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The effect of titanium dioxide nanoparticles on pulmonary surfactant function and ultrastructure

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Abstract

Background: Pulmonary surfactant reduces surface tension and is present at the air-liquid interface in the alveoli where inhaled nanoparticles preferentially deposit. We investigated the effect of titanium dioxide (TiO₂) nanosized particles (NSP) and microsized particles (MSP) on biophysical surfactant function after direct particle contact and after surface area cycling *in vitro*. In addition, TiO₂ effects on surfactant ultrastructure were visualized.

Methods: A natural porcine surfactant preparation was incubated with increasing concentrations (50-500 µg/ml) of TiO₂ NSP or MSP, respectively. Biophysical surfactant function was measured in a pulsating bubble surfactometer before and after surface area cycling. Furthermore, surfactant ultrastructure was evaluated with a transmission electron microscope.

Results: TiO₂ NSP, but not MSP, induced a surfactant dysfunction. For TiO₂ NSP, adsorption surface tension (γ_{ads}) increased in a dose-dependent manner from 28.2 ± 2.3 mN/m to 33.2 ± 2.3 mN/m ($p < 0.01$), and surface tension at minimum bubble size (γ_{min}) slightly increased from 4.8 ± 0.5 mN/m up to 8.4 ± 1.3 mN/m ($p < 0.01$) at high TiO₂ NSP concentrations. Presence of NSP during surface area cycling caused large and significant increases in both γ_{ads} (63.6 ± 0.4 mN/m) and γ_{min} (21.1 ± 0.4 mN/m). Interestingly, TiO₂ NSP induced aberrations in the surfactant ultrastructure. Lamellar body like structures were deformed and decreased in size. In addition, unilamellar vesicles were formed. Particle aggregates were found between single lamellae.

Conclusion: TiO₂ nanosized particles can alter the structure and function of pulmonary surfactant. Particle size and surface area respectively play a critical role for the biophysical surfactant response in the lung.

Background

High amounts of ambient particulate matter (PM) exist in our atmosphere, and it is known that a high proportion of these particles are nanosized particles (NSP) with a diameter of ≤ 100 nm. NSP can be found in the air as a result of combustion processes such as automobile engines and fires. In addition, the rapidly developing field of nanotechnology is becoming a potential source for human exposure to NSP. Titanium dioxide (TiO_2) NSP e.g. are widely produced for industrial processes since several years [1].

Importantly, PM exposure is linked with the occurrence of cardio-respiratory disease as well as mortality [2,3]. Epidemiological and experimental data suggest a relationship between PM and e.g. asthma [4], chronic obstructive pulmonary disease [5], and cystic fibrosis [6,7]. Unfortunately, the exact mechanism by which PM induces or aggravates airway disease is still unknown.

Dependent on their size, particles preferentially deposit in different compartments of the lung. Importantly, most of the nanosized particles have a high alveolar deposition rate [8]. In the alveoli, these particles come into contact with the pulmonary surfactant layer that covers the entire alveolar region. Surfactant decreases the surface tension at the air-liquid interface and thereby prevents alveolar collapse. Surface activity is mainly accomplished by surfactant phospholipids and the specific surfactant proteins (SP)-B, and -C. Morphologically, surfactant exists in different subfractions. The surface active fraction consists of lamellar bodies and tubular myelin whereas the less surface active fraction is comprised of unilamellar vesicles. By ultracentrifugation, lamellar bodies and tubular myelin can be pelleted and are thereby called large aggregates (LA). In contrast, unilamellar vesicles remain in the supernatant and are defined as small aggregates (SA). Conversion of LA into SA occurs during respiration [9].

It has been demonstrated that particles of anthropogenic origin are able to directly interact with pulmonary surfactant components [10-13]. Further, it has been shown that nanosized particles can disturb surfactant function [14,15]. However, a systematic comparison of nanosized and microsized particles (MSP) of different composition has not been made. Moreover, it is unclear whether particle-surfactant interactions during dynamic conditions of surface area cycling aggravate the biophysical surfactant dysfunction. Therefore, we investigated the effect of increasing concentrations of TiO_2 NSP and TiO_2 MSP, as model particles, on pulmonary surfactant function by means of a pulsating bubble surfactometer both under native conditions and following surface area cycling. For comparison reasons, the effect on surfactant function was investigated for nanosized and microsized polystyrene particles as well as for quartz particles. Furthermore, we

studied the effect of nanosized TiO_2 particles on surfactant ultrastructure by transmission electron microscope (TEM). In order to elaborate on the in-vivo relevance, rats were exposed to TiO_2 NSP versus TiO_2 MSP, lungs were fixed and lung tissue blocks were prepared for electron microscopy. The ultrastructure and distribution of the different subtypes of intra-alveolar surfactant was observed.

Methods

Particles

Nanosized and microsized titanium dioxide particles (Alfa Aesar, Karlsruhe, Germany; product numbers: 44689 & 42681) were used in this study. For comparison, polystyrene particles (Micromod, Rostock-Warnemuende, Germany), Sikron SF 800 quartz particles (Quarzwerke, Frechen, Germany) as well as citrate coated nanosized gold particles (Plano, Wetzlar Germany; product number: EM.GC5) were studied [for details see additional file 1]. Particle stock solutions were prepared in sterilized bidistilled water at a concentration of 25 mg/ml or 50 mg/ml. Particles were sonicated prior to each experiment.

