Evaluation of health risks of nanoparticles – a contribution to a sustainable development of nanotechnology

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Abstract. The increasing use of nanoparticles makes it necessary to check up possible toxicological risks of this new materials class. In this paper we describe on two nanopowders (tungsten carbide, titanium nitride) which methods and parameters of a chemical-physical characterization are needed in forefront of toxicological experiments. This includes investigation on the powder itself as well as on particles suspended in water and physiological media, respectively. The most important result is that nanoparticles agglomerate in serum-free medium within minutes, whereas in the present of serum an agglomeration is inhibited. Hence, we have physiological suspensions with well-distributed stabilized particles which allow performing toxicological testing under reproducible conditions. Furthermore we could prove that tungsten carbide particles were taken up into cells, but no acute toxicity was found determined by means of in vitro viability tests with different cells.

Introduction

Nanoparticles, which are much smaller than mammalian cells, are already being used in many products, such as cosmetics, paints or tires. An early forecast of the effects of synthetic nanoparticles on humans and the environment represents an important precondition for the sustainable development of nanotechnology. Therefore it is necessary to reveal possible risks which occur due to the small dimension of nanomaterials [1, 2]. First investigations demonstrate that nanoparticles cause enhanced toxicity compared to larger-sized particles with the same chemical composition [3]. Besides toxicological testing, investigations on the unusual properties on nanoparticles have to be done. This includes for instance parameters such as particle size and size distribution, specific surface area, morphology, cristallinity and chemical composition. Furthermore, the chemical-physical characterization should be as close as possible to conditions of the toxicological experiments [4].

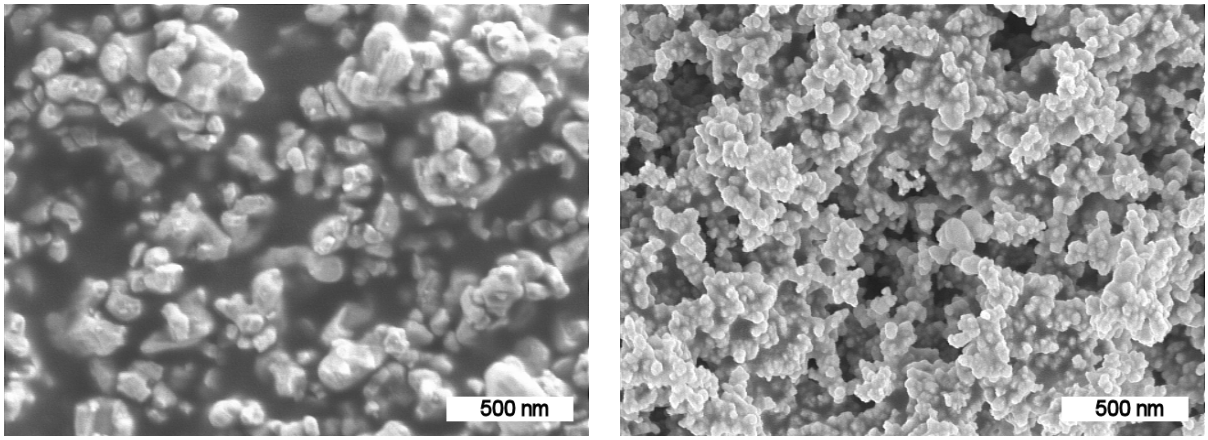
In this paper we report investigations on tungsten carbide (WC) and titanium nitride (TiN) nanoparticles. These special ceramic particles are used for the production of hard metals which are also known as cemented carbides. The main applications of hard metals are cutting tools who withstand higher temperatures than steel tools. The focus of our work is the combination of chemical-physical characterization and of toxicological testing which allows a better assessment of health effects of nanomaterials.

