Screening strategy to avoid toxicological hazards of inhaled nanoparticles for drug delivery: the use of α-quartz and nano zinc oxide particles as benchmark

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Abstract: Nanotechnology is a broad, revolutionary field with promising advantages for new medicine. In this context the rapid development and improvement of so called nanocarriers is of high pharmaceutical interest and some devices are already on the market. In our project we aim to develop well characterized nanoscaled drug delivery systems for an inhalative application. To this end, we focus on the most adverse side-effects within the lung, the cytotoxic and the proinflammatory responses to these nanoparticles (NPs). Before performing any animal experiments, we start with an in vitro screening for analyzing the cytotoxic and proinflammatory effects of the investigated particles on two murine lung target cell lines, the alveolar epithelial like typ II cell line (LA4) and the alveolar macrophage cell line (MH-S). Three different endpoints were estimated, (i) cellular metabolic activity, determined by the WST-1 assay, (ii) membrane integrity, by detection of LDH release and hemolytic activity, and (iii) secretion of inflammatory mediators. To analyze the relative particle toxicity we choose two reference particles as benchmarks, (i) fine α-quartz, and (ii) ultrafine ZnO particles. The investigation of dose-response and kinetics of proinflammatory and toxic effects caused to the named cell lines provide an insight to a close evaluation of our cell based screening strategy. α-quartz is well known for its inflammatory and toxic potential caused by inhalation, and nanosized ZnO particles - used in a broad field of nanotechnology like electronics, but also cosmetics and pharmaceuticals - is to a high degree cytotoxic and proinflammatory in vitro. Preliminary experiments indicated not only particle and cell specific inflammatory responses, but also different susceptibilities of the cell types being exposed to our benchmark particles regarding their size and surface activities. Exposure to the µm-sized α-quartz particles affected the viability of epithelia cells less than that of macrophages, pointing to the impact of particle uptake by phagocytosis. In contrast, the nanosized ZnO particles caused much stronger decrease in cell viability and higher levels of LDH in the macrophage cell line compared to epithelial cells, even though the hemolytic activity was much higher for the α-quartz particles than for the nanosized ZnO. For the proinflammatory effects, we observed a clear dose-dependent release of acute phase cytokines (TNF-α, IL-6, G-CSF> CXCL10>CCL2) for both alveolar cell lines after Min-U-Sil exposure. After ZnO treatment the cytokine responses were negligible compare to control cells. In conclusion, our data attach value to the use of different cell types to detect different pathways of toxicity generated by different particle properties.
Therefore, we will establish both lung target cell lines for an in vitro screening to analyze proinflammatory and cytotoxicity effects of nanocarriers. The implementation of the two reference particles facilitate the validated classification of the cytotoxic responses caused by the NPs investigated.

1. Introduction:
In the field of nanotechnology the emerging developments make demands more and more on considerations of ‘nano-hazards’ from nano-devices, -application or combustion-derived nanoparticles (NPs). To ensure the safety of such a material or device and to categorize nanomaterial regarding its toxic potential, many researchers focus now on the most crucial issues, namely cytotoxicity and proinflammatory effects, especially for the lung exposure [1]. These issues are of particular interest since instilled non-biodegradable nanospheres have been shown to induce pulmonary inflammation [2]. In addition, upon pulmonary delivery, for similar particles even systemic effects like enhanced thrombosis have been observed [3]. The commonly accepted model suggests, that the toxicity of particulate matter (PM) is related to their ability to generate free radicals and induce oxidative stress and inflammation. Inflammation sounds crucial for PM-exposure related diseases like exacerbations of airways disease and cardiovascular disease [4]. Since sub-micron sized particles could inherently be more toxic due to their increased surface area, this drawback has to be kept in mind [5].

In our project we aim to develop well characterized nanoscaled drug delivery systems for an pulmonary application. A lot of studies dealt with the physicochemical characterization and application behavior of these material [6], but only a few studies focus on the risk assessment [7].

In the literature it has been well described that particle size and surface area are the most important and toxicity driving parameters, and it is thus generally accepted, that at a given mass, smaller sized particles are more toxic than larger sized particles.

