Implications of the stability behavior of zinc oxide nanoparticles for toxicological studies

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Abstract The increasing use of zinc oxide (ZnO) nanoparticles in sunscreens and other cosmetic products demands a risk assessment that has to be done in toxicological studies. Such investigations require profound knowledge of the behavior of ZnO in cell culture media. The current study was performed to get well-dispersed suspensions of a hydrophilic (ZnO-hydro) and a lipophilic coated (ZnO-lipo) ZnO nanomaterial for use in in vitro tests. Therefore, systematic tests were carried out with common dispersants (phosphate, lecithin, proteins) to elucidate chemical and physical changes of ZnO nanoparticles in water and physiological solutions (PBS, DMEM). Non-physiological stock suspensions were prepared using ultrasonication. Time-dependent changes of pH, conductivity, zeta potential, particle size and dissolution were recorded. Secondly, the stock suspensions were added to physiological media with or without albumin (BSA) or serum (FBS), to examine characteristics such as agglomeration and dissolution. Stable stock suspensions were obtained using phosphate as natural and physiological electrolytic stabilizing agent. Lecithin proved to be an effective wetting agent for ZnO-lipo. Although the particle size remained constant, the suspension changed over time. The pH increased as a result of ZnO dissolution and formation of zinc phosphate complexes. The behavior of ZnO in physiological media was found to depend strongly on the additives used. Applying only phosphate as additive, ZnO-hydro agglomerated within minutes. In the presence of lecithin or BSA/serum, agglomeration was inhibited. ZnO dissolution was higher under physiological conditions than in the stock suspension. Serum especially promoted this process. Using body-related dispersants (phosphate, lecithin) non-agglomerating stock suspensions of hydrophilic and lipophilic ZnO were prepared as a prerequisite to perform meaningful toxicological investigation. Both nanomaterials showed a non-negligible dissolution behavior that strongly depended on the surrounding conditions. Agglomeration of ZnO particles in physiological media is a complex function of particle coating, used dispersants and serum proteins if supplemented. The present study gives a clear guideline how to prepare and handle suspensions with ZnO for in vitro testing and allows the correlation between the chemical-physical particles behavior with findings from toxicological tests.

Keywords Zinc oxide · Nanoparticles · Toxicity · Dispersion · Dissolution · Particle agglomeration

Background

Zinc oxide (ZnO) and titanium dioxide (TiO2) have intrinsic UV-absorbing properties and are therefore used as ingredients in sunscreens. Moreover, ZnO absorbs UV radiation more effectively than TiO2 does, particularly in the UVA range. With a sufficiently small particle size, ZnO can absorb and scatter UV radiation, making sunscreens transparent. Potential for human exposure to ZnO exists in the workplace and in the private sphere. ZnO nanoparticles can also be released in the environment by the production process or usage by consumers. Therefore, a thorough risk
assessment at each stage from production to use of the product and disposal of ZnO is indispensable [1].

The changed chemical and physical parameters of nanoparticles can lead to increased biological activity and thus trigger unexpected reactions with biological systems. One related question is whether nanoparticles are more toxic than their larger counterparts [2, 3]. Hsiao and Huang [4], for example, were able to demonstrate that the cytotoxicity of ZnO is size-dependent. In contrast, Lin et al. [5] examined the differences between nanoscale and submicron zinc oxides (70 and 420 nm) in human lung epithelial cells (A549) and found nearly identical EC50 values of 13.6 and 14.2 μg/ml. Despite different primary particle sizes, the same hydrodynamic diameter for both of the ZnO types were measured in cell culture media. Whether this is a cause of the same toxicity or not, is not explained in more detail. A second aspect of Lin’s study was to determine the influence of free zinc ions. At Zn2+ concentrations of 2–8 μg/ml, no significant cytotoxicity compared with a blank test was found in their study. According to Lin, the free zinc ions are not responsible for the toxicity of ZnO. Hence, the dissolution of ZnO is a second aspect discussed in relation with the toxicity of these particles. Contrary to Lin’s study, Xia et al. [6] demonstrated that dissolution can play an important role in cytotoxicity. The ZnO dissociation disrupts cellular zinc hemostasis, leading to lysosomal and mitochondrial disruption, and finally cell death. In Xia’s experiments, a maximum dissolved zinc concentration of 225 μM (corresponding to 14.7 μg/ml Zn2+) in DMEM supplemented with FBS was noticed. Pujalte and coworkers [7] hypothesized that ROS production, oxidative stress, and cytotoxicity induced by ZnO NPs are partly linked to the release of Zn2+ ions. Their experiments were performed in RPMI 1640 media without serum. It should be noted here that particles show different behavior in media with serum. Brunner et al. [8] also stated that the toxic effect of ZnO could be attributed to the release of Zn2+ ions. They found a sharp concentration dependence of the cytotoxic effects based on a critical Zn2+ concentration in the cell culture medium. All cells died above a ZnO concentration of 15 ppm. Moreover, the presence or absence of serum in in vitro experiments with ZnO nanoparticles has impact on the biological identity of nanomaterials such as numerous studies show [15, 18, 19]. The link between protein corona and ZnO, particularly in view of its solubility should be further investigated. All in all, comprehensive characterization of the materials used is necessary for interpretation of toxicological results, as mentioned in numerous reviews [3, 20–22]. Especially the behavior of nanoparticles under physiological conditions must be considered to fully understand the interactions with components of the culture media [20]. To obtain meaningful results, a clearly structured procedure in the dispersion is required.

In the present study, a hydrophilic (ZnO-hydro) and a lipophilic coated (ZnO-lipo) ZnO powder were studied. A main aspect of this work was the preparation of stable, i.e. non-agglomerating stock suspensions for use in in vitro testing. A two-stage procedure (1, suspension preparation in non-physiological media; and 2, transferring and testing in physiological media), as used by many researchers, e.g., Bihari et al. [23] and Taurozzi et al. [24], was used. The proven approach from previous studies [25–28] was adapted to the requirements of the ZnO powders. The approach includes the destruction of existing agglomerates down to the smallest dispersible units of the given powder. Different dispersing agents were tested to yield an
optimized stock suspension exhibiting stability over time. The behavior of ZnO particles under physiological conditions was studied through addition of the stock suspensions to phosphate buffer (PBS and PBS with BSA) and cell culture media (DMEM and DMEM/FBS). Extensive investigations were performed on both the stock suspensions and the particles in DMEM and in DMEM/FBS. For this purpose, pH, conductivity, particle size, zeta potential, and dissolved zinc ion concentrations were measured over several days to record changes in the samples. Finally, the goal was to record the changes in the kinetics of ZnO as agglomeration and dissolution under different test conditions in order to improve toxicological studies on ZnO. For example, it is necessary to know about the actual zinc ion and zinc oxide concentration in test media, as the basis for dose–response curves. Last but not least, the Brunauer–Emmett–Teller (BET) surface area was determined and particle morphology, aggregation degree, and other properties were characterized using scanning electron microscopy.

