Moreover, the data suggest an enhanced toxicity of larger particles (size-stratification indicated by solid lines), which is a metric-related bias due to the increase in surface area per particle for larger particles.

instillation instead of inhalation. Naturally, inhalation studies are considered the gold standard for inhalation risk assessment. Hence, it is important to compare our findings to results from inhalation studies. Numerous chronic (> 14 weeks of exposure), sub-chronic (1–14 weeks) and acute (< 1 weeks) animal inhalation studies are also providing evidence for surface area as the biologically most relevant dose metric for non-soluble particles. For instance, the lung tumor prevalence occurring in animal models after chronic inhalation of toner, coal dust, talk, titanium dioxide, diesel soot and carbon black particles was found to be well correlated with pulmonary surface area dose (Maynard & Kuempel, 2005). Another retrospective-analysis of animal data on the carcinogenicity of inhaled particles found elevated toxicity of particles smaller than 100 nm for mass as dose metric, but no size-dependence for surface area or volume dose (Gebel, 2012). Also short-term inhalation studies with different sized silver NPs implied surface area as the biologically most relevant dose metric for acute pulmonary inflammation (Braakhuis et al., 2015). The toxicological impact of surface area may be rationalized by the fact that only molecules on the particle surface area are in direct contact with bodily fluids and tissue. Hence, effects such as the rate of dissolution of toxic components, formation of reactive oxygen species, or depletion of proteins/lipids in bodily fluids due to the formation of a protein or lipid corona and subsequent adverse effects on cell homeostasis are likely to depend on particle surface area dose (Fubini, Ghiazza, & Fenoglio, 2010; Stoeger et al., 2009; Xia et al., 2006).

However, there are also inhalation studies championing volume as biologically most relevant dose metric. In a retrospective-analysis of sub-chronic inhalation studies (4–13 weeks exposure) with a wide variety of particle types (e.g. AlOOH, Fe_2O_3, TiO_2, multi-walled carbon nanotubes – in entangled, agglomerated form), it was shown that PMN influx in the lung was best correlated with the volume burden of the particles in the lung (Pauluhn, 2009, 2011). The dose–response curves were consistent with the well-known overload paradigm of particle toxicity, which claims that particles without inherent toxicity are non-toxic in chronic inhalation scenarios unless the pulmonary particle volume burden exceeds 6% of the macrophage volume of the lung (Pauluhn, 2011). Once this dose threshold, known as the overload condition, is reached the mobility of alveolar macrophages and their ability of clearing particles from the alveolar surface is retarded leading to chronic inflammation and associated adverse health effects (Pauluhn, 2011). A more in-depth discussion of lung particle overload and its role for deriving occupational exposure levels for nanoparticles has been presented by an editorial and two companion papers recently published in *Particle Fibre Toxicology* (Borm, Cassee, & Oberdorster, 2015; Morfeld et al., 2015; Pauluhn, 2014).

In addition to surface area and volume, particle number has been identified as biologically effective dose metric for stiff, biopersistent, fiber-like particles such as asbestos or other fiber-like particles. While short fiber-like particles can be easily phagocytized, fiber-like particles longer than 5 μm (some say longer than 15–20 μm) are too long for macrophages to completely engulf them. This *frustrated phagocytosis* results in persistent inflammatory stimulation leading to fibrotic or carcinogenic tissue degradation (Archer, 1979; Murphy, Schinwald, Poland, & Donaldson, 2012). As each fiber can eliminate at least one macrophage, particle number is the biologically most effective dose metric for this mode of action.

In light of the large number of engineered nanomaterials being developed, chronic inhalation studies are too time consuming and expensive for rigorous risk assessment. Hence, intratracheal instillation animal studies on acute lung inflammation may serve as cost effective, reliable, early indicator of potential adverse health effects of inhaled particles. Fig. 1A confirms that both inter-species (mouse and rat) and inter-laboratory variability is sufficiently small to obtain consistent data sets from rodent studies performed in different laboratories. In the present retrospective-analysis we investigated the moments of the particle size distribution (0th, 1st, 2nd and 3rd moment correspond to the number-, length-, surface-area-, volume-/mass-weighted particle size distribution, respectively) for their biological relevance for acute lung inflammation in animal models.

