Not ready to use – overcoming pitfalls when dispersing nanoparticles in physiological media

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ABSTRACT

We rigorously characterized the interaction of nanoparticles with physiological media for in-vitro nanotoxicology experiments. Beyond adsorption of proteins on metal oxide and polymeric nanoparticles, we quantified nanoparticle desagglomeration due to adsorbing proteins acting as protection colloids. We report on previously neglected, but indispensable testing of sterility and measures to ensure it. Our findings result in a checklist of pre-requirements for dispersion of nanoparticles in physiological media and for reliable attribution of potential toxic effects.
Nanoparticles are widely used in electronic, aerospace, textile, pharmaceutical or plastics industry and research, and nanoparticle (NP) products become available to the end-user. The discipline of nanotoxicology emerges from the need to assess potential environmental and health risks due to the nano-size and extremely large surface areas of NPs\(^1\). However, industrial NPs were not developed to be compatible with in vitro cell culture assays: nano-suspensions use non-physiological pH values or cytotoxic stabilizing agents; their tendency to disaggregate or aggregate is strongly sensitive to pH and ionic strength. For the use of NPs in in-vitro assays, the dispersions must be isotonic, adapted to a pH of 7.4 and applicable in the presence of divalent ions and protein mixtures. Individual aspects of NP dispersion and protein adsorption have been investigated recently\(^2\)\(^-\)\(^7\).

Here we rigorously characterized the interaction of nanoparticles with physiological media for in-vitro nanotoxicology experiments and quantified the drastic dispersing action of proteins with appropriate fractionating methods. We also report on previously neglected, but indispensable testing of sterility and measures to ensure it.

Before studying the specific pitfalls with metal oxide NPs, we established our test systems by characterization of model NPs (polystyrene NPs from Fluoresbrite, carboxylated, Ø 50 nm; c50) in size and ζ-potential via Dynamic Light-Scattering (Zetasizer Nano ZS from Malvern Instruments, see Supporting Information) in different dispersion media with increasing complexity. In Millipore water, the hydrodynamic diameters of the particle were 49.5 ± 1.7 nm and had a ζ-potential of -64.4 ± 4.2 mV. In phosphate buffer (0.05 M; ionic strength 0.116 M), and isotonic phosphate buffer (0.05 M; ionic strength 0.154 M) the c50 particle showed a slightly higher size of 67 ± 3.5 nm or 57.5 ± 1.7 nm, respectively. The ζ-potentials increased to -58.6 ± 0.8 mV and to -51.2 ± 3.9 mV when isotonized. When dispersed in Krebs-Ringer-Buffer (KRB, see Supporting Information), the c50 agglomerated and showed a size of more than 3000 nm. Also, the ζ-potential increased nonlinear to -29 ± 1.4 mV (fig. 1). Since the presence of Ca\(^{2+}\) ions is a significant difference between phosphate buffers and KRB, CaCl\(_2\)-titrations in water and both phosphate buffers were performed and the sizes of the particles at
concentrations of 0.5 mM Ca\(^{2+}\) determined. The experiments revealed the Ca\(^{2+}\) as main factor in the agglomeration of c50 in KRB. At CaCl\(_2\) concentrations from 0.5 to 1.5 mM Ca\(^{2+}\) (CaCl\(_2\) concentration in KRB: 1.4 mM), particles agglomerated in all of the dispersion media tested (data not shown).

Addition of bovine serum albumin (1 %) lead to complete disagglomeration (hydrodynamic diameter decreased to 70.2 ±1 nm) and a further \(\zeta\)-potential increase up to -10.4 ± 2.7 mV (for comparison: 1 % of BSA in KRB without particles had a \(\zeta\)-potential of -5.6 ± 2.7 mV). The slight increase in diameter compared to the values in water and the similar \(\zeta\)-potential in relation to the \(\zeta\)-potential of BSA in KRB without particles suggested a protein coating of the particles.

To prove this hypothesis, a density gradient ultracentrifugation\(^8\) was performed (see Supporting Information). The density gradient fractionated the sample according to the buoyant density and hence was direct evidence of the chemical composition, but insensitive to diameters. In repeated experiments,
we detected no signal at the density of pure polystyrene (PS, 1.57 g/cm³), but a strong signal at 1.26±0.06 g/cm³, corresponding to composite particles of ~40/60 BSA/PS (fig. 2). The width of the distribution indicated a distribution of densities; hence the adsorption was not uniform. The non-uniform adsorption was reflected also by the significant increase and broadening of the particle sedimentation velocities upon mixing with BSA (data not shown). From the velocity increase, we deduced an adsorption of 20 – 60 wt% BSA on the PS core, in good agreement with the density gradient approach. Finally, the binding of protein onto the carboxylated particle surface was monitored by a loss of freely dispersed BSA monomer and dimer as quantified by their characteristic sedimentation coefficients (data not shown).