Methods

ZnO powders and their characterization

The investigations were conducted with ZnO Z-COTE® (abbreviated as “ZnO-hydro”) and ZnO Z-COTE® HPI (abbreviated as “ZnO-lipo”). Both powders were produced by BASF SE, Ludwigshafen, Germany, and were provided by the National Physical Lab, Teddington, UK. The surface of ZnO-lipo was coated with triethoxycaprylylsilane, whereas ZnO-hydro was uncoated.

For both powders, specific surface area, crystallinity, size, and morphology were determined. Nitrogen adsorption was determined using the BET method with an ASAP 2010 accelerated surface area and porosimetry analyzer (Micromeritics GmbH, Mönchengladbach, Germany). Phase compositions were determined by X-ray diffraction (XRD 7, Seifert-FPM, Germany). Powder imaging was performed using a Zeiss ULTRA 55 (Carl Zeiss SMT, Oberkochen, Germany). SEM images of the raw, untreated powders were first obtained. Then samples were taken from the suspension for investigation of the deagglomeration progress after sonication. For this purpose, a small amount of suspension was filtered through a 50-nm filter, dried, and analyzed in the SEM.

Zeta potential

The zeta potential was obtained through measurement of the electrophoretic mobility of the particles using a Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). Capillary cells DTS1060C from Malvern Instruments were used for the electrophoresis measurements. All investigations were performed at 25 °C. The electrophoretic mobility was converted into the Smoluchowski zeta potential using the integrated Malvern software DTS. For each test, at least ten records were obtained and then averaged.

Dynamic light scattering (DLS)

Measurements of particle size and size distribution were performed using a Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). The device operates on the principle of DLS, according to which time-dependent fluctuations in the intensity of light scattered from suspended particles are recorded. The Malvern software permits different size calculation modes. The cumulant method described in ISO 22412 yields the mean particle diameter, x_{DLS}, and the polydispersity index PI. This index describes the polydispersity of the investigated system (0, monodisperse; 1, polydisperse). In addition to the cumulant method, particle size distributions (PSD) were calculated using the software integrated general purpose mode. A refractive index of 2.0 was taken for calculation of the PSD [29]. Due to the fast agglomeration process under physiological conditions found in some cases, the change in particle size in the physiological media was only evaluated by the cumulant method. All size measurements were performed at 25 °C.

Suspension preparation

All suspensions were prepared in pure water (resistivity ≥18 MΩ cm; Wilhelm Werner GmbH, Leverkusen, Germany). For uncoated ZnO-hydro, the zeta potential was recorded as a function of pH. For this purpose, a suspension of 100 μg/ml with a 1 mM KNO₃ background was prepared by sonication for three minutes with 80 % amplitude (Disintegrator UDS 751 equipped with a 14 mm titanium probe tip, Topas GmbH, Dresden, Germany). Using this tip and an amplitude of 80 % results in a theoretical power of around 130 W. The pH value was adjusted with KOH or HNO₃ of differing molarity (both Merck, Darmstadt, Germany). Due to the low absolute values of the zeta potential in the measured pH range, no stable suspension could be achieved by pH adjustment. Suspensions were prepared using phosphate ions as a dispersing agent. The phosphate salts used are based on physiological PBS, which consists of two salts with total 12 mM PO₄. Pure water with 10 mM Na₂HPO₄·12H₂O (Fluka Analytical, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 2 mM KH₂PO₄ (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were taken without the addition of NaCl and KCl, both also...
found in physiological PBS. The pH was then adjusted to 7.4 using 1 M HCl (Merck, Darmstadt, Germany). Because the salt concentration had to remain low for stability reasons, the final stock suspensions contained only a tenth of this solution (=1.2 mM phosphate). In other words, 0.1× PBS without KCl and NaCl was taken. Furthermore, some suspensions included the addition of 100 µg/ml soy lecithin (BDH Prolabo, VWR International GmbH, Darmstadt, Germany). Lecithin acted as a wetting agent for ZnO-lipo. For comparison with ZnO-lipo, ZnO-hydro suspensions with phosphate and lecithin were also prepared and examined. All nanoparticle suspensions (500 µg/ml ZnO) were prepared by sonication using the ultrasonic disintegrator UDS 751 (14 mm probe tip, 80% amplitude). For all suspensions a dispersion time of 4 min was taken. Preinvestigations monitored with DLS showed that longer sonication times did not result in a further deagglomeration progress. To avoid an excessive heating of the particles during the sonication process the suspensions were placed in an ice bath. The long-time behavior of stock suspensions was examined over a period of 14 days, with size parameters (x_{DLS} and PI), zeta potential, pH value, conductivity, and dissolution behavior being recorded. For analysis of the influence of phosphate and lecithin on ZnO, additional ZnO-hydro suspensions with just water were prepared. Due to the instability in pure water, particle size could not be examined in this case.

Behavior in physiological media

Investigations on the nanoparticle behavior under physiological conditions were performed according to our established step-by-step procedure [25–28]. For the measurements, 10% (v/v) nanoparticle stock suspension was added to 90% (v/v) physiological media. For investigations at different concentrations, stock suspensions were diluted with background solution (1.2 mM phosphate or 1.2 mM phosphate with 100 µg/ml lecithin). The physiological media used were PBS with pyruvate and l-glutamine (Invitrogen GmbH, Karlsruhe, Germany), DMEM high glucose with pyruvate and l-glutamine (Invitrogen GmbH, Karlsruhe, Germany), and DMEM supplemented with 2% (v/v) or 10% (v/v) FBS (Biochrom, Berlin, Germany). The particle size and the zeta potential were measured with the Zetasizer Nano ZS, as described above.