Acute Effects on Biophysical Surfactant Function

A natural porcine surfactant preparation (Curosurf[®], Asche Chiesi, Hamburg, Germany) was used as a standard and was adjusted to 1.5 mg/ml phospholipids in Ringer's solution. Particles at increasing concentrations were added (50 $\mu\text{g}/\text{ml}$ - 500 $\mu\text{g}/\text{ml}$) and biophysical surfactant function was assessed with a pulsating bubble surfactometer (PBS) (Electronetics, Buffalo, NY, USA) as described below.

Surface Area Cycling

Surface area cycling is a standardized method to simulate the *in vivo* conversion of surface active surfactant subtypes (lamellar bodies, tubular myelin) to inferior surfactant subtypes (unilamellar vesicles) *in vitro* [16-20]. We measured the biophysical surfactant function following surface area cycling in the presence or absence of particles in order to assess the effect of particles during the conversion process. Curosurf[®] was adjusted to 1.5 mg/ml phospholipids in ringer solution with or without particles in increasing concentrations (50 $\mu\text{g}/\text{ml}$ - 500 $\mu\text{g}/\text{ml}$). Aliquots were placed in 12 \times 75 mm capped plastic tubes (Falcon 2058) and rotated end over end for 8 hours at 0.43 Hz and 37°C in the dark. Thereby, surface area changed from 1.1 cm^2 to 9 cm^2 twice per cycle. After surface area cycling biophysical surfactant function was measured in a pulsating bubble surfactometer as described below.

Surface Activity Evaluated with the Pulsating Bubble Surfactometer

Surface activity of pulmonary surfactant was measured with a PBS. Forty μl of the surfactant mixture were filled into the sample chamber. The surface tension used for sta-

tistical analysis of this study was the value at minimum bubble size (γ_{\min}) registered after 330 seconds of pulsation at a rate of 20 cycles/min and a temperature of 37°C. In addition, adsorption surface tension (γ_{ads}) was evaluated by determining surface tension 10 s after formation of a bubble under static bubble conditions. All data were digitalized and recorded by computer. All assays were performed in duplicate and the mean value was reported. The PBS was calibrated and checked with reference substances for proper operation before starting the measurements on each day.

Transmission Electron Microscope

Surfactant was fixed in Eppendorf tubes with 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.15 M Hepes buffer. The samples were stored in the fixative for 1 hour at room temperature and at least 24 hours at 4°C. Afterwards, samples were centrifuged at 10,000 g to obtain a surfactant pellet. After several washings in buffer, the samples were subsequently postfixated in osmium tetroxide and half-saturated aqueous uranyl acetate, dehydrated in an ascending acetone series and embedded in Epon at 60°C. The Eppendorf cups were removed and ultrathin 50 nm sections were cut using an ultramicrotome. The sections were analyzed with a Philips CM12 transmission electron microscope (FEI Co. Philips Electron Optics, Zürich, Switzerland).

Exposure of rats to particles and assessment of surfactant ultrastructure

Female Wistar rats (162 - 200 g) were randomly exposed once for 6 hours to either TiO₂ NSP (P25; Evonik Degussa, Essen, Germany), TiO₂ MSP (Bayertitan T, Bayer, Leverkusen, Germany), or clean air, respectively (n = 5 per group). The exposure atmosphere was adjusted to either 10 mg TiO₂ MSP/m³ or 25 mg TiO₂ NSP/Na₂HPO₄/m³ (60% Na₂HPO₄, 40% TiO₂ NSP). Since the TiO₂ MSP and TiO₂ NSP/Na₂HPO₄ droplets in the atmosphere were approximately of the same size, a similar alveolar deposition rate of 60 µg TiO₂ particles per animal was accomplished [21]. Rats were sacrificed by pentobarbital overdose at the end of the exposure and the lungs were perfusion-fixated as described before [22]. Surfactant ultrastructure was assessed on ultrathin sections by electron microscopy and surfactant subtype conversion was studied semiquantitatively.

Statistical Analysis

Values are given as means ± SEM. Statistical analysis was performed using GraphPad Prism®, Version 4.03. The one-way ANOVA was used for statistical analysis of the data. A Bonferroni correction was used throughout. P values < 0.05 were considered to be significant.