**figure 2.** Density gradient measurement of the density of polystyrene NP c50, dispersed in KRB + 1 % BSA. The left margin corresponds to the meniscus at a density of 1.07 g/cm³, the right to the boundary of the cell at 1.36 g/cm³, i.e. the force is directed to the right. The particles accumulate at their isopycnic point, where their density corresponds to the density of the gradient medium, in this experiment around 1.20 g/cm³. Unprocessed CCD image in the inset: The Schlieren-AUC measures \( \frac{dn}{dR} (R) \) and a gaussian distribution of density fractions induces a positive-to-negative modulation (original) superimposed with the positive-slope signal from the rising concentration of the gradient medium. (see Supporting Information)
Taken together, this data were the first direct and quantitative in-solution evidence of adsorption of proteins on (polymeric) NPs (Fig. 2) with ensuing dispersing action, as seen by DLS and AUC in excellent agreement. (Fig. 1a).

It is known that electrostatically stabilized NP dispersions with a zeta potential converging to zero become unstable\(^9\). That is what happens when dispersing the c50 in KRB containing 1.4 mM CaCl\(_2\): the electrostatic stabilization is not sufficient to avoid agglomeration anymore. Presumably, the deprotonated, negatively charged carboxyl groups at the particle surface interact directly with the positively charged Ca\(^{2+}\)-ions. The increase in size of c50 when dispersed in KRB containing 1% of BSA was due to covering the particles with albumin. The coating of particles with BSA was shown with colloidal gold\(^{10}\), polymer colloids\(^{11}\) and Aluminum hydroxide\(^{12}\) particles already. The originally electrostatically stabilized particles can be dispersed in media containing Ca\(^{2+}\) via a switch to a steric stabilization by addition of albumine\(^9\).

These results, generated from the model particles, were then transferred to much less defined metal oxide NPs. In cell culture, addition of serum to the culture medium, mostly Fetal Calf Serum (FCS), is mandatory for many cell lines to guarantee an ideal cell growth\(^13\). Therefore, FCS replaced BSA as the protein component in our test systems. Dry CeO\(_2\) and TiO\(_2\) particles (both used as manufactured without any modifications; this condition will be referred to as ‘naked’) and predispersed ZrO\(_2\) (organically modified and stabilized with an organic acid with a molecular weight of 180 g/mol) (see Supporting Information for detailed intrinsic properties of the nanoparticles) were dispersed in different physiological media (see Supporting Information) and Millipore water to test which medium suits best for \textit{in vitro} assays. Primary particle diameters are 21 nm (TiO\(_2\)), 14 nm (ZrO\(_2\)), 30 nm (CeO\(_2\)) (TEM shown in Supporting Information). The NPs were weighed into a snap-on lid glass, covered with the dispersion medium and stirred for 24 h without any ultrasound treatment (see Supporting Information for complete protocol). Particles sedimented immediately in media free of proteins. However, dispersions were stable in protein-containing media for the \(\zeta\)-potential measurement period. The \(\zeta\)-potentials of all particles in protein containing buffered media were very similar to the \(\zeta\)-potentials of
particles in pure buffered media. Particles in ion free water showed completely different \( \zeta \)-potentials (fig. 3).

The \( \zeta \)-potential measurements gave no direct evidence for a protein coating of the inorganic industrial particles tested. However the stabilization of particle dispersions by added proteins indicated a coating. The switch of the originally positive \( \zeta \)-potentials of CeO\(_2\) and ZrO\(_2\) in Milipore water to negative values in media containing 10 % FCS was in good correlation to the findings of Limbach et al., but they postulated that this switch leads to agglomeration of CeO\(_2\) and also other metal oxide NPs because of a loss of electrostatical stabilization. Contrary to these results we could correlate particle size and protein concentration in our dispersions.