Dissolution experiments

For analysis of the dissolution behavior of ZnO-hydro, suspensions containing just water, 1.2 mM phosphate, or 1.2 mM phosphate with 100 µg/ml lecithin were prepared. For coated ZnO-lipo, only suspensions with 1.2 mM phosphate and 100 µg/ml lecithin were prepared. Dissolution of ZnO-hydro and ZnO-lipo was quantified through measurement of the concentration of free zinc ions after a defined suspension aging time. For that, samples were stored at room temperature. First, the suspension was centrifuged twice at 15,000 g for 20 min in a Sigma 4K15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode, Germany). Next, the clear supernatant was drawn off, and the zinc concentration was determined using an Ultima ICP-OES (Horiba Jobin Yvon GmbH, Unterhaching, Germany). Dissolution experiments were performed with ZnO stock suspensions as well as with ZnO in DMEM and in DMEM with 10% FBS.

Results

Powder analysis

ZnO-hydro and ZnO-lipo were very similar in terms of specific surface and appearance, as visualized by electron microscopy (Fig. 1). The specific surface area of ZnO-lipo (14.5 m²/g) was slightly larger than that of ZnO-hydro (11.7 m²/g). Both powders consisted of agglomerates or aggregates several micrometers in diameter. SEM images also showed fine-structured particles within the agglomerates. To describe the aggregation degree of the primary particles, a deagglomeration step was required (see below). XRD analysis of both ZnO powders gave the same results, namely, the presence of a ZnO phase only.

Preparation of NP stock suspension

One way to prepare a stable suspension is to adjust the pH to regions with high electrostatic repulsive forces between the particles. Therefore, the zeta potential of ZnO-hydro was determined as a function of pH (Fig. 2). Dispersion of the ZnO-hydro suspension resulted in a self-adjusting pH of 8 and a zeta potential of 28 mV. This value was slightly below the threshold of 30 mV, which is considered as the minimum zeta potential for electrostatically stabilized suspensions [30]. Lowering the pH increased the zeta potential, but the pH was instable and drifted back to the initial pH value of 8. Additionally, ZnO dissolved very well under acidic conditions, inhibiting zeta potential measurement at pH values of <7. Titration to alkaline conditions was possible, but after the isoelectric point was passed at a pH of ~10 the zeta potential did not fall below the critical ~30 mV. Higher electrostatic repulsion forces between the particles were obtained through use of phosphate salts as dispersing agents. One advantage of phosphates is that they are an integral part of physiological media. As a result, a zeta potential of ~50 mV for ZnO-
hydro in just 1.2 mM phosphate background was obtained. For ZnO-lipo, the nontoxic lecithin was used as a wetting agent. When only soy lecithin was used, the zeta potential was $-23 \text{ mV}$, which is too low for electrostatic stabilization. Suspensions prepared with lecithin and phosphate also had a zeta potential of $-50 \text{ mV}$. Although lecithin was not necessary for uncoated ZnO-hydro, suspensions with lecithin and phosphate were prepared for comparison purposes. They also yielded a zeta potential of around $-50 \text{ mV}$. Four minutes of probe sonication were identified as the time beyond which no further reduction of the particle size for both materials occurs. Once optimum dispersing conditions were found, stable suspensions could be prepared by sonication. Table 1 gives the mean particle size $\xi_{\text{DLS}}$ and polydispersity index PI. There were no marked differences in size between ZnO-hydro and ZnO-lipo. The hydrodynamic diameter and the PI of ZnO-hydro in the suspension with soy lecithin and phosphate were slightly higher than those of the ZnO-hydro suspension without lecithin. Thus, the increased size parameters indicated adsorption of lecithin onto the surfaces of the nanoparticles.

Long-time behavior of stock suspensions

Before ZnO from the stable stock suspension was brought into contact with the physiological media, the long-time behavior of the stock suspensions was studied. For uncoated ZnO-hydro, three types of suspensions were prepared: (1) in water, (2) in 1.2 mM phosphate, and (3) in 1.2 mM phosphate with 100 $\mu$g/ml lecithin. Figure 3 shows the changes in pH, conductivity, zeta potential, and particle size occurring over a period of 14 days (Fig. 3a). All suspensions containing phosphate showed an increase in pH from 7.4 into a more alkaline region. The strongest increase of up to pH 9 was exhibited by ZnO-hydro suspensions with phosphate and phosphate/lecithin. In contrast, the pH of ZnO-hydro in pure water shifted from 7.9 to 7.4 over the 14-day period. As expected, the conductivity was higher in suspensions with phosphate and phosphate/lecithin than for ZnO-hydro in pure water (Fig. 3b). In all cases, the conductivity was found to increase slightly at the beginning of the experiment before reaching a constant value. The zeta potential of ZnO-hydro in water had values between 20 and 28 mV, too low for electrostatic stabilization of the suspensions (Fig. 3c). Hence, useful size
Archive of SID measurements could not be performed by means of DLS. All suspensions containing 1.2 mM phosphate had negative zeta potential values due to the adsorbing phosphate ions. Values were between $-50$ and $-40$ mV, sufficient for electrostatic suspension stabilization. The electrostatic stabilization of the particles was reflected in a constant particle size for ZnO-hydro and ZnO-lipo over the whole time (Fig. 3d). The $\xi_{\text{DLS}}$ was $\sim 250$ nm for all types of suspension. Slight variations in size were found only for ZnO-hydro with phosphate/lecithin.

The results of the dissolution experiments exhibited a significant difference in the dissolved ZnO quantity between ZnO particles in water and those in phosphate-containing suspensions (Fig. 4). For ZnO-hydro in water, the amount of dissolved zinc was found to be 3.5–4.3 µg/ml. With the concentration of the stock suspension (500 µg/ml ZnO) being taken into account, the amount of ZnO dissolved in pure water was determined to be around 1 % of the total particle mass. Interestingly, the amount of ZnO dissolved in the suspension with phosphate and lecithin was $<0.3$ %. For ZnO-hydro in the phosphate-containing suspension without lecithin, zinc concentrations with a dissolved amount below 0.01 % were measured. Independently of the suspension composition, no
significant increase in the amount of zinc oxide dissolved was observed over time.