Results

Direct Effect of Particles on Pulmonary Surfactant Function

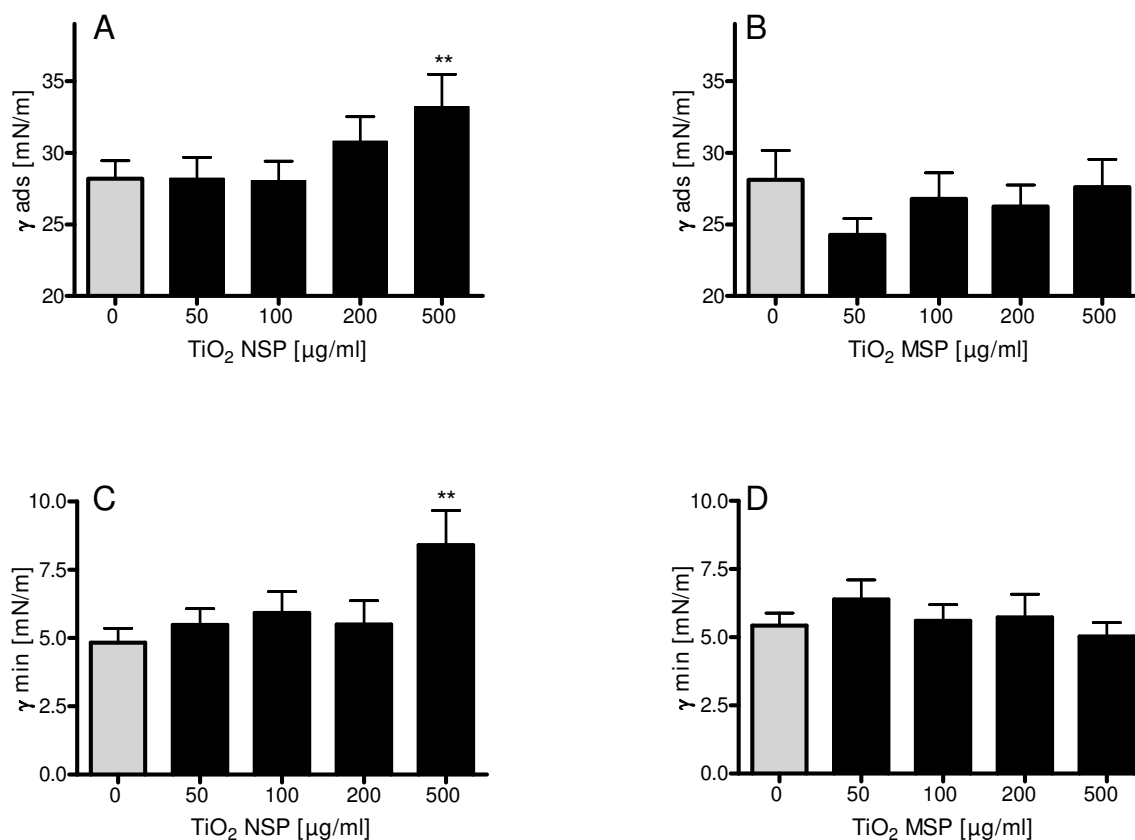
To assess the direct effect of particles on surfactant function, surface tension was measured after addition of particles. Pure surfactant showed an adsorption surface tension of ~28 mN/m and addition of TiO₂ NSP in concentrations up to 200 µg/ml did not affect this active surface tension (Figure 1A). However, further increase of the particle dose up to 500 µg/ml led to a significant increase in adsorption surface tension to 33.2 ± 2.3 mN/m. In contrast, surface tension was unaffected by treatment with the same mass concentrations of TiO₂ MSP (Figure 1B). TiO₂ NSP slightly increased γ_{\min} from ≤ 5 mN/m, which denotes active surfactant, up to 8.4 ± 1.3 mN/m at 500 µg/ml (Figure 1C). Again, TiO₂ MSP showed no effect on surface tension in this concentration range (Figure 1D). However, at very high particle concentrations of TiO₂ MSP (~10 mg/ml) that deliver a similar surface area compared to TiO₂ NSP γ_{\min} increased to 15.9 ± 1.3 mN/m (n = 6, p < 0.01).

As for the TiO₂ particles similar results were observed for the other particles. Whereas polystyrene NSP significantly increased adsorption surface tension for 500 µg/ml, polystyrene and quartz MSP did not influence the surface tension up to a concentration of 500 µg/ml (Table 1). In addition, nanosized polystyrene particles increased surface tension at minimum bubble size significantly at 500 µg/ml up to 6.8 ± 1.2 mN/m, whereas microsized particles did not influence surfactant function in this concentration range (Table 1). Again, MSP (Quartz) at a very high concentration (~10 mg/ml) that deliver a similar surface area compared to TiO₂ NSP increased γ_{\min} to 15.5 ± 1.8 mN/m (n = 5, p < 0.05).

Furthermore, we tested commercially available gold NSP with citrate coating (5 nm) in single experiments. At 200 µg/ml and 500 µg/ml, gold NSP increased γ_{\min} to 7.7 ± 2.8 and 13.2 ± 5.3, respectively (n = 4).

Effects of Particles Following Surface Area Cycling

Surface area cycling alone led to an increase in adsorption surface tension from ~28 to ~45 mN/m (Figure 2A and 2B). The presence of TiO₂ NSP in concentrations of 200 µg/ml and 500 µg/ml during the cycling process led to a further increase of adsorption surface tension to 53.3 ± 1.3 mN/m and 63.6 ± 0.4 mN/m, respectively (Figure 2A). TiO₂ MSP concentrations up to 500 µg/ml did not affect adsorption surface tension (Figure 2B). The influence of TiO₂ NSP on surface tension at minimum bubble size was pronounced (Figure 2C). TiO₂ NSP at 100 µg/ml led to a significant increase in surface tension from 1.1 ± 0.1 mN/m up to 8.4 ± 3.1 mN/m. Further increase of particle dose induced a strong surfactant dysfunction with γ_{\min} of 18.0 ± 1.6 mN/m and 21.1 ± 0.4 mN/m after incubation with