![Figure 3](image)

**Figure 3.** \( \zeta \)-potential of CeO\(_2\), TiO\(_2\) and ZrO\(_2\) in different dispersion media. Except in water, the \( \zeta \)-potentials of each particle is more or less the same. No difference can be seen between KRB and DMEM with or without addition of proteins, i.e. from this measurement no adsorption of the proteins onto the particle surface can be concluded. DMEM: Dulbecco's Modified Eagle's Medium.
Size characterization constitutes a major pitfall due to the enormous colloidal polydispersity. Ultrafine particles (NPs < 100 nm in diameter)\textsuperscript{15} and agglomerates have to be quantified in a hundredfold excess of proteins with 5 nm diameter (BSA monomer) plus salts. We cross-checked the most wide-spread

![Graph](image)

**Figure 4.** Diameters of CeO\textsubscript{2} (green triangles), TiO\textsubscript{2} (black squares) and ZrO\textsubscript{2} (red dots) in DMEM with different concentrations of FCS. \(d_{50}\) values represent the weight-average agglomerate size by turbidity-AUC except otherwise indicated. In complete reproductions of preparation and measurement the values scatter by ± 30%. In all cases, the particle size decreases with increasing concentration of FCS, which shows its disagglomerating properties, leading to significant fractions of ultrafine particles in the dispersions (lines are guides to the eye). Grey diamonds come from inappropriate methods, black diamonds show differing results from several valid approaches, all on TiO\textsubscript{2} in 100% FCS.
methods of characterization on the example of TiO$_2$ in pure FCS (Fig. 4, diamonds). The nominal working range of DLS does cover all components, but even after prefiltering (450 nm pore size) DLS fails to detect the proteins that constitute 99% of the sample. The same is true for laser diffraction, whose lower working limit is exceeded. TEM images the NPs and agglomerates, but with comparably low statistics. Even without particles, drying and/or cryo-preparation of FCS induces artefacts. Optical AUC$^{8,14}$ (see Supporting Information) is the only method that detects also the sub-10-nm proteins with correct molar masses and concentrations, but in the ultrafine particle range turbidity-AUC has its uncertainties due to the Mie correction of intensities. X-Ray-AUC needs no such correction and detects selectively inorganic components, but requires exceedingly high concentrations of particles (~1%). Note that only accelerations above 1000g enable the detection of ultrafine components in sedimentation (fulfilled for X-ray-AUC fast (6000 rpm), not for slow (1500 rpm)) (Fig. 4). It is beyond the scope of this letter to settle the size characterization issue. Our results suggest that DLS, laser diffraction and slow-speed sedimentation cannot determine whether an ultrafine fraction is present or not. Valid methods confirm that FCS desagglomerates particles to a significant fraction of ultrafine particles with low energy input by stirring only.

The desagglomeration scales with the protein content. In Dulbecco's Modified Eagle's Medium (DMEM), all of the tested NPs were strongly agglomerated and no significant fraction of ultrafine particles could be determined (fig. 4). With increasing concentration of FCS, the mean particle size distribution ($d_{50}$) decreased, and a significant effect could be seen even in the presence of 5% FCS. Also, large agglomerates were disintegrated, indicated by the declining $d_{90}$ (data not shown).

The ZrO$_2$ used was stabilized and coated with an organic acid and well dispersible in ion free water, but agglomerated in DMEM, due to a change in pH. In 100% FCS, the dispersion was as good as in pure water (fig. 5a). This behaviour was very similar to the dispersion pattern of carboxylated polystyrene particle: well dispersible in water, strong agglomeration in the presence of salts and dispersible again by addition of proteins. The naked TiO$_2$ P25 particle revealed a different dispersion pattern: hardly dispersible in water, wetted but still agglomerated in DMEM (fig. 5b). With increasing
FCS-concentration, particles disagglomerated and at FCS-concentrations above 40%, an ultrafine dispersion was reached. Two different dispersion patterns are evident: a) the naked NPs (CeO$_2$, TiO$_2$) and b) the particles modified with an organic acid (ZrO$_2$ and polystyrene latex). Dispersion patterns seem to depend mostly on the particle’s surface modification, irrespective of the particle’s bulk material.

It has been reported previously that metal oxide NPs mixed with a BSA solution showed a uniform Zeta-potential, irrespective of their 'naked' chemical surface modification. This effect was thought to result from a BSA NP surfaces coverage$^5$. The Hussain group studied NP suspensions in physiological media by the less sensitive DLS method and observed a generally similar but much weaker trend of FCS to prevent agglomeration$^6$. No systematic study of the correlation of protein adsorption with disagglomeration tendency has been published yet. Natural organic matter act as effective wetting and dispersing agents for carbon nanotubes, often more effective than synthetic head-tail surfactants$^2$. The wetting and dispersing effect of interface-active proteins (among them HSA) has very recently been shown to be effective to debundle agglomerated single walled carbon nanotubes in aqueous suspension,