SEM images of ZnO-hydro and ZnO-lipo particles in stock suspensions showed primary particles with sizes of around 200 nm and below (Fig. 5). In many cases, they were connected to larger aggregates. Arrangements of the single particles were more difficult to recognize in the coated ZnO-hydro than in the uncoated ZnO-hydro. Interestingly, cubic particles with an edge length of around 1 µm were found for the ZnO-hydro sample in 1.2 mM phosphate. EDX analysis revealed that these cubic particles had a different composition from that of the ZnO particles (Fig. 5a). In the case of the ZnO particles, the zinc and oxygen peaks had the highest intensities in the EDX spectrum. Determination of the quantity of every elemental peak yielded 75 % (w/w) zinc and 24 % (w/w) oxygen, in good agreement with the theoretical values for ZnO of 80 % zinc (w/w) and 20 % (w/w) oxygen. In contrast, the EDX spectrum of the cubic particles showed characteristic peaks for C, O, Zn, and P. The high intensity of the carbon peak was surely a contribution of the filter and not of the cubic particles. With the carbon peak omitted, the following weight concentrations were found: 43 % (w/w) oxygen, 47 % (w/w) zinc, and 10 % (w/w) phosphor. ZnO-hydro and ZnO-lipo in suspensions with phosphate and lecithin exhibited ZnO particles only.

Behavior under physiological conditions

After the stock suspension was added to the physiological media, the particle sizes of ZnO-hydro and ZnO-lipo were recorded by means of DLS. Figure 6 shows the changes in particle size over time on the basis of the x_DLS at a ZnO concentration of 50 µg/ml. The measurements revealed that ZnO-hydro particles from the stock suspension with 1.2 mM phosphate agglomerated after being added to PBS. In contrast, ZnO-hydro and ZnO-lipo particles from samples in 1.2 mM phosphate with lecithin remained stable in PBS. The same experiments were performed in DMEM to reveal possible differences due to the different compositions of DMEM and PBS. Again, particles from suspensions with lecithin retained their initial size, whereas ZnO-hydro particles without lecithin agglomerated (data not shown). The only difference was that ZnO-hydro particles without lecithin agglomerated faster in PBS than in DMEM. For precise quantification of the differences in behavior of ZnO-hydro without lecithin in PBS and in DMEM, studies were conducted at a concentration of 10 µg/ml. Agglomeration was slower at 10 than at 50 µg/ml (Fig. 7). Additionally, the assumption of faster agglomeration in PBS was confirmed at a concentration of 10 µg/ml. Samples with ZnO-hydro and ZnO-lipo containing lecithin did not agglomerate in PBS or DMEM at a particle concentration of 10 µg/ml either (data not shown).
Further experiments were performed on DMEM supplemented with 2 or 10% serum (FBS). Due to the fact that lecithin-containing ZnO-hydro and ZnO-lipo samples showed no differences in terms of their behavior in PBS and in DMEM, investigations in DMEM with serum were limited to stock suspensions with ZnO-hydro in phosphate and ZnO-lipo in phosphate/lecithin. Figure 8 shows $x_{\text{DLS}}$ of ZnO-hydro and ZnO-lipo in DMEM supplemented with 2 or 10% FBS over a period of 3 days. For ZnO-lipo in DMEM with 2% FBS, the particle size remained constant within the first 24 h, after which time the size increased slightly, finally reaching a $x_{\text{DLS}}$ of around 350 nm after 3 days. In contrast, the particle size of ZnO-lipo in DMEM with 10% FBS remained constant, even decreasing slightly. The uncoated ZnO-hydro particles did not agglomerate in DMEM with 2% FBS or in DMEM with 10% FBS. Furthermore, the mean size eventually dropped below the initial value.

Besides size measurements, the zeta potentials in PBS, DMEM, and DMEM supplemented with FBS were recorded (Table 2). After the stock suspensions were added to the physiological media, the zeta potential increased from $-50 \text{ mV}$ to values above $-30 \text{ mV}$, considered to be the stability threshold [30]. In DMEM with FBS, zeta potential values were between $-8.7 \pm 1.3$ and $-9.2 \pm 0.9$ mV. The polydispersity of all tested samples was increased in DMEM with FBS (PI from 0.2 to 0.4) as compared to values of the stock suspension (Table 1). When using the general purpose mode, in some cases, a peak of protein in addition to the actual peak of particles were detected at the same time.

The extent of dissolution of ZnO was higher in DMEM and DMEM supplemented with FBS than in the stock suspensions, although the particle concentration in the

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**Table 2** Zeta potential in mV of ZnO-hydro and ZnO-lipo in PBS, DMEM, and DMEM supplemented with FBS

| Particle          | PBS | DMEM | DMEM with 2% FBS | DMEM with 10% FBS |
|-------------------|-----|------|------------------|-------------------|
| ZnO-hydro         | $-16.2 \pm 1.2$ | $-7.4 \pm 1.4$ | $-8.7 \pm 1.3$   | $-9.2 \pm 0.9$    |
| ZnO-lipo          | $-24.3 \pm 2.7$ | $-21.7 \pm 2.6$ | $-10.4 \pm 1.2$  | $-9.1 \pm 1.1$    |

The particle concentration in physiological media was 50 µg/ml. The stock suspensions used were ZnO-hydro in 1.2 mM phosphate and ZnO-lipo in 1.2 mM phosphate with lecithin. Zeta potential values are given as mean ± standard deviation values.
physiological media was only a tenth of those in the stock suspensions (Fig. 9). Furthermore, the extent of dissolution of ZnO-hydro and ZnO-lipo was higher in the presence of serum, and the process in serum-containing media seemed to be material-independent. In serum-free DMEM, ZnO-hydro dissolved better than coated ZnO-lipo did.

**Discussion**

One aim of the present study was to prepare stable stock suspensions from a hydrophilic and a lipophilic ZnO powder as a prerequisite for performing toxicological in vitro testing. Investigations were also carried out in different physiological fluids to improve the understanding and interpretation of the results of toxicological experiments. For this purpose, the ZnO-containing stock suspensions were combined with the physiological fluids such as was previously done in numerous other studies [25–28].

Phosphate salts are suitable for electrostatic suspension stabilization

Besides the preparation and characterization of the suspension, comprehensive powder characterization is essential for further investigations [20]. Concerning their powder properties, hydrophilic ZnO-hydro and hydrophobic ZnO-lipo were very similar. This was not surprising because they both consisted of the same initial powder, but ZnO-lipo was coated with triethoxycaprylylsilane. Both powders consisted of micrometer-sized agglomerates with submicron- and nanometer-sized primary particles. After sonication the agglomerates could be broken up into smaller particles with a mean size x_{DLS} of around 250 nm. For use in toxicological experiments, the particles of the stock suspension must be stable over time and may not agglomerate. One stabilization mechanism is based on the electrostatic repulsion of the particles. A simple method of achieving this is through adjustment of the pH to regions with high absolute values of the zeta potential. For ZnO-hydro, a pH below 7.5 is suitable, but if the original pH of 8 is decreased to lower values, the dissolution tendency increases. Thus, lowering of the pH by addition of acid was not useable in this case. Instead, phosphate salts, which are main components of nearly all physiological media and hence biocompatible, were added. The phosphate salts used were chosen to have suspensions with a physiological pH of 7.4. Further phosphates and polyphosphates are known to act as dispersants [31, 32]. Due to the use of phosphates, zeta potential values of around −50 mV were obtained. This resulted in good electrostatic stabilization of the suspension, as clearly demonstrated by the constant particle size values.