**Figure 1**

Surface activity evaluated with the pulsating bubble surfactometer. A) Adsorption surface tension (γ_{ads}) after incubation with TiO_2 nanosized particles (NSP) at a static bubble condition. B) Influence of TiO_2 microsized particles (MSP) on γ_{ads} . C) Influence of TiO_2 NSP on surface tension at minimal bubble size (γ_{min}) during pulsation. D) γ_{min} after incubation with TiO_2 MSP. Values are given as means of at least 4 experiments \pm SEM. ** indicates p values < 0.01 compared with the control at 0 $\mu\text{g/ml}$ particle concentration.

200 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ TiO_2 NSP, respectively. TiO_2 MSP led to a slight but non-significant increase in γ_{min} (Figure 2D).

Polystyrene NSP led to a slight increase in adsorption surface tension from 45.7 ± 1.0 mN/ml up to 51.4 ± 0.9 mN/ml which was only significant at a concentration of 500 $\mu\text{g/ml}$ polystyrene NSP (Table 1). All other MSP did not influence adsorption surface tension (Table 1). Surface tension at minimum bubble size was also unaffected by polystyrene MSP and quartz MSP (Table 1), while polystyrene NSP induced a strong surfactant dysfunction at minimum bubble size. Incubation with 500 $\mu\text{g/ml}$ polystyrene NSP during the cycling process led to a surface tension of 17.5 ± 1.4 mN/m (Table 1).

Influence of Nanosized TiO_2 Particles on Surfactant Ultrastructure

Natural porcine surfactant used in this study consisted mostly of lamellar body-like forms. Unilamellar vesicles were hardly present (Figure 3A and 3B). After addition of 100 $\mu\text{g/ml}$ TiO_2 NSP, lamellar body-like forms were decreased in size and deformed (Figure 3C). In addition, an increase in the amount of unilamellar vesicles appeared (Figure 3C). Interestingly, small TiO_2 NSP aggregates accumulated between lamellae of the lamellar body-like forms (Figure 3D). Rotation of the pure surfactant in the absence of particles readily led to a conversion of lamellar body-like forms to unilamellar vesicles (Figure 3E). Rotation in the presence of TiO_2 NSP did not further change subtype conversion (Figure 3F). However, large

Table 1: Surface activity evaluated with the pulsating bubble surfactometer.

	Polystyrene NSP	Polystyrene MSP	Quartz MSP
Direct effects - γ_{ads} [mN/m]			
0 $\mu\text{g/ml}$	29.2 \pm 1.5	28.9 \pm 2.7	25.9 \pm 1.5
50 $\mu\text{g/ml}$	28.3 \pm 1.4	28.3 \pm 2.2	25.0 \pm 1.6
100 $\mu\text{g/ml}$	30.8 \pm 2.2	28.6 \pm 2.3	25.0 \pm 2.0
200 $\mu\text{g/ml}$	32.4 \pm 2.1	30.1 \pm 3.0	26.3 \pm 2.9
500 $\mu\text{g/ml}$	34.1 \pm 2.2**	28.3 \pm 2.3	25.2 \pm 1.5
Direct effects - γ_{min} [mN/m]			
0 $\mu\text{g/ml}$	4.0 \pm 0.9	2.4 \pm 1.2	4.9 \pm 0.5
50 $\mu\text{g/ml}$	5.1 \pm 0.9	2.2 \pm 0.9	4.1 \pm 0.9
100 $\mu\text{g/ml}$	4.0 \pm 1.0	2.8 \pm 1.8	4.6 \pm 0.8
200 $\mu\text{g/ml}$	4.7 \pm 1.1	3.1 \pm 1.3	4.1 \pm 0.9
500 $\mu\text{g/ml}$	6.8 \pm 1.2**	3.0 \pm 1.5	5.0 \pm 0.8
Surface area cycling - γ_{ads} [mN/m]			
Control	27.1 \pm 1.5	27.4 \pm 2.7	28.1 \pm 0.8
0 $\mu\text{g/ml}$	45.7 \pm 1.0	46.4 \pm 1.3	43.7 \pm 0.8
50 $\mu\text{g/ml}$	42.4 \pm 2.2	43.7 \pm 2.2	47.1 \pm 0.9
100 $\mu\text{g/ml}$	47.1 \pm 1.3	43.1 \pm 0.8	45.8 \pm 2.8
200 $\mu\text{g/ml}$	44.6 \pm 2.1	45.3 \pm 2.3	45.4 \pm 3.1
500 $\mu\text{g/ml}$	51.4 \pm 0.9*	42.7 \pm 1.7	48.4 \pm 1.5
Surface area cycling - γ_{min} [mN/m]			
Control	1.1 \pm 0.2	1.1 \pm 0.2	1.2 \pm 0.4
0 $\mu\text{g/ml}$	1.2 \pm 0.2	1.1 \pm 0.2	1.7 \pm 0.4
50 $\mu\text{g/ml}$	2.1 \pm 0.4	1.1 \pm 0.4	0.9 \pm 0.3
100 $\mu\text{g/ml}$	1.5 \pm 0.5	1.2 \pm 0.2	2.1 \pm 0.9
200 $\mu\text{g/ml}$	6.3 \pm 2.8	1.8 \pm 0.5	2.8 \pm 0.7
500 $\mu\text{g/ml}$	17.5 \pm 1.4***	1.9 \pm 0.5	1.0 \pm 0.3