**figure 5.** Size distribution of a) ZrO$_2$ (stabilized with an organic acid) and b) TiO$_2$ (unmodified) by turbidity-AUC. The ZrO$_2$ particle is well dispersible in water (yellow line) and strongly agglomerating in the high salt DMEM (black line), whereas the TiO$_2$ is hardly dispersible in water, at all. With increasing concentration of FCS, the particle sizes decrease at both particles.
which was technically a very demanding task before\textsuperscript{4}. Similar to model nanoparticles, where we provided direct evidence of both adsorption (fig. 2) and ensuing dispersion (fig. 1), we identified adsorbed protein functioning as protection colloids as mechanism for the observed dispersion effect (figs. 4-5)\textsuperscript{5}. Especially albumines are interface active and show at least five different binding sites for an entire variety of molecules (inorganic minerals, proteins, polar organic molecules, fats, and chiral centers)\textsuperscript{16}. Our findings are in agreement with and complementary to the previous reports of protein adsorption to NPs and carbon nanotubes.

The dispersion of NPs in buffered solutions of proteins and nutrients allows the growth of contaminating microorganisms. Since these organisms as bacteria, yeast or other fungi and their metabolism products interfere with most of the toxicity test systems, NP preparations have to be sterilized.

For the optimization of the sterilization procedure we first analyzed the germ load of the used industrially produced and processed NPs. Dispersions of ZrO\textsubscript{2}, TiO\textsubscript{2}, and CeO\textsubscript{2} were tested aerobically and anaerobically for sterility according standard procedures applying fluid and solid media under long-term cultivation\textsuperscript{17}. The prominent bacteria identified were \textit{Micrococcus luteus}\textsuperscript{18,19} and \textit{Bacillus sp.}\textsuperscript{19,20}. \textit{M. luteus} is a Gram positive, spherical, saprotrophic spore-forming bacterium that belongs to the \textit{Micrococcaceae}\textsuperscript{21}. Bacteria of the genus \textit{Bacillus} are Gram-positive, rod-shaped, spore-forming, and are either facultative or obligate anaerobes.

Due to the presence of microorganisms in the NPs analysed, we applied different standard methods for sterilization of cell culture materials to aliquots of dispersed ZrO\textsubscript{2}, or TiO\textsubscript{2} and CeO\textsubscript{2}. In order to reduce the microorganism burden of the particles, we chose the addition of antibiotics/antimycotics, chemical sterilisation (sodiumazide, NaN\textsubscript{3}), heat sterilisation (134 °C, 2,5 bar), and sterilisation by ionizing irradiation (30 Gy). The sterilisation method should be effective to kill microorganisms without altering NP properties and without influencing the biological test systems.

NPs sterilized with the various methods were dispersed in cell culture medium and tested for contaminating microorganisms as described above. According to these standardized analyses, only
\( \gamma \) irradiation was capable of eliminating all microorganisms identified in dispersions of untreated NPs. It has been shown that spores are often more resistant to \( \gamma \) irradiation than metabolically active bacteria\(^{22}\), however, the underlying mechanism is still unclear\(^{23}\). The amount of endospores and the general level of contamination in our set-up appear to be sufficiently low to be eliminated by \( \gamma \) irradiation.

As \( \gamma \) rays have the capacity to ionise atoms, the effect of irradiation on nanomaterial properties was characterized. Irradiated and untreated dispersed ZrO\(_2\), or TiO\(_2\) and CeO\(_2\) powders were analysed with regard to surface chemistry (\( \zeta \)-potential and X-ray Photoelectron Spectroscopy, XPS), bulk structure (X-ray diffraction, XRD) and dispersion behaviour (AUC). None of the four methods detected any significant change after irradiation, hence \( \gamma \)-rays can serve as standard sterilisation procedure since neither surface nor the structure of our particles were altered (for details, see supporting information).

Overall, we have identified \( \gamma \) irradiation as method of choice to efficiently sterilize dispersed and powder NPs for the use in physiological media without changing material properties and without disturbing the \textit{in vitro} test systems.

Beside microorganism contamination itself test systems can also be affected by endotoxins, biologically highly active bacterial molecules often present in materials after a bacterial contamination. In 1894, endotoxin was first described as a heat-stable toxic substance that was released upon disruption of microbial envelopes\(^{24}\). Today, endotoxin is widely defined as component of the outer cell membrane of Gram negative bacteria being made up by lipopolysaccharides with lipid A being the bioactive component\(^{25}\). Inoue et al. (2006) have reported that endotoxins aggravate the inflammatory effect of carbon NPs in mice when applied simultaneously\(^{26}\) on the one hand while nanometre sized TiO\(_2\) has been shown to adsorb endotoxins and cause proinflammatory reactions\(^{27}\). Furthermore, the determination of endotoxin concentration is a standard procedure in characterizing particulate matter or dust when studying health risks resulting from environmental\(^{28}\) and work-place exposure.