Lecithin can be used as a biocompatible wetting agent for hydrophobic powders

For lipophilic ZnO-lipo, a wetting agent is a prerequisite for particle suspension. In this study, soy lecithin was taken as a wetting agent. Lecithins are phospholipids, which include, e.g., the surfactant constituent dipalmitoylphosphatidylcholine (DPPC). Several studies showed that DPPC is an excellent and nontoxic dispersant [33, 34]. Preparation of ZnO-lipo suspension with only lecithin had a zeta potential of −23 mV, which was not sufficient for electrostatic stabilization. Additional phosphate shifted the zeta potential of ZnO-lipo to values of ≈ −50 mV. Furthermore, no differences in zeta potential compared with ZnO-hydro in 1.2 mM phosphate and those in 1.2 mM phosphate with lecithin were found. Therefore, the dominating component for electrostatic stabilization of the suspension was concluded to be phosphate.

Suspensions exhibit long-time stability, but complex dissolution processes occur

The two different ZnO-hydro stock suspensions and the ZnO-lipo stock suspension remained stable over the test period of 14 days, as demonstrated by the constant mean particle size of around 250 nm. Due to the use of different ZnO-hydro suspension types, the influence of the various additives could be studied well. In water, suspended ZnO-hydro particles were not stable and agglomerated as a result of the low electrostatic repulsion forces. Remarkably, the extent of dissolution in water was low (3.5–4.3 μg/ml zinc), corresponding to just 1 % ZnO. The values were in good agreement with those reported by Dimkpa and colleagues [35]. His investigations were performed at an identical ZnO concentration and resulted in 4.9 μg/ml (ZnO NP) and 4.6 μg/ml (ZnO Bulk) of dissolved zinc at a pH of 7. Dissolution in water was much higher compared with ZnO-hydro in phosphate with lecithin (0.3 %) and with ZnO-hydro in phosphate (0.03 %). At first glance, it was not immediately apparent as to why the dissolution of ZnO-hydro in the phosphate-containing media was lower. Another unclear aspect was the influence of lecithin, since dissolution of ZnO increased further in the presence of this wetting agent. It was supposed that the measured zinc concentration in the supernatant of phosphate- and phosphate/lecithin-containing samples did not represent the real amount of dissolved ZnO particles. Proof of a greater extent of ZnO dissolution was given by the pH increase from 7.4 to 9. Although phosphate had pH-buffering properties, the pH value increased notably, reflecting the release of numerous hydroxide ions according to the equation

\[
\text{ZnO} + \text{H}_2\text{O} \rightarrow \text{Zn}^{2+} + 2 \text{OH}^- \tag{36}
\]
Furthermore, SEM analysis of particles obtained from the phosphate-containing ZnO-hydro suspension without lecithin revealed cubic particles with an edge length of ~1 μm. These cubic particles consisted of about equal mass fractions of Zn and O and also contained 10% P, as shown by the EDX analysis. Thus, the formed crystals corresponded to a zinc phosphate compound. Consequently, the phosphate ions in the suspensions were consumed by both the released hydroxyl ions and the resulting zinc phosphate compound. Hence, not enough phosphate ions were available to act as pH buffers. Dissolution equilibrium was presumably reached when the pH stopped increasing. In contrast to these findings, Michelmore et al. [37] described the reaction of solid ZnO with water and phosphate ions to form a hydroxyphosphato complex according to the equation ZnO + H₂O + HPO₄²⁻ → Zn(OH)₂(HPO₄)²⁻. In Michelmore’s study, investigations with ZnO and polyphosphate yielded zinc polyphosphate complexes that precipitated on the ZnO surfaces. They further found that the concentration of dissolved zinc increases with increasing polyphosphate concentration. It is therefore conceivable that similar processes can even occur when simple phosphate is used. If such complexes of zinc and phosphate were formed and were bound to the particle surface, they would be separated during centrifugation. Consequently, the measured zinc concentration in the supernatant would be less than expected. Another aspect described for TiO₂, is that surface hydroxyl groups can be exchanged with phosphate ions [38]. The same substitution might occur on the ZnO surface.

Remarkably, the cubic zinc phosphate crystals were only found for ZnO-hydro suspensions in 1.2 mM phosphate. For ZnO-hydro and ZnO-lipo suspensions with phosphate and soy lecithin, such crystals were absent. In the case of the uncoated ZnO-hydro, the two suspensions differed only in terms of the presence or absence of lecithin. Due to the fact that lecithins are phospholipids, their phosphate group could bind the dissolved zinc ions as well. If binding of zinc ions to lecithin occurred preferentially, no or a smaller amount of zinc phosphate compound would precipitate. The assumption that the dissolved zinc ions were bound to lecithin was supported by the higher zinc concentrations measured in the centrifugal supernatant. The ZnO particles as well as the 1-μm cubic zinc phosphate crystals could be separated from the dissolved zinc ions by centrifugation, but the zinc-loaded lecithin could not be separated. The molecular weight was too small to be affected by the centrifugal forces.

Stock suspensions with phosphate and lecithin can be used for reproducible toxicological experiments

Finally, suspensions with deagglomerated, stabilized ZnO particles are available, but the addition of dispersant is necessary. Phosphate and lecithin are biocompatible and effective dispersants in terms of electrostatic stabilization and wetting, respectively. Both dispersants affect the behavior of the suspended ZnO particles, especially the dissolution. Because phosphate is generally present in physiological environments and phospholipids are essential components of surfactants, reactions taking place in the stock suspension would proceed naturally later on in a physiological surrounding. The advantage of the present method is that these reactions have already been completed, and constant conditions for further investigation, e.g., in toxicological studies, are therefore given.

