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Morphological impact of zinc oxide particles on the antibacterial activity and human epithelia toxicity

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ABSTRACT

ZnO nanoparticles are utilized in an ever growing number of products and can, therefore, be readily encountered in our everyday life. Human beings' outermost tissues consist of different epithelia and are, therefore, the most exposed to materials from the environment. In this paper, *Caco-2* and *Calu-3* cell lines were used, having been previously broadly applied for *in vitro* modelling of intestinal and respiratory epithelia, respectively. The toxicity of synthesized micro-, submicro- and nanoparticulate ZnO on these epithelia was measured and compared to the efficacy of the same ZnO particles as antibacterial agents. An approximately four-fold excess in antibacterial activity of ZnO nanoparticles over ZnO granulate was observed. The results of this paper reveal a sharp distinction between toxic nanoparticulate ZnO and safe ZnO particles of larger sizes in intestinal and airway *in vitro* epithelial models. In contrast, ZnO of larger particle sizes had only modestly lower antibacterial activity, which can be compensated for with higher dosing. These results show that nanoparticulate ZnO requires critical *in vivo* assessment before application.

1. Introduction

A number of fabricated metal oxides, when in nanoparticulate form, exhibit certain unique properties that can be the basis for the development of new products in the fields of technology and medicine [1]. Zinc oxide (ZnO) is one such favoured material since it can be prepared in numerous forms and is available in large quantities. Different forms of ZnO have been utilized as additives for a wide range of products, including composite materials such as glass, ceramics, plastics, lubricants, paints, cement, adhesives, sealants, batteries, fire retardants and medical products [2–5]. The food supplements industry offers ZnO based products, because Zn is an essential micronutrient, serving important roles in human and animal growth, development and well-being [4,6]. Due to its excellent UV absorption and reflective abilities, nano-sized ZnO is also found in personal care products in cosmetics and sunscreens [7,8]. Furthermore, ZnO particles have the potential to become a next generation biocide or disinfecting agent, due to their stability and lower probability of developing resistances in comparison to their organic-based counterparts [9–11]. The antimicrobial properties of ZnO have been suggested to serve as a means of packaged food preservation [12–14]. Such intentive intake makes ZnO toxicity an issue of the highest importance.

Nanoparticles share some unique physiochemical properties, such as very small size, a large active surface area, and associated reactivity. As a consequence, the properties of nanoparticles may differ substantially from larger particles of chemically identical materials. The novel properties of nanoparticles could lead to adverse biological effects resulting in acute toxicity [15–18]. The current trends of increased production and utilization of nanoparticles lead to larger exposure of humans and the environment to their actions [19]. It can, therefore, be concluded that studies on the safety and (eco)toxicity of nanoparticles are of the highest importance and should precede the commercialization of these materials [20]. Several reports have already addressed the harmful impact of nanomaterials on eukaryotic cells, although as far as ZnO is considered, lower concentrations of ZnO are thought to be non-toxic [21–24]. Zinc oxide is generally considered to be a safe and bio-compatible material for use in humans and animals [25].

Several studies have suggested that size and morphologically different zinc oxide particles exhibit different concentration-dependent antibacterial activity [26–29] and differ in toxicity for human cells [21, 30–32]. The main routes of human exposure to nanoparticles are *via* inhalation, dermal contact and gastrointestinal tract absorption [15]. Several studies found no risk in skin exposure to ZnO nanoparticles [33–35]. Therefore, we have chosen *in vitro* models of respiratory and intestinal epithelium for toxicity testing. These models can mimic native mucosa in many aspects [36,37] and are likely to aid in the development and adequate estimate of the latter's sensitivity to these agents.

We prepared a series of ZnO particles of well-reproductive size and shape. In tests, these nano- to micro-particulate ZnO particles were compared to randomly granulated ZnO of analytical grade. Such a ZnO is generally recognized to be a safe material [38] and was therefore

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used as a reference in epithelia toxicity testing. We characterize our novel ZnO nanoparticles as well as commercially available ZnO materials with field emission scanning electron microscopy (FE SEM), X-ray diffraction (XRD), FT infrared (FTIR) spectrometer, dynamic light scattering (DLS) and with the standard Brunauer–Emmett–Teller (BET) technique. Their antibacterial activities were evaluated through their effects on *Escherichia coli* (*E. coli*) culture growth. The enhanced antibacterial effects of ZnO nanoparticles when compared to larger ZnO particles have been described previously. ZnO particles are available in different shapes, and their effects on bacteria may depend on their specific surface area (SSA) rather than merely on the particle diameter. Studies published thus far are often difficult to compare due to differences in the preparation of the ZnO particles and differences in bacteria culture toxicity-testing procedures.

In this work, we compare the antibacterial activity on *E. coli* and epithelial toxicity on *Caco-2* and *Calu-3* cell lines of the same particles, varying in morphologies and sizes, in order to determine safe and efficient concentrations for potential antibacterial applications.

2. Materials and methods

2.1. Materials

ZnO particles were prepared from chemicals of analytical grade as follows: zinc nitrate hexahydrate $Zn(NO_3)_2 \cdot 6H_2O$, 98% and urea 99% were from Sigma-Aldrich, sodium hydroxide NaOH, >99% p.a, and ethylene glycol EG, 99.5% p.a. were from Merck. ZnO-10 and the obtained ZnO-granulate were from IBU-tec Advanced Materials AG Germany (215–222-5) and Sigma-Aldrich Germany (205532-NOG), respectively.

2.1.1. Synthesis of ZnO nanoparticles

Particulate ZnOs of various sizes were used, of which three different forms (ZnO-1, ZnO-2 and ZnO-3) were synthesized in our laboratory, and two samples were from commercial source.