To determine the endotoxin concentration of dispersed and powder NPs we made use of the Limulus amebocyte lysate (LAL) test (Kinetic-QCL\textsuperscript{\textregistered} Kinetic Chromogenic Assay; Lonza). Dispersions of ZrO\(_2\), TiO\(_2\), and CeO\(_2\) (32 \( \mu \)g/ml) were first tested with and without added standard endotoxin concentrations.
to determine the influence of the nanomaterial on the enzymatic assay. The ZrO$_2$ surface modifier was tested likewise.

Nanoparticles reduced the measured endotoxin concentrations by more than 50% (TiO$_2$, CeO$_2$) or about 70% (ZrO$_2$, organically modified). As the particles do not interfere with absorption at 405 nm, this effect must be due to a partially inhibition of the enzymatic reaction (data not shown). Also the ZrO$_2$ surface modifier (OM) contributes to the effect on the endotoxin test, as an amount of OM diluted in cell culture media equivalent to the amount present in the tested particle dispersions completely abolishes a detectable reaction. However, the particle bound surface modifier showed a minor effect on the endotoxin test system.

The endotoxin concentration measured in particle dispersions is well below the detection limit of the assay (0.005EU/ml ≈ 0.5 pg/ml). Even taking into account the inhibitory effect of nanoparticles on the test system the endotoxin concentrations of NP dispersions were below a critical limit. According to Friberg (1987)$^{29}$, the average endotoxin concentration in human plasma is 6 pg/ml (0.07 EU/ml) which is half of the theoretical endotoxin concentration during a pyrogen reaction. We can therefore conclude that all three nanomaterials do not contain endotoxin in an extent detectable by the assay and exceeding the average concentration in human plasma.

Taken together our data particles should be tested on endotoxins for in vivo and in vitro testing to prevent endotoxin dependent results of toxicity tests. The applied Kinetic-QCL endotoxin assay is suitable for measurement of NPs endotoxin concentration. NPs and possible surface modifiers can influence the results of the endotoxin assay which needs to be considered when interpreting absorption data. Based on our date and the relevant literature we recommend the use of NPs only if the endotoxin contamination is below 0.5 pg/ml, the detection limit of the assay.

Concluded from our results, many facets of preparation of nanoparticle dispersions have to be taken into account before physiological assays with NPs can be correctly interpreted. It is not sufficient to characterize the intrinsic properties of the nanomaterial – such as chemical composition and
crystallinity, primary particle size and morphology, surface chemistry and charge, organic modification and water solubility (compare supporting info).

We summarized the necessary information beyond intrinsic properties that one will need when working with NPs in physiological media in a checklist:

Dispersion issues
- dispersion protocol
- agglomerate size distribution and agglomeration state
- zeta-potential
- wettability and tendency to agglomerate/disagglomerate due to adsorption of solvent compounds
- adsorption of solvent compounds with possible influence on passivation, solubility, recognition.

Microbiological issues
- sterility
- endotoxin concentration
- endotoxin test reliability

By application of these topics characterized dispersions of particles for in vitro toxicity tests become available and reliable experiments can be performed.

To summarize our results, we quantified the drastic dispersing activity of fetal calf serum mixed with various types (polymer and metal oxide) of NPs that are either naked or organically modified. In pure serum, NPs were desagglomerated down to significantly nanosized dispersions, presumably by adsorption of proteins functioning as protection colloids. Beyond scientific implications, specific proteins may serve in nanotechnology as biocompatible, yet extremely efficient wetting and dispersing agents for some of the most abundantly used metal-oxides. To complement this result, we reported the first in-solution quantitative evidence of adsorption of proteins on (polymeric) NPs with ensuing dispersing action, in excellent agreement of several fractionating and integral methods.
Furthermore we provided evidence of inadvertent microbial contamination of standard NP test substances, a source of false interpretation of supposed nano-effects. We identified γ-irradiation as appropriate sterilization treatment. We proposed a comprehensive but feasible checklist to guarantee reproducible and reliable in-vitro studies with inorganic NPs.

The recommendations we express here are based on experimental findings and have implications for cell culture nanotoxicology. Do observed effects truly arise from the particle itself? With the above checklist, the pitfalls arising when dispersing NPs in physiological media to use them in in-vitro assays can be overcome.

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SUPPORTING INFORMATION PARAGRAPH

Materials: Intrinsic properties of nanoparticles (TEM, XRD, XPS); further materials. Protocols for sterilization and dispersion. Methods: measurements of size and ζ-potential via DLS and AUC, determination of endotoxin concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


24. Pfeiffer, R. Z Hyg 1894, (18), 1-16.