In this study, we choose two particles as benchmarks, (i) fine α-quartz, and (ii) ultrafine ZnO particles to estimate the related particle toxicity. Min-U-Sil-5 possess well characterized physico-chemical properties, is of high purity (98.3% silicium dioxide), and has over many years been extensively studied for its acute inflammatory and cytotoxic lung response [8-11]. Since this fine sized quartz might not reflect the toxic aspects inherent to nanomaterials we additionally included 70nm ZnO particles, a well known lung-toxic nanoparticle from occupational medicine [12-14], as reference. Both particles are form high purity and well characterized by the manufacturers for their physico-chemical properties, all these attributes mandatory for any ‘reference material’ being applied as positive control. To minimize animal experiments we start with an in vitro screening of the two different reference particles described above for better understanding of the involved toxicity driven pathways.

2. Materials and Methods:

2.1. Cell culture
Cell culture experiments were carried out using the murine alveolar epithelial – like type II cells (LA4; ATCC No. CCL-196™) and the murine alveolar macrophages (MH-S; ATCC No. CRL-2019). LA4 cells were grown in HAM’s F12 medium with stable Glutamax containing 15% fetal bovine serum (FBS, Gibco, Germany) and 1% non essential amino acids and 100U/ml penicillin and 100mg/ml streptomycin, MH-S cells were cultured in Dulbecco’s modified Eagle’s medium with stable Glutamax supplemented with 10% fetal bovine serum (FBS, Gibco, Germany) and 100U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO2. All cells were passaged every 2-3 days. All reagents were obtained from Biochrom AG, Seromed, Germany or otherwise signed.
2.2. Particle preparation
Min-U-Sil 5 (α-quartz) was obtained from U.S. Silica Company, Berkeley Springs, WV, USA, with a median diameter of 1.7µm declared on the datasheet from the manufacturer. Zinc oxide (CAS-No: 1314-13-2) was obtained from Alfa Aesar (A Johnson Matthey Company, Karlsruhe, Germany) and the manufacturer declares a average diameter of 70nm. Stock suspensions (10mg/ml) of each particle were prepared in sterile, double-distilled water. Each suspension was sonicated for 15min. prior to exposure and subsequently diluted in sterile, double-distilled water to reach a range of particle concentration from 20-1000µg/ml.

2.3. Cell viability
Cell viability was determined using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Germany) according to the method of Mosmann [15]. Briefly, LA4, MH-S cells were seeded at a density of 0.25*10⁶ cells/well/2cm² in 24-well-plate in cell culture medium containing FBS and grown overnight in an incubator at 37°C and 5%CO₂. For the treatment, the cell culture medium was replaced by freshly, pre-warmed, serum-reduced (2% FBS) cell culture medium without antibiotics. Particles were exposed to the cells for 2h, 6h and 24h and the relative viability [%] related to control samples (untreated cells) was calculated by following equation: Cell viability = ( ODsample/ODcontrol ) × 100. All data represent at least three independent experiments.

2.4. Cytotoxicity
For detection of the cytosolic enzyme lactate dehydrogenase (LDH) characteristically for membrane damage effects we used the Cytotoxicity Detection Kit (Roche Diagnostics, Germany) according to the manufacturer protocol. The experiments were carried out according to the conditions at the WST-1 assay. After three different time points (2h, 6h, and 24h), the LDH concentration in the cell culture supernatant was spectrophotometrically determined in an ELISA reader (Labsystems iEMS Reader MF) at a wavelength of 492nm. As control served cells treated with 2% (w/v) Triton X-100 according to the manufacturer protocol and set as maximum of LDH release (100%). The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells (high control). Less than 10% LDH release were regarded as non-toxic effect level in our experiments [16]. All data represent at least three independent experiments.

2.5. Hemolysis test
The hemolytic activity was determined according to [17]. Fresh blood were taken from human volunteers and collected in heparinized tubes. Blood was centrifuged at 700g at 4°C for 10min, and washed several times with PBS until the supernatant was colorless. The supernatant was removed and a 2.5% (v/v) suspension of erythrocytes was prepared. 500µl of the 2.5%(v/v) suspension of erythrocytes was mixed with 500µl of the appropriate particle dilution in eppendorf cups. After 60min. incubation time in a shaking water bath, the red blood cells were removed by centrifugation, and the supernatants were investigated spectroscopically at a wavelength of λ=540nm for detection of the release of hemoglobin. As references served PBS (negative) and 0.2% Triton X-100 solution (positive). All experiments were performed in triplicates.