Lecithin and serum proteins inhibit particle agglomeration in physiological media

For the addition of stabilized ZnO-hydro particles to physiological media, stock suspensions with phosphate were a prerequisite, whereas ZnO-lipo particles required the addition of lecithin as a wetting agent. In protein-free physiological media, the particle behavior depended on the presence of lecithin in the stock suspension. ZnO-hydro particles previously stabilized with phosphate agglomerated in PBS and DMEM. As expected, the agglomeration velocity increased with increasing particle concentration. More particles in the same volume meant an increased probability of particle–particle contact. These findings were in agreement with studies on other particles in physiological media [25, 26, 39] as well as in media used for ecotoxicological purposes [40]. Electrostatic repulsion forces were lower in physiological media due to the high electrolyte content, demonstrated by the low absolute values of the zeta potential. The values were outside the stability range of ≤−30 mV or greater +30 mV [30]. However, ZnO-hydro without added lecithin had a negative zeta potential in both PBS (−16.2 mV) and DMEM (−7.4 mV), verifying the statement of Cho et al. [14] that ZnO is negatively charged in physiological media. Thus, ZnO and other metal oxide NPs should not simply be regarded as positively charged NPs, as has been done in other studies based on the positive zeta potential value in water [4, 12, 13]. Especially for PBS, it can be concluded that the adsorbed phosphate ions were responsible for the negative charge. The only other negatively charged ion in PBS was chloride, which is not known to have a high adsorbing ability, in contrast to phosphate, which is known as a strongly adsorbing species [41]. In contrast, DMEM is a very complex media, making an attribution to one adsorbing species difficult. Adsorbed phosphate ions presumably codetermine the surface charge of ZnO. Interestingly, the zeta potential was significantly less negative in PBS than in DMEM. This also confirmed the domination of adsorbed phosphate in PBS.
ZnO particles, whether coated or not, did not agglomerate in physiological fluids when the stock suspensions previously contained lecithin. The particles remained the initial size of the stock suspension in PBS and DMEM even though the zeta potentials were outside the stable region. Therefore, the mechanism of stabilization had to be sterical or electrosterical. This can only be explained by the fact that lecithin was adsorbed on the particle surface, and thus the particles were stabilized. Other studies also described a stabilizing effect of lecithin and DPPC [33, 34, 42].

In DMEM supplemented with FBS, agglomeration of ZnO-hydro and ZnO-lipo particles was inhibited through the presence of serum proteins. The ZnO-hydro particles without lecithin in the stock suspension also remained stable, eliminating the need for lecithin as a stabilizing agent in protein-containing media. The findings confirmed the results of our own studies [25–28] and those of other groups [23, 39, 43] that proteins, especially BSA, stabilize different kinds of nanoparticles under physiological conditions. Furthermore, Tantra et al. [44] were able to demonstrate a stabilizing effect of BSA for ZnO particles, but these investigations were carried out in deionized water and not in physiological media.

Stabilization of particles through serum depends on the amount of available proteins. The particle size of ZnO-lipo increased slightly in DMEM with only 2 % FBS, whereas 10 % FBS permitted full stabilization over 3 days. For ZnO-hydro, no differences due to the different FBS quantities were found. Furthermore, protein adsorption and the related stabilization mechanisms strongly depend on the particle’s hydrophilicity and hydrophobicity, respectively. This, in turn, could possibly suggest the formation of other adsorption layers. The slight decrease in the mean particle size \( x_{\text{DLS}} \) of ZnO-hydro and ZnO-lipo (DMEM with 10 % FBS) below values obtained in stock suspensions could not be indicative of further progression of deagglomeration because the stock suspension contained indestructible aggregates. The size decrease must have resulted from sedimentation of the ZnO particles within the investigation period of 3 days. In all samples tested, an increased PI was observed that is certainly a consequence of the parallel presence of particles and proteins. The cumulant analysis is restricted in this case as it only yields an average particle diameter and no multiple peaks. A particle and protein peak were monitored using the general purpose mode, but this complex analysis model tends to have stronger deviations. All in all, DLS is limited in these complex fluids.

The zeta potential of ZnO in DMEM with FBS was between \(-8.7\) and \(-10.4\) mV regardless of the FBS amount and the ZnO particles used. This conformity suggests that serum proteins adsorbed onto the particle surface, with the result that protein-coated ZnO particles appeared electrostatically to be like the free serum proteins. Such zeta potential values of protein-covered particles in physiological media are often found and seem to be particle-independent [23, 45, 46].

The increased dissolution in DMEM and DMEM/FBS compared with the stock suspension could possibly have been caused by the high electrolyte content in the physiological media. In the presence of serum, an additional increase in dissolution was detected, confirming Hsiao’s and Huang [4] finding that more ions are released in media with serum than in media without serum. This seems to be a general fact, because SiO₂ also showed a higher dissolution under biological media conditions as compared to water alone [47]. According to Nel et al. [15], two mechanisms explain the increased dissolution of ZnO and other nanomaterials due to the protein availability. Free ions released from the material’s surface form complexes with proteins. Organic material can also extract surface metal atoms from the nanoparticle surface. In Xia’s work [6], the maximum total dissolved zinc concentration in DMEM with FBS was 225 \( \mu \text{M} \), or 14.7 \( \mu \text{g/ml} \) \text{Zn}^{2+}, in the range of our dissolved zinc measurements of 13.4 \( \mu \text{g/ml} \) (\( >3.4 \) % dissolved ZnO) to 20.0 \( \mu \text{g/ml} \) (\( >5.0 \) % dissolved ZnO). This match could be an indication of a saturation concentration of dissolved zinc in a cell culture medium supplemented with serum.

Conclusions

In this study, a feasible method for preparing stable, non-physiological suspensions containing hydrophilic or lipophilic ZnO particles was developed. Because stabilization via pH adjustment was not possible, phosphate was taken as a dispersant and lecithin as a wetting agent for the lipophilic ZnO. The resulting suspensions showed constant values with respect to particle size and zeta potential over a test period of 14 days, thus meeting a prerequisite for further toxicological studies. However, reactions occurred between ZnO and the phosphate ions, in turn affecting the dissolution of the powders. Although these reactions took place in a non-physiological fluid, they nevertheless yielded conclusions about the influence of complex cell culture media, which always contain phosphate ions. ZnO underwent extensive dissolution, detected not directly through the zinc ion concentration but rather through the pH, which showed an increase. The dissolved zinc ions formed complexes with phosphate and lecithin, which contained a phosphate group in its molecular structure.