Direct effects were measured immediately after addition of particles, effects after surface area cycling were measured following 8 hour rotation at 0.43 Hz with or without particles. Adsorption surface tension (γ_{ads}) was obtained from the value of a static bubble. Surface tension at minimal bubble size (γ_{min}) was recorded during pulsation. Values are given as means \pm SEM of at least 4 experiments. * indicates p values < 0.05; ** indicates p values < 0.01; *** indicates p values < 0.001; all compared with the control without particles (0 $\mu\text{g/ml}$). In case of surface area cycling, the 0 $\mu\text{g/ml}$ control was rotated for 8 hours at 0.43 Hz. NSP - nanosized particles; MSP - microsized particles.

TiO₂ aggregates were found after rotation (Figure 3F). These aggregates were larger in size than the TiO₂ aggregates in the non-rotated sample (Figure 3D).

Effect of inhaled particles on surfactant ultrastructure in rats

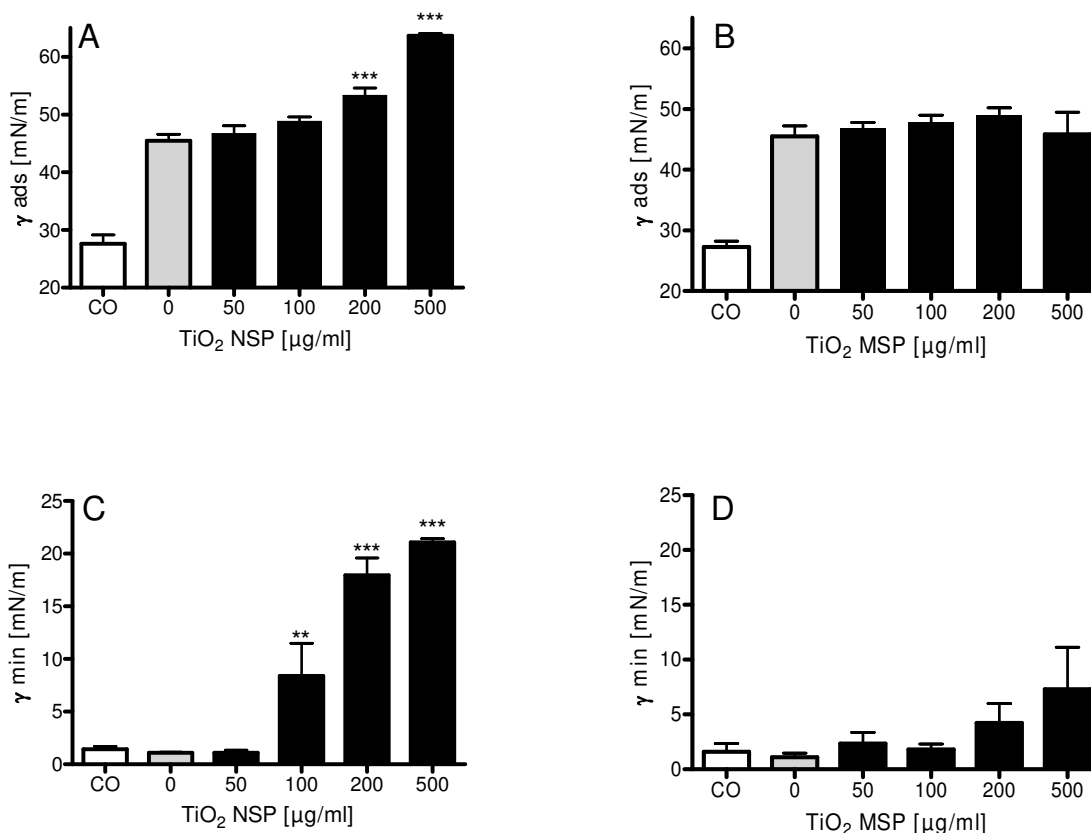
Semiquantitative analysis of intra-alveolar active (tubular myelin and lamellar bodies) and inactive surfactant subtypes (unilamellar vesicles) did not differ between both groups. There were no signs of alveolar oedema or inflammation.

Discussion

The present data show that nanosized particles, but not microsized particles, induce a dysfunction of pulmonary surfactant. Nanosized titanium dioxide as well as nano-

sized polystyrene particles at high concentrations can induce a slight pulmonary surfactant dysfunction *in vitro*. Interestingly, surface area cycling *in vitro* aggravated the surfactant dysfunction induced by nanoparticles, both by TiO₂ NSP and by polystyrene NSP. In addition, biophysical alterations of pulmonary surfactant by TiO₂ NSP were accompanied by changes of the surfactant ultrastructure indicating increased surfactant subtype conversion.