2.6. Enzyme-linked immunosorbent assay
22 cytokines/chemokines were detected simultaneously in the cell culture supernatant by using Luminex technology (Linco Research, St. Charles, MO). In this study, the secretion of following cytokines/chemokines was investigated: IL-1α, IL-18, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, TNF-alpha, INF-γ, G-CSF, GM-CSF, CXCL1, CXCL10, CCL2, CCL3, and CCL5. The assay was performed as described previously [18]. The mean fluorescence intensity (MFI) was detected by the Multiplex plate reader (Luminex System, Bio-Rad Laboratories, Germany) for each sample (50µl) with a minimum of 50 beads per region being analyzed. The raw data (MFI) were
captured using the Multiplex plate reader software (Bioplex Manager, Version 2.0). For data analysis, a 4-parameter logistic curve fit was applied to each standard curve and sample.

2.7. Statistics
All values are presented as means±standard error (SEM) of at least three independent experiments. Significant differences between two groups were evaluated by Student’s t-test or between more than two groups by one-way ANOVA followed by Tukey’s multiple comparison test. Statistical analysis was performed using the program STATGRAPHICS PLUS Version 5.0 (Statpoint, Inc., Virginia, US).

Figure 1: Cell viability as determined by WST-1 Cell Proliferation Assay. Values are expressed as means±SEM (n=4-5). Five different concentrations (20, 100, 400, 500, and 1000µg/ml) of Min-U-Sil 5 (left figures), and ultrafine ZnO (right figures) were exposed to alveolar epithelial cells (LA4, black bars) and alveolar macrophages (MH-S, open bars). Differences to control (untreated cells) with (p)<0.05 were considered statistically significant and marked with an asterisk.
3. Results and Discussion:

3.1. Cell viability
In both alveolar cell lines the cell viability decreased in a time- and dose-dependent manner, which was more prominent in the alveolar macrophages (MH-S). Regarding the two different particle exposures, namely the $\alpha$-quartz particle with a mean diameter of 1.2 µm and the ultrafine ZnO particles with an average diameter of 70 nm, we observed a statistically significant higher decrease in metabolic activity after ZnO treatment (Figure 1).

Already after 2h particle exposure the cell viability decreased statistically significant in both cell lines in a dose-dependent manner. After $\alpha$-quartz treatment the cell viability in the alveolar epithelial cells remained constantly around 80%, even after 24h the cell viability was around 60% with the highest quartz dose. Nevertheless, in the alveolar macrophages we detected a statistically significant decrease in cell viability with a dose more than 100 µg/ml Min-U-Sil. Remarkably, after 6h and 24h exposure of the two particles to the alveolar cell lines we could distinguished between a dramatically loss of metabolic activity after ultrafine ZnO treatment and a sustained decrease in cell viability after $\alpha$-quartz exposure, especially for the alveolar epithelial cells. In all cases the alveolar macrophages (MH-S) were more susceptible than the alveolar epithelial cells. The different particle effects could be explain in general because of the different sizes and surface areas of these particles.

3.2. Cytotoxicity (LDH release)
In agreement to the WST-1 data, the $\alpha$-quartz exposure caused much less membrane damage detected by LDH release in the supernatants than the ultrafine ZnO exposure and again, the alveolar macrophages were more sensitive to these particles (Figure 2).