In physiological media with higher electrolyte contents and more phosphate ions, the extent of dissolution of ZnO increased. Additional serum increased the dissolution rate even further. Different additives clearly had different effects on the stability of ZnO particles in physiological...
media. Phosphate-stabilized ZnO particles were found to agglomerate in PBS and DMEM in a process depending on the mass concentration and the physiological media. In comparison, ZnO particles suspended in water with phosphate/lecithin did not agglomerate after being added to physiological media. It is believed that lecithin adsorbs on the particle surface, thereby effecting sterical or electropherical stabilization. In the presence of serum in the media, particle agglomeration was effectively prevented regardless of the stabilization variants of the stock suspension (phosphate or phosphate/lecithin). The protein adsorption was reflected in a shift in the zeta potential to about $-10$ mV, corresponding to the value for the free serum proteins.

In summary, the approach described here allows for the preparation of non-agglomerating, well-characterized ZnO suspensions with particles that are dispersed to the smallest achievable dimension. This is a prerequisite for the use in in vitro testing. Furthermore, comprehensive time-dependent characterization of particle behavior under non-physiological as well as physiological conditions gave better insight into the dissolution properties of ZnO, which can be useful for interpretation of the results of toxicological studies of ZnO (nano)particles. This allows the correlation between the chemical-physical particle behavior with findings from toxicological tests.

For ecotoxicological assessment of ZnO and other nanomaterials, our approach has to be adapted despite media in ecotoxicological tests differ in their composition compared with cell culture media. Phosphate is undesirable in many cases for use in ecotoxicological media and free proteins are not available either. But also the environment has lots of dispersants, e.g. humic substances that are suitable for the stabilization of nanomaterials.

Conflict of interest The authors declare that they have no competing interests.

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References

1. Osmond, M.J., McCall, M.J.: Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. Nanotoxicology 4(1), 15–41 (2010)
2. Oberdörster, G., Oberdörster, E., Oberdörster, J.: Nanotoxicology: an emerging discipline evolving from studies of ultratrace particles. Environ. Health Perspect. 113(7), 823–839 (2005)
3. Dhawan, A., Sharma, V., Parmar, D.: Nanomaterials: a challenge for toxicologists. Nanotoxicology 3(1), 1–9 (2009)
4. Hsiao, I.L., Huang, Y.J.: Effects of various physicochemical characteristics on the toxicities of ZnO and TiO$_2$ nanoparticles toward human lung epithelial cells. Sci. Total Environ. 409, 1219–1228 (2011)
5. Lin, W., Xu, Y., Huang, C.C., Ma, Y., Shannon, K., Chen, D.R., Huang, Y.W.: Toxicity of nano- and micro-sized ZnO particles in human lung epithelial cells. J. Nanopart. Res. 2008(11), 23–39 (2008)
6. Xia, T., Kovochich, M., Liong, M., Mäddler, L., Gilbert, B., Shi, H., Yeh, J.I., Zink, J.I., Nel, A.E.: Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2, 2121–2134 (2008)
7. Pujalé, I., Passagne, I., Brouilhau, B., Tréguer, M., Durand, E., Ohayon-Courtès, C., L’Azou, B.: Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. Part. Fibre Toxicol. 8, 10 (2011)
8. Brunner, J.T., Wick, P., Manser, P., Spohn, P., Grass, R.N., Limbach, L.K., Bruniink, A., Stark, W.J.: In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. Environ. Sci. Technol. 40(14), 4374–4381 (2006)
9. Corradi, S., Gonzalez, L., Thomassen, L.C.J., Bilanícová, D., Birkedal, R.K., Pojana, G., Marcomini, A., Jensen, K.A., Leyns, L., Kirsch-Volders, M.: Influence of serum on in situ proliferation and genotoxicity in A549 human lung cells exposed to nanomaterials. Mutat Res 745, 21–27 (2012)
10. Cho, W.S., Duffin, R., Howie, S., Scotton, C., Wallace, W., MacNee, W., Bradley, M., Megson, I., Donaldson, K.: Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn$^{2+}$ dissolution inside lysosomes. Part. Fibre Toxicol. 8, 27 (2011)
11. Osaka, T., Nakashima, T., Shannugam, S., Takahama, S., Zhang, H.: Effect of surface charge of magnetite nanoparticles on their internalization into breast cancer and umbilical vein endothelial cells. Colloids Surf. B Biointerfaces 71, 325–330 (2009)
12. Murdock, R.C., Braydich-Stolle, L., Schrand, A.M., Schlager, J.J., Hussain, S.M.: Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scatering technique. Toxicol. Sci. 101(2), 239–253 (2008)
13. Berg, J.M., Romoser, A., Banerjee, N., Zebda, R., Sayes, C.M.: The relationship between pH and zeta potential of $\sim 30$ nm metal oxide nanoparticle suspensions relevant to in vitro toxicological evaluations. Nanotoxicology 3(4), 276–283 (2009)
14. Cho, W.S., Duffin, R., Thielbeer, F., Bradley, M., Megson, I.L., MacNee, W., Poland, C.A., Tran, C.L., Donaldson, K.: Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal-oxide nanoparticles. Toxicol. Sci. 126, 469–477 (2012)
15. Nel, A.E., Mäddler, L., Velegol, D., Xia, T., Hoek, E.M.V., Somasundaran, P., Klaessig, F., Castranova, V., Thompson, M.: Understanding biophysicochemical interactions at the nano-bio interface. Nat. Mater. 8, 543–557 (2009)
16. Horie, M., Nishio, K., Fujita, K., Endoh, S., Miyauchi, A., Saito, Y., Iwahashi, H., Yamamoto, K., Murayama, H., Nakano, H., Nanashima, N., Niki, E., Yoshida, Y.: Protein adsorption of ultratine metal oxide and its influence on cytotoxicity toward cultured cells. Chem. Res. Toxicol. 22, 543–553 (2009)
17. Kathiravan, A., Paramaguru, G., Renganathan, R.: Study on the binding of colloidal zinc oxide nanoparticles with bovine serum albumin. J. Mol. Struct. 934, 129–137 (2009)
18. Monopoli, M.P., Aberg, C., Salvati, A., Dawson, K.A.: Biomolecular coronas provide the biological identity of nanosized materials. Nat. Nanotechnol. 7, 779–786 (2012)
19. Casals, E., Pfänder, T., Duschl, A., Oostingh, G.J., Puntes, V.: Hardening of the nanoparticle-protein corona in metal (Au, Ag) and oxide (Fe$_3$O$_4$, CoO, and CeO$_2$) nanoparticles. Small 7(24), 3479–3486 (2011)
20. Powers, K.W., Brown, S.C., Krishna, V.B., Wasdo, S.C., Moudgil, B.M., Roberts, S.M.: Research strategies for safety
evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol. Sci. 90(2), 296–303 (2006)

