Suspension characterization as important key for toxicological investigations

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Abstract. To assess potential health risks of nanoparticles by means of in vitro or in vivo assays and to determine dose-action curves a defined and reproducible method of particle administration is required. The interpretation of the toxicological results should be based on a comprehensive chemical-physical characterization of the particles used. Therefore, we developed a method to suspend nanoparticles stably and homogenously in physiological media. Our approach consists of three steps: (1) physical-chemical characterization of the powders as delivered, (2) preparation and characterization of a non-physiological electro-statically stabilized nanoparticle suspension and (3) assessment of the nanoparticles behaviour in physiological media with or without proteins. This approach is demonstrated on a titanium dioxide and a tungsten carbide nanopowder. Results showed that particles agglomerate in protein-free medium within minutes, whereas in the presence of bovine serum albumin or foetal bovine serum an agglomeration is hindered.

1. Introduction
Due to the increasing use of nanomaterials, the assessment of health risks of nanoparticles become urgent. It is a central task to reveal possible risks that can emerge through the small dimension of nanostructured materials [1-3]. For a complete risk and safety assessment of nanoscaled materials, such as nanoparticles, nanotubes and nanoplates, a comprehensive chemical-physical characterization of these substances is necessary. Chemical composition, cristallinity, morphology, particle shape and size as well as the specific surface area of the powders in the as-delivered state are key properties. But, for a correct interpretation of in vivo and in vitro the particles behaviour under physiological conditions has also to be understood. Physical parameters like the size of nanoparticles, for instance, should be measured in cell culture media under conditions as close as possible to the toxicological testing [4]. Particle-particle interactions resulting in agglomeration processes and reactions between particles and serum proteins have to be taken into account. In cell culture media, proteins are rapidly adsorbed on the nanoparticle surface. The resulting protein corona modifies the appearance of the particles and their interactions with biological structures. For example, the cellular uptake of nanoparticles can be influenced by adsorbed serum proteins [5, 6].

As a result, the characterization of suspended particles in physiological media is one of the most difficult operations in a complete biological and chemical-physical assessment of nanoparticles toxicity, but it is one of the most important ones [4, 7]. Without an adequate material characterization the obtained toxicological results can hardly be interpreted correctly [8]. The toxicity of nanoparticles
may be elucidated only through the combination of physico-chemical characterization and toxicological testing [9].

In this paper we describe an approach of preparing and using stable nanoparticle suspensions for toxicological testing. Instructions for a correct measurement of parameters such as particle size, size distribution and zeta potential of suspended particles in water as well as in physiological media are given. The preparation of a stable initial nanoparticle suspension is explained with the example of a titanium dioxide (TiO$_2$) and a tungsten carbide (WC) powder, respectively. Subsequently the initial suspensions were added to different physiological media to study the agglomeration behaviour of the nanoparticles and the influence of proteins to the stability of the suspensions. The main result of our investigations is that the agglomeration of particles in physiological media can be perfectly inhibited in the presence of bovine serum albumin (BSA) or foetal bovine serum (FBS). This enhances the knowledge of nanoparticles actions in culture media and gives toxicologist a helpful tool to work with well-defined suspensions.

2. Materials and Methods

Investigations were done using a TiO$_2$ (Aeroxide P25, Evonik) and a WC powder. The specific surface area was determined with the N$_2$-BET method. Cristallinity of both powders was analysed by X-ray diffraction (XRD). Primary particle size, morphology and aggregation degree of the particles were determined by means of scanning electron microscopy (SEM).

Before preparing a stable initial suspension, a titration of a TiO$_2$ and a WC suspension was performed to get the zeta potential as a function of pH-value. Suspension background was 1 mM NaCl, and HCl or NaOH at different molarity was used for titration. Resulting from the titration, WC was dispersed in pure water whereas TiO$_2$ was dispersed in pure water which was adjusted to pH 4 with HCl to have stable initial suspensions. All zeta potential values were obtained from electrophoretic mobility measurements using Smoluchowski equation. The initial suspensions were prepared by sonication with an ultrasound horn. Sonication duration was as long as no further de-agglomeration progress was achieved. For that, particle size and size distribution was measured by means of dynamic light scattering.

Furthermore, these initial suspensions were added to physiological media to record the agglomeration behaviour by determining particle size and zeta potential. Physiological media were phosphate buffered saline (PBS) or PBS supplemented with bovine serum albumin (BSA) as well as Dulbecco's Modified Eagle Medium (DMEM) or DMEM supplemented with 5% foetal bovine serum (FBS).

3. Results

3.1. Powder Characterization and preparation of initial suspension

Diffraction analysis shows that TiO$_2$ consists of anatase and rutile whereas the WC powder consists of a pure tungsten carbide phase. Both powders differ strong in their specific surface area obtained from BET and also in their size and aggregation grade which can be estimated from electron micrographs (Figure 1). The specific surface area (BET) of TiO$_2$ is 55.9 m$^2$/g and 6.9 m$^2$/g for WC, respectively. The primary particles of TiO$_2$ are below 50 nm, nearly spherical and highly aggregated, while particles of WC are in the range of approximately 50 to 200 nm, having different shapes and are more isolated.
In order to get stable initial suspension, high absolute values of zeta potentials are necessary for electro-statically stabilized particles. In general, a suspension is stable when the zeta potential value is less than -30 mV or greater than +30 mV [10]. Figure 2 shows the result of the zeta potential titration of both suspensions. For TiO$_2$ high enough zeta potential values were obtained in the acid region at a pH lower than 4. Thus, TiO$_2$ was suspended in 10$^{-5}$ M HCl which yields a zeta potential of about 39 mV. Titration of the WC suspension indicates values between -30 mV and -40 mV over the whole measured pH-range which is acceptable for an electrostatic stabilization. A pH adjustment is not necessary. WC was just dispersed in water which results in a pH of 5 and a zeta potential of -35 mV.