Materials and Methods

In the forefront of toxicological testing, an intensive physico-chemical characterization was performed. The specific surface area was determined using BET method. Additional scanning electron microscopy was done to describe primary particles size, aggregation degree and morphology of the particles. The powders were also dispersed in water to measure the zeta potential and aggregate size by means of dynamic light scattering. Beforehand, dispersions were prepared by sonication with an ultrasound horn. Furthermore, these suspensions were added to different physiological media to study the agglomeration behaviours under blood-like conditions.
To reduce animal testing, *in vitro* cell tests were carried out. On that account cell cultures were chosen which represent the most important routes of particle uptake into humans, namely lung (A549 cells), skin (HaCaT cells) and digestive tract (CaCo-2 cells). It is known that nanoparticles can also penetrate the brain, either through the blood circulation and by subsequentially passing the blood-brain-barrier or directly via the olfactory nerve [3, 5]. Therefore, *in vitro* experiments were also performed using different cell types of the rat brain (oligodendrocytes and primary cultures of astrocytes and neurons). After finishing toxicological experiments, cells were fixed for scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDX) investigations.

Results

Physico-chemical characterization
The characterization of the tungsten carbide and titanium nitride powder shows great differences in their physical parameters. The specific surface area of WC is 6.9 m^2/g whereas that of TiN is 42.0 m^2/g. These findings are reflected in the images taken by electron microscopy (Figure 1). The mean primary particles size of WC is the range of 100 to 200 nm, while that of TiN is approximately 20 to 50 nm. As one can see, both powders show highly aggregated primary particles which are typically for nano-scaled powders. After sonication of the suspensions high values of the zeta potential were measured (WC -35 mV, TiN -37 mV). These high zeta potentials guarantee strong electrostatic repulsive forces. Hence, we have stable and homogenous initial suspensions that can be added to physiological solution for agglomeration investigations. In this instance no pH adjustment or addition of dispersant agents is necessary to obtain the required stability. The mean particle size of the initial suspensions was 145 nm for WC and 160 nm for TiN. The particle size obtained for the WC suspension is in good agreement with value from the SEM micrograph. After sonication, the WC suspension contains mainly primary particles. In the case of TiN, the size measured by means of dynamic light scattering is between three- and eightfold higher compared to the primary particle size determined by SEM. The TiN aggregates cannot be destroyed through sonication due to the high surface energy holding the primary particles together.



a) b)
Fig. 1. SEM micrographs of a) WC and b) TiN

The next step in our investigations was to study the behaviour of these primary particles and aggregates in physiological media. All physiological solutions are marked through their high electrolyte content which yields to a compression of the electrochemical double layer of the particles. As a result, the electrostatic repulsion forces are reduced and the particles come in contact. Finally the van-der-Waals forces dominate the particles interaction and let the particles agglomerate. Figure 2 shows these agglomerations in Dulbecco's Modified Eagle Medium (DMEM), a standardized solution which is taken for *in vitro* experiments. For cell culture experiments serum is supplemented because it includes different growth factors cells require. In the absence of serum, particles agglomerate within a short time. Interestingly, the kinetics of TiN is much faster compared to WC. The zeta potentials are too low with -23 mV (WC) and -9 mV (TiN) to have strong enough electrostatic repulsion. When serum is present in DMEM, agglomeration of WC and also TiN is fully inhibited. Now the zeta potentials of both particles are identical with -11mV. The stabilization can only occur through steric effects of proteins adsorbing on the particle surface. This assumption can be confirmed by the identical zeta potentials we found. Furthermore, the adsorption of proteins on particles is a well known accepted fact [6, 7]. Two important aspects can be deduced. First, we have particles coated by serum proteins which could influence the biological response in toxicological testing [8]. The second aspect is that we still have well-distributed stabilized particles in now physiological media which avoids typical dosimetry problems like agglomeration or sedimentation.