After both particle treatments LDH release was negligible after 2h, less than 10%, which is considered as a threshold for membrane toxic behavior [16]. Upon $\alpha$-quartz exposure the LDH release remained under 10%, but yielded some statistically significance compare to control (untreated cells). In contrast, after the ultrafine ZnO treatment high levels of LDH were observed in the alveolar macrophages after 6h and 24h, and the maximum was reached after 6h, but not in a dose-dependent manner. In the alveolar epithelial cells the LDH release was under the 10% threshold for membrane toxicity and were therefore negligible similar to the $\alpha$-quartz exposure. Again, we could show that the ultrafine ZnO particles caused a much higher toxicity, which we correlate to the higher surface area of these particles in comparison to the $\alpha$-quartz particles according to [19].
Figure 2: Influence of cell membrane integrity was observed by measuring LDH in the cell culture supernatant after three different time points (2h, 6h, and 24h) in the alveolar epithelial cell line (LA4, black bars) and the alveolar macrophages (MH-S, open bars). Cells were exposed to Min-U-Sil 5 (left figures) and ultrafine ZnO (right figures) with five different concentrations (20, 100, 400, 500, and 1000µg/ml). Values are expressed as means ± SEM (n=4-5). Probability (p)<0.05 was considered as significant compared to control (untreated cells) and indicated with an asterisk.

3.3. Hemolytic activity
In addition, we investigated the hemolytic activity of both particles. After 60min. treatment of human red blood cells we observed a dose-dependent elevated release of hemoglobin upon α-quartz treatment, whereas the ultrafine ZnO particles indicated a sustained release of hemoglobin independent from the dose. Both particles caused significantly high hemolytic activity, but in a different manner with regard to the dose.

Remarkably, the hemolytic activity of Min-U-Sil particles was much higher and dose-dependent, whereas Min-U-Sil particles released less LDH, which indicated only few membrane damage effect. In contrast, the ultrafine ZnO particles caused very high sustained LDH release already after 6h as well as a very high sustained release of hemoglobin, but much lower compare to the hemolytic activity of the α-quartz particles. The different underlying mechanism regard to cell death need to be observed in further experiments.
3.4. Cytokine response

The cytokine secretion of 22 proinflammatory cytokines were detected by multiplex technique. From this cytokine profiling five cytokines, mainly acute phase cytokines (TNF-α, IL-6, G-CSF, CXCL10, and CCL2) were induced more than 2-fold compared to controls (untreated cells), see Table 1. For Min-U-Sil we observed the most significant changes in a dose-dependent manner in both cell lines. In contrast, ZnO exposure caused only slightly elevated cytokine levels, but without any statistically significance. Due to very high cytotoxic effects we observed the proinflammatory effects of ZnO particles only for two non toxic concentrations (1.25µg/ml and 2.5µg/ml), where the cell viability is more than 80% and the LDH release is negligible after 24h treatment (data not shown).

Table 1: Relative cytokine secretion in LA4 and MH-S cells after 24h treatment of Min-U-Sil (20µg/ml,100µg/ml, and 400µg/ml) and ultrafine ZnO (1.25µg/ml, and 2.5µg/ml) determined by multiple analyte detection immunoassay. Means were normalized to control cells (untreated cells). Values represent mean ± SEM, n=2-3; b.d.l. = below detection limit; statistically significant (p<0.05) changes compared to control levels (untreated cells) are marked with an asterisk.
4. Conclusion:
In this study, we detected different time- and dose-dependent cytotoxic and proinflammatory responses of the two different particles. The ultrafine ZnO particles caused high, but sustained membrane damage effects confirming to a high decrease in metabolic activity, whereas the α-quartz particles revealed much less decrease in cell viability and no significant LDH release, but higher hemolytic activity in a dose dependent manner. Regarding the two different cell lines cytotoxic effects were more prominent in the alveolar macrophage cell line (MH-S). For the proinflammatory effects, we observed a clear dose-dependent release of acute phase cytokines (TNF-α, IL-6, G-CSF, CXCL10, CCL2), and to a higher extent in the macrophages, and only after Min-U-Sil exposure. After ZnO treatment the cytokine responses were negligible compare to control cells, but this might be because of the very low doses (1.25µg/ml and 2.5µg/ml).

In summary, we aimed to established two positive reference particles for our in vitro assessment of nanoscaled pulmonary drug delivery and discovered high cytotoxic effects in a dose- and time-dependent manner for both particles, most prominent in macrophages and for the ultrafine ZnO particles. Interestingly, the proinflammatory effects seem to be more pronounced after Min-U-Sil treatment. Thus, we concluded that for further in vitro assessment of nanocarriers for pulmonary applications it is necessary to estimated additionally the effects of two positive reference particles such as fine Min-U-Sil and ultrafine ZnO.

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