A direct interaction between particles and the surfactant constituents is the most likely explanation for the observed surfactant dysfunction. It is well known that phospholipids bind to particles [10,14,23] and to TiO₂ structures [24,25]. In this respect, surface area seems to be the major determinant of the observed biophysical and ultrastructural changes. Accordingly, particles with the

**Figure 2**

Surface activity evaluated with the pulsating bubble surfactometer following 8 hour rotation at 0.43 Hz. A) Influence of TiO_2 NSP on adsorption surface tension (γ_{ads}) at a static bubble condition. B) Influence of TiO_2 MSP on γ_{ads} . C) Influence of TiO_2 NSP on surface tension at minimal bubble size (γ_{min}) during pulsation D) TiO_2 MSP effect on γ_{min} . Values are given as means \pm SEM of at least 4 experiments. * indicates p values < 0.01; *** indicates p values < 0.001; both compared with the rotated 0 $\mu\text{g/ml}$ particle concentration (grey columns). CO/white columns - control surfactant which was placed for 8 hours in an incubator without rotation.

highest surface area - TiO_2 NSP and also reference polystyrene NSP - induced the most prominent alterations. Microsized particles with a relatively low surface area did not induce a surfactant dysfunction in our study.

In separate experiments, we compared equal surface areas by testing very high microparticle mass concentrations. With concentrations of ~ 10 mg/ml TiO_2 MSP and quartz MSP, we observed a strong surfactant dysfunction. However, the experimental conditions were limited because microsized particles at this very high concentration aggregated and rapidly sedimented to the bottom of the test capillary. By this segregation, the phospholipid concentra-

tion was not stable which limits the comparison of NSP and MSP at similar surface areas.

Bakshi and coworkers demonstrated a potent pulmonary surfactant dysfunction at low concentrations of ~ 2 $\mu\text{g/ml}$ gold nanoparticles [14]. In contrast, much higher concentrations of TiO_2 NSP were required to induce an increase of surface tension in our experiments. In addition, the degree of surfactant dysfunction was less with TiO_2 NSP in our study compared to the gold nanoparticles used by Bakshi et al. Differences in 1) the measuring system, 2) the surfactant preparation and concentration, or 3) the nanoparticles themselves might account for the discrepancy.