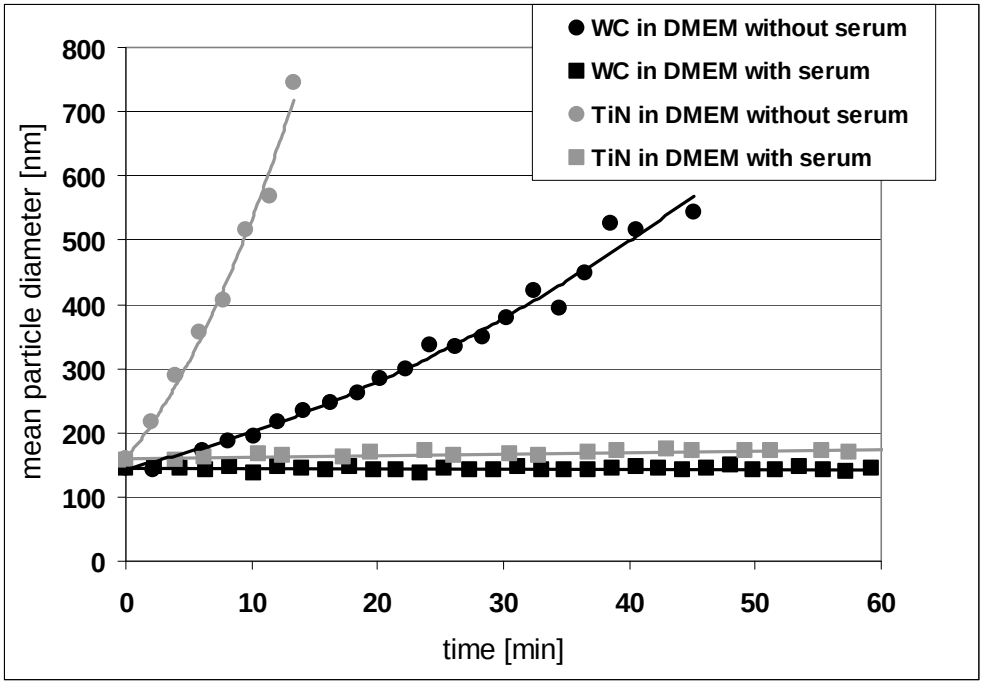


Fig. 2. Agglomeration behaviour of WC and TiN at a particle concentration of 10 µg/ml in DMEM and in DMEM supplemented with 10% serum, respectively.

Toxicological investigations and visualisation

The following text segment will only show results for WC, because toxicological as well as localization experiments with TiN are not finished, yet. By the use of SEM in combination with EDX we could prove that WC nanoparticles can penetrate cells. Entered particles were found as primary particles or as agglomerates. Up to now, WC particles were not detected within the nucleus (Fig 3).