Clearly, primary particle number and joint length proved to be inferior to particle mass and volume (Fig. 3). This assessment is not only based on the poor correlation coefficient of number and length, but also on the size-stratification of the data (larger particles appear more toxic), which is most pronounced for number and length as the dose metric, but also present in the volume and mass data (not shown in Fig. 3A and B), although to a much smaller degree. Historically, most of the particle toxicity studies were performed with mass as the dose metric. While mass is an adequate dose metric for soluble materials, it is inadequate for non- and low-solubility particles used here. In fact using mass as dose metric has led to the false conclusion that smaller particles are inherently more toxic than larger ones (Gebel, 2012). On the other hand, smaller particles appear to be less toxic than larger ones, if number or length is chosen as the dose metric. This apparent dependence of toxicity on particle size vanishes, if surface area is used as the dose metric as shown by our and numerous other studies (Braakhuis et al., 2015).

Volume dose displayed similarly mediocre correlation with acute inflammation as mass. For none of the cases investigated here, the overload dose (6% of macrophage volume) was exceeded (Fig. 3B). Hence, the observed significant acute inflammatory response for some cases cannot be explained with the overload paradigm. In fact the overload paradigm refers to chronic inflammation where long term retention of the alveolar deposited particles and subsequent epithelial irritation is of significance and not to acute effects. Hence, this mode of action is not expected to be relevant for the data analyzed here.

The retrospective-analysis of acute lung inflammation data indicates that surface area is the biologically most effective dose metric with no significant difference between BET or geometric surface area (Fig. 2B), which is consistent with the fact that the materials investigated here had smooth surfaces without significant microporous structures. This can be seen from the ratios of geometric and BET surface area ranging from 0.41 to 1.5 with most of the particles clustering about unity (Table 1). For microporous and non-spherical particle shapes (except fibers) BET rather than geometric surface area is expected to be the biologically more effective (or more easily determined) dose metric. In contrast to geometric surface area, there is currently no real-time measurement method for BET surface area. Thus, the fact that for spherical particles with smooth surfaces the geometric surface area can be used as biologically most relevant dose metric has severe practical implications for exposure measurements. Using recently described multi-instrument approaches it is possible to determine all moments (0th–3rd moment) of nanoparticle size distributions for both spherical and agglomerated (smooth) spherical NPs as required for nanoparticle inhalation risk assessment (Shin et al., 2010; Wang et al., 2010).

The close proximity of the dose–response curves of the four different types of LSLT materials (polystyrene, TiO_2, carbonaceous materials, smooth quartz) depicted in Fig. 2 indicates that these materials have similar surface-specific toxicity possibly due to similar catalytic activity of the surface. These types of materials are considered materials without intrinsic toxicity. On the other hand, it is well known that certain transition metal oxides and milled quartz have enhanced toxicity. Fig. 2 shows evidence for 12-fold enhanced surface-specific toxicity of Co-, Ni- and Zn-oxide compared to LSLT materials. This can be attributed to the production of highly toxic free OH-radicals from hydrogen peroxide (Fenton reaction) and uptake of particles in intracellular compartments (the endo-lysosomal system) with low pH, where partial dissolution leads to enhanced release of high (toxic) concentrations of metal ions in the cells. This so-called Trojan horse effect can also lead to an imbalance of the intracellular homeostasis of otherwise tightly regulated metal ions and subsequent inflammatory response. These additional modes of action account for the enhanced surface-specific toxicity of transition metal compared to LSLT NPs. Our study also shows that synthesized, nano-sized α-crystalline silica (α-quartz) belongs to the LSLT toxicity class (Fig. 2). This may seem surprising as numerous studies report elevated surface-specific toxicity for quartz particles such as Min-U-Sil (Warheit, Webb, Colvin, Reed, & Sayes, 2007), which was not included here because of its irregular particle shape and its primary particle size outside the nano-scale range (typically much larger than 1 μm diameter). For grinded crystalline quartz (like Min-U-Sil), it has been hypothesized that the sharp-edged particles can cause lysosomal membrane destabilization, which contributes to the elevated inflammatory toxicity (Sun, Wang, Ji, Li, & Xia, 2013). In contrast, the hydrothermally synthesized nano-quartz included here does not activate lysosomal destabilization due to its smooth surface structure (Warheit et al., 2007). In absence of this toxicity mechanism, nano-quartz shows the same toxicity level as all the other LSLT particles.