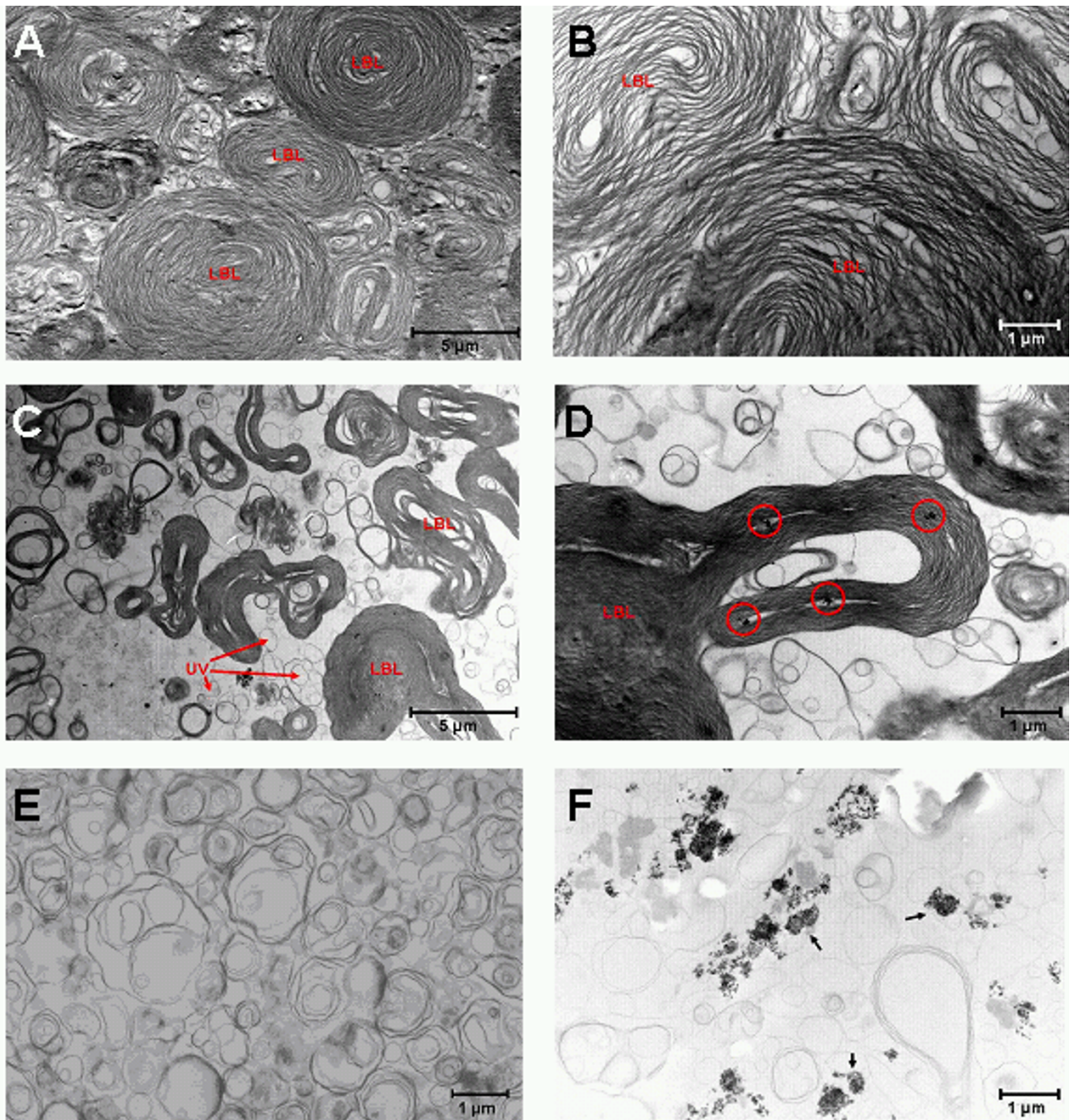


Figure 3

Representative transmission electron microscope pictures of the surfactant ultrastructure. A) and B) untreated control surfactant. C) and D) porcine surfactant after addition of 100 $\mu\text{g/ml}$ TiO_2 nanosized particles. Red circles show small particle aggregates. E) Control surfactant after 8 hours rotation at 0.43 Hz and 37°C. F) Surfactant after 8 hours rotation at 0.43 Hz and 37°C in the presence of 100 $\mu\text{g/ml}$ TiO_2 nanosized particles. Black arrows show large particle aggregates; lbl - lamellar body like forms; ulv - unilamellar vesicles.

Both, the pulsating bubble surfactometer (PBS) and the captive bubble surfactometer (CPS) are able to evaluate low surface tensions [26] while the CPS is regarded to yield even lower surface tensions [27] which makes differences in the device an unlikely explanation. Regarding surfactant preparation and concentration, we used Curosurf[®], a natural surfactant derived from minced porcine lungs [28] while a semisynthetic surfactant composed of two phospholipids plus SP-B was used by Bakshi. It is unlikely that differences in the surfactants are solely responsible for the different effects seen with gold nanoparticles and TiO₂ NSP. Both surfactants have been demonstrated to have excellent surface activity and to achieve very low surface tensions under compression at the concentrations used. The most likely explanation for the potent dysfunction in the study by Bakshi seems related to the material properties (size/surface) of the gold nanoparticles. Since the gold NSP had citrate groups on their surface, aggregation is mostly avoided [29]. In contrast, pure TiO₂ nanoparticles highly aggregate. Although the surface area is not known for the gold NSP from Bakshi's study, it is likely that the surface area per mass unit is higher for the citrate coated gold NSP than for the TiO₂ NSP. This could explain the more potent induction of surfactant dysfunction by gold NSP compared to TiO₂ NSP because surfactant components could be bound to the large gold nanoparticle surface area making them unavailable for lowering surface tension at the air-liquid interface.

In an attempt of direct comparison between TiO₂ NSP and the gold nanoparticles by Bakshi (~15 nm), we tested commercially available gold NSP with citrate coating (5 nm) in single experiments. Interestingly, at equal mass the surfactant dysfunction by gold NSP was stronger compared with TiO₂ NSP. However, the dysfunction was less compared with data from Bakshi et al., but this discrepancy can be accounted to differences in surface area of the gold NSP or the surfactant preparations used in both studies.

The *in vivo* conversion of surface active LA to inferior SA can be simulated *in vitro* by surface area cycling [16]. By this technique, the impact of meconium, serum proteins, or surfactant proteins during the surfactant conversion process have been studied [19,30-32]. We assessed the effect of TiO₂ NSP on the conversion process. Importantly, a dose-dependent increase in surface tension was obtained. Remarkably, this effect was much stronger than the direct biophysical effect of TiO₂ NSP without cycling. TEM pictures demonstrated that the occurrence of unilamellar vesicles was independent from NSP presence. Possibly, binding of NSP to SP-B and subsequent loss of SP-B from the air-liquid-interface could explain the loss of surface activity following surface area cycling in the presence of NSP. *In vivo*, SP-B becomes cleaved by a serine active

carboxylesterase called convertase [33-35]. However, Curosurf[®] is prepared by chloroform extraction and hence does not contain convertase [20]. Therefore, intact SP-B should be present in Curosurf[®] following surface area cycling. We speculate that free SP-B could interact with TiO₂ NSP which in turn becomes depleted leading to disturbed surfactant function. High ability of SP-B to bind to surfaces during surface area cycling was shown before when binding of SP-B to tube walls was investigated during surface area cycling [18]. Unfortunately, we were not able to provide direct evidence of binding of SP-B to TiO₂ NSP by TEM due to methodological limitations.

Admittedly, inhaled particles act directly on the surfactant layer at the air-liquid interface and not primarily through the subphase as in our *in vitro* experiments. However, after deposition at the air-liquid interface the particles subsequently become dissolved in the epithelial lining layer and interfere with the dynamic process of phospholipid arrangement at the interface. Therefore, the assay system with the pulsating bubble surfactometer is at least capable to demonstrate differential effects of nanoparticles versus microparticles in phospholipid suspensions when particles interfere with the formation of the surfactant layer from the hypophase. It is very well conceivable that the initial effect of particles might even be greater when they are reaching the interface directly.