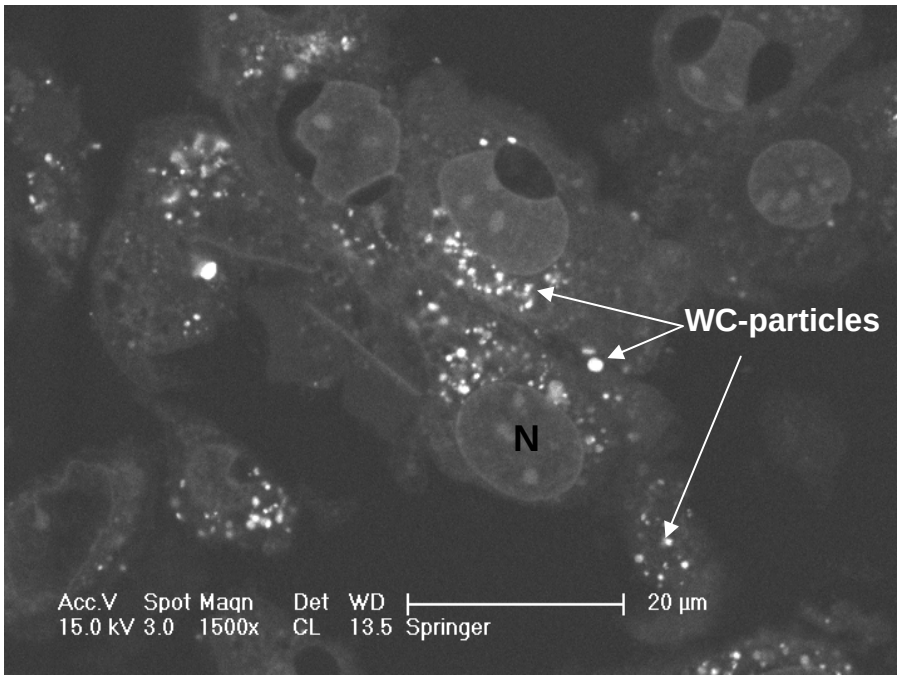


Fig 3. SEM figure of skin cells (HaCaT) after 2 days exposition in cell culture medium (RPMI with 5% serum) with WC particles at a concentration of 30 µg/ml. Note: N = nucleus.

The viabilities of lung (A549), skin (HaCaT) and digestive tract (CaCo-2) cells were determined at WC concentrations of 7.5, 15 and 30 µg/ml. Cells were exposed for different time periodes, namely 3 h and 3 d and changes in metabolic activity were assessed. Although the nanoparticles were able to enter the cells we found no decrease in the viability of the cells which means that no acute toxicity appears at the tested concentrations (Fig. 3).

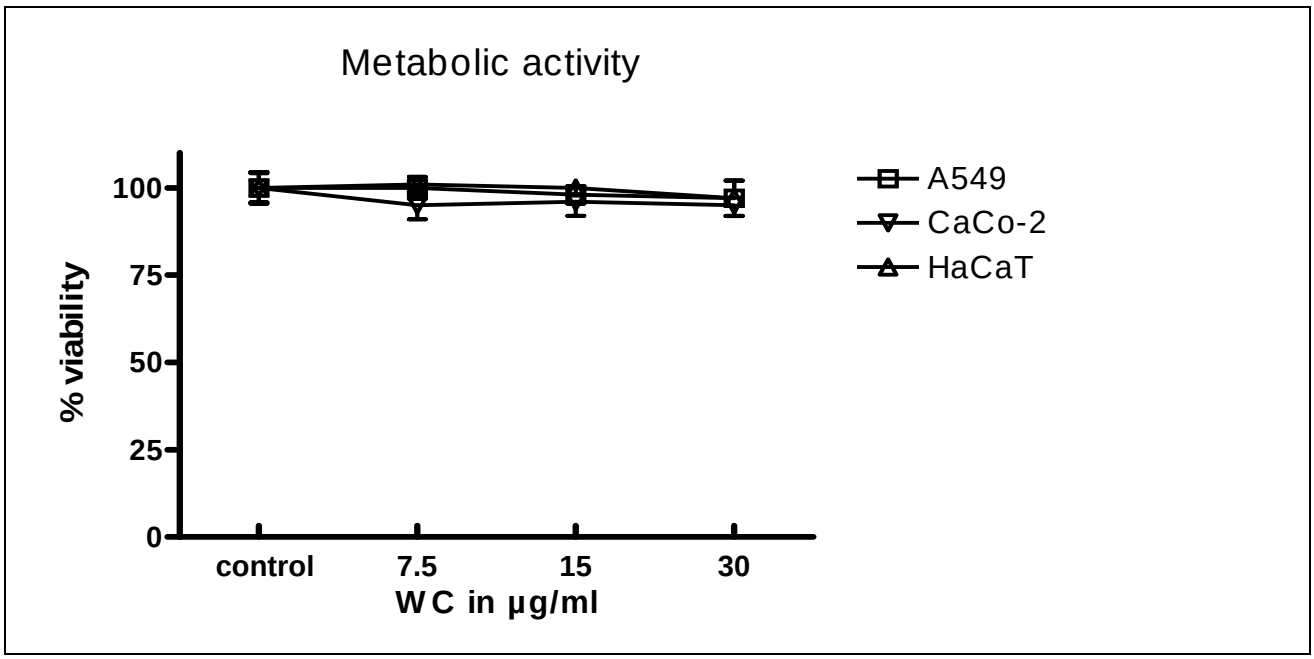


Fig. 4 Viability of WC exposed human lung (A549), colon (CaCo-2) and skin (HaCaT) cells. After an exposure period of 3 days metabolic activity was not influenced by WC nanoparticles.