The volume-weighted size distributions of both sonicated suspensions are very similar, although the primary particles of tungsten carbide are significantly greater than those of titanium dioxide (Figure 3). Interestingly, the distribution of WC particles is shifted to smaller sizes compared to TiO$_2$. The particle size for the WC suspension agrees with the values from electron microscopy. This means that the WC suspension contains mainly primary particles after sonication. For TiO$_2$, sonication is not able to destroy the strong bridges between the primary particles detected by SEM. For that reason, the TiO$_2$ suspension includes aggregates and no primary particles.
3.2. Behaviour under physiological conditions
In the next step we studied the agglomeration kinetics of the particles after adding the initial suspension (containing the particles) to the appropriate physiological media. The final mixture consists of 10% nanoparticle suspension (v/v) and 90% (v/v) media. The high percentage of media guarantees that pH and salt content of the media are not changed in a critical way. This was confirmed by in vitro viability tests using different cell types. The control solution which contained 90% media and 10% water or appropriate background solution without particles did not reduce the cell viability [11, 12]. Figure 4 shows the agglomeration behaviour of TiO$_2$ and WC at a mass concentration of 10 µg/ml. In the absence of BSA or FBS, both types of nanoparticles agglomerate quickly, whereas the agglomeration of TiO$_2$ is much faster compared to WC. Furthermore, the cluster formation seems to be independent of the used physiological media. The zeta potentials of the particles change radical in the physiological environment. We obtained -23 mV (TiO$_2$) and -22 mV (WC) in PBS and -8 mV (TiO$_2$) and -23 mV (WC) in DMEM. All values are outside the electrostatic stable area which gives an explanation of the agglomeration. In analogy to blood, cells are cultivated in physiological media containing proteins. So our agglomeration investigations were extended to study the nanoparticles activities in PBS including BSA (data not shown in the diagram) and in DMEM supplemented with FBS. For the tested protein concentrations tested we found that BSA (0.5 mg/ml) and FBS (5% FBS/DMEM) prevent the particles from agglomeration. BSA and the proteins from serum adsorb on the particles surface and stabilize the suspensions. BSA is the major component in FBS. We assume that the serum albumin is mainly adsorbed on the particle surface causing a stabilization of the particles. Proof can be given by the results from zeta potential measurements. We determined -11 mV in DMEM with FBS for both nanoparticle types and in PBS with BSA the zeta potentials are -15 mV for TiO$_2$ and -13 mV for WC. These values are very similar and close to that of free BSA with -11 mV (measured in PBS). An electrostatic stabilization of the formed nanoparticle-BSA-complexes is not possible at these very low absolute values of the zeta potential. Therefore, the stabilization effect can only be of steric or electro-steric nature.
Figure 4. Agglomeration behaviour of TiO$_2$ and WC nanoparticles at a concentration of 10 µg/ml in PBS, DMEM and DMEM supplemented with 5% FBS. Results in PBS containing 500µg/ml BSA have the same curve shape as DMEM with FBS and are not shown for clarity reason.

One question rising from the agglomeration curves remains unanswered. Why does TiO$_2$ agglomerate so much faster then WC although the mass concentration is identical and the size distributions (see Figure 3 again) are very similar? The surface of nanomaterials is often considered as appropriate dose quantity in toxicological testing and research into the health effects of nanoparticles [13, 2]. Therefore, we carried out agglomeration experiments with TiO$_2$ and WC with the same particle surface area per unit volume instead of the same mass (Figure 5). The curves received differ only slightly. That demonstrates that the surface area offered by the particles in the suspension has a strong effect on the agglomeration rate. The exposure of nanoparticles to cells in *in vitro* tests should be compared by a surface based dosage concentration.

Figure 5. Agglomeration behaviour of TiO$_2$ and WC nanoparticles at a particle surface concentration of 2.5 cm$^2$/ml in PBS.
4. Conclusions
In this paper the chemical-physical characterization of nanoparticles prior to toxicological studies is described. Resulting from our investigations we have derived the following guidelines for preparing a homogenous physiological nanoparticle suspension. First of all, a stable initial suspension is developed which will be later mixed with the corresponding cell culture media. Stability of the initial suspension is ensured by electrostatic forces and can be quantified by zeta potential measurements. If necessary, suspensions have to be adapted by pH adjustment or addition of a non-toxic dispersant to get a sufficiently high absolute value of the zeta potential. In protein-free cell culture medium nanoparticles agglomerate rapidly in order to reduce surface area and the surface energy. Therefore, exposure concentrations in toxicity testing should be related to the surface area of the nanoparticles. But, in many biological liquids like blood proteins are present in high concentrations. The protein BSA or BSA containing serum like FBS hinder agglomeration. They act as natural dispersant aids and stabilize the nanoparticles in their original size. Finally, the nanoparticles in physiological media are homogenous suspended, naturally stabilized through proteins and their size distribution is retained unchanged. This approach gives the toxicologists a helpful tool to assess health risks in in vitro investigations. The method has been successfully tested by our partners. The procedure we demonstrated for two different powders is recommended to become a standard preparation and operation procedure in nanoparticles risk assessment.

Acknowledgements
This work was partially financed by the German Federal Ministry of Education and Research within the INOS project (funding number 03X0013).

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