Although we have demonstrated that TiO₂ NSP elicited biophysical and structural changes of surfactant *in vitro*, the *in vivo* relevance has to be scrutinized because the particle concentrations that we found effective *in vitro* can hardly occur *in vivo*. With the human alveolar surface area of ~100 m² and assuming an average thickness of the alveolar lining fluid of approximately 200 nm [36], the amount of alveolar lining fluid can be assessed as ~20 ml. In accordance, the epithelial lining fluid has been suggested to be 6 ml/L total lung capacity, resulting in 40 ml in man [37]. With the assumption of a particle concentration of 100 µg/m³, which can occur in polluted inner cities, and an alveolar deposition rate as high as 50%, the amount of particles deposited per day would be ~360 µg. At steady state, this would result in a concentration of ~10 µg nanoparticles per ml alveolar lining fluid. This particle concentration is far below what has been demonstrated to cause a surfactant dysfunction in our study. In addition, clearance of particles and secretion of newly synthesized surfactant would further improve this particle/surfactant ratio and consequently question whether nanoparticles can cause a surfactant alteration under these conditions *in vivo*. This view is supported by our experimental evidence in rats. Following inhalation of TiO₂ particles that were aerosolized and adjusted to result in the highest technically possible alveolar deposition of 60 µg particles per animal, surfactant ultrastructure was found unaffected *in*

in vivo. Assuming an alveolar lining fluid in rats of approximately 70 μL [36], the *in vivo* particle concentration in the epithelial lining fluid would have been approximately 53.5 $\mu\text{g}/\text{mL}$ (normalized to 1.5 mg/ml phospholipids and assuming static conditions). Noteworthy, the local concentration at the air-liquid interface was probably much higher suggesting that no changes of surfactant ultrastructure occur *in vivo* under acute maximal TiO_2 particle exposure.

Although these considerations suggest that the impact of TiO_2 NSP on surfactant function in the human lung is highly unlikely to cause adverse effects in healthy individuals, in diseased subjects, however, additive effects of NSP on pulmonary surfactant function and ultrastructure have to be taken into account. For example, it has been demonstrated that a pulmonary surfactant dysfunction can be found in various airway diseases like asthma [38], cystic fibrosis [39], or after lung transplantation [40]. In particular, leakage of plasma proteins into the airway lumen is known to induce a surfactant dysfunction [41,42]. Importantly, NSP can induce [43-45] or enhance [46,47] pulmonary inflammation which is accompanied by protein leakage. This in turn could lead to a surfactant dysfunction *in vivo*. Moreover, NSP are able to induce oxidative stress and lipid peroxidation [48-50]. Oxidative stress with lipid peroxidation can induce an increase in surface tension [51,52]. In addition, NSPs emitted by engines are contaminated with alkanes and sulfates [53] and it is known, that eicosane, a specific n-alkane constituent of diesel exhaust NSPs, can affect the biophysical surfactant function [54]. Therefore, nanoparticles might amplify alterations of the pulmonary surfactant system, particularly under predisposed conditions of airway inflammation.

Conclusion

Taken together, TiO_2 NSP induce biophysical and structural alterations of pulmonary surfactant *in vitro*. Under dynamic conditions of surface area cycling, this interfering impact is aggravated. Although our data do not suggest that inhalation of nanoparticles cause a significant disturbance of the pulmonary surfactant system *in vivo*, nanoparticles might be detrimental in patients with preexisting airway disease.

Abbreviations

CPS: Captive bubble surfactometer; CO: Control; DPPC: Dipalmitoylphosphatidylcholine; LA: Large aggregates; lbl: Lamellar body-like forms; MSP: Microsized particles; NSP: Nanosized particles; PBS: Pulsating bubble surfactometer; PM: Particulate matter; SA: Small aggregates; SEM: Standard error of the mean; SP: Surfactant protein; TEM: Transmission electron microscope; TiO_2 : Titanium dioxide; ulv: Unilamellar vesicles; γ_{min} : surface tension at minimum bubble size; γ_{ads} : adsorption surface tension

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CS planned the concept and study design, performed the surface area cycling as well as pulsating bubble surfactometer experiments, interpreted the results and wrote major parts of the manuscript. CM performed the electron microscopic analyses of the surfactant ultrastructure and interpreted the results. KP performed the electron microscopic analyses of the particles and interpreted the results. AS performed the electron microscopic analyses of the surfactant ultrastructure and interpreted the results. MN participated in characterization of the particles and interpreted the results. HDL made substantial contributions to the analysis and interpretation of the data. AB made substantial contributions to the analysis and interpretation of the data. NK made substantial contributions to the analysis and interpretation of the data. VJE participated in planning the study design and made substantial contributions to the analysis and interpretation of the data. JMH planned the concept and study design, made substantial contributions to the analysis and interpretation of the data and wrote major parts of the manuscript. All of the authors have critically read the manuscript and approved its submission.

Additional material

Additional file 1

Characterization of the particles. Representative transmission electron microscopic pictures of the particles [see figure S1 in additional file 1] as well as a table with the characteristics of the particles [see table S1 in additional file 1] are shown.

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