The experiments with the brain cells confirmed these findings. Following two days of treatment with up to 30 µg/ml WC viability of oligodendrocytes (OLN 93), astrocytes and neurons was not reduced (Fig. 5).

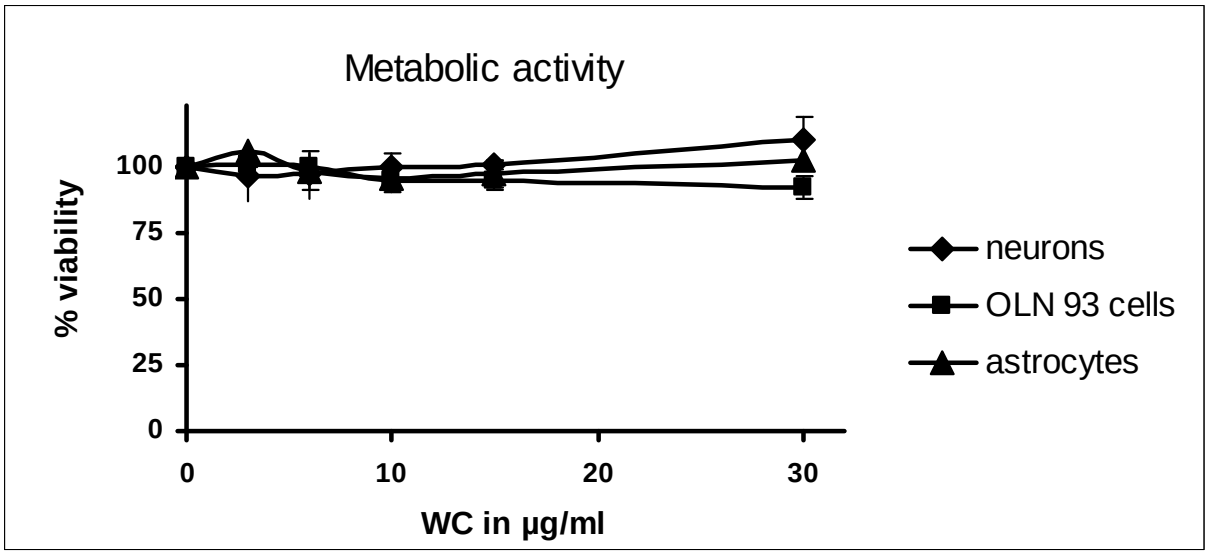


Fig. 5 Viability of WC exposed rat brain cells. After two days of exposure metabolic activity was not affected by nanoscaled WC particles.

Conclusions

Our investigations demonstrate that a physico-chemical characterization is very important prior to toxicological testing. Powders with nano-scaled particles form aggregates that cannot always be destroyed when preparing suspensions. The aggregate size and size distribution of particles can only be measured meaningful from a stable initial suspension that implies to have a high enough zeta potential. Adding this initial suspension to physiological media allows performing agglomeration studies and toxicological experiments under well-defined states. In serum-free medium particles agglomerate within minutes, but in the presence of serum, particles can be stabilized through steric effects of serum proteins. This guarantees a homogenous suspension under conditions present in *in vitro* experiments. Following this procedure, toxicological viability tests with WC nanoparticles were preformed. We found that WC particles are able to enter cells but the do not provoke an acute toxic response. To date, the mechanism of uptake and the role of serum proteins in the uptake process are unknown and subject of future studies. Further work is necessary to reveal possible long-time effects.

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