21. Teeguarden, J.G., Hinderliter, P.M., Orr, G., Thrall, B.D., Pounds, J.G.: Particokinetics in vitro: dosimetry Considerations for in vitro nanoparticle toxicity assessments. Toxicol. Sci. 95(2), 300–312 (2007)

22. Warheit, D.B.: How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol. Sci. 101(2), 183–185 (2008)

23. Bihari, P., Vippola, M., Schultes, S., Praetner, M., Khandoga, A., Reichel, C., Coester, C., Tuomi, T., Rehberg, M., Krombach, F.: Optimized dispersion of nanoparticles for biological in vitro and in vivo studies. Part. Fibre Toxicol. 5, 14 (2008)

24. Taurozzi, J.S., Hackley, V.A., Wiesner, M.R.: A standardised approach for the dispersion of titanium dioxide nanoparticles in biological media. Nanotoxicology 7(4), 389–401 (2013)

25. Meißner, T., Kühnel, A., Busch, W., Oswald, S., Richter, V., Michaelis, A., Schirmer, K., Potthoff, A.: Physical-chemical characterization of tungsten carbide nanoparticles as a basis for toxicological investigations. Nanotoxicology 4(2), 196–206 (2010)

26. Meißner, T., Potthoff, A., Richter, V.: Physico-chemical characterization in the light of toxicological effects. Inhal. Toxicol. 21, 35–39 (2010)

27. Potthoff, A., Meißner, T., Richter, V., Busch, W., Kühnel, D., Bastian, S., Iwe, M., Springer, A.: Evaluation of health risks of nanoparticles—a contribution to a sustainable development of nanotechnology. Solid State Phenom. 151, 183–189 (2009)

28. Bastian, S., Busch, W., Kühnel, D., Springer, A., Meißner, T., Holke, R., Scholz, S., Iwe, M., Pompe, W., Gellinsky, M., Potthoff, A., Richter, V., Ikonomidou, H., Schirmer, K.: Toxicity of tungsten carbide and cobalt-doped tungsten carbide nanoparticles in mammalian cells in vitro. Environ. Health Perspect. 117(4), 530–536 (2009)

29. Malvern Instruments.: Sample and dispersion refractive index guide. Man0396, Issue 1.0 (2007)

30. International Organisation for Standardization. ISO 14887:2000. Sample preparation—dispersing procedures for powders in liquids (2000)

31. Müller, R.H.: Zeta-potential und Partikelladung in der Laborpraxis. APV paperback series, vol. 37. Wissenschaftliche Verlagsgesellschaft, Stuttgart (1996)

32. Jiang, J., Obendorfer, G., Biswas, P.: Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. J. Nanopart. Res. 11, 77–89 (2009)

33. Sager, T.M., Porter, D.W., Robinson, V.A., Lindsley, W.G., Schwelger-Berry, D.E., Castranova, V.: Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity. Nanotoxicology 1(2), 188–129 (2007)

34. Porter, D., Sriram, K., Wolfarth, M., Jefferson, A., Schwelger-Berry, D., Andrew, M.E., Castranova, V.: A biocompatible medium for nanoparticle dispersion. Nanotoxicology 2(3), 144–154 (2008)

35. Dimkpa, C.O., Calder, A., Britt, D.W., McLean, J.E., Anderson, A.J.: Responses of a soil bacterium, Pseudomonas chlororaphis O6 to commercial metal oxide nanoparticles compared with responses to metal ions. Environ. Pollut. 159(7), 1749–1756 (2011)

36. Fruhwirth, O., Herzog, G.W., Hollerer, L., Reitsamer, G.: ZnO dissolution kinetics by means of a 65Zn tracer method. Surf. Technol. 15, 43–50 (1982)

37. Michelmore, A., Jenkins, P., Ralston, J.: The interaction of linear polyphosphates with zincite surfaces. Int. J. Min. Process. 68, 1–16 (2003)

38. Healy, K.E., Ducheyne, P.: Hydration and preferential molecular adsorption on titanium in vitro. Biomaterials 13(8), 553–561 (1992)

39. Allouni, Z.E., Cimpan, M.R., Hol, P.J., Skodvin, T., Gjerdet, N.R.: Agglomeration and sedimentation of TiO2 nanoparticles in cell culture medium. Colloids Surf. B Biointerfaces 68, 83–87 (2009)

40. Baalousha, M.: Aggregation and disaggregation of iron oxide nanoparticles: influence of particle concentration, pH and natural organic matter. Sci. Tot. Environ. 407(6), 2093–2101 (2009)

41. Kosmulska. M.: Chemical Properties of Material Surfaces. In: Schäck, M.J. (ed.) Surfactant science series, vol. 102, pp. 310–326. Marcel Dekker Inc., New York (2001)

42. Chibowski, E., Holysz, L., Terpilowski, K., Wiacek, A.E.: Influence of ionic surfactants and lecithin on stability of titanium dioxide in aqueous electrolyte solution. Croat. Chem. Acta 80, 395–403 (2007)

43. Butford, M., Hamilton, R., Holian, A.: A comparison of dispersing media for various engineered carbon nanoparticles. Part. Fibre Toxicol. 4, 6 (2007)

44. Tantra, R., Tompkins, J., Quincey, P.: Characterisation of the deagglomeration effects of bovine serum albumin on nanoparticles in aqueous suspension. Colloids Surf. B Biointerfaces 75, 275–281 (2010)

45. Limbach, L.K., Grass, R.N., Brunner, T.J., Hintermann, M.A., Stark, W.J., Li, Y., Gunther, D., Muller, M.: Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. Environ. Sci. Technol. 39(23), 9370–9376 (2005)

46. Landsiedel, R., Ma-Hock, L., Kroll, A., Hahn, D., Schnekenburger, J., Wiencz, K., Wohleben, W.: Testing metal-oxide nanomaterials for human safety. Adv. Mater. 2010(22), 2601–2627 (2010)

47. Mahon, E., Hristov, D.R., Dawson, K.A.: Stabilising fluorescent silica nanoparticles against dissolution effects for biological studies. Chem. Commun. 48(64), 7970–7972 (2012)