2.1.1.1. ZnO-1. ZnO nanoparticles were prepared in two steps. In the first step, fresh stock solutions were prepared from $Zn(NO_3)_2 \cdot 6H_2O$ and NaOH in Milli-Q (Millipore) water to avoid hydrolysis upon storage. The total concentration of zinc ions and sodium hydroxide in the 800 mL reaction mixture was kept constant at 0.1 mol/L. The experiment was carried out in a 1 L laboratory bottle, at room temperature for 4 h. The obtained precipitate was filtered, washed with distilled water and ethanol to remove soluble impurities, and dried in an oven at 60 °C for 12 h. In the second step, the as-prepared precipitate (40 mg) was heat-treated in EtOH (25 mL) in an autoclave in an oven at 150 °C for 2 h. The obtained white precipitate was isolated *via* centrifugation (Hettich Rotina 38, Tuttingen) at 8000 rpm for 6 min, washed 3 times, followed by repeated centrifugation with ethanol and dried in an oven at 60 °C for 12 h.

2.1.1.2. ZnO-2. The first step was the same as in ZnO-1. In the second step, the conversion of precipitate in ZnO was performed in an autoclave (100 mg, at 100 °C for 2 h) in a mixture of EG/water (25 mL) in a ratio of 1:1. The sample was washed as described for the ZnO-1.

2.1.1.3. ZnO-3. ZnO nanoparticles were prepared in two steps. In the first step, hydrozincite was prepared from $Zn(NO_3)_2 \cdot 6H_2O$ and urea in Milli-Q [39]. In the experiments, the concentrations of Zn^{2+} ions and urea were kept constant at 0.1 mol/L and 0.5 mol/L, respectively. The experiments were carried out in 1 L laboratory bottles at 90 °C for 6 h. The precipitate was filtered off on 200 nm filter paper (Millipore), washed two times with distilled water to remove soluble impurities and dried in an oven at 60 °C for 12 h. To obtain zinc oxide, the products were heat-treated in an oven at 600 °C for 1 h under a static atmosphere of air.

2.2. Characterization of ZnO nanoparticles

All samples were characterized with respect to their morphological and chemical properties.

2.2.1. FE-SEM

The samples were characterized by FE-SEM (Zeiss Supra 35 VP) in order to define their size and morphology. For FE-SEM measurement, suspensions of ZnO-1, ZnO-2, ZnO-10, and ZnO-granulate were prepared in MQ and sonicated with an ultrasonicator (Transsonic Digitals Elma Singen, Germany) for 10 min. ZnO-3 was only dispersed by pipetting to avoid damaging of particle porous structure. ZnO suspensions were transferred to 0.2 μm filter (Millipore), having previously been covered with Au, and were air-dried at room temperature. After being dried, samples were analysed at 1 kV and aperture 30 nm.

2.2.2. FTIR

IR-spectra were performed on an FTIR spectrometer (Bruker IFS 66/S) in the spectral range between 4000 cm^{-1} and 400 cm^{-1} at a spectral resolution of 4 cm^{-1} in the transmittance mode. The KBr pellet technique was used for the sample preparation.

2.2.3. DLS

Size distribution measurements were performed with a Zetasizer Nano ZS laser scattering system with a 633 nm laser source (Malvern Instruments, UK). The intensity of scattered light was detected at 173° to minimize the effect of multiple scattering. The investigated water suspensions with nanoparticles concentration of 1.25 mg/mL were measured in 10 mm diameter polystyrene cuvettes (Brand, Germany). All analyses were run at 25 °C in 10–20 repetitions (the number was defined according to the overall quality of the sample, which was automatically evaluated each time a fresh cuvette had been inserted into the system). Powder ZnO-1, ZnO-2, ZnO-10 and ZnO-granulate particles were dispersed in MQ water and were sonicated with an ultrasonicator (Elma, Germany) for 15 min at 40 kHz. Concentrations of the ZnO samples were 1.25 mg/mL.

2.2.4. BET

The specific surface area (SSA) of the samples was determined using the BET technique with a Micrometrics Gemini II 2370 Surface Area Analyser. The samples were dried and degassed at 90 °C. The analyser performed a fully automated analysis in ultra-high purity N_2 (99.9995), collected data and performed calculations to obtain the BET surface area.

2.3. Antibacterial activity of ZnO particles

Gram-negative bacteria *E. coli* strain DH5α was used to test antibacterial properties of ZnO. The bacteria were grown overnight in Luria–Bertani (LB) medium at 37 °C on a shaking platform at 160 rpm. The 500-fold dilution of the overnight culture was prepared and incubated until the culture reached exponential phase. From this culture, working suspensions of bacteria were prepared with an optical density (OD) of 0.01 measured at 600 nm (OD_{600}). The OD_{600} value of 0.5 corresponded to 4×10^8 CFU/mL (CFU; colony forming unit).

All ZnO suspensions were prepared prior to the experiments in sterile MQ water to a stock of 40 mg/mL, and all samples except ZnO-3 were sonicated with an ultrasonicator (Elma, Germany) for 15 min at 40 kHz. ZnO-3 particles were previously found to be sensitive to ultrasound, but of low aggregation tendency. Stock suspensions were diluted to the required final densities in LB medium.

Microtiter plates (96-well) containing LB medium supplemented with ZnO particles in a broad concentration range were used to test antibacterial activity. The final volume of 200 μL in a well contained 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0312 or 0.0156 mg/mL of ZnO particles and an inoculum of bacteria. Each condition on the plate was tested in triplicate along with a corresponding control; the identical treatment without bacteria as a