The analysis presented here also indicates that primary particle size has no effect on surface-specific particle toxicity. As caveat we add that this study was not designed to study effects of primary particle size on surface-specific toxicity. Hence, we cannot rule out that size-dependent effects do occur especially for very small primary particle sizes, where quantum effects may come into play. Moreover, many toxicologically relevant modes of action such as endocytic cellular uptake of non-professional phagocytic cells depend on agglomerate rather than primary particle size. The means of application (intratracheal instillation) suggests that rather large agglomerates had formed either in the particle suspension or on the lung epithelium. Thus, we can only conclude that for agglomerated LSLT particles the combined effect of primary particle size and material type on acute lung toxicity is less than a factor of 4.8 ($2\sigma_g$ of EC50; see Fig. 1B).

For aerosol exposure measurements it is important to note that not surface area concentration, but the toxicologically effective, lung-deposited surface area concentration is expected to be the biologically most effective dose metric for non-soluble NPs. This is evident from Eq. (4), describing the relationship between the *effective lung-deposited surface area dose* ($Dose_{SAeff}$) and the surface area (or mass) exposure level of the aerosolized nanoparticles

$$Dose_{SA,eff} = f_{tox}\dot{V}\, t \int [m(D)sa(D)Dep(D)]dD, \tag{4}$$

where $m(D)=dM/dD$ is the differential particle mass distribution (D=particle diameter, M=aerosol mass per volume air), $Dep(D)$ is the size-dependent fraction of inhaled particles deposited onto the lung epithelium (see e.g. ICRP, 1994; Londahl et al., 2014), sa is the mass-specific surface area of the particles, t is the exposure time and $\dot{V}$ is the ventilation rate of the lung (inhaled air volume per time). To account for different relative toxicities (high and low toxicity class) the toxicological weighting factor f_{tox} is introduced which accounts for differences in surface-specific toxicity, which can be defined as the *inverse* of the normalized surface-based EC_{50} value, where the LSLT EC_{50} value is a suitable normalization constant (i.e. $f_{tox}=12$ and 1 for transition metal oxide and LSLT nanoparticles having EC_{50} values of 15 and 175 cm^2/g-lung, respectively). If any of the parameters in Eq. (4) is time-dependent, time averages have to be used or the right hand side of Eq. (4) has to be integrated over time as well. For long-term exposures, time-dependent particle clearance rates (from the lung) may have to be included as well (ICRP, 1994).

The integral term in Eq. (4) represents the lung-deposited surface area concentration, which – under certain conditions – can be measured in real-time by electrometer-based measurement techniques (e.g. NSAM or AeroTrak by TSI, Partector by Naneos; (Fissan et al., 2007)). These devices provide reliable lung-deposited surface area concentrations for smooth, compact particles with diameters between about 20 and 300 nm, i.e. they do not necessarily adequately account for effects of shape, agglomeration state or surface-porosity on surface area (Wang et al., 2010). These instruments do also not account for hygroscopic particle growth in the lung making the results unreliable for (partially) water-soluble particles. Thus, for non-soluble compact particles only are electrometer-based measurement techniques expected to provide a valuable tool for real-time exposure measurement of the biologically most effective particle dose (lung-deposited surface area dose). Moreover, for agglomerated spherical NPs, multi-instrument approaches can be used for real-time measurement of both primary particle size and particle number which can then be converted into derived surface area (for smooth surfaces) and volume concentration (Shin et al., 2010; Wang et al., 2010) and ultimately into lung-deposited dose by accounting for the known size-dependent lung deposition fraction (ICRP, 1994; Londahl et al., 2014).

As seen in Fig. 2 spherical nanoparticles can be stratified into surface-based classes of toxicity. As suggested in Eq. (4) these differences in surface-specific toxicity can be incorporated into risk assessment by defining appropriate toxicity weighting factors. For instance our retrospective-analysis suggests that transition metal oxides and LSLT particles can be presented by $f_{tox}=12$ and 1, respectively, based on the ratios of the corresponding EC_{50} values. Recently, there have been attempts of defining equivalent toxicological doses using the concept of lung-deposited surface area and appropriate weighting factors accounting for enhanced health risks due to e.g. highly toxic materials, surface roughness and the occurrence of frustrated phagocytosis (Simko, Nosske, & Kreyling, 2014).

As discussed above the EC_{50} doses presented here are expected to provide a valuable tool for comparing the *relative* toxicity of different types of nanoparticles, but they are *not* suitable as sole basis for recommended exposure limits of nanoparticles (*absolute* toxicity). The latter is mainly because exposure limits should be based on chronic inhalation studies rather than on acute instillation studies as presented here. Nevertheless, from the perspective of exposure assessment it is instructive to relate the EC_{50} values derived here to corresponding exposure concentrations. Assuming a worst case exposure scenario, the EC_{50} values of 175 and 15 m^2/g-lung for nanoparticles belonging to the low and high toxicity class defined in Fig. 2, respectively, are allometrically equivalent to the lung-deposited surface area dose received by a person during an 8-h work shift at an aerosol mass concentration of 360 and 30 $\mu g/m^3$, respectively. Here, the following worst case assumptions were made: The worker conducted heavy work during the entire 8 h shift (3 m^3/h ventilation rate, (ICRP, 1994), the lung deposited aerosol fraction in the alveoli is 60% (ICRP, 1994) and the mass-specific surface area of the nanoparticles is 1000 m^2/g. Allometric scaling of the lung deposited particle dose from mouse/rat to man was based on an assumed lung surface area of 100 m^2 and 600 cm^2 (for a 0.18 g mouse lung) for man and mouse, respectively (Kida et al., 1989; Paur et al., 2011; Stone, Mercer, Gehr, Stockstill, & Crapo, 1992). Thus, the rodent-derived EC_{50} doses of 175 and 15 m^2/g-lung can be converted into corresponding human EC_{50} doses of 530 and 45 cm^2/m^2-lung (surface area NP per surface area of lung epithelium), respectively. We reiterate that these exposure levels should not be interpreted as guidance for NP workplace exposure limits, because of the difficulties of relating acute pulmonary effects observed after instillation of NP into the lung to adverse health effects due to chronic inhalation scenarios.

5. Conclusions

The retrospective-analysis provided here showed that allometrically scaled animal data from mice and rats provide species- and laboratory-independent information on the pulmonary toxicity of intratracheally instilled nanoparticles (NPs). For low- and poorly-soluble spherical NPs, (lung-deposited) particle surface area emerged as the biologically most effective

dose metric for acute pulmonary inflammation. As expected, there was no significant difference between BET and geometric surface area as dose metric for NPs with relatively smooth surface structure (no micro-pores).

Using surface area as the dose metric, allowed identification of material-based toxicity classes independent of particle size. Materials with no intrinsic toxicity like polystyrene, carbonaceous materials, titanium dioxide and synthesized (smooth) α-quartz (but not for grinded sharp-edged quartz) showed similar dose response curves with EC_{50} values (half-maximum response level: here 30% PMN influx into the lungs) clustering in a narrow dose range between 85 and 405 m^2/g-lung (geometric mean at 175 m^2/g-lung; geometric standard deviation of 2.2). This toxicity class was referred to as low solubility, low toxicity (LSLT) class. It is noteworthy that not only (aged) diesel soot, but also all other five types of carbonaceous materials considered here belong to the LSLT class suggesting that also ambient urban ultrafine particles might belong to the LSLT class at least in the absence of highly toxic material adsorbed onto the core of these particles. For transition metal oxides (here: Co, Ni, Zn), which are considered materials with intrinsic toxicity, a surface-specific toxicity enhancement factor of about 12 compared to LSLTS particles was found (EC_{50} = 15 m^2/g-lung).

As caveat we add that applying NP suspensions rather than freshly generated aerosols may have an effect on the observed surface-specific toxicity due to interactions of the dispersion medium with the particles. However, the notion that surface area is the most relevant dose metric for particle toxicity has also been shown by numerous inhalation studies. Depending on the mode of action, dose metrics other than surface area may be more effective. For instance, volume and number are biologically more effective dose metrics for high particle lung burden (overload conditions; more than 6% of alveolar macrophage volume is filled with particles) and long, stiff fiber-like particles (frustrated phagocytosis). In addition, mass should be included as dose metric due to its historic and regulatory relevance for NP toxicity.

With these limitations in mind lung-deposited particle surface area dose can be expected to be the toxicologically most relevant dose metric for inhaled, spherical NPs. Hence, future aerosol exposure measurements related to health effects of inhaled particles should strive to include lung-deposited particle surface area concentration as main exposure